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A postmortem study

Zhang, L.

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Molecular Pathology of Suicide

A postmortem study



Lin Zhang

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Molecular Pathology of Suicide: A postmortem study

ACADEMISCH PROERSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. P.P.C.C. Verbeek

ter overstaan van een door het College voor Promoties ingestelde commissie,
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Promotores:	Prof. dr. D.F. Swaab	Zhejiang University
	Prof. dr. P.J. Lucassen	Universiteit van Amsterdam
Overige leden:	Prof. dr. I. Huitinga	Universiteit van Amsterdam
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	Prof. dr. E.M.A. Aronica	Universiteit van Amsterdam

Faculteit: Faculteit der Natuurwetenschappen, Wiskunde en Informatica

“Whoever Saves One Life, Saves the World Entire.”

— *Talmud*

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE PRESENT THESIS

GENERAL INTRODUCTION

Suicide is a worldwide health problem that terminates approximately 700,000 reported human lives every year, which may even be an underestimation and according to the WHO, many more people likely commit suicide (WHO, 2021). It is one of the leading causes of death during the period of late adolescence (WHO, 2021). Suicide rates in European countries have been gradually rising and, in the Netherlands, the number of suicides is increasing 3–6% annually. The effect of preventive interventions is hard to establish and almost half of the suicides in Netherlands are committed by individuals who are receiving psychiatric therapies.

Suicide and comorbid psychiatric illnesses

Among the various risk factors for suicide, the occurrence of pre-existing psychiatric disorders increase the chance to attempt suicide (CDC, 2021). Suicide risks are generally associated with the presence of mental disorders, in particular when multiple ones are involved. Individuals with a tendency for suicide e.g. have a 6 times higher chance to combine multiple psychiatric illnesses when compared to control subjects (Préville et al., 2005). The type of co-existing psychiatric disorders varies substantially, however, (Qin et al., 2014; Tidemalm et al., 2008) with in particular (combinations of) major depressive disorder (MDD), bipolar disorder (BD) and schizophrenia (SCZ) having a stronger relationship than other disorders (de Medeiros Alves et al., 2015; San Too et al., 2019).

Epidemiological studies have shown that adult patients with psychiatric illnesses have robust tendencies for suicide, relative to those without mental diagnoses (Choi et al., 2020; Sorenson and Golding, 1988). However, two topics in this respect deserve our attention. First, geographical variations due to culture specificities and lack of medical care reduce the proportion of reported mental disorders among suicide cases. East Asian countries such as China and India, that are generally short of psychiatric interventions, report a lower prevalence of mental disorders in individuals with suicide compared to North America and Europe (Cho et al., 2016; Milner et al., 2013). Adolescent and young adult suicide attempters without a diagnosable psychiatric disorder are generally linked to substance misuse that impacts their mental states adversely (Cao et al., 2015; Marttunen et al., 1998). In addition, subclinical psychiatric symptoms that did not meet the full diagnostic criteria of a mental disorder also appeared to be present in a great majority of individuals who died by suicide (Joiner Jr et al., 2017). These unnoticed and ‘subthreshold’ forms of psychiatric disorders that coexist in approximately half of the suicide attempters should be taken into account when considering prevalence and the relation between psychiatric health and suicide risk (Balázs et al., 2000).

In addition, among individuals with mental comorbidity, death by suicide is not only considered the final outcome of the mental illness, but is also underpinned by independent genetic features (Zhao et al., 2019). Clinical studies have shown that individuals with a first-

degree relative who had mental disorders and died by suicide, are more likely to commit suicide (Qin et al., 2002). This effect can be even stronger in the case of a parental history of suicide, regardless of the presence or absence of a psychiatric disorder (Ballard et al., 2019; Sørensen et al., 2009). Genetic studies further support the idea that the etiology of suicidal behavior cannot be solely explained by psychiatric background (Erlangsen et al., 2018; Zhang et al., 2020a, b; Zhao et al., 2015; Zhao et al., 2018). We hypothesize that pre-existing mental disorders may contribute to genetic vulnerability of suicide, instead of being a deterministic factor in suicide pathophysiology.

Age-associated suicide portraits

Throughout the whole lifespan, personal, familial and/or societal events may have varying effects on people in terms of mental factors related to suicide. For instance early life circumstances and related adversities, including a higher birth order, childhood exposure to psychological trauma (violence, sexual abuse or neglect), difficulties at school, a younger maternal age and adult internalizing disorders, are early signs for later mental disorders, which will eventually enhance the risk of suicide in adulthood (Geoffroy et al., 2018; Ligier et al., 2020; Park et al., 2015; Rossi et al., 2020). In addition, it is suggested that these early life stressful events, particularly in patients with MDD, BD or SCZ, can predict the aggregation of a mental condition, including mental extremes such as suicide attempts (Adigüzel et al., 2019; Alli et al., 2019; Tanti et al., 2017; Tasmim et al., 2020). Mental disorders that represent the largest risk for suicide in teenagers and young adults typically include posttraumatic stress disorder, dysthymia, MDD and borderline personality disorder (Lesage et al., 1994; Miché et al., 2018). For individuals who do not have specific mental diagnoses, comorbidities involving substance abuse (alcohol, nicotine or drug dependency) can also contribute to psychiatric illnesses and are frequently associated with suicide attempts (Cao et al., 2015; Lesage et al., 1994; Miché et al., 2018; Shafii et al., 1988). The processes that lead to suicide completion from the moment of ideation onset, appear to be relatively short, which obviously limits opportunities for therapeutic interventions (Marttunen et al., 1998).

Compared to the young population, diagnosable mental disorders are more often seen in elderly individuals who commit suicide (de Raykeer Pascal et al., 2018; Henriksson et al., 1995). Age appears less important in assessing suicide risks when compared to personal or socioeconomic factors, like personality traits, physical illness, domestic violence, poverty, recent bereavement and public disgrace (Harwood et al., 2001, 2006; Maselko and Patel, 2008; Pridmore, 2015).

Gender differences in suicidal behaviors

Suicidal behavior show a prominent gender difference, that starts from adolescence and persists throughout the lifespan. Worldwide suicide rates in males are 2-3 times higher than in females,

and a long-term risk of suicide in the context of mental illness shows a similar sex difference, particularly in subjects above thirty (Babanejad et al., 2014; Gunnell et al., 2002; Organization, 2014). The most prevalent mental disorder and risk factor in males is substance abuse, while MDD and anxiety disorder are the most common ones in females (Liu et al., 2018; Taylor et al., 2004). In addition, suicide attempts were independently associated with sexual orientation minority status. Homosexual and bisexual populations have much higher lifetime prevalence of suicide attempts than heterosexual populations (Bolton and Sareen, 2011). Interestingly, in older adults, the presence of mental disorders that are related to an elevated risk of suicide was more common in women (Vasiliadis et al., 2017), which seems to imply that the impact of mental health on suicide tendency is stronger in this group.

Of concern, however, in aged males, either before or after suicide attempts, fewer psychiatric disorders are detected, most likely due to a more limited mental health follow-up than for females of the same age (Niederkrotenthaler et al., 2019; Schmutte and Wilkinson, 2020). The sex-biased variables mentioned above that take effect from childhood to adolescence have been linked to higher suicide tendencies throughout the age range, but their influence appears to be reduced in males of advanced age.

Suicide prevention

Efforts to prevent suicide have consistently shown a poor response, even when compared to the pharmaceutical treatment of mental disorders. According to a complete follow-up of the patient records, more than 5% of the patients with MDD, BD or SCZ continue to be at the risk for suicide even when they are receiving active mental health service (Nordentoft et al., 2011). Death by suicide occurs most frequently within the first three months after the initial psychiatric diagnosis and during the first year after hospital discharge (Orme et al., 2020; Randall et al., 2014), indicating that switching therapeutic approaches may possibly trigger psychobiological alterations that could perhaps contribute to suicide behaviors ('treatment-emergent suicidality') (Jick et al., 2004).

Apart from their improvement of psychotic symptoms, most antipsychotics are similar in their effect and not different from placebo treatment when it comes to suicide prevention (Garlow et al., 2013; Khan et al., 2001; Tauscher-Wisniewski et al., 2007). Among the selective serotonin reuptake inhibitors, fluoxetine, when considered in relation to suicide prevention, is more effective in females (Beasley et al., 1991; Milane et al., 2006), but paroxetine treatment resulted in an increased intensity of attempted suicides (Aursnes et al., 2005). As to serotonin-norepinephrine reuptake inhibitors, milnacipran appears to induce a rapid improvement from suicidality (Kirino and Gitoh, 2011). In contrast, a higher risk of suicide has been consistently linked to the prescription of venlafaxine, regardless of whether the patient suffered from severe mental illnesses (Mines et al., 2005; Rubino et al., 2007). It is thus important to be aware of the

differences and possible links between the prescription of specific antidepressants and the risk of suicidality.

Tricyclic antidepressants and benzodiazepines have proved to alleviate suicidal thoughts in adults with anxiety disorders, posttraumatic stress disorder, and in adolescents under chronic stress, on the condition that it is prescribed alone (Boggs et al., 2020; Soreni et al., 1999). Clozapine and olanzapine have demonstrated improvements in preventing suicide risk among patients with schizophrenia and schizoaffective disorder, especially when this coincided with other public health and psychosocial interventions (Bastiampillai et al., 2017; Meltzer et al., 2003; Ringbäck Weitoft et al., 2014). However, a possible link between benzodiazepines over-prescription and increased potential for suicide attempts in males and adolescents is also worth discussing (Neutel and Patten, 1997; Niederkrotenthaler et al., 2019). A concurrent use with antidepressants like zolpidem may have increased the suicide risk compared to the use of zolpidem solo (Sung et al., 2019). Of note, there are cases that document a relationship between abrupt clozapine discontinuation and the subsequent rise in suicide events (Patchan et al., 2015).

Lithium has been broadly prescribed in patients with mental health diagnoses (MDD and BD in particular) and a history of suicide ideation and attempts (Roberts et al., 2017; Smith and Cipriani, 2017; Terao et al., 2018). Its discontinuation within six months has been linked to increased suicide death rates (Oquendo et al., 2011; Smith et al., 2014). Since the majority of antipsychotics do not reverse suicidal ideations or behaviors, and can even trigger suicide tendencies in some circumstances, it is concluded that novel anti-suicide treatments should focus on understanding of the molecular mechanisms specific for suicide, rather than for the pre-existing mental disorders per se.

LEGALIZED EUTHANASIA AND PHYSICIAN-ASSISTED SUICIDE

Euthanasia and physician-assisted suicide (PAS) are legally practiced in the Netherlands but concern a relatively small number of people and is performed only under very strict regulations. Euthanasia is defined as the administration by a physician of a medication, such as a sedative and neuromuscular relaxant, to intentionally end a patient's life upon the explicit and repeated request of the mentally competent patient (Emanuel et al., 2016). For the practice of euthanasia, the euthanatic agents are administered intravenously. First, a coma is introduced by thiopental (2000 mg) or propofol (1000 mg). Subsequently, when the patient is determined to be in a medically induced coma, a neuromuscular blocker, such as rocuronium (150 mg), atracurium (100 mg) or cisatracurium (30 mg) is administered. This paralyzes all striated muscles, with the exception of the heart and cause the patient to die (KNMG/KNMP, 2012).

PAS is defined when the physician provides medication to a patient at his or her explicit request with the understanding that the patients intends to use the medications to end his or

her life (Emanuel et al., 2016). With PAS, the patient takes the euthanistic agents him/herself. A sufficiently high dose of an orally administered barbiturate (pentobarbital or secobarbital) results in depression of the respiratory system, causing respiratory acidosis. This coupled with vascular and/or cardiogenic shock results in death (KNMG/KNMP, 2012).

Studies based on human brain tissue have so far never investigated subjects who died by legal euthanasia and PAS. In our following studies, individuals who died of legal euthanasia will be subdivided into patients with mood disorders (MD) (to compare the differences between suicidal behaviors and suicidal ideations) and controls without a neuropsychiatric disorder (to correct for the effect of drug overdose and death ideation, that occurred in patients with MD who died of legal euthanasia and controls who died of incurable physical diseases such as cancer).

BRAIN REGIONS IMPLICATED IN SUICIDE

Prefrontal cortex

The prefrontal cortex (PFC) has been implicated as the executive center, in processes like planning, decision making, short-term memory, personality expression, and the moderation of social behavior and control of speech and language (Siddiqui et al., 2008). Both structural and functional abnormalities of the PFC have been linked to suicide and mental disorders. In patients with MDD, suicide attempters have shown alterations in functional connectivity and cortical protein markers in the dorsolateral prefrontal cortex (DLPFC), as compared to non-suicide attempters (Dean et al., 2019; Wang et al., 2019). Suicide attempters with MDD or BD also share specific structural alterations, suggesting there could be common neural pathways for suicide in mood disorders (Wei et al., 2020; Zhao et al., 2018).

Apart from suicide, neuroimaging alterations in the PFC are often related to psychosis. Information processing in the DLPFC, which has been related to the etiology of psychotic features (McIntosh et al., 2008), was preferentially reduced in psychotic BD patients. This suggests that psychosis might be accompanied by a disruption of the prefrontal control network which could possibly result in a relative stronger activation of the default network (Baker et al., 2014). In addition, abnormal brain activation in the ACC, as measured by functional magnetic resonance imaging (fMRI), has been related to impairments in emotional processing, attention and neurotransmitter levels in pediatric and euthymic individuals with BD (Lee et al., 2018; Li et al., 2018; Soeiro-de-Souza et al., 2018).

Consistent with this, patients in their first manic episode with psychosis also had a significant volume reduction of the entire ACC (Keramatian et al., 2016), whereas the right dorsal ACC volume was found to be increased in recent-onset psychosis (de Azevedo-Marques Perico et al., 2011). Similarly, in SCZ, a frontal cortex-targeted MRI study showed that an ACC-

based disturbance in cognitive control was related to long-term suicidal ideations and behaviors, whereas no such relation was present in the DLPFC (Minzenberg et al., 2014). In addition, transcranial magnetic stimulation that targeted the PFC can improve clinical symptoms and have anti-suicidal effects in patients with SCZ (George et al., 2014; Linsambarth et al., 2019; Mehta et al., 2019). The abovementioned observations indicate that the PFC is associated with the pathogenesis of suicidal behavior in conditions of mental illness. This implies that therapies targeting the PFC may be potentially effective for suicide prevention.

Hypothalamus

The hypothalamus is part of the limbic system and links the nervous system to the endocrine system. One of its major functions is its involvement in the stress response. This thesis will focus on the paraventricular nucleus (PVN) and supraoptic nucleus (SON), which are two primary drivers of the hypothalamo-pituitary-adrenocortical axis, and the infundibular nucleus (INF), a nucleus exposed outside the blood-brain barrier, that regulates hormone release and provide inputs to the other hypothalamic nuclei. So far, only a few studies that focused on the human hypothalamus have addressed the neurobiological alterations in relation to suicide (Merali et al., 2006). However, the functions dominated by the hypothalamus strongly indicate that this structure may play a critical role in the pathophysiology of suicide.

Hippocampus

The human hippocampus contains a high density of glucocorticoid receptors, which make it more vulnerable to long-term stress than other brain areas (Wang et al., 2013). Many structural and functional imaging studies have shown that the hippocampus is dynamically and volumetrically altered in patients with mental disorders who have suicidal ideations or attempts (Zhang et al., 2022). Of note, MDD, as a major comorbidity of suicide, is associated with structural changes to the hippocampus. Previous meta-analyses have shown a reduction in the volume of the hippocampus in subjects with MDD, which was absent in bipolar disorder (BD) (Campbell et al., 2004). Depressed patients with suicidal ideation did not show volumetric alterations, but did display decreased functional dynamics (determined by the amplitude of low-frequency fluctuations) in the hippocampus (Jiao et al., 2019; Lan et al., 2019). Different from suicide ideators, suicide attempters, independent of their prior psychiatric disorder, exhibited a reduced regional homogeneity in their left hippocampus, indicating hippocampal dysfunction in the predisposition to suicidal behaviors (Cao et al., 2015). For example, suicide attempters in MDD displayed a higher hippocampal functional connectivity (Wagner et al., 2021; Weng et al., 2019). MDD patients with repeated or acute suicide attempts showed much smaller hippocampal volumes than patients with a first suicide attempt, non-suicidal patients or healthy controls (Colle et al., 2015; Kang et al., 2020; Sarkinaite et al., 2021). In addition, suicidal attempters with BD had significantly reduced gray matter volume in the hippocampus (Johnston et al.,

2017; Niu et al., 2019). However, this suicide attempt-related cortical atrophy did not appear in schizophrenia (Spoletini et al., 2011), suggesting that reduced hippocampal volume promises to be a possible neuroimaging marker to predict suicidality in MDD.

Furthermore, as whole-brain functional neuroimaging studies have shown that the connectivity of the hippocampus with the hypothalamus and PFC specifically predicts the subjective sensation of stress in humans (Goldfarb et al., 2020), we decided to include these three brain regions in our research.

SCOPE OF THE PRESENT THESIS

Previously, our group has studied changes in glutamate- and GABA-related gene expression in the PFC in relation to stress, specific neurotransmitters, suicide and MDD (Zhao et al., 2015; Zhao et al., 2018; Zhao et al., 2016). However, very few studies have examined glial genes in patients with mental disorders and controls, accounting for confounding factors like suicide, gender and psychosis. Therefore, in **Chapter 2**, we studied whether suicide and psychotic features, either as parts of the symptoms of MDD, or as separate entities, are characterized by differences in glial gene expression in the human PFC. In **Chapter 3**, we focus on the question whether suicide and psychotic features are characterized by glia alterations in the human PFC in BD, and whether glia genes are differently expressed between genders in relation to suicide and psychotic features. As an extension of Chapter 3, we review in **Chapter 4** the evidence on sex differences of BD in terms of epidemiology, cognition, clinical manifestation, neuroimaging, neurobiological mechanisms, and highlight the role of the DLPFC as a key brain region in providing molecular landscape. In **Chapter 5**, we study whether suicide, either as part of the symptoms of schizophrenia, or as a specific entity, is characterized by differences in glia expression in the human PFC.

Chapter 6 focuses on the hypothalamus in relation to a possible hormonal risk factor for suicide. In this chapter, we hypothesize, on the basis of epidemiological data, that progesterone in the hypothalamus may play a role in suicide behaviors. In order to validate our hypothesis, two experiments were performed. Firstly, we investigated the distribution of the progesterone receptor (PR) in the human hypothalamus and determined whether some common parameters (age and gender) affect its expression in specific hypothalamic nuclei. This first human hypothalamic map of PR distribution allows to localize the most probable site for progesterone action at the central level. Secondly, we studied alterations in PR/proopiomelanocortin (POMC)- and PR/neuro-peptide Y (NPY)-co-expressing neuronal densities, and their ratios in the INF and compared MD, suicide and control subjects. Two groups of individuals with euthanasia, i.e., MDs with euthanasia and controls (i.e., donors without psychiatric disorders) who also died by legal euthanasia, were included in order to distinguish the changes going together with the possible differences between a completed suicide and suicide ideation, and the pharmacological

and possible other effects of the euthanasia. Subsequently, corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) positive neurons were quantified, in order to explore differential projections of the POMC and NPY innervation of the PVN in brains of suicide and MD cases. A better understanding of the molecular mechanisms underlying the involvement of progesterone in suicide pathophysiology may have future consequences for the prescription of oral anticonception types.

Brain imaging and postmortem studies in suicide have demonstrated a reduction in the volume of the hippocampus as well as alterations in its cytobiology. Studies in the human hippocampus of suicide victims have shown alterations in neuronal-glial morphology and composition. A better understanding of the cellular and molecular mechanisms that underlie suicidal behavior and pharmacological responses may lead to the identification of novel targets that might make diagnoses and therapies more personalized. **Chapter 7** therefore contains a review that discusses several leading theories of the neurobiological factors in the hippocampus that may contribute to suicide, including the serotonin system, Wnt signaling pathway, brain-derived neurotrophic factor/tropomyosin receptor kinase B (BDNF/TrkB) signaling and the androgen receptors. We review in detail evidence for the role of these factors in suicide and in treatment response. In addition, cognitive dysfunction is discussed as a risk factor for suicide since novel evidence hints at a role for cognitive therapies in decreasing suicidal ideations, in addition to antidepressant therapy.

Chapter 8 is based on the finding that medications that regulate purine metabolism have antidepressant and anti-suicidal effects, indicating a pathophysiological involvement of purinergic disturbance in depression and suicide. Since the different psycho-pharmacological effects involve separate pathways, differences in purinergic receptor expression or activation may relate to suicide and MD, respectively. In addition, a hypothalamic involvement in suicide, MD and legal euthanasia has not been reported before, even though this subcortical region is driving key roles in emotion and stress regulation. Thus, in this chapter, we investigate in detail the expressions of stress-associated purinergic receptors in key stress regions of the human brain: the hippocampus, hypothalamus and PFC.

On the basis of our research findings shown in the above chapters, we discuss in **Chapter 9**, the feasibility and significance of using bio-samples from donors who died by legal euthanasia as potent novel research materials in suicide research. We also propose detailed strategies in both antemortem and postmortem studies in relation to this research cohort.

In **Chapter 10** and **11**, we provide an overview of the main findings, and hypothesize how the different brain areas and systems studied here and, in the literature, may lead to a better understanding of the mechanisms underlying depression and different aspects of suicidality. In all, our research aims to unravel the novel pharmacological targets that are widely applicable to suicidal populations, independent of their psychiatric diagnoses, which sheds light on the direct control of global suicides.

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CHAPTER 2

CHANGES IN GLIAL GENE EXPRESSION IN THE PREFRONTAL CORTEX IN RELATION TO MAJOR DEPRESSIVE DISORDER, SUICIDE AND PSYCHOTIC FEATURES

Zhang L, Verwer RWH, Zhao J, Huitinga I, Lucassen PJ, Swaab DF.

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ABSTRACT

Background: To establish whether major depressive disorder (MDD), suicidal behaviors and psychotic features contribute to glial alterations in the human prefrontal cortex.

Materials and Methods: We compared mRNA expression using real-time qPCR of 17 glia related genes in the dorsolateral prefrontal cortex (DLPFC) and the anterior cingulate cortex (ACC) between 24 patients with MDD and 12 well-matched controls without psychiatric or neurological diseases. The MDD group was subdivided into i) MDD who died of suicide (MDD-S) or natural causes (MDD-NS) and ii) MDD with or without psychotic features (MDD-P and MDD-NP). The results were followed up with confounder factor analysis.

Results: Astrocyte gene aldehyde dehydrogenase-1 L1 (ALDH1L1) showed an increased expression in the DLPFC of MDD-NS and the ACC of MDD-NP. S100 calcium-binding protein B (S100B) was upregulated in the DLPFC of MDD compared to the controls. Microglial markers CD11B and purinergic receptor 12 (P2RY12) both showed decreased expression in the ACC of MDD-NS. CD68 was increased in the DLPFC of MDD in both, MDD-S and MDD-P, compared to the controls. In addition, there was increased translocator protein (TSPO) expression in the DLPFC of MDD, especially MDD-NS. In the ACC, this gene had a lower expression in MDD-P than in MDD-NP. Myelin basic protein (MBP) mRNA in the DLPFC increased in MDD, in relation to psychotic features, but not to suicide.

Limitations: Sample volumes are relatively small.

Conclusions: Different glial functions in MDD were related to specific brain area, suicide or psychotic features.

Keywords

Major depressive disorder; suicide; psychotic features; glia; prefrontal cortex

INTRODUCTION

One-third of individuals with major depressive disorder (MDD) attempt suicide during their lifetime (Dong et al., 2019). Suicidal behavior is often associated with psychotic symptoms (Ma et al., 2019), while the presence of psychotic features in MDD predicts a double mortality rate, and a 2–5 times higher completion of suicide attempts during the acute episodes (Nelson et al., 2018; Suominen et al., 2009; Zalpuri and Rothschild, 2016). This elevated suicide mortality rate does not respond to antidepressants (Suominen et al., 2009), while tricyclic antidepressants have been reported to have a low impact but potentially exacerbate psychotic symptoms in patients with MDD (Glassman et al., 1975; Kantrowitz and Tampi, 2008). In addition, while family history of depression remains unclear among patients with MDD and psychotic features, family members of patients with psychotic features have a higher vulnerability to develop psychotic features (Nelson et al., 2018), which points to independence of psychotic features and MDD. Thus, the evidence above indicates that suicide and psychotic features can be considered as two heterogeneous conditions (Cardinal and Bullmore, 2011; Holma et al., 2010) that may be to a certain degree independent factors in MDD severity.

Based on previous work on neuronal abnormalities in both dorsolateral prefrontal cortex (DLPFC) and anterior cingulate cortex (ACC), changes in neuron-glia interactions have been implicated as the neurobiological underpinning of MDD (Zhao et al., 2015; Zhao et al., 2018; Zhao et al., 2016). In our studies, glial-driven functional brain changes have been explored in MDD pathophysiology, and several suicide-/psychotic features-related specific glial alterations have been described in bipolar disorder and schizophrenia (SCZ) (Zhang et al., 2020a, b). Within this framework, the pathological studies on the two regions that reported glial abnormalities required some follow up. Firstly, they mainly focused on the white, and not the grey matter, and are thus, intuitively, difficult to align to the neuronal changes in the cortex (Gittins and Harrison, 2011; Mosebach et al., 2013; Rajkowska et al., 2015; Schnieder et al., 2014; Schnieder et al., 2018; Torres-Platas et al., 2014; Webster et al., 2005). Secondly, age-dependent glial changes, that may indicate a compensatory response to, or underlying cause of, the neuronal dysfunction, have been found that complicate the pattern of glial pathology in MDD (Miguel-Hidalgo et al., 2000; Rajkowska and A Stockmeier, 2013; Vostrikov and Uranova, 2011). Our earlier studies pointed out that MDD and suicide may differ in the alterations of key stress-related genes transcripts (Wang et al., 2019). However, in studies on MDD so far, patients who died of suicide or non-suicidal causes were often lumped together (Ernst et al., 2011; Mosebach et al., 2013; Rajkowska et al., 2013). Additionally, suicide victims were compared to control subjects losing sight of the fact that the far majority of suicide cases have an underlying psychiatric illness (Klempner et al., 2009a; Lutz et al., 2017; Nagy et al., 2015; Schnieder et al., 2018; Torres-Platas et al., 2014; Wang et al., 2019). Moreover, none of the glia-related studies has so far analyzed psychotic features as

a subgroup of patients with MDD. This may at least partly be an explanation for inconsistencies in the literature.

Therefore, we investigated here the expression levels of 17 glia-related genes in the prefrontal cortex (PFC) in MDD, taking into account suicide and psychotic features as separate subsets.

MATERIALS AND METHODS

Brain samples

RNA samples were obtained from the Stanley Medical Research Institute (SMRI, Bethesda, MD, USA). Permission for the use of brain material was provided by the next of kin. Diagnoses were based on the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV (American Psychiatric Association, 1994). All brain regions were microscopically examined to exclude patients with pathological signs of neuro-degeneration or other lesions. Exclusion criteria included subjects over age 70, subjects with a history of seizures or other neurological disorders that might affect brain pathology. The cause of death for 17 of 24 patients with MDD was suicide. Eleven patients with MDD had a history of psychotic features. The other cases and all control subjects died of natural causes or accidents.

The SMRI provided us with RNA from the isolated grey matter of the DLPFC (Brodmann area 46) and ACC (Brodmann area 24). They were obtained from patients with MDD (MDD, N = 24) and matched controls (Ctr, N = 12) without a history of suicidal behavior or any major psychiatric diagnosis. We distinguished the following subsets within the MDD group: (1) patients with MDD who died of suicide (MDD-S) or natural causes (MDD-NS) and (2) patients with or without psychotic features (MDD-P or MDD-NP). All groups were matched for age, brain pH, post-mortem interval, brain weight, history and severity of alcohol/substance abuse (**Table 1** and **Table S1**). All demographic information and medical data, including any lifetime use of antidepressants or a history of substance abuse, were provided by the SMRI. All analyses were performed by investigators unaware of the diagnosis.

Quantitative real-time PCR

RNA isolation and cDNA synthesis were performed as described before (Zhao et al., 2018). RNA integrity value (RIN), an indicator of human post-mortem tissue RNA quality (Stan et al., 2006), did not show any significant difference between the diagnostic groups in the SMRI material (RIN value of the ACC/DLPFC from MDD-S: 7.69 ± 0.66 ; MDD-NS: 7.49 ± 0.56 ; MDD-P: 7.56 ± 0.26 ; MDD-NP: 7.69 ± 0.10 and the control group: 7.73 ± 0.95 , mean $\pm$ SEM). For cDNA synthesis, an equal quantity of RNA (300 ng) for each sample was used for the synthesis of cDNA, mixed with a 4.1 ml mixture of oligo dT (100 mg ml⁻¹) and 10 x hexanucleotide (Roche, Basel, Switzerland)

(40:1 for the oligo-dT and hexanucleotide mixture), heated to 80 °C for 10 min, after which the tubes were quickly transferred to ice. Then 1 ml reverse transcriptase Superscript II RT (Invitrogen Life Technologies) was added together with a mixture of 5 ml 5 first-strand buffer, 2.5 ml 100 mM dithiothreitol, 1.5 ml 10 mM dNTPs and 0.5 ml RNase inhibitor. The synthetic reaction was allowed to proceed for 1 h at 42 °C, after which cDNA was stored at -20 °C or used immediately. Additional information on all tested genes and the sequences for each primer pair is shown in **Table S2**. cDNA template (equivalent to 5 ng of total RNA) was amplified in a final volume of 20 µl using an SYBR Green PCR master mix (Applied Biosystems, CA, USA) and a mixture of forward and reverse primers (each 2 pmol/µl).

Table 1. Demographic information on the control and MDD subjects.

	Ctrl	MDD	F or χ^2	P
Age (years, range)	47 (24–63)	42 (24–63)	1.23	0.64
Gender (M/F)	8/4	13/11	0.51	0.47
Race	11W, 1H	23W, 1H	0.26	0.61
PMD (hours, range)	25.3 (9–40)	29.7 (13–65)	1.35	0.30
Brain pH	6.6 (6.31–6.91)	6.6 (6.3–6.9)	1.47	0.81
Brain Weight (gram, range)	1445 (1200–1595)	1469 (1170–1780)	1.69	0.63
Side of Brain Frozen (L/R)	6/6	15/9	0.51	0.47
Psychotic Features	–	11		
Alcohol hx	6	16	0.94	0.33
Severity of Alcohol abuse ¹	2.08 (0–5)	1.92 (0–5)	1.07	0.82
Drug hx	4	8	0.00	1.00
Severity of Substance abuse ¹	0.75 (0–4)	1.04 (0–4)	1.35	0.59
Fluphenazine (lifetime, range)	–	1121 (0–6500)		

Abbreviation: Ctrl, control; F, female; hx, history; L, left; M, male; MDD, major depressive disorder; PMD, postmortem delay; R, right.

¹ Substance abuse and alcohol abuse was rated on a scale of 0–5.

Data were acquired and processed automatically by the Applied Biosystems 7300 Real-Time PCR System. The specificity of amplification was checked by melting curve analysis and electrophoresis of the products on an 8% polyacrylamide gel. Sterile water and RNA samples without the addition of reverse transcriptase during cDNA synthesis served as negative controls. The linearity of each qPCR assay was tested by preparing a series of dilutions of the same stock cDNA in multiple plates. To reduce the effect of sample variability, reference genes were selected based on expression stability measurement (Vandesompele et al., 2002) and the proportion

of explained variability regarding all target genes. The higher stability of a reference gene means that its variance was relatively lower than the other considered reference genes. Further, if the expression of a reference gene reflects the variability of the samples well, it is expected that its application should reduce the overall variability of the target genes. Briefly, we used the following reference genes: actin beta (ACT β), glyceraldehyde-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), tubulin alpha (TUB α), tubulin beta (TUB β) and ubiquitin C (UBC). For the comparisons in the DLPFC, the specific selection was ACT β , TUB α , TUB β and UBC; in the ACC, the corresponding selection was ACT β , TUB β and UBC.

Statistical analysis

Statistical analysis was conducted with IBM SPSS (version 20, SPSS) and TIBCO S+ software (version 8.2.0, TIBCO, Seattle, WA, USA). The gene expression values were ¹⁰log-transformed before they were further processed for statistical analysis. The transformation of the essentially exponential expression data facilitates the application of reference gene correction and enables the application of conventional statistical methods. Thus, the residuals of the combination of selected reference genes can be simply subtracted from the original target gene values to obtain corrected gene expression values.

Given the higher variability in human postmortem data, that may not sufficiently conform to commonly assumed theoretical distributions of test statistics, we applied a non-parametric resampling procedure (Zhao et al., 2018). Resampling was performed without replacement in two-group comparisons, thereby providing an alternative for the t-test. We generated 9999 replicates consisting of randomly reallocated patients over the two groups in each comparison to obtaining a permutation NULL distribution of the test statistic. The obtained NULL distribution mimics the hypothesis that the patients from either group belong to one and the same group (Davison and Hinkley, 1997). As test statistic, we used $T = (\text{mean}(\text{group2}) - \text{mean}(\text{group1})) / \text{se}$. In this formula, se is the standard error of the difference in means. If our data are normal with unknown, unequal variances in group2 and group1, while group1 and group2 observations belong to the same group, T should approximate the t-distribution with Welch modified degrees-of-freedom. In that case, the observed T-value would be sufficient to decide whether the two groups should be considered as one or not. If it is uncertain how appropriate the t-distribution is, the 9999 permutation T-values together with the real observed T-value constitute a sample of 10,000 “resampled observations”, that can be used to estimate how unusual the observed T-value is (Davison and Hinkley, 1997). Since the observed T-value can be on either side of the mean, testing was done two-sided (Zhao et al., 2018). P-values were corrected for multiple testing using the Benjamini-Hochberg criterion (Benjamini and Hochberg, 1995). After statistical analysis, group mean expression levels were back-transformed and expressed as fold-changes to

compare their mutual differences (relative mRNA values in the figures). Figures were made with Graphpad Prism 8.1.2.

RESULTS

Gene transcripts encoding astrocytic markers

As an indication for changes in astrocyte-related functions in the PFC of patients with MDD, we analyzed qPCR values of three gene transcripts: aldehyde dehydrogenase 1 family, member L1 (ALDH1L1), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B).

In the DLPFC, S100B was the only gene that showed a significant increase (fold change = 1.25, $P = 0.037$) in patients with MDD. In suicide- or psychotic features-related subset comparisons, however, no significance was found. A trend for an increase was found for ALDH1L1 (fold change = 1.32, $P = 0.061$) in patients with MDD. The elevated transcript level of this gene was significant in the MDD-NS group (fold change = 1.60, $P = 0.023$). Psychotic features did not significantly confound the detected change in astrocytic transcripts (see **Table 2B**).

For the ACC, no changes were found between patients with MDD and controls, neither following subgrouping for suicide. However, in patients with MDD who did not have psychotic features, ALDH1L1 expression was higher than those with psychotic features (fold change = 1.38, $P = 0.034$) and the control subjects (fold change = 1.43, $P = 0.034$).

Gene transcripts encoding microglial markers

To assess whether MDD, suicide and psychotic features may be accompanied by gene expression changes of proteins characteristic for microglia, we tested the expression levels of the following 10 microglial markers. They are CD11B (CR3), CD45, CD68, chemokine (C-X3-C motif) receptor 1 (CX3CR1), human leukocyte antigen-DR alpha chain (HLA-DRA), ionized calcium-binding adapter molecule 1 (IBA1), purinergic receptor 12 (P2RY12), transmembrane protein 119 (TMEM119), triggering receptor expressed on myeloid cells 2 (TREM2) and translocator protein (TSPO).

Among these genes, both CD68 and TSPO revealed more than 20% increases in MDD in the DLPFC (CD68: fold change = 1.22, $P = 0.037$; TSPO: fold change = 1.25, $P = 0.020$), and for CD68 in the suicide completers (MDD-S vs. Ctr: fold change = 1.23, $P = 0.033$). TSPO expression in MDD-NS (MDD-NS vs. Ctr: fold change = 1.40, $P = 0.014$) significantly increased compared to the controls. For CD68, another elevation is present in patients with MDD and psychotic symptoms relative to the controls (fold change = 1.32, $P = 0.015$).

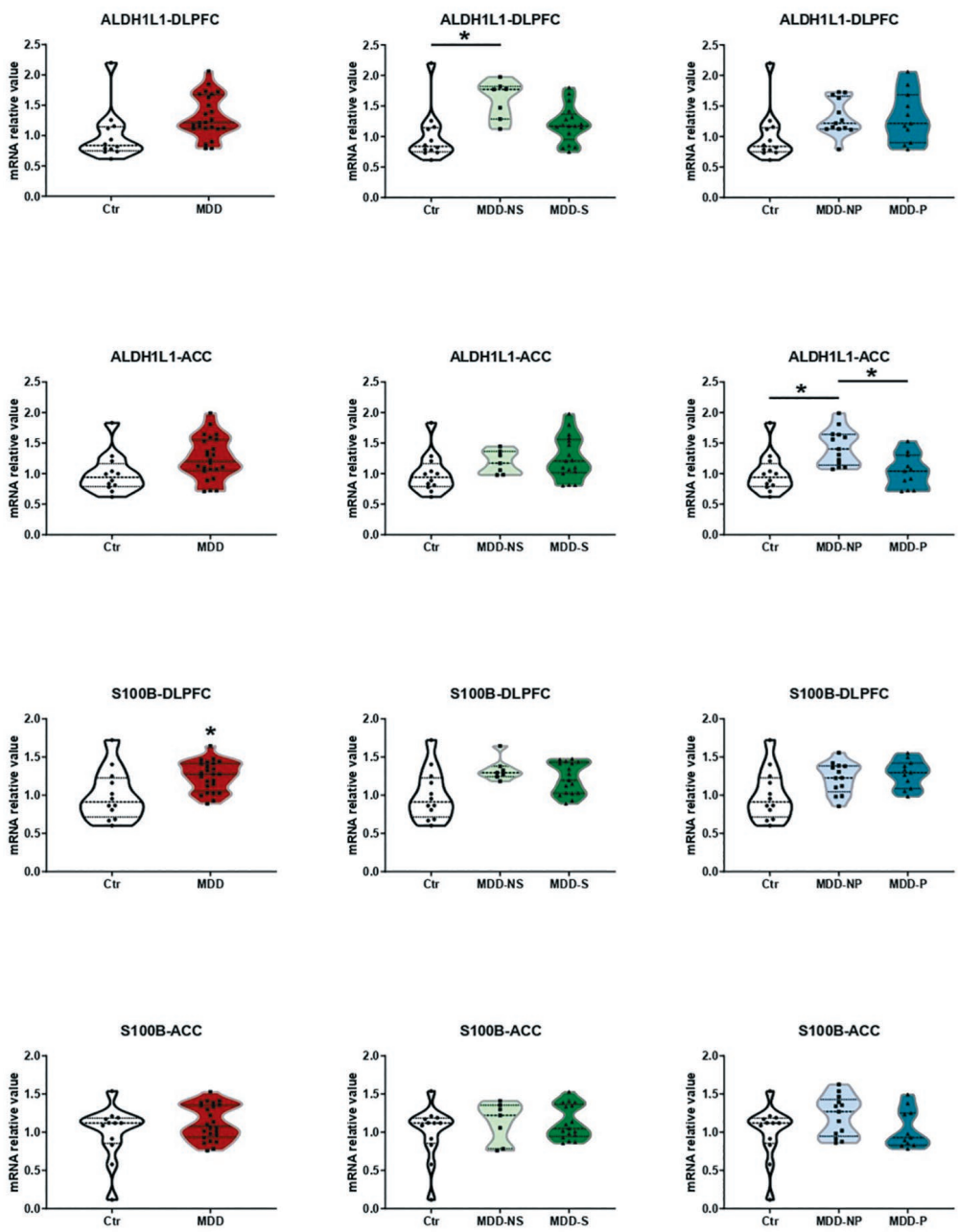


Figure 1. Transcript levels of astrocyte related genes (ALDH1L1 and S100B) in the DLPFC and ACC in controls (Ctr) and patients with major depressive disorder (MDD) that died of suicide (MDD-S) or other causes than suicide (MDD-NS), and patients with (MDD-P) or without psychotic features (MDD-NP).

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; * indicates $0.01 \leq P < 0.05$.

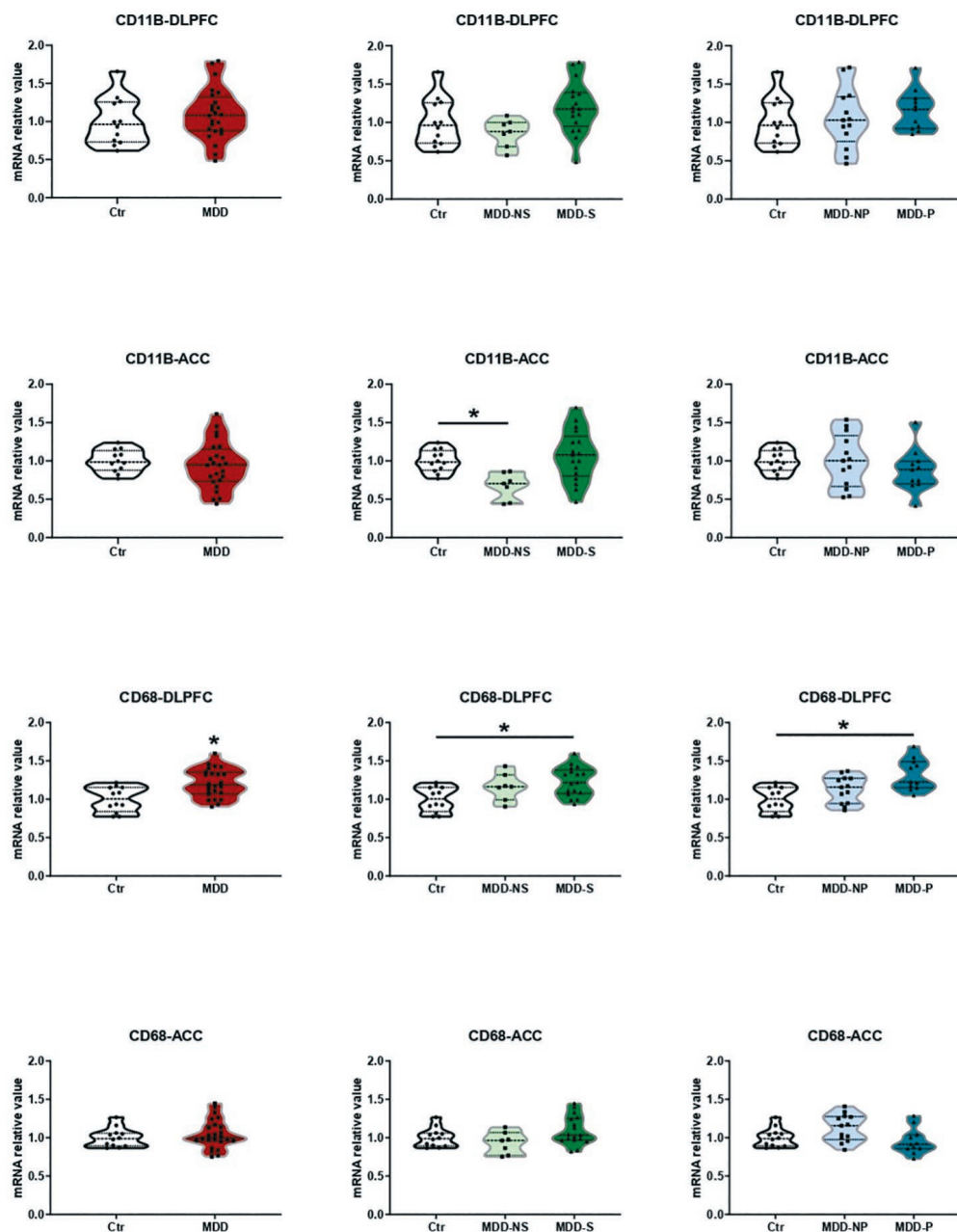


Figure 2. Transcript levels of microglia related genes (**Figure 2A** CD11B and CD68; **Figure 2B** P2RY12 and TSPO) in the DLPFC and ACC in controls (Ctr) and patients with major depressive disorder (MDD) that died of suicide (MDD-S) or other causes than suicide (MDD-NS), and patients with (MDD-P) or without psychotic features (MDD-NP).

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; * indicates $0.01 \leq P < 0.05$.

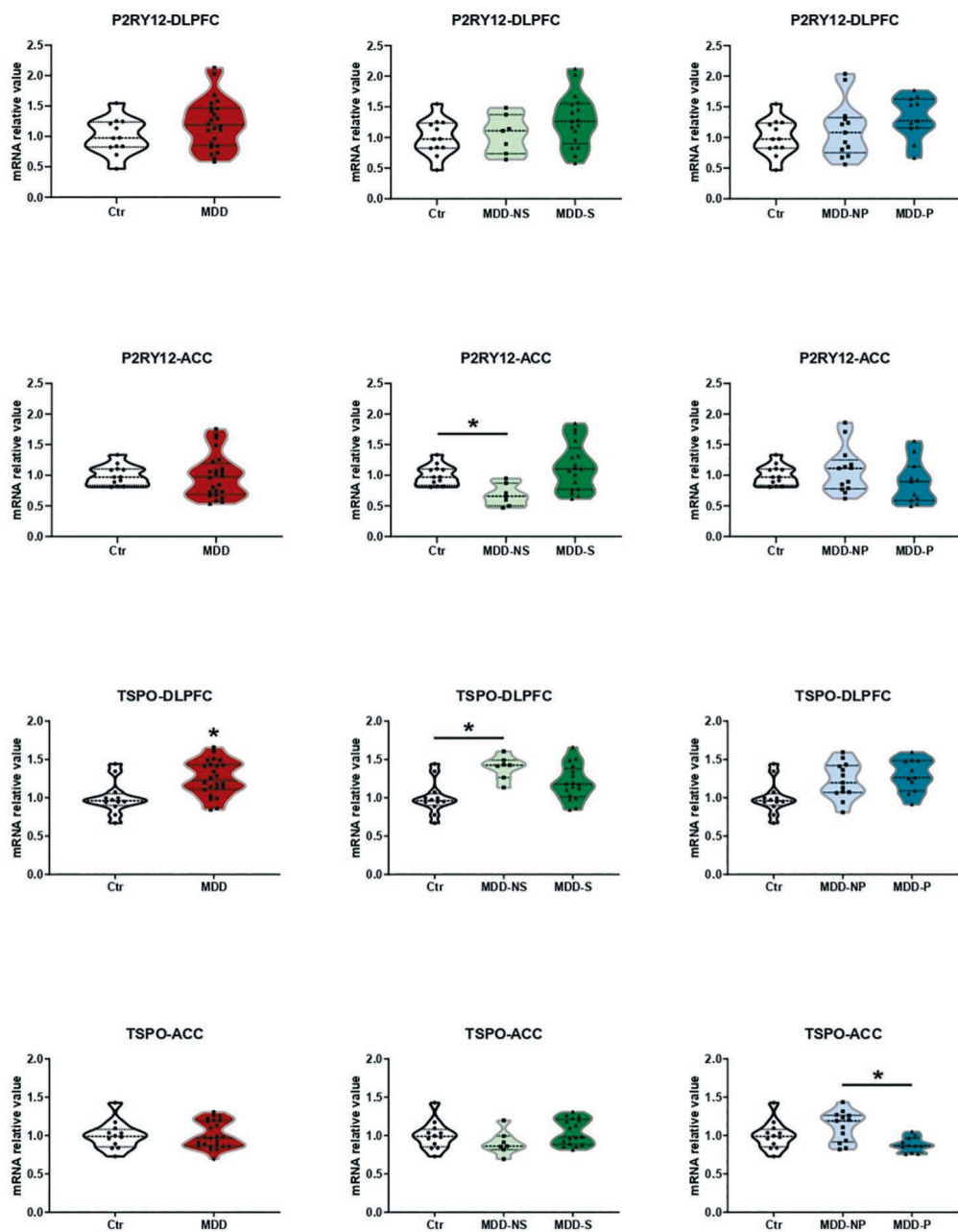


Figure 2. Continued

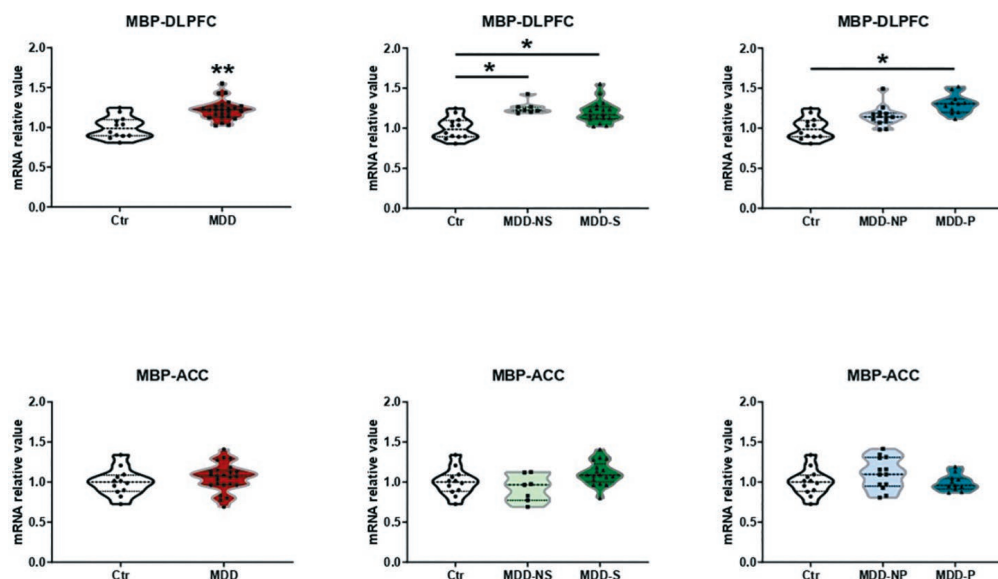


Figure 3. Transcript levels of oligodendrocyte related genes (MBP) in the DLPFC and ACC in controls (Ctr) and patients with major depressive disorder (MDD) that died of suicide (MDD-S) or other causes than suicide (MDD-NS), and patients with (MDD-P) or without psychotic features (MDD-NP).

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; * indicates $0.01 \leq P < 0.05$, ** indicates $0.001 \leq P < 0.01$.

In the ACC, no alterations were observed in the two-group comparisons. However, we found some genes differently expressed between MDD subgroups. For example, both CD11B and P2RY12 were approximately reduced by half in non-suicidal patients with MDD, but not in suicide completers compared to the controls (CD11B: MDD-NS vs. Ctr: fold change = -1.49, $P = 0.031$; MDD-S vs. MDD-NS: fold change = 1.59, $P = 0.065$. P2RY12: MDD-NS vs. Ctr: fold change = -1.47, $P = 0.046$; MDD-S vs. MDD-NS: fold change = 1.68; $P = 0.065$). Additionally, TSPO showed opposite expressions between patients with and without psychotic features (MDD-P vs. MDD-NP: fold change = -1.27, $P = 0.034$). These variable alterations per subgroup account for the unchanged transcript levels between MDD and controls.

Gene transcripts encoding oligodendrocytic markers

In addition, we investigated the following 4 oligodendrocyte genes: myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), oligodendrocyte transcription factor 2 (OLIG2) and myelin proteolipid protein (PLP).

In the DLPFC, MDDs showed increased MBP expression compared to the controls (fold change = 1.22, $P = 0.003$), but the subgroup of suicide completers did not specially contribute to this elevation (MDD-S vs. Ctr: fold change = 1.20, $P = 0.023$; MDD-NS vs. Ctr: fold change = 1.26, $P = 0.023$; MDD-S vs. MDD-NS: $P = 0.289$). Interestingly, the expression of this gene in patients with psychotic features increased 30% compared to the controls (fold change = 1.30, $P = 0.010$).

No changes were detected for oligodendrocyte markers from two- or three-group comparisons regarding MDD, suicide or psychotic features in the ACC.

An overview of significant changes is presented in **Figures 1-3**. For more details, we refer to **Tables 1** and **2A-C**.

Sex differences

We did not find significant differences between the sexes, neither in the MDD nor in the matched control group.

Confounder analysis

The P -values for matching for possibly confounding factors are given in **Table 1**, **Table S1B** and **S1C**. There were no significant differences. Correlations between glia expression and non-matched factors, i.e., age of onset, disease duration and lifetime antipsychotic use, have been done and these factors did not alter our conclusions.

Table 2A. Alterations in glia gene expression in MDD as compared to their matched control group.

	DLPFC			ACC		
	Fold change	P-perm	BHadj-p	Fold change	P-perm	BHadj-p
Astrocyte genes						
ALDH1L1	1.32	0.018	0.061	1.25	0.030	0.503
GFAP	1.16	0.072		1.05	0.696	
S100B	1.25	0.009	0.037	1.13	0.197	
Microglia genes						
CD11B	1.10	0.416		-1.06	0.237	
CD45	1.22	0.403		1.20	0.391	
CD68	1.22	0.003	0.020	1.04	0.453	
CX3CR1	1.07	0.777		-1.03	0.564	
HLA-DRA	1.33	0.135		-1.01	0.804	
IBA1	1.13	0.305		1.01	0.718	
P2RY12	1.22	0.129		1.00	0.506	
TMEM119	1.44	0.025	0.070	1.07	0.509	
TREM2	1.13	0.413		1.07	0.618	
TSPO	1.25	0.004	0.020	1.01	0.929	
Oligodendrocyte genes						
MBP	1.22	0.000	0.003	1.05	0.368	
MOG	1.18	0.141		1.07	0.524	
OLIG2	1.06	0.426		1.00	0.941	
PLP	1.22	0.065		-1.06	0.640	

Note: ACC: anterior cingulate cortex; BHadj-p: P value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsolateral prefrontal cortex; MDD: major depressive disorder; P-perm: P value of permutation test.

Table 2B. Alterations in glia gene expression in MDD in relation to suicide.

	Fold change						P-perm		BHadj.p	
	MDD-S/ MDD-NS		MDD-S/ Ctr		MDD-NS/ MDD-NS		MDD-S/ Ctr		MDD-S/ Ctr	
	MDD-S/ MDD-NS	Ctr	MDD-S/ Ctr	Ctr	MDD-NS/ MDD-NS	Ctr	MDD-S/ Ctr	Ctr	MDD-S/ Ctr	MDD-NS/ Ctr
DLPFC-astrocyte genes										
ALDH1L1	-1.34	1.20	1.60		0.009		0.077	0.002	0.061	0.224 0.023
GFAP	-1.09	1.13	1.24		0.407		0.127	0.073		
S100B	-1.10	1.21	1.34		0.120		0.030	0.008	0.267	0.139 0.061
DLPFC-microglia genes										
CD11B	1.38	1.19	-1.16		0.015		0.136	0.352	0.087	0.267 0.485
CD45	1.15	1.26	1.10		0.587		0.335	0.852		
CD68	1.06	1.23	1.16		0.420		0.003	0.079	0.522	0.033 0.420
CX3CR1	1.27	1.14	-1.12		0.134		0.394	0.388		
HLA-DRA	1.29	1.42	1.10		0.345		0.112	0.542		
IBA1	1.33	1.21	-1.10		0.043		0.113	0.652	0.167	0.267 0.722
P2RY12	1.21	1.28	1.05		0.233		0.076	0.742		
TMEM119	1.38	1.57	1.14		0.106		0.010	0.514	0.267	0.061 0.610
TREM2	1.18	1.17	-1.00		0.325		0.264	0.882		
TSPO	-1.16	1.20	1.40		0.033		0.024	0.001	0.142	0.121 0.014
DLPFC-oligodendrocyte genes										
MBP	-1.04	1.20	1.26		0.185		0.001	0.001	0.349	0.014 0.014
MOG	1.06	1.20	1.13		0.702		0.123	0.310		
OLIG2	1.11	1.10	-1.01		0.338		0.285	0.976		
PLP	1.10	1.26	1.14		0.513		0.051	0.231		

	Fold change				P-perm				BHadj,p			
	MDD-S/ MDD-NS		MDD-S/ Ctr		MDD-S/ MDD-NS		MDD-S/ Ctr		MDD-S/ MDD-NS		MDD-S/ Ctr	
	MDD-S/ MDD-NS		MDD-S/ Ctr		MDD-S/ MDD-NS		MDD-S/ Ctr		MDD-S/ MDD-NS		MDD-S/ Ctr	
ACC-astrocyte genes												
ALDH1L1	1.06		1.26		1.19		0.687		0.876		0.218	
GFAP	1.08		1.06		-1.02		0.516		0.907			
S100B	1.00		1.13		1.13		0.923		0.491			
ACC-microglia genes												
CD11B	1.59		1.07		-1.49		0.005		0.801		0.930	
CD45	1.40		1.30		-1.07		0.161		0.195		0.760	
CD68	1.17		1.09		-1.07		0.058		0.138		0.307	
CX3CRI	1.33		1.05		-1.27		0.093		0.876		0.081	
HLA-DRA	1.55		1.12		-1.38		0.049		0.521		0.147	
IBA1	1.50		1.12		-1.34		0.006		0.188		0.012	
P2RY12	1.68		1.14		-1.47		0.004		0.399		0.065	
TMEM119	1.17		1.12		-1.05		0.402		0.392		0.9798	
TREM2	1.47		1.19		-1.24		0.008		0.115		0.0428	
TSPO	1.16		1.05		-1.10		0.080		0.445		0.2662	
ACC-oligodendrocyte genes												
MBP	1.20		1.11		-1.08		0.017		0.074		0.3378	
MOG	-1.04		1.05		1.10		0.908		0.472		0.7442	
OLIG2	1.05		1.04		-1.04		0.685		0.839		0.8374	
PLP	1.05		-1.05		-1.10		0.555		0.805		0.5188	

Note: ACC: anterior cingulate cortex; BHadj-p: P value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsolateral prefrontal cortex; MDD: major depressive disorder; MDD-NS: MDD patients died of non-suicidal reasons; MDD-S: MDD patients died of suicide; P-perm: P value of permutation test.

Table 2C. Alterations in glia gene expression in MDD in relation to psychotic features.

	Fold change				p-perm		BHadj.p			
	MDD-P/ MDD-NP		MDD-P/ Ctr		MDD-P/ Ctr		MDD-P/ MDD-NP		MDD-P/ Ctr	
	MDD-P/ MDD-NP	MDD-P/ Ctr	MDD-NP/ Ctr	MDD-NP/ MDD-NP	MDD-P/ Ctr	MDD-P/ MDD-NP	MDD-P/ MDD-NP	MDD-P/ Ctr	MDD-P/ Ctr	MDD-NP/ Ctr
DLPFC-astrocyte genes										
ALDH1L1	1.00	1.32	1.31	0.855	0.053	0.014	0.099	0.172		0.891
GFAP	1.00	1.17	1.16	0.985	0.106	0.094				
S100B	1.04	1.28	1.22	0.477	0.013	0.033	0.626	0.099		0.152
DLPFC-microglia genes										
CD11B	1.10	1.16	1.05	0.305	0.140	0.856				
CD45	1.25	1.37	1.10	0.207	0.133	0.928				
CD68	1.17	1.32	1.13	0.021	0.001	0.077	0.105	0.015		0.217
CX3CR1	1.21	1.18	-1.02	0.157	0.193	0.588				
HLA-DRA	1.19	1.46	1.22	0.295	0.055	0.491				
IBA1	1.21	1.25	1.03	0.275	0.185	0.640				
P2RY12	1.17	1.32	1.13	0.193	0.036	0.535	0.339	0.152		0.665
TMEM119	-1.21	1.29	1.56	0.290	0.162	0.019	0.445	0.330		0.105
TREM2	1.30	1.29	-1.01	0.057	0.047	0.717	0.172	0.172		0.813
TSP0	1.05	1.29	1.22	0.452	0.004	0.017	0.623	0.075		0.105
DLPFC-oligodendrocyte genes										
MBP	1.13	1.30	1.15	0.008	0.000	0.007	0.078	0.010		0.078
MOG	-1.06	1.14	1.21	0.559	0.299	0.104				
OLIG2	-1.07	1.07	1.10	0.485	0.736	0.314				
PLP	-1.02	1.21	1.23	0.762	0.178	0.057				

	Fold change				p-perm		BHadj.p			
	MDD-P/ MDD-NP		MDD-P/ Ctr		MDD-P/ Ctr		MDD-P/ MDD-NP		MDD-P/ Ctr	
	MDD-P/ MDD-NP	Ctr	MDD-NP/ Ctr	Ctr	MDD-P/ MDD-NP	Ctr	MDD-NP/ Ctr	MDD-NP	MDD-P/ Ctr	MDD-NP/ Ctr
ACC-astrocyte genes										
ALDH1L1	-1.38	1.04	1.43	0.002	0.698	0.002	0.034	0.831	0.034	0.374
GFAP	-1.32	-1.12	1.18	0.011	0.200	0.108	0.138	0.425		
S100B	-1.17	1.04	1.21	0.104	0.663	0.110				
ACC-microglia genes										
CD11B	-1.14	-1.14	1.00	0.404	0.128	0.646				
CD45	-1.57	-1.09	1.43	0.015	0.611	0.034	0.149	0.820	0.245	
CD68	-1.18	-1.05	1.13	0.022	0.392	0.055	0.189	0.572	0.341	
CX3CR1	-1.15	-1.12	1.03	0.335	0.305	0.983				
HLA-DRA	1.02	1.00	-1.02	0.928	0.863	0.792				
IBA1	-1.19	-1.10	1.09	0.162	0.228	0.599				
P2RY12	-1.22	-1.12	1.08	0.151	0.186	0.701				
TMEM119	-1.25	-1.06	1.18	0.159	0.930	0.248				
TREM2	-1.19	-1.03	1.16	0.197	0.645	0.308				
TSPO	-1.27	-1.14	1.12	0.001	0.060	0.139	0.034	0.341	0.403	
ACC-oligodendrocyte genes										
MBP	-1.12	-1.01	1.11	0.105	0.991	0.166				
MOG	-1.15	-1.01	1.14	0.270	0.948	0.260				
OLIG2	-1.21	-1.11	1.09	0.082	0.383	0.400				
PLP	-1.20	-1.17	1.03	0.102	0.247	0.726				

Note: ACC: anterior cingulate cortex; BHadj-p: P value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsolateral prefrontal cortex; MDD: major depressive disorder; MDD-NP: MDD patients did not report psychotic features; MDD-P: MDD patients reported psychotic features; P-perm: P value of permutation test.

DISCUSSION

We have found alterations in gene expression in astrocytes, microglia and oligodendrocytes in the DLPFC and ACC in relation to MDD, suicide and psychotic features. As discussed below, altered neuronal functions behind these changed glial transcripts may include dopamine (DA) and gamma-aminobutyric acid (GABA) signaling, blood-brain barrier (BBB) permeability and central nervous system (CNS) injury, microglia phagocytosis, innate immune response and neuroinflammation, platelet coagulation and myelin stability.

In our previous study, we have found that patients with SCZ who died of non-suicidal causes presented elevated ALDH1L1 mRNA in the DLPFC (Zhang et al., 2020a). This present data show an increased expression of this gene in MDD, an effect that was not confounded by age (Barley et al., 2009). Although dopaminergic impairments may trigger psychotic symptoms (Pai et al., 2019), lower DA metabolites have been found in body fluids of suicide attempters (Roy et al., 1989; Roy et al., 1992), a change that was notably unrelated to the underlying psychiatric disorder (Pitchot et al., 2001). One possible explanation could be the stronger affinity of immunoglobulin G for DA found in the cerebral spinal fluid (CSF) of subjects with suicide attempts (Bergquist et al., 2002). However, although D2 dopaminergic dysfunction was reported to be related to DAD2 receptor (DAD2R) polymorphisms in suicide attempters (Suda et al., 2009), this possibility was not supported by other studies showing an unchanged binding in subcortical regions known to control DA synthesis, comparing suicide cases with controls (Allard and Norlen, 1997; Allard and Norlén, 2001; Fitzgerald et al., 2017; Pare et al., 1969). One could thus assume that the binding of DA to DAD2R in the DLPFC of patients with suicide is not as strong as in non-suicides. In addition, in the ACC, we found an exclusive increase of ALDH1L1 expression in nonpsychotic MDDs, suggesting that region-specific changes in DAD2R could be involved in MDD pathogenesis (Larisch et al., 1997). On basis of these observations, we presume that the ACC, but not the DLPFC, may serve as a specific DA binding site that presents different binding abilities when comparing MDD and psychotic features per se (Suhara et al., 2002; Takahashi et al., 2006).

The elevated S100B expression we observed in MDD brain samples may contribute to the increased S100B levels reported in peripheral blood (Gos et al., 2013; Hamidi et al., 2004; L Schroeter et al., 2013). However, CSF S100B was reported to remain unchanged in MDD, likely reflecting different alterations among different brain areas (Schmidt et al., 2015). An alternative possibility, i.e. that a compromised BBB permeability could affect the DLPFC of depressed patients, awaits further study (Loftis et al., 2018). Klempan et al. reported reduced levels of S100B transcripts in the DLPFC of MDD-S relative to controls (Klempan et al., 2009b), while in our comparison, we did not find any significant alteration. However, a previous rodent study showed that drug overdose increased S100B concentration in CSF (Vicente et al., 2007) and there

are five suicide victims in our collection who died of a drug overdose, this may have contributed to a higher S100B expression in the MDD-S abolishing significance.

CD11B expression decreased in MDD, confirming observations in rodent models. In both depression models, neonatal (early life stress) and adult (social defeat stress), CD11B expression was found to be decreased in the PFC (Ambrée et al., 2018; Giridharan et al., 2019). Our data indicate that there is no increased neuro-inflammation in non-suicidal individuals with MDD. Given the unchanged CD68 expression found after our three-group comparison, we deduce that reduced innate inflammatory state and synapse pruning may be present in the ACC in patients with MDD who died naturally (Michailidou et al., 2015). In human postmortem studies, complement C3b was found to be deposited in peripheral capillaries of suicide completers (Thorell, 1989; Varis et al., 1993). Our current data showed a trend for an increase in CD11B, a C3 receptor, in MDD-S compared to the rest of the patients, which supports this finding. Additionally, a reduced hypo-thrombotic state has been found in peripheral plasma of patients with MDD relative to suicide attempters and healthy controls and independent of suicidality. It should be noted that this condition went together with a higher pro-coagulant activity, solely in MDDs who had presented suicidal behaviors. In our ACC data, P2RY12, a key regulator in platelet aggregation (Dorsam and Kunapuli, 2004), showed a similar crosstalk suggesting that, in suicide, an increased platelet aggregation may be induced by capillary injury that activated complement system (Yang et al., 2016).

We found an increase of CD68 mRNA in the DLPFC in suicide completers. This is an extension of the finding that some types of microglia in the prefrontal white matter were associated with suicide (Schnieder et al., 2014). These observations also agree with previous studies reporting a peripheral and cerebral increase of monokines, that is mainly produced by monocytes and macrophages, and are indicative of microglia activation in suicide. For instance, plasma tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) were both increased in MD patients with suicidality (Li et al., 2013; Monfrim et al., 2014), while TNF- α levels in patients who attempted suicide seem to be even higher compared to those who only displayed suicidal ideation (Janelidze et al., 2011). In addition, in the PFC of suicidal patients, an increased expression of cytokines, that promote monocyte-macrophage activation (IL-4 and IL-13), or are centrally secreted by macrophages (IL-1 β and TNF- α), supports a CD68-driven microglial activation in suicide completers with MDD (Pandey et al., 2012; Tonelli et al., 2008). Of note, Steiner et al. reported an increased microgliosis in the DLPFC of patients who accomplished suicide and included both patients with MDD and SCZ (Steiner et al., 2008). Since we previously found an unchanged CD68 expression in the PFC of SCZ patients that committed suicide, their observation may be influenced by a large number of MDD-S cases. In addition, enhanced microglial phagocytosis has been assumed to participate in the pathophysiology of psychotic diseases that could be inhibited by antipsychotics (Chen et al., 2013; Rački et al., 2016). We here

provide additional molecular proof showing that CD68 was increased in the DLPFC of patients with MDD and psychotic features.

A positron emission tomography (PET) study showed that TSPO was increased in the frontal cortex in patients with MDD who did not present with severe suicidality (Li et al., 2018). This is in line with our results in the DLPFC, but not the ACC. TSPO, as a benzodiazepine receptor, was believed to be associated with the effects of GABA at the GABA_A receptors (Costa et al., 2011). These receptor subunits were already shown to be increased more in MDD-NS than in MDD-S versus controls in the same cohort (Zhao et al., 2018). Another PET study, however, presented a suicidality-specific TSPO elevation in the ACC, but not in the DLPFC (Holmes et al., 2018). We tend to consider the suicidal category (ideation in their patients, but actual completion in ours) and brain region (middle frontal cortex includes BA9 and BA46) to be the main reasons explaining why our results differ. In addition, a distinction was noticed related to the psychotic symptoms in MDD. In vivo, a psychotic features-dependent reduction in frontal grey matter volume was noticed in TSPO imaging, which indicates that MDD pathophysiology may at least in part be driven by microglial deficits (Collste et al., 2017; Selvaraj et al., 2018). In our data, the DLPFC did not show psychotic features-related alterations in TSPO expression, which is consistent with an imaging study on patients with a high risk for psychotic features (Hafizi et al., 2018a; Hafizi et al., 2018b).

Our data together with the findings of others indicate that changes in oligodendrocyte related transcripts in MDD may be Brodmann area-specific and differ between the grey and white matter (Mosebach et al., 2013; Pantazatos et al., 2017; Rajkowska et al., 2015). We did not find evidence to support the possibility that suicide is an independent factor in oligodendrocyte expression. This is in agreement with two Canadian studies indicating that suicide does not contribute to altered oligodendrocytic activities in the PFC (Lutz et al., 2017; Tanti et al., 2017). Data from blood samples report higher MBP transcripts in patients with first-episode psychosis compared to healthy controls (Ota et al., 2015; Santoro et al., 2015). However, postmortem studies in the human brain did not reveal elevated oligodendrocyte-related gene expression in relation to psychosis (Aston et al., 2005; Barley et al., 2009; Chandley et al., 2013; Matthews et al., 2012). Thus, the increased MBP expression we report in the DLPFC may be the first possible cortical region from where the increased plasma MBP levels are derived. This suggests stronger demyelination in patients with MDD and psychotic features, especially when MBP increases BBB permeability by inducing cerebral inflammation (D'Aversa et al., 2013).

In conclusion, differential changes were observed in the expression of several glial transcripts, in relation to MDD, suicide and psychotic features that were region-specific. Overall, MDD shows glia-related changes that may be relevant for BBB integrity, microglial phagocytosis, DA and GABA signaling, and myelination. While both suicide and psychotic features confound microglial phagocytosis, only psychotic features impact myelin stability.

LIMITATIONS

Inherent to postmortem studies is the sample volumes in this study relatively small. However, subjects of the collection studied have been well matched to reduce the output variabilities. Correlation analysis between glia expression and fluphenazine lifetime dosage has been performed but we did not find significance.

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Table S1A. Clinico-pathological information of patients with MDD and control subjects (SMRI collection).

	Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Medication	Psychotic features	Cause of Death
MDD1	F	48	24	6.36	1330	8.0	L DLPFC, L ACC	TCA	Yes	SUIC: OD
MDD2	F	40	49	6.72	1450	8.8	L DLPFC, L ACC	SSRI	Yes	SUIC: HANGING
MDD3	F	56	15	6.59	1370	8.4	L DLPFC, L ACC	antidepressant	No	BURNS
MDD4	M	28	26	6.7	1780	6.9	R DLPFC, R ACC	SSRI	Yes	SUIC: HANGING
MDD5	M	35	19	6.6	1335	8.2	R DLPFC, R ACC	-	Yes	SUIC: HANGING
MDD6	F	32	19	6.7	1280	8.9	L DLPFC, L ACC	-	Yes	SUIC: HANGING
MDD7	F	32	19	6.8	1470	8.0	R DLPFC, R ACC	non-TCA, SSRI	No	SUIC: HANGING
MDD8	M	63	31	6.6	1540	7.8	L DLPFC, L ACC	non-TCA	Yes	SUIC: HANGING
MDD9	F	51	36	6.3	1440	7.3	L DLPFC, L ACC	MAOI, TCA	Yes	UNKNOWN
MDD10	M	35	36	6.6	1710	6.3	L DLPFC, L ACC	-	Yes	SUIC: GSW
MDD11	M	44	24	6.52	1550	7.9	R DLPFC, R ACC	TCA, SSRI	No	CARDIAC
MDD12	M	56	38	6.59	1365	7.3	L DLPFC, L ACC	SNRI, anxiolytics	No	SUIC: OD
MDD13	M	33	25	6.86	1640	7.6	R DLPFC, R ACC	-	No	SUIC: HANGING
MDD14	M	34	24	6.79	1425	8.2	R DLPFC, R ACC	SSRI	No	SUIC: JUMPED
MDD15	F	45	29	6.9	1350	7.4	L DLPFC, L ACC	SSRI	No	CARDIAC
MDD16	M	53	21	6.64	1520	7.5	L DLPFC, L ACC	-	No	CARDIAC
MDD17	M	62	65	6.57	1490	7.5	R DLPFC, R ACC	non-TCA	No	SUIC: STABBED
MDD18	F	36	32	6.74	1270	7.3	R DLPFC, R ACC	SARI, TCA	Yes	PULM EMBOL
MDD19	M	40	52	6.48	1590	6.6	L DLPFC, L ACC	-	Yes	OD
MDD20	F	28	40	6.68	1430	7.1	L DLPFC, L ACC	-	Yes	SUIC: OD
MDD21	M	45	29	6.75	1514	7.9	L DLPFC, L ACC	SARI	No	SUIC: HANGING

Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Medication	Psychotic features	Cause of Death	
MDD22	M	24	21	6.61	1737	7.4	R DLPFC, R ACC	SNRI	No	SUIC; OD
MDD23	F	45	13	6.58	1170	7.2	L DLPFC, L ACC	TCA	No	SUIC; OD
MDD24	F	47	25	6.88	1495	7.7	L DLPFC, L ACC	TCA	No	SUIC; GSW
Ctr1	M	48	12	6.51	1410	8.6	R DLPFC, R ACC	-	No	CARDIAC
Ctr2	F	50	35	6.31	1520	7.9	L DLPFC, L ACC	anxiolytics	No	CARDIAC
Ctr3	M	50	11	6.5	1530	8.6	L DLPFC, L ACC	-	No	CARDIAC
Ctr4	M	63	37	6.5	1530	8	L DLPFC, L ACC	-	No	CARDIAC
Ctr5	M	24	17	6.6	1595	8.1	L DLPFC, L ACC	-	No	MVA
Ctr6	M	44	27	6.82	1410	7.2	R DLPFC, R ACC	-	No	ACUTE ALCOHOL POISONING
Ctr7	M	35	31	6.59	1520	8	R DLPFC, R ACC	-	No	MVA
Ctr8	M	63	40	6.91	1410	8.2	R DLPFC, R ACC	-	No	CARDIAC
Ctr9	M	34	9	6.56	1535	5.9	R DLPFC, R ACC	-	No	MVA
Ctr10	F	56	29	6.78	1278	8.9	L DLPFC, L ACC	-	No	CARDIAC
Ctr11	F	56	31	6.66	1400	6.1	L DLPFC, L ACC	-	No	OBESITY
Ctr12	F	39	24	6.88	1200	7.3	R DLPFC, R ACC	-	No	CARDIAC

Notes: ACC, anterior cingulate cortex; BW, brain weight; Ctr, control; DLPFC, dorsolateral prefrontal cortex; F, female; GSW, gunshot wound; L, left; M, male; MAOI, monoamine oxidase inhibitor; MVA, motor vehicle accidents; OD, overdose drugs; PMD, postmortem delay; R, right; RIN, RNA integrity number; SARI, serotonin antagonist and reuptake inhibitor; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; SUIC, suicide; TCA, tricyclic antidepressant.

Table S1B. Demographic information on the control and MDD subjects (MDD-NS and MDD-S).

	Ctrl	MDD-NS	MDD-S	F or χ^2	P
Age (years, range)	47 (24–63)	46 (36–56)	40 (24–63)	1.404	0.260
Gender (M/F)	8/4	3/4	10/7	1.034	0.596
Race	11W, 1H	7W	16W, 1H	0.529	0.744
PMD (hours, range)	25.3 (9–40)	29.9 (15–52)	29.6 (13–65)	0.541	0.587
Brain pH	6.64 (6.31–6.91)	6.60 (6.30–6.90)	6.67 (6.36–6.88)	0.565	0.574
Brain Weight (gram, range)	1445 (1200–1595)	1441 (1270–1590)	1480 (1170–1780)	0.295	0.747
Side of Brain Frozen (L/R)	6/6	5/2	10/7	0.838	0.658
Psychotic Features ¹	–	3	8	0.100	0.752
Alcohol hx	6	5	11	1.029	0.598
Severity of Alcohol abuse ²	2.08 (0–5)	1.29 (0–5)	2.18 (0–5)	0.509	0.606
Drug hx	4	3	5	0.403	0.817
Severity of Substance abuse ²	0.75 (0–4)	1.43 (0–4)	0.88 (0–4)	0.471	0.629
Fluphenazine (lifetime, range) ³	–	1314.29 (0–3000)	1041.18 (0–6500)	0.121	0.732

Abbreviation: Ctrl, control; F, female; hx, history; L, left; M, male; MDD-NS, patients with major depressive disorder who died of non-suicidal causes; MDD-S, patients with major depressive disorder who died of suicide; PMD, postmortem delay; R, right.

¹ Psychotic Features tested without controls

² Substance abuse and alcohol abuse was rated on a scale of 0–5

³ Fluphenazine tested without controls

Table S1C. Demographic information on the control and MDD subjects (MDD-NP and MDD-P).

	Ctr	MDD-NP	MDD-P	F or χ^2	P
Age (years, range)	47 (24–63)	44 (24–62)	40 (28–63)	1.193	0.316
Gender (M/F)	8/4	8/5	5/6	1.148	0.563
Race	11W, 1H	13W	10W, 1H	1.203	0.548
PMD (hours, range)	25.3 (9–40)	26.8 (13–65)	33.1 (19–52)	1.435	0.253
Brain pH	6.64 (6.31–6.91)	6.70 (6.52–6.90)	6.59 (6.30–6.74)	1.504	0.237
Brain Weight (gram, range)	1445 (1200–1595)	1469 (1170–1737)	1469 (1270–1780)	0.112	0.895
Side of Brain Frozen (L/R)	6/6	7/6	8/3	1.388	0.500
Suicide ¹	–	9	8	0.035	0.851
Alcohol hx	6	8	8	1.249	0.536
Severity of Alcohol abuse ²	2.08 (0–5)	1.69 (0–5)	2.18 (0–5)	0.198	0.821
Drug hx	4	4	4	0.084	0.956
Severity of Substance abuse ²	0.75 (0–4)	0.85 (0–4)	1.27 (0–4)	0.383	0.685
Fluphenazine (lifetime, range) ³	–	315.38 (0–3000)	2072.73 (0–6500)	2.861	0.009

Abbreviation: Ctr, control; F, female; hx, history; L, left; M, male; MDD-NP, patients with major depressive disorder who did not have psychotic features; MDD-P, patients with major depressive disorder and psychotic features; PMD, postmortem delay; R, right.

¹ Suicide tested without controls

² Substance abuse and alcohol abuse was rated on a scale of 0–5

³ Fluphenazine tested without controls

Table S2. Reference gene and target gene sequence.

Gene	Full name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
Reference Gene					
ACTβ	Actin beta		NM_001101	CCCAGCCATGTACGTT GCTA	TCACCGGAGTCCATCACGAT
GAPDH	Glyceraldehyde-phosphate dehydrogenase		NM_002046	CAAATTCCATGGCACC GTC	TCTCGCTCCTGGAAAGATGGT
HPRT1	Hypoxanthine phosphoribosyl-transferase 1		NM_000194	GGACAGGACTGAACG TCTTGC	ATAGCCCCCCTTGAGGACAC
TUBα	Tubulin alpha		NM_006082	CTTTGAGCCAGCCAACC AGA	GTACAACAGGCAGCAAGCCAT
TUBβ	Tubulin beta		NM_006087	GGGCCAAAGTTTGGG AGGT	CACTGTCCCCCATGGTATGTGC
UBC	Ubiquitin C		NM_021009	GCTGCTCATAAGACTCG GCC	GTCACCCAAAGTCCCGTCCTA
Target Gene	Full name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
Astrocyte					
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1	Neuron-glia interaction (dopaminergic system)	NM_001270364	GAACACAGTGGTGATCAA GCC	GGAGGACGTTAACCCACACCTT
GEAP	Glial fibrillary acidic protein	Astrocyte activation	NM_001363846	CCCACTCTGCTTT GACTGAGC	CCTTCTTCGGCCTTAGAGGG
S100B	S100 calcium binding protein B	Blood-brain barrier (BBB) permeability	NM_006272	TGGAAAAAGCAACTCCA TCAGAA	GAATCGCATGGGTCACGG

Target Gene	Full name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
Microglia					
CD11B	Cluster of differentiation 11B	Innate inflammatory state	NM_000632	TGCTTCCTGTTTGGATC CAACCTA	AGAAGGCAATGTCAC TATCCTCTTGA
CD45	Cluster of differentiation 45	Microglia activity downregulation	NM_002838	GCAGCTAGCAAGTG GTTTGTTT	AAACAGCATGCGTCCTTTTCTC
CD68	Cluster of differentiation 68	Microglia phagocytosis	NM_001040059	CTACCAGGCCCTCTGAGC	GGCGTGTGCGAGGAAATAAGG
CX3CR1	Chemokine (C-X3-C motif) receptor 1	Microglia homeostasis	NM_001337	TTGGCCTGGTGG GAAATTGT	AGGAGGTAAATGTCGGTGA CACT
HLA-DRA	Human leukocyte antigen-DR alpha chain	Microglia activation and neuroinflammation	NM_019111	CCCAGGGAAGACCAC CTTT	CACCCTGCAGTCGTAAACGT
IBA1	Ionized calcium-binding adapter molecule 1	Microglia activation and neuroinflammation	NM_032955	AGGTGTCCAGTGGCTCC GGG	TGGCAGATCTCTTGCCACAGCA
P2RY12	Purinergic receptor 12	Microglia homeostasis, ATP signaling and coagulation state	NM_176876	TTCAAACCCCTCCAGAA TCAACAG	GTGCACAGACTGGTGTTACC
TMEM119	Transmembrane protein 119	Microglia identity	NM_181724	CCACTCTCGCTCCATTCC	CAGCAACAGAAGGATGAGGA
TREM2	Triggering receptor expressed on myeloid cells 2	Microglial metabolism and anti-inflammatory activity	NM_018965	CCACCCACTTCCATCC TTCT	GTCCCTGGCTTCTGTCCAT

Target Gene	Full name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
TSPO	Translocator protein	Neuron-glia interaction (GABAergic system)	NM_000714	TCTGGAAAGAGCTGG GAGG	TTGTGCGGCACCAAAAGAAG
Oligodendrocyte					
MBP	Myelin basic protein	Myelin stability and BBB permeability	NM_001025101	AAATGCCGAGAAAGGCC AGTAC	CCCATTTGTTCTGTGTTTCGCA
MOG	Myelin oligodendrocyte glycoprotein	Myelin sheath maintenance	NM_002433	TGAGAGGAAAAACTTCG AGCAG	CGGCACAATTACAAAACAGGG
OLIG2	Oligodendrocyte transcription factor 2	Neural repair	NM_005806	GTGTCCAGCGCCTCTCT	GGCAGCAGACGGGAGACT
PLP	Myelin proteolipid protein	Myelin formation and maintenance	NM_000533	CCTCACTGGGCACAGA AAAGCT	GGCCCCATAAAGGAAGAA GAA

CHAPTER 3

SEX DIFFERENCE IN GLIA GENE EXPRESSION IN THE DORSOLATERAL PREFRONTAL CORTEX IN BIPOLAR DISORDER: RELATION TO PSYCHOTIC FEATURES

Zhang L, Verwer RWH, Lucassen PJ, Huitinga I, Swaab DF.

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ABSTRACT

Background: Suicide, psychotic features and gender influence the epidemiology and clinical prognosis of bipolar disorder (BD). Differences in glial function between the genders might contribute to these clinical variables. Here we studied expression of glial genes in human post-mortem prefrontal cortex of BD and control subjects in relation to suicide, psychotic features and sex.

Methods: Real time PCR was used to detect transcriptional alterations of 16 glia-related genes in two brain areas, the dorsolateral prefrontal cortex (DLPFC) and anterior cingulate cortex (ACC), from 30 patients with BD subdivided by suicide and psychotic features, and from 34 well-matched control cases.

Results: We found no evidence of immune activation in BD. Instead, we found three microglial genes to be downregulated in the DLPFC of non-suicidal individuals with BD, i.e., CD68, triggering receptor expressed on myeloid cells 2 (TREM2) and purinergic receptor 12 (P2RY12). A remarkable sex difference was observed in the DLPFC of patients with BD: 14 glia-related genes were expressed at significantly higher levels in males, including all three glial cell types. A subset analysis showed that the sex differences were closely associated with the presence of psychotic features.

Conclusions: No evidence of immune activation was found in these two brain regions in BD. The sex-specific differences in glial gene expression in BD, found particularly in patients with psychotic features, may be associated with the potential co-existence of mania and psychotic features and could potentially contribute to the gender-biased characteristics in BD.

Keywords

Bipolar disorder; dorsolateral prefrontal cortex; glia; suicide; sex differences; psychotic features

INTRODUCTION

Bipolar disorder (BD) is a multidimensional mood disorder with a strong genetic component in up to 85% of the patients (Goodwin and Jamison, 2007). Suicide rates in BD are approximately 20–30 fold higher, compared with the general population, and these cases occur mainly in a depressive episode (Gonda et al., 2012; Plans et al., 2018). As a heritable illness with neurodevelopmental impairments, this disorder often presents with diversified psychotic symptoms that are referred to approximately one-fifth of the lifetime suicidality in mood disorders and to more than 50% of the suicide ideations, independent of individual therapies or mood disorder classifications (Gesi et al., 2016; SanSegundo et al., 2018; Schaffer et al., 2015; Tsai et al., 2002). The descendants of people with psychosis and self-harm behaviors have a two to three times higher genetic risk for suicide than the general population (Andersen and Hynnekleiv, 2007). Suicide prevention thus has a high priority, but this requires a better understanding of the molecular mechanisms involved.

Sex difference may play an important role in the pathophysiology, cognitive and behavior phenotypes of BD. Clinical observations have revealed that, in females with BD, depressive episodes are a more frequent occurrence, and more often a previous family and personal history of suicidal behaviors is present (Nivoli et al., 2011). Male individuals with BD show higher risks of violent suicide attempts and psychotic features than females. Genetic data have further shown that elderly female individuals with BD without a family history of psychiatric disorders tended to have a later age of disease onset (Grigoriu-Serbanescu et al., 2005) but a higher accumulation of cortical mitochondrial DNA (mtDNA) deletions than elderly men (Fuke et al., 2008). In particular, females with BD and psychotic features possessed more neuropsychological impairments than males suffering from the same conditions (Bräunig et al., 2009; Zanelli et al., 2013). Moreover, BD females treated with lithium tended to be more vulnerable to manic and depressive episodes than men (Queissner et al., 2018), but their cognitive performance showed more improvement after physical training (Fellendorf et al., 2017). However, the mechanisms behind the sex-differential vulnerability and prognosis remain unknown.

Regional differences in volume have been implicated in the dorsolateral prefrontal cortex (DLPFC) and anterior cingulate cortex (ACC) of patients with BD. Information processing in the DLPFC, which has been related to the etiology of psychotic features, was preferentially reduced in psychotic BD, suggesting that psychosis might be accompanied by a disruption of the prefrontal control, which may result in the activation of the default network (Baker et al., 2014). Differences in the ACC, as measured by functional magnetic resonance imaging, have further been related to emotional processing, attentional and neurotransmitter impairments in pediatric and euthymic individuals with BD (Lee et al., 2018; Li et al., 2018; Soeiro-de-Souza et al., 2018). Patients in their first-episode mania with psychosis were further found to have a significant volume reduction of the entire ACC (Keramatian et al., 2016), whereas the right dorsal ACC

volume was found to be increased in recent onset psychosis (de Azevedo-Marques Perico et al., 2011).

Interestingly, the volumetric alterations found in patients with mood disorders have been linked to stress-induced changes in glia (Czéh et al., 2007). Such structural glia alterations, indicative of hyper- or hypofunction of these cells, may possibly reflect measures of central inflammatory processes ongoing in the BD brain. In the periphery, elevated levels of inflammation-related markers have indeed been found in patients with BD (Jakobsson et al., 2015; Rolstad et al., 2015). For instance, the cytokine interleukin 10 was elevated in plasma in first-episode bipolar patients with psychotic features (Lesh et al., 2018), indicating potential monocyte/macrophage involvement in the psychotic etiology of BD. However, studies on postmortem frontal tissues have reported reductions in glial cell density in sublayers of both the DLPFC and ACC of patients with BD (Gittins and Harrison, 2011; Rajkowska et al., 2001). Pathologic changes in dendritic spines and reductions in oligodendrocyte specific proteins have been described in the isolated layers of the prefrontal cortex (PFC) in individuals with BD (Konopaske et al., 2014; Wesseling et al., 2014). Whether there is an immune process going on in the brain of patients with BD is thus still an open question.

Even though changes in peripheral immune markers were thought to point to an immune involvement in BD etiology, so far, the reported alterations in glia in the postmortem brain remain fragmentary and they vary per glial type, brain region, and the presence/absence of suicide, psychotic features and gender. The aim of our study was, therefore, to explore in patients with BD in a systematic way the transcriptional changes of markers of astrocytes, microglia and oligodendrocytes in BD, in relation to suicide, psychotic features and sex. Given their clear involvement in BD as outlined above, we focused on the DLPFC and ACC.

MATERIALS AND METHODS

Human brain samples from the Stanley Medical Research Institute (SMRI)

The Stanley Medical Research Institute (SMRI, Bethesda, MD, USA, Director: Dr. Maree J. Webster) provided 128 brain samples (25 male and 9 female controls and 15 male and 15 female patients with BD) for this study. The next of kin provided permission for the use of brain material. Diagnoses were made according to the Diagnostic and Statistical Manual of Mental Disorder IV (DSM-IV). The SMRI formulated exclusion criteria for all specimens, and all brain regions included were examined microscopically to exclude subjects with pathological signs of neurodegeneration or other lesions. The cause of death for 13 patients with BD was suicide (7 males and 6 females); the other cases and all control subjects died from natural causes or accidents. Sixteen patients with BD had psychotic features (7 males and 9 females) while 12 patients had no such features (for clinico-pathological details see **Table 1**).

The SMRI provided us with RNA from isolated grey matter of two brain areas: the DLPFC (Brodmann area 46) and ACC (Brodmann area 24) of each subject. The controls did not have suicidal behaviors or any major psychiatric diagnosis. Groups for comparison were well matched for sex, age, postmortem delay (PMD), month of death (MOD), and brain weight (BW) (see Table 1 and Table S2A-G). Demographic information and medical data were provided by SMRI. All analyses were performed by investigators unaware of the grouped information.

Quantitative real-time PCR

cDNA synthesis was performed as described by us before (Wang et al., 2008). The RNA integrity value (RIN), an indicator of tissue RNA quality, did not show any significant difference between the diagnostic groups ($P = 0.76$). Our selection strategy towards the detected glial genes and their sequences for each primer pair are shown in Table S1. cDNA template (equivalent to 5 ng of total RNA) was amplified in a final volume of 10 μ l using a SYBR Green PCR master mix (Applied Biosystems, CA, USA) and a mixture of forward and reverse primers (each 2 pmoL/ μ l). Data were acquired and processed automatically by the Applied Biosystems 7300 Real-time PCR System. The specificity of amplification was checked by melting curve analysis. Sterile water and RNA samples without the addition of reverse transcriptase during cDNA synthesis served as negative controls. The linearity of each qPCR assay was tested by preparing a series of dilutions of the same stock of cDNA in multiple plates. Stable reference genes were selected to reduce the effect of sample variability (Vandesompele et al., 2002). The initial set of reference genes was: actin beta (ACT β), glyceraldehyde-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), tubulin alpha (TUB α), tubulin beta (TUB β) and ubiquitin C (UBC). For the comparisons in the ACC, ACT β , TUB β and GAPDH were selected. For DLPFC the selection of ACT β , TUB α , TUB β and UBC appeared to be the most appropriate.

Statistical analysis

S+ software (version 8.2, TIBCO, Seattle, WA, USA) was used for statistical analysis. The Chi-square test was used for analysis of categorical data (gender). For interval data, the Mann-Whitney test (2 samples) or the Kruskal-Wallis test with multiple comparisons (3 samples) was used (Conover, 1980). Before processing of gene expression data, the values were ¹⁰log-transformed to enable simple reference gene correction and conventional statistical procedures. The reason for this transformation is that the observed Ct values used in order to quantify gene expression, appear as exponents of the PCR efficiency. Application of the log-transformation yields an additive statistical model and, after all statistical procedures have been finished, the data are back-transformed and presented as fold-changes. In multiple testing situations the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) of P -values was applied. When the Kruskal-Wallis test was used in combination with the Benjamini-Hochberg correction, we proceeded in a 2-step way. As multiple comparisons in the Kruskal-Wallis test are only allowed

if the global $P < 0.05$ (Conover, 1980), we first corrected the global P values and then selected for further analysis only those genes for which this requirement was met. For each appropriate comparison, the corresponding P -values were pooled and corrected according to Benjamini-Hochberg. All tests were 2-sided.

Table 1. Demographic information.

	Ctr	BD	<i>P</i>
Age (year) ¹	45 (31–60)	44 (19–64)	0.66
Gender (M/F)	25/9	15/15	0.05
PMD (hour) ¹	28.5 (9–58)	33.5 (12–84)	0.13
Brain pH ¹	6.69 (6.00–7.03)	6.50 (5.92–6.97)	0.03
Brain weight (gram) ¹	1413 (1120–1900)	1420 (1170–1670)	0.49
Hemisphere	16 L/18 R	13 L/17 R	0.77
Age of onset (year) ¹	–	22.5 (14–48)	–
Duration of illness (year) ¹	–	18 (2–45)	–
Suicide	–	13	–
Psychotic features	–	16	–
Fluphenazine equivalents	–	19	–

Notes: Ctr, control; F, female; L, left; M, male; BD, bipolar disorder; L, left; PMD, postmortem delay; R, right. ¹Data were shown as median with range.

RESULTS

Altered microglia mRNA expression in BD

An overview of mRNA expression in genes is presented in **Figure 1** and **Tables 2A** to **2C**.

In the DLPFC, CD68 transcripts were downregulated in patients with BD compared to matched controls (Fold change = -1.15, $P = 0.006$). For the three group comparisons, we found that CD68 was only reduced in patients with BD who did not die by suicide (BD-NS vs. Ctr: $P < 0.0001$; BD-NS vs. BD-S: $P = 0.01$), whereas no difference was found between the controls and patients with BD that died from suicide ($P = 0.23$). Both BD with and without psychotic features revealed reduced CD68 gene expression compared to the controls (BD-NP vs. Ctr: $P = 0.002$; BD-P vs. Ctr: $P = 0.004$), while no difference was found for CD68 between BD with and without psychosis ($P = 0.70$).

In addition, the transcript level of TREM2 in the DLPFC was significantly reduced in patients with BD (Fold change = -1.31, $P = 0.01$). When compared to controls, a reduction of the expression of this gene was only found in patients with BD without completed suicide ($P = 0.0004$), while no difference was present between individuals with BD who died by suicide and controls ($P = 0.23$), nor between patients with BD with and without completed suicide ($P = 0.09$). TREM2 downregulation was present in patients with BD both with and without psychotic features, compared to the controls (BD-NP vs. Ctr: $P = 0.03$; BD-P vs. Ctr: $P = 0.002$). No significant changes were present between patients with and without psychosis ($P = 0.42$).

Moreover, we found a down-regulation in P2RY12 transcripts in patients with BD who died of natural causes relative to controls (Fold change = -1.41, $P = 0.01$) and between non-suicide patients with BD and suicide completers (Fold change = -1.69, $P = 0.004$). However, the differences between suicide completers and their controls, or between all BD subjects and their controls, were not significant (BD-S vs. Ctr: $P = 0.23$; BD vs. Ctr: $P = 0.35$).

None of the genes studied revealed any significant differences in the ACC.

Sex differences in BD in the DLPFC

A striking sex difference of the glia-related gene expression was found in individuals with BD in the DLPFC (see **Figure 2** and **Table 3**), with males having a higher expression of most detected genes. In a subset analysis, we found that these changes were more obvious in patients with psychotic features in their clinical history than in the other subsets. In addition, we noticed several other types of differences: GLT1, GS and P2RY12 showed similar significances in patients who had died of natural causes. Both CD68 and MOG revealed changes in the suicide subset. MBP exhibited this alteration in all subsets. No sex-specific change was found in the controls, and not in the BD group in the ACC for the two brain areas studied.

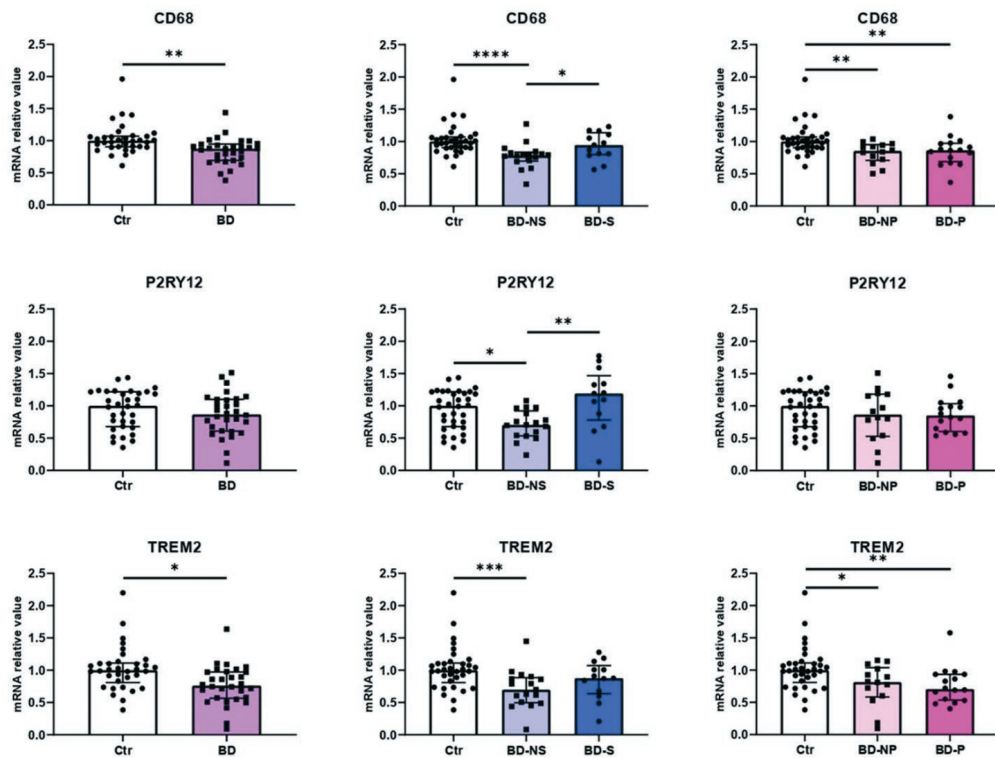


Figure 1. Transcript levels of microglia related genes (CD68, P2RY12 and TREM2) in the dorsolateral prefrontal cortex (DLPFC) in controls (Ctr, $n = 34$) and patients with bipolar disorder (BD, $n = 30$) that died of suicide (BD-S, $n = 13$) or causes other than suicide (BD-NS, $n = 17$), and with or without psychotic features (BD-P, $n = 16$ or BD-NP, $n = 14$). Data shown in this graph were irrespective of gender and plotted as median with interquartile range.

Note: * indicates $0.01 < P \leq 0.05$, ** indicates $0.001 < P \leq 0.01$, *** indicates $0.001 < P \leq 0.0001$, **** indicates $0.001 < P \leq 0.0001$.

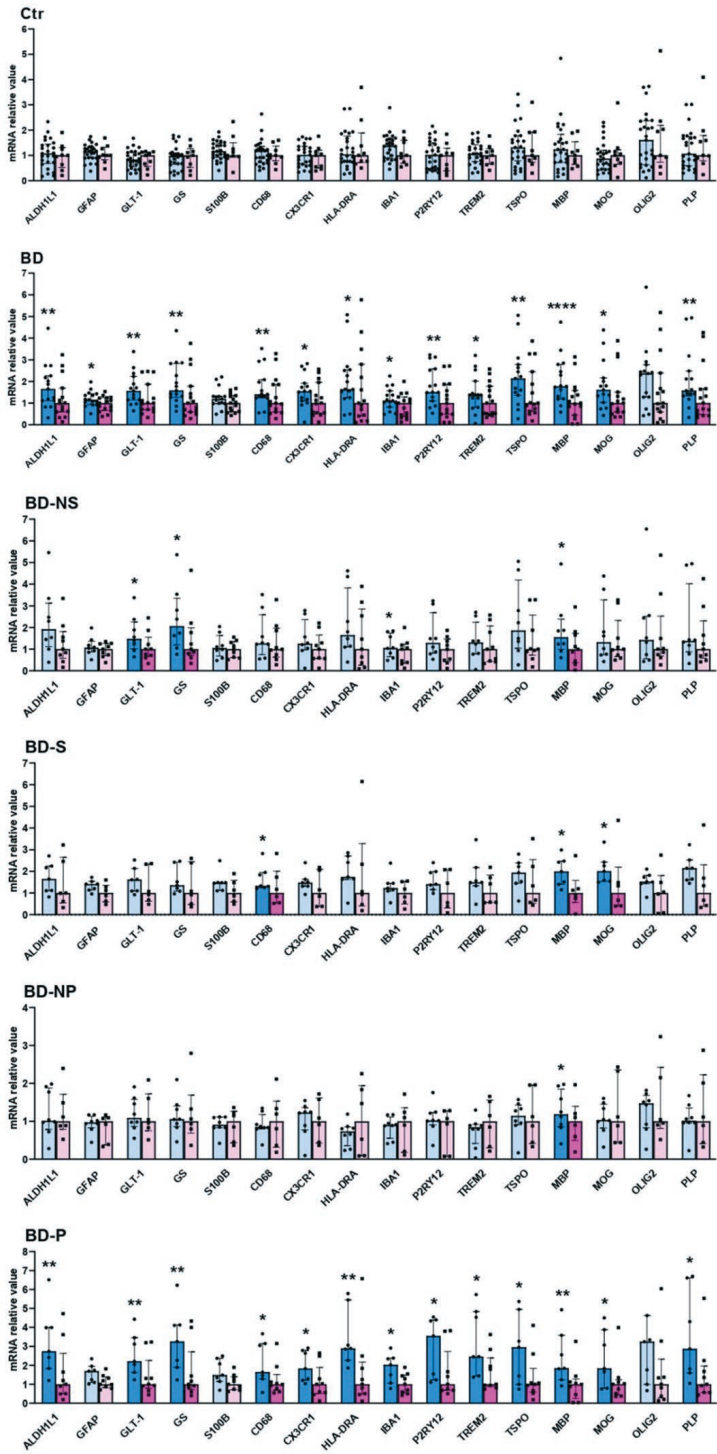


Figure 2. Transcript levels of glial genes in the DLPFC in controls and patients with BD and their subsets between males and females. Sample volumes: Ctr-male: n = 25, Ctr-female: n = 9; BD-male: n = 15, BD-female: n = 15; BD-NS-male: n = 8, BD-NS-female: n = 9; BD-S-male: n = 7, BD-S-female: n = 6; BD-NP-male: n = 8, BD-NP-female: n = 6; BD-P-male: n = 7, BD-P-female: n = 9. Data were plotted as median with interquartile range.

Note: * indicates $0.01 < P \leq 0.05$, ** indicates $0.001 < P \leq 0.01$, *** indicates $0.001 < P \leq 0.0001$, **** indicates $0.001 < P \leq 0.0001$.

Table 2A. Expression of glial genes in BD compared to their matched controls in the DLPFC and ACC.

	DLPFC		ACC			
	Fold change	P value	BHadj-p	Fold change	P value	BHadj-p
Astrocyte genes						
ALDH1L1	1.20	0.63		1.27	0.03	0.18
GFAP	-1.16	0.14		1.00	0.78	
GLT1	1.11	0.35		1.17	0.28	
GS	1.05	0.56		1.18	0.22	
S100b	-1.04	0.55		1.04	0.32	
Microglia genes						
CD68	-1.15	0.000	0.006	1.06	0.73	
CX3CR1	-1.21	0.05		-1.09	0.49	
HLA-DRA	-1.11	0.48		-1.21	0.41	
IBA1	-1.38	0.01	0.05	-1.42	0.02	0.13
P2RY12	-1.15	0.35		-1.09	0.55	
TREM2	-1.31	0.002	0.013	-1.24	0.008	0.12
TSPO	-1.09	0.47		1.10	0.11	
Oligodendrocyte genes						
MBP	-1.08	0.90		-1.02	0.35	
MOG	-1.05	0.35		1.10	0.09	
OLIG2	1.00	0.80		1.22	0.15	
PLP	-1.15	0.38		1.05	0.29	

Notes: ACC: anterior cingulate cortex; BD: bipolar disorder; BHadj-p: P value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsolateral prefrontal cortex.

Table 2B. Expression of glial genes in BD with or without suicide compared to their matched controls in the DLPFC and ACC.

	Fold change				Global-p	BHadj-p	BHadj,p	
	BD-S/BD-NS	BD-S/Ctr	BD-NS/Ctr	BD-S/BD-NS			BD-S/Ctr	BD-NS/Ctr
DLPFC-astrocyte genes								
ALDH1L1	1.23	1.37	1.12	0.34				
GFAP	1.07	-1.07	-1.14	0.20				
GLT1	1.14	1.21	1.06	0.32				
GS	1.12	1.10	-1.02	0.33				
S100b	1.05	-1.01	-1.06	0.64				
DLPFC-microglia genes								
CD68	1.20	-1.05	-1.27	0.000	0.003	0.01	0.23	0.000
CX3CR1	1.35	-1.02	-1.34	0.02	0.06			
HLA-DRA	-1.11	-1.08	1.02	0.72				
IBA1	1.31	-1.18	-1.55	0.003	0.07			
P2RY12	1.69	1.19	-1.41	0.005	0.03	0.004	0.23	0.01
TREM2	1.25	-1.14	-1.43	0.002	0.02	0.09	0.23	0.000
TSPO	1.28	1.04	-1.22	0.47				
DLPFC-oligodendrocyte genes								
MBP	1.39	1.13	-1.23	0.04	0.06			
MOG	1.00	-1.13	-1.13	0.58				
OLIG2	1.36	1.18	-1.15	0.42				
PLP	1.11	-1.03	-1.14	0.43				

	Fold change				Global-p	BHadj-p	BHadj-p	
	BD-S/BD-NS	BD-S/Ctr	BD-NS/Ctr	BD-S/BD-NS			BD-S/Ctr	BD-NS/Ctr
ACC-astrocyte genes								
ALDH1L1	-1.29	1.06	1.38	0.03	0.11			
GFAP	1.04	1.01	-1.03	0.85				
GLT1	-1.04	1.14	1.19	0.45				
GS	-1.08	1.16	1.25	0.33				
S100b	1.09	1.09	1.00	0.61				
ACC-microglia genes								
CD68	1.07	1.11	1.04	0.79	0.11			
CX3CR1	1.06	-1.07	-1.14	0.35				
HLA-DRA	1.04	-1.19	-1.24	0.69				
IBA1	1.22	-1.22	-1.48	0.02				
P2RY12	1.12	1.00	-1.12	0.84				
TREM2	1.24	-1.07	-1.32	0.01	0.11			
TSPO	-1.15	1.01	1.16	0.08				
ACC-oligodendrocyte genes								
MBP	1.00	-1.02	-1.01	0.64	0.11			
MOG	-1.28	-1.05	1.21	0.03				
OLIG2	-1.29	-1.03	1.24	0.09				
PLP	-1.31	-1.13	1.16	0.08				

Notes: ACC: anterior cingulate cortex; BD: bipolar disorder; BD-NS: bipolar disorder – non suicide; BD-S: bipolar disorder – suicide; BHadj-p: *P* value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsolateral prefrontal cortex.

Table 2C. Expression of glial genes in BD with and without psychotic features and their matched controls in the DLPFC and ACC.

	Fold change				BHadj-p	
	BD-P/BD-NP	BD-P/Ctr	BD-NP/Ctr	Global-p	BD-P/Ctr	BD-NP/Ctr
DLPFC-astrocyte genes						
ALDH1L1	-1.04	1.16	1.20	0.80		
GFAP	-1.02	-1.17	-1.14	0.31		
GLT1	1.02	1.08	1.06	0.66		
GS	-1.17	-1.01	1.16	0.48		
S100b	-1.05	-1.08	-1.03	0.68		
DLPFC-microglia genes						
CD68	1.01	-1.16	-1.17	0.002	0.03	0.004
CX3CR1	-1.04	-1.22	-1.18	0.16		
HLA-DRA	1.37	1.13	-1.22	0.24		
IBA1	-1.07	-1.43	-1.34	0.03	0.16	
P2RY12	-1.02	-1.17	-1.15	0.62		
TREM2	-1.15	-1.42	-1.23	0.005	0.04	0.03
TSP0	-1.13	-1.15	-1.02	0.55		
DLPFC-oligodendrocyte genes						
MBP	-1.14	-1.16	-1.02	0.24		
MOG	1.07	-1.04	-1.11	0.59		
OLIG2	-1.32	-1.22	1.08	0.09		
PLP	-1.01	-1.14	-1.13	0.63		

		Fold change		Global-p		BHadj-p		BHadj-p	
		BD-P/BD-NP	BD-P/Ctr	BD-NP/Ctr		BD-P/BD-NP	BD-P/Ctr	BD-NP/Ctr	
ACC-astrocyte genes									
ALDH1L1	1.48	1.52		1.03	0.007				0.09
GFAP	1.14	1.06		-1.07	0.41				
GLT1	1.38	1.28		-1.08	0.03				0.09
GS	1.35	1.44		1.06	0.02				0.09
SI00b	1.33	1.21		-1.10	0.19				
ACC-microglia genes									
CD68	1.16	1.14		-1.02	0.22				
CX3CR1	1.28	1.02		-1.25	0.15				
HLA-DRA	-1.15	-1.23		-1.06	0.76				
IBA1	-1.17	-1.40		-1.19	0.03				0.09
P2RY12	1.12	-1.02		-1.14	0.41				
TREM2	-1.14	-1.31		-1.15	0.04				0.09
TSPO	-1.04	1.07		1.12	0.05				
ACC-oligodendrocyte genes									
MBP	1.15	1.05		-1.09	0.32				
MOG	1.35	1.27		-1.06	0.04				0.09
OLIG2	1.21	1.31		1.09	0.04				0.09
PLP	1.43	1.26		-1.14	0.05				

Notes: ACC: anterior cingulate cortex; BD: bipolar disorder; BD-NP: bipolar disorder – nonpsychotic features; BD-P: bipolar disorder – psychotic features; BHadj-p: P value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsolateral prefrontal cortex.

Table 3. Sex differences in glial gene expression (male/female) in Ctr and BD (and its subgroups) in the DLPFC.

Ctr		BD		BD-NS		BD-S		BD-NP		BD-P							
Fold change	P value	BH-adj-p	Fold change	P value	BH-adj-p	Fold change	P value	BH-adj-p	Fold change	P value	BH-adj-p						
Astrocytic genes																	
ALDH1L1	1.08	0.55	1.66	0.004	0.008	1.91	0.02	0.06	1.65	0.05	1.01	0.90	2.73	0.001	0.008		
GFAP	1.16	0.71	1.15	0.02	0.03	1.07	0.25		1.41	0.02	0.05	0.25	1.69	0.04	0.070		
GLT1	0.83	0.68	1.56	0.001	0.005	1.47	0.009	0.046	1.61	0.06	1.09	0.70	2.21	0.001	0.008		
GS	0.98	0.92	1.58	0.0006	0.003	2.06	0.003	0.02	1.35	0.12	1.05	0.80	3.25	0.001	0.008		
S100B	1.17	0.63	1.20	0.05		1.05	0.70		1.48	0.02	0.06	0.44	1.48	0.03	0.06		
Microglial genes																	
CD68	1.19	0.63	1.41	0.002	0.006	1.27	0.05		1.33	0.01	0.046	0.25	1.64	0.01	0.03		
CX3CR1	1.02	0.20	1.54	0.02	0.02	1.25	0.34		1.47	0.05	1.23	1.00	1.83	0.003	0.01		
HLA-DRA	1.04	0.80	1.64	0.01	0.02	1.64	0.02	0.06	1.75	0.20	1.36	0.90	2.89	0.001	0.008		
IBA1	1.37	0.38	1.10	0.008	0.01	1.05	0.04	0.07	1.22	0.09	1.11	0.37	2.03	0.004	0.01		
P2RY12	1.02	0.27	1.51	0.0003	0.002	1.29	0.002	0.02	1.41	0.03	1.02	0.03	0.06	3.54	0.005	0.01	
TREM2	1.07	0.34	1.37	0.02	0.03	1.30	0.34		1.49	0.05	1.19	0.90	2.46	0.004	0.01		
TSPO	1.31	0.60	2.14	0.003	0.007	1.86	0.04	0.07	1.93	0.03	1.14	0.52	2.95	0.01	0.02		
Oligodendrocytic genes																	
MBP	1.24	0.47	1.77	0.0000	0.000	1.55	0.001	0.02	1.99	0.003	0.02	1.18	0.007	0.02	1.85	0.001	0.008
MOG	0.87	0.80	1.61	0.03	0.03	1.32	0.29		2.00	0.01	0.046	1.03	0.80	1.84	0.007	0.02	
OLIG2	1.61	0.65	2.37	0.09		1.42	0.70		1.52	0.02	0.05	1.47	0.61	3.25	0.04	0.07	
PLP	1.06	0.83	1.57	0.004	0.008	1.38	0.15		2.15	0.02	0.05	1.02	0.70	2.86	0.003	0.01	

Notes: BD: bipolar disorder; BD-NP: BD without psychotic features; BD-NS: patients with BD died of non-suicidal causes; BD-P: BD with psychotic features; BD-S: patients with BD died of suicide; BHadj-p: P value of Benjamini-Hochberg's adjustment; Ctr: controls.

DISCUSSION

To explore potential central immunological processes in BD and investigate whether they may be associated with suicide and/or psychotic features, we analyzed mRNA expression in the DLPFC and ACC from individuals with BD and matched controls. We also paid special attention to possible gender differences. Some alterations in microglia-related gene expression in the DLPFC were only present in patients who did not attempt suicide (i.e. a decreased expression of the microglial genes CD68, P2RY12 and TREM2). We did not find psychotic features confound our data. A striking finding was the prominent sex difference in glial gene expression in the same region. The clear sex differences in glia-related genes in the DLPFC were only observed in this brain region and only in BD, especially in patients with psychotic features.

Microglia plays an important role in central immune responses. Upon activation, microglia can aggravate neural inflammation by releasing specific pro-inflammatory cytokines. Functional abnormalities of microglia have been implicated in the onset of some mood disorders and have therefore been proposed as novel therapeutic targets. Even though pro-inflammatory cytokines are elevated in the peripheral circulation in BD, there are only limited studies that have analyzed microglia in the BD brain. Our data on unaltered microglial transcripts in BD agree with those from Sneeboer et al. (Sneeboer et al., 2019). In addition, we found a down-regulation of CD68-, P2RY12- and TREM2-mRNA expression in the DLPFC in individuals with BD who did not attempt suicide. De Baumont et al. have reported changes in CD68 and TREM2 expression in BD compared to schizophrenia (SCZ) (de Baumont et al., 2015). As an M2 microglia marker, this reduced TREM2 expression reminded us of a reduced anti-inflammatory activity in non-suicidal BD cases as reported before (Zhang et al., 2018). Interestingly, TREM2 has a more significant reduction in psychotic victims, indicating that a decrease in TREM2-mRNA expression may be related more to the psychotic features than to BD *per se*. Indeed, psychotic symptoms might be associated with increases in microglia indoleamine 2,3 dioxygenase expression following TREM2 deficiency, as was suggested based on TREM2 knock down studies (Penberthy, 2007); this was thought to result from increases in pro-inflammatory cytokine release in the periphery in BD (Anderson et al., 2016; Takahashi et al., 2005).

CD68 and P2RY12 both showed an elevated expression in suicide completers with BD relative to those non-suicidal patients and had levels that met those of the control subjects, suggesting that microglia are activated during suicidal behaviors. In our previous study, we found an increase in P2RY12 expression in suicide completers with SCZ (Zhang et al., 2020), which is consistent with a disturbed purine metabolism in the PFC in suicide (Pantazatos et al., 2017). In major depressive disorder (MDD), we also found an upregulation of CD68 transcript in this cohort, but not in patients who died of non-suicidal causes, as compared to controls (unpublished data), suggesting an enhanced microglial phagocytosis in suicide completers

among individuals who are suffering from depression-related psychiatric diseases, and that this increase might be related to the ATP signaling activation.

Human postmortem brain studies on the relationship between glial expression and sex differences in BD are of general interest because of the clear sex-specific differences in the vulnerability to develop psychiatric disorders and in this case in particular because of the sex differences in BD symptoms. To our best knowledge, and for the first time, we here report higher levels of gene expression of the three types of glia in the DLPFC of male individuals relative to females with BD, in particular in patients with BD and psychotic features. Interestingly, also in autism spectrum disorder (ASD) in the postmortem frontal cortex, astrocyte and microglia gene expression levels were significantly higher in adult males compared to females (Werling et al., 2016). A higher glial cell density in male patients with BD has further been proposed to sustain an increased gray matter volume by anti-apoptotic or proliferative/supportive effects of glia (Keshavarz, 2017). Further, a seasonal pattern of manic episodes has been mentioned in relation to psychotic BD in males (Hochman et al., 2016). One may presume that this seasonal pattern may also be, as shown in animal studies, glia-mediated and possibly sex hormone determined (Forlano and Bass, 2005a, b). We performed gender analysis of our transcriptional glia data in the DLPFC and ACC in both MDD and SCZ, but no sex related changes were found (Zhang et al., 2020). We thus deduced that these glia differences in relation to sex are more specific when mania is accompanied by psychotic features.

In our study, sex-based differences appeared mainly in mature astrocytes expressing GS and GLT1. These are both biochemical parameters relevant for glutamate signaling. Sex differences in the glutamate pathway were recently reported to contribute to psychiatric diseases such as MDD, SCZ, ASD and attention deficit hyperactivity disorder (Wickens et al., 2018). Interestingly, we found that P2RY12 shared a similar sexually dimorphic expression pattern in non-suicidal individuals with BD, indicating that glial-dependent purinergic signaling may be disturbed by stress-related diseases and involved in the regulation of glutamate transmission. On the other hand, the immature astrocytic marker S100b did not reveal any significant differences between the sexes, suggesting that astrocytes in the DLPFC are sexually differentiated to the same extent in patients with BD as in controls.

Microglia-related sex differences seem to be brain region and species specific. The enhanced microglia density and soma size that were found before in the somatosensory cortex, hippocampus and amygdala of male adult mice (Guneykaya et al., 2018) were not recapitulated in our human prefrontal data in controls. Following a restrained stress exposure, adult rodents showed different states of microglial activity and proliferation, i.e. the proportion of ramified microglia was increased more in females than in males (Bollinger et al., 2016). However, when males were exposed to social defeat stress they displayed higher phagocytic activity and proliferation of microglia than females (Lehmann et al., 2016). Importantly, there was a shift from an activated state to an anti-inflammatory state in female prefrontal cortical microglia following

stress exposure (Bollinger et al., 2016). The strongly lowered microglia mRNA expression in such female mice may predict a weaker innate immune function than in males. When lithium is prescribed to BD females, the total glial cell numbers increased (Keshavarz, 2017) and more manic and depressive episodes were reported than in males (Queissner et al., 2018).

In MDD, opposite changes in gene expression were reported for both sexes in the DLPFC: male patients showed an increase, and females a decrease in oligodendrocyte expression relative to controls (Seney et al., 2018). Notably, OLIG2, a gene determining the differentiation and proliferation of oligodendrocytes, was not significantly different between the sexes in our data. But in our study, MBP and PLP1, two genes closely interacting with each other, showed an, approximately, double increase in BD males compared to females, suggesting the presence of sex differences in the process of myelination, which could include myelin sheath compaction, stabilization, maintenance and/or neuronal survival. Our data provide evidence for a clear sex difference in immature oligodendrocyte expression in BD. In addition, the sex difference in expression of CD68 and MOG supported the possibility of increases in microglial activation/phagocytosis that may occur in response to myelin injury and regeneration in the DLPFC of suicide cases.

A few limitations in our study should be mentioned. Firstly, the relatively small sample size in the subsets (BDNS and BDS, BDNP and BDP) precluded a further exploration of sex differences in patients with BD with and without suicide and psychotic features. Secondly, antipsychotic medication may, at least in theory, have affected P2RY12 mRNA expression in both the DLPFC and ACC. Information on such medication was provided by SMRI as a lifetime dosage in fluphenazine equivalents for 19 individuals with BD. A positive correlation was present between this medication equivalent and P2RY12 expression in both the DLPFC ($\rho = -0.49$, $P = 0.041$) and ACC ($\rho = -0.54$, $P = 0.026$). However, since there was no significant alteration in the DLPFC ($P = 1.00$) or ACC ($P = 0.26$) among the three groups (control and subjects with BD with and without an intake of fluphenazine), it is unlikely that antipsychotics have influenced our main conclusions.

CONCLUSIONS

Different expression patterns are present in BD when comparing suicide as a confounder. In our data, different genes (decreased CD68, P2RY12 and TREM2) were decreased in patients with BD who did not die by suicide, while individuals with BD who died from suicide showed significantly higher expression of CD68 and P2RY12 mRNA than patients who died of other causes. These findings indicate that in individuals with BD, a stronger prefrontal microglial phagocytosis activity may be present, together with an ATP signaling activation, and a stronger purinergic metabolism in suicide cases. A remarkable sex difference was present in the expression

of these three types of glia in BD, especially in those with psychotic features. These changes were observed in particular in the DLPFC. No such changes were found in our SCZ and MDD studies.

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Contributors

Lin Zhang, Inge Huitinga and Dick F. Swaab designed the protocol. Lin Zhang undertook data collection. Ronald W. H. Verwer performed statistical analysis. Lin Zhang wrote the first draft. Dick F. Swaab and Paul J. Lucassen amended the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of Interest Statement

None of the authors has anything to disclose.

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Table S1. Reference genes and target genes sequences.

Gene	Full Name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
Reference Gene					
ACTβ	Actin beta		NM_001101	CCCAGCCATGTACGT TGCTA	TCACCGGAGTCCATCAGGAT
GAPDH	Glyceraldehyde- phosphate dehydrogenase		NM_002046	CAAATTCCATGGCACCGTC	TCTCGCTCCTTGGAAGATGGT
HPRT1	Hypoxanthine phosphori- bosyltransferase 1		NM_000194	GGACAGGACTGAACGT CTTGC	ATAGCCCCCCTTGAGCACAC
TUBα	Tubulin alpha		NM_006082	CTTTGAGCCAGCCAAACC AGA	GTACAACAGGCAGCAAGCCAT
TUBβ	Tubulin beta		NM_006087	GGGCCAAGTTTTTGGG AGGT	CACTGTCCCCATGGTATGTGC
UBC	Ubiquitin C		NM_021009	GCTGCTCATAAGACTCG GCC	GTCACCCAAGTCCCGTCCTA
Target Gene	Full Name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
Astrocyte					
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1	Neuron-glia interaction (dopaminergic system)	NM_001270364	GAACACAGTGGTGATC AAGCC	GGAGGACGTTAACCACACCTT
GFAP	Glial fibrillary acidic protein	Astrocyte activation	NM_001363846	CCCACCTCTGCTTTG ACTGAGC	CCTTCTTCGGCCTTAGAGGG
GLT1	Glutamate transporter 1	Neuron-glia interaction (glutamate-glutamine signaling)	NM_001195728	ATACCATTGACTCC CAGCATCG	GAGTTGCTTTCCCTGTG- GTTCTT

Target Gene	Full Name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
GS	Glutamine synthetase	Neuron-glia interaction (glutamate-glutamine signaling)	NM_001033056	TGTGTGGAAGAGTTG CCTGAG	TGGCAGCAGGCACGAGATAC
S100b	S100 calcium binding protein b	Blood-brain barrier (BBB) permeability	NM_006272	TGGAAGAAAGCAACTC CATCAGAA	GAATCGCATGGGTACGG
Microglia					
CD68	Cluster of differentiation 68	Microglia phagocytosis	NM_001040059	CTACCAGGCCCTCTGAGC	GGCGTGTGAGGAAATAAGG
CX3CR1	Chemokine (C-X3-C motif) receptor 1	Microglia homeostasis	NM_001337	TTGGCCTGGTG GGAAATTGT	AGGAGGTAAATGTCGGT GACACT
HLA-DRA	Human leukocyte antigen-DRA	Microglia activation and neuroinflammation	NM_019111	CCCAGGGAAGACCAC CTTT	CACCCTGCAGTCGTAAACGT
IBA1	Ionized calcium-binding adapter molecule 1	Microglia activation and neuroinflammation	NM_032955	AGGTGTCCAGTGGCT CCGGG	TGGCAGATCTCTTGCCCCAGCA
P2RY12	Purinergic receptor 12	Microglia homeostasis, ATP signaling and coagulation state	NM_176876	TTCAAAACCTCCAGAA TCAACAG	GTGCACAGACTGGTGTACC
TREM2	Triggering receptor expressed on myeloid cells 2	Microglial metabolism and anti-inflammatory activity	NM_018965	CCACCCACTTCCATCC TTCT	GTCCCTGGCTTCTGTCCAT
TSPO	Translocator protein	Neuron-glia interaction (GABAergic system)	NM_000714	TCTGGAAGAGCTGG GAGG	TTGTGCGGCAACCAAGAAG

Target Gene	Full Name	Selection Strategy	Accession Code	Forward Primer	Reverse Primer
Oligodendrocyte					
MBP	Myelin basic protein	Myelin stability and BBB permeability	NM_001025101	AAATGCCGAGAAAGG CCAGTAC	CCCATTGTTCTGGTTTCGCA
MOG	Myelin oligodendrocyte glycoprotein	Myelin sheath maintenance	NM_002433	TGAGAGGGAACCTTC GAGCAG	CGGCACAATTACAAACA GGG
OLIG2	Oligodendrocyte transcription factor 2	Neural repair	NM_005806	GTGTCCAGCGCCTCTCT	GGCAGCAGACGGA GACT
PLP1	Myelin proteolipid protein 1	Myelin formation and maintenance	NM_000533	CCTCACTGGCA CAGAAAAGCT	GGCCCCATAAA GGAAAGAAGAA

Table S2A. Clinico-pathological information (Ctr-BD).

Group	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR1	F	44	28	5	6.59	1330	8.3	R DLPFC, R ACC	3	0	No	0	CARDIAC
CTR2	M	49	46	7	6.5	1605	8.3	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR3	M	53	9	12	6.4	1500	9.1	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR4	M	37	13	3	6.5	1600	8.3	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR5	M	51	31	4	6.7	1400	7.3	R DLPFC, R ACC	1	0	No	0	CARDIAC
CTR6	M	53	28	6	6	1340	8.4	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR7	F	38	33	7	6	1120	9.7	R DLPFC, R ACC	3	0	No	0	CARDIAC
CTR8	F	38	28	9	6.7	1350	8.8	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR9	M	60	47	10	6.8	1460	8.4	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR10	M	35	52	6	6.7	1700	8.7	R DLPFC, R ACC	0	0	No	0	MYOCARDITIS
CTR11	M	34	22	11	6.48	1480	8.2	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR12	M	47	21	1	6.81	1550	8.7	L DLPFC, L ACC	0	0	No	0	CARDIAC

Group	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR13	M	45	29	2	6.94	1405	8.5	R DLPFC, R ACC	1	0	No	0	CARDIAC
CTR14	F	34	24	2	6.87	1255	6.6	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR15	M	42	37	3	6.91	1340	7.8	L DLPFC, L ACC	4	3	No	0	CARDIAC
CTR16	F	44	10	3	6.2	1305	8.4	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR17	M	45	18	4	6.81	1585	8.0	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR18	M	49	23	6	6.93	1390	9.5	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR19	M	32	24	7	7.03	1415	8.3	L DLPFC, L ACC	2	0	No	0	CARDIAC
CTR20	M	55	31	8	6.7	1515	8.3	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR21	F	49	45	9	6.72	1435	8.6	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR22	F	33	29	2	6.52	1360	8.3	L DLPFC, L ACC	0	1	No	0	ASTHMA
CTR23	M	48	31	3	6.86	1580	7.0	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR24	M	50	49	3	6.75	1645	8.0	R DLPFC, R ACC	1	0	No	0	CARDIAC

Group	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR25	M	32	13	8	6.57	1410	9.0	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR26	M	47	11	8	6.6	1495	8.3	L DLPFC, LACC	0	1	No	0	CARDIAC
CTR27	M	46	31	9	6.67	1360	8.6	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR28	M	40	38	11	6.67	1498	8.7	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR29	M	51	22	11	6.71	1900	8.7	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR30	M	31	11	1	6.13	1335	8.4	R DLPFC, RACC	1	1	No	0	PULM EMBOL
CTR31	M	48	24	1	6.91	1321	8.6	R DLPFC, RACC	1	1	No	0	CARDIAC
CTR32	F	39	58	2	6.46	1260	8.4	R DLPFC, RACC	4	0	No	0	CARDIAC
CTR33	M	47	36	6	6.57	1535	8.0	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR34	F	41	50	8	6.17	1290	7.3	R DLPFC, RACC	0	0	No	0	CARDIAC
Median	-	45	28.5	-	6.69	1413	8.40	-	-	-	-	-	-
BD1	M	29	48	12	6.39	1570	9.2	R DLPFC, RACC	3	3	Yes	9000	SUIC;jUMPED
BD2	M	29	60	1	6.7	1430	8.5	R DLPFC, RACC	5	5	No	0	SUIC:CO

Group	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
BD3	M	45	28	1	6.35	1480	9.1	L DLPFC, L ACC		5	Yes	10000	CARDIAC
BD4	M	41	70	1	6.71	1625	7.5	R DLPFC, R ACC	5	4	No	0	SUIC:OD
BD5	F	29	62	2	6.74	1330	7.8	R DLPFC, R ACC	3	5	Yes	0	OD
BD6	M	44	19	10	6.74	1660	9.3	L DLPFC, L ACC	2	1	No	0	SUIC:HANGING
BD7	F	48	18	5	6.5	1205	8.3	R DLPFC, R ACC	1	3	No	0	CARDIAC
BD8	M	42	32	6	6.65	1470	8.4	L DLPFC, L ACC	5	5	No	0	DROWNING
BD9	M	35	35	7	6.3	1490	7.8	R DLPFC, R ACC	1	2	Yes	30000	CARDIAC
BD10	F	59	53	7	6.2	1410	8.5	L DLPFC, L ACC	1	0	No	0	SUIC:OD
BD11	M	54	44	10	6.5	1510	9.2	R DLPFC, R ACC	2	2	No	0	SUIC:OD
BD12	F	35	17	11	6.1	1250	7.9	L DLPFC, L ACC	1	0	Yes	3000	SUIC:CO
BD13	F	42	49	2	6.65	1335	8.0	R DLPFC, R ACC	4	3	Yes	15000	OD
BD14	F	58	35	8	6.5	1440	9.5	R DLPFC, R ACC	1	1	Yes	12000	SUIC:GSW
BD15	M	64	16	6	6.1	1340	7.8	L DLPFC, L ACC	0	0	Yes	130000	PNEUMONIA

Group	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
BD16	M	59	84	7	6.65	1300	7.3	L DLPFC, L ACC	1	0	No	500	SLEEP APNEA
BD17	M	51	23	3	6.67	1590	9.5	L DLPFC, L ACC	3	0	Yes	1200	CARDIAC
BD18	F	63	32	4	6.97	1290	8.9	R DLPFC, R ACC	0	0	No	0	CARDIAC
BD19	F	44	37	5	6.37	1200	7.3	L DLPFC, L ACC	0	0	Yes	30000	MYOCARDITIS
BD20	F	56	26	5	6.58	1170	8.2	R DLPFC, R ACC	5	4	No	25000	DROWNING
BD21	F	43	39	5	6.74	1505	9.3	R DLPFC, R ACC	2	1	Yes	4500	SUIC:OD
BD22	M	35	22	6	6.58	1390	8.5	L DLPFC, L ACC	2	2	Yes	2000	DROWNING
BD23	F	50	62	8	6.51	1400	8.3	R DLPFC, R ACC	1	2	Yes	15000	SUIC:OD
BD24	F	49	38	9	6.39	1190	8.5	L DLPFC, L ACC	5	1	Yes	0	OD
BD25	F	33	24	11	6.51	1450	8.6	R DLPFC, R ACC	5	2	No	3000	SUIC:HANGING
BD26	F	41	28	2	6.44	1360	8.7	R DLPFC, R ACC	4	4	No	3000	CARDIAC
BD27	F	43	57	10	5.92	1340	6.3	R DLPFC, R ACC	4	4	Yes	10000	OD
BD28	M	56	23	2	6.07	1670	9.3	L DLPFC, L ACC	4	0	Yes	10000	SUIC:OD

Group	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
BD29	M	48	23	3	6.9	1466	8.4	R DLPFC, R ACC	5	2	No	0	SUIC:HANGING
BD30	M	19	12	5	5.97	1484	7.9	L DLPFC, L ACC	2	5	No	2000	OD
Median	-	44	33.5	-	6.50	1420	8.45	-	-	-	-	-	-
P value	0.05	0.66	0.13	NS	0.03	0.49	0.76	0.77	-	-	-	-	-

Notes: ACC, anterior cingulate cortex; BD, bipolar disorder; BW, brain weight; CSF, cerebrospinal fluid; CTR, control; DLPFC, dorsolateral prefrontal cortex; F, female; L, left; M, male; MOD, month of death; N.A., not mention; NS, not significant; OD, overdose drugs; PMD, postmortem delay; R, right; RIN, RNA integrity number; SUIC, suicide. Scale of alcohol and drug use: 0, little or none; 1, social; 2, moderate present; 3, moderate past; 4, heavy past; 5, heavy present.

Table S2B. Demographic information (Ctr-BD-NS-BD-S).

	Ctr	BD-NS	BD-S	<i>P</i>
Age (year, range)	45 (31–60)	44 (19–64)	44 (29–59)	0.90
Gender (M/F)	25/9	8/9	7/6	0.14
PMD (hour, range)	28.5 (9–58)	32 (16–84)	39 (17–70)	0.23
Brain pH	6.69 (6.00–7.03)	6.50 (6.10–6.97)	6.51 (6.1–6.74)	0.07
Brain weight (gram, range)	1413 (1120–1900)	1340 (1170–1590)	1466 (1250–1670)	0.01
Hemisphere	16 L/18 R	9 L/8 R	4 L/9 R	0.46
Fluphenazine equivalents	–	12	7	–

Notes: BD-NS, patients with bipolar disorder who died of non-suicidal reasons; BD-S, patients with bipolar disorder who died of suicides; Ctr, control; F, female; L, left; M, male; PMD, postmortem delay; R, right.

Table S2C. Demographic information (Ctr-BD-NP-BD-P).

	Ctr	BD-NP	BD-P	<i>P</i>
Age (year, range)	45 (31–60)	46 (19–63)	43.5 (29–64)	0.83
Gender (M/F)	25/9	8/6	7/9	0.11
PMD (hour, range)	28.5 (9–58)	30 (12–84)	36 (16–62)	0.29
Brain pH	6.69 (6.00–7.03)	6.62 (5.97–6.97)	6.39 (5.92–6.74)	0.02
Brain weight (gram, range)	1413 (1120–1900)	1440 (1170–1660)	1395 (1190–1670)	0.76
Hemisphere	16 L/18 R	5 L/9 R	8 L/8 R	0.70
Fluphenazine equivalents	–	5	14	–

Notes: BD-NP, patients with bipolar disorder but without psychotic features; BD-P, patients with bipolar disorder and psychotic features; Ctr, control; F, female; L, left; M, male; PMD, postmortem delay; R, right.

Table S2D. Demographic information (Ctr-M vs. Ctr-F).

	Ctr-M	Ctr-F	<i>P</i>
Age (year, range)	47 (31–60)	39 (33–49)	0.06
Gender (M/F)	25	9	–
PMD (hour, range)	28 (9–52)	29 (10–58)	0.32
Brain pH	6.70 (6.00–6.94)	6.52 (6.00–6.87)	0.09
Brain weight (gram, range)	1495 (1321–1900)	1305 (1120–1435)	0.0003
Hemisphere	11 L/14 R	7 L/2 R	0.08

Notes: Ctr-F, control female; Ctr-M, control male; L, left; PMD, postmortem delay; R, right.

Table S2E. Demographic information (BD-M vs. BD-F).

	BD-M	BD-F	<i>P</i>
Age (year, range)	44 (19–64)	44 (29–63)	0.62
Gender (M/F)	15	15	–
PMD (hour, range)	28 (12–84)	37 (17–62)	0.35
Brain pH	6.58 (5.97–6.74)	6.50 (5.92–6.97)	0.85
Brain weight (gram, range)	1484 (1300–1670)	1335 (1170–1505)	0.0005
Hemisphere	9 L/6 R	4 L/11 R	0.07
Psychotic features	7	9	–
Suicide	7	6	–
Fluphenazine equivalents (lifetime dosage, mg)	1200 (0–130000)	3000 (0–30000)	0.41

Notes: BD-F, female patients with bipolar disorder; BD-M, male patients with bipolar disorder; L, left; PMD, postmortem delay; R, right.

Table S2F. Demographic information (Ctr-M vs. BD-M).

	Ctr-M	BD-M	<i>P</i>
Age (year, range)	47 (31–60)	44 (19–64)	0.46
PMD (hour, range)	28 (9–52)	28 (12–84)	0.40
Brain pH	6.70 (6.00–6.94)	6.58 (5.97–6.74)	0.03
Brain weight (gram, range)	1495 (1321–1900)	1484 (1300–1670)	0.95
Hemisphere	11 L/14 R	9 L/6 R	0.33
Psychotic features	–	7	–
Suicide	–	7	–
Fluphenazine equivalents	–	9	–

Notes: BD-M, male patients with bipolar disorder; Ctr-M, male control subjects; L, left; PMD, postmortem delay; R, right.

Table S2G. Demographic information (Ctr-F vs. BD-F).

	Ctr-F	BD-F	<i>P</i>
Age (year, range)	39 (33–49)	44 (29–63)	0.11
PMD (hour, range)	29 (10–58)	37 (17–62)	0.53
Brain pH	6.52 (6.00–6.87)	6.50 (5.92–6.97)	0.88
Brain weight (gram, range)	1305 (1120–1435)	1335 (1170–1505)	0.61
Hemisphere	7 L/2 R	4 L/11 R	0.02
Psychotic features	–	9	–
Suicide	–	6	–
Fluphenazine equivalents	–	10	–

Notes: BD-F, female patients with bipolar disorder; Ctr-F, female control subjects; L, left; PMD, postmortem delay; R, right.

CHAPTER 4

SEX DIFFERENCES IN BIPOLAR DISORDER: THE DORSOLATERAL PREFRONTAL CORTEX AS AN ETIOPATHOGENIC REGION

Zhang L and Swaab DE.

Under review.

ABSTRACT

Bipolar disorder (BD) is a prevalent mental illness worldwide and a leading risk factor for suicide. In recent three decades, it has been discovered that sexual dimorphisms exist throughout the panorama of BD, but the etiologic regions and mechanisms that generate such dimorphisms remain poorly characterized. As an aberrant mental state, a history of psychotic features in BD patients has been associated with a series of epidemiological and clinical features, which have been identified as high-risk factors for suicide, particularly in women. Available evidence strongly indicates that the dorsolateral prefrontal cortex (DLPFC), a critical region that controls higher-order cognitive processing and mood regulation, has provided biological disparities between male and female patients with BD, which are highly correlated with the co-occurrence of psychotic symptoms. To speculate about the potential connection between the DLPFC and sexual dimorphisms, this review addresses evidence on sex differences in BD concerning epidemiology, cognitive impairments, clinical manifestations, neuroimaging abnormalities, and neurobiological factors, indicating that the DLPFC is a key structure for the explanation of the biological differences between males and females in BD. We highlight the need to address the transcriptomic profiles in the DLPFC of patients, regarding the patterns of divergence between individuals with BD and non-psychiatric controls. By integrating bulk RNA sequencing and single-nucleus RNA sequencing, a comprehensive profile can be portrayed to delineate the sexually dimorphic differences, explain sex-biased symptomatic features, and provide novel targets for sex-specific therapeutics.

Key words

Bipolar disorder; sex differences; dorsolateral prefrontal cortex; psychotic features

INTRODUCTION

Bipolar disorder (BD) is a severe mood disorder that is categorized as extreme mood swings that include emotional highs (mania or hypomania) and lows (depression). According to the intensity of mood elevation, bipolar I disorder (mania) and bipolar II disorder (hypomania) are two major forms of BD. In 2019, over 40 million people experienced BD (Evaluation). Approximately half to 70% of BD patients develop psychotic features during their lifetime (Van Bergen et al., 2019). Such a history has been associated with earlier onset of BD, more frequent hospitalizations, severer cognitive impairments, lower response to lithium, and worse psychosocial prognosis, particularly for manic episodes (Glahn et al., 2007; Maj, 2003; Maj et al., 2002; Van Bergen et al., 2019). As a major hazard of psychiatric illnesses, life expectancy in patients with BD has been reported to be decreased by 10 to 20 years, with suicide serving as the primary culprit (Kessing et al., 2015). Suicidal behaviors are prevalent among patients with BD, as 20% to 60% of them attempt suicide at least once in their lifetime (Rihmer et al., 2017). The incidence of suicide death among patients with BD can be more than 20 times higher than in the general population, particularly when untreated (Grande et al., 2016). In addition, the lethal index, a parameter defined as the ratio of suicidal attempts to suicide completion, is more than 10 times lower for BD patients (3:1) than for the general population (35:1) (Dome et al., 2019).

A distinct feature that underpins the high suicidality in BD is its sex difference. Among bipolar patients, suicide attempters were 35% more frequent in women than in men, regardless of the diagnostic subtypes (Tondo et al., 2016). When patients had manic episodes, women were 2.4 times more likely to commit suicide than men (Bhattacharya et al., 2011). This gender-related suicide rate was up to 3 times higher in female patients with first-episode psychosis than in male counterparts, which could be remitted after therapeutic interventions (Chang et al., 2011; Tseliou et al., 2017). These data suggest that the presenting manic episode and psychotic features are major risk factors for suicide. The higher prevalence of suicide rate in females with BD, and the observation that suicidality increased with the development of psychotic symptoms and was reduced after medical care, have led to the hypothesis that unraveling the neurobiological basis of sex differences in BD, particularly in patients with psychosis comorbid (bipolar psychosis), may provide novel therapeutic targets for extending the lifespan of BD populations.

The main pathophysiological mechanisms underlying BD are considered to be an immune/inflammatory-mediated alteration in neurotransmitter signaling involving the limbic network (Magoncalda and Martino, 2022). The limbic circuits are, therefore, key loci to search for biological evidence for sex differences underpinning BD. This review explores the literature on sex differences in BD with respect to epidemiology, clinical presentations, and brain region-specific genetics. As major specifiers of BD, the sex differences in psychotic features were together discussed. Of note, literature concerning psychotic features in schizophrenia is excluded in this review, in order to decrease the biological homogeneity of psychosis. Supported by these human

studies, we conclude that the dorsolateral prefrontal cortex (DLPFC) is a putative brain region with respect to the pathophysiological contexts in BD research.

SEX DIFFERENCES IN THE EPIDEMIOLOGY OF BIPOLAR DISORDER

Sex dimorphisms in BD displayed distinct patterns in terms of the incidence and the longitudinal course. According to epidemiologic and clinical studies, the prevalence of BD is approximately equal between the genders in the general population but is twice higher amongst female as amongst male adolescents (Mitchell et al., 2018). Seasonality is also a well-documented feature. A population-based study has shown that, in admissions, the seasonality in BD was significant in females for depressive episodes that peaked in winter, and in males for manic episodes that peaked in spring and summer (Yang et al., 2013). The same study also presented data showing that young adults displayed a higher degree of seasonality for acute admissions than middle-aged adults, which indicated that age-related sex hormones might act on the pathogenesis of seasonality in BD (Yang et al., 2013). Compared to female patients, male patients experience the onset of symptoms earlier, including the development of mood, mania, and hospitalization (Kennedy et al., 2005; Mitchell et al., 2020).

The sex difference in epidemiological vulnerability can be even complicated when specific psychiatric conditions, e.g., psychosis or early life adversity, are comorbid. Although the overall risk of psychosis was higher in men than in women, the incidence may vary according to their subtypes. For example, a meta-analysis that identified 33 reports has shown that, compared to patients with non-affective disorders, females with mood disorders have a nearly two times higher incidence of psychosis than male counterparts (Castillejos et al., 2018). On the contrary, among patients with psychotic features, the incidence of BD among women was 6.6-fold lower than among men (Scully et al., 2002). In comparison with the general population, male psychotic patients were more exposed than females to childhood physical abuse and developed more legal issues in adulthood, whereas both childhood sexual abuse and recent life events were overrepresented among female psychotic patients (Mansueto and Faravelli, 2022; Muntaner et al., 1998). This evidence indicates that women with a pre-existing diagnosis of BD or psychological trauma are more prone to develop psychotic symptoms than men.

SEX DIFFERENCES IN THE COGNITIVE IMPAIRMENT OF BIPOLAR DISORDER

Cognitive deficits are strongly associated with a worse course and outcome in BD, which presents as complex sexual variabilities. When compared to healthy controls, BD patients exhibit three main forms of gender-biased cognitive dysfunctions. First, BD disrupts sex-related variations as they are present in healthy controls. For example, clinical analyses of the neurocognitive variables have shown that healthy women outperformed healthy men in the emotional and affective aspects of social cognition, which was absent in BD patients (Navarra-Ventura et al., 2021). On the other hand, male controls were significantly better at performing spatial working memory tasks than female controls, which also disappeared as the disorder progresses (Barrett et al., 2008; Solé et al., 2022; Tournikioti et al., 2018). Second, BD has specific sex-related variations in neurocognitive deficits. In the early phase of BD, for example in first-diagnosed and drug-naïve patients, males had worse cognitive dysfunctions compared to females in verbal learning, attention, and immediate and delayed memory (Carrus et al., 2010; Solé et al., 2022; Xu et al., 2021). Third, in one sex-only comparison, BD patients have significant cognitive disturbances compared to the controls of the same gender, which is absent in the other gender. It has been reported that male BD patients had poorer spatial working memory scores than male controls, which was insignificant in female patients (Suwalska and Łojko, 2014). In addition, the presence of gender-biased cognitive deficiencies may be strongly associated with the development and prognosis of mania. It has been shown that cognitive deficits, e.g., worse attention and delayed memory performance, were correlated with mania severity only in BD males (Xu et al., 2021). Following their recovery from mania, these patients may regain the gender differences in cognitive functions that are shown in non-psychiatric controls (Bücker et al., 2014). Taken together, male patients with BD have lost their cognitive strengths that appeared in healthy controls and developed more severe early symptoms, and their cognitive deficits were associated with the severity of manic symptoms. These features are insignificant in female patients.

Cognitive impairment is also a key feature of psychosis and is expected to be a treatment target in early intervention. Individuals who are at a high-risk mental state for psychosis have already developed gender-biased differences in cognitive functions. For example, men at ultra-high risk for developing a psychotic disorder exhibited significantly poorer social functioning and more severe asociality of negative symptoms than women (Willhite et al., 2008). At the early phase of psychosis, patients can still retain some sex-related cognitive abilities occurring in control populations. Large-cohort cognitive functioning analyses have shown that, from healthy control subjects to patients with first-episode psychosis, women generally performed better in verbal learning and memory than men (Ittig et al., 2015). However, parts of sex-associated cognitive functions, such as better auditory attention observed in control females than in control males can be disrupted by the aggravation of psychotic symptoms (Ruiz-Veguilla et al., 2017).

Early life adversity also plays a negative role in cognitive deterioration in male patients. In the first episode and early-onset psychosis, men had greater impairments of their premorbid functioning, which was associated with sex differences in childhood trauma experience (Fresan et al., 2003; Good et al., 2007; Pruessner et al., 2019; Smelror et al., 2021).

Compared to male patients, cognitive impairments in female psychotic patients are mainly focused on internal functions. Clinical observations have reported more severe psychotic symptoms in females than males, especially in perceptual abnormalities and lowered self-certainty (Hu et al., 2022; Penney et al., 2020). Among adult-onset psychosis patients, women reported a significantly increased emotional reactivity to daily life stress compared to men, which is shown by both, an increase in negative affect and a decrease in positive affect (Myin-Germeys et al., 2004). They also underwent more profound deterioration in personal identity than men, including higher dejection-related emotions and lower facial emotion recognition (Andric Petrovic et al., 2019; García-Mieres et al., 2020). In bipolar psychosis, the aforementioned gender differences became more complicated. Among these patients, men were more likely than women to exhibit suicidal ideations and hallucinatory behaviors, which was the opposite when psychotic symptoms were absent (Dell'Osso et al., 2021).

SEX DIFFERENCES IN THE CLINICAL MANIFESTATIONS OF BIPOLAR DISORDER

The clinical features of BD are covered by differences between the sexes. Men and women with BD differed in the episodes at the onset. The first episode in males was mostly mania, whereas the majority of females had a first episode of depression (Pillai et al., 2021). Throughout their lifespan, the number of manic episodes was significantly more in BD males, while female patients presented a higher rate of rapid cycling, depressive polarity, mixed episodes, and suicide attempts (Dell'Osso et al., 2021; Pillai et al., 2021). Therefore, bipolar II disorder, which is predominated by depressive episodes, also appears to be more common in women than in men (Arnold, 2003). Interestingly, the occurrence of depression and mixed symptoms were associated with the presence and severity of hypomania in women (Suppes et al., 2005).

In addition, the two sexes differ in the type of comorbid illnesses. Female patients with BD have a much higher burden of medical comorbidity and hazard of recurrence than male patients. With respect to physical diseases, women with BD had higher odds of comorbid metabolic disorders and inflammatory diseases than males (Patel et al., 2018). One exception is the prevalence of hyperhomocysteinemia, which is higher in male than in female patients (Mu et al., 2020). In view of psychiatric disorders, males were more likely to present with obsessive-compulsive disorder, substance abuse, pathological gambling, and conduct disorder (Benedetti et al., 2007; Kawa et al., 2005). Subsequently, more men than women patients had a history of

legal problems (Baldassano et al., 2005). Less female than male patients develop substance use disorder, partially because the heavy load of some long-term substance use, such as cannabis intake, has worsened the quality of life in BD females, particularly on bodily pain and mental health subscales (de La Fuente-Tomas et al., 2020). These health harms are not obvious in male patients (de La Fuente-Tomas et al., 2020). However, female patients have a much higher likelihood of combining post-traumatic stress disorder, panic disorder, and anxiety disorders compared to males (Benedetti et al., 2007; Patel et al., 2018). Female BD patients are older than males at clinical intake but have more frequent psychiatric visits, longer inpatient length, and more inpatient episodes (Buoli et al., 2019; Fellingner et al., 2018; Mitchell et al., 2020; Ragazan et al., 2019).

Gender differences in the clinical features of psychosis already appear when people are at high risk or experiencing subclinical psychosis (Schultze-Lutter et al., 2020; Stainton et al., 2021). Research suggests that men at the stage of clinical high risk for psychosis displayed more pronounced negative symptoms, higher rates of past substance abuse disorders, and higher deficits in social functioning than women (Rietschel et al., 2017). Such male-biased gender differences persist throughout the process of psychosis. In general, men have an earlier onset of psychotic symptoms than women. In first episode psychosis, more women have affective and positive symptoms and past suicide attempts, but they have higher levels of cognitive functioning than men (Chang et al., 2011; Pruessner et al., 2019). Childhood trauma is strongly associated with the gender differences in the clinical manifestations of first episode psychosis in both sexes. Drastic early life trauma has been related to severe depressive symptoms with a reduced age of onset in women and to the presence of psychotic symptoms in men (Comacchio et al., 2019; Pruessner et al., 2019). Particularly in male patients, emotional abuse predicted positive symptom severity and impaired global functioning, while emotional neglect predicted more severe negative symptoms (Pruessner et al., 2019). Patients with early onset psychosis presented with similar sex differences as in first-episode psychosis in adults (Carter et al., 2022; Garcia et al., 2016). In adult-onset psychotic features, women manifested a greater admixture of symptoms than men, especially concerning mixed affective symptoms and less aggressive behaviors (Bachetti et al., 2020; Copolov et al., 1990). Of note, substance-dependent women are more vulnerable than their male counterparts to report psychotic symptoms (Mahoney III et al., 2010). However, in men who were diagnosed with psychotic disorders, there was a higher subsequent comorbidity of substance abuse, indicating that the signal pathways and molecular mechanisms between addiction and psychosis might be opposite between genders (Pence et al., 2022). Compared to depressive episodes, there seems to be a sex-biased connection between manic episodes and psychosis. Among individuals in a pure mania episode, men had significantly increased and worse psychotic symptoms than women (Miquel et al., 2011). However, in patients at acute manic episodes, women exhibited a specific pattern of psychotic features, appearing to be associated with greater severity of the acute episode, more mixed states, and a more severe

course of BD (Bräunig et al., 2009). A specific molecular basis may have underpinned these sex differences among bipolar patients who develop psychotic symptoms.

HORMONAL ORIGINS OF SEX DIFFERENCES IN BIPOLAR DISORDER

The higher prevalence of bipolar II/hypomania, rapid cycling, and mixed episodes, as well as an earlier age of mania or depression onset in women than men during the reproductive years is thought to result from the differential effects of gonadal hormones. Epigenetic modifications establish and maintain sex differences. A study has examined the association of over 14,000 X-chromosome single nucleotide polymorphisms (SNPs) with sex-associated BD traits in almost 2,000 samples and discovered that the X-chromosome variant rs5932307 is associated with BD with a stronger effect in females than males (Jons et al., 2019). Gonadal steroid hormones determine sex differences. Evidence has been accumulating to support the relationship between hormonal oscillations due to menstruation, reproduction, and menopause and the course of BD (Gogos et al., 2019). Therefore, a link between female sex hormones (progesterone and estrogens) and the pathways of neuroprotection/neurodegeneration/ neuroinflammation may be implicated in the psychobiological mechanisms of BD in women (Frey and Dias, 2014). It has been reported that euthymic BD women had increased circulating progesterone and allopregnanolone (an active metabolite of progesterone) during the luteal phase of the menstrual cycle (Hardoy et al., 2006). A small clinical trial also highlighted the efficacy of using synthetic progesterone for the treatment of acute mania (Kulkarni et al., 2014).

As a major product of progesterone, cortisol, one of the key stress hormones, has distinctly different responses in BD between the genders. In men, the release of cortisol after mental challenges was blunted in BD patients compared to non-psychiatric controls, while in female patients, the cortisol fluctuations were associated with the number of depressive episodes (Steen et al., 2011). Since the immune system response to stress can be suppressed by cortisol, inflammatory cell components in peripheral blood have also presented gender differences. For example, the neutrophil-to-lymphocyte ratio is regarded to be a peripheral biomarker of (hypo) mania, however, its usefulness seemed to be exclusive to men, as the same role of platelet values exclusive to women (Fusar-Poli et al., 2021). In response to inflammatory cells, the C-reactive protein that modulates cognition and real-world functioning further showed sex-specific associations. In males with BD, the C-reactive protein level in peripheral blood was related to psychosocial dysfunction (interpersonal relationships and financial functioning), while in their female counterparts, it was correlated with cognitive performance (immediate and delayed verbal learning, and verbal fluency) (Sanchez-Autet et al., 2018).

The potential associations between corticosteroids, gonadal hormones, and gender differences in psychotic features are worth further discussion. In the psychosis-risk period,

males, but not females, who carried a catechol-O-methyltransferase (COMT) polymorphism rs4680 are more prone to develop psychotic-like symptoms (de Castro-Catala et al., 2015). It has been proven that the COMT polymorphisms may alter the HPA axis function and cortisol secretion (Oswald et al., 2004). Male patients with first-episode psychosis showed significantly lower cortisol awakening response (CAR) compared to male controls and female patients, which was related to the presence of positive symptoms and functional deficits (Pruessner et al., 2015). On the other hand, females with early psychosis had an increased CAR, which was related to poorer cognitive performance such as speed and verbal memory (Labad et al., 2016).

Elevated plasma prolactin in psychosis is another biological marker that shows sex-dependent characteristics in clinical symptoms. In women with emerging psychosis, the increased prolactin level was related to enhanced stress reactivity and higher vulnerability to developing psychotic features, possibly due to the modulation of progesterone and estradiol (Camilletti et al., 2019; Ittig et al., 2017). As an adverse drug event during psychosis treatments, the incidence of hyperprolactinemia was more frequent in women than in men (Sørup et al., 2020). Compared to female patients who presented prolactin fluctuations in the emerging phase, males with first episode early psychosis had higher prolactin levels and experienced more severe symptoms, impaired cognitive processing, and more intensive treatments (Hidalgo-Figueroa et al., 2022; Montalvo et al., 2018). The biological connections of cortisol and prolactin to psychosis displayed two gender-specific patterns, suggesting that psychotic features might drive distinct molecular systems depending on sex (Montalvo et al., 2018).

MAJOR PATTERNS OF SEX DIFFERENCES IN BIPOLAR DISORDER

Sexual dimorphisms penetrate every pathophysiological aspect of BD. In the literature, two main categories of sex differences in BD became apparent (**Figure 1**). It should be noted that other psychiatric disorders may potentially display comparable patterns. One is the diagnosis-based divergences between genders, such as physical gender differences that were present in non-psychiatric controls disappeared in individuals with BD, or vice versa (**Figure 1a** and **1b**). The second type shows gender-based divergences between BD and controls, which can be divided into six subsets. First, only one gender shows alterations in biological hallmarks in BD compared to the controls (**Figure 1c**). Second, the two genders show opposite alterations in one biological symbol in BD compared to the controls (**Figure 1d**). Third, with the emergence of a particular comorbid symptom, pre-existing sex differences in BD can be reversed (**Figure 1e** and **1f**). Fourth, two interrelated factors were altered in both genders in BD, but their sequences of occurrence are switched between the two genders (**Figure 1g**). Fifth, when compared to controls, both genders displayed the same changes in BD, but these changes were linked to different clinical symptoms in each gender (**Figure 1h**). Sixth, both genders displayed the same

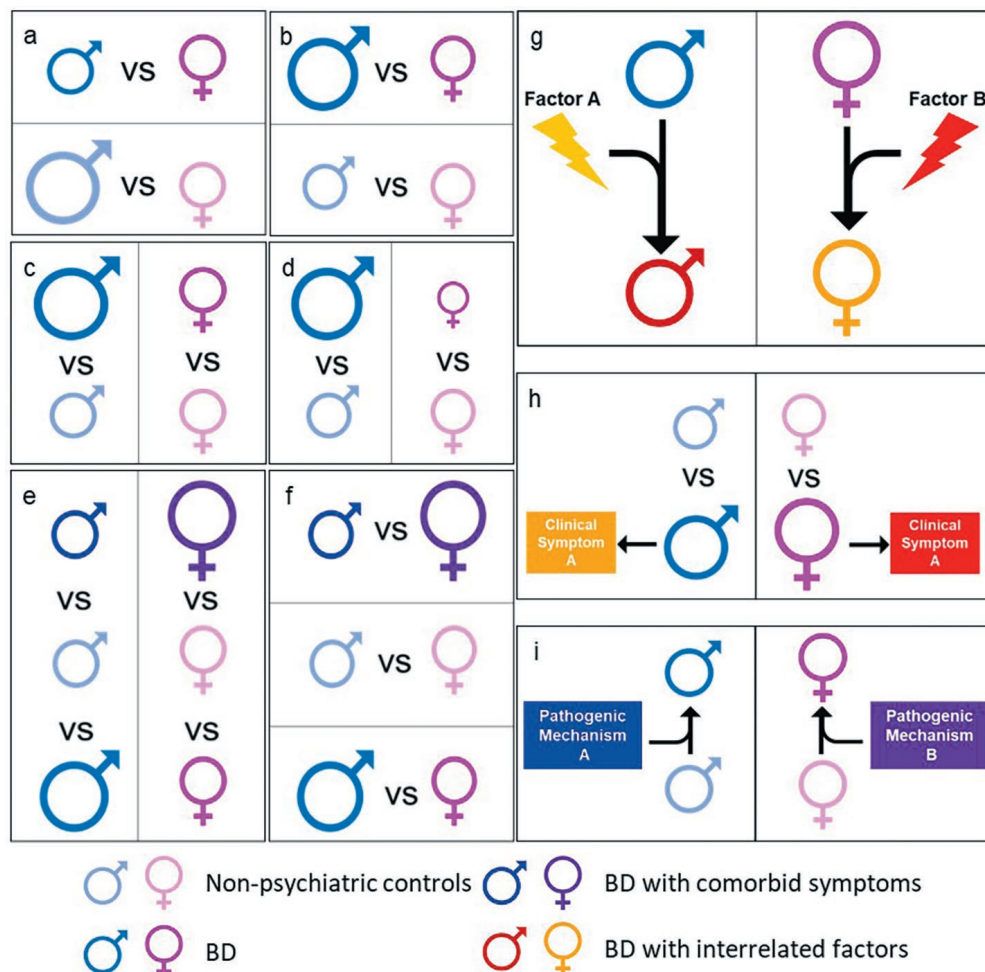


Figure 1. Major patterns of sex differences in bipolar disorder. Nine forms of sex differences are summarized as follows: **(a)** Physical gender differences that were present in non-psychiatric controls disappeared in individuals with BD. **(b)** Gender differences that were present in BD were absent in non-psychiatric controls. **(c)** Only one gender shows alterations in biological hallmarks between patients with BD and the controls. **(d)** The two genders show opposite alterations in one biological symbol between patients with BD and the controls. **(e)** and **(f)** Pre-existing sex differences in BD can be reversed when a particular comorbid symptom emerges. **(g)** Two interrelated factors were altered in both genders in BD, but their sequences of occurrence are switched between the two genders. **(h)** When compared to controls, both genders displayed the same molecular changes in BD, but these changes were linked to different clinical symptoms in each gender. **(i)** Both genders displayed the same alteration in BD, but they were driven by different pathogenic mechanisms.

alterations in BD, but they might be driven by different pathogenic mechanisms (**Figure 1i**). However, the molecular mechanisms stem from etiological cell types and key brain regions that explained the sex differences in BD, which provides essential evidence for divergent therapeutics between genders, are sparsely reported.

DORSOLATERAL PREFRONTAL CORTEX: A SEX-DEPENDENT ETIO-PATHO-GENIC REGION IN BIPOLAR DISORDER

Sex-associated cognitive and clinical alterations in psychiatric disorders have recorded their biological fingerprints in the human brain. Structural and functional neuroimaging studies have proposed that the DLPFC is a promising region involved in the etiopathogenesis of sex differences in BD. Recent studies have investigated individuals with BD by functional magnetic resonance imaging (fMRI), focusing on sex-by-diagnosis interactions on patterns of brain activation obtained during tasks of working memory, incentive decision-making, and facial affect processing (Jogia et al., 2012). They demonstrated that the prefrontal regions, which were integrated into the neurocircuits that regulated emotion and reward, were implicated in the crosstalk between gender and diagnosis in BD (Jogia et al., 2012). In addition, by measuring the gray matter volume with structural MRI, male patients, rather than female patients, showed that normative sex differences in the brain structures involved in the prefrontal network may be disrupted in BD (Mitchell et al., 2018). A resting-state fMRI study further discovered that, so far, the DLPFC was the only cerebral structure that had a main effect on sex-by-diagnosis interactions involved in the dysfunction of the cortico-limbic neural system in BD (Jiang et al., 2021). The specific role of the DLPFC seems to be preserved even when psychotic features are comorbid. Structural neuroimaging studies of voxel-based morphometry have detected significant reductions in the gray matter of fronto-temporo-limbic structures in subjects at vulnerable states for psychosis, as well as the role of prefrontal abnormalities in relation to early risk and subsequent disease transition (Koutsouleris et al., 2009), which has been shown to be more pronounced in male patients than female patients (Rapado-Castro et al., 2015).

A related line of evidence supports the relationship between gender and BD therapies. In clinical practice, treatments with repetitive transcranial magnetic stimulation (rTMS) over the DLPFC are becoming promising options for patients with BD. A meta-analysis of 54 sham-controlled studies published across 16 years has shown that the antidepressant properties of rTMS may depend on sex (Kedzior et al., 2014). Recent studies further investigated the clinical and neuroimaging biomarkers associated with responsiveness to high-frequency rTMS over the DLPFC in a large cohort of patients with treatment-resistant depression, showing that women than men, and patients with BD than patients with major depressive disorder (MDD) are proven to respond better (Harika-Germaneau et al., 2022). More evidence also indicated

that these region-specific gender differences may be preferably sensitive to BD versus MDD or schizophrenia (Huber et al., 2003; Maniam et al., 2020; Zhang et al., 2020a; Zhang et al., 2021).

An obvious explanation for the gender differences mentioned above is the sex-specific genetic factors underlying the DLPFC of BD. In the largest genome-wide genotype-by-sex analysis of mood and psychotic disorders to date, significant sex-dependent effects were found to be enriched for genes related to immune, neuronal development, and vascular functions in BD (Blokland et al., 2022). Our transcriptional analysis additionally validated this hypothesis by measuring the expression of glial genes in the DLPFC of BD versus the controls (Zhang et al., 2020b). A remarkable sex difference was observed that most were expressed at significantly higher levels in male patients versus female patients, including all three glial cell types (astrocyte, microglia, and oligodendrocyte) that were involved in a wide range of biomechanisms. A subset analysis additionally showed that the sex differences were closely correlated with the presence of psychotic features. In addition, evidence also indicated that the significant glial markers could be related to suicide death or even independent of confounding factors. These findings implied that dysfunctional genomics is stronger in males than females with BD, which may explain the clinically well-known sexual dimorphisms.

Existing publications have provided substantial evidence supporting the molecular basis of sex difference in the DLPFC of BD patients, including but not limited to serotonin and dopamine transmission (aldehyde dehydrogenase 1 family member L1, *ALDH1L1*), purine (purinergic receptor *P2RY12*) and lipid metabolism (triggering receptor expressed on myeloid cells 2, *TREM2*, proteolipid protein, *PLP*), and cell proliferation. In addition, prolactin may be a pleiotropic factor in distinguishing BD males from females on glial expression, especially when psychotic features are comorbid. It has been shown that prolactin modulates glial cell proliferation by acting on its prolactin receptors expressed in glia (Anagnostou et al., 2018; Gregg et al., 2007; Ramos-Martinez et al., 2021). The higher level of prolactin in male versus female psychotic patients has suggested its effect on a general upregulation of glial cells in the DLPFC.

In mechanistic genomics, we compiled a number of scenarios that contributed to these sex dimorphisms. For example, the risk for BD has gender-specific associations with the *serotonin transporter-linked polymorphic region gene (5-HTTLPR)*, depending on its specific variants. In males with BD, only the ll (+) polymorphism was found to be associated with the *dopamine D2 receptor* variant (Wang et al., 2014), while in female counterparts, the ss genotype was associated with higher impulsivity severity (Boscutti et al., 2022). Compared to the ll genotype which can express sufficient serotonin transporters (5HT-T), it has been shown that the ss genotype expresses less 5HT-T on the presynaptic sites (Shinozaki, 2012). Consequently, excess extracellular serotonin may have been absorbed by glial cells in BD females that mediate programmed glial cell death (Persico et al., 2003; Shinozaki, 2012). In addition, linkage and association studies have implicated that the dopamine transporter gene *DAT1* in the etiopathophysiology of BD

is specific to males, indicating gender dimorphism with respect to predisposing polymorphic alleles and susceptibility to affection (Ohadi et al., 2007).

Available evidence suggests a special involvement of purinergic metabolism in sex differences in bipolar patients. A nuclear magnetic resonance-based metabonomic study has measured the urinary metabolite biomarkers of BD patients versus non-psychiatric controls and found that male and female BD patients have distinct biomarkers in urine, e.g., choline and formate in males versus oxalacetate and acetone in females (Chen et al., 2014). Such increased anabolism of choline and formate in male patients may elevate the concentration of tetrahydrofolate for purine synthesis, which is in line with our finding that the microglia-expressing purinergic receptor P2RY12 has a higher expression in the DLPFC of males versus females with BD (Lamarre et al., 2013; Zhang et al., 2020b). Additional evidence refers to a large cohort genotyping study that the effects of purinergic receptor *P2RX7* variants (rs1621388 and rs2230912) on BD may be sex-specific, with increased P2X7 activity substantially elevating the risk for BD only in females (Winham et al., 2019).

Cognitive impairments in women with BD have been related to GABAergic deficits. It has been suggested that low plasma GABA was a trait-related feature in episodes of BD (Petty et al., 1995). A clinical observation discovered that pretreatment plasma GABA was negatively correlated with the severity of manic symptoms, which was stronger in women (Swann et al., 1999). As a major component that is strongly expressed in GABAergic interneurons, Reelin expression is decreased in the prefrontal cortex of BD (Torrey et al., 2005; Vilchez-Acosta et al., 2022). Genotyping analyses have provided preliminary support that genetic variation in Reelin is associated with susceptibility to BD and, in particular, to BD in females (Goes et al., 2010). The major types of glial cells are known to express functional metabotropic and ionotropic GABA receptors (Fraser et al., 1994; Liu et al., 2016; Serrano-Regal et al., 2020). Such an overall decrease in GABAergic signaling in neuron-glia interaction may be linked to the less expression of glia-related genes in women with BD.

Clinical symptoms and peripheral biomarkers of people with BD differ by gender, yet there are discrepancies. As opposed to female patients who are more susceptible to concomitant metabolic diseases, males with BD have shown overall altered biosignatures in peripheral blood toward metabolic risks. A sex-specific genetic association has been found between the polymorphisms of D-element binding protein, a candidate genetic marker for metabolic risk, and the severity of metabolic syndrome or obesity in male patients with BD (Kim et al., 2016). Plasma profiles of lipids have reported that, compared to female patients, male patients with BD had a higher prevalence of disturbed lipid metabolism, also indicating a metabolic involvement underlying the BD etiology (Vemuri et al., 2011). Similarly, this relationship between metabolic factors and psychiatric symptoms in individuals with first-episode psychosis was sex-dependent, as a female-specific negative correlation was found between serum high-density lipoprotein levels and negative symptoms (Gjerde et al., 2021). The peripheral biosignatures of oxidative stress,

which were implicated as both causative and consequential to concurrent metabolic disorders in adults with BD, are found to be significantly higher in BD males than their female counterparts (Bengesser et al., 2015). Therefore, further evidence is needed to determine whether these sex-related changes were brain-derived.

A FUTURE PERSPECTIVE

Evidently, the limited knowledge merely provides a glimmer of explanation for the existing sex-dependent impact on bipolar patients, particularly on bipolar psychosis patients. An observation of the gene expression patterns and their interaction networks at the cellular and subcellular level is therefore essential for in-depth sex-directing cell biology in BD. Only a few studies have found transcriptional or proteomic signatures for sexual dimorphisms and affective pathways with respect to human samples from donors with psychiatric disorders, with the majority of the findings being related to MDD or schizophrenia (Lobentanzer et al., 2019; Maitra et al., 2022; Talishinsky et al., 2022; Tiihonen et al., 2019). Our previous findings have strongly indicated that the pathophysiology of BD differs between males and females, emphasizing the necessity to gain basic knowledge of specific molecular changes. Given recent technical innovations, sex-related cerebral transcriptomics resulting from BD-perturbed biological networks can be captured with advanced measurement techniques such as bulk RNA sequencing (bulk RNA-seq) and single-nucleus RNA sequencing (snRNA-seq). However, there is no one-size fits all approach due to the low sensitivity of snRNA-seq data and the reduced specificity of bulk RNA-seq (Barrett et al., 2022).

Earlier bulk RNA-seq analysis of DLPFC specimens from BD patients has put forward dysregulation of neuroplasticity, circadian rhythms, and GTPase binding as putative target pathways for therapeutic intervention (Akula et al., 2014). Yet, such tissue-level approaches do not provide insights into the putatively differential involvement of distinct cellular (sub) populations in these observations. Moreover, altered cell type composition may impact the observed gene expression changes between BD and control individuals (Ramaker et al., 2017). Employing snRNA-seq, significant associations with BD genetic risk were recently reported in excitatory, layer-specific DLPFC subpopulations, providing initial proof-of-concept that distinct cell type-specific molecular signatures may differentially contribute to BD pathophysiology (Tran et al., 2021). Of note, none of the studies described here has assessed the dependency of these findings on gender-specific variables; reversely, female subjects have been even underrepresented (Ramaker et al., 2017). Following such strategies, the differential expression patterns of sex-related transcripts in the DLPFC of BD patients and their matched controls can be profiled and analyzed (**Figure 2**). Apart from the promising gender differences to be reported

between males and females with BD, other three types of differential gene expression can be examined (Figure 2).

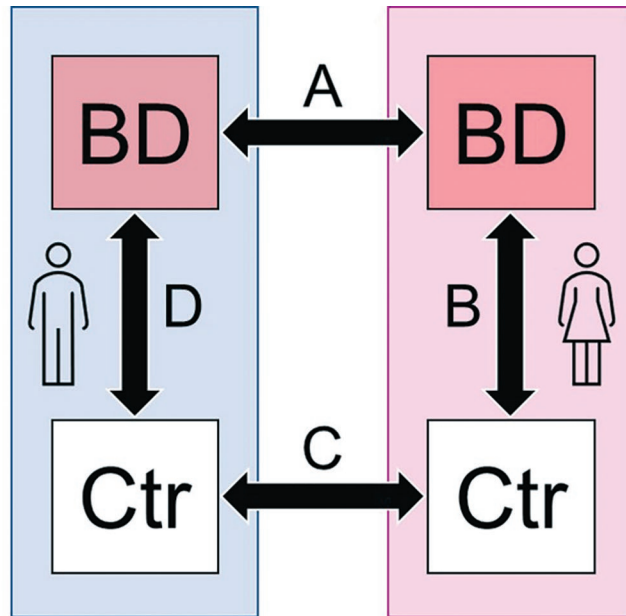


Figure 2. Divergent transcriptomes in the dorsolateral prefrontal cortex in males and females with BD Differential gene expression results were dually compared: BD versus Ctr or male versus female: (A) BD-biased genes diverge between males and females. (B) Female-biased genes diverge between BD and Ctr. (C) Ctr-biased genes diverge between males and females. (D) Male-biased genes diverge between BD and Ctr.

CONCLUSIONS

Due to the asymmetry in research intensity between the clinical and biological literature that has controlled for sex, at present, we could merely provide our speculative explanation for the origin of gender disparities in clinical symptoms and cognitive impairments in BD. While there are highly promising preliminary findings, research on the pathophysiology of BD has yet to fully analyze the confounding factors, particularly psychotic features and suicide in relation to gender. Transcriptomic approaches to studying the neurobiology of BD in both sexes have been discussed; these techniques place a strong emphasis on discovering the key cells and molecules across psychiatric diagnoses as well as comorbidities based on diverse clusters of symptoms.

Despite the fact that the effect of female sex hormones and neurosteroids on BD is beginning to be understood, studies should evaluate treatments in terms of women's hormonal fluctuations, such as entering puberty, menstrual cycle stage, menopausal stage, or use of hormonal contraception. It should be clinical and laboratorial consensus that, in BD research at multi-dimensional levels, data are analyzed including sex as a confounder.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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CHAPTER 5

PREFRONTAL CORTEX ALTERATIONS IN GLIA GENE EXPRESSION IN SCHIZOPHRENIA WITH AND WITHOUT SUICIDE

Zhang L, Verwer RWH, Lucassen PJ, Huitinga I, Swaab DF.

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ABSTRACT

Background: Patients with schizophrenia (SCZ) run a lifelong risk of suicide. Alterations in glia activities in the prefrontal cortex (PFC) have been reported in relation to suicide in patients with SCZ. While immune processes in the CNS have been related to the susceptibility and course of SCZ, there are hardly any direct comparisons between individuals with SCZ, both those who died of natural causes and those that committed suicide, and healthy controls.

Materials and Methods: We compared mRNA expression using real time qPCR of 16 glia-related genes in the dorsal lateral prefrontal cortex (DLPFC) and the anterior cingulate cortex (ACC) between 35 patients with SCZ (7 suicide completers and 28 patients who died of natural causes) and 34 well-matched controls without psychiatric or neurological disease.

Results: We found an increased expression of the astrocytic gene aldehyde dehydrogenase-1 family member L1 (ALDH1L1) mRNA, a marker involved in dopaminergic activity, in SCZ versus controls. Excluding individuals with SCZ that committed suicide resulted in an elevated expression in the DLPFC of both ALDH1L1 and glutamine synthetase (GS) genes in patients with SCZ, compared to suicide completers and non-psychiatric controls. Regarding microglia genes: in the ACC, homeostatic markers such as chemokine (C-X3-C motif) ligand 1 (CX3CR1) mRNA expression was increased in SCZ without suicide as compared to suicide completers, while no change was found when compared to controls. Another, purinergic receptor 12 (P2RY12) mRNA was exclusively elevated in the ACC of suicide completers, compared to either other group. Triggering receptor expressed on myeloid cells 2 (TREM2) expression, which maintains microglial metabolism, was reduced in non-suicide patients with SCZ, compared to suicide victims and control subjects.

Conclusions: Differential changes are found in astrocyte and microglia genes in PFC subregions in relation to schizophrenia and suicide, indicating possible disturbances of glia homeostasis in these conditions.

Key words

Schizophrenia; prefrontal cortex; glia; suicide

INTRODUCTION

Patients with schizophrenia (SCZ) run a high risk of committing suicide. Although the 5–15% incidence of completed suicides in individuals with SCZ is lower than that of major depressive disorder (MDD) and bipolar disorder (BD) patients (respectively 15% and 25%), 20–40% of the individuals with SCZ had suicidal ideations and did one or more attempts at suicide (Association, 2013; Bachmann, 2018; Ko et al., 2018). In terms of suicide risk; sex differences are well documented in the population with SCZ: males commit suicide 3–9 times more frequently than females (Johns et al., 1986; Karvonen et al., 2007). This is in line with the observation that a longer duration of the disorder (related to poor prognosis) is more prevalent in males (Association, 2013). On the other hand, females were more likely to go through with a suicide attempt during a sudden aggravation of the disease (Heila et al., 1997).

Various neurobiological observations and neuroimaging studies, point to the possible involvement in suicide of the dorsolateral prefrontal cortex (DLPFC, Brodmann area 46) and anterior cingulate cortex (ACC, Brodmann area 24), independent of the underlying psychiatric cause. In MDD patients who committed suicide, specific changes were observed in the expression of neuronal and glia-related genes as compared to MDD patients that did not commit suicide (Zhao et al., 2019; Zhao et al., 2018; Zhao et al., 2016). In contrast, an exclusive reduction in serotonin receptor 2A (HTR2A) expression was observed in the DLPFC of suicide victims with SCZ as compared to non-suicidal cases with SCZ and controls (Garbett et al., 2008). This observation is in accordance with the lower serotonin transmitter binding reported in the DLPFC in patients with SCZ who completed suicide (Underwood et al., 2018). Moreover, a frontal cortex-targeted magnetic resonance imaging (MRI) study in patients with SCZ showed that ACC-based cognitive control disturbance in the ACC was related to long-term suicidal ideations and behaviors, whereas such a connection was not present in the DLPFC (Minzenberg et al., 2014). In addition, it was shown that noninvasive prefrontal stimulation can improve clinical symptoms and may have anti-suicidal effects in patients with SCZ (George et al., 2014; Linsambarth et al., 2019; Mehta et al., 2019). This indicates that these brain areas may play a causal role in these signs and symptoms of SCZ.

Glia cells, implicated in SCZ pathophysiology, have been widely investigated in different cortical regions and glial subtypes, and an elevation of astrocyte-related glutamate transporter-related transcripts was found in, e.g., the DLPFC in SCZ (Toker et al., 2018). Glial fibrillary acidic protein (GFAP) expression was also increased in SCZ, at both the messenger and protein level, although this appeared to occur mainly when there was mention in their clinical files of neuroinflammation, old age or dementia (Arnold et al., 1996; Catts et al., 2014; Martins-de-Souza et al., 2009). In the ACC, no differences were reported in astrocytic genes in SCZ, except for some marker reductions in the deep cortical layers (Katsel et al., 2011). Microglial density based on immunocytochemical detection of human leukocyte antigen-DR (HLA-DR)

was significantly increased in the DLPFC and ACC in individuals with SCZ who committed suicide, independent of their underlying psychiatric condition (Steiner et al., 2008; Steiner et al., 2006). This observation suggests that also microglial alterations may be related to suicide in SCZ. Another aspect of altered glia responses, i.e. myelin basic protein (MBP), did not change in the DLPFC in SCZ according to one study (Baruch et al., 2009), whereas a reduction of this marker was observed by proteomic analysis in another study in elderly individuals with SCZ (Martins-de-Souza et al., 2009). In these postmortem studies, research was predominately carried out in samples with neurodegenerative or neuroinflammatory changes, and it is thus not clear whether the alterations occur specifically in patients with SCZ or in patients who committed suicide.

So far, studies referring to suicide-specific alterations in SCZ are limited to the few mentioned above. Therefore, we investigate here whether suicidal and non-suicidal patients with SCZ differ from each other in terms of glial gene expression in the PFC. Quantitative real-time PCR (qPCR) was performed on isolated grey matter of the DLPFC and ACC, using a panel of common markers for astrocytes, microglia and oligodendrocytes in patients with SCZ who had died from either suicide or from natural causes and who were compared to well-matched controls.

MATERIALS AND METHODS

Brain samples

One hundred and thirty-eight brain samples, from 25 male and 9 female controls and 26 male and 9 female patients with SCZ, were provided by the Stanley Medical Research Institute (SMRI, Bethesda, MD, USA. Director: Dr. Maree J. Webster. For demographic and clinical information see **Table 1** and **SI Table 2A** and **2B**). The next of kin provided permission for the use of brain material for scientific research. Diagnoses were made by senior psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorder IV (DSM-IV) (American Psychiatric Association, 1994). Diagnoses of unaffected controls were based on structured interviews with family member(s) by a senior psychiatrist to rule out Axis I diagnoses. Non-psychiatric controls had not displayed any suicidal behaviors, nor had they suffered from any psychiatric or neurological disorder.

The brain regions included were microscopically examined to exclude subjects with pathological signs of neurodegeneration or other obvious lesions. The SMRI formulated exclusion criteria for all specimens, which comprised; (a) significant structural brain pathology on postmortem examination by a qualified neuropathologist or by ante-mortem imaging; (b) history of significant focal neurological signs ante-mortem; (c) history of a central nervous system disease that could be expected to permanently alter gene expression; d) documented IQ < 70; e) poor RNA quality as indicated by an RNA integrity value (RIN) of lower than 7; f)

additional exclusion criteria for unaffected controls, including age under 30 (thus, still in the period of maximum risk for SCZ) and substance abuse within 1 year before death or significant alcohol-related changes in the liver. The cause of death for 7 patients with SCZ was suicide (SCZ-S); the other cases (SCZ-NS) and all control subjects (Ctr) died from non-suicidal medical conditions or accidents (for clinico-pathological details and matching for confounding factors see **Tables SI 2A and 2B**).

Two brain areas were studied: the DLPFC (Brodmann area 46) and ACC (Brodmann area 24). Both groups were matched for sex, age, postmortem delay (PMD), month of death (MOD), RIN value and brain weight (BW). RNA from isolated grey matter and the corresponding demographic information. Medical data were provided by the SMRI. All analyses were performed by investigators ignorant of the patient information.

Quantitative real-time PCR

cDNA synthesis was performed as described by us before (Zhao et al., 2018; Zhao et al., 2016). RIN values did not show any significant differences between the diagnostic groups ($P = 0.30$). The investigated genes were as follows: *astrocyte-related genes*, i.e. astrocytic gene aldehyde dehydrogenase-1 family, member L1 (ALDH1L1), GFAP, glutamate transporter 1 (GLT1), glutamine synthetase (GS) and S100 calcium binding protein b (S100b); *microglia-related genes*, i.e. cluster of differentiation 68 (CD68), chemokine (C-X3-C motif) receptor 1 (CX3CR1), human leukocyte antigen-DRA (HLA-DRA), ionized calcium-binding adapter molecule-1 (IBA1), purinergic receptor 12 (P2RY12), triggering receptor expressed on myeloid cells 2 (TREM2) and translocator protein (TSPO); *oligodendrocyte-related genes*, i.e. MBP, myelin oligodendrocyte glycoprotein (MOG), oligodendrocyte transcription factor 2 (OLIG2) and myelin proteolipid protein 1 (PLP1). Additional information on all tested genes and the sequences for each primer pair are shown in **SI Table 1**.

A cDNA template (equivalent to 5 ng of total RNA) was amplified in a final volume of 10 μ l using SYBR Green PCR master mix (Applied Biosystems, CA, USA) and a mixture of forward and reverse primers (each 2 pmol/ μ l). Data were acquired and processed automatically by an Applied Biosystems 7300 Real-time PCR System. The specificity of amplification was checked by melting curve analysis. Sterile water and RNA samples without the addition of reverse transcriptase during cDNA synthesis served as negative controls. The linearity of each qPCR assay was tested by preparing a series of dilutions of the same stock cDNA in multiple plates. In this way, also the efficiency of the polymerase reaction of each gene could be estimated and applied to calculate the expression values. Reference genes were selected to reduce the effect of sample variability (Vandesompele et al., 2002; Zhao et al., 2018). The initial set of reference genes was: actin beta (ACT β), glyceraldehyde-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase 1 (HPRT1), tubulin alpha (TUB α), tubulin beta (TUB β) and ubiquitin C (UBC).

For the comparisons in DLPFC, the specific selection was ACT β , TUB β and UBC; in ACC, the corresponding selection was ACT β , GAPDH and TUB β .

Statistical analysis

S+ software (version 8.2, TIBCO, Seattle, WA, USA) was used for statistical analysis. To analyze categorical data, the Mann-Whitney test (2 samples), the Kruskal-Wallis test with multiple comparisons (3 samples) or Chi-square test was used. A Mardia-Watson-Wheeler test was performed for month-of-death analyses (Batschelet, 1981; Zar, 1999). For interval data the Mann-Whitney test (2 samples) or the Kruskal-Wallis test with multiple comparisons (3 samples) was used (Conover, 1980). Before processing the gene expression data, the values were ¹⁰log-transformed to enable simple reference gene correction and conventional statistical procedures. In multiple testing situations the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) of *P*-values was applied. When the Kruskal-Wallis test was used in combination with the Benjamini-Hochberg correction we used 2-step analysis. As multiple comparisons in the Kruskal-Wallis test are only allowed if the global *P* < 0.05 (Conover, 1980), we first corrected the global *P*-values and then selected for further analysis those genes for which this requirement was met. For each appropriate comparison the corresponding *P*-values were pooled and corrected according to Benjamini-Hochberg. All tests were 2-sided.

Table 1A. Demographic information for the SMRI array collection (Ctr-SCZ).

	Ctr	SCZ	<i>P</i>
Age (year, range)	45 (31–60)	43 (19–59)	0.558
Gender (M/F)	25/9	26/9	0.943 ¹
PMD (hours, range)	28.5 (9–58)	31.3 (9–80)	0.631
Brain pH	6.69 (6.00–7.03)	6.50 (5.90–6.90)	0.015
Brain weight (gram, range)	1413 (1120–1900)	1420 (1170–1670)	0.972
Hemisphere	16 L; 18 R	17 L; 18 R	0.900 ¹
Age of onset	–	20 (9–34)	–
Duration of illness	–	24 (1–45)	–
Suicide	–	7	–
Psychotic features	–	35	–
Fluphenazine	–	35	–

Abbreviation: Ctr, control; F, female; L, left; M, male; PMD, postmortem delay; R, right; SCZ, schizophrenia.

¹ Chi-square test.

Table 1B. Demographic information for the SMRI array collection (Ctr-SCZNS-SCZS).

	Ctr	SCZ-NS	SCZ-S	P
Age (year, range)	45 (31–60)	44 (19–59)	39 (24–45)	0.118
Gender (M/F)	25/9	22/6	4/3	0.512 ¹
PMD (hours, range)	28.5 (9–58)	30.0 (9–80)	27.0 (15–65)	0.806
Brain pH	6.69 (6.00–7.03)	6.46 (5.90–6.90)	6.52 (6.19–6.80)	0.043
Brain weight (gram, range)	1413 (1120–1900)	1440 (1170–1670)	1480 (1340–1510)	0.837
Hemisphere	16 L; 18 R	15 L; 13 R	2 L; 5 R	0.492 ¹
Age of onset	–	19 (9–31)	29 (20–34)	–
Duration of illness	–	24 (1–45)	5 (3–18)	–
Suicide	–	–	7	–
Psychotic features	–	28	7	–
Fluphenazine	–	28	7	–

Abbreviation: Ctr, control; F, female; L, left; M, male; PMD, postmortem delay; R, right; SCZ-NS, schizophrenia patients who died of other causes than suicide; SCZ-S, schizophrenia patients who committed suicide.

¹ Chi-square test.

RESULTS

Confounder analysis

We used the Spearman test to examine the correlations between brain pH, antipsychotics and glia genes expression. We did not find a significant correlation between CSF pH and glial gene expression in either the DLPFC or the ACC. As for the use of antipsychotics, which was recorded as fluphenazine equivalents in a lifetime dosage (see **SI Table 2A** and **2B**), a significant positive correlation was found between this parameter and CX3CR1 expression ($\rho = -0.544$, $P = 0.024$) and P2RY12 expression ($\rho = -0.483$, $P = 0.039$) in the ACC. These differences were not observed in the subgroup of individuals with SCZ who had either died naturally (CX3CR1: $P = 0.080$; P2RY12: $P = 0.091$) or from suicide (CX3CR1: $P = 0.912$; P2RY12: $P = 1.000$).

Glial alterations in the DLPFC and ACC in SCZ and suicide

An overview of mRNA expression in genes is presented in **Tables 2A** and **2B**.

In the DLPFC, the expression of the astrocytic gene ALDH1L1 was significantly higher in patients with SCZ than in controls (Fold change = 1.42, $P = 0.017$). Another two astrocytic genes, which are related to the glutamate (Glu)- glutamine (Gln) cycle, i.e. GLT1 and GS, showed an upward trend in their mRNA levels, but no significant effects were present after correction

for multiple testing ($P = 0.099$). When the patients who had completed suicide were studied as a separate group, both ALDH1L1 and GS showed elevated expression only in SCZ-NS, compared to the SCZ-S and controls (ALDH1L1: SCZ-NS vs. SCZ-S, fold change = 1.27, $P = 0.010$; SCZ-NS vs. controls, fold change = 1.43, $P < 0.001$. GS: SCZ-NS vs. SCZ-S, fold change = 1.34, $P = 0.024$; SCZ-NS vs. controls, fold change = 1.19, $P = 0.001$). GLT1 increases in SCZ-NS only showed a trend compared to the controls ($P = 0.065$). No changes were found in the DLPFC between control subjects and patients with SCZ with or without suicide in terms of microglia or oligodendrocyte-related gene expression.

Table 2A. Results of glial target gene expression in the DLPFC and ACC between patients with SCZ and matched controls.

	DLPFC			ACC		
	Fold change	<i>P</i> value	BHadj-p	Fold change	<i>P</i> value	BHadj-p
Astrocyte genes						
ALDH1L1	1.42	0.001	0.017	1.38	0.003	0.047
GFAP	1.05	0.606		1.20	0.719	
GLT1	1.27	0.019	0.099	1.10	0.302	
GS	1.16	0.013	0.099	1.20	0.517	
S100b	1.21	0.100		1.22	0.199	
Microglia genes						
CD68	1.11	0.119		1.06	0.952	
CX3CR1	1.13	0.313		-1.10	0.394	
HLA	1.25	0.885		1.00	0.285	
IBA1	1.00	0.876		-1.06	0.749	
P2RY12	-1.03	0.838		-1.04	0.471	
TREM2	-1.05	0.581		-1.12	0.095	
TSPO	1.16	0.103		1.08	0.254	
Oligodendrocyte genes						
MBP	1.10	0.259		-1.05	0.494	
MOG	1.08	0.464		1.09	0.130	
OLIG2	1.10	0.486		1.00	0.792	
PLP	1.00	0.525		-1.03	0.548	

Note: ACC: anterior cingulate cortex; BHadj-p: *P* value of Benjamini-Hochberg's adjustment; DLPFC: dorsal lateral prefrontal cortex.

Table 2B. Results of glial target gene expression in the DLPFC and ACC between suicide and non-suicide patients with SCZ and their matched controls.

	Fold change			<i>P</i> value	BHadj-p	BHadj-p (2-step)		
	SCZ-S/ SCZ-NS	SCZ-S/ Ctr	SCZ-NS/ Ctr			SCZ-S/ SCZ-NS	SCZ-S/ Ctr	SCZ-NS/ Ctr
DLPFC-astrocyte genes								
ALDH1L1	-1.27	1.16	1.47	0.000	0.004	0.019	0.991	0.000
GFAP	-1.28	-1.18	1.08	0.191				
GLT1	-1.22	1.08	1.31	0.012				
GS	-1.33	-1.12	1.19	0.004				
S100b	1.02	1.24	1.21	0.215	0.031	0.024	0.991	0.001
DLPFC-microglia genes								
CD68	1.05	1.16	1.11	0.292				
CX3CR1	1.22	1.31	1.08	0.054				
HLA	1.11	1.25	1.13	0.986				
IBA1	1.18	1.15	-1.03	0.751				
P2RY12	1.30	1.21	-1.08	0.450				
TREM2	1.00	-1.05	-1.05	0.722				
TSPO	-1.15	1.04	1.20	0.202				
DLPFC-oligodendrocyte genes								
MBP	1.09	1.20	1.10	0.258				
MOG	1.59	1.59	1.00	0.243				
OLIG2	1.18	1.30	1.10	0.648				
PLP	1.45	1.38	-1.03	0.127				
ACC-astrocyte genes								
ALDH1L1	1.07	1.43	1.33	0.014	0.056			
GFAP	-1.45	-1.22	1.19	0.796				
GLT1	1.23	1.35	1.10	0.515				
GS	1.17	1.35	1.15	0.703				
S100b	-1.33	-1.12	1.19	0.343				
ACC-microglia genes								
CD68	1.19	1.19	1.00	0.185				
CX3CR1	1.57	1.31	-1.19	0.008	0.040	0.003	0.056	0.066
HLA	1.60	1.33	-1.20	0.121				
IBA1	1.48	1.30	-1.14	0.297				

	Fold change			<i>P</i> value	BHadj-p	BHadj-p (2-step)		
	SCZ-S/ SCZ-NS	SCZ-S/ Ctr	SCZ-NS/ Ctr			SCZ-S/ SCZ-NS	SCZ-S/ Ctr	SCZ-NS/ Ctr
P2RY12	1.65	1.43	-1.15	0.002	0.027	0.001	0.027	0.066
TREM2	1.25	1.11	-1.12	0.006	0.040	0.006	0.227	0.024
TSPO	1.23	1.28	1.04	0.111				
ACC-oligodendrocyte genes								
MBP	1.22	1.16	-1.05	0.243				
MOG	1.08	1.21	1.12	0.284				
OLIG2	1.22	1.22	1.00	0.908				
PLP	1.06	-1.02	-1.08	0.653				

Note: ACC: anterior cingulate cortex; BHadj-p: *P* value of Benjamini-Hochberg's adjustment; Ctr: control; DLPFC: dorsal lateral prefrontal cortex; SCZ: schizophrenia; SCZ-NS: schizophrenia – non suicide; SCZ-S: schizophrenia – suicide.

In the ACC, ALDH1L1 expression was also elevated in SCZ (Fold change = 1.38, $P = 0.047$), but when we included suicide as a covariate the difference decreased to trend level ($P = 0.056$). Two or three-group comparisons did not yield any changes in the expression of microglia-related genes. However, an almost 60% increased expression in CX3CR1 mRNA was found in SCZ-S patients compared to SCZ-NS patients (fold change = 1.57, $P = 0.003$), but not when compared to the controls. No difference was found between SCZ-NS and controls. P2RY12 was found to be exclusively elevated in the SCZ-S compared to the SCZ-NS group (fold change = 1.65, $P < 0.001$; SCZ-S vs. controls, fold change = 1.43, $P = 0.027$). A difference in this gene between SCZ-NS patients and control subjects was absent. In addition, the reduction in TREM2 mRNA was significant in SCZ-NS patients compared to SCZ-S (fold change = -1.25, $P = 0.006$; SCZ-NS vs. controls, fold change = -1.12, $P = 0.024$). This significance was absent in the SCZ-S group compared to controls.

Within the two Brodmann areas, neither two nor three group comparisons revealed any significant alterations in oligodendrocyte-related gene expression levels. Moreover, in the glia markers studied, sex differences were absent in both SCZ and control subjects.

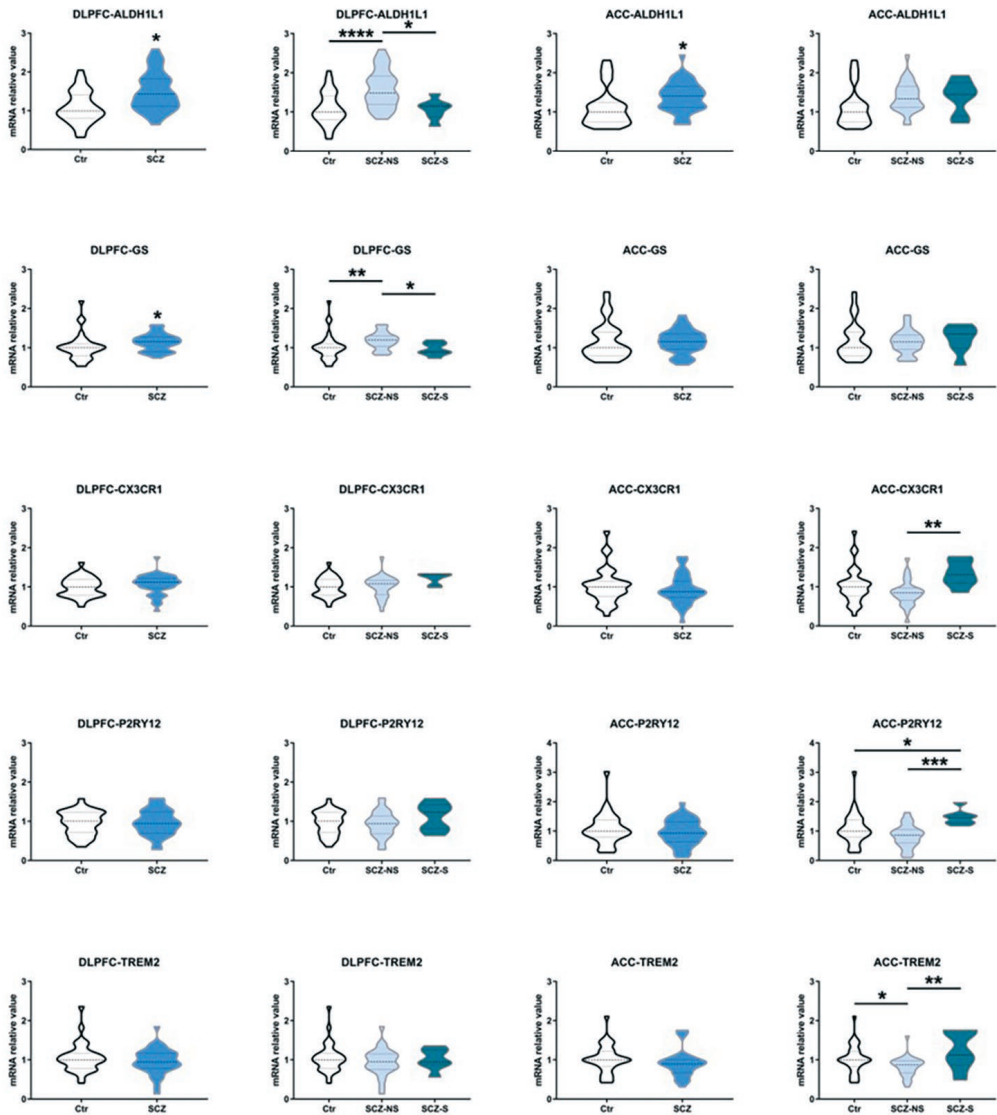


Figure 1. Results were plotted with violin plots. Transcript levels of astrocyte (ALDH1L1 and GS) and microglia related genes (CX3CR1, P2RY12 and TREM2) in the DLPFC and ACC in controls (Ctr) and patients with schizophrenia (SCZ) that died of suicide (SCZ-S) or other causes than suicide (SCZ-NS).

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsal lateral prefrontal cortex; * indicates $0.01 < P \leq 0.05$, ** indicates $0.001 < P \leq 0.01$, *** indicates $0.001 < P \leq 0.0001$, **** indicates $0.001 < P \leq 0.0001$.

DISCUSSION

The present study revealed regionally different glia-related changes in individuals with SCZ who either died as a result of suicide or of other causes. In both the DLPFC and ACC, an astrocytic gene *ALDH1L1*, which represents dopaminergic activity, was found to be elevated in SCZ, especially in the DLPFC of patients who died of non-suicidal causes. In the DLPFC, we noticed similar differences in *GS*, an enzyme involved in the glutamate-glutamine cycle. In the ACC of individuals with SCZ, the expression of three microglial genes (*CX3CR1*, *P2RY12* and *TREM2*) was increased in suicide completers than the others. Among them, *CX3CR1* expression was strongly increased in suicidal patients with SCZ compared to those who died of other causes. *P2RY12* showed increased expression exclusively in suicide victims. In addition, the only significant reduction in mRNA expression was observed in *TREM 2* in non-suicidal SCZ. In view of the sex differences in terms of prevalence, signs and symptoms in SCZ, it is surprising that no sex differences were found in relation to the genes investigated. Our results suggest that functional disturbances in dopamine-glutamate interaction, microglia phagocytosis and purine metabolism are involved in SCZ.

Dysfunctional astrocyte activity has been implicated in the pathogenesis and pathophysiology of schizophrenia before (Mei et al., 2018; Xia et al., 2016). This relationship is supported by glia-related molecular alterations on both the transcriptional and translational level and by rare genetic variants in different astrocytic genes associated with an increased risk for SCZ (Catts et al., 2014; González-Peñas et al., 2019). *ALDH1L1* has been regarded as a dopamine-related astrocytic gene, not only because it was reported to be expressed in astrocytes, but also since it is present in, and functionally correlated with, human dopamine (DA) neurons (Galter et al., 2003). *ALDH1L1* is one of the isoenzymes of *ALDH1*, which plays a main role in supporting acetaldehyde dehydrogenase (*ALDH1*) activities (Shen et al., 2016). A study based upon sub-cortical microdissection reported an overall increase of *ALDH1L1* transcripts in the anteroventral, mediodorsal thalamic nucleus, internal capsule and putamen in SCZ, all components of cortico-striato-thalamic circuits, from which especially the mediodorsal thalamic nucleus projects directly to the DLPFC (Barley et al., 2009).

To our knowledge, this is the first time that *ALDH1L1* is reported to be increased in the cortex of patients with SCZ, although not in suicide cases. In suicidal individuals with SCZ, *ALDH1L1* transcripts may possibly be replaced by other members of the *ALDH* family (Fiori et al., 2011; Hishimoto et al., 2010; Monson et al., 2017) or by single nucleotide polymorphisms (SNPs), that indeed go together with a higher incidence in suicide cases (Erlangsen et al., 2018). An RNA-sequencing study showed significant increases in the expression of specific astrocyte-related genes in the cingulate cortex of patients with SCZ that were medication independent (González-Peñas et al., 2019). The brain samples in that study were from the same collection as used in our present study and our data of increased anterior cingulate cortical *ALDH1L1*

mRNA expression in our data thus confirmed, with different techniques, their finding. In our study, similarly enhanced astrocytic expression in the DLPFC was observed using the markers ALDH1L1 and GS in non-suicidal subjects with SCZ, implicating the involvement of dopaminergic and glutamatergic overexposure in SCZ. The presence of such alterations has further been supported by functional MRI of the DLPFC in genetic risk variants for SCZ that were related to DA and Glu, which predicted the incidence and clinical manifestations of SCZ (Nixon et al., 2011; Tost and Meyer-Lindenberg, 2011). Our present data also supports our previous work, showing that astrocyte-related genes involved in the glutamate-glutamine cycle are only elevated in the DLPFC of non-suicidal MDD patients (Zhao et al., 2016).

The enhanced trend of GLT1 transcripts, another astrocytic gene involved in the glutamate-glutamine cycle that was observed in SCZ-NS patients, further supports this finding. Consistent with this, a microarray analysis reported a reduction of GS mRNA in the PFC of suicidal cases with SCZ (Kim et al., 2007). The observation that increased GS mRNA was also present in the thalamus of patients with SCZ in subregions that are connected to the DLPFC and show enhanced expression of ALDH1L1 (Bruneau et al., 2005) further supports our observation. One exception is the reduced GS expression that was reported in an older SCZ study (Burbaeva et al., 2003). However, as this analysis was based upon combined DLPFC and anterior prefrontal cortex (APFC, BA10) samples, regional differences in the APFC samples may have contributed to this finding.

Functional alterations of microglia in the prefrontal cortex of patients with SCZ are quite heterogeneous, as are these brain immune cells themselves (Böttcher et al., 2019), which may represent the different roles they play in SCZ (Notter and Meyer, 2017). For our study, we selected seven microglia-related genes reflecting microglia activation, phagocytosis, migration, antigen presentation, angiogenetic regulation, purine meta-bolism and inflammatory response. There were no significant alterations among them for the total group of individuals with SCZ as compared to controls. However, different expression levels between suicide victims and patients who died from other causes were found in the ACC. In suicide victims with SCZ, increased CX3CR1 expression was present when compared to patients with SCZ who did not commit suicide. As a member of the chemokine family, the ligand of CX3CR1, chemokine (C-X3-C motif) ligand 1 (CX3CL1, also known as fractalkine), has not been explicitly mentioned in relation to suicide or SCZ before, but it has been mentioned in relation to bipolar mood disorder (Stuart and Baune, 2014). In addition, one study reported that the expression of this gene was dramatically increased in moderate to severe MDD patients (Merendino et al., 2004), who had a high suicide risk.

Strikingly, we found more than 50% enhancement of P2RY12 mRNA only in the group of suicidal individuals with SCZ in the ACC, indicating that an abnormal metabolism in purinergic signaling may be related to suicide, probably independent of the illness. As a member of the P2RY family, P2RY12 activity is inhibited by ADP during purine metabolism (Tulapurkar et

al., 2005). In body fluids, such as CSF and urine, lower levels of hypoxanthine, a spontaneous de-amination product of adenine, were present in severely affected MDD patients that had suicide ideations or had completed suicide (Agren et al., 1983; Lis et al., 1975). In addition, the serotonin augmentation index, assessed by measuring the serotonin-mediated enhancement of ADP-induced platelet aggregation, was impaired in patients with a high risk for suicide (Mann et al., 1992). This may explain the reduced ADP activity in SCZ-suicidal behaviors. We may, therefore, assume that higher P2RY12 expression in suicidality might be due to the partially lifted inhibition from ADP inactivation. In addition, our results showed upregulations of both P2RY12 and CX3CR1, two homeostatic microglial genes, that indicated a sign of imbalanced and activated homeostasis in suicidal individuals with SCZ (Landsman et al., 2009).

A reduction of TREM2-mRNA was found in the ACC only in non-suicidal cases with SCZ. While TREM2 was repeatedly reported to be expressed at higher mRNA levels and at lower DNA methylation rates, e.g. in peripheral leukocytes from patients with SCZ (Mori et al., 2015; Yoshino et al., 2016; Yoshino et al., 2017), our results now show opposite changes in the brain. This difference may be explained by the presence of different inflammatory responses in peripheral neutrophils and central monocytes/macrophages. Indeed, on the basis of molecular changes observed in TREM2 knockdown mice, it was proposed that a TREM2 deficiency due to genetic mutations might rapidly induce psychotic symptoms (Penberthy, 2007), which is in agreement with similar manifestations and common psychotic features in SCZ. As a marker that sustains microglia metabolism and its response towards amyloid β plague pathology in Alzheimer's disease, one can indicate that functionally deficient TREM2 expression may be in relation to the high risk of psychotic symptoms in some neurodegenerative status (Ropacki and Jeste, 2005; Ulland and Colonna, 2018). Furthermore, it was proven that TREM2 is necessary for synapse elimination, which is accompanied by modulating excitatory neurotransmission and changes in long-range functional connectivity (Filipello et al., 2018; Sellgren et al., 2019). Indeed, increased numbers of glutamatergic axons and axo-spinous synapses were found in the cingulate cortex of patients with SCZ (Harrison, 1999), supporting the idea that hyperfunctional glutamatergic synapses might be a primary genetic abnormality in SCZ etiology (Owen et al., 2005). We may further speculate that the elevated synaptic density in ACC is due to microglia deficiency, which may be mediated by the reduction in TREM2, known to disrupt adequate synapse regulation.

We did not find changes in oligodendrocyte markers in SCZ. Morphological studies of oligodendrocyte densities gave variable results and oligodendrocyte densities in the DLPFC were reported to be unchanged in the BA 9 (Hercher et al., 2014) but reduced in layer III, V and VI in BA 10 (Kolomeets and Uranova, 2018; Vostrikov and Uranova, 2011; Vostrikov and Uranova, 2018), indicating regional and even layer-specific changes. In the ACC, previous data did not report changes in MBP expression, either on the mRNA or protein level in patients with

SCZ (Dracheva et al., 2006; Haroutunian et al., 2007). Our results confirm these data, now also for the patients that committed suicide.

One of the possible confounding factors in this postmortem study is medication. A relation between fluphenazine equivalents, as a measure for the amount of dopamine receptor D1 and D2 antagonists used during life, and microglia activities has not been shown before. Our data indicate that CX3CR1 and P2RY12 may play a novel role as microglia-related genes that are targeted towards dopaminergic neurons. We also observed, for the first time, a dose-effect relationship of fluphenazine on microglia suppression in the subgroup of SCZ-NS. However, these negative correlations were disturbed in suicidal SCZs compared to the other patients, and may imply that the elevated expression of CX3CR1 and P2RY12 in suicide completers seems to counteract the microglia-suppressing effect of fluphenazine.

In conclusion, our present study highlights the heterogeneity in glia gene alterations in patients with SCZ in relation to suicide. A glutamate-related astrocytic gene, ALDH1L1, was the only one that was significantly elevated in both the DLPFC and ACC, which supports the possible presence of a hyperfunctional dopaminergic involvement in SCZ. Different, or even opposite, astrocytic and microglia alterations were found in individuals with SCZ, depending on whether they had died from natural causes or from suicide. Moreover, functional disturbances in glutamate-dopamine interaction, microglia phagocytosis and purinergic metabolism seem to participate in the pathophysiology of SCZ. Therefore, we want to emphasize that, in future research, it is of crucial importance to separately study groups of non-suicidal and suicidal patients with SCZ. The same holds for other psychiatric disorders that go together with suicide (Zhao et al., 2019).

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Contributors

L. Zhang, I. Huitinga and D.F. Swaab designed the research protocol. L. Zhang undertook data collection. R.W.H. Verwer performed the statistical analysis. L. Zhang wrote the first draft, D.F. Swaab, I. Huitinga and P.J. Lucassen amended the manuscript. All authors have approved and contributed to the final manuscript. D.F. Swaab provided the financial support.

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SI Table 1. Reference genes and target gene sequences.

Gene	Full name	Accession Code	Forward Primer	Reverse Primer
Reference Gene				
ACTβ	Actin beta	NM_001101	CCCAGCCATGTACGTTGCTA	TCACCGGAGTCCATCACGAT
GAPDH	Glyceraldehyde-phosphate dehydrogenase	NM_002046	CAAAATTCATGGCACCGTC	TCTCGCTCCTGGAAGATGGT
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194	GGACAGGACTGAACGTCTTGC	ATAGCCCCCCTTGAGCACAC
TUBα	Tubulin alpha	NM_006082	CTTTGAGCCAGCCCAACCAGA	GTACAAACAGGACGAAAGCCAT
TUBβ	Tubulin beta	NM_006087	GGGCCAAAGTTTGGGAGGT	CACGTGTCCCCCATGGTATGTGC
UBC	Ubiquitin C	NM_021009	GCTGCTCATAAGACTCGGCC	GTCACCCCAAGTCCCGTCCTA
Target Gene				
	Full name	Accession Code	Forward Primer	Reverse Primer
Astrocyte				
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1	NM_001270364	GAACACAGTGGTGATCAAGCC	GGAGGACGTTAACCACACCTT
GFAP	Glial fibrillary acidic protein	NM_001363846	CCCACCTCTGCTTTGACTGAGC	CCTTCTTCGGCCTTAGAGGG
GLT1	Glutamate transporter 1	NM_0011195728	ATACCATTGACTCCCAAGCATCG	GAGTTGCTTTCCCTGTGGTTCTT
GS	Glutamine synthetase	NM_001033056	TGTGTGGAAGAGTTGCCTGAG	TGGCAGCAGGCACGAGATAC
S100b	S100 calcium binding protein b	NM_006272	TGGAAAAAGCAAACTCCATCAGAA	GAATCGCATGGGTCACGG

Target Gene	Full name	Accession Code	Forward Primer	Reverse Primer
CD68	Cluster of differentiation 68	NM_001040059	CTACCAGGCCCTCTGAGC	GGCGTGTGAGGAAATAAGG
CX3CR1	Chemokine (C-X3-C motif) receptor 1	NM_001337	TTGGCCTGGTGGGAAATTTGT	AGGAGGTAAATGTCGGTGACACT
HLA-DRA	Human leukocyte anti-gen-DRA	NM_019111	CCCAGGGAAGACCACCTTT	CACCCCTGCAGTCGTAAACGT
IBA1	Ionized calcium-binding adapter molecule 1	NM_032955	AGGTGTCCAGTGGCTCCGGG	TGGCAGATCTCTTGCCCAGCA
P2RY12	Purinergic receptor 12	NM_176876	TTCAAACCCTCCAGAATCAACAG	GTGCACAGACTGGTGTTACC
TREM2	Triggering receptor expressed on myeloid cells 2	NM_018965	CCACCCACTTCCATCCTTCT	GTCCCCTGGCTTCTGTCCAT
TSPO	Translocator protein	NM_000714	TCTGGAAAGAGCTGGGAGG	TTGTCTGGGCACCAAAAGAAAG
Oligodendrocyte				
MBP	Myelin basic protein	NM_001025101	AAATGCCGAGAAGGCCAGTAC	CCCATTGTTCTGGTTCGCA
MOG	Myelin oligodendrocyte glycoprotein	NM_002433	TGAGAGGAAAAACTTCGAGCAG	CGGCACAATTACAAACAGGG
OLIG2	Oligodendrocyte transcription factor 2	NM_005806	GTGTCCAGGCCTCTCT	GGCAGCAGACGGGAGACT
PLP1	Myelin proteolipid protein 1	NM_000533	CCTCACTGGCACAGAAAAGCT	GGCCCCATAAAGGAAGAAAGAA

SI Table 2A. Clinico-pathological information (CTR-SCZ).

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR1		F	44	28	5	6.59	1330	8.3	R DLPFC, RACC	3	0	No	0	CARDIAC
CTR2		M	49	46	7	6.5	1605	8.3	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR3		M	53	9	12	6.4	1500	9.1	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR4		M	37	13	3	6.5	1600	8.3	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR5		M	51	31	4	6.7	1400	7.3	R DLPFC, RACC	1	0	No	0	CARDIAC
CTR6		M	53	28	6	6	1340	8.4	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR7		F	38	33	7	6	1120	9.7	R DLPFC, RACC	3	0	No	0	CARDIAC
CTR8		F	38	28	9	6.7	1350	8.8	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR9		M	60	47	10	6.8	1460	8.4	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR10		M	35	52	6	6.7	1700	8.7	R DLPFC, RACC	0	0	No	0	MYOCARDITIS
CTR11		M	34	22	11	6.48	1480	8.2	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR12		M	47	21	1	6.81	1550	8.7	L DLPFC, LACC	0	0	No	0	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR13		M	45	29	2	6.94	1405	8.5	R DLPFC, RACC	1	0	No	0	CARDIAC
CTR14		F	34	24	2	6.87	1255	6.6	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR15		M	42	37	3	6.91	1340	7.8	L DLPFC, LACC	4	3	No	0	CARDIAC
CTR16		F	44	10	3	6.2	1305	8.4	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR17		M	45	18	4	6.81	1585	8.0	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR18		M	49	23	6	6.93	1390	9.5	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR19		M	32	24	7	7.03	1415	8.3	L DLPFC, LACC	2	0	No	0	CARDIAC
CTR20		M	55	31	8	6.7	1515	8.3	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR21		F	49	45	9	6.72	1435	8.6	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR22		F	33	29	2	6.52	1360	8.3	L DLPFC, LACC	0	1	No	0	ASTHMA
CTR23		M	48	31	3	6.86	1580	7.0	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR24		M	50	49	3	6.75	1645	8.0	R DLPFC, RACC	1	0	No	0	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR25		M	32	13	8	6.57	1410	9.0	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR26		M	47	11	8	6.6	1495	8.3	L DLPFC, L ACC	0	1	No	0	CARDIAC
CTR27		M	46	31	9	6.67	1360	8.6	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR28		M	40	38	11	6.67	1498	8.7	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR29		M	51	22	11	6.71	1900	8.7	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR30		M	31	11	1	6.13	1335	8.4	R DLPFC, R ACC	1	1	No	0	PULM EMBOL
CTR31		M	48	24	1	6.91	1321	8.6	R DLPFC, R ACC	1	1	No	0	CARDIAC
CTR32		F	39	58	2	6.46	1260	8.4	R DLPFC, R ACC	4	0	No	0	CARDIAC
CTR33		M	47	36	6	6.57	1535	8.0	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR34		F	41	50	8	6.17	1290	7.3	R DLPFC, R ACC	0	0	No	0	CARDIAC
Median	-	-	45	28.5	-	6.69	1413	8.40	-	-	-	-	-	-
SCZ1	295.9	M	43	26	12	6.42	1620	7.5	L DLPFC, L ACC	0	0	YES	180000	PNEUMONIA
SCZ2	295.3	M	31	33	6	6.20	1480	8.5	R DLPFC, R ACC	5	4	YES	35000	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
SCZ4	295.9	M	40	34	12	6.18	1480	8.8	R DLPFC, R ACC	3	3	YES	75000	PNEUMONIA
SCZ5	295.9	M	51	43	1	6.63	1390	8.6	L DLPFC, L ACC	3	1	YES	130000	CARDIAC
SCZ6	295.9	M	19	28	4	6.73	1465	9.0	L DLPFC, L ACC	1	5	YES	2500	OD
SCZ7	295.9	F	53	13	7	6.49	1345	8.8	L DLPFC, L ACC	5	0	YES	15000	CARDIAC
SCZ8	295.9	M	37	30	10	6.80	1550	9.0	R DLPFC, R ACC	5	5	YES	20000	CARDIAC
SCZ9	295.1	M	52	10	1	6.10	1450	8.5	R DLPFC, R ACC	0	0	YES	100000	CARDIAC
SCZ11	295.9	M	44	9	6	5.90	1415	9.4	L DLPFC, L ACC	3	3	YES	350000	EXHAUSTIVE MANIA/NMS
SCZ12	295.3	M	39	80	11	6.60	1355	8.4	L DLPFC, L ACC	1	1	YES	120000	MVA
SCZ13	295.9	M	33	29	1	6.50	1470	9.1	L DLPFC, L ACC	1	0	YES	20000	CARDIAC
SCZ14	295.3	M	50	9	5	6.20	1400	8.4	R DLPFC, R ACC	0	0	YES	34000	CARDIAC
SCZ15	295.9	M	43	18	5	6.30	1520	8.2	R DLPFC, R ACC	5	5	YES	90000	CIRRHOIS
SCZ17	295.3	M	35	47	6	6.40	1370	7.2	R DLPFC, R ACC	0	0	YES	200000	CARDIAC
SCZ18	295.3	M	44	32	8	6.67	1560	7.7	L DLPFC, L ACC	5	2	YES	20000	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
SCZ19	295.9	M	47	13	3	6.30	1310	8.3	L DLPFC, LACC	2	1	YES	300000	ACUTE PANCREAT
SCZ20	295.9	M	45	35	6	6.66	1390	7.6	L DLPFC, LACC	5	2	YES	50	CARDIAC
SCZ22	295.9	M	54	38	10	6.17	1400	8.3	R DLPFC, RACC	2	0	YES	120000	CARDIAC
SCZ23	295.9	F	54	42	11	6.65	1170	8.4	R DLPFC, RACC	0	0	YES	400000	PNEUMONIA
SCZ24	295.9	F	44	26	3	6.58	1490	8.7	L DLPFC, LACC	0	0	YES	50000	POSS PULM THROMB
SCZ25	295.9	F	47	30	4	6.47	1430	8.7	R DLPFC, RACC	1	0	YES	15000	OD
SCZ27	295.9	M	38	35	9	6.68	1210	8.4	L DLPFC, LACC	5	5	YES	15000	OD
SCZ28	295.9	M	41	54	9	6.18	1629	7.9	L DLPFC, LACC	0	0	YES	115000	CARDIAC
SCZ31	295.3	F	47	35	1	6.50	1575	9.0	L DLPFC, LACC	1	0	YES	90000	CARDIAC
SCZ32	295.3	M	42	19	1	6.48	1310	9.3	R DLPFC, RACC	4	4	YES	18000	CARDIAC
SCZ33	295.9	M	46	30	1	6.72	1630	8.8	L DLPFC, LACC	2	3	YES	200000	PNEUMONIA
SCZ34	295.9	F	59	38	2	6.93	1515	7.6	R DLPFC, RACC	4	0	YES	30000	CARDIAC
SCZ35	295.9	M	52	16	2	6.52	1340	7.7	R DLPFC, RACC	1	0	YES	60000	PNEUMONIA

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
SCZ3	295.3	F	45	52	11	6.51	1510	9.0	R DLPFC, R ACC	4	4	YES	20000	SUIC:JUMPED
SCZ10	295.9	M	24	15	1	6.20	1505	8.4	R DLPFC, R ACC	5	5	YES	12000	SUIC:OD
SCZ16	295.9	F	32	36	6	6.80	1340	8.8	L DLPFC, L ACC	0	0	YES	10000	SUIC:JUMPED
SCZ21	295.9	F	36	27	7	6.49	1480	8.4	L DLPFC, L ACC	0	2	YES	600	SUIC:HANGING
SCZ26	295.9	M	39	26	7	6.80	1470	8.2	R DLPFC, R ACC	0	0	YES	48000	SUIC:HANGING
SCZ29	295.9	M	43	65	10	6.67	1490	9.6	R DLPFC, R ACC	1	1	YES	70000	SUIC:HANGING
SCZ30	295.9	M	42	26	12	6.19	1410	8.3	R DLPFC, R ACC	5	5	YES	10000	SUIC:JUMPED
Median	-	-	43	30	-	6.50	1465	8.47	-	-	-	-	-	-
P value	-	0.94	0.56	0.63	NS	0.02	0.97	0.30	0.90	-	-	-	-	-

Note: BW, brain weight; CSF, cerebrospinal fluid; CTR, control; F, female; L, left; M, male; MOD, month of death; MVA, motor vehicle accident; NMS, neuroleptic malignant syndrome; NS, not significant; OD, overdose drugs; PMD, postmortem delay; POSS, possible; PULM EMBOL, pulmonary embolism; R, right; RIN, RNA integrity number; SCZ, schizophrenia; SUIC, suicide. Scale of alcohol and drug use: 0, little or none; 1, social; 2, moderate past; 3, moderate present; 4, heavy past; 5, heavy present.

SI Table 2B. Clinico-pathological information (CTR-SCZ suicide versus non-suicide).

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR1		F	44	28	5	6.59	1330	8.3	R DLPFC, R ACC	3	0	No	0	CARDIAC
CTR2		M	49	46	7	6.5	1605	8.3	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR3		M	53	9	12	6.4	1500	9.1	L DLPFC, L ACC	1	0	No	0	CARDIAC
CTR4		M	37	13	3	6.5	1600	8.3	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR5		M	51	31	4	6.7	1400	7.3	R DLPFC, R ACC	1	0	No	0	CARDIAC
CTR6		M	53	28	6	6	1340	8.4	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR7		F	38	33	7	6	1120	9.7	R DLPFC, R ACC	3	0	No	0	CARDIAC
CTR8		F	38	28	9	6.7	1350	8.8	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR9		M	60	47	10	6.8	1460	8.4	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR10		M	35	52	6	6.7	1700	8.7	R DLPFC, R ACC	0	0	No	0	MYOCARDITIS
CTR11		M	34	22	11	6.48	1480	8.2	R DLPFC, R ACC	0	0	No	0	CARDIAC
CTR12		M	47	21	1	6.81	1550	8.7	L DLPFC, L ACC	0	0	No	0	CARDIAC
CTR13		M	45	29	2	6.94	1405	8.5	R DLPFC, R ACC	1	0	No	0	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR14		F	34	24	2	6.87	1255	6.6	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR15		M	42	37	3	6.91	1340	7.8	L DLPFC, LACC	4	3	No	0	CARDIAC
CTR16		F	44	10	3	6.2	1305	8.4	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR17		M	45	18	4	6.81	1585	8.0	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR18		M	49	23	6	6.93	1390	9.5	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR19		M	32	24	7	7.03	1415	8.3	L DLPFC, LACC	2	0	No	0	CARDIAC
CTR20		M	55	31	8	6.7	1515	8.3	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR21		F	49	45	9	6.72	1435	8.6	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR22		F	33	29	2	6.52	1360	8.3	L DLPFC, LACC	0	1	No	0	ASTHMA
CTR23		M	48	31	3	6.86	1580	7.0	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR24		M	50	49	3	6.75	1645	8.0	R DLPFC, RACC	1	0	No	0	CARDIAC
CTR25		M	32	13	8	6.57	1410	9.0	R DLPFC, RACC	0	0	No	0	CARDIAC
CTR26		M	47	11	8	6.6	1495	8.3	L DLPFC, LACC	0	1	No	0	CARDIAC
CTR27		M	46	31	9	6.67	1360	8.6	L DLPFC, LACC	1	0	No	0	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
CTR28		M	40	38	11	6.67	1498	8.7	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR29		M	51	22	11	6.71	1900	8.7	L DLPFC, LACC	1	0	No	0	CARDIAC
CTR30		M	31	11	1	6.13	1335	8.4	R DLPFC, RACC	1	1	No	0	PULM EMBOL
CTR31		M	48	24	1	6.91	1321	8.6	R DLPFC, RACC	1	1	No	0	CARDIAC
CTR32		F	39	58	2	6.46	1260	8.4	R DLPFC, RACC	4	0	No	0	CARDIAC
CTR33		M	47	36	6	6.57	1535	8.0	L DLPFC, LACC	0	0	No	0	CARDIAC
CTR34		F	41	50	8	6.17	1290	7.3	R DLPFC, RACC	0	0	No	0	CARDIAC
Median	-	-	45	28.5	-	6.69	1413	8.40		-	-	-	-	-
SCZ1	295.9	M	43	26	12	6.42	1620	7.5	L DLPFC, LACC	0	0	YES	180000	PNEUMONIA
SCZ2	295.3	M	31	33	6	6.20	1480	8.5	R DLPFC, RACC	5	4	YES	35000	CARDIAC
SCZ4	295.9	M	40	34	12	6.18	1480	8.8	R DLPFC, RACC	3	3	YES	75000	PNEUMONIA
SCZ5	295.9	M	51	43	1	6.63	1390	8.6	L DLPFC, LACC	3	1	YES	130000	CARDIAC
SCZ6	295.9	M	19	28	4	6.73	1465	9.0	L DLPFC, LACC	1	5	YES	2500	OD
SCZ7	295.9	F	53	13	7	6.49	1345	8.8	L DLPFC, LACC	5	0	YES	15000	CARDIAC

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
SCZ8	295.9	M	37	30	10	6.80	1550	9.0	R DLPFC, RACC	5	5	YES	20000	CARDIAC
SCZ9	295.1	M	52	10	1	6.10	1450	8.5	R DLPFC, RACC	0	0	YES	100000	CARDIAC
SCZ11	295.9	M	44	9	6	5.90	1415	9.4	L DLPFC, LACC	3	3	YES	350000	EXHAUSTIVE MANIA/NMS
SCZ12	295.3	M	39	80	11	6.60	1355	8.4	L DLPFC, LACC	1	1	YES	120000	MVA
SCZ13	295.9	M	33	29	1	6.50	1470	9.1	L DLPFC, LACC	1	0	YES	20000	CARDIAC
SCZ14	295.3	M	50	9	5	6.20	1400	8.4	R DLPFC, RACC	0	0	YES	34000	CARDIAC
SCZ15	295.9	M	43	18	5	6.30	1520	8.2	R DLPFC, RACC	5	5	YES	90000	CIRRHOISIS
SCZ17	295.3	M	35	47	6	6.40	1370	7.2	R DLPFC, RACC	0	0	YES	200000	CARDIAC
SCZ18	295.3	M	44	32	8	6.67	1560	7.7	L DLPFC, LACC	5	2	YES	20000	CARDIAC
SCZ19	295.9	M	47	13	3	6.30	1310	8.3	L DLPFC, LACC	2	1	YES	300000	ACUTE PANCREAT
SCZ20	295.9	M	45	35	6	6.66	1390	7.6	L DLPFC, LACC	5	2	YES	50	CARDIAC
SCZ22	295.9	M	54	38	10	6.17	1400	8.3	R DLPFC, RACC	2	0	YES	120000	CARDIAC
SCZ23	295.9	F	54	42	11	6.65	1170	8.4	R DLPFC, RACC	0	0	YES	400000	PNEUMONIA

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
SCZ24	295.9	F	44	26	3	6.58	1490	8.7	L DLPFC, LACC	0	0	YES	50000	POSS PULM THROMB
SCZ25	295.9	F	47	30	4	6.47	1430	8.7	R DLPFC, RACC	1	0	YES	15000	OD
SCZ27	295.9	M	38	35	9	6.68	1210	8.4	L DLPFC, LACC	5	5	YES	15000	OD
SCZ28	295.9	M	41	54	9	6.18	1629	7.9	L DLPFC, LACC	0	0	YES	115000	CARDIAC
SCZ31	295.3	F	47	35	1	6.50	1575	9.0	L DLPFC, LACC	1	0	YES	90000	CARDIAC
SCZ32	295.3	M	42	19	1	6.48	1310	9.3	R DLPFC, RACC	4	4	YES	18000	CARDIAC
SCZ33	295.9	M	46	30	1	6.72	1630	8.8	L DLPFC, LACC	2	3	YES	200000	PNEUMONIA
SCZ34	295.9	F	59	38	2	6.93	1515	7.6	R DLPFC, RACC	4	0	YES	30000	CARDIAC
SCZ35	295.9	M	52	16	2	6.52	1340	7.7	R DLPFC, RACC	1	0	YES	60000	PNEUMONIA
Median	-	-	44	30	-	6.46	1440	8.5	-	-	-	-	-	-
SCZ3	295.3	F	45	52	11	6.51	1510	9.0	R DLPFC, RACC	4	4	YES	20000	SUIC;JUMPED
SCZ10	295.9	M	24	15	1	6.20	1505	8.4	R DLPFC, RACC	5	5	YES	12000	SUIC;OD
SCZ16	295.9	F	32	36	6	6.80	1340	8.8	L DLPFC, LACC	0	0	YES	10000	SUIC;JUMPED
SCZ21	295.9	F	36	27	7	6.49	1480	8.4	L DLPFC, LACC	0	2	YES	600	SUIC;HANGING

Group	DSM-IV	Sex	Age (y)	PMD (hr)	MOD	CSF pH	BW (g)	RIN value	Hemisphere	Alcohol use	Drug use	Psychotic features	Fluphenazine equivalents (mg)	Cause of death
SCZ26	295.9	M	39	26	7	6.80	1470	8.2	R DLPFC, R ACC	0	0	YES	48000	SUIC:HANGING
SCZ29	295.9	M	43	65	10	6.67	1490	9.6	R DLPFC, R ACC	1	1	YES	70000	SUIC:HANGING
SCZ30	295.9	M	42	26	12	6.19	1410	8.3	R DLPFC, R ACC	5	5	YES	10000	SUIC:JUMPED
Median	-	-	39	27	-	6.52	1480	8.4	-	-	-	-	-	-
P value	-	0.51	0.11	0.80	NS	0.04	0.84	0.48	0.49	-	-	-	-	-

Note: BW, brain weight; CSF, cerebrospinal fluid; CTR, control; F, female; L, left; M, male; MOD, month of death; MVA, motor vehicle accident; NMS, neuroleptic malignant syndrome; NS, not significant; OD, overdose drugs; PMD, postmortem delay; POSS, possible; PULM EMBOL, pulmonary embolism; R, right; RIN, RNA integrity number; SCZ, schizophrenia; SUIC, suicide. Scale of alcohol and drug use: 0, little or none; 1, social; 2, moderate past; 3, moderate present; 4, heavy past; 5, heavy present.

CHAPTER 6

PROGESTERONE RECEPTOR DISTRIBUTION IN THE HUMAN HYPOTHALAMUS AND ITS ASSOCIATION WITH SUICIDE

Zhang L, Verwer RWH, van Heerikhuize J, Lucassen PJ, Nathanielsz PW,
Hol EM, Aronica E, Dhillon WS, Meynen G, Swaab DF.

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ABSTRACT

Background: The human hypothalamus modulates mental health by balancing interactions between hormonal fluctuations and stress responses. Stress-induced progesterone release activates progesterone receptors (PR) in human brain and triggers neuropeptides/neurotransmitters alterations.

Methods: As recent epidemiological studies have associated peripheral progesterone levels with human suicide risk, we mapped PR distribution in the human hypothalamus in relation to age and gender and characterized its (co-)expression in specific cell types.

Results: The infundibular nucleus (INF) is the primary hypothalamic structure via which progesterone modulates stress-related neural circuitry. An elevation of pro-opiomelanocortin (POMC, an endogenous opioid precursor) neurons in the INF, which was due to a high proportion of POMC neurons that co-expressed PR, was related to suicide in patients with mood disorders (MD). MD donors who died of legal euthanasia were for the first time enrolled in a postmortem study to investigate the molecular signatures related to suicidal ideations. They have higher proportioned PR-expressing POMC neurons than MD patients who died naturally. This indicates that the onset of endogenous opioid activation in MD with suicide tendency may be progesterone-associated. We revealed that both MD and suicidality might disturb the reciprocal correlations in control subjects between PR co-expressing neurons in the INF, and peptidergic neurons in the paraventricular nucleus.

Conclusions: Our findings have important implications for users of progesterone-enriched contraceptives who also have MD and suicidal tendencies. Considering this widespread impact on the general population, research aimed at elucidating the involvement of progesterone in the opioid-associated neuronal circuit in suicide should have a high priority.

Key words

Progesterone receptor; hypothalamus; suicide; depression; pro-opiomelanocortin; neuropeptide Y

INTRODUCTION

Progesterone plays a key function in the stress responses in the human hypothalamus. Recent studies have shown a biphasic and dynamic relationship between fluctuating progesterone levels and the risk and severity of suicidality. Both clinical and forensic studies reported that suicidal behaviors in fertile women were more prevalent during the peri-menstrual period when peripheral progesterone levels were high (Behera et al., 2019; Papadopoulou et al., 2018), and in menopausal and amenorrheic women when peripheral progesterone levels diminished (Baca-Garcia et al., 2010). Moreover, a nationwide prospective analysis has reported that, among contraception users, the suicide risks increased with the enrichment of progesterone in the contraceptive (Skovlund et al., 2017). In addition, higher plasma progesterone, but not estrogen levels, were found in women who had attempted suicide repeatedly (Mousavi et al., 2014). Until now, however, studies on progesterone effects on the human central nervous system, and in relation to suicidal behaviors, are lacking (Ho et al., 2021). In humans, the effects of progesterone are mediated by progesterone receptors (PR). An imaging study reported that progesterone-containing oral contraceptives decreased hypothalamic volumes (Chen et al., 2021), but the localization of PR was largely unknown. Therefore, we mapped the distribution of PR in the human hypothalamic nuclei with respect to age and gender and investigated its functionally neurobiological signatures in glia and peptidergic neurons. Subsequently, we studied the possible relationship between changes in PR-expressing peptidergic neurons and suicide, focusing on a key stress-regulating and PR-dense area in the hypothalamus, the infundibular nucleus (INF; in rodents the arcuate nucleus, ARC).

The INF contains two distinct populations of neurons, the pro-opiomelanocortin (POMC)-expressing neurons and the neuropeptide Y (NPY)-expressing neurons, that are both involved in mood disorders (MD) and suicide but exert opposing actions. Preclinical stress studies have revealed that POMC neurons in the ARC are involved in depressive-like behaviors (Qu et al., 2020). Concomitantly, strong selective serotonin reuptake inhibitor (SSRI) antidepressants showed high binding affinity to serotonin receptor 2A that localized on ARC POMC neurons (Martin-Gronert et al., 2016; Pälvimäki et al., 1996). Clinical studies have shown selective epigenetic alterations in the POMC gene of adolescents who suffered from depression and self-injuries (Zheng et al., 2020). Adults with major depressive disorder (MDD) have higher plasma levels of POMC, and their responses to antidepressants depend on the presence of certain POMC haplotypes (Chang et al., 2015; Leake et al., 1989). Naltrexone, a competitive antagonist of opioid receptors that can cause a strong increase in POMC activity in the INF, attenuates the anti-suicidality effects of ketamine (Billes et al., 2014; Williams et al., 2019). In addition, it has been shown that individuals who poorly responded to ketamine treatment had more POMC transcripts in blood than responders (Shenoda et al., 2016). In postmortem observations, higher pituitary POMC expression has been found in suicide completers compared to controls (López

et al., 1992). Thus, excessive POMC activity in the INF and pituitary seems to implicate the onset of suicidality. Contrary to POMC, rodent models of depression reported lower levels of NPY in plasma, cerebral spinal fluid, frontal-limbic regions and ARC (Desai et al., 2014; Husum et al., 2001; Melas et al., 2012). Interestingly, in these compartments, NPY levels were normalized by traditional antidepressant treatments such as SSRIs and electroconvulsive therapy (Christiansen et al., 2011; Jiménez-Vasquez et al., 2007). Together, these preclinical observations pointed to marked antidepressant effects of NPY, with prominent involvement in the ARC. In humans, declining NPY expression levels in the periphery and the central limbic system were also reported in MD (Kuromitsu et al., 2001; Ozsoy et al., 2016; Widerlöv et al., 1988). Intranasal NPY administration to patients with MDD elicits rapid antidepressant effects (Mathé et al., 2020). However, in most previous literature, interpretations on neurobiological alterations respectively linked to either psychiatric disorders or suicide remain unclear since their data were derived from samples of individuals with psychiatric disorders who merged accomplished suicide.

Rodent studies showed that ARC-originating POMC and NPY fibers innervated multiple stress-responsive intra- and extra-hypothalamic regions (Betley et al., 2013; Qu et al., 2020), including the paraventricular nucleus (PVN), where corticotropin-releasing hormone (CRH)- and thyrotropin-releasing hormone (TRH)-expressing neurons played different roles in modulating stress, mood and anxiety (Fekete et al., 2000; Liposits et al., 1988; Toni et al., 1990). Previous research showed that NPY adapted the effects of CRH in the human hypothalamus during the depression by playing an inhibitory role in emotional modulation after stress stimulation (Heilig et al., 1994; Thorsell, 2010). In addition, paraventricular TRH neurons can be innervated by both NPY and POMC neurons (Mihály et al., 2000).

Based on the background discussed above, we hypothesized that progesterone played an important role in MD and suicide by affecting the activity of stress-related neurons that co-express PR and innervate other stress-related neurons. In this study, we first investigated the distribution of PR in the human hypothalamus. In addition, we characterized PR co-expression in cell types across hypothalamic nuclei. To investigate whether PR was especially associated with MD, fatal death ideations or suicidal behaviors, we further quantified the total numbers of POMC immunoreactive (-ir), NPY-ir neurons and their co-localization with PR in the INF, and CRH-ir and TRH-ir neurons in the PVN of individuals with MD and well-matched controls.

METHODS AND MATERIALS

Human brain materials

Sixty tissue blocks containing the entire hypothalamus were obtained from the Netherlands Brain Bank (NBB, Amsterdam, the Netherlands, Director: Prof. dr. Inge Huitinga). The donors provided permission in advance for the autopsy, use of brain material, and clinical documentation

for research. For PR mapping throughout the hypothalamus, a total of 12 control subjects (6 males and 6 females) were selected from fetal, childhood, adolescent, young, middle ages and elderly ages (**Table S1a**). To detect the presence of PR in early development, 3 fetal hypothalami (22, 30 and 37 gestational weeks) were included (**Table S1b**). Secondly, for observing the potential relationship between PR and MD, 45 subjects were collected and divided into two main groups: 28 MDs and 17 matched controls (CTR) without a neuropsychiatric disorder (**Table 1, S1c and S1d**). Patients with MD were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorder (DSM)-III, IIIR and IV criteria. A neuropathologist systematically examined each brain to detect unrelated neuropathological alterations. Subjects with evidence of neurological disorders or with Alzheimer's (Braak) stage III or higher were excluded (Braak and Braak, 1991). Included subjects had no significant history of substance abuse in the 10 years before death.

Matching for potentially confounding factors was non-parametrically tested (**Table S1c and S1d**). These factors were age, sex, post-mortem delay (PMD), clock time of death (CTD), brain pH and brain weight (BW). Of notable mention, PMDs of suicide cases were estimated values. MD donors who died of legal euthanasia were for the first time enrolled in a postmortem study to investigate the molecular signatures related to fatal death ideations (Zhang et al., 2022). Since legal euthanasia cases in the Netherlands were mostly performed during working hours, CTDs of the euthanasia groups differed significantly from the other groups. There were three sub-groups distinguished in the MD group and two sub-groups in the CTR group. The MD group contained 10 subjects with MD who died by suicide (MDS), 7 brain donors with MD who died of legal euthanasia (MDE), and 11 subjects with MD who had not reported suicidality within 5 years before their natural death (MDN). The CTR group contained 12 subjects who had no primary neurological or psychiatric disorders and died of natural causes (CN) and 5 subjects who had no primary neurological or psychiatric disorders and died of legal euthanasia due to physical diseases (CE), with strong death ideations recorded in their medical history.

Hypothalamic tissue was dissected at autopsy, fixed, dehydrated, cleared and embedded in paraffin. Coronal serial sections (6 μ m) were cut from the level of the lamina terminalis to mamillary bodies. Every 100th section was collected and stained with thionine for orientation.

Immunocytochemical staining

Details on antibodies used in this study, their manufacturer, specificity and staining protocol were presented in supplementary tables (**Table S2 and S3**). In brief, after deparaffinization and rehydration, the sections were washed, treated for antigen retrieval and/or pre-incubated to block background staining. Subsequently, the sections were incubated with primary antibody, then with biotinylated secondary antibody (1:400; Vector Labs), followed by avidin-biotin-peroxidase complex (ABC; 1:800; Vector Labs). Finally, sections were incubated in a solution of 0.5 mg/mL 3, 3'-diaminobenzidine (Sigma) in a total volume of 15 mL TBS containing 5 μ L

H₂O₂ 30% (Merck) and 0.035 g ammonium nickel sulfate (DAB-Ni), at room temperature for color development. For double labelling (DAB-Ni vs. DAB), ammonium nickel sulphate was not included in the second DAB development. The enzyme reaction was stopped in distilled water. Subsequently, the sections were dehydrated, cleared and cover slipped with Entellan (Merck).

Localization of POMC-ir and NPY-ir neurons

The localization of the INF was detected by performing POMC or NPY staining in the hypothalamus from the level of the optic tracts to the level of the corpus mamillare (one section per 100th). The fornix, optic tract and median eminence were used as anatomical landmarks. POMC neurons in the INF were labelled by an antibody directed against its major peptide, alpha-melanocyte stimulating hormone (α -MSH) (Goldstone et al., 2002).

Localization of PR/POMC-ir and PR/NPY-ir neurons

Double staining of PR with POMC or NPY was performed on sections adjacent to those with single POMC/NPY neuronal staining throughout the INF. In brief, sections after PR-DAB-Ni development were incubated with 20 μ g/mL AffiniPure Fab fragment goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA) then incubated with the POMC/NPY antibody, followed by a secondary antibody and ABC incubation, and DAB development.

Localization of CRH-ir and TRH-ir neurons

Based on thionine staining, we performed CRH and TRH staining in the hypothalamus in the region to localize the part of the PVN containing these cells. Every 100th section in this region was stained.

Quantification of immunocytochemical staining

Six main classes of cell profiles for quantification were determined, i.e. (1) total POMC-ir neurons, (2) nuclear PR-cytoplasmic POMC double-stained neurons (PR/POMC-ir neurons), (3) total NPY-ir neurons, (4) nuclear PR-cytoplasmic NPY double-stained neurons (PR/NPY-ir neurons) in the INF, (5) total CRH-ir neurons and (6) total TRH-ir neurons in the PVN.

A 2.5 $\times$ objective (Plan-Neofluar) was used on a Zeiss (Jena, Germany) Axioskop microscope mounted with a Sony (Tokyo, Japan) black/white CCD camera (model XC77CE), connecting to software package Image-Pro Plus 6.3 (Media Cybernetics Inc, Rockville, MD, USA). The total volume of the INF/PVN containing a particular cell type was calculated based upon Cavalieri principle (Gundersen et al., 1988). The contour of the entire INF/PVN field occupied by the targeted neurons was manually outlined by the operator with the cursor. Subsequently, the imaging analysis system overlaid a grid of rectangular fields representing a 40 $\times$ objective within the outlined cross-sectional area. To prevent double-counting, in single staining, only neurons containing a nucleolus were counted (Fronczek et al., 2008). For analysis, 100% of the

rectangular fields of the mask area were counted. All visible neurons with a nucleus containing a nucleolus, and within the exclusion lines, were examined using a 40× objective. In double-staining, since the nucleolus was not visible, nuclear and cytoplasmic double-staining neurons were counted instead. By dividing the total number of cell profiles by the whole volume, an estimate of the cellular density was calculated for each INF or PVN area, and the total number of the cell profiles summed up the multiplying the INF or PVN volume with the neuronal density of every level.

Statistical analysis

S+ software (version 8.2, TIBCO, Seattle, WA, USA) was used for statistical analysis. The chi-square test was used for the analysis of categorical data (gender). For interval data, the Mann-Whitney test (2 samples) or the Kruskal-Wallis test with multiple comparisons (5 samples) was used (Conover, 1998). In multiple testing situations, the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) of *P* values was applied. When the Kruskal-Wallis test was used in combination with the Benjamini-Hochberg correction, we proceeded in a two-step manner. As multiple comparisons in the Kruskal-Wallis test are only allowed if the global *P* < 0.05 (Conover, 1998), we first corrected the global *P* values and then selected for further analysis only those markers for which this requirement was met. Then, the similar multiple comparisons of the allowed markers were combined and corrected with Benjamini-Hochberg. Subsequently, the corrected multiple comparisons were recombined for each marker for presentation in the tables. Correlations were determined using Spearman's test. CTD was also used to divide subjects into day (06:00–17:59) and night (18:00–05:59) groups. Differences in CTD and MOD (circular parameters) between controls and patients with MD were tested with the Mardia-Watson-Wheeler test (Batschelet, 1981). For each appropriate comparison, the corresponding *P* values were pooled and corrected according to Benjamini-Hochberg. All tests were 2-sided. Results were plotted in GraphPad Prism 8.

RESULTS

Progesterone receptor distribution in the human hypothalamus in relation to age and gender

From rostral to caudal in the hypothalamus and adjacent structures, cells were present with PR-ir nuclei in the triangular septal nucleus, dorsal periventricular nucleus, anteroventral periventricular nucleus, periventricular nucleus, bed nucleus of the stria terminalis, suprachiasmatic nucleus, pre-optic area (POA), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), PVN and INF (**Figure 1A, 1B** and **Figure S1A** and **S1B**). The presence of PR-ir cells in the INF was weak but detectable at 22 gestational weeks (**Figure S1C**). In addition, cells

Table 1. Demographic information.

Diagnosis - Cause of death	Cases, n	Psychiatric diagnosis (MDD/BD)	Reason for euthanasia application (physical diseases/ MD) ¹	Age (y) ²	Gender (M/F)	PMD (h) ²	Brain pH ²	BW (g) ²
Mood disorder	28	22/6	-	66 (38–94)	19/9	8:55 (3:30–62:55)	6.70 (5.61–7.55)	1291 (985–1670)
- Suicide	10	9/1	-	60 (39–90)	6/4	20:35 (3:55–62:55)	6.53 (6.18–6.80)	1300 (1120–1670)
- Legal euthanasia	7	6/1	4/5 ³	61 (38–81)	5/2	6:40 (3:30–9:40)	6.78 (6.70–7.07)	1283 (1120–1525)
Died of natural causes	11	7/4	-	70 (58–94)	8/3	8:55 (4:50–60:20)	6.37 (5.61–7.55)	1295 (985–1510)
Control	17	-	-	72 (39–88)	11/6	7:15 (3:20–41:00)	6.60 (5.37–7.28)	1259 (1115–1629)
- Legal euthanasia	5	-	5/0	79 (49–83)	3/2	5:45 (3:20–6:30)	6.80 (6.15–7.28)	1173 (1121–1364)
Died of natural causes	12	-	-	70 (39–88)	8/4	8:30 (4:35–41:00)	6.45 (5.37–6.95)	1372 (1115–1629)

Abbreviations: BD, bipolar disorder; BW, brain weight; F, female; g, gram; h, hour; M, male; MD, mood disorder; MDD, major depressive disorder; PMD, postmortem delay; y, year.

Note:

¹ In the Netherlands, legal euthanasia will be approved to end unbearable suffering with no prospect of improvement in physical/psychiatric diseases (<https://www.government.nl/topics/euthanasia/is-euthanasia-allowed>). For the practice of legal euthanasia in the Netherlands see <https://www.knmp.nl/downloads/guidelines-for-the-practice-of-euthanasia.pdf>.

² Values are provided with medians within ranges.

³ In subjects who died of legal euthanasia, two individuals applied due to a combination of physical diseases and MD. The others were approved either because of physical diseases or mood disorders.

in the ependymal layer and subventricular zone along the lateral and third ventricle showed positive nuclear staining (**Figure 1C**). Ependymal cells along the third ventricle revealed a higher density of PR-ir cells than the lateral ventricle. In the choroid plexus, both cuboidal and columnar cells showed cytoplasmic staining (**Figure 1D**). In addition, a very high density of PR-ir cells was present in the pars tuberalis of the pituitary gland from the fetal period throughout the entire life (**Figure 1E**).

A summary of PR expression in the hypothalamus across ages, in different nuclei and between genders is depicted in a preliminary map in **Figure S1D**. We found that the presence of PR started in the fetal hypothalamus and is maintained throughout the entire lifespan. The heat map indicates that the POA, INF, VMN and DMN revealed higher densities of expression relative to the other positive nuclei. Surprisingly, the POA, which was functionally involved in sexual and parental behaviors in adults, showed robust PR expression in the fetal brains at the end of pregnancy; however, this expression was reduced in early childhood regardless of gender. Unexpectedly, the hypothalami of elderly subjects showed overall increases in PR staining compared to the younger subjects.

Of interest, cells in the ependymal layer, subventricular zone and choroid plexus did not present positive signals before birth but thereafter maintained a lifelong and sex-biased expression (consistent lower density in females than in males). In the choroid plexus, the columnar cells in females had mild to moderate cytoplasmic signals while both cuboidal and columnar cells in males revealed strong expression (**Figure S1E**).

We performed nuclear-cytoplasm double staining to investigate the functional involvements of PR-ir cells. Using double labelling with glial fibrillary acidic protein (GFAP) or ionized calcium-binding adaptor molecule 1 (Iba1), we did not find PR/GFAP astrocytes or PR/Iba1 microglia (**Figure 2A, a and b**). The general absence of PR-ir cells in the main white matter structures, including the fornix, anterior commissure and optic nerve showed that there was no PR expression in oligodendrocytes (figures not shown). However, in the SVZ, we found PR-ir cells co-labelled with GFAP- δ and nestin that detected the neural progenitors (Van Den Berge et al., 2010) (**Figure 2A, c and d**). In the INF, we found binuclear PR and POMC co-localized (PR/POMC-ir) neurons (**Figure 2A, e**).

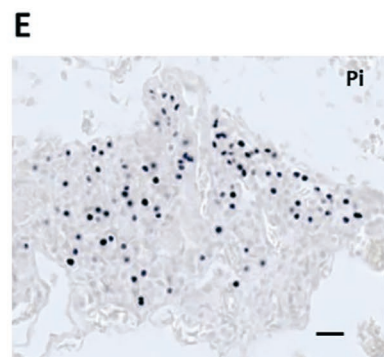
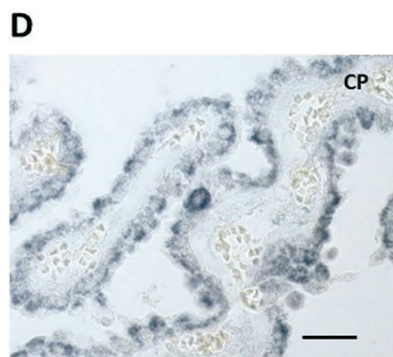
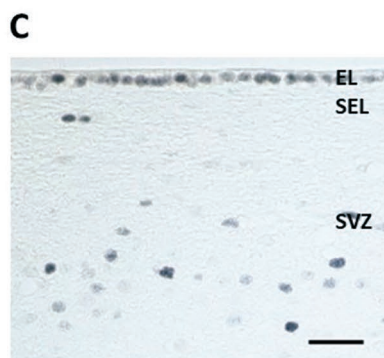
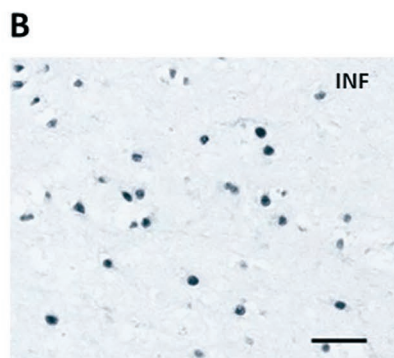
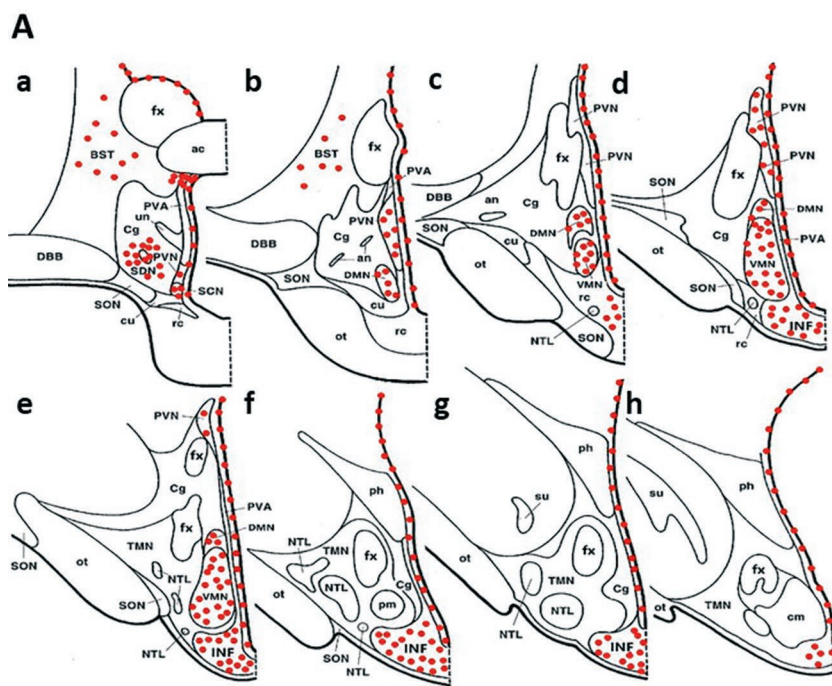


Figure 1. Progesterone receptor distribution in the human hypothalamus and adjacent regions. (A) Schematic illustration of PR mapping throughout the human hypothalamus (a-h from rostral to caudal). (B) Nuclear PR expression in the hypothalamic nuclei (displayed as the INF). (C) Nuclear PR expression in cells of the EL and SEL, and SVZ along lateral ventricle. (D) Cytoplasmic PR expression in the cuboidal and columnar epithelium of the choroid plexus. (E) Nuclear PR expression in the pars tuberalis of the pituitary. Scale bars: 100 μ m.

Abbreviations: ac, anterior commissure; an, accessory neurosecretory nucleus; BST, bed nucleus of the stria terminalis; Cg, chiasmatic grey; cm, corpus mamillare; CP, choroid plexus; cu, cuneate nucleus; DBB, nucleus of the diagonal band (of Broca); DMN, dorsomedial nucleus; EL, ependymal layer; fx, fornix; INF, infundibular nucleus; NTL, lateral tuberal nucleus; ot, optic tract; ph, posterior hypothalamic nucleus; Pi, pituitary; pm, posteromedial nucleus; PVA, periventricular area; PVN, paraventricular nucleus; rc, retrochiasmatic nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic (or intermediate) nucleus; SEL, subependymal layer; SON, supraoptic nucleus; su, subthalamic nucleus; SVZ, subventricular zone; TMN, tuberomammillary nucleus; un, unicate nucleus; VMN, ventromedial nucleus.

Progesterone receptor expression in hypothalamic neural progenitors and peptidergic neurons

Concerning the neuronal hypothalamic markers, double staining for PR was performed with CRH, TRH, oxytocin (OXT), arginine-vasopressin (AVP), somatostatin (SOM), galanin (GAL), kisspeptin (KISS1), tyrosine hydroxylase (TH), dynorphin (DYN), POMC and NPY. In the PVN (**Figure 2B, a**), we did not find PR co-expression in CRH-ir, TRH-ir, OXT-ir, AVP-ir, SOM-ir, TH-ir or DYN-ir neurons (**Figure 2B, b-h**), but we found PR/KISS1-ir neurons in elderly males (**Figure 2B, i**) (Taziaux et al., 2016). In the periventricular nucleus (**Figure S2A, a**), PR co-labelled CRH and TH neurons (**Figure S2A, b and c**), but not DYN neurons (**Figure S2A, d**). In the INF (**Figure 2C, a**), a considerable proportion of PR-ir cells co-expressed POMC-ir (PR/POMC-ir) and SOM-ir (PR/SOM-ir) neurons (**Figure 2C, b and c**) and a small proportion of co-expressing NPY-ir (PR/NPY-ir) neurons were present (**Figure 2C, d**). Besides, we found a high proportion of PR/KISS1 neurons in the INF of elderly females (**Figure 2C, e**) (Rumpler et al., 2021). Few DYN-ir neurons co-labelled PR in the INF (**Figure 2C, f**). Occasionally observed CRH neurons in this region did not co-label with PR (**Figure 2C, g**). Neither did we detect PR/AVP-ir or PR/DYN-ir neurons in the suprachiasmatic nucleus nor PR/GAL-ir neurons in the sexually dimorphic nucleus (also known as the intermediate nucleus, INAH 1) (**Figure S2B and S2C**).

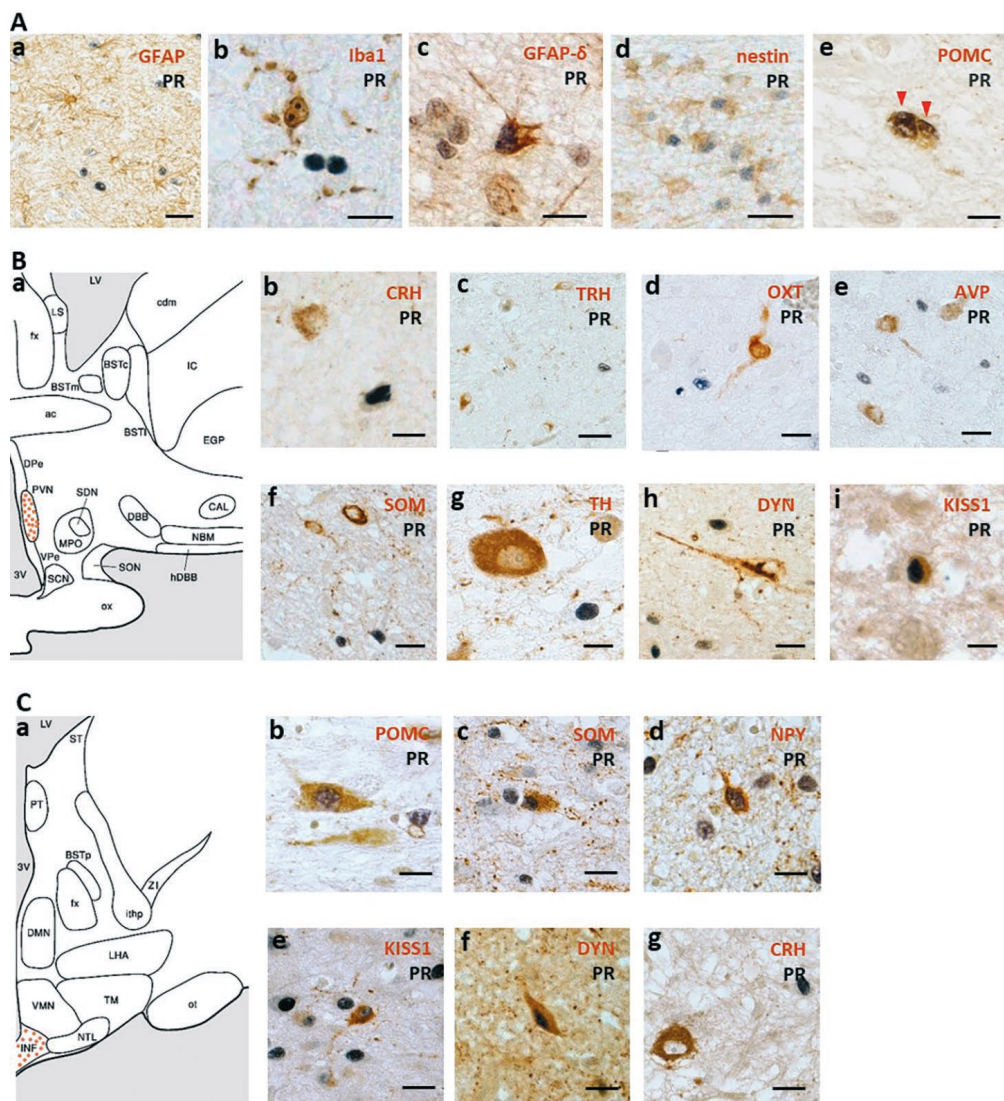


Figure 2. Characterization of progesterone receptor immunoreactive (-ir) cells in the paraventricular nucleus and infundibular nucleus. (A) PR expression in neural progenitors and immature neurons. (a and b) Absent co-localization of PR in astrocytes (GFAP) or microglia (Iba1). (c and d) Co-localization of PR in neural progenitors (GFAP- δ and nestin). (e) Co-localization of PR in an immature POMC neuron (two nuclei shown by arrowheads). (B) PR expression in the PVN. a Schematic illustration of the PVN in the human hypothalamus. (b-h) Absent expression of PR in CRH-ir, TRH-ir, OXT-ir, AVP-ir, SOM-ir, TH-ir or DYN-ir neurons. (i) Co-localization of PR in KISS1-ir neurons. (C) PR expression in the INF. (a) Schematic illustration of the INF in the human hypothalamus. (b-f) PR expression in POMC-ir (~50%), NPY-ir (~20%), SOM-ir (~50%), KISS1-ir (~70% in elderly males) and DYN-ir neurons in the INF. (g) Absent co-localization of PR in CRH-ir neurons in the INF. Scale bars: (A) a and d, 50 μ m; b, c and e, 20 μ m. (B) b, g and i, 10 μ m; c-f and h, 30 μ m. (C) b-d, 20 μ m, e-g, 30 μ m.

Abbreviations: AVP, arginine-vasopressin; CRH, corticotropin-releasing hormone; DYN, dynorphin; GFAP, glial fibrillary acidic protein; GFAP- δ , glial fibrillary acidic protein-delta; Iba1, ionized calcium-binding adaptor molecule 1; INF, infundibular nucleus; ir, immunoreactive; KISS1, kisspeptin; NPY, neuropeptide Y; OXT, oxytocin; POMC, pro-opiomelanocortin; PR, progesterone receptor; PVN, paraventricular nucleus; SOM, somatostatin; TH, tyrosine hydroxylase; TRH, thyrotropin-releasing hormone (for other abbreviations see legend Figure 1).

Elevated PR/POMC-ir neurons contribute to the increased POMC-ir neurons in depressed suicides

We quantified the total number of POMC-ir and PR/POMC-ir neurons in the INF (Figure 3A). No difference was observed between MD patients and controls (Figure 3B, a and c). Of note, some POMC-ir neurons with two nucleoli were present in both MD and CTR (Figure S3A, also binucleated SOM-ir neurons were present, Figure S3B). The number of PR and POMC double-staining neurons was determined on adjacent sections but did not differ between MD patients and controls (Figure 3B, e and g). The number of POMC-ir neurons that did not co-localize PR (sPOMC-ir neurons) was not different between MD and controls (Figure 3B, i).

In a subset analysis, however, a robust increase in the total number of POMC-ir neurons was observed in suicide cases (MDS) compared to the other MD subsets and CN subjects (Figure 3A, a and b; 3B, b). Total volumes of the POMC-ir neuron cluster were not different within the five subgroups (Figure 3B, d), indicating that, in brains of those who died by suicide, an elevation of POMC-ir neurons was the major explanation for the higher POMC-ir density in these volumetrically stable INFs.

By analysing PR/POMC-ir neuron number as well as its INF volume, we observed an exclusive increase in MDS relative to the other subgroups (Figure 3A, c and d; 3B, f and h), indicating that increased PR/POMC neurons were recruited within the INF. The number of sPOMC-ir neurons remained unchanged within the five groups (Figure 3B,

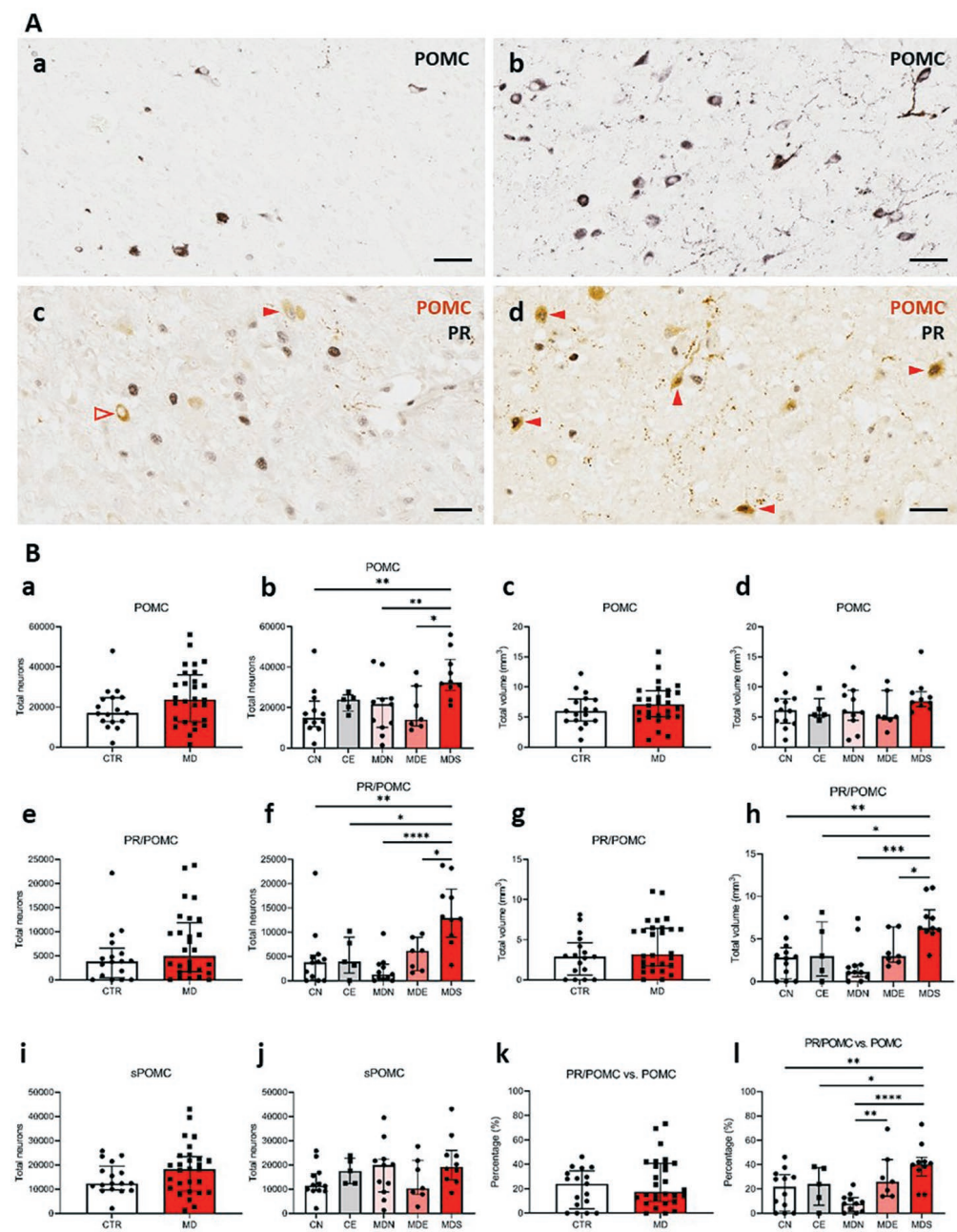


Figure 3. Increased numbers of PR/POMC-ir neurons contribute considerably to the POMC-ir neuronal increase in depressed suicides. (A) POMC-ir and PR/POMC-ir neurons in the INF of a control subject (a and c) and a patient with MD who died of suicide (b and d). Solid arrowheads point at PR/POMC-ir neurons. Empty arrowheads point at sPOMC-ir neurons. (B) Analyses between CTR and MD subjects (two-group comparison), and their subsets (five-group comparison) on total neurons and volume of POMC (a-d), PR/POMC (e-h), total neurons of sPOMC (i-j) and the proportion of PR/POMC neurons in POMC neurons (k-l). Note that patients with MD who died of suicide or legal euthanasia presented higher proportions of PR/POMC-ir neurons in the INF than patients who died by natural causes (l). Scale bars: 100 μ m.

Abbreviations: CE, control subjects who died of legal euthanasia; CN, control subjects who died of natural causes; CTR, control subjects; INF, infundibular nucleus; -ir, immunoreactive; MD, mood disorders; MDE, subjects with mood disorders who died of legal euthanasia; MDN, subjects with mood disorders who died of natural causes; MDS, subjects with mood disorders who died of suicide; POMC, pro-opiomelanocortin; PR, progesterone receptor; PR/POMC, neurons co-labelling progesterone receptor and pro-opiomelanocortin; sPOMC, pro-opiomelanocortin neurons that did not express progesterone receptor.

Note: Lines with asterisks on the graphs indicate that the two groups under them are significantly different. * indicates $0.01 \leq P < 0.05$, ** indicates $0.001 \leq P < 0.01$, *** indicates $0.0001 \leq P < 0.001$, **** indicates $0.00001 \leq P < 0.0001$, Global- $P < 0.05$.

j). In addition, a higher proportion of POMC-ir neurons that co-labelled PR was detected in MDE versus MDN (**Figure 4 3B, l**), which suggested that in individuals with MD, the ratio of POMC neurons that co-labelled PR might be an indicator of suicidality, including suicidal behaviours and even be sensitive to suicidal ideations. We concluded that the elevated sum of POMC-ir neurons in the INF of suicidal cases was due to an increased number of PR/POMC co-expressing neurons.

Progesterone receptor is not associated with reduced NPY expression in mood disorders.

A marked reduction of the total number of NPY-ir neurons was found in subjects with MD (**Figure 4A, a and b; Figure 4B, a and c**). Subset analysis showed that individuals with MD who died of euthanasia had the lowest NPY-ir neuronal counts among the five subgroups (**Figure 4B, b and d**). Strikingly, control subjects who died of euthanasia presented the most NPY-ir neurons, in sharp contrast to MDE subjects, which indicated that NPY expression in the INF could be seriously inhibited in MD.

The number of PR/NPY-ir neurons remained unchanged in both two- and five-group comparisons (**Figure 4A, c and d; Figure 4B, e-h**). Similar changes with total NPY neurons have been found in non-PR-expressing NPY-ir (sNPY-ir) neurons (**Figure 4B, i and j**), suggesting that PR may be unrelated to these decreases in MD. In contrast to the high proportion of POMC-ir neurons that co-expressed PR, NPY-ir neurons that showed co-localization with PR were generally less than 10% of the total number of NPY neurons (**Figure 4B, k and l**).

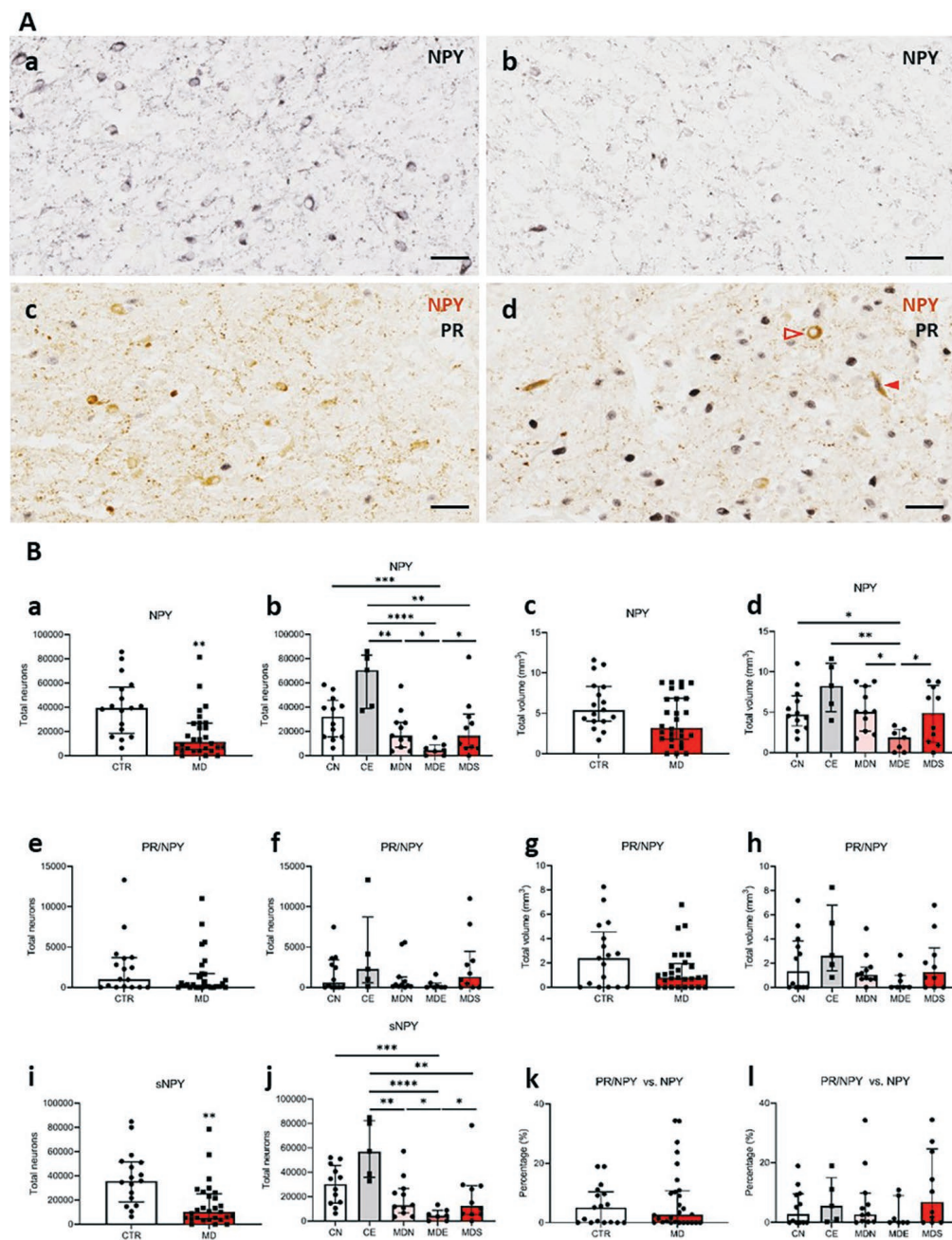


Figure 4. Reduction of the total number of NPY-ir neurons in the infundibular nucleus of mood disorders is not associated with progesterone receptor. (A) NPY-ir and PR/NPY-ir neurons in the INF of a control subject (a and c) and an individual with MD (b and d). Solid arrowheads point at PR/ NPY-ir neurons. Empty arrowheads point at sNPY-ir neurons. (B) Analyses between CTR and MD subjects, and their subsets on total neurons and volume of NPY (a-d), PR/NPY (e-h), total neurons of sNPY (i-j) and the proportion of PR/NPY neurons in NPY neurons (k-l). Note the sharp drop of NPY expression in MDE compared to CE (b and j). Scale bars: 100 μ m.

Abbreviations: CE, control subjects who died of legal euthanasia; CN, control subjects who died of natural causes; CTR, control subjects; INF, infundibular nucleus; ir, immunoreactive; MD, mood disorders; MDE, subjects with mood disorders who died of legal euthanasia; MDN, subjects with mood disorders who died of natural causes; MDS, subjects with mood disorders who died of suicide; NPY, neuropeptide Y; PR, progesterone receptor; PR/NPY, neurons co-labelling progesterone receptor and neuropeptide Y; sNPY, neuropeptide Y neurons that did not express progesterone receptor.

Note: Lines with asterisks on the graphs indicate that the two groups under them are significantly different. * indicates $0.01 \leq P < 0.05$, ** indicates $0.001 \leq P < 0.01$, *** indicates $0.0001 \leq P < 0.001$, **** indicates $0.00001 \leq P < 0.0001$, Global- $P < 0.05$.

Mood disorders and suicidality disrupt interactions between the infundibular nucleus and paraventricular nucleus

To investigate the interactions between POMC-ir and NPY-ir neurons in the INF, and CRH-ir and TRH-ir neurons in the PVN, we performed double staining for these peptides to reveal their morphology and the distribution of neuronal elements. As shown in **Figure 5A**, cell bodies of POMC-ir and NPY-ir neurons, CRH-ir and TRH-ir neurons were located in the INF and PVN respectively, while their axonal varicosities were distributed in both hypothalamic nuclei. We observed NPY-ir, CRH-ir and TRH-ir neurons that received considerable POMC contacts on both perikarya and dendrites, while NPY-ir terminals densely targeted the other neurons with well-defined juxtapositions. Similarly, both CRH-ir and TRH-ir fiber varicosities were surrounding other peptidergic neurons with multiple contacts.

A negative correlation was found between PR/POMC-ir and TRH-ir neuron numbers (**Figure 5B, a**), and a positive correlation between PR/NPY-ir and CRH-ir neurons in control subjects who died naturally (**Figure 5B, b**). Such findings were not detected in the other groups or subgroups. Neither two- nor five-group comparisons showed significant alterations in CRH expression in individuals with MD versus the controls (**Figure 6A and 6B**). In addition, we found an absence of CRH-ir neurons in the anterior PVN (APVN) that contributed to the lower expression in the euthanasia subgroups (**Figure S4**). On the contrary, a striking increase in the total number of TRH-ir neurons together with their increased distribution area was found in MDE compared to the other subsets except CE, which was due to a larger number of TRH-ir neurons throughout the hypothalamus (**Figure 6C, 6D and S4**).

An overview of data graphed in **Figure 3, 4 and 6** is presented in **Table S4**.

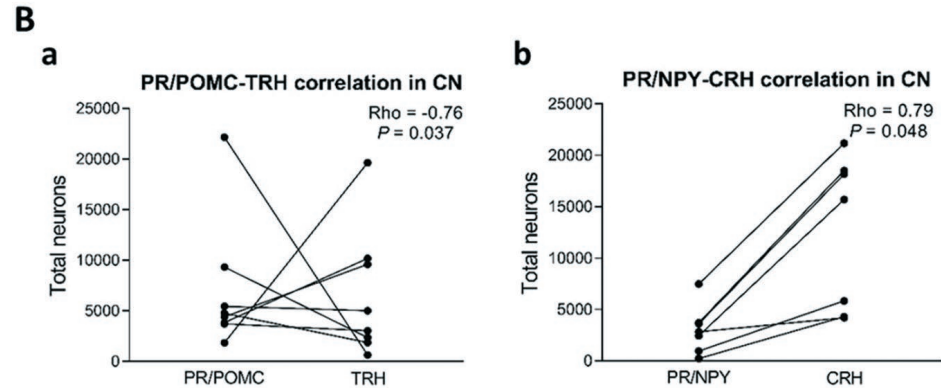
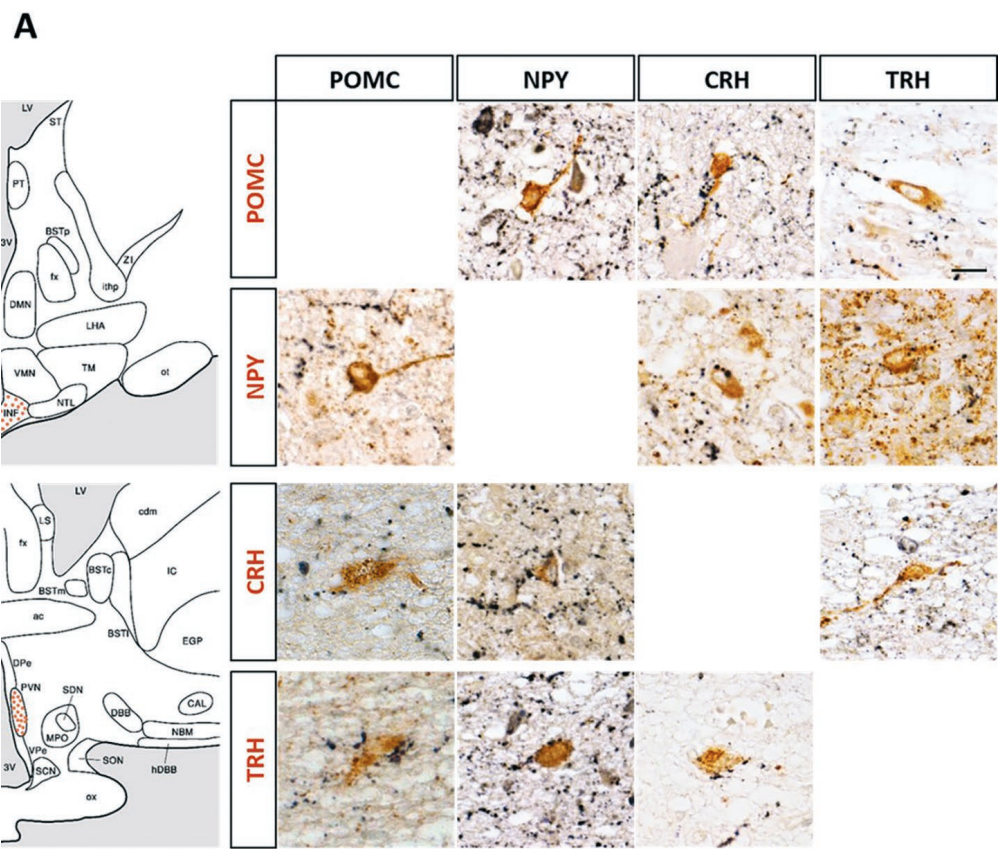


Figure 5. Reciprocal innervations of POMC-ir, NPY-ir, CRH-ir and TRH-ir neurons in the infundibular nucleus and paraventricular nucleus. (A) Neurons are indicated in horizontal rows and brown, fiber innervation is indicated in vertical columns and black. (B) Correlations between PR/POMC-ir neurons in the INF and the TRH-ir neurons in the PVN (a), and between PR/NPY-ir neurons in the INF and CRH-ir neurons in the PVN (b) of controls subjects who died by natural causes. Due to the ceiling effect that zero values may mislead the correlations, subjects who displayed zero values after quantification were excluded from the correlation analysis. Scale bar: 20 μ m.

Abbreviations: CRH, corticotropin-releasing hormone; INF, infundibular nucleus; ir, immunoreactive; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PR/NPY, neurons co-labelling progesterone receptor and neuropeptide Y; PR/POMC, neurons co-labelling progesterone receptor and pro-opiomelanocortin; PVN, paraventricular nucleus; TRH, thyrotropin-releasing hormone.

Analysis of putative confounders

We included age, gender, CTD, PMD, BW and brain pH in the analysis of factors that might confound our results. There were no differences in the NPY expression between the two sexes within MD and CTR separately (**Figure S5A**). However, the sharp reduction of NPY-ir and sNPY-ir neurons in MD versus CTR was significant only in males (**Figure S5A**). The other detected neuropeptides did not reveal gender-biased alterations (**Figure S5B-D**). Since we have matched our (sub)groups for the factors mentioned above, the observed correlations did not affect our conclusions.

Of note, individuals who died of legal euthanasia showed significantly different CTDs. A similar situation also held for patients with MD who died of suicide, who had a trend for particularly long PMDs compared to other subgroups. However, we did not find evidence that these two parameters might have driven any of the reported effects.

MDD and bipolar disorder are two major classifications of MD. We further compared the peptide neurons between them but did not find significant differences. In addition, psychoactive substances were used in controls subjects for treatments of physical illnesses three months before death, but they did not seem to confound our results.

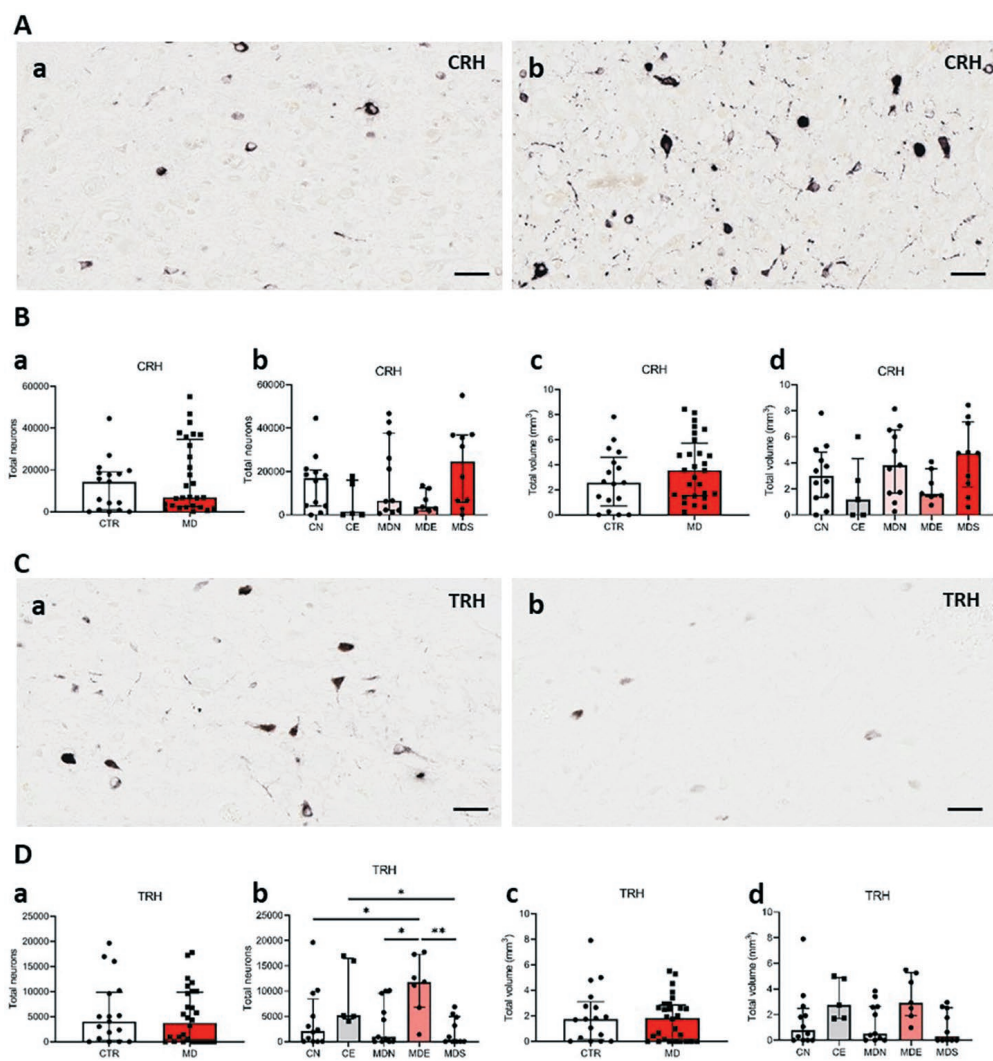


Figure 6. CRH and TRH expression in the paraventricular nucleus of controls and patients with mood disorders. (A) CRH-ir neurons in the PVN of a control subject (a) and a patient with MD who died of suicide (b). (B) Comparison of CTR and MD subjects, and their subsets on the total number of neurons and volume of CRH (a-d). (C) TRH-ir neurons in the PVN of a control subject (a) and a patient with MD who died of suicide (b). (D) Comparison of CTR and MD subjects, and their subsets on total neurons and volume of TRH (a-d). Scale bars: 100 μ m.

Abbreviations: CE, control subjects who died of legal euthanasia; CN, control subjects who died of natural causes; CRH, corticotropin-releasing hormone; CTR, control subjects; ir, immunoreactive; MD, mood disorders; MDE, subjects with mood disorders who died of legal euthanasia; MDN, subjects with mood disorders who died of natural causes; MDS, subjects with mood disorders who died of suicide; PVN, paraventricular nucleus; TRH, thyrotropin-releasing hormone.

Note: Lines with asterisks on the graphs indicate that the two groups under them are significantly different. * indicates $0.01 \leq P < 0.05$, ** indicates $0.001 \leq P < 0.01$, *** indicates $0.0001 \leq P < 0.001$, **** indicates $0.00001 \leq P < 0.0001$, Global- $P < 0.05$.

DISCUSSION

The human hypothalamus plays a major role in mood and stress modulation, but the hypothalamic pathophysiologic systems that differentiate suicidality from MD are poorly understood and continue to be debated. Here, we show for the first time, strong PR-associated changes in a specific group of peptidergic neurons in relation to suicide. Our major findings are: (1) hypothalamic PR-containing cells were concentrated in the nuclei adjacent to the third ventricle in a relatively stable pattern in both genders throughout the lifespan, (2) various peptidergic neurons, as well as putative neural progenitors co-expressed PR, (3) the number of POMC neurons in the INF was increased in suicide completers, and this elevation was primarily due to PR/POMC co-expressing neurons, (4) NPY neuron number in the INF was sharply reduced in patients with MD, but PR/NPY co-expressing neurons did not participate in this decrease, (5) in the control subjects who died of natural causes, the number of PR/POMC and PR/NPY neurons in the INF correlated with the number of CRH and TRH neurons in the PVN, which seemed to be disturbed by MD and suicide tendencies. We also reported a sex difference: A reduced number of NPY neurons was found in MD compared to controls in males but not in females. Together, these findings add to the growing evidence supporting an involvement of POMC neurons in suicide and suggest that progesterone-mediated, hypothalamic peptide alterations are an important molecular basis of suicide.

We showed the distribution of PR cells throughout the lifespan in the human hypothalamus, which was generally in accordance with animal studies (Wagner, 2006). PR was present in specific hypothalamic nuclei known to have diverse hormone-mediating and neurodevelopmental functions in, e.g. the anxiety and stress response, neurogenesis, sexually dimorphic behaviors, circadian rhythm, feeding and metabolism, drinking, parental behavior and sleep (Swaab et al.,

2021). This widespread distribution suggests that progesterone via hypothalamic PR may affect a multitude of neuroendocrine regulators. The largely identical distribution pattern between the genders over the ages thereby seems to be independent of the progesterone plasma levels, which differ between males and females. Central progesterone could be derived from progesterone entering the brain after being synthesized in the periphery as a sex hormone, or it could be locally produced by astrocytes as a brain-derived neuroactive steroid (Schumacher et al., 2004). Interestingly, the ependymal layer, subventricular zone and choroid plexus did not show PR cells in the fetal hypothalamus, but these structures were positively stained in children. In contrast, the tuberal part of the pituitary expressed PR-ir cells from the fetal period throughout the lifespan, which suggested that the placenta and fetal hypothalamic astrocytes, rather than the fetal adrenal gland, were two major resources for fetal brain progesterone (Sinchak et al., 2003; Zwain and Yen, 1999). In addition, PR expression in the choroid plexus was weaker in females than males during their reproductive ages but became more similar after menopause, indicative of a sex hormone-specific sensitivity in PR in a brain region that was part of the blood-cerebrospinal fluid barrier. The preoptic area, a region involved in sexual and parental behaviors, showed robust PR staining in the fetal hypothalamus, suggesting that fetal progesterone levels were positively associated with the maternal levels (Wagner, 2006). Of note, the density of PR-ir cells along the third ventricle decreased from the ventromedial to the dorsolateral hypothalamus, indicating that the INF, a nucleus that was anatomically located outside the blood-brain barrier, might be a major structure through which peripheral progesterone entered the brain. Positive PR expression in the epithelial cytoplasm, but not in the nuclei of choroid plexus, supports this possibility, which also suggests that both peripherally synthesized and neuroactive progesterone inputs may regulate emotional and metabolic integration (Magoul et al., 1994).

The presence of PR in neural progenitors and specific populations of peptidergic neurons potentially determines the neurobiological substrate that is modulated by progesterone in the human hypothalamus. Selective co-expression of PR and particular neuropeptides, e.g., CRH, SOM and TH, in different human hypothalamic nuclei indicates that progesterone regulation of the neuroendocrine system has region- and function-heterogeneous targets. PR also presented co-localization with neural progenitors in the subventricular zone and INF, which were considered to be the main neurogenic niches in the adult human brain (Van Den Berge et al., 2010), indicating that progesterone might be involved in human adult neurogenesis (Ponti et al., 2018). In addition, the presence of some binuclear POMC and SOM neurons showed the late phase of mitosis and post-mitotic maturation of neurogenesis in the INF. Among these PR-ir nuclei, INF not only receives peripheral humoral stimuli (Magoul et al., 1994), but it is also connected to multiple regions in the limbic system via which the INF can be associated with the pathogenesis of neuropsychiatric disorders, as supported by murine models of depression (Chronwall, 1985; Desai et al., 2014; Qu et al., 2020).

Studies have demonstrated that endogenous opioids play important roles in modulating stress-related behaviors (Bali et al., 2015; Nobile et al., 2020). As a classic precursor, the current elevated POMC expression in suicide completers indicated the involvement of the endogenous opioid system activation (Williams et al., 2019). The possibility that progesterone participated in the modulation of this cell population was reinforced by the higher proportion of PR/POMC co-expressing neurons in MDS. Because progesterone is increased following stress (Ladisich, 1977), one may hypothesize that, in individuals with MD who are experiencing suicidal thoughts, progesterone may modulate POMC synthesis, leading to opioid activation and subsequent suicidal acts (Lutz et al., 2020; Yang and Lim, 1995). Among subjects with MD, the numbers of POMC-ir and PR/POMC-ir neurons were increased in MDS, and the proportion of PR/POMC-ir neurons increased both in MDS and MDE, suggesting that progesterone might be associated with endogenous opioid activation in MD patients with suicidality. A similar increase of PR/POMC-ir neurons was absent in the controls who died by legal euthanasia, supporting the assessment that comorbid mental disorders were of higher risk for suicide attempts. In addition, an animal study reported that progesterone administration could elicit long term POMC elevation (Petersen et al., 1993), which was consistent with the observation that women had a higher tendency of suicide during menstrual and postpartum periods when their peripheral progesterone levels dropped sharply (Baca-Garcia et al., 2010; Behera et al., 2019; Papadopoulou et al., 2018).

An overall reduction of NPY expression in individuals with MD and their sub-groups showed that NPY levels in the INF were low, independent of suicidality. Our earlier research has displayed that the duration of an agonal state before death in controls was accompanied by a decreased NPY expression (Fliers et al., 2001a). For this reason, we examined brain donors who underwent legal euthanasia as they had very short agonal states. By comparing NPY-ir neurons in CE versus MDE and CN, we found that a reduction in numbers was present only in MD. Our data agreed with an antidepressant effect of NPY, and we concluded that MD, rather than euthanasia, could play a major role in impairing hypothalamic NPY reserves. On the other hand, both MD and control subjects who died of legal euthanasia possessed strong suicidal ideations, but they showed opposite numbers of NPY-ir neurons in the INF, indicating that decreased hypothalamic NPY expression might be rather related to MD than to suicide. Although NPY in the plasma of suicide completers has been found increased compared to non-psychiatric controls (Lu et al., 2019), it is not known whether this elevation was caused by the NPY released from other regions of the brain or other organs. Moreover, there was a small proportion of PR/NPY-ir neurons that showed no changes in the different conditions. We have no evidence to relate this population to MD or suicidality.

Neurons in the INF control behaviors by synaptic regulations across neural circuits. We investigated an important step in this network, i.e., the relationship between the INF and PVN. The observations that POMC-ir, NPY-ir, CRH-ir and TRH-ir neurons terminated on each

other have provided a morphological substrate for their bidirectional interactions. In addition, we quantified the total number of CRH-ir and TRH-ir neurons in the PVN under different conditions and investigated their correlations with PR/POMC and PR/NPY neurons. The correlations in CN suggested that progesterone might inhibit TRH expression by upregulating POMC-ir neuronal projections or activate CRH-ir neurons by NPY innervation. However, these correlations did not persist in the subgroups of MD and death ideations, indicating that the INF-PVN circuit might be disturbed in this condition. Alternatively, terminations of POMC neuron axon projections, to the other hypothalamic nuclei, thalamus, amygdala, or brainstem that have been associated with the opioid system in depression (Lutz et al., 2020; Qu et al., 2020; Russell et al., 2014; Zan et al., 2021), may be the targeted regions that respond to negative stimuli. Tracing the INF projections of this opioid population may help to identify brain areas interconnected to the neuropathology of death ideations and suicidal behaviors.

Previous studies have proposed that suicide is associated with an overall CRH activation in the frontal-limbic system (Austin et al., 2003; Merali et al., 2006; Zhao et al., 2015). Our results and previous studies have revealed that CRH-ir neurons received POMC projections and processed opioid receptors (Reyes et al., 2007; Reyes et al., 2017), further indicating a possible involvement of opioid system activation in suicide. However, in the present study, we did not find alterations in the total number of CRH-ir neurons in the PVN of MD versus CTR, partly because CRH-ir neurons were absent in the APVN of euthanasia subjects. We point out that a lethal dosage of barbiturate administration, as performed in euthanasia, may prevent CRH-induction required neuronal stimulation by its anti-anxiety effect, which may be an explanation for the trend of lower CRH in euthanasia. In addition, we did not find an altered number of CRH-ir neurons in the PVN of MDNs compared to the other subgroups, which seems largely to be due to the data variation. In a previous study in a mixed group of patients with MD and suicide, we did find elevated numbers of CRH-ir neurons in patients with depression (Raadsheer et al., 1994).

In a previous study, we reported a strong reduction of TRH mRNA in the PVN in depressed patients and subjects with death ideations and depressive symptoms (Alkemade et al., 2003), which was in line with our current findings in MDN and MDS. A drop of the hypothalamus-pituitary-thyroid axis activity, including decreased thyroid-stimulating hormone (TSH), thyroid hormones and their negative correlations with suicide risk has been found in depressed patients and suicide attempters (Corrigan et al., 1992; Duval et al., 2017; Jokinen et al., 2008; Linkowski et al., 1984). The absence of CRH neurons in the APVN of euthanized subjects suggests that there may be diminished stress compared to the other patients with MD. Reversely, significantly more TRH neurons were found in MDE and CE compared to subjects who died of natural causes. In individuals who died of natural causes, the APVN has substantially fewer TRH neurons than the posterior PVN (Fliers et al., 1994), while euthanasia subjects in this study reveal a large number of TRH-ir neurons throughout their entire PVN. In humans, glucocorticoid administration

downregulates TRH expression (Alkemade et al., 2005). Considering the CRH innervation on TRH-ir neurons and their opposite expressions in the APVN across different groups, we propose that CRH negatively modulates the TRH activity.

It is important to notice that only MD males, but not MD females have fewer NPY-ir neurons in the INF. To the best of our knowledge, studies that showed hypothalamic NPY reductions in depression models and normalization of depressive-like behaviors after treatments used only male animals (Desai et al., 2014; Husum et al., 2003; Jiménez-Vasquez et al., 2007). Previous research showed that NPY expression in the INF was activated by testosterone only in male animals (Urban et al., 1993) when testosterone deficiency was accompanied by a higher incidence of depression, especially in males after midlife (Shores et al., 2005). As the subjects in this study had a median age over 60, decreases in testosterone might underlie the observed NPY reduction.

There are a few aspects of our study that require consideration. Menstrual information was not recorded in primary clinical data. Since the number of younger women is small, this will likely not influence our general conclusions. Secondly, the sample size is relatively small, limited by the unique cases of legal euthanasia and the strict inclusion and exclusion criteria we used. Another limitation is that we could not stratify for the way every subject accomplished suicide. For instance, information about violent or non-violent categories was not available in the clinical records. In addition, our study employed human postmortem brain tissue which made it inevitable to consider the possible role of antidepressants. A recent clinical study has examined thyroid function in first-episode drug-naïve patients with MDD and reports that suicide attempters had higher levels of TSH than non-suicidal individuals (Shen et al., 2019). Therefore, we distributed the antidepressant users, in relation to the pharmacologic effects and dosages in MD, among the subsets.

In conclusion, we systematically examined the anatomical and functional organization of PR in the human hypothalamus and addressed its association with suicide by demonstrating an increased proportion of PR/POMC neurons in the INF in patients with MD and fatal suicidality. This indicates that the possible role of progesterone in suicide may at least partially depend on the opioid system activation that disrupts the physiological connections with the PVN. The identification of distinct neuronal subpopulations involved in the effects of progesterone on suicidality will help better understand the molecular mechanisms underlying MD and suicide. We suggest that special attention should be paid to females with MD, who use hormonal contraception containing a high concentration of progesterone. Given PR/POMC-ir neurons present in both genders and throughout the entire lifespan, the implications may be wide-reaching and relevant for the general population and thus deserve additional studies. Last but not the least, we showed for the first time that on a molecular level difference can be found between legal euthanasia and accomplished suicide in the human brain, indicating that there might be distinct neurobiological mechanisms underlying fatal suicidality and suicide, respectively.

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Disclosures

The authors have no conflicts of interest to declare.

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Table S1a. Clinico-pathological information (PR mapping).

Sample number	Age	Age group	Sex	PMD (h)	CTD	CSF pH	BW (g)	Cause of death
CM1	40gw	Perinatal period	M	14:05	19:55	N.A.	320	Respiratory failure
CM2	4y	Childhood	M	23:55	9:35	N.A.	1565	Sepsis
CM3	17y	Adolescence	M	41:00	0:00	N.A.	1499	Myocarditis
CM4	27y	Youth	M	N.A.	N.A.	N.A.	N.A.	Drowning
CM5	55y	Middle age	M	7:15	11:05	N.A.	1393	Intestinal ischemia
CM6	79y	Elderly age	M	6:30	10:00	6.71	1121	Legal euthanasia
CF1	40gw	Perinatal period	F	30:45	3:30	N.A.	380	Asphyxia
CF2	7y	Childhood	F	9:45	7:15	N.A.	1320	Respiratory failure
CF3	15y	Adolescence	F	20:00	13:00	N.A.	1480	Lung edema
CF4	26y	Youth	F	6:35	2:25	N.A.	1156	Respiratory arrest
CF5	54y	Middle age	F	5:35	15:15	6.40	1118	Liver cirrhosis
CF6	83y	Elderly age	F	3:20	16:30	6.80	1173	Legal euthanasia

Abbreviations: BW, brain weight; CF, control females; CM, control males; CSF, cerebral spinal fluid; CTD, clock time of death; F, female; g, gram; gw, gestational week; h, hour; M, male; N.A., not available; PMD, postmortem delay; y, year.

Table S1b. Clinico-pathological information (fetal hypothalami).

Sample	Age (gw)	Sex	PMD (h)	CTD	BW (g)	Cause of death
1	22	M	15:16	17:44	N.A.	N.A.
2	30	M	17:00	0:00	150	Pneumonia
3	37	M	17:00	0:00	400	Congenital heart syndrome

Abbreviations: BW, brain weight; CTD, clock time of death; g, gram; gw, gestational week; h, hour; M, male; N.A., not available; PMD, postmortem delay.

Table S1c. Clinico-pathological information (two-group comparison).

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
MD1	MDD	50	M	N.A.	N.A.	N.A.	1380	Yes	N.A.	Suicide
MD2	MDD	39	M	41:00	00:00	N.A.	1670	Yes	TCA, SSRI	Suicide
MD3	BD	39	M	48:00	11:30	N.A.	1220	Yes	BZD, RIMA, SSRI	Suicide: intoxication
MD4	MDD	79	M	21:10	17:50	N.A.	1530	Yes	BZD, SSRI	Suicide: high fall
MD5	MDD	74	M	62:55	17:05	N.A.	1444	Yes	BZD, Cisordinol, SSRI	Suicide: hanging
MD6	MDD	62	F	20:35	00:00	6.53	1199	Yes	BZD	Suicide: suffocation
MD7	MDD	56	F	10:30	14:00	6.42	1120	Yes	BZD	Suicide: intoxication
MD8	MDD	62	F	11:40	16:15	6.18	1155	Yes	BZD	Suicide: strangulation
MD9	MDD	58	M	4:30	9:40	6.80	1470	Yes	SGA, SSRI, TCA	Suicide: intoxication
MD10	MDD	90	F	3:55	11:20	6.62	1220	No	BZD, INN	Suicide: intoxication
MD11	BD	81	M	6:40	20:00	6.70	1283	No	BZD, Li, TCA, VPA	Legal euthanasia
MD12	MDD	77	F	8:40	11:20	6.77	1120	Yes	BZD, INN, SSRI	Legal euthanasia
MD13	MDD	50	M	9:40	13:00	6.80	1420	No	BZD	Legal euthanasia
MD14	MDD	38	M	6:15	11:45	6.79	1525	No	BZD	Legal euthanasia
MD15	MDD	61	F	8:40	15:35	7.07	1205	No	BZD	Legal euthanasia
MD16	MDD	61	M	5:45	14:05	N.A.	1210	No	SSRI	Legal euthanasia
MD17	MDD	67	M	3:30	11:10	6.72	1445	No	BZD, Hal	Legal euthanasia
MD18	MDD	70	M	20:00	19:00	N.A.	1500	No	BZD, DA stabilizer, SSRI	Heart attack
MD19	MDD	73	F	22:00	19:00	N.A.	1287	No	BZD, TCA	Bronchopneumonia
MD20	MDD	71	M	13:30	22:30	N.A.	1109	No	BZD, Li, MAOI, PTZ	Respiratory insufficiency
MD21	MDD	61	M	60:20	4:40	N.A.	1424	No	PTZ	Pneumonia

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
MD22	MDD	81	M	6:00	15:30	6.50	1280	No	Hal	Renal insufficiency
MD23	MDD	68	M	8:55	5:45	6.82	1510	Yes	BZD, SSRI	Sudden death
MD24	BD	70	M	6:50	21:00	6.19	1275	No	BZD, LTG, VPA	Pulmonary infection and renal insufficiency
MD25	BD	73	M	13:45	19:45	6.24	1480	No	BZD	Infection
MD26	BD	94	F	5:10	4:00	7.55	985	No	BZD, SSRI	Respiratory and cardiac failure
MD27	MDD	58	F	7:20	23:00	5.61	1295	No	BZD, DA stabilizer, SSRI	Renal insufficiency
MD28	BD	65	M	4:50	18:30	N.A.	1305	No	BZD, Li, SGA	Colon carcinoma
Median	-	66	-	08:55	14:05	6.70	1291	-	-	-
CTR1	-	68	F	5:45	12:15	6.97	1135	No	None	Legal euthanasia
CTR2	-	83	F	3:20	16:30	6.80	1173	No	None	Legal euthanasia
CTR3	-	49	M	6:15	17:30	6.15	1364	No	BZD	Legal euthanasia
CTR4	-	79	M	6:30	10:00	6.71	1121	No	BZD	Legal euthanasia
CTR5	-	83	M	5:45	19:35	7.28	1195	No	None	Legal euthanasia
CTR6	-	41	M	17:00	00:00	N.A.	1150	No	None	Renal insufficiency
CTR7	-	72	F	17:30	16:30	N.A.	N.A.	No	None	Bronchopneumonia
CTR8	-	49	M	21:40	19:20	N.A.	1629	No	None	Cardiac infarction
CTR9	-	74	F	7:25	9:50	6.95	1167	No	BZD	Intestinal necrosis
CTR10	-	66	M	41:00	00:00	N.A.	1461	No	INN	Septic shock
CTR11	-	88	F	5:55	7:00	6.05	1115	No	BZD	Cardiac arrest
CTR12	-	39	M	16:30	00:30	N.A.	1400	No	None	Cardiac infarction

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
CTR13	-	55	M	7:15	11:05	N.A.	1393	No	BZD, INN	Intestinal ischemia
CTR14	-	78	F	4:35	22:40	6.41	1226	No	BZD, INN	Bronchopneumonia
CTR15	-	73	M	8:00	16:15	5.37	1553	No	BZD, INN	Pneumonia
CTR16	-	83	M	5:15	11:15	6.60	1372	No	BZD	Cardiac infarction
CTR17	-	67	M	9:00	15:05	6.48	1292	No	BZD, INN	Aortic aneurysm
Median	-	72	-	07:15	12:15	6.60	1259	-	-	-
P value	-	0.45	0.83	0.34	n.sig.	0.75	0.47	-	-	-

Abbreviations: BD, bipolar disorder; BW, brain weight; BZD, benzodiazepine; CTD, clock time of death; CTR, control; DA, dopamine; F, female; g, gram; h, hour; Hal, haloperidol; INN, nifedipine; Li, lithium; LTG, lamotrigine; M, male; MAOI, monoamine oxidase inhibitor; MD, mood disorders; MDD, major depressive disorder; N.A., not available; PMD, postmortem delay; PTZ, pentylentetrazole; RIMA, reversible inhibitor of monoamine oxidase-A; SGA, second-generation antipsychotics; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressants; VPA, valproate; y, year.

Table S1d. Clinico-pathological information (five-group comparison).

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
MDS1	MDD	50	M	N.A.	N.A.	N.A.	1380	Yes	N.A.	Suicide
MDS2	MDD	39	M	41:00	00:00	N.A.	1670	Yes	TCA, SSRI	Suicide
MDS3	BD	39	M	48:00	11:30	N.A.	1220	Yes	BZD, RIMA, SSRI	Suicide: intoxication
MDS4	MDD	79	M	21:10	17:50	N.A.	1530	Yes	BZD, SSRI	Suicide: high fall
MDS5	MDD	74	M	62:55	17:05	N.A.	1444	Yes	BZD, Cisordinol, SSRI	Suicide: hanging
MDS6	MDD	62	F	20:35	00:00	6.53	1199	Yes	BZD	Suicide: suffocation
MDS7	MDD	56	F	10:30	14:00	6.42	1120	Yes	BZD	Suicide: intoxication
MDS8	MDD	62	F	11:40	16:15	6.18	1155	Yes	BZD	Suicide: strangulation
MDS9	MDD	58	M	4:30	9:40	6.80	1470	Yes	SGA, SSRI, TCA	Suicide: intoxication
MDS10	MDD	90	F	3:55	11:20	6.62	1220	No	BZD, INN	Suicide: intoxication
Median	-	60	-	20:35	11:30	6.53	1300	-	-	-
MDE1	BD	81	M	6:40	20:00	6.70	1283	No	BZD, Li, TCA, VPA	Legal euthanasia
MDE2	MDD	77	F	8:40	11:20	6.77	1120	Yes	BZD, INN, SSRI	Legal euthanasia
MDE3	MDD	50	M	9:40	13:00	6.80	1420	No	BZD	Legal euthanasia
MDE4	MDD	38	M	6:15	11:45	6.79	1525	No	BZD	Legal euthanasia
MDE5	MDD	61	F	8:40	15:35	7.07	1205	No	BZD	Legal euthanasia
MDE6	MDD	61	M	5:45	14:05	N.A.	1210	No	SSRI	Legal euthanasia
MDE7	MDD	67	M	3:30	11:10	6.72	1445	No	BZD, Hal	Legal euthanasia
Median	-	61	-	6:40	13:00	6.78	1283	-	-	-

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
MDN1	MDD	70	M	20:00	19:00	N.A.	1500	No	BZD, DA stabilizer, SSRI	Heart attack
MDN2	MDD	73	F	22:00	19:00	N.A.	1287	No	BZD, TCA	Bronchopneumonia
MDN3	MDD	71	M	13:30	22:30	N.A.	1109	No	BZD, Li, MAOI, PTZ	Respiratory insufficiency
MDN4	MDD	61	M	60:20	4:40	N.A.	1424	No	PTZ	Pneumonia
MDN5	MDD	81	M	6:00	15:30	6.50	1280	No	Hal	Renal insufficiency
MDN6	MDD	68	M	8:55	5:45	6.82	1510	Yes	BZD, SSRI	Sudden death
MDN7	BD	70	M	6:50	21:00	6.19	1275	No	BZD, LTG, VPA	Pulmonary infection and renal insufficiency
MDN8	BD	73	M	13:45	19:45	6.24	1480	No	BZD	Infection
MDN9	BD	94	F	5:10	4:00	7.55	985	No	BZD, SSRI	Respiratory and cardiac failure
MDN10	MDD	58	F	7:20	23:00	5.61	1295	No	BZD, DA stabilizer, SSRI	Renal insufficiency
MDN11	BD	65	M	4:50	18:30	N.A.	1305	No	BZD, Li, SGA	Colon carcinoma
Median	-	70	-	8:55	19:00	6.37	1295	-	-	-
CE1	-	68	F	5:45	12:15	6.97	1135	No	None	Legal euthanasia
CE2	-	83	F	3:20	16:30	6.80	1173	No	None	Legal euthanasia
CE3	-	49	M	6:15	17:30	6.15	1364	No	BZD	Legal euthanasia
CE4	-	79	M	6:30	10:00	6.71	1121	No	BZD	Legal euthanasia
CE5	-	83	M	5:45	19:35	7.28	1195	No	None	Legal euthanasia

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
Median	-	79	-	5:45	16:30	6.80	1173	-	-	-
CN1	-	41	M	17:00	00:00	N.A.	1150	No	None	Renal insufficiency
CN2	-	72	F	17:30	16:30	N.A.	N.A.	No	None	Bronchopneumonia
CN3	-	49	M	21:40	19:20	N.A.	1629	No	None	Cardiac infarction
CN4	-	74	F	7:25	9:50	6.95	1167	No	BZD	Intestinal necrosis
CN5	-	66	M	41:00	00:00	N.A.	1461	No	INN	Septic shock
CN6	-	88	F	5:55	7:00	6.05	1115	No	BZD	Cardiac arrest
CN7	-	39	M	16:30	00:30	N.A.	1400	No	None	Cardiac infarction
CN8	-	55	M	7:15	11:05	N.A.	1393	No	BZD, INN	Intestinal ischemia
CN9	-	78	F	4:35	22:40	6.41	1226	No	BZD, INN	Bronchopneumonia
CN10	-	73	M	8:00	16:15	5.37	1553	No	BZD, INN	Pneumonia
CN11	-	83	M	5:15	11:15	6.60	1372	No	BZD	Cardiac infarction
CN12	-	67	M	9:00	15:05	6.48	1292	No	BZD, INN	Aortic aneurysm
Median	-	70	-	8:30	11:10	6.45	1372	-	-	-
P value	-	0.43	0.97	0.07	Sig.	0.21	0.47	-	-	-

Abbreviations: BD, bipolar disorder; BW, brain weight; BZD, benzodiazepine; CTD, clock time of death; CTR, control; DA, dopamine; F, female; g, gram; h, hour; Hal, haloperidol; INN, nefiritide; Li, lithium; LTG, lamotrigine; M, male; MAOI, monoamine oxidase inhibitor; MD, mood disorders; MDD, major depressive disorder; N.A., not available; PMD, postmortem delay; PTZ, pentylenetetrazole; RIMA, reversible inhibitor of monoamine oxidase-A; SGA, second-generation antipsychotics; Sig., significance; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressants; VPA, valproate; y, year.

Table S2. Specification of the antibodies used.

Antibody	Species	Manufacturer	Catalogue number and specificity
PR	rabbit monoclonal	Abcam	ab32085
α -MSH	rabbit polyclonal	NIN	α -MSH 4372 #23.04.75 (Swaab and Visser, 1977)
NPY	rabbit polyclonal	NIN	Niepke #26.11.88 (Fliers et al., 2001b)
CRH	rat monoclonal	Gift NIN	PFU 83 (Raadsheer et al., 1993)
TRH	rabbit polyclonal	Novus Biologicals	NBP2-34014
GFAP	rabbit polyclonal	Agilent DAKO	GA524
Iba1	rabbit polyclonal	FUJIFILM Wako	019-19741
GFAP- δ	rabbit polyclonal	Gift NIN	100501 (Roelofs et al., 2005)
Nestin	mouse monoclonal	Chemicon	MAB5326
OXT	mouse monoclonal	Gift	OT-A-I-28 (Dai et al., 2017)
AVP	mouse monoclonal	Gift	VPIII-D-7 (Wu et al., 2006)
SOM	rabbit polyclonal	NIN	Somaar (Van de Nes et al., 1994)
GAL	rabbit polyclonal	NIN	Gaaltje (Garcia-Falgueras et al., 2011)
KISS1	sheep polyclonal	Gift	GQ2 (Dhillon et al., 2005)
TH	mouse monoclonal	Sigma-Aldrich	MAB318
DYN	rabbit polyclonal	Gift	Dynorphin (1-8) #73 (Sherman et al., 1988)

Abbreviations: α -MSH, alpha-melanocyte-stimulating hormone; AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; DYN, dynorphin; GAL, galanin; GFAP, glial fibrillary acidic protein; GFAP- δ , glial fibrillary acidic protein-delta; NIN, Netherlands Institute for Neuroscience; HLA-DP, DQ, DR, human leukocyte antigen-DP, DQ, DR isotypes; KISS1, kisspeptin; NPY, neuropeptide Y; OXT, oxytocin; PR, progesterone receptor; SOM, somatostatin; TH, tyrosine hydroxylase; TRH, thyrotropin-releasing hormone.

Note: 1 Specificity of commercial antibodies were provided by the manufacturers.

2 POMC-ir neurons were stained by an α -MSH antibody (Goldstone et al., 2002).

Anti-PR antibody used in this study detects PR isoform B, which is the positive regulator of progesterone effects.

Anti-CRH antibody is a kind gift from Prof. Dr. F.J.H. Tilders.

Anti-GFAP- δ antibody is a kind gift from Prof. Dr. E.M. Hol.

Anti-OXT antibody is a kind gift from Prof. Dr. F.W. van Leeuwen who obtained it from Dr. A. Hou Yu.

Anti-AVP antibody is a kind gift from Prof. Dr. F.W. Van Leeuwen who obtained it from Dr. A. Silverman.

Anti-KISS1 antibody is a kind gift from Prof. Dr. W.S. Dhillon.

Anti-DYN antibody is a kind gift from Prof. Dr. S.J. Watson.

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Table S3a. Immunohistochemistry for single staining.

Antibody	Washing buffer	Antigen retrieval	Blocking buffer	Incubating buffer	Dilution	Incubating time
PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI ¹	1:100	72 h
α-MSH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI-HS ²	1:8000	24 h
NPY	1×TBS	–	1×TBS–5% milk	SUMI-milk ³	1:1000	24 h
CRH	1×TBS	–	–	SUMI	1:100000	24 h
TRH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI-HS	1:4000	24 h

Abbreviations: α-MSH, alpha-melanocyte-stimulating hormone; CRH, corticotropin-releasing hormone; NPY, neuropeptide Y; PR, progesterone receptor; TBS, tris-buffered saline; TRH, thyrotropin-releasing hormone.

Note:

¹ SUMI: supermix, 0.25 g gelatin in 100 ml 1×TBS, pH 7.6 heat the gelatin, mix until it dissolves, add Triton-X 0.5 ml. Store in fridge.

² SUMI-HS: supermix-high salt, 0.25 g gelatin in 100 ml 3×TBS, pH 7.6 heat the gelatin, mix until it dissolves, add Triton-X 0.5 ml. Store in fridge.

³ SUMI-milk: add 5 g milk powder in 100 ml SUMI, mix until it dissolves. Prepare before use.

Table S3b. Immunohistochemistry for double staining.

Combo	Antibodies	Washing buffer	Antigen retrieval	Blocking buffer	Incubating buffer	Dilution	Incubating time	Colour development
PR+α-MSH	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	α-MSH	3×TBS	–	–	SUMI–HS	1:4000	24 h	DAB
PR+NPY	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	NPY	1×TBS	–	1×TBS–5% milk	SUMI–milk	1:500	24 h	DAB
PR+CRH	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	CRH	1×TBS	–	–	SUMI	1:50000	24 h	DAB
PR+TRH	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	TRH	3×TBS	–	–	SUMI–HS	1:2000	24 h	DAB
PR+GFAP	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	GFAP	1×TBS	–	–	SUMI	1:10000	24 h	DAB
PR+ Iba1	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	Iba1	1×TBS	–	–	SUMI	1:500	24 h	DAB
PR+GFAP–δ	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	GFAP–δ	1×TBS	–	–	SUMI	1:250	24 h	DAB

Combo	Antibodies	Washing buffer	Antigen retrieval	Blocking buffer	Incubating buffer	Dilution	Incubating time	Colour development
PR+nestin	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	nestin	1×TBS	–	–	SUMI	1:200	24 h	DAB
PR+OXT	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	OXT	1×TBS	–	–	SUMI	1:500	24 h	DAB
PR+AVP	PR	1×TBS	0.05M Tris–HCl buffer (pH 9.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	AVP	1×TBS	–	–	SUMI	1:100	24 h	DAB
PR+SOM	PR	1×TBS	0.05M Tris–HCl buffer (pH 9.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	SOM	3×TBS	–	–	SUMI–HS	1:800	24 h	DAB
PR+GAL	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	GAL	1×TBS	–	–	SUMI	1:800	24 h	DAB
PR+KISS1	PR	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	KISS	–	–	–	SUMI	1:50000	24 h	DAB
PR+TH	PR	1×TBS	0.05M Tris–HCl buffer (pH 9.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	TH	1×TBS	–	–	SUMI	1:750	24 h	DAB
PR+DYN	PR	1×TBS	0.05M Tris–HCl buffer (pH 9.0) microwave 800 w 20 min	–	SUMI	1:100	72 h	DAB–Ni
	DYN	1×TBS	–	–	SUMI	1:200	24 h	DAB

Combo	Antibodies	Washing buffer	Antigen retrieval	Blocking buffer	Incubating buffer	Dilution	Incubating time	Colour development
α -MSH+NPY	α -MSH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI-HS	1:8000	24 h	DAB-Ni
	NPY	1×TBS	-	1×TBS-5% milk	SUMI-milk	1:500	24 h	DAB
α -MSH+CRH	α -MSH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI-HS	1:8000	24 h	DAB-Ni
	CRH	1×TBS	-	-	SUMI	1:50000	24 h	DAB
α -MSH+TRH	α -MSH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI-HS	1:8000	24 h	DAB-Ni
	TRH	3×TBS	-	-	SUMI-HS	1:2000	24 h	DAB
NPY+ α -MSH	NPY	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	1×TBS-5% milk	SUMI-milk	1:1000	24 h	DAB-Ni
	α -MSH	3×TBS	-	-	SUMI-HS	1:4000	24 h	DAB
NPY+CRH	NPY	1×TBS	-	1×TBS-5% milk	SUMI-milk	1:1000	24 h	DAB-Ni
	CRH	1×TBS	-	-	SUMI	1:50000	24 h	DAB
NPY+TRH	NPY	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	1×TBS-5% milk	SUMI-milk	1:1000	24 h	DAB-Ni
	TRH	3×TBS	-	-	SUMI-HS	1:2000	24 h	DAB
CRH+ α -MSH	CRH	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI	1:100000	24 h	DAB-Ni
	α -MSH	3×TBS	-	-	SUMI-HS	1:4000	24 h	DAB
CRH+NPY	CRH	1×TBS	-	-	SUMI	1:100000	24 h	DAB-Ni
	NPY	1×TBS	-	1×TBS-5% milk	SUMI-milk	1:500	24 h	DAB

Combo	Antibodies	Washing buffer	Antigen retrieval	Blocking buffer	Incubating buffer	Dilution	Incubating time	Colour development
CRH+TRH	CRH	1×TBS	0.01M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI	1:100000	24 h	DAB-Ni
	TRH	3×TBS	-	-	SUMI-HS	1:2000	24 h	DAB
TRH+α-MSH	TRH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI-HS	1:4000	24 h	DAB-Ni
	α-MSH	3×TBS	-	-	SUMI-HS	1:4000	24 h	DAB
TRH+NPY	TRH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI-HS	1:4000	24 h	DAB-Ni
	NPY	1×TBS	-	1×TBS-5% milk	SUMI-milk	1:500	24 h	DAB
TRH+CRH	TRH	3×TBS	0.03M citrate buffer (pH 6.0) microwave 800 w 20 min	-	SUMI-HS	1:4000	24 h	DAB-Ni
	CRH	1×TBS	-	-	SUMI	1:50000	24 h	DAB

Note:

¹SUMI: supermix, 0.25 g gelatin in 100 ml 1×TBS, pH 7.6 heat the gelatin, mix until it dissolves, add Triton-X 0.5 ml. Store in fridge.

²SUMI-HS: supermix-high salt, 0.25 g gelatin in 100 ml 3×TBS, pH 7.6 heat the gelatin, mix until it dissolves, add Triton-X 0.5 ml. Store in fridge.

³SUMI-milk: add 5 g milk powder in 100 ml SUMI, mix until it dissolves. Prepare before use.

Abbreviations: α-MSH, alpha-melanocyte-stimulating hormone; AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; DAB, 3,3'-diaminobenzidine; DAB-Ni, 3,3'-diaminobenzidine-nickel; DYN, dynorphin; GAL, galanin; GFAP, glial fibrillary acidic protein; GFAP-δ, glial fibrillary acidic protein-delta; HLA-DP, DQ, DR, human leukocyte antigen-DP, DQ, DR isotypes; KISS1, kisspeptin; NPY, neuropeptide Y; OXT, oxytocin; PR, progesterone receptor; SOM, somatostatin; TBS, tris-buffered saline; TRH, thyrotropin-releasing hormone.

Table S4. Alterations in peptide neuron expression in the INF (POMC-ir, PR/POMC-ir, sPOMC, NPY-ir, PR/NPY-ir and sNPY) and PVN (CRH-ir and TRH-ir) neurons in relation to MD, legal euthanasia and suicide.

	Comparison	Fold change	Global-P	BHadj-P
POMC-TN	MD/CTR	1.40	0.15	
	CE/CN	1.60	0.01	0.36
	MDE/CE	-1.70	0.01	0.69
	MDN/CE	-1.11	0.01	0.47
	MDS/CE	1.35	0.01	0.09
	MDE/CN	-1.06	0.01	0.82
	MDN/CN	1.45	0.01	0.84
	MDS/CN	2.16	0.01	0.002
	MDE/MDN	-1.53	0.01	0.81
	MDS/MDE	2.29	0.01	0.02
	MDS/MDN	1.49	0.01	0.008
POMC-TV	MD/CTR	1.18	0.27	
	CE/CN	-1.13	0.33	
	MDE/CE	-1.08	0.33	
	MDN/CE	1.09	0.33	
	MDS/CE	1.41	0.33	
	MDE/CN	-1.21	0.33	
	MDN/CN	-1.03	0.33	
	MDS/CN	1.26	0.33	
	MDE/MDN	-1.18	0.33	
	MDS/MDE	1.53	0.33	
	MDS/MDN	1.30	0.33	
PR/POMC-TN	MD/CTR	1.29	0.28	
	CE/CN	1.04	0.001	0.58
	MDE/CE	1.57	0.001	0.81
	MDN/CE	-3.02	0.001	0.20
	MDS/CE	3.30	0.001	0.02
	MDE/CN	1.63	0.001	0.42
	MDN/CN	-2.91	0.001	0.44
	MDS/CN	3.43	0.001	0.002
	MDE/MDN	4.74	0.001	0.06
	MDS/MDE	2.10	0.001	0.03
	MDS/MDN	9.97	0.001	0.00004

	Comparison	Fold change	Global-P	BHadj-P
PR/POMC-TV	MD/CTR	1.10	0.25	
	CE/CN	1.06	0.003	0.58
	MDE/CE	1.00	0.003	0.81
	MDN/CE	-2.73	0.003	0.21
	MDS/CE	2.11	0.003	0.04
	MDE/CN	1.06	0.003	0.39
	MDN/CN	-2.57	0.003	0.53
	MDS/CN	2.24	0.003	0.002
	MDE/MDN	2.72	0.003	0.06
	MDS/MDE	2.11	0.003	0.04
	MDS/MDN	5.76	0.003	0.0002
sPOMC-TN	MD/CTR	1.48	0.26	
	CE/CN	1.52	0.22	
	MDE/CE	-1.70	0.22	
	MDN/CE	1.14	0.22	
	MDS/CE	1.10	0.22	
	MDE/CN	-1.12	0.22	
	MDN/CN	1.73	0.22	
	MDS/CN	1.67	0.22	
	MDE/MDN	-1.93	0.22	
	MDS/MDE	1.87	0.22	
	MDS/MDN	-1.03	0.22	
NPY-TN	MD/CTR	-3.45	0.0007	0.005
	CE/CN	2.19	0.0007	0.12
	MDE/CE	-15.87	0.0007	0.00002
	MDN/CE	-4.32	0.0007	0.008
	MDS/CE	-4.31	0.0007	0.008
	MDE/CN	-7.27	0.0007	0.0006
	MDN/CN	-1.97	0.0007	0.40
	MDS/CN	-1.97	0.0007	0.15
	MDE/MDN	-3.68	0.0007	0.02
	MDS/MDE	3.69	0.0007	0.03
	MDS/MDN	1.00	0.0007	0.93
NPY-TV	MD/CTR	-1.69	0.05	
	CE/CN	1.74	0.01	0.20
	MDE/CE	-4.46	0.01	0.001
	MDN/CE	-1.63	0.01	0.20

	Comparison	Fold change	Global-P	BHadj-P
	MDS/CE	-1.68	0.01	0.06
	MDE/CN	-2.55	0.01	0.01
	MDN/CN	1.07	0.01	0.98
	MDS/CN	1.04	0.01	0.55
	MDE/MDN	-2.74	0.01	0.02
	MDS/MDE	2.65	0.01	0.03
	MDS/MDN	-1.03	0.01	0.67
PR/NPY-TN	MD/CTR	-3.03	0.39	
	CE/CN	3.85	0.19	
	MDE/CE	N.A.	0.19	
	MDN/CE	-7.02	0.19	
	MDS/CE	-1.75	0.19	
	MDE/CN	N.A.	0.19	
	MDN/CN	-1.82	0.19	
	MDS/CN	2.20	0.19	
	MDE/MDN	N.A.	0.19	
	MDS/MDE	N.A.	0.19	
	MDS/MDN	4.01	0.19	
PR/NPY-TV	MD/CTR	-3.03	0.20	
	CE/CN	1.96	0.15	
	MDE/CE	N.A.	0.15	
	MDN/CE	-2.51	0.15	
	MDS/CE	-2.10	0.15	
	MDE/CN	N.A.	0.15	
	MDN/CN	-1.29	0.15	
	MDS/CN	-1.07	0.15	
	MDE/MDN	N.A.	0.15	
	MDS/MDE	N.A.	0.15	
	MDS/MDN	1.20	0.15	
sNPY-TN	MD/CTR	-3.45	0.0004	0.004
	CE/CN	1.88	0.0006	0.12
	MDE/CE	-12.91	0.0006	0.00002
	MDN/CE	-4.38	0.0006	0.008
	MDS/CE	-4.60	0.0006	0.008
	MDE/CN	-6.85	0.0006	0.0006
	MDN/CN	-2.33	0.0006	0.39
	MDS/CN	-2.44	0.0006	0.10

	Comparison	Fold change	Global-P	BHadj-P
	MDE/MDN	-2.95	0.0006	0.02
	MDS/MDE	2.81	0.0006	0.03
	MDS/MDN	-1.05	0.0006	0.91
CRH-TN	MD/CTR	-2.08	0.44	
	CE/CN	-23.36	0.17	
	MDE/CE	5.10	0.17	
	MDN/CE	9.00	0.17	
	MDS/CE	33.95	0.17	
	MDE/CN	-4.58	0.17	
	MDN/CN	-2.60	0.17	
	MDS/CN	1.45	0.17	
	MDE/MDN	-1.76	0.17	
	MDS/MDE	6.65	0.17	
	MDS/MDN	3.77	0.17	
CRH-TV	MD/CTR	1.38	0.21	
	CE/CN	-2.55	0.21	
	MDE/CE	1.39	0.21	
	MDN/CE	3.27	0.21	
	MDS/CE	4.04	0.21	
	MDE/CN	-1.83	0.21	
	MDN/CN	1.29	0.21	
	MDS/CN	1.59	0.21	
	MDE/MDN	-2.35	0.21	
	MDS/MDE	2.90	0.21	
	MDS/MDN	1.23	0.21	
TRH-TN	MD/CTR	-1.08	0.76	
	CE/CN	2.46	0.008	0.12
	MDE/CE	2.27	0.008	0.78
	MDN/CE	-5.55	0.008	0.13
	MDS/CE	-11.48	0.008	0.02
	MDE/CN	5.58	0.008	0.01
	MDN/CN	-2.26	0.008	0.98
	MDS/CN	-4.67	0.008	0.40
	MDE/MDN	12.61	0.008	0.02
	MDS/MDE	-26.01	0.008	0.005
	MDS/MDN	-2.07	0.008	0.64

	Comparison	Fold change	Global-P	BHadj-P
TRH-TV	MD/CTR	1.05	0.78	
	CE/CN	3.51	0.07	
	MDE/CE	1.05	0.07	
	MDN/CE	-5.48	0.07	
	MDS/CE	-8.56	0.07	
	MDE/CN	3.76	0.07	
	MDN/CN	-1.56	0.07	
	MDS/CN	-2.43	0.07	
	MDE/MDN	5.86	0.07	
	MDS/MDE	-9.15	0.07	
	MDS/MDN	-1.56	0.07	

Note: N.A., data incalculable as PR/NPY-TN and PR/NPY-TV in MDE are 0.

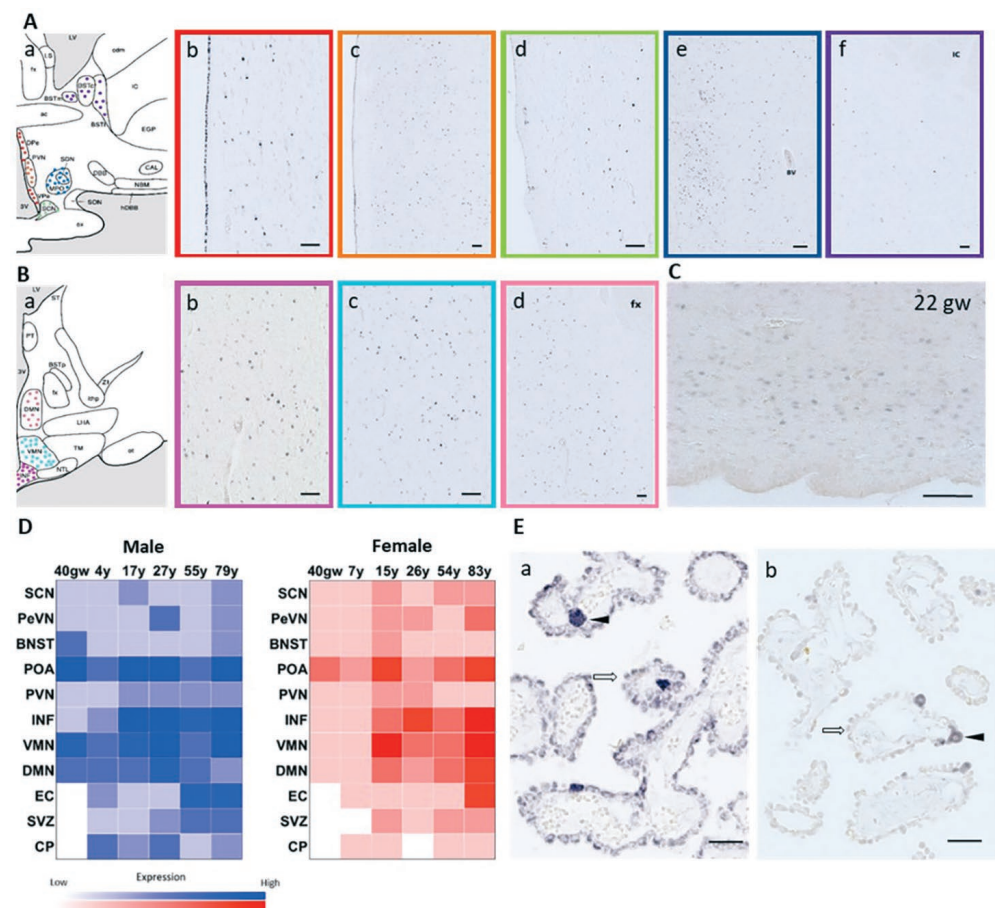


Figure S1. Progesterone receptor in the human hypothalamic nuclei. (A) Nuclear PR expression in the anterior hypothalamus. Higher magnification of the areas plotted in schematic figure **a** are shown in (b-f). Colors of the dots correspond to the frames. (B) Nuclear PR expression in the posterior hypothalamus. Higher magnification of the areas plotted in schematic figure **a** are shown in (b-d). Colors of the dots correspond to the frames. (C) Nuclear PR expression in the infundibular nucleus of 22 gestational weeks. (D) A brief plotting of PR expression in the hypothalamic nuclei across age and gender. (E) Cytoplasmic PR expression in the CP between a control male (a) and a control female (b). Empty arrows reveal the cuboidal epithelium while solid arrowheads show the columnar epithelium. As columnar epithelium represents an earlier stage of choroid plexus development than cuboidal epithelium, we thus assume that progesterone receptor has a preference for selective expression on more immature cells in males. Scale bars: 100 μm .

Abbreviations: 3V, third ventricle; ac, anterior commissure; BST, bed nucleus of the stria terminalis; BSTc, central nucleus of the bed nucleus of the stria terminalis; BSTl, lateral nucleus of the bed nucleus of the stria terminalis; BSTm, medial nucleus of the bed nucleus of the stria terminalis; BSTp, posterior nucleus of the bed nucleus of the stria terminalis; BV, blood vessel; CAL, accumbens nucleus, lateral part; cdm, medial caudate nucleus; CP, choroid plexus; DBB, diagonal band of Broca; DMN, dorsomedial nucleus; DPe, dorsal periventricular nucleus; EC, ependymal cells; EGP, external globus pallidus; fx, fornix; gw, gestational weeks; hDBB, horizontal limb of the diagonal band of Broca; IC, internal capsule; INF, infundibular nucleus; ithp, inferior thalamic peduncle; LHA, lateral hypothalamic area; LS, lateral septum; LV, lateral ventricle; MPO, medial preoptic area; NBM, nucleus basalis of Meynert; NTL, nucleus tuberalis lateralis; ot, optic tract; ox, optic chiasm; PeVN, periventricular nucleus; POA, preoptic area; PR, progesterone receptor; PT, paratenial thalamic nucleus; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic (or intermediate) nucleus; SON, supraoptic nucleus; ST, stria terminalis; SVZ, subventricular zone; TM, tuberomammillary hypothalamic nucleus; VMN, ventromedial nucleus; VPe, ventral periventricular nucleus; y, years; ZI, zona incerta.

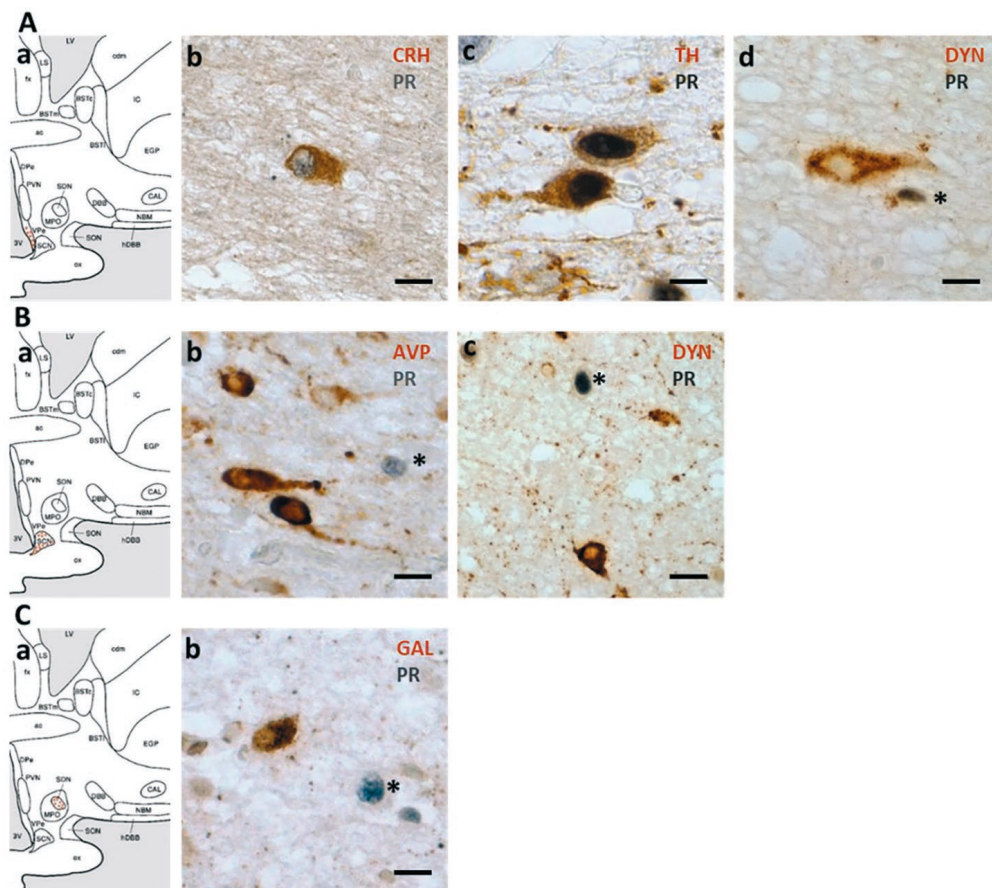


Figure S2. Double staining of PR-ir cells with different neuropeptides in the VPe, SCN and SDN. (A) (a) Schematic illustration of VPe in the human hypothalamus. PR-ir cells in the VPe express CRH (b) and TH (c), but not DYN (d, shown by *). (B) (a) Schematic illustration of SCN in the human hypothalamus. PR-ir cells in the SCN did not express AVP (b) or DYN (c, shown by *). (C) (a) Schematic illustration of SDN in the human hypothalamus. PR-ir cells in the SDN did not express GAL (b, shown by *). Scale bars: 20 μ m.

Abbreviations: AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; DYN, dynorphin; GAL, galanin; ir, immunoreactive; PR, progesterone receptor; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic (or intermediate) nucleus; TH, tyrosine hydroxylase; VPe, ventral periventricular nucleus.

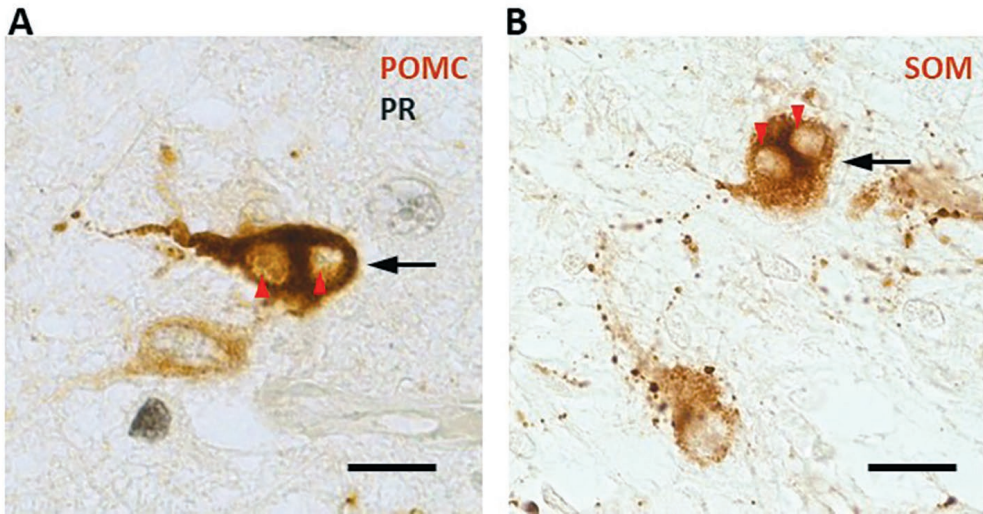


Figure S3. POMC-ir and SOM-ir neurons in the INF. A bi-nuclear POMC neuron (A) and a bi-nuclear SOM neuron (B). Arrowheads point at the two nucleoli in one neuron. Scale bars: 20 μ m.

Abbreviations: INF, infundibular nucleus; POMC, pro-opiomelanocortin; PR, progesterone receptor; SOM, somatostatin.

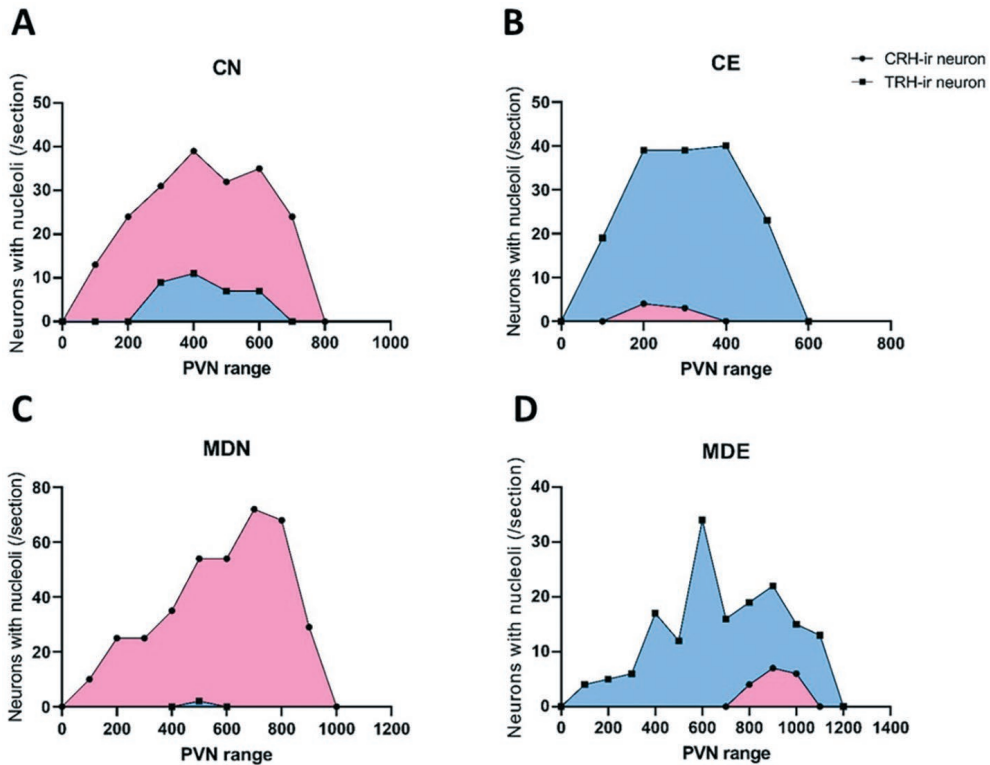


Figure S4. Sample distribution patterns of CRH-ir and TRH-ir neurons in the PVN. (A) A control subject who died by natural causes (CN). (B) A control subject who died of legal euthanasia (CE). (C) A patient with MD who died by natural causes (MDN). (D) A patient with MD who died of legal euthanasia (MDE). For each section, the total numbers of CRH-ir and TRH-ir neurons were determined by calculating the areas under the curve. A subject has been included only when the complete CRH or TRH cell areas are contained within the measured slides.

Abbreviations: CRH, corticotropin-releasing hormone; PVN, paraventricular nucleus; TRH, thyrotropin-releasing hormone.

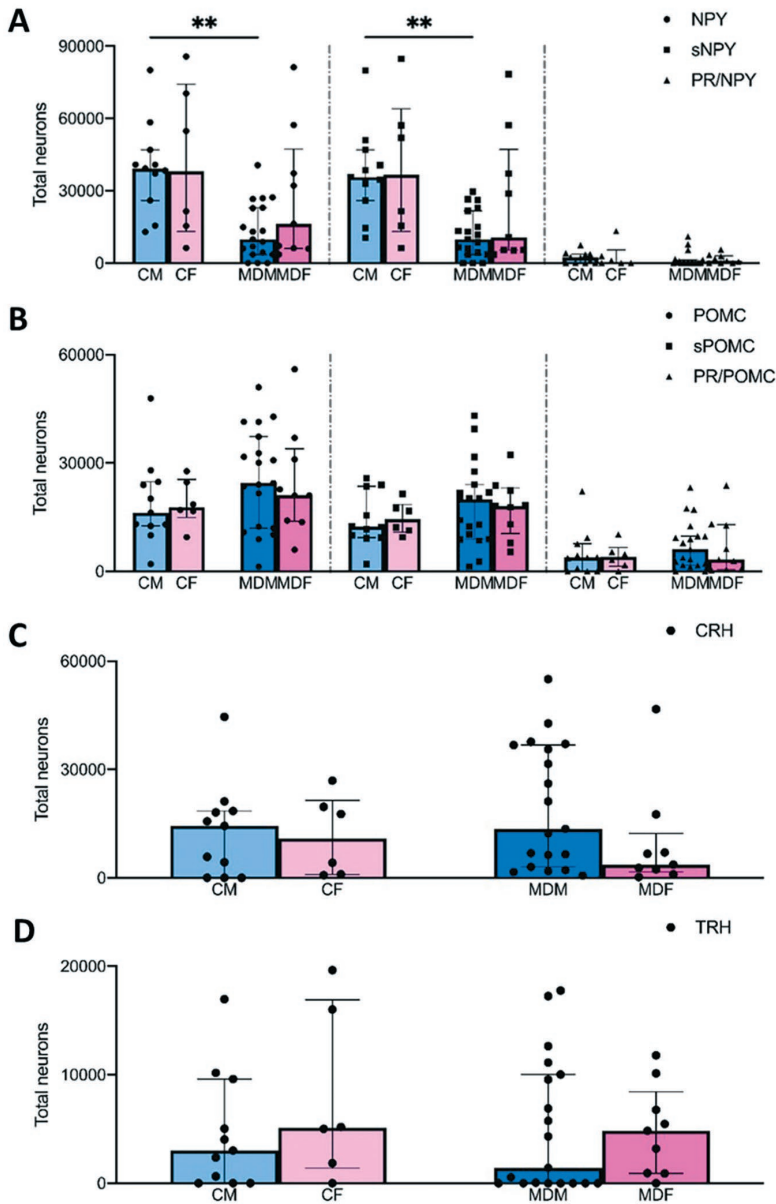


Figure S5. Total number of NPY (A) and POMC (B) neurons and their subsets, CRH (C) and TRH (D) total neurons in the controls and patients with MD between males and females.

Abbreviations: CF, control females; CM, control males; CRH, corticotropin-releasing hormone; MD, mood disorders; MDF, female patients with mood disorders; MDM, male patients with mood disorders; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; sNPY, NPY neurons that did not express PR; sPOMC, POMC neurons that did not express PR; TRH, thyrotropin-releasing hormone. Note: ** indicates $0.001 \leq P < 0.01$.

CHAPTER 7

HIPPOCAMPAL NEUROPATHOLOGY IN SUICIDE: GAPS IN OUR KNOWLEDGE AND OPPORTUNITIES FOR A BREAKTHROUGH

Zhang L, Lucassen PJ, Salta E, Verhaert PDEM, Swaab DF.

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ABSTRACT

Suicide is a major global hazard. There is a need for increasing suicide awareness and effective and evidence-based interventions, targeting both suicidal ideation and conduct. However, anti-suicide pharmacological effects are unsatisfactory. The human hippocampus is vulnerable to neuropsychiatric damages and subsequently releases psychobiological signals. Human hippocampal studies of suicide completers have shown mechanistic changes in neurobiology, which, however, could not reflect the neuropathological ‘fingerprints’ of fatal suicide ideations and suicide attempts. In this review, we provide several leading theories of suicide, including the serotonergic system, Wnt pathway and brain-derived neurotrophic factor/tropomyosin receptor kinase B signalling, and discuss the evidence for their roles in suicide and treatment. Moreover, the cognitive dysfunctions associated with suicide risk are discussed, as well as the novel evidence on cognitive therapies that decrease suicidal ideation. We highlight the need to apply multi-omics techniques (including single-nucleus RNA sequencing and mass spectrometry histochemistry) on hippocampal samples from donors who died by suicide or legal euthanasia, to clarify the aetiology of suicide and propose novel therapeutic strategies.

Keywords

Suicide; human hippocampus; neuropathology; cognition; legal euthanasia; single-nucleus RNA sequencing; mass spectrometry histochemistry.

INTRODUCTION

Suicide is a worldwide major public health and societal problem that involves over 700,000 human lives lost every year (WHO, 2021), while twenty times more people attempt suicide. The suicide rate in European countries has been gradually rising. In the Netherlands, the number of suicides is increasing by between 3 and 6 % annually. Preventive measures are lacking efficacy (Zalsman et al., 2016), and almost half of the suicide cases in the Netherlands are conducted by individuals receiving psychotropic drugs. Suicide is also the fourth leading cause of death among teenagers and adolescents (WHO, 2021). Belgium has the highest suicide rates among the European countries, where almost a quarter of the young population has had suicidal thoughts or attempted suicide in the last 12 months (Belgium, 2021). Psychiatric disorders, such as mood disorder (MD) and repeated suicide attempts, are the main risk factors for suicide completion (Beghi et al., 2021; Turecki et al., 2019). While suicide is often considered the worst outcome or consequence of psychiatric disorders, little attention has been paid to its independent, and likely unique, molecular-genetic background (Wang et al., 2019).

The hippocampus (HC) is a subcortical brain region that is highly sensitive to stress and richly endowed with glucocorticoid receptors (GR) in humans (Wang et al., 2013). It is part of the limbic-cortical-hypothalamic circuit, which is implicated in the pathophysiology of suicide (Lutz et al., 2017; Mahar et al., 2017). Neuroimaging studies have discovered a smaller HC in suicide, along with significant histopathological changes. Hippocampal subregions were shown to be anatomically and functionally distinct, which was also reflected in suicide-specific changes. For example, fewer granular neurons and more glia with larger nuclei by age were reported in the dentate gyrus (DG), while more neurons but fewer astrocytes were found in the *Cornu Ammonis* (CA) 2/3 subareas of the HC in individuals with major depressive disorder (MDD) who died of suicide (Boldrini et al., 2019; Chen et al., 2020; Cobb et al., 2016; Cobb et al., 2013), suggesting that components involved in the basic circuits of HC may perform functionally distinct roles in suicide development. In addition, antidepressants increased neural progenitor cells in the DG of subjects with MDD, which seems to be more specific to suicidal death than non-suicidal death (Boldrini et al., 2009; Lucassen et al., 2010).

While the neuropathology of suicidality was so far based upon HCs from subjects who had all completed suicide, a major problem for those studies is that it is hard to distinguish the molecular similarities and differences between suicide ideation, suicide attempts (single/repeated), and completed suicide. Strikingly, recent magnetoencephalographic studies showed that a specific type of electrophysiological connectivity and dynamics in the left HC was linked to suicidal thoughts in patients with MDD (Jiao et al., 2019; Nugent et al., 2020). While this novel finding points towards a putative neurobiological substrate and possible diagnostic marker for (the onset of) suicidality, it awaits future confirmation.

This review discusses the clinical and neuropathological manifestations in the human HC in association with suicidality. We searched the PubMed database until November 2021 using the medical subject headings search terms of hippocampus and suicide. In addition, we discuss the current antidepressants, which are prescribed with the hope of reverting the hippocampal alterations found in suicide, and their adverse effects in suicide intervention. We further discuss the unique opportunity of the HC from brain donors who died of legal euthanasia, to study differences between suicide ideations and attempts. Finally, we propose the employment of single-nucleus RNA sequencing and mass spectrometry histochemistry (MSHC) as future key spatial ‘-omics’ technologies to investigate the biomolecular fingerprints of different aspects of suicide severity. The objective is to obtain a better understanding of the cellular and molecular mechanisms that underlie suicide ideation and suicidal behaviour, and of potential novel targets for anti-suicidal therapies.

SUICIDE AND HIPPOCAMPAL ATROPHY

Various structural and functional imaging studies have reported that the HC is dynamically and volumetrically disorganized in patients with psychiatric disorders and suicidality. Of note, MDD, as the condition most frequently linked to suicide, is associated with structural changes in the HC. Previous meta-analyses suggest a reduction in the hippocampal volume of subjects with MDD, which is absent in bipolar disorder (BD) (Campbell et al., 2004). Depressed patients with suicidal ideations show no volumetric alterations but do show a decreased dynamic activity in the HC compared to non-suicidal individuals with depression (Jiao et al., 2019; Lan et al., 2019). Different from suicide ideators, suicide attempters exhibit a regional asymmetry in their left HC, independent of their psychiatric disorder (Cao et al., 2015). For example, suicide attempters with MDD show evidence of higher hippocampal functional connectivity (Wagner et al., 2021; Weng et al., 2019).

MDD patients with repeated or acutely strong suicide attempts further show much smaller hippocampal volumes than patients with a first suicide attempt, non-suicidal patients, or healthy controls (Colle et al., 2015; Kang et al., 2020; Sarkinaite et al., 2021). Reversely, in adolescent and young populations, suicide attempters with MDD have larger white matter subfields than non-suicidal patients (Zhang et al., 2021b). Suicidal attempters with BD also appear to have significantly reduced grey matter volume in the HC as compared to non-suicidal BD patients (Johnston et al., 2017; Niu et al., 2019). Hippocampal volume is reduced in schizophrenia (SCZ) (Nelson et al., 1998), but suicidality-associated hippocampal atrophy does not appear in SCZ (Spoletini et al., 2011), suggesting that reduced hippocampal volume may be a promising selective neuroimaging marker to predict suicidality in MD.

SUICIDALITY AND COGNITIVE IMPAIRMENT

Suicide is regarded to be a cognitive disorder because it strongly disturbs the mental processes involved in gaining knowledge and comprehension. The human HC is a crucial structure for such cognitive abilities and processing. Suicide-associated cognitive impairments have posited differences across ages. In psychiatrically hospitalized children, a sluggish cognitive tempo is associated with their increased suicide risk (Becker et al., 2016). These children also show decreases in episodic memory, which probably extends into adolescence and adulthood, where suicide attempts are also correlated with decreased memory performance (Huber et al., 2020). In adolescents, there is a significant association between specific cognitive characteristics and suicidal behaviours, with self-harm being among the strongest risk factors for eventual suicide death (Sinyor et al., 2020). Youths who attempt suicide are at high risk for repeated attempts and subsequently diagnosed mental health problems. They show general deficits in cognitive control towards emotional stimuli, a potential marker for adolescent suicide tendencies (Stewart et al., 2017). An increased risk for suicide completion in young males was associated with extreme cognitive abilities and poor social functioning (Weiser et al., 2017), while in young females, sleep disturbances are more likely linked to the onset of suicidal ideations (Bozzay et al., 2016).

Adults with suicide ideations can display social alienation due to perceived burdensomeness and thwarted belongingness, cognitive distortions, high reactivity to hopelessness, and aggression (Antypa et al., 2010; Fazakas-DeHoog et al., 2017; Jahn et al., 2015; Jankowski et al., 2020). In addition, susceptibility to suicidal thoughts seems to be induced via increasing anxiety sensitivity and rising cognitive concerns, robust predictors of suicide risk among patients with mental illnesses (Capron et al., 2013; Oglesby et al., 2015; Tucker et al., 2016). Compared to suicide ideators, people who have attempted suicide perform significantly worse on mental-state examinations including having a stronger self-blame, rumination, catastrophizing impulsiveness, and thus developing lethal behaviours (Abdollahpour Ranjbar et al., 2021; Gilbert et al., 2011). They also have less sense of acceptance, lower attention control, and hampered abilities of learning and memory (Abdollahpour Ranjbar et al., 2021; Alacreu-Crespo et al., 2020). In addition, relative to individuals who had either suicide ideations or undertook one single attempt, people who attempted suicide repeatedly showed worse neuropsychological performance (Delaney et al., 2012), suggesting that repeated suicidal behaviours might be related to impaired cognition. On the other hand, some studies seemed to show that the cognitive status of suicide attempters might not simply reflect a graver condition compared to the cognition of suicide ideators. For example, while cognitive inflexibility was prevalent among suicide ideators (Miranda et al., 2012; Roush et al., 2019), cognitive flexibility remained intact in suicide attempters (Brokke et al., 2020). Furthermore, regardless of their prior psychiatric disorder, suicide attempters have higher cognitive functions, such as normative planning and choice consistency (Alacreu-

Crespo et al., 2020; Moniz et al., 2017). The above findings suggest that there might be distinct neurobiological mechanisms underlying suicidal ideations and suicide attempts, respectively.

In the aged population, people diagnosed with a recent mild cognitive impairment or dementia are at an increased risk of attempting suicide (Günak et al., 2021), when suicide attempters demonstrate a pattern of deficits involving poorer abstract thinking and conceptual reasoning (McGirr et al., 2012; Olsson et al., 2016). Of note, suicide-associated cognitive dysfunction, such as decision-making impairments, were also apparent in healthy first-degree relatives of suicide completers (Hoehne et al., 2015). This may be related to the observation that individuals with relatives who completed suicide are more likely to attempt suicide (Qin et al., 2002). Thus, for every age group, the profiling of molecular patterns in association with the monitoring of cognitive performance and the pertinent brain morphology in the HC will be critical in a better understanding of the pathological mechanisms of suicide, as well as in improving the diagnosis and treatments.

Importantly, the cognitive deficits we summarized above were more prominently connected with the HC in structure and function (Brambilla et al., 2013; Chung et al., 2021; Hanseeuw et al., 2016; Khoury et al., 2019; Tang et al., 2021; Wixted et al., 2014) as compared to the other brain regions that were also reactive to (the early phase of) suicidality, such as the dorsolateral prefrontal cortex and insula (Nugent et al., 2020). In addition to the neuroimaging observations, HC-associated cognitive manifestations in clinical practice could be more sensitive in predicting an early phase of suicidality, suggesting that the HC might be a pertinent and regionally specific target for suicide-related cognition repair and early prevention.

BIOLOGICAL MECHANISMS IN THE HIPPOCAMPUS THAT ARE ASSOCIATED WITH SUICIDE

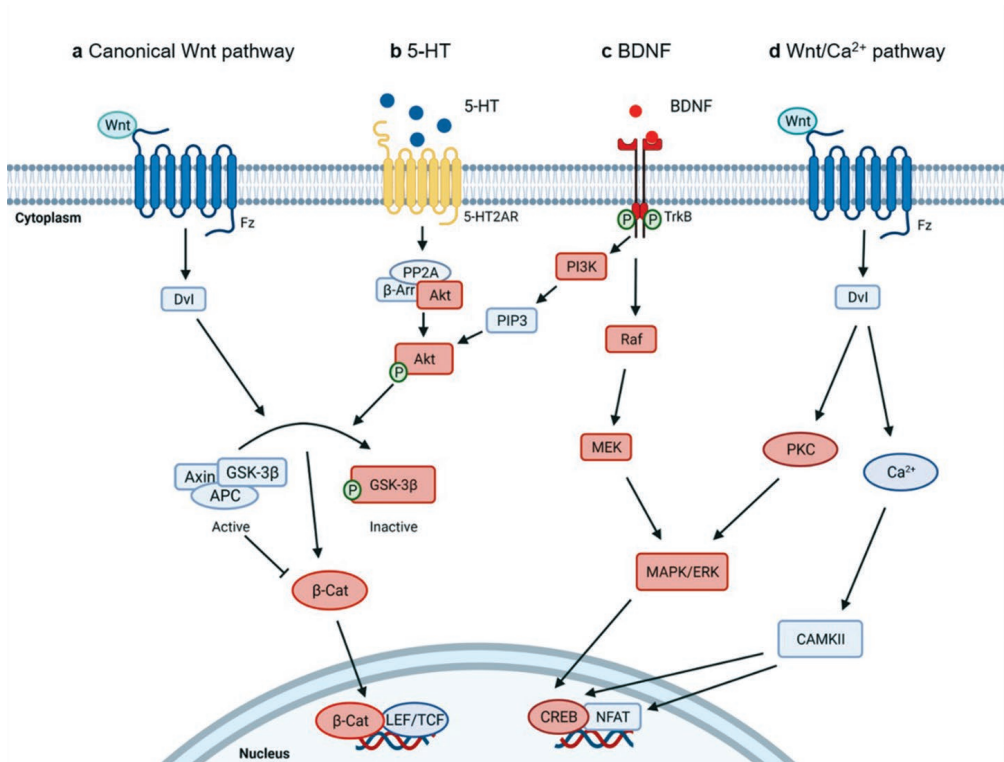


Figure 1. Schematic illustration of altered molecules in 5-HT, BDNF and Wnt pathways in the homogenate HC of depressed suicide. Molecules in red show down-regulation. Molecules in blue show, so far to our knowledge, no alterations have been reported. The molecule in yellow reveals up-regulation in signal cascades. By summing up this review, we considered that an excessive release of intracellular neuronal inhibitor dopamine may trigger a hyperpolarization of the postsynaptic membrane and thus activate postsynaptic 5-HT2AR, which produces a long-lasting depression to the downstream molecules. The figure is based on the literature of the present review and partly on modified figures by Voleti and Duman (Voleti and Duman, 2012).

Abbreviations: Akt, protein kinase B; APC, adenomatous polyposis coli; β-Cat, β-catenin; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding; Dvl, Dishevelled; ERK, extracellular signal-regulated kinase; 5-HT, serotonin; 5-HT2AR, serotonin receptor 2A; Fz, frizzled; GSK-3β, glycogen synthase kinase β; HC, hippocampus; LEF, lymphoid enhancer-binding factor; MAPK, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; NFAT, nuclear factor of activated T-cells; p, phosphorylation; PI3K, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKC, protein kinase C; PP2A, protein phosphatase; TCF, T-cell factor; TrkB, tyrosine kinase receptor B. (Figure 1 is created with BioRender.com. Agreement number: QO22Q3OPVD)

Epigenetic marks of suicide in the hippocampus

As one of the main mechanisms through which environmental and genetic factors interact with each other, epigenetic modifications in the HC are assumed to contribute to the development of suicidal behaviours (Kouter et al., 2019). Heritable phenotypic alterations, e.g. via DNA methylation and histone modification, reflect the multifactorial and polygenic state of suicide. A genome-wide methylation study on hippocampal tissue comparing suicide completers with non-psychiatric control subjects revealed a series of methylated DNA promoters that were functionally involved in learning and memory, and neuronal communication, such as synaptic transmission (Labonté et al., 2013). In addition, risk factors of suicide can alter the epigenetic status of specific genes. A specific genotype of the somatostatin receptor 4, a receptor that is predominantly found in the CA1, is involved in memory formation and related to an increased risk of suicide in individuals with alcohol dependence (Berent et al., 2017). Moreover, early life adversity is known as a relationship-associated suicidality factor, increasing the risk for various psychiatric disorders and posing a significant risk for attempting suicide. In particular, decreased levels of several human GR variants were found in the HC of suicide completers with a history of childhood abuse versus suicides without such history (Labonté et al., 2012). Also, hypermethylation of the ribosomal RNA gene promoter U13369 was consistent with reduced ribosome RNA expression in suicide subjects with a history of early childhood neglect/abuse (McGowan et al., 2008).

Gene expression and signal pathways concerning suicide

Serotonin receptor 2A (5-HT_{2A})

The human HC has a particular role in regulating the serotonergic system. Serotonin (5-hydroxytryptamine, 5-HT) is involved in various mental processes such as cognition, memory, anxiety and mood by activating its specific receptors that are regionally distributed throughout the brain. Both depression and suicide are accompanied by modulation of distinct 5-HT receptor subtypes in the human HC. It is widely accepted that an overall downregulation of the 5-HT_{1A} system is characteristic for depression (Yohn et al., 2017), without an influence of suicide (López et al., 1998). Where the concentrations of 5-HT and its turnover and transporter were not altered by suicidality (Anisman et al., 2008; Cheetham et al., 1989; Little et al., 1997), its main metabolite, i.e. 5-hydroxyindoleacetic acid, was increased in the HC of depressed patients who died by suicide (Cheetham et al., 1989; Owen et al., 1986).

Also, 5-HT_{2A}R is abundant in the HC of patients who completed suicide. Previous studies have associated polymorphisms of the 5-HT_{2A}R gene with a biological susceptibility to suicide attempts (Vaquero-Lorenzo et al., 2008). The expression of 5-HT_{2A}R is also higher in the HC of suicide victims, while its binding potential, combining receptor density and the affinity of serotonin, was not altered (Pandey et al., 2002; Roth et al., 1990; Soloff et al., 2007). Others showed that the binding sites of 5-HT₂ receptors in the HC were diminished in antidepressant-

free individuals who died by suicide compared to control subjects (Cheetham et al., 1988). This is likely due to a reduction of 5-HT_{2A} binding sites accompanied by higher serotonin affinity (Rosel et al., 2004; Rosel et al., 1998). Moreover, while the presynaptic uptake of serotonin was decreased in suicide cases as compared to the controls, the 5-HT_{2A} postsynaptic receptor was hypersensitive in line with a high affinity of this receptor subtype (Rosel et al., 2000; Rosel et al., 1997).

Taken together, the above evidence indicates that suicide may possibly trigger a synaptopathy in hippocampal cells. Functionally, a microarray analysis has revealed alterations of synaptic neurotransmission in the hippocampus of subjects who died by suicide (Sequeira et al., 2009). In synaptic structures, decreases of synapsin/synaptophysin ratio and postsynaptic density protein 95 have been found in the hippocampus of suicide victims (Sowa-Kućma et al., 2013; Vawter et al., 2002), suggesting that suicide may have affected synaptic components and plasticity. One of the major results is 5-HT_{2AR}-mediated postsynaptic hyper-excitability during serotonin transmission. Downstream of the 5-HT receptors, a decreased activity of protein kinase B (PKB or Akt) has been reported in the HC of suicide victims with depression compared to non-psychiatric control subjects (**Figure 1**) (Dwivedi et al., 2010). In addition, 5-HT receptor ligands and purine nucleotides were shown to have molecular similarities in terms of their receptor regulatory properties, such as crosstalk between the 5-HT_{2A} receptor and P2Y purinoceptor 12 in 5-HT storage during platelet aggregation, and in suicide as well (Vaquero-Lorenzo et al., 2008; Zhang et al., 2020a, b; Zhang et al., 2021a). Other depression-associated receptors, such as 5-HT_{1A}, 5-HT_{1C}, 5-HT_{2C}, and 5-HT₄ did not display alterations in the HC of suicide completers (López et al., 1998; Lowther et al., 1997; Pandey et al., 2006; Rosel et al., 2004; Roth et al., 1990). Of note, the above findings were derived from suicide victims who had suffered from depression as well and were analyzed by comparing them to non-psychiatric controls, indicating that the possible influence of the psychiatric disorders could have been overlooked (Zhao et al., 2019).

Brain-derived neurotrophic factor system

The brain-derived neurotrophic factor (BDNF) is highly expressed in the human HC and it is a critical mediator of physical activities. Among others, BDNF supports neuronal survival and promotes the growth and differentiation of immature neurons and synapses (Duman et al., 2021; Marlatt et al., 2012; Notaras and van den Buuse, 2020). As a well-known genetic locus of risk for mental disorders, BDNF is associated with stress and stress-related disorders (Castrén and Rantamäki, 2010; Molendijk et al., 2014; Shirayama et al., 2002). The presence of BDNF Val66Met polymorphism is even proposed as an independent predictor of high lethality in suicide attempts of depressed patients (Schenkel et al., 2010).

In conditions of prolonged stress, the influx of increased glucocorticoids into the HC activates the mitogen-activated protein kinase (MAPK) pathway via phosphorylation of its

receptor tropomyosin-related kinases B (TrkB), and thereby enhances negative memory formation (Notaras and van den Buuse, 2020). Dysfunctional BDNF in depression and suicide probably share common pathways and may procure similar hippocampal malfunctions (**Figure 1**). Previous studies have reported an overall breakdown of BDNF as well as the downstream-regulated molecules in the HC of depressed patients (Molendijk et al., 2014). At the extracellular and membrane levels, reduced BDNF results in lower levels of the transmembrane receptor TrkB, its activation resulting in decreased intracellular signalling among the phosphatidylinositol-3-kinase/Akt and MAPK-ERK kinase/MAPK pathways (Banerjee et al., 2013; Dwivedi et al., 2001; Dwivedi et al., 2006; Dwivedi et al., 2003b; Dwivedi et al., 2008; Dwivedi et al., 2009a; Dwivedi et al., 2009b, 2010; Karege et al., 2005; Pandey et al., 2008). Consequently, intra-nuclear expression and functional characteristics of cyclic adenosine monophosphate response element-binding protein, a transcription factor that triggers expression of various genes involved in neurogenesis and mood regulation, has been found decreased in the HC (Dwivedi et al., 2003a). Decreased histone acetylation and increased levels of factors related to de-acetylation and methylation processes also have been reported to lower BDNF, which may trigger subsequent alterations (Misztak et al., 2020).

Of note, the above-mentioned studies all were performed on hippocampal tissue from patients who died by suicide. The data were compared to healthy controls, and, thus, the contribution of the underlying psychiatric disorders themselves was not considered as a putative confounder (Zhao et al., 2019).

Wnt signalling

A role for the Wnt pathway in suicidal behaviours has been reported in several studies on the human postmortem HC. Generally, Wnt signalling is a pivotal regulator of cell proliferation, specification and migration. Molecules activated by the BDNF pathway are also involved in the modulation of Wnt signalling (**Figure 1**). Three different Wnt signalling pathways have been described: a canonical pathway and two non-canonical ones. In the canonical pathway, the activity of Akt is influenced by serotonergic and BDNF systems. Both Wnt and BDNF signalling are coupled to activation of intracellular signalling cascades that involve phosphorylation of glycogen synthase kinase β (GSK-3 β) (Voleti and Duman, 2012). This key downstream enzyme controls the translocation of β -catenin to the nucleus and activates the transcription of Wnt target genes. In the HC of suicide victims, pGSK-3 β -ser⁹, the phosphorylated isoform of Ser9 that exhibits a reduced activity, and β -catenin, both showed decreased expression relative to the controls (Pandey et al., 2009). This indicates aberrations in the downstream cascades of the Wnt signalling pathway, whereas GSK-3 β itself does not show marked changes in expression (Ren et al., 2013).

On the other hand, reduced activity of protein kinase C (PKC) levels has been reported in the HC of suicide cases compared to non-psychiatric control subjects. PKC is a midstream

molecule involved in the non-canonical Wnt pathway that downregulates the MAPK/ERK signals and subsequent cascades (Dwivedi et al., 2003a; Voleti and Duman, 2012). This is in accordance with a decreased phosphorylation of its substrate in the HC (Pandey et al., 2003). It also supports the synergistic effect between BDNF and non-canonical Wnt pathways (**Figure 1**). Taken together, this evidence emphasizes that data obtained after mixing suicide and depression in the same comparison may be interpreted with more prudence.

Glucocorticoid receptor

Long-term, stress-induced glucocorticoid elevation impairs hippocampal neuroplasticity and neurogenesis, and is involved in functional (feedback) abnormalities of the hypothalamus-pituitary-adrenal (HPA) axis that can, to some extent, be normalized by antidepressant drugs (Anacker et al., 2018; Brummelte and Galea, 2010; Lucassen et al., 2013; Lucassen et al., 2015; Snyder et al., 2011; Surget et al., 2011). A recent retrospective analysis has shown that oral glucocorticoids were associated with a robust increased risk of suicide in a dose-dependent manner (Laugesen et al., 2021). Although previous findings did not reveal obvious alterations in the cellular integrity or total transcripts of the human HC in relation to depression or glucocorticoid treatment, we later observed an increased expression of the GR in hippocampal CA1 of MDD patients, which interestingly, appeared higher in females than in males (Klok et al., 2011; Müller et al., 2001; Wang et al., 2012). In addition, glucocorticoid stress hormones that act on the GR have been proven to interact with the BDNF Val66Met polymorphism to determine hippocampus-gated fear and spatial memory (Notaras et al., 2016). In contrast, current studies did not reveal changes in the HC in molecules involved in the HPA circuitry in relation to suicide, including corticotropin-releasing hormone, its receptors and binding protein, or GR (isoforms) (Medina et al., 2013; Pandey et al., 2019; Pandey et al., 2013). Other studies hypothesize that early life adversity, but not suicide, was the primary cause of subfield volume and plasticity changes, and dysregulation of the GR gene in the HC (Alt et al., 2010; Boldrini et al., 2019; Labonté et al., 2012; McGowan et al., 2009; Teicher et al., 2012; Youssef et al., 2019). However, these hippocampal measurements were performed in bulk hippocampal preparations, which might preclude the profiling of the unique effects of glucocorticoids on specific cell populations in the hippocampus.

Androgen receptor

Androgens are steroid hormones involved in adult hippocampal neurogenesis and mood regulation by selectively binding to androgen receptor (AR) subtypes (Hamson et al., 2013; Zhang et al., 2010). Circulating levels of testosterone have been positively associated with suicidal behaviours in patients with depression (Sher et al., 2012). Postmortem studies have shown that the HC of suicide subjects has a stronger binding potential of AR- α 2, as evidenced by increased receptor density and binding affinity relative to non-suicidal depressed patients and

non-psychiatric controls (González et al., 1994; Meana et al., 1992). In contrast, AR- β expression in the HC did not exhibit alterations between suicide and control (Gurguis et al., 1999), yet the asymmetric expression of AR- β 2 between hemispheres in the control group was absent in suicidal cases (Joyce et al., 1992). So far, the mechanisms of androgen involvement in suicidality remain elusive.

ANTI-SUICIDAL INTERVENTIONS ASSOCIATED WITH COGNITIVE IMPROVEMENT

Cognitive-behavioural therapy

Cognitive-behavioural therapy (CBT) has been widely applied, and its efficacy towards suicide prevention has been proven across ages and following various adverse life triggers, including self-injury, post-traumatic stress disorder, sexual assault, and cognitive degeneration. Early intervention with CBT, via education and support to access community treatment, is crucial for decreasing suicide thoughts and self-injury, especially in young people at risk of suicide, who are, however, frequently not seeking treatment (Asarnow et al., 2017; Hetrick et al., 2017; Weinstein et al., 2018).

In adults, CBT is effective in preventing suicide attempts for patients with depression and/or a history of recent suicide attempts (Brown et al., 2005). Brief CBT and mindfulness-based cognitive therapy have been shown to reduce suicide risk by regulating cognitive reactivity, including measures of hopelessness and suicide beliefs (Chesin et al., 2016; Roberge et al., 2019). Given that mild cognitive impairment and dementia have been implicated in part of the aged suicide attempters, supportive services and cognitive reappraisal intervention may prevent the elderly from being hospitalized for suicidality (Günak et al., 2021; Lin et al., 2019). Of note, cognitive and working memory deficits may result directly from polysubstance use in the elderly and contribute to current serious suicidal ideation and attempts (Pompili et al., 2007). However, CBT was not beneficial in reducing suicidality when this occurred as a comorbidity of substance abuse (Morley et al., 2014), indicating that addiction-suicide interactions can have an independent impact on the pathogenesis and therapeutic barrier of suicide. Thus, cognitive improvement, possibly via CBT, plays an important role and marks the favourable prognosis of anti-suicidal therapies.

As we summarized in **Figure 1**, a decrease of neurotrophic intracellular molecules has been associated with alterations in several signalling pathways. A correction of BDNF levels may therefore improve the deficiency of downstream cytoplasmic molecules and, eventually, reverse intranuclear transcriptional regulation. A clinical trial has shown that plasma levels of BDNF can serve as an indicator of treatment response associated with the recovery of suicide ideations (Grunebaum et al., 2017). In addition, therapeutic effects of CBT are dependent on the BDNF

Val66Met polymorphism (Peters et al., 2020). Therefore, we hypothesize that the serum increase of BDNF could be a long-term effect of CBT therapies (Kobayashi et al., 2005), and the increase itself may be antisuicidal.

Serotonin receptor 2A ligands

The observations discussed above indicate that increased post-synaptic 5-HT_{2A}R level in the human HC may be involved in the pathogenesis of suicide. One may thus assume that either agonists or antagonists of the 5-HT_{2A}R could provide an anti-suicidal effect. So far, however, most 5-HT_{2A}R agonists or antagonists did not yield an improvement of the onset or progression of suicidality. However, some prescribed formulations have even been found to be associated with elevated suicide risk, possibly due to initial responses and adaptations in the 5-HT system. Among the agonists, for example, clinical application of 25I-NBOMe that maps the cerebral localization of 5-HT_{2A}R was found to be associated with more suicide attempts (Nichols and Grob, 2018; Suzuki et al., 2014). In addition, partial agonists of 5-HT_{2A}R caused more lethal and negative psychiatric effects. Among human immunodeficiency virus-infected adults treated with efavirenz, an antiretroviral compound that is also a 5-HT_{2A}R agonist, an increased risk of suicidal ideation was related to its plasma level (Mollan et al., 2014; Mollan et al., 2017; Ophinni et al., 2020). Furthermore, exposure to mefloquine, an antimalarial drug, has also been associated with violent acts and suicidal behaviours (Nevin, 2012; Ritchie et al., 2013). Moreover, selective 5-HT_{2A}R antagonists that inhibit serotonin reuptake, such as trazodone and mirtazapine, were both related to higher rates of suicide ideation, attempts, and self-harm behaviours (Coupland et al., 2015; Lavigne et al., 2019; Tubbs et al., 2021). The atypical antipsychotic olanzapine, whether or not it was associated with higher suicide-related events or the prevention of suicide, was found to be dependent on the underlying psychiatric illnesses of patients (Delapaz et al., 2021; Reutfors et al., 2013). A 5-HT_{2A}R antagonist is more likely to play a positive role in suicide intervention when it also blocks the dopaminergic system, as it is in the case of risperidone or olanzapine (Reutfors et al., 2013). Similarly, cyproheptadine is a preventive measure to reduce the adverse effects triggered by efavirenz-included antiretroviral therapy (Dabaghzadeh et al., 2013). It seems thus that simply activating or blocking the activity of the 5-HT_{2A}R does not relieve suicidality, but synergism with other neurotransmitters and neuromodulators may be effective. Evidently, more studies are needed to illustrate the mechanistic involvement of 5-HT_{2A}R in suicide.

Ketamine

In recent years, ketamine has become a novel drug in suicide interventions. Many studies have confirmed that ketamine can rapidly alleviate suicide thoughts regardless of the administered frequency (single/repeated) or mode (intravenous/intranasal/oral) (Beaudequin et al., 2020; Domany and McCullumsmith, 2021; Grunebaum et al., 2017; Grunebaum et al., 2018).

Ketamine has been broadly prescribed to patients with MD, SCZ, or non-psychiatric individuals with recent suicide ideations (Domany et al., 2020; Fan et al., 2017; Grunebaum et al., 2017; Grunebaum et al., 2018). The benefit of the significant reduction in suicide ideations induced by ketamine is enhanced by its being safe and well-tolerated. Its anti-suicidal effect is partly independent of the underlying psychiatric disorders or mood dysregulation (Ballard et al., 2014; Kang et al., 2021).

Interestingly, improvements in BDNF levels and memory, especially working memory, emerge as promising markers for the anti-suicide efficacy of ketamine treatment (Chen et al., 2021; Grunebaum et al., 2017). In clinical practice, short-term ketamine administration has pro-cognitive effects, e.g. in improving executive function and emotional processing (Lee et al., 2016). To date, both animal and human studies profiling neurobiological phenotypes and mechanisms have demonstrated that short-term ketamine intake is associated with reduced neuroinflammation, normalized glutamatergic hyperexcitability and synaptic plasticity (including BDNF, GSK-3, and TrkB receptor), modified dopamine synthesis and parvalbumin expression, and activation of the opioid system in the HC (Al Jurdi et al., 2015; Kokkinou et al., 2020; Luo et al., 2020; Nowak et al., 2019; Williams et al., 2019; Zanos and Gould, 2018). In addition, acute ketamine administration has been found to induce dopamine release in the rodent brain, further supporting its antidepressant potential (Kokkinou et al., 2018). This observation also suggests that reducing the intracellular dopamine content may be a putative anti-suicidal target. Moreover, MDD patients who bear a BDNF Val66Met polymorphism showed a weakened antidepressant response of ketamine as compared to non-Met carriers (Laje et al., 2012). Interestingly, this allele also impairs ketamine-stimulated synaptogenesis in mice (Liu et al., 2012).

However, our knowledge of safety data on long-term ketamine use, such as unresponsive cases, tolerance, possible adverse effects associated with discontinuation, and potential abuse is worth mentioning. For example, compared to individuals with acute and moderate suicidality, those who had a chronic and strong suicide ideation responded with less anti-suicidal effects to ketamine (Ballard et al., 2018; Zhan et al., 2019). In addition, suicide attempts during maintenance ketamine treatment were also reported (Cusin et al., 2020). Strikingly, it has been shown that long-term ketamine use could produce cognitive deficits partly because it restrains synaptic signalling (Luo et al., 2020). Therefore, understanding the specific mechanistic alterations in subjects who have suicide ideations or attempted suicide, but did not die by suicide, can help in the future to avoid or modify risk factors and adverse actions that occur in suicide interference. The study of hippocampal brain samples of such individuals may be particularly useful in that regard and in further developing novel pertinent therapeutic strategies.

THE FUTURE OF SUICIDE RESEARCH: A PROMISE AND A FOCUS ON NOVEL TECHNOLOGIES

Opportunities using the hippocampus of legal euthanasia donors

Suicide is a progressive disorder and we presume that suicide thoughts, suicide attempts and their severity, and suicide completion correlate with increasing neuropathological alterations in the human brain. Aberrant signal pathways summarized above have indicated the presence of a molecular diversity implicated in the HC in relation to suicidality. However, the neurobiological changes exhibited by subjects who completed suicide are considered as strong molecular features of the underlying mental illnesses. Our previous studies were devoted to unravelling the neuropathology of psychiatric diseases and suicide as separate entities that have revealed different molecular changes in relation to MD and suicide (Zhang et al., 2020a, b; Zhang et al., 2021a; Zhao et al., 2019).

In addition to completed suicide, we recently introduced, for the first time, brain samples from individuals who died of legal euthanasia (people who had strong death ideation or even attempted suicide but were eventually euthanized) to explore the stages of suicidality in different ways (unpublished results). First, with euthanasia samples, we could classify the pathogenesis of suicide thoughts and suicidal behaviours as single or repeated attempts, which may ultimately be essential for personalized medication applications according to severity and complications. Second, individuals who died of legal euthanasia may have comorbid psychiatric illnesses or be non-psychiatric ‘controls’, e.g. cancer patients. This way, we could distinguish biomarkers between subjects with psychiatric disorders, who died of natural causes, versus subjects who died of legal euthanasia. The same holds for the control cases, which will enable us to analyze whether differential gene expression may be due to suicide tendency per se, or to the underlying psychiatric disorders. Third, by comparing subjects who died of legal euthanasia but did not attempt suicide throughout their lifespan, and subjects who died of natural causes but had suicidal ideations during their lifetime, we can profile vulnerability or resilience gene signatures that explain the potential conversion from suicide thoughts to actual suicides. Fourth, by comparing the differential gene expression patterns derived from subjects with psychiatric illnesses, versus controls in the euthanasia group, and differential patterns derived from the same subset analysis within natural deaths, we hope to identify the target genes of high suicidality among patients with psychiatric disorders.

Application of single-nucleus RNA sequencing

Evidently, mapping the cellular and molecular complexity of the events that underlie the conversion from suicide ideation to suicide completion will be key to identifying novel and putatively druggable regulators and pathways. Only a handful of studies assaying the human ‘suicide’ brain in an unbiased, genome-wide manner, and in relation to MDs, or rather independently of them,

have been published to date (Glavan et al., 2021; Jabbi et al., 2020; Pantazatos et al., 2017; Punzi et al., 2019; Zhou et al., 2018). Strikingly, a common theme emerging from all these reports is the involvement of immune and inflammatory responses in the transcriptomic signatures related to suicide. These responses were associated not only with neuronal cells and pathways but also with astrocytes and microglia, highlighting the significance of multi-cellular crosstalk in shaping behaviours linked to suicide. Moreover, these findings suggest that decoding hub regulators driving suicide-associated behaviours cannot be achieved by bulk approaches and necessitates single-cell resolution. Even though no such evidence currently exists with respect to suicide subjects, single-nucleus RNA sequencing has been successfully applied to a wide range of human brain disorders, ranging from neurodegenerative to neuropsychiatric illnesses (Al-Dalahmah et al., 2020; Davila-Velderrain et al., 2021; Del-Aguila et al., 2019; Mathys et al., 2019; Nagy et al., 2020). While some concerns have been reported with respect to how representative the nuclear transcriptome is for the cellular transcriptomic in the human postmortem brain (Thrupp et al., 2020), studies directly comparing gene expression data from single nuclei versus single whole cells have demonstrated a high degree of concordance (Bakken et al., 2018; Lake et al., 2017; Olah et al., 2020), while disease-specific cellular states in the human brain also have been reported (Davila-Velderrain et al., 2021; Leng et al., 2021; Olah et al., 2020). Taken together, these observations may pave the way for the application of single-nucleus omics technologies to profile the cellular and molecular signatures of suicidal behaviours, and thereby identify novel targets for therapeutic intervention.

Application of mass spectrometry histochemistry

The above indicates that genes and proteins involved in specific neuronal signalling are strongly linked to suicidal tendencies. In addition, localization of molecular alterations in the brain is crucial for the interpretation of their functional consequences.

Besides the conventional ‘-omics’ technologies, we, therefore, envision a promising role in future HC studies for analytical methods, which allow the imaging of signalling biomolecules directly on histological sections. In this context, a reactive matrix-assisted laser desorption and ionization mass spectrometry imaging (MSI) method for visualizing neurotransmitters has been shown to work on cryosections (Shariatgorji et al., 2019). Moreover, recently technology has been developed based on high-resolution MSI for the localization of neuropeptides in historic collections of well-documented formaldehyde-fixed and paraffin-embedded (FFPE) tissue samples (Paine et al., 2018). Such FFPE tissues are abundantly stored in biobanks all over the globe, including the Netherlands Brain Bank (NBB). This FFPE method for localizing neuropeptides was designated MSHC. Unlike immunohistochemistry which requires specific antibodies for biomolecule detection/localization, MSHC allows the study of multiple molecules simultaneously, combining detained localization with a very high molecular specificity. Indeed, methodological aspects like the capacity to detect low expressed proteins and genes deserve

attention. Techniques like MSHC together with new developments in spatial transcriptomics, RNAscope and high-plex RNA imaging (Longo et al., 2021; Strack, 2021) allow for complete molecular structure characterization of tissue, combining high accuracy mass measurements with tandem mass spectrometry primary structure confirmation, and as such includes all the features necessary to evolve into a powerful non-targeted biomolecular discovery tool.

CONCLUSIONS

We have here synthesized the existing knowledge regarding the functional association of the hippocampal formation with suicide. Two major concerns merge from this review. First, the psychopathological basis of psychiatric disorders and suicide should be distinguished and studied separately. Neglecting this may very well be the reason for the so far less satisfactory effects of antidepressant or antipsychotic treatment for suicide prevention. Second, unravelling the neurobiological alterations in carefully stratified subject cohorts that fall under different categories of suicide classifications is key to ultimately enable the development of novel individual suicide treatment and prevention strategies. Considering the heterogeneity of the suicide alterations and their regional specificity in the human brain, we presented an overview of the current state of knowledge regarding pathological alterations, focusing on the HC since hippocampal atrophy and specific cognitive dysfunction are two major parameters implicated in suicidality. We have, therefore, initiated a comparative study of donors from the NBB (a scientific infrastructure that is capable to support a comprehensive analysis of the different stages of suicidality and selecting the right samples) who died of legal euthanasia, and those who completed suicide, in an attempt to detect in the human HC the neuropathological grading from the onset of suicide ideations, over single suicide attempts, to recurrent suicidal behaviours. These and other similar future studies employing multi-omics techniques on tissue samples from donors with different stages of suicidality will hopefully provide novel mechanistic insights into the pathogenesis of suicide across different stages.

Declaration of interest

The authors have no conflicts of interest to declare.

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CHAPTER 8

STRESS-ASSOCIATED PURINERGIC RECEPTORS CODE FOR FATAL SUICIDALITY IN THE HIPPOCAMPAL-HYPOTHALAMIC- PREFRONTAL CIRCUIT

Zhang L, Verwer RWH, van Heerikhuize J, Balesar R,
Correa-da-Silva F, Slabe Z, Lucassen PJ, Swaab DF.

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ABSTRACT

Imbalanced purine metabolism is a key neurological basis for suicide and mood disorders (MD), wherein purinergic receptors in stress-sensitive cerebral regions are thought to be differentially activated. A hippocampal network that links the hypothalamus and prefrontal cortex implements an affective sensation of stress. We discovered that the hippocampus encoded fatal suicidal ideations in the dentate gyrus (DG) by a considerable amount of the granule cell nuclei with P2X purinoceptor 7 (P2RX7) expression, irrespective of the underlying MD. Compared to controls, patients with MD showed microglial dyshomeostasis throughout the hippocampal formation. Strikingly, P2Y purinoceptor 12 (P2RY12)-expressing microglia with segmented processes were remarkably present in the superficial layers of the medial entorhinal cortex (mEnt) in individuals with fatal suicidality. In the hypothalamic stress-sensitive nuclei, P2RY12⁺ microglia were more expressed in the supraoptic nucleus in MD and even higher when fatal suicidality was present. In the prefrontal cortex, P2RX7 transcripts sharply dropped in suicidal individuals, possibly removing the prefrontal inhibition of the hippocampus and hypothalamus. Confounder analysis showed that the suicide-specific molecular features faded when the postmortem delay was prolonged. Our findings imply that fatal suicidality presents unique neuropathological alterations. The DG and mEnt are two crucial areas for deciphering the suicidal consequences. By including brain samples from legal euthanasia donors, suicide-specific biosignatures can be maximally retained. Decoding the bioactive framework through key genes, brain regions and neurological processes involved in suicide neuropathology may provide therapeutic strategies for suicidal individuals who are beyond the reach of mental health care.

Keywords

Suicide; legal euthanasia; hippocampus; hypothalamus; prefrontal cortex; purinergic receptors

INTRODUCTION

Suicide is a leading cause of death worldwide and has significant emotional, physical, and economic impact on sufferers and their relatives (WHO, 2021). It frequently occurs in the context of a mood disorder (MD), but these two conditions have at least partially distinct molecular signatures (Zhao et al., 2019). Disturbed purine metabolism is a key component in the pathophysiology of suicide (Klempner et al., 2009; Punzi et al., 2022; Zhang et al., 2020a, b; Zhang et al., 2021). A large-scale pharmacoepidemiologic study has shown that the prescription of folic acid, which plays a key role in purine biosynthesis, is linked to lower rates of suicide attempts in adults (Gibbons et al., 2022; Huennekens, 1969). Measuring the expression of purinergic receptors can predict both extracellular purine activities and intracellular biological responses in suicide and MD. One potent candidate is P2X purinoceptor 7 (P2RX7), an adenosine triphosphate (ATP)-gated ion channel, which is implicated as a novel therapeutic target for depression (Deussing and Arzt, 2018). Human genetic studies have linked the highly polymorphic *P2RX7* gene to MD (Bhattacharya et al., 2016). In peripheral blood mononuclear cells of suicide completers, P2RX7 mRNA levels were lower as compared to non-suicidal controls (Zhang et al., 2011). The other is a microglia-expressed adenosine diphosphate (ADP) chemoreceptor, P2Y purinoceptor 12 (P2RY12), which is found to be more expressed in the prefrontal cortex (PFC) of suicide completers (Punzi et al., 2022; Zhang et al., 2020a, b; Zhang et al., 2021). In addition to the PFC, animal models of depressive symptoms have further shown disturbed purine metabolism in the hypothalamus and hippocampus (Kongsui et al., 2014; Wu et al., 2017).

The hippocampus, which is incorporated in a limbic-cortical-hypothalamic circuit, has been functionally implicated in the connection between stress and cognition. Whole-brain functional neuroimaging studies have shown that hippocampal connectivity with the hypothalamus and PFC specifically predicts the subjective sensation of stress in humans (Goldfarb et al., 2020). While studies have discrepancies in summarizing brain circuits associated with suicidal behaviors (Malhi et al., 2020; Stange et al., 2020; Thompson et al., 2022), there are no follow-up studies that focus on people who later died by suicide, which is essential for studying the neurocircuitry of strong and determined suicide intent. One possibility is that intense and predetermined suicide tendencies (fatal suicidality) would be better detectable than transient or sporadic risks (non-fatal suicidality) by imaging-based indicators. Therefore, biological insights into suicide are more likely to be implanted in the candidate circuits of people with fatal suicidality.

To address the issue, we collected 321 samples (including 77 hippocampi, 44 hypothalami, and 200 PFCs) from donors who had MD, suicidality or accomplished suicide, and their matched controls. We examined the transcriptional and translational alterations of stress-related purinergic receptors (P2RX7 and P2RY12), as well as two glial markers (GFAP and CD68) to probe the neuroinflammatory and microglial phagocytic implications in suicide and MD. In contrast to the earlier measurements that were carried out in bulk tissue preparation, which

might have overlooked the specific role of selective subregions, we quantified five subdivisions in the hippocampal formation separately (dentate gyrus (DG), *cornu ammonis* (CA) 1, CA2-4, subiculum (Sub) and entorhinal cortex (Ent)), and two stress-sensitive hypothalamic nuclei (supraoptic nucleus, SON and paraventricular nucleus, PVN). Based on our previous studies (Zhang et al., 2020b; Zhang et al., 2021), we examined P2RX7 transcripts in the dorsolateral prefrontal cortex (DLPFC) and anterior cingulate cortex (ACC), to address the question of whether P2RX7 expression profiles were regionally modulated by completed suicide or coexistent psychiatric diagnoses.

MATERIALS AND METHODS

Human brain materials

Hippocampus

Seventy-seven paraffin-embedded tissue blocks containing the anterior or middle hippocampus were obtained from the Netherlands Brain Bank (NBB, Amsterdam, the Netherlands, Director: Prof. Dr. Inge Huitinga). The donors or their next of kin provided permission for the autopsy, use of brain material, and clinical documentation for research. The samples were divided into two main groups: 56 MDs and 21 matched non-psychiatric controls (Ctr) without a neurological disorder. There were three subsets in the MD group and two subsets in the Ctr group. The MD group contained 10 subjects with MD who completed suicide (MDS), 21 subjects with MD and fatal suicidality that led to legal euthanasia (MDE), and 25 subjects with MD who died of natural causes (MDN). For this cohort, we carefully selected a matched group that contained 10 subjects who displayed fatal suicidality and died of legal euthanasia for incurable diseases (CE), and 11 subjects who died of natural causes (CN). According to the suicidal consequences, subjects were regrouped into three subsets. Twenty-three subjects who did not report suicidality lifelong and died of natural causes (NS, no suicidality), 13 subjects who had a clinical history of suicide tendency but died of natural causes (NFS, non-fatal suicidality), and 41 subjects who died of completed suicide or legal euthanasia (FS, fatal suicidality). After we introduced the classifications of suicidality into grouping, which was documented as suicidal ideations and suicide attempts, subjects were subdivided into non-fatal suicidal ideation (NFSI) and suicide attempts (NFSA), and fatal suicidal ideations (FSI) and suicide attempts (FSA).

Hypothalamus

Forty-four tissue blocks containing the entire hypothalamus were obtained from the NBB according to the same procedure as described above. Subjects were collected and divided into two main groups: 28 MDs and 16 Ctrs. The MD group contained 10 MDS, 7 MDE, and 11 MDN subjects. The Ctr group contained 5 CE and 11 CN subjects.

Prefrontal cortex

Two hundred RNA samples, together with their demographic information and medical data, were obtained from the Depression and Array Collections of the Stanley Medical Research Institute (SMRI, Bethesda, MD, the USA, Director: Dr. Maree J. Webster). The SMRI provided us with RNA from the isolated grey matter of the DLPFC (Brodmann area 46) and ACC (Brodmann area 24). They were obtained from patients with MD (N = 54; major depressive disorder, MDD N = 24; bipolar disorder, BD N = 30) and matched controls (Ctr, N = 36) without a history of suicidal behaviors or any major psychiatric diagnosis. The cause of death for 30 of 54 patients with MD was suicide. Twenty-nine patients with MD had a history of psychotic features. The other cases and all control subjects died of natural causes or accidents. We distinguished the following subsets within the MD group: (1) patients with MDD or BD; (2) patients with MDD/BD who died of suicide (MDDS/BDS) or non-suicidal causes (MDDNS/BDNS); and (3) patients with or without a history of psychotic features (MDDP/BDP or MDDNP/BDNP).

Clinical details of every donor can be found in the Supplementary Information (**Table S1**).

Characterizing and counting P2RX7⁺ nuclei in the hippocampal formation

First, the density of the granule cells in the DG of every section was determined to assess structural stability. Second, P2RX7 and GFAP immunohistochemical staining was performed to investigate their expression in the hippocampal subfields. The distribution of P2RX7⁺ nuclei and P2RX7⁺ astrocytes, and the total expression of P2RX7 and GFAP in the hippocampus were determined and their associations with suicide and MD were established. Bright-field images were acquired by a ZEISS Axio Scanner and analyzed using QuPath (see Supplementary Information).

Recalling microglial functions and morphological profiles in the hippocampus

P2RY12 and CD68 immunohistochemical and immunofluorescent staining were performed as markers for microglial homeostatic and phagocytic capacity. Bright-field images were analyzed using QuPath and ImageJ for expression and morphological measurements. Fluorescent images were captured by a Leica laser-scanning microscope SP8 and processed with Imaris for 3D reconstruction. The density and radius of the microglial cell body, the size of the microglial cell body with processes and fragments (microglial segments that were disconnected from the cell body) were determined (see Supplementary Information).

Profiling the expression of purinergic receptors and glia in the hypothalamic nuclei

First, the volumes of the hypothalamic SON and PVN were determined based on the thionine staining. Second, P2RY12, CD68, P2RX7 and GFAP immunohistochemical staining were performed to investigate their expression in the SON and PVN. Images were analyzed with Image-Pro. CD68 and P2RY12 immunofluorescent staining and image processing were performed with the same protocols mentioned above (see Supplementary Information).

Transcriptional analysis of P2RX7 expression in the PFC

cDNA synthesis of the PFC-RNA was performed for real-time qPCR studies (see Supplementary Information). The transcriptional data of prefrontal P2RY12, GFAP, and CD68 expression in relation to suicide and MD have been included in our previous publications (Zhang et al., 2020b; Zhang et al., 2021).

Statistical analysis

S+ software (version 8.2, TIBCO, Seattle, WA, USA) was used for statistical analysis. The Chi-square test was used for the analysis of categorical data (suicidal classification and gender). For interval data, the Mann-Whitney test (2 samples) or the Kruskal-Wallis test with multiple comparisons (more than 2 samples) was used (Conover, 1980). Before processing real-time qPCR data, the values were $^{10}\log$ -transformed to enable simple reference gene correction and conventional statistical procedures. The reason for this transformation was that the observed Ct values used in order to quantify gene expression are exponents of the PCR efficiency. Application of the log-transformation yields an additive statistical model and, after all, statistical procedures have been finished, the data are back-transformed and presented as fold-changes. In the case of multiple testing, a Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) of p values was applied. When the Kruskal-Wallis test was used in combination with the Benjamini-Hochberg correction, we proceeded in a 2-step way. As multiple comparisons in the Kruskal-Wallis test are only allowed if the global $p < 0.05$ (Conover, 1980), we first corrected the global p -values and then selected for further analysis only those biomarkers for which this requirement was met. For each appropriate comparison, the corresponding p values were pooled and corrected according to Benjamini-Hochberg. All tests were 2-sided. Figures were prepared with GraphPad Prism (statistical graphs) or BioRender (illustrative graphs).

RESULTS

A prevalence of P2RX7-positive nuclei in the hippocampal formation mark fatal suicidality

In the hippocampus of individuals who died of natural causes, P2RX7 was generally expressed by glial cells (**Figure 1d** and **1l**, **S1d** and **S1h**, and **S2a** and **S2i**). In the DG, granule cell densities were similar between these groups (**Figure 1c**). Strikingly, in individuals who died by suicide or legal euthanasia, a considerable number of granule cells expressed P2RX7 within their nuclei (**Figure 1e-g**). Characteristic features of the P2RX7⁺ nuclei were their chromatin condensation and marginalization (**Figure 1e-g**). To qualify this finding, we determined the percentage of over 5% of P2RX7⁺ nuclei within the total granule cells to be a proportioned positive subject, in which P2RX7⁺ nuclei were at detectable levels in microscopy practice. The donors who died by

legal euthanasia or suicide had much higher proportions than those who died of natural causes (**Figure 1h**). Quantitative results presented similar enhancements in the ratio of P2RX7⁺ nuclei to the total number of nuclei in the granule cell layer (CE vs. CN: $p = 0.0008$, MDE vs. MDN: $p < 0.0001$, MDS vs. MDN: $p = 0.0008$, MDS vs. CN: $p = 0.014$, MDE vs. CN: $p = 0.0002$; **Figure 1i**). No differences were found between MD and controls following legal euthanasia (MDE vs. CE: $p = 0.74$), or in natural death cases (MDN vs. CN: $p = 0.35$). In MD, suicide cases did not show alterations compared to legal euthanasia (MDS vs. MDE: $p = 0.71$).

The samples were re-stratified according to the suicidal classification and consequence as mentioned above. We found that in subjects with fatal suicidality, a much higher ratio of P2RX7⁺ nuclei was present than in subjects with a history of non-fatal suicidality or subjects without an inclination for suicide (FS vs NFS: $p < 0.0001$; FS vs. NS: $p < 0.0001$; **Figure 1j**). The ratio of P2RX7⁺ nuclei in the group of non-fatal suicidality subjects was not different from the ratio in non-suicidal subjects (NFS vs. NS: $p = 0.77$). Subsequently, fatal and non-fatal suicidalities were subdivided into suicidal ideations and suicide attempts to see whether the suicidal outcome might affect these changes. Relative to non-fatal suicidal ideations, subjects with fatal suicidal ideations showed a significant increase of P2RX7⁺ nuclei compared to non-suicidal cases (FSI vs. NFSI: $p = 0.0007$; **Figure 1k**). Similarly, increased ratios were found in fatal suicidal attempters compared to non-fatal suicidal attempters (FSA vs. NFSA: $p = 0.007$; **Figure 1k**). Subjects with suicidal ideations or with a history of suicide attempt(s) did not show differences within the same prognosis (NFSA vs. NFSI: $p = 0.87$; FSA vs. FSI: $p = 0.96$; **Figure 1k**). In addition, when individuals with fatal suicide attempts were subdivided into single attempts and repeated attempts, no differences were found between these groups ($p = 0.31$). Similar changes were observed for P2RX7⁺ nuclei densities (**Figure S1a-c**).

In contrast to the DG, no differences were present in the CA1 of the controls who died by natural causes as compared to legal euthanasia (CN vs. CE: $p = 0.28$; **Figure 1o**). P2RX7⁺ nuclei in the CA2-4 presented increased density in individuals who died by legal euthanasia compared to natural causes (**Figure S1j**). In the Sub and Ent, such differences were found only within MD subsets (**Figure S2d and S2m**). Apart from the P2RX7⁺ nuclei, we quantified P2RX7⁺ astrocytes in the hippocampal subregions (CA1 and Sub), but we did not find differences in relation to suicide or MD (**Figure 1r-t**). Detailed information about the quantification of P2RX7⁺ nuclei in the hippocampal subareas is presented in Supplementary **Figure S1-S2**. The total expression of P2RX7 and GFAP in the hippocampal subfields did not show differences (**Figure S3 and S4**).

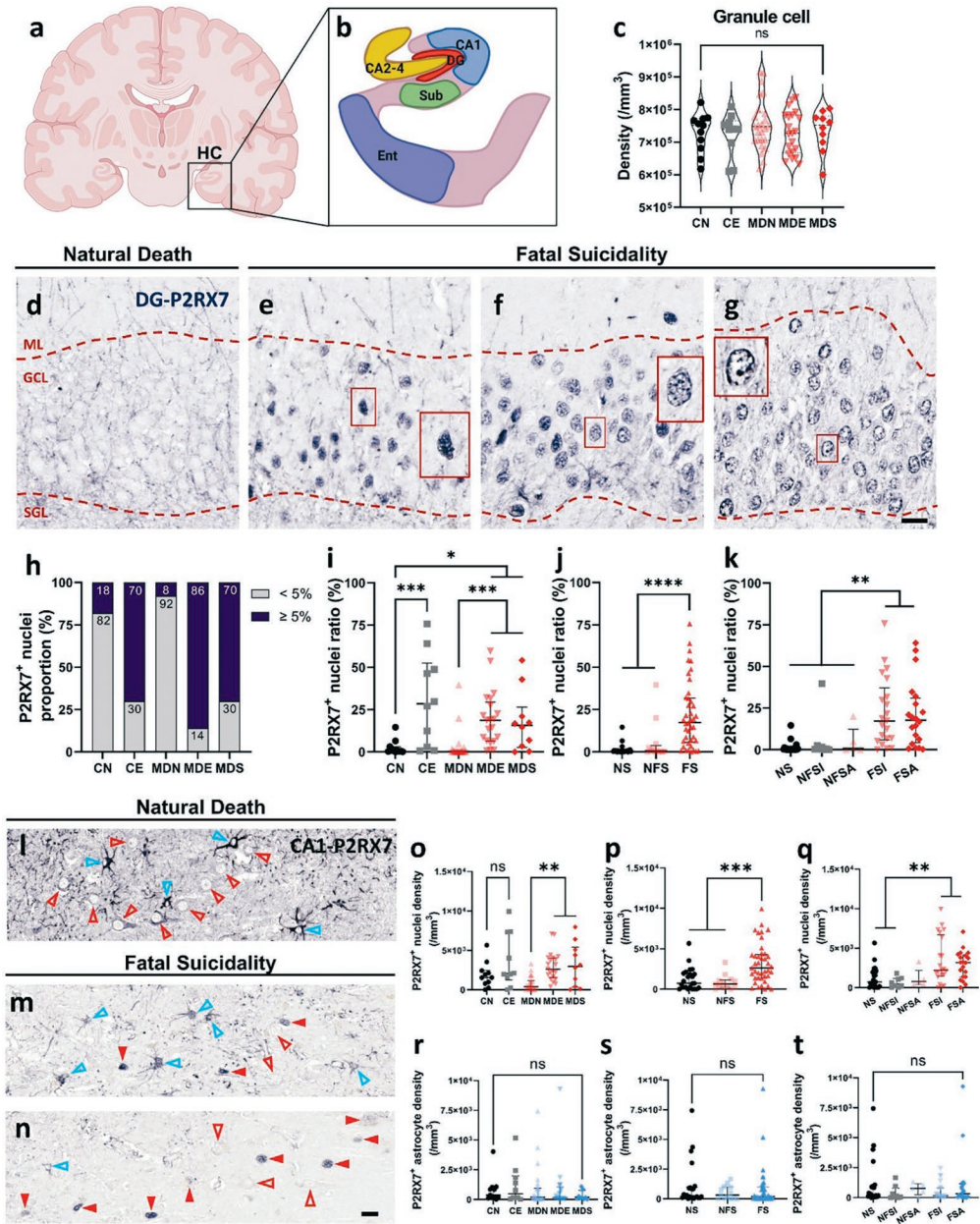


Figure 1. P2RX7⁺ nuclei in the hippocampal formation code for fatal suicidality. Anatomical scheme (a) of the hippocampal subregions (b) employed in this study. c Granule cell density in the DG of different groups is stable. (d-k) P2RX7 expression in the DG of individuals without (d) and with fatal suicidality (e-g). Notably, e-g present a progressively increased number of P2RX7⁺ nuclei accompanied by elevated chromatin condensation and marginalization. The boxes magnify the positive staining patterns of P2RX7⁺ nuclei in the GCL. (h) shows higher proportions of individuals with over 5% granule cells that express nuclear P2RX7, and relatively more granular nuclei express P2RX7 in both the Ctr and MD groups in relation to fatal suicidality (i). (j and k) show a dramatic increase of P2RX7⁺ nuclei in subjects who had fatal suicidality, compared to no or non-fatal suicidality. (l-t) P2RX7 expression in the CA1 of a control subject (l) and two depressed patients with fatal suicidality (m and n). Red solid arrowheads point to neurons with P2RX7⁺ nuclei. Red empty arrowheads point to neurons without nuclear P2RX7 staining. Blue empty arrowheads point to P2RX7⁺ astrocytes. (o) shows the increases of P2RX7⁺ nuclei in the CA1 in MD patients with fatal suicidality, but there is no difference between non-psychiatric control subsets. (p and q) reveal dramatic increases of P2RX7⁺ nuclei in subjects with fatal suicidality compared to no or non-fatal suicidality. (r-t) P2RX7-expressing astrocytes in the CA1 did not show differences in relation to suicide or MD. Scale bar, 15 μ m.

Abbreviations: CA, *cornu ammonis*; CE, control subjects who died by legal euthanasia; CN, control subjects who died by natural causes; DG, dentate gyrus; Ent, entorhinal cortex; FS, fatal suicidality; FSA, suicide attempters who died by suicide or legal euthanasia; FSI, suicide ideators who died by suicide or legal euthanasia; GCL, granule cell layer; HC, hippocampus; MDE, individuals with MD who died by legal euthanasia; MDN, individuals with MD who died by natural causes; MDS, individuals with MD who died by suicide; ML, molecular layer; NFS, non-fatal suicidality; NFSA, non-fatal suicide attempts; NFSI, non-fatal suicidal ideations; NS, no suicidality; ns, no significance; SGL, subgranular layer; Sub, subiculum. Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, *** indicates $0.0001 \leq p < 0.001$, **** indicates $p < 0.0001$.

Microglial priming in the hippocampal formation correlates with suicidal consequences in mood disorders

Suicide is strongly linked to disrupted microglial homeostasis and phagocytosis in the human brain. To estimate the phagocytotic capacity versus microglial homeostasis, we determined the ratio of the phagosome-to-soma IOD CD68/P2RY12. This ratio was generally decreased throughout hippocampal subregions of MD patients as compared to the controls (**Figure 2a-e**). Subset analysis showed that, except for the Sub, these reduced ratios in the other subfields mainly resulted from depressed patients with fatal suicidality (**Figure 2f-j**). Since their average age was younger than non-suicidal controls (**Table S1**), we split the data into middle age and old age (below or above 70 years old) and compared them separately with the controls who died of natural causes. The significant ratio reductions persisted, except for the Sub (**Figure S5**).

Surprisingly, approximately half of the individuals with fatal suicidality showed in the superficial layers of the medial Ent (mEnt) widely distributed P2RY12⁺ microglia with segmented processes and microspheres (**Figure 2k-m** and **Figure S6a-c**). Subgroup analysis showed that

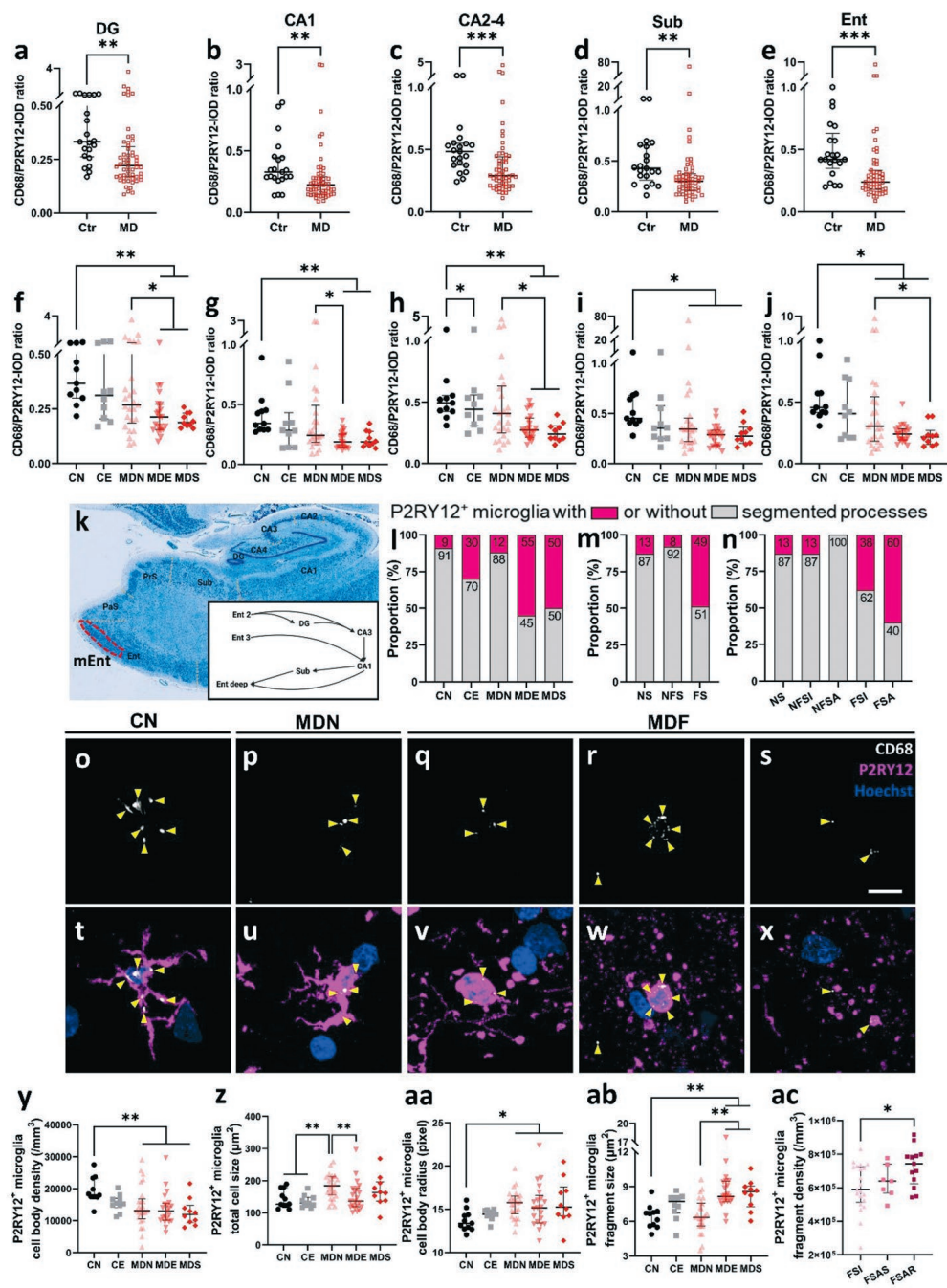


Figure 2. Microglial priming in the hippocampus reflects the suicidal process in mood disorders. (a-j) Quantitative analysis of the ratio of CD68 to P2RY12 shows an overall decline throughout the hippocampal subregions of MD patients (a-e), which is mainly due to the reduction in depressed patients who had fatal suicidality (f-j). k Anatomical localization of the mEnt (superficial layers) following thionine staining. The hippocampal tri-synaptic circuit is schematically miniaturized in the frame. l-n display the distribution of deformed P2RY12⁺ microglia in different subgroups in relation to MD and suicidality. Confocal images of CD68 and P2RY12 immunostaining in the mEnt exhibit diminished CD68 expression in MD (p-s) versus Ctr (o). Co-labeled P2RY12⁺ microglia show typical morphological phenotypes from ramified microglia (t) in CN, reactive microglia characterized by enlarged cell bodies and bushy processes (u) in the MDN, swelling microglia with segmented processes (v) or with disconnected microspheres and debris (w), and microspheres and debris without microglial cell bodies (x) in MD patients with fatal suicidality. Yellow solid arrowheads point to CD68⁺ phagosomes. Imaging analyses show an overall decline of microglial density in the mEnt of patients with depression (y). (z) By quantifying the size of microglia, there is an increase in the MDN relative to the controls, and a reduction in the MDE compared to the MDN. (aa) shows an overall increase of P2RY12⁺ microglial cell body radius in MD versus CN. (ab) reveals that the size of microglial fragments is larger in depressed patients with fatal suicidality in comparison to the individuals who died naturally. ac In individuals with fatal suicidality, the density of microglial fragments is significantly higher in repeated suicide attempters than in suicidal ideators. Scale bar, s 15 μ m.

Abbreviations: FSAR, FS with repeated suicide attempts; FSAS, FS with a single suicide attempt; MDF, MD patients with fatal suicidality; mEnt, medial entorhinal cortex; PaS, parasubiculum; PrS, presubiculum. Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, *** indicates $0.0001 \leq p < 0.001$.

these heteromorphic microglia were more often detected in fatal suicide attempters than in fatal suicidal ideators ($p = 0.002$, **Figure 2n**). The morphological distinctions between ramified microglia, reactive microglia, and swollen microglia with segmented processes were confirmed by 3D immunofluorescence reconstructions (**Figure 2o-x, S6d-g**).

We subsequently quantified the P2RY12⁺ microglia in the mEnt and found their numbers to be reduced in MD compared to CN ($p = 0.004$, **Figure 2y**). However, the size of the microglia was larger in MDN than in the other subgroups ($p = 0.003$, **Figure 2z**). To further estimate the extent of cellular deformations, we measured the radius of microglial cell bodies and found longer radii in MD than in CN ($p = 0.01$, **Figure 2aa**). We also determined the size of microglial fragments and found that they were larger in depressed patients who had fatal suicidality than in subjects with natural death ($p < 0.0001$, **Figure 2ab**). Notably, among individuals with fatal suicidality, the density of microglial fragments was higher in people who attempted suicide repeatedly than in those who only had suicidal thoughts ($p = 0.01$, **Figure 2ac**). These malformed microglia were absent in the other hippocampal subareas of the same cohort. Detailed information referring to the quantification is provided in Supplementary **Figure S6**.

Selective activation of purinergic glia in the supraoptic and paraventricular nuclei in suicide and mood disorders

The volumes of selected hypothalamic nuclei were determined based on the thionine-delineated magnocellular neurons in the SON, and on both magnocellular and parvocellular neurons in the PVN. Surprisingly, in MD, individuals who died of legal euthanasia or completed suicide exhibited decreases in the SON volumes of 24.8% and 39.3%, respectively, compared to those who died of natural causes (MDE vs. MDN: $p = 0.028$; MDS vs. MDN: $p = 0.005$, **Figure 3c**). By contrast, the PVN volume between the five groups was not different ($p = 0.72$, **Figure 3d**). The volume of the PVN and SON showed no difference between patients with MD and the controls (PVN: $p = 0.64$; SON: $p = 0.97$, **Figure S7a** and **S7b**).

Increases of P2RY12 expression were found in the SON and PVN of MD patients relative to the controls (SON: ratio = 2.59, $p = 0.03$, PVN: ratio = 1.41, $p = 0.049$, **Figure 3e**, **3f**, **3i** and **S7c**). The microscopy of these P2RY12⁺ microglia revealed increases in size, thickened and bushy processes with retractions. In addition to MD, a higher expression of P2RY12⁺ microglia was present in the SON of subjects with fatal suicidality, which was absent in the PVN (**Figure 3l** and **S7f**). We further incorporated CD68 expression and found a decreasing trend of CD68/P2RY12 IOD ratio in MD ($p = 0.052$, **Figure 3k**), which was mainly contributed by subjects who had fatal suicidality compared to the controls without suicidal intention (**Figure 3n**). However, the expression of CD68 in the hypothalamic nuclei did not change in relation to suicide or depression (**Figure 3j** and **3m**).

In the PVN of depressed patients, P2RX7 showed over three times higher expression than the controls ($p = 0.008$, **Figure 3o-s**). In subset analyses, we found female MD patients had two times higher P2RX7 expression in the PVN than their male counterparts (MDF vs. MDM: $p = 0.02$, **Figure 3t**). This sex difference was not observed in the controls (CF vs. CM: $p = 0.78$). P2RX7 expression in the SON did not show changes (**Figure S7i** and **S7j**).

GFAP-positive astrocytes also presented higher expression in the PVN of depression ($p = 0.049$, **Figure 3u-w**). Elevated expression was found in subjects who died by suicide compared to those who died of natural causes (MDS vs. MDN: ratio = 1.79, $p = 0.01$; MDS vs. CN: ratio = 2.22, $p = 0.0002$, **Figure 3x**). However, no differences were shown due to the presence of MD or suicidal ideation. By contrast, GFAP in the SON did not show changes (**Figure S7k** and **S7l**). For more detailed glial expression in the hypothalamus see Supplementary **Figure S7**.

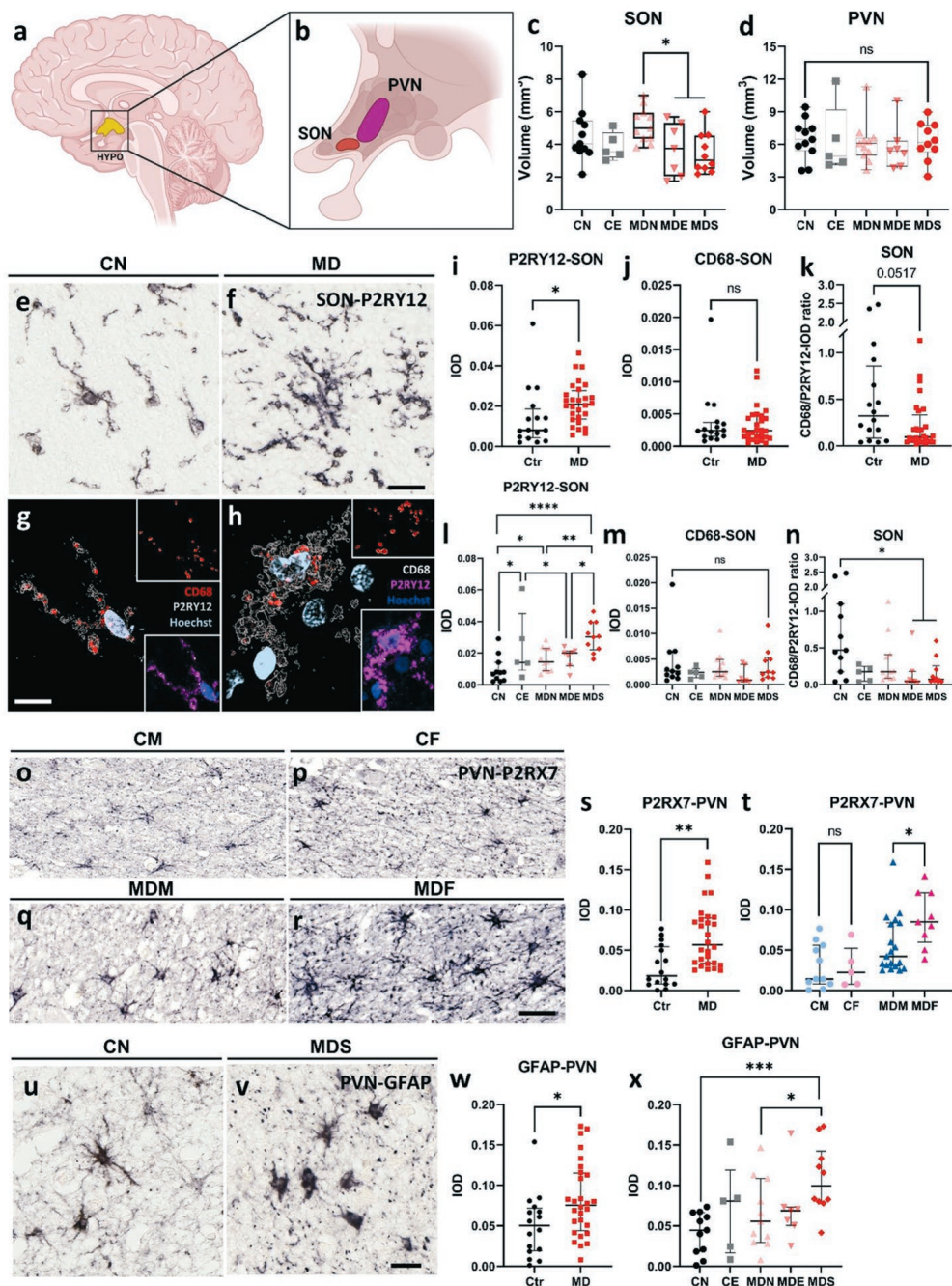


Figure 3. Activated purinergic glia in hypothalamic nuclei are specific to suicide or mood disorders. The anatomical scheme (a) of the hypothalamic nuclei studied (b). In MD, patients with fatal suicidality show a volumetric shrinkage in the SON (c), but not PVN (d). (e-h) P2RY12⁺ microglia in the SON of a control subject (e) and an MD patient (f). Spatial reconstruction specifies CD68⁺ phagosomes in microglia of a non-suicidal control (g) and a depressed patient who died of suicide (h). Images miniaturized in the top right reveal the reconstructed surfaces of CD68⁺ phagosomes. Images miniaturized in the bottom right show their raw morphology. (i-n) The quantitative analysis in relation to MD, suicidality, and completed suicide. (o-r) display the increase of paraventricular P2RX7 expression in MD (s) with a gender preference for females over males, which is absent among the controls (t). (o CM, p CF, q MDM, r) MDF. (u and v) show GFAP⁺ astrocytes in the PVN of a control subject (quiescent, u) and a depressed patient who completed suicide (reactive, v). (w and x) are the quantitative analyses that demonstrate a specific increase in GFAP expression in MD, which is largely due to accomplished suicide compared to subjects who did not have suicidality. Scale bar, 20 μ m, except for 10 μ m in **Figure 3g**.

Abbreviations: HYPO, hypothalamus; CF, non-psychiatric females; CM, non-psychiatric males; MDF, female patients with mood disorders; MDM, male patients with mood disorders; PVN, paraventricular nucleus; SON, supraoptic nucleus. Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, *** indicates $0.0001 \leq p < 0.001$, **** indicates $p < 0.0001$.

P2RX7 expression decreased in the prefrontal cortex in suicide and major depressive disorder

The expression of P2RX7 in the PFC (DLPFC and ACC) of MD subjects was determined at the transcriptional level. MDD, but not BD patients, had lower prefrontal P2RX7 expression compared to the controls (DLPFC: MDD vs. Ctr: fold change = -1.24, $p = 0.002$, MDD vs. BD: fold change = -1.29, $p = 0.03$, BD vs. Ctr: $p = 0.41$; ACC: MDD vs. Ctr: fold change = -1.81, $p < 0.0001$, MDD vs. BD: fold change = -1.81, $p < 0.0001$, BD vs. Ctr: $p = 0.78$; **Figure 4b** and **4c**). In addition to completed suicide, the possible impact of psychotic features, which are linked to a clinically high risk for suicide attempts, was examined by subset analyses. Decreased expression of P2RX7 appeared to be more related to suicide in the DLPFC than in the ACC (DLPFC: MDDNS vs. Ctr: $p = 0.14$; ACC: MDDNS vs. Ctr: fold change = -1.85, $p < 0.0001$; **Figure 4d** and **4e**). The presence of psychotic features did not affect the P2RX7 expression in the MDD cohort, including the DLPFC and ACC (**Figure 4f** and **4g**). We did not find significant alterations in P2RX7 transcripts related to suicide or psychotic features in BD (**Figure 4h-k**).

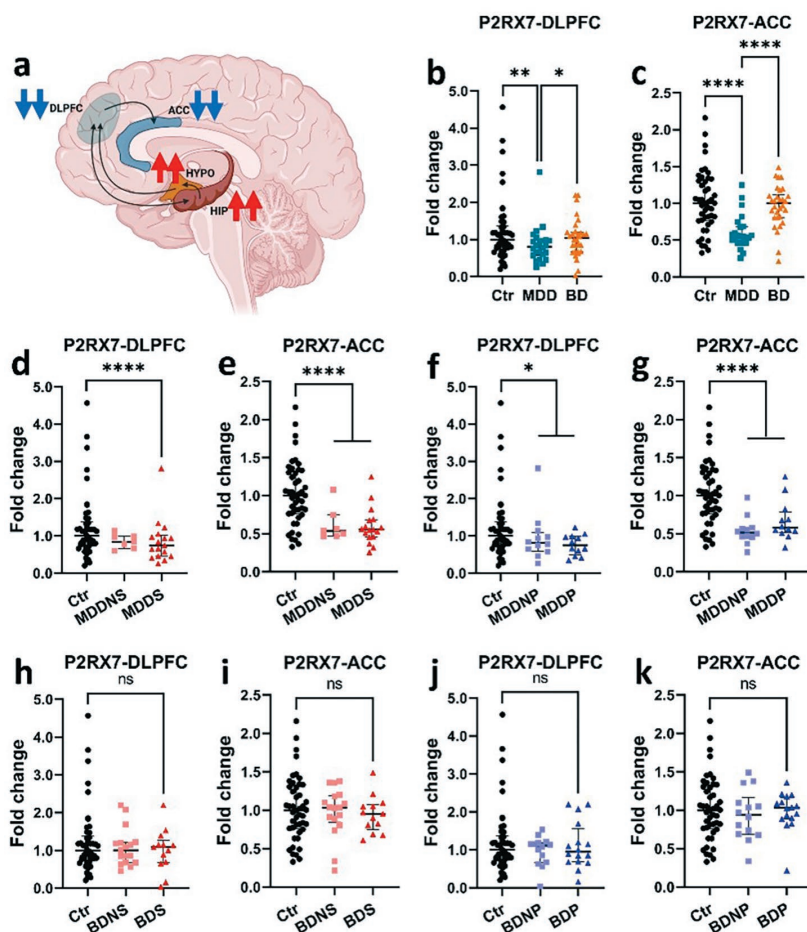


Figure 4. Diminished P2RX7 transcripts in the dorsolateral prefrontal cortex of major depressive disorder are associated with completed suicide. (a) Anatomical scheme of the DLPFC and ACC, and the unified neurocircuitry map of stress-associated purinergic receptors in suicide. Blue/red arrows indicate reduced/elevated purinergic receptor expression, respectively. (b and c) show that P2RX7 expression in the PFC is reduced in MDD but not in BD. In the DLPFC of MDD, this reduction is related to completed suicide (d), while in the ACC, both MDD and completed suicide present declined P2RX7 expression (e). (f and g) show that psychotic features do not confound the results. (h-k) shows that the P2RX7 transcripts in the PFC of BD are not altered in completed suicide or psychotic features.

Abbreviations: ACC, anterior cingulate cortex; BD, bipolar disorder; BDNP, bipolar disorder without psychotic features; BDNS, bipolar disorder without suicide; BDP, bipolar disorder with psychotic features; BDS, bipolar disorder with suicide; Ctr, non-psychiatric controls; DLPFC, dorsolateral prefrontal cortex; MDD, major depressive disorder; MDDNP, major depressive disorder without psychotic features; MDDNS, major depressive disorder without suicide; MDDP, major depressive disorder with psychotic features; MDDS, major depressive disorder with suicide; PFC, prefrontal cortex. Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, **** indicates $p < 0.0001$.

Putative confounders

Confounder analysis was performed for age, sex, postmortem delay (PMD), clock time of death, and brain pH. Significantly, the presence of P2RX7⁺ nuclei sharply dropped with prolonged PMDs in the hippocampal subfields of donors with fatal suicidality, which was barely detectable 20 hours after death (**Figure S8a-e**). These negative correlations were absent in donors who died of natural causes (**Figure S8f-o**). We investigated whether this negative correlation could be alleviated by excluding the intervals over 12h, 9h, and 6h, separately. However, in the DG, P2RX7⁺ nuclei diminished by the PMDs remained evident, as the share of P2RX7⁺ nuclei in the granule cells of fatal suicidality increased more within shorter postmortem intervals (**Figure S8p-r**). In addition, CD68/P2RY12 ratios throughout the hippocampus of subjects with fatal suicidality were found to increase with age (**Figure S9a-e**). In individuals who had temporary or transient suicide tendencies, CD68 expression in the CA2-4, Sub and Ent positively correlated with age, with the Ent showing the highest significance (**Figure S9f-j**). These age-related correlations did not affect our conclusions.

For the PFC, we examined the parameters used to match observations over groups and found that in BD subjects, P2RX7 expression was positively correlated with age in the ACC ($Rho = 0.497$, $p = 0.006$; **Figure S9k**), which seemed to be explained by patients with psychotic features who died naturally ($Rho = 0.670$, $p = 0.006$; **Figure S9l-o**). In addition, there was a positive correlation between P2RX7 mRNA and lifetime fluphenazine equivalents intake in the ACC of individuals with MD ($Rho = 0.594$, $p = 0.0004$), which was absent in the DLPFC.

DISCUSSION

Biological indicators grounded in key brain regions distinguish suicide neuropathology from comorbid psychiatric disorders. Our current findings revealed four possibilities concerning the differences and similarities between suicide and MD. (1) Fatal suicidality has distinct molecular characteristics, which are independent of the psychiatric background. In this study, it was typically shown by the granule cell nuclei in the DG that expressed P2RX7; (2) When a neuropsychiatric disorder is comorbid, suicide/suicidality-associated molecular alterations occur as symbols of disease progression. For instance, we observed nuclear P2RX7 expression in the CA1 and P2RY12⁺ microglia with segmented processes in the mEnt of MD patients with fatal suicidality; (3) Molecules are sensitive to both suicide and psychiatric disorders, for example, the P2RY12 expression in the SON; (4) Completed suicide or the development of suicidality do not confound molecular changes related to pre-existing psychiatric disorders, as shown by the expression of P2RX7 and P2RY12 in the PVN.

In this study, the most important finding is the extensive and ultra-high presence of P2RX7⁺ nuclei in the GCL of individuals who had intense and persistent suicidal ideations. This

striking rise appeared not only in MD patients but also in non-psychiatric controls who had a record of fatal suicidality (i.e., individuals who died by legal euthanasia), indicating that suicide might have a profile of genes homogenously rooted in non-psychiatric populations as well as psychiatric patients. By targeting these key genes and brain regions involved in suicide, therapies may be designed for suicidal individuals who are unable of receiving mental care.

Psychological stress is sensed by the innate immune system in the brain via the ATP/P2RX7 pathway (Iwata et al., 2016). Aberrant P2RX7 expression in neural cells dysregulates neuronal-glial homeostasis and cognition, which may trigger the emergence of depressive symptoms (Díaz-Hernandez et al., 2008; Drevets et al., 2022; Khan et al., 2019; Kongsui et al., 2014; Liu et al., 2017; Papp et al., 2004). The DNA promoter of the *P2RX7* gene has a series of binding sites for nuclear transcription factors that biologically adapt and modify gene expression (Bergamin et al., 2021). Animal studies have demonstrated that guanosine administration, through its binding site on *P2RX7*, can inhibit ATP activity and display antidepressant and pro-neurogenic effects in the hippocampus (Bergamin et al., 2021; McCarthy et al., 2019; Morton and Parsons, 1977). In the plasma of treatment-resistant depressed patients, a subpopulation at a high risk of suicide, *P2RX7* transcripts were found to correlate with the glucocorticoid receptor, a key stress-responsive transcription factor (Cattaneo et al., 2020). We suggest that the *P2RX7* expression in the granule cell nuclei may provide a target for the interactions between neuroinflammation and glucocorticoids (Reutfors et al., 2021; Wang et al., 2012). In addition, chromatin condensation and marginalization in the dentate *P2RX7*⁺ nuclei may be pathological features of neurodegeneration. *P2RX7* initiates programmed cell death by activating caspases (Nishida et al., 2012). Given that the overall density of granule cells in the DG remains unchanged, future research should focus on the earlier stages of neuronal apoptosis in the DG of fatal suicidal cases.

Of note, there were no variations in the dentate *P2RX7*⁺ nuclei between the two fatal suicidal phenotypes (i.e., ideations or attempts). For years, the development of suicidal thoughts was believed to indicate an aggravation of the pre-existing psychiatric disorder (Fazel and Runeson, 2020). In our study, however, legal euthanasia donors who did not have diagnosable mental illnesses, but requested euthanasia due to their malignant diseases, also presented a remarkable proportion of *P2RX7*⁺ nuclei in the DG. Nine out of ten of these donors had specified suicidal ideations but not behaviors. The only attempter in this subset did not contribute an extreme value of *P2RX7*⁺ nuclei. It has been proven that the heterogeneity of the *P2RX7* gene was strongly linked to cognitive deficits, a key factor in the suicide processes (Backlund et al., 2011; Zhang et al., 2022). Therefore, the distinctive presence of *P2RX7*⁺ nuclei in the DG may provide a pointcut for deciphering the suicide-specific cognitive inflexibility.

An unavoidable question is whether the high-proportioned *P2RX7*⁺ nuclei in granule cells of fatal suicidal cases serve as a surrogate for one particular cell group or a proxy for several subdivisions that respond to lethal suicidal thoughts. After all, nuclear transcriptional factors and mechanistic insights implicated in these suicide-specific nuclei remain unknown. Assuming

neurological substrates of the DG form a molecular fingerprint of fatal suicidality, the question is whether any P2RX7⁺ nuclei subset(s) could distinguish suicidal phenotypes or consequences. As the presence of P2RX7⁺ nuclei rapidly decreased with the extension of the PMD, technological approaches that profile transcriptomic alterations at single-cell resolution may provide a feasible alternative for overcoming PMD-dependent protein degradation in postmortem human DG (Zhou et al., 2022).

Microglial homeostasis in human tissue can be mimicked by biomarkers that monitor phagocytic capacity and morphological indices. In this study, the ratio of CD68 to P2RY12, used to reflect the relation between microglial phagocytosis and homeostasis, generally declined in the hippocampus of MD patients (Cserép et al., 2020). Subset analysis showed that this dramatic decrease was more associated with fatal suicidality. Additionally, lesioned processes in entorhinal microglia were implicated in suicidal classifications and severity. First, microglial cell bodies in the mEnt of the entire MD cohort that showed reductions in density together with soma enlargement suggested microglial degeneration. Second, the total size of microglia was larger only in depressed patients who died from non-suicidal causes compared to other subsets, while microglial fragments were smaller in these patients than in those with fatal suicidality. This suggests that a significant number of microglial processes were disconnected from cell bodies. Third, the density of these fragments in the fatal suicidality cohort was much higher in repeated suicide attempters than in suicidal ideators, indicating that entorhinal microglial lesions reflect the repetition of suicidal behaviors. Similar segmented processes in microglia have been found in a marmoset model of autism, a neurological disorder with suicide as a leading cause of premature death (Cassidy and Rodgers, 2017; Sanagi et al., 2019). In clinical practice, cellular deformability was reported in peripheral blood monocytes, a myelomonocytic relative of microglia, in patients with persistent MDD, in whom the rate of lifetime suicide attempts is over three times higher than in non-chronic MDD (Nübel et al., 2020; Walther et al., 2022). Taken together, these data suggest that the pathogenic mechanisms of psychiatric disorders may contribute to dystrophic transformation of microglia (Neumann et al., 2022). The heteromorphic microglia that mark alterations in energy metabolism and immune homeostasis are extensively distributed in the superficial layers of the Ent (Ent2 and Ent3), which contain the major stimulating afferents to the DG that effectuate the impact of antidepressants (Šimić et al., 2022; Yun et al., 2018). Our next step is to determine certain groups of entorhinal neurons that associate with suicidal severity and explore their functional connectivity within the Ent-DG circuitry.

So far, neuroimaging strategies cannot precisely identify the hypothalamic nuclei because their anatomical boundaries lack contrast. We discovered that MD patients with fatal suicidality had reduced SON volumes compared to non-suicidal MD patients. The SON is generally homogeneous and primarily composed of vasopressin neurons, which have been linked to enhanced suicide risk (Merali et al., 2006). However, this activation probably lacks effective regulation of microglial phagocytosis, as CD68 expression was unchanged. We assume that

the entire process of suicide may drive persistent hyperactivity in the hypothalamic-pituitary-adrenal axis and the consequent strengthening of negative cortisol feedback to the SON, which may reduce its volume (Berardelli et al., 2020; Erkut et al., 1998). In the PVN, both P2RX7 and P2RY12 were highly expressed in depressed subjects, indicating a significant extracellular release of ATP and ADP. When compared to non-suicidal individuals, cases of completed suicide had an additionally increased GFAP expression in the PVN, suggesting that hypothalamic astrocytes may have a distinct role in suicide pathophysiology.

In addition, we observed a sex-specific increase of P2RX7 expression in the PVN of female depressed patients. Genetic associations between *P2RX7* mutations and the higher risk of BD occurrence have been reported in females but not males (Winham et al., 2019). In animal stress models, a higher P2RX7 expression in the PVN may likely induce a gender-biased elevation of FosB, a transcription factor that modulates several target genes implicated in depression (Gajewski et al., 2016; Gavala et al., 2010; Sterrenburg et al., 2012). Again, sex may be a mechanism-modifying variable with important treatment implications.

In contrast to the subcortical purinergic receptors that were overactivated in suicide, P2RX7 transcripts in the PFC of MDD subjects, but not BD subjects, showed sharp declines when compared to the controls. In MDD, this decrease in the DLPFC may have been caused by P2RX7 depletion only in cases of suicide, which is consistent with other studies (Punzi et al., 2022; Zhang et al., 2011). There were no differences in the ACC between patients with or without actual suicides. Neither did prefrontal P2RX7 expression in BD change in relation to suicide. However, P2RY12 transcripts in the DLPFC of BD donors, as reported in our earlier findings, were lower in non-suicidal patients than in suicide victims and non-psychiatric controls (Zhang et al., 2020b). Since the PFC is involved in negative feedback regulation of the hypothalamus-pituitary-adrenal axis and hippocampal responses to stress (Bian et al., 2019; Sullivan and Gratton, 2002), the findings above suggest that purinergic hypoactivity in the PFC together with hyperactivity in the hippocampus and hypothalamus could reflect a diminished inhibitory connection between cortical and subcortical components. Further, recent studies have found that a *P2RX7* variant plays a role in the vulnerability to develop psychotic features, which have a markedly increased likelihood of subsequent suicide death (Boks et al., 2020). Our results, however, did not provide evidence that this observation could be related to the prefrontal regions.

There are some limitations to this study. First, the detectable number of P2RX7⁺ nuclei in the hippocampus of fatal suicidality subjects significantly dropped over PMD. This has weakened the analysis of possible impacts, such as suicidal classifications, consequences, or reasons for legal euthanasia (physical versus psychiatric diseases). The PMDs among suicide completers were relatively long and recorded by estimation. Practically speaking, some critical molecules may have been degraded, which confines the brain sample inclusion for further research. To retain these suicide biomarkers, we introduced brain samples from donors who

died by legal euthanasia and emphasized the imperative role of their short and precise PMDs. Second, P2RX7⁺ nuclei were densely packed in the DG but loosely distributed in the other hippocampal subregions, which resulted in increased data variation and decreased statistical significance. Therefore, we could not determine whether regional specificity or methodological constraints were to blame for the lack of significance between fatal and non-fatal suicidality in the Sub or Ent (**Figure S2d** and **S2m**). These two hurdles can be technically overcome by follow-up studies using single-nucleus transcriptomics. Third, PFC samples were obtained from the SMRI, from which legal euthanasia cases were not available. We do not have information on prefrontal alterations in relation to fatal suicidal thoughts or attempts. Fourth, the average age of the prefrontal collection was thirty years younger than the other brain cohorts. However, our recent proteomic studies have reported sharply diminished energy metabolism in the PFCs of elderly depressed patients in the NBB cohort, which supported our conclusions (Qi et al., 2022).

The biological hallmarks of fatal suicidality are molecule-, cell type- and region-specific. They may be either independent or (partially) overlapping with the changes observed in the comorbid psychiatric disorders. We investigated a network linked to suicidality, which was mediated by a mini neurocircuit in the hippocampus, and a major circuit involving the hypothalamus and PFC. Our discovery of a specific group of hippocampal granule cells exclusively associated with fatal suicidality indicates that the DG may contain cellular and molecular signatures for intense and determined suicidal ideations (Zhang et al., 2022). Importantly, this suicide-specific feature sharply dropped over PMD, even within our very short intervals. This phenomenon urged the application of brain samples from donors who died by legal euthanasia, to enable the study of suicide biosignatures that were insignificant in previous research. Microglial dyshomeostasis and morphological traits in the mEnt of depression cases were found to be related to the suicidal categories. Similar findings were presented in the hypothalamic SON. Our results suggest that, in individuals with intense and determined suicidality, certain subcortical regions may exhibit increased energy metabolism, which may be due to the deficiency of prefrontal inhibition. Overall, unraveling the molecular basis that identifies suicide can provide therapeutic targets for suicidal individuals with or without previous mental care.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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SUPPLEMENTARY MATERIALS AND METHODS

Neuropathological admittance and sample matching

NBB collections

Patients with MD had been diagnosed by licensed psychiatrists during their lifetime according to the Diagnostic and Statistical Manual of Mental Disorder (DSM)-III, IIIR, and IV. A neuropathologist systematically examined each brain to exclude pathological alterations. Subjects with evidence of neurological disorders or with Braak stage III or higher for Alzheimer's were excluded¹. Subjects had no significant history of substance dependence 10 years before death. Matching for potentially confounding factors was non-parametrically tested. This included age, sex, post-mortem delay (PMD), month of death, clock time of death (CTD), brain weight, and the pH of cerebrospinal fluid. We noticed that ages of subjects with MD who died of suicide or legal euthanasia were younger than the other subjects. Since legal euthanasia is generally performed during working hours, CTDs of euthanasia groups were, therefore, significantly different from the other groups. In addition, PMDs of suicide completers were estimated values, while they were precise values in the other subsets. For demographic and clinic-pathological information see **Table S1a-d**.

SMRI collections

Permission for the use of brain material was provided by the next of kin. Diagnoses were based on the DSM-IV. All brain regions were microscopically examined to exclude patients having pathological signs of neurodegeneration or other lesions. Exclusion criteria included subjects with a history of seizures or other neurological disorders that might affect brain pathology. All groups were matched for age, brain pH, PMD, brain weight, history, and severity of alcohol/substance abuse. For demographic information see **Table S1e-f**.

Table S1a. Demographic information of Suicide-HIPPOCAMPUS collection (NBB).

Diagnosis - Cause of death	Cases, n	Psychiatric diagnosis (MDD/BD)	Reason for euthanasia (Physical diseases/ MD) ¹	Age (y) ²	Sex (M/F)	PMD (h) ²	Brain pH ²	BW (g) ²
MD	56	37/19	–	66.5 (23–93)	21/35	6:58 (3:55–20:35)	6.62 (5.61–7.30)	1220 (912–1525)
- Suicide	10	7/3	–	58.5 (43–90)	2/8	9:52 (3:55–20:35)	6.58 (6.18–6.93)	1215 (1120–1475)
- Legal euthanasia	21	18/3	9/13 ³	61 (23–83)	6/15	6:20 (4:10–12:45)	6.80 (6.48–7.30)	1210 (1045–1525)
Natural causes	25	12/13	–	70 (45–93)	13/12	7:00 (4:20–10:20)	6.50 (5.61–6.95)	1237 (912–1510)
Ctr	21	–	–	83 (51–94)	8/13	5:45 (4:15–12:50)	6.56 (6.05–7.00)	1259 (1054–1590)
- Legal euthanasia	10	–	10/0	82.5 (51–92)	2/8	5:40 (4:25–7:50)	6.63 (6.36–6.92)	1206 (1090–1390)
Natural causes	11	–	–	83 (73–94)	6/5	5:50 (4:15–12:50)	6.49 (6.05–7.00)	1275 (1054–1590)

Abbreviations: BD, bipolar disorder; BW, brain weight; Ctr, non-psychiatric controls; F, female; g, gram; h, hour; M, male; MD, mood disorders; MDD, major depressive disorder; NBB, Netherlands Brain Bank; PMD, postmortem delay; y, year.

Note:

1 In the Netherlands, legal euthanasia will be approved to end unbearable suffering with no prospect of improvement in physical/psychiatric diseases (<https://www.government.nl/topics/euthanasia/is-euthanasia-allowed>). For the practice of legal euthanasia in the Netherlands see <https://www.knmp.nl/downloads/guidelines-for-the-practice-of-euthanasia.pdf>.

2 Values are provided with medians (and ranges).

3 One individual who died of legal euthanasia applied on basis of a combination of physical diseases and MD. The others were approved either because of physical diseases or MD.

Table S1b. Clinico-pathological information of patients with suicide, legal euthanasia, and control subjects (Suicide-HIPPOCAMPUS collection, NBB).

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicidality	Antipsychotics in last three months	Cause of death
MD1	MDD	59	F	20:35	00:00	6.53	1199	Repeated attempts	BZD	Suicide
MD2	MDD	56	F	10:30	14:00	6.42	1120	Repeated attempts	BZD	Suicide, intoxication
MD3	MDD	62	F	11:40	16:15	6.18	1155	Repeated attempts	BZD	Suicide, strangulation
MD4	BD	83	M	15:25	10:15	6.5	1401	Single attempt	TeCA	Suicide, strangulation
MD5	MDD	58	M	04:30	09:40	6.8	1470	Single attempt	TCA, SGA, SSRI	Suicide, intoxication
MD6	BD	46	F	05:45	16:45	N.A.	1370	Suicidal ideations	BZD	Suicide, intoxication
MD7	MDD	85	F	04:30	12:00	6.93	1200	Suicidal ideations	AA, BZD, TCA	Suicide, intoxication
MD8	MDD	43	F	11:20	10:00	N.A.	1475	Suicidal ideations	BZD, SSRI	Suicide, hanging
MD9	BD	55	F	09:15	09:45	6.83	1210	Suicidal ideations	AA, BZD, DPBP, VPA	Suicide, intoxication
MD10	MDD	90	F	03:55	11:20	6.62	1220	Suicidal ideations	BZD, INN	Suicide, intoxication
MD11	MDD	60	F	04:20	16:10	N.A.	1074	Suicidal ideations	Hal	Legal euthanasia
MD12	MDD	83	F	04:05	14:25	6.75	1066	Single attempt	BZD, TeCA	Legal euthanasia
MD13	MDD	47	F	06:05	15:00	N.A.	1264	Repeated attempts	AA, BZD, SARI	Legal euthanasia
MD14	MDD	73	F	05:45	15:30	N.A.	1205	Suicidal ideations	TeCA	Legal euthanasia
MD15	MDD	66	F	07:55	14:35	N.A.	1165	Repeated attempts	AA, BZD, SNRI	Legal euthanasia
MD16	MDD	47	M	05:55	11:35	6.67	1390	Repeated attempts	N.A.	Legal euthanasia
MD17	MDD	66	F	10:40	17:00	6.71	1045	Suicidal ideations	VPA	Legal euthanasia
MD18	MDD	55	M	04:19	16:11	6.48	1190	Repeated attempts	N.A.	Legal euthanasia
MD19	MDD	77	F	08:40	11:20	6.77	1120	Repeated attempts	BZD, INN, nBZD, SSRI	Legal euthanasia
MD20	MDD	50	M	09:40	13:00	6.8	1420	Repeated attempts	BZD	Legal euthanasia

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicidality	Antipsychotics in last three months	Cause of death
MD21	MDD	38	M	06:15	11:45	6.79	1525	Single attempt	BZD	Legal euthanasia
MD22	BD	45	F	07:30	13:00	6.93	1325	Repeated attempts	SSRI	Legal euthanasia
MD23	MDD	67	F	08:40	15:35	7.07	1205	Suicidal ideations	BZD	Legal euthanasia
MD24	MDD	23	F	08:35	15:45	N.A.	1415	Repeated attempts	AA, BZD, NDRI, TCA	Legal euthanasia
MD25	MDD	48	M	12:45	10:15	6.76	1350	Single attempt	MAOI	Legal euthanasia
MD26	MDD	61	M	05:45	14:05	N.A.	1210	Suicidal ideations	SSRI	Legal euthanasia
MD27	MDD	74	F	06:20	13:45	6.96	1313	Single attempt	AA, BZD, nBZD, TCA	Legal euthanasia
MD28	BD	81	F	07:35	15:25	7.3	1250	Repeated attempts	BZD	Legal euthanasia
MD29	BD	59	F	06:35	11:35	6.92	1132	Single attempt	BZD	Legal euthanasia
MD30	MDD	72	F	04:30	19:30	6.88	1168	Suicidal ideations	BZD, SSRI, TCA	Legal euthanasia
MD31	MDD	61	F	04:45	10:35	6.81	1290	Suicidal ideations	BZD, SSRI	Legal euthanasia
MD32	BD	70	M	04:50	02:45	6.26	1442	No	N.A.	Cardiac arrest
MD33	MDD	45	M	07:00	02:30	6.55	1412	Suicidal ideations	SSRI	Brain hemorrhage
MD34	MDD	81	M	06:00	15:30	6.5	1280	No	Hal	Renal insufficiency
MD35	MDD	70	M	07:15	08:00	6.5	1380	Single attempt	BZD, MAOI, SGA, SNRI, TCA	Respiratory insufficiency
MD36	BD	80	F	09:30	09:30	6.33	1140	No	SSRI, VPA	Acute cardiac arrest
MD37	MDD	93	F	04:20	04:55	6.8	1023	Suicidal ideations	BZD, SSRI	Pneumonia
MD38	MDD	80	F	06:40	16:30	6.62	1237	Suicidal ideations	BZD, TA, TCA	Sudden death
MD39	MDD	68	M	08:55	05:45	6.82	1510	Repeated attempts	BZD, SSRI	Sudden death
MD40	BD	66	M	07:35	16:40	5.83	1320	Repeated attempts	BZD, Li	Acute myeloid leukemia
MD41	BD	58	M	09:15	12:40	N.A.	1380	Suicidal ideations	TCA	Atrial fibrillation
MD42	MDD	69	M	04:40	20:10	6.63	1145	Suicidal ideations	BZD, SSRI	Multiorgan failure

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicidality	Antipsychotics in last three months	Cause of death
MD43	MDD	88	F	10:00	09:00	6.26	1160	No	BZD, SSRI	Burns, dehydration
MD44	BD	70	M	06:50	21:00	6.19	1275	No	BZD, LTG, VPA	Pulmonary infection
MD45	BD	64	F	05:00	19:15	6.76	1170	No	BZD, TCA	CVA
MD46	MDD	69	F	06:55	09:15	6.28	1470	No	nBZD, SSRI, TeCA	Congestive heart failure
MD47	MDD	58	F	07:20	23:00	5.61	1295	Suicidal ideations	BZD, DA stabilizer, SSRI	Renal insufficiency
MD48	BD	65	M	04:50	18:30	N.A.	1305	No	BZD, Li, SGA	Metastatic colon carcinoma
MD49	MDD	86	F	07:30	08:30	6.57	912	No	AA, BZD	Metastatic ovarian cancer
MD50	BD	62	M	07:30	09:05	6.4	1005	No	AA, BZD	Palliative sedation
MD51	BD	82	F	10:15	18:55	6.6	1220	Suicidal ideations	AA, BZD, Li, LTG	Myocardial infarction
MD52	BD	70	F	06:00	00:35	6.95	971	No	BZD	Kidney failure
MD53	BD	78	F	05:45	03:30	6.5	1010	Suicidal ideations	N.A.	Acute kidney injury
MD54	MDD	91	M	06:10	00:30	6.42	1064	No	BZD, MAOI, SGA, VPA	Respiratory insufficiency
MD55	BD	70	F	07:00	14:10	6.37	985	Repeated attempts	BZD, Li, SGA, VPA	Sepsis
MD56	BD	68	M	10:20	01:40	6.41	1430	No	BZD	CVA
Median	-	66.5	-	06:58	-	6.62	1220	-	-	-
Ctr1	-	62	F	07:55	11:15	6.4	1115	Suicidal ideations	BZD	Legal euthanasia
Ctr2	-	51	F	05:36	17:59	6.92	1090	Suicidal ideations	TCA	Legal euthanasia
Ctr3	-	89	F	06:10	13:35	6.36	1199	Suicidal ideations	-	Legal euthanasia
Ctr4	-	89	F	04:45	16:15	6.67	1259	Suicidal ideations	TCA	Legal euthanasia
Ctr5	-	75	F	05:25	18:10	6.75	1320	Suicidal ideations	BZD	Legal euthanasia
Ctr6	-	92	F	04:25	13:25	6.65	1212	Repeated attempts	-	Legal euthanasia
Ctr7	-	92	F	07:45	10:00	6.71	1200	Suicidal ideations	-	Legal euthanasia

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicidality	Antipsychotics in last three months	Cause of death
Ctrl8	-	65	M	05:45	18:25	6.55	1390	Suicidal ideations	BZD, LEV	Legal euthanasia
Ctrl9	-	91	M	05:05	10:20	6.55	1175	Suicidal ideations	-	Legal euthanasia
Ctrl10	-	76	F	06:55	16:50	6.61	1295	Suicidal ideations	-	Legal euthanasia
Ctrl11	-	82	M	12:55	05:00	6.21	1406	No	-	Sudden death.
Ctrl12	-	83	M	05:45	03:00	6.35	1590	Single attempts	BZD, Hal, SSRI, VPA	Pneumonia
Ctrl13	-	91	F	04:15	13:20	6.5	1054	No	-	Heart infarction
Ctrl14	-	82	F	05:15	18:55	6.34	1221	No	BZD	Thoracic aortic dissection
Ctrl15	-	73	M	04:25	05:05	7	1285	No	BZD, TCA	Pneumonia
Ctrl16	-	82	F	06:20	08:30	N.A.	1275	No	-	Physical deterioration
Ctrl17	-	94	M	05:30	16:15	6.57	1229	No	BZD	Metastatic lung cancer
Ctrl18	-	87	M	06:20	13:10	6.47	1275	No	BZD	Renal insufficiency
Ctrl19	-	85	F	06:50	09:20	6.84	1190	No	BZD, Li	Metastatic lung cancer
Ctrl20	-	82	M	05:55	18:45	6.05	1580	No	BZD	Prostate cancer
Ctrl21	-	84	F	07:50	17:55	6.59	1345	No	BZD	Colon cancer
Median	-	83	-	05:45	-	6.56	1259	-	-	-
P value	-	<0.0001	0.96	0.053	n.sig	0.38	0.53	-	-	-

Abbreviations: AA, atypical antidepressant; BD, bipolar disorder; BW, brain weight; BZD, benzodiazepine; CTD, clock time of death; CTR, control; CVA, cardiovascular arrest; DA, dopamine; DPPB, diphenylbutylpiperidine; F, female; g, gram; h, hour; Hal, haloperidol; INN, nemiifide; LEV, levetiracetam; Li, lithium; LTG, lamotrigine; M, male; MAOI, monoamine oxidase inhibitor; MD, mood disorders; MDD, major depressive disorder; N.A., not available; nBZD, nonbenzodiazepines; NDRI, norepinephrine-dopamine reuptake inhibitor; n.sig, non-significant; PMD, postmortem delay; SARI, serotonin antagonist and reuptake inhibitor; SGA, second-generation antipsychotics; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TA, typical antipsychotics; TCA, tricyclic antidepressants; TeCA, tetracyclic antidepressants; VPA, valproate; y, year.

Table S1c. Demographic information of Suicide-HYPOTHALAMUS collection (NBB).

Diagnosis - Cause of death	Cases, n	Psychiatric diagnosis (MDD/BD)	Reason for euthanasia (Physical diseases/ MD) ¹	Age (y)	Sex (M/F)	PMD (h)	Brain pH	BW (g)
MD	28	22/6	–	66 (38–94)	19/9	8:55 (3:30–62:55)	6.70 (5.61–7.55)	1291 (985–1670)
- Suicide	10	9/1	–	60 (39–90)	6/4	20:35 (3:55–62:55)	6.53 (6.18–6.80)	1300 (1120–1670)
- Legal euthanasia	7	6/1	4/5	61 (38–81)	5/2	6:40 (3:30–9:40)	6.78 (6.70–7.07)	1283 (1120–1525)
Natural causes	11	7/4	–	70 (58–94)	8/3	8:55 (4:50–60:20)	6.37 (5.61–7.55)	1295 (985–1510)
Ctr	16	–	–	72 (39–88)	11/5	7:15 (3:20–41:00)	6.60 (5.37–7.28)	1259 (1115–1629)
- Legal euthanasia	5	–	5/0	79 (49–83)	3/2	5:45 (3:20–6:30)	6.80 (6.15–7.28)	1173 (1121–1364)
Natural causes	11	–	–	67 (39–88)	8/3	9:00 (4:35–41:00)	6.00 (5.37–6.60)	1383 (1115–1629)

Abbreviations: BD, bipolar disorder; BW, brain weight; Ctr, non-psychiatric controls; F, female; g, gram; h, hour; M, male; MD, mood disorders; MDD, major depressive disorder; NBB, Netherlands Brain Bank; PMD, postmortem delay; y, year.

Table S1d. Clinico-pathological information of patients with suicide, legal euthanasia, and control subjects (Suicide-HYPOTHALAMUS collection, NBB).

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
MD1	MDD	50	M	N.A.	N.A.	N.A.	1380	Yes	N.A.	Suicide
MD2	MDD	39	M	41:00	00:00	N.A.	1670	Yes	TCA, SSRI	Suicide
MD3	BD	39	M	48:00	11:30	N.A.	1220	Yes	BZD, RIMA, SSRI	Suicide, intoxication
MD4	MDD	79	M	21:10	17:50	N.A.	1530	Yes	BZD, SSRI	Suicide, high fall
MD5	MDD	74	M	62:55	17:05	N.A.	1444	Yes	BZD, Cisordinol, SSRI	Suicide, hanging
MD6	MDD	59	F	20:35	00:00	6.53	1199	Yes	BZD	Suicide, suffocation
MD7	MDD	56	F	10:30	14:00	6.42	1120	Yes	BZD	Suicide, intoxication
MD8	MDD	62	F	11:40	16:15	6.18	1155	Yes	BZD	Suicide, strangulation
MD9	MDD	58	M	4:30	9:40	6.80	1470	Yes	SGA, SSRI, TCA	Suicide, intoxication
MD10	MDD	90	F	3:55	11:20	6.62	1220	No	BZD, INN	Suicide, intoxication
MD11	BD	81	M	6:40	20:00	6.70	1283	No	BZD, Li, TCA, VPA	Legal euthanasia
MD12	MDD	77	F	8:40	11:20	6.77	1120	Yes	BZD, INN, SSRI	Legal euthanasia
MD13	MDD	50	M	9:40	13:00	6.80	1420	No	BZD	Legal euthanasia
MD14	MDD	38	M	6:15	11:45	6.79	1525	No	BZD	Legal euthanasia
MD15	MDD	61	F	8:40	15:35	7.07	1205	No	BZD	Legal euthanasia
MD16	MDD	61	M	5:45	14:05	N.A.	1210	No	SSRI	Legal euthanasia
MD17	MDD	67	M	3:30	11:10	6.72	1445	No	BZD, Hal	Legal euthanasia
MD18	MDD	70	M	20:00	19:00	N.A.	1500	No	BZD, DA stabilizer, SSRI	Heart attack
MD19	MDD	73	F	22:00	19:00	N.A.	1287	No	BZD, TCA	Bronchopneumonia
MD20	MDD	71	M	13:30	22:30	N.A.	1109	No	BZD, Li, MAOI, PTZ	Respiratory insufficiency
MD21	MDD	61	M	60:20	4:40	N.A.	1424	No	PTZ	Pneumonia

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
MD22	MDD	81	M	6:00	15:30	6.50	1280	No	Hal	Renal insufficiency
MD23	MDD	68	M	8:55	5:45	6.82	1510	Yes	BZD, SSRI	Sudden death
MD24	BD	70	M	6:50	21:00	6.19	1275	No	BZD, LTG, VPA	Pulmonary infection and renal insufficiency
MD25	BD	73	M	13:45	19:45	6.24	1480	No	BZD	Infection
MD26	BD	94	F	5:10	4:00	7.55	985	No	BZD, SSRI	Respiratory and cardiac failure
MD27	MDD	58	F	7:20	23:00	5.61	1295	No	BZD, DA stabilizer, SSRI	Renal insufficiency
MD28	BD	65	M	4:50	18:30	N.A.	1305	No	BZD, Li, SGA	Colon carcinoma
Median	–	66	–	08:55	–	6.70	1291	–	–	–
CTR1	–	68	F	5:45	12:15	6.97	1135	No	–	Legal euthanasia
CTR2	–	83	F	3:20	16:30	6.80	1173	No	–	Legal euthanasia
CTR3	–	49	M	6:15	17:30	6.15	1364	No	BZD	Legal euthanasia
CTR4	–	79	M	6:30	10:00	6.71	1121	No	BZD	Legal euthanasia
CTR5	–	83	M	5:45	19:35	7.28	1195	No	–	Legal euthanasia
CTR6	–	41	M	17:00	00:00	N.A.	1150	No	–	Renal insufficiency
CTR7	–	72	F	17:30	16:30	N.A.	N.A.	No	–	Bronchopneumonia
CTR8	–	49	M	21:40	19:20	N.A.	1629	No	–	Cardiac infarction
CTR9	–	66	M	41:00	00:00	N.A.	1461	No	INN	Septic shock
CTR10	–	88	F	5:55	7:00	6.05	1115	No	BZD	Cardiac arrest
CTR11	–	39	M	16:30	00:30	N.A.	1400	No	–	Cardiac infarction
CTR12	–	55	M	7:15	11:05	N.A.	1393	No	BZD, INN	Intestinal ischemia

Sample number	Psychiatric diagnosis	Age (y)	Sex	PMD (h)	CTD	CSF pH	BW (g)	Suicide attempt	Antipsychotics in last three months	Cause of death
CTR13	-	78	F	4:35	22:40	6.41	1226	No	BZD, INN	Bronchopneumonia
CTR14	-	73	M	8:00	16:15	5.37	1553	No	BZD, INN	Pneumonia
CTR15	-	83	M	5:15	11:15	6.60	1372	No	BZD	Cardiac infarction
CTR16	-	67	M	9:00	15:05	6.48	1292	No	BZD, INN	Aortic aneurysm
Median	-	70	-	6:52	-	6.54	1292	-	-	-
P value	-	0.54	0.95	0.35	n.sig.	0.50	0.60	-	-	-

Abbreviations: BD, bipolar disorder; BW, brain weight; BZD, benzodiazepine; CTD, clock time of death; CTR, control; DA, dopamine; F, female; g, gram; h, hour; Hal, haloperidol; INN, nemiifide; Li, lithium; LTG, lamotrigine; M, male; MAOI, monoamine oxidase inhibitor; MD, mood disorders; MDD, major depressive disorder; N.A., not available; PMD, postmortem delay; PTZ, pentylenetetrazole; RIMA, reversible inhibitor of monoamine oxidase-A; SGA, second-generation antipsychotics; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressants; VPA, valproate; y, year.

Table S1e. Demographic information of Suicide-PFC collection (SMRI).

Diagnosis - Cause of death	Cases, n	Psychiatric diagnosis (MDD/BD)	Age (y)	Sex (M/F)	PMD (h)	Brain pH	BW (g)	Psychotic features (NP/P)	Fluphenazine (mg)
MD	54	24/30	44 (19–64)	28/26	29 (12–84)	6.60 (5.92–6.97)	1440 (1170–1780)	28/26	850 (0–130000)
- Suicide	30	17/13	42 (24–63)	17/13	27.5 (13–70)	6.61 (6.07–6.90)	1468 (1170–1780)	15/15	100 (0–15000)
Natural causes	24	7/17	44.5 (19–64)	11/13	30.5 (12–84)	6.55 (5.92–6.97)	1355 (1170–1590)	13/11	2000 (130000)
Ctr	46	-	45.5 (24–63)	33/13	28.5 (9–58)	6.67 (6.00–7.03)	1413 (1120–1900)	-	-

Abbreviations: NP, individuals without a history of psychotic features; P, individuals with a history of psychotic features.

Table S1f. Clinico-pathological information of patients with suicide, MD, and control subjects (Suicide-PFC collection, SMRI).

Sample number	Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Psychotic feature	Fluphenazine (mg)	Cause of Death
MDD1	F	48	24	6.36	1330	8.0	L DLPFC, L ACC	Yes	6500	SUIC: OD
MDD2	F	40	49	6.72	1450	8.8	L DLPFC, L ACC	Yes	1000	SUIC: HANGING
MDD3	F	56	15	6.59	1370	8.4	L DLPFC, L ACC	No	0	BURNS
MDD4	M	28	26	6.7	1780	6.9	R DLPFC, R ACC	Yes	3000	SUIC: HANGING
MDD5	M	35	19	6.6	1335	8.2	R DLPFC, R ACC	Yes	2000	SUIC: HANGING
MDD6	F	32	19	6.7	1280	8.9	L DLPFC, L ACC	Yes	100	SUIC: HANGING
MDD7	F	32	19	6.8	1470	8.0	R DLPFC, R ACC	No	0	SUIC: HANGING
MDD8	M	63	31	6.6	1540	7.8	L DLPFC, L ACC	Yes	4000	SUIC: HANGING
MDD9	F	51	36	6.3	1440	7.3	L DLPFC, L ACC	Yes	700	UNKNOWN
MDD10	M	35	36	6.6	1710	6.3	L DLPFC, L ACC	Yes	0	SUIC: GSW
MDD11	M	44	24	6.52	1550	7.9	R DLPFC, R ACC	No	3000	CARDIAC
MDD12	M	56	38	6.59	1365	7.3	L DLPFC, L ACC	No	1000	SUIC: OD
MDD13	M	33	25	6.86	1640	7.6	R DLPFC, R ACC	No	0	SUIC: HANGING
MDD14	M	34	24	6.79	1425	8.2	R DLPFC, R ACC	No	0	SUIC: JUMPED
MDD15	F	45	29	6.9	1350	7.4	L DLPFC, L ACC	No	0	CARDIAC
MDD16	M	53	21	6.64	1520	7.5	L DLPFC, L ACC	No	0	CARDIAC
MDD17	M	62	65	6.57	1490	7.5	R DLPFC, R ACC	No	0	SUIC: STABBED
MDD18	F	36	32	6.74	1270	7.3	R DLPFC, R ACC	Yes	2500	PULM EMBOL
MDD19	M	40	52	6.48	1590	6.6	L DLPFC, L ACC	Yes	3000	OD
MDD20	F	28	40	6.68	1430	7.1	L DLPFC, L ACC	Yes	0	SUIC: OD
MDD21	M	45	29	6.75	1514	7.9	L DLPFC, L ACC	No	0	SUIC: HANGING

Sample number	Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Psychotic feature	Fluphenazine (mg)	Cause of Death
MDD22	M	24	21	6.61	1737	7.4	R DLPFC, R ACC	No	0	SUIC: OD
MDD23	F	45	13	6.58	1170	7.2	L DLPFC, L ACC	No	100	SUIC: OD
MDD24	F	47	25	6.88	1495	7.7	L DLPFC, L ACC	No	0	SUIC: GSW
BD1	M	29	48	6.39	1570	9.2	R DLPFC, R ACC	Yes	9000	SUIC: JUMPED
BD2	M	29	60	6.7	1430	8.5	R DLPFC, R ACC	No	0	SUIC: CO
BD3	M	45	28	6.35	1480	9.1	L DLPFC, L ACC	Yes	10000	CARDIAC
BD4	M	41	70	6.71	1625	7.5	R DLPFC, R ACC	No	0	SUIC: OD
BD5	F	29	62	6.74	1330	7.8	R DLPFC, R ACC	Yes	0	OD
BD6	M	44	19	6.74	1660	9.3	L DLPFC, L ACC	No	0	SUIC: HANGING
BD7	F	48	18	6.5	1205	8.3	R DLPFC, R ACC	No	0	CARDIAC
BD8	M	42	32	6.65	1470	8.4	L DLPFC, L ACC	No	0	DROWNING
BD9	M	35	35	6.3	1490	7.8	R DLPFC, R ACC	Yes	30000	CARDIAC
BD10	F	59	53	6.2	1410	8.5	L DLPFC, L ACC	No	0	SUIC: OD
BD11	M	54	44	6.5	1510	9.2	R DLPFC, R ACC	No	0	SUIC: OD
BD12	F	35	17	6.1	1250	7.9	L DLPFC, L ACC	Yes	3000	SUIC: CO
BD13	F	42	49	6.65	1335	8.0	R DLPFC, R ACC	Yes	15000	OD
BD14	F	58	35	6.5	1440	9.5	R DLPFC, R ACC	Yes	12000	SUIC: GSW
BD15	M	64	16	6.1	1340	7.8	L DLPFC, L ACC	Yes	130000	PNEUMONIA
BD16	M	59	84	6.65	1300	7.3	L DLPFC, L ACC	No	500	SLEEP APNEA
BD17	M	51	23	6.67	1590	9.5	L DLPFC, L ACC	Yes	1200	CARDIAC
BD18	F	63	32	6.97	1290	8.9	R DLPFC, R ACC	No	0	CARDIAC
BD19	F	44	37	6.37	1200	7.3	L DLPFC, L ACC	Yes	30000	MYOCARDITIS

Sample number	Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Psychotic feature	Fluphenazine (mg)	Cause of Death
BD20	F	56	26	6.58	1170	8.2	R DLPFC, R ACC	No	25000	DROWNING
BD21	F	43	39	6.74	1505	9.3	R DLPFC, R ACC	Yes	4500	SUIC:OD
BD22	M	35	22	6.58	1390	8.5	L DLPFC, L ACC	Yes	2000	DROWNING
BD23	F	50	62	6.51	1400	8.3	R DLPFC, R ACC	Yes	15000	SUIC:OD
BD24	F	49	38	6.39	1190	8.5	L DLPFC, L ACC	Yes	0	OD
BD25	F	33	24	6.51	1450	8.6	R DLPFC, R ACC	No	3000	SUIC:HANGING
BD26	F	41	28	6.44	1360	8.7	R DLPFC, R ACC	No	3000	CARDIAC
BD27	F	43	57	5.92	1340	6.3	R DLPFC, R ACC	Yes	10000	OD
BD28	M	56	23	6.07	1670	9.3	L DLPFC, L ACC	Yes	10000	SUIC:OD
BD29	M	48	23	6.9	1466	8.4	R DLPFC, R ACC	No	0	SUIC:HANGING
BD30	M	19	12	5.97	1484	7.9	L DLPFC, L ACC	No	2000	OD
Median	-	44	29	6.60	1440	8.0	-	-	850	-
Ctr1	M	48	12	6.51	1410	8.6	R DLPFC, R ACC	No	0	CARDIAC
Ctr2	F	50	35	6.31	1520	7.9	L DLPFC, L ACC	No	0	CARDIAC
Ctr3	M	50	11	6.5	1530	8.6	L DLPFC, L ACC	No	0	CARDIAC
Ctr4	M	63	37	6.5	1530	8	L DLPFC, L ACC	No	0	CARDIAC
Ctr5	M	24	17	6.6	1595	8.1	L DLPFC, L ACC	No	0	MVA
Ctr6	M	44	27	6.82	1410	7.2	R DLPFC, R ACC	No	0	ACUTE ALCOHOL POI-SONING
Ctr7	M	35	31	6.59	1520	8	R DLPFC, R ACC	No	0	MVA
Ctr8	M	63	40	6.91	1410	8.2	R DLPFC, R ACC	No	0	CARDIAC
Ctr9	M	34	9	6.56	1535	5.9	R DLPFC, R ACC	No	0	MVA

Sample number	Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Psychotic feature	Fluphenazine (mg)	Cause of Death
Ctr10	F	56	29	6.78	1278	8.9	L DLPEC, L ACC	No	0	CARDIAC
Ctr11	F	56	31	6.66	1400	6.1	L DLPEC, L ACC	No	0	OBESITY
Ctr12	F	39	24	6.88	1200	7.3	R DLPEC, R ACC	No	0	CARDIAC
Ctr13	F	44	28	6.59	1330	8.3	R DLPEC, R ACC	No	0	CARDIAC
Ctr14	M	49	46	6.5	1605	8.3	R DLPEC, R ACC	No	0	CARDIAC
Ctr15	M	53	9	6.4	1500	9.1	L DLPEC, L ACC	No	0	CARDIAC
Ctr16	M	37	13	6.5	1600	8.3	L DLPEC, L ACC	No	0	CARDIAC
Ctr17	M	51	31	6.7	1400	7.3	R DLPEC, R ACC	No	0	CARDIAC
Ctr18	M	53	28	6	1340	8.4	L DLPEC, L ACC	No	0	CARDIAC
Ctr19	F	38	33	6	1120	9.7	R DLPEC, R ACC	No	0	CARDIAC
Ctr20	F	38	28	6.7	1350	8.8	R DLPEC, R ACC	No	0	CARDIAC
Ctr21	M	60	47	6.8	1460	8.4	R DLPEC, R ACC	No	0	CARDIAC
Ctr22	M	35	52	6.7	1700	8.7	R DLPEC, R ACC	No	0	MYOCARDITIS
Ctr23	M	34	22	6.48	1480	8.2	R DLPEC, R ACC	No	0	CARDIAC
Ctr24	M	47	21	6.81	1550	8.7	L DLPEC, L ACC	No	0	CARDIAC
Ctr25	M	45	29	6.94	1405	8.5	R DLPEC, R ACC	No	0	CARDIAC
Ctr26	F	34	24	6.87	1255	6.6	R DLPEC, R ACC	No	0	CARDIAC
Ctr27	M	42	37	6.91	1340	7.8	L DLPEC, L ACC	No	0	CARDIAC
Ctr28	F	44	10	6.2	1305	8.4	R DLPEC, R ACC	No	0	CARDIAC
Ctr29	M	45	18	6.81	1585	8.0	L DLPEC, L ACC	No	0	CARDIAC
Ctr30	M	49	23	6.93	1390	9.5	L DLPEC, L ACC	No	0	CARDIAC
Ctr31	M	32	24	7.03	1415	8.3	L DLPEC, L ACC	No	0	CARDIAC

Sample number	Sex	Age (y)	PMD (h)	Brain pH	BW (g)	RIN	Brain Region	Psychotic feature	Fluphenazine (mg)	Cause of Death
Ctr32	M	55	31	6.7	1515	8.3	L DLPFC, L ACC	No	0	CARDIAC
Ctr33	F	49	45	6.72	1435	8.6	L DLPFC, L ACC	No	0	CARDIAC
Ctr34	F	33	29	6.52	1360	8.3	L DLPFC, L ACC	No	0	ASTHMA
Ctr35	M	48	31	6.86	1580	7.0	R DLPFC, R ACC	No	0	CARDIAC
Ctr36	M	50	49	6.75	1645	8.0	R DLPFC, R ACC	No	0	CARDIAC
Ctr37	M	32	13	6.57	1410	9.0	R DLPFC, R ACC	No	0	CARDIAC
Ctr38	M	47	11	6.6	1495	8.3	L DLPFC, L ACC	No	0	CARDIAC
Ctr39	M	46	31	6.67	1360	8.6	L DLPFC, L ACC	No	0	CARDIAC
Ctr40	M	40	38	6.67	1498	8.7	L DLPFC, L ACC	No	0	CARDIAC
Ctr41	M	51	22	6.71	1900	8.7	L DLPFC, L ACC	No	0	CARDIAC
Ctr42	M	31	11	6.13	1335	8.4	R DLPFC, R ACC	No	0	PULM EMBOL
Ctr43	M	48	24	6.91	1321	8.6	R DLPFC, R ACC	No	0	CARDIAC
Ctr44	F	39	58	6.46	1260	8.4	R DLPFC, R ACC	No	0	CARDIAC
Ctr45	M	47	36	6.57	1535	8.0	L DLPFC, L ACC	No	0	CARDIAC
Ctr46	F	41	50	6.17	1290	7.3	R DLPFC, R ACC	No	0	CARDIAC
Median	-	45.5	28.5	6.67	1413	8.3	-	-	-	-
P value	0.04	0.62	0.18	0.16	0.80	0.21	0.69	-	-	-

Abbreviations: ACC, anterior cingulate cortex; BD, bipolar disorder; BW, brain weight; CO, carbon monoxide intoxication; Ctr, control; DLPFC, dorsolateral prefrontal cortex; F, female; GSW, gunshot wound; L, left; M, male; MVA, motor vehicle accident; OD, overdose drugs; PMD, postmortem delay; PULM EMBOL, pulmonary embolism; R, right; RIN, RNA integrity number; SUIC, suicide.

Sample preparation

Tissues for immunohistochemical and immunofluorescent staining

Tissues from the hippocampi and hypothalami were dissected at autopsy, fixed, dehydrated, cleared, and embedded in paraffin. Coronal serial sections (6 μm) were made from the anterior or middle hippocampus and hypothalamus from the level of the lamina terminalis to mamillary bodies. Every 100th section from the hypothalamus and one section from the hippocampus were collected and stained with thionine for orientation. One section from the hippocampus was collected and stained with hematoxylin for cell nucleus counting in the dentate gyrus.

Tissues for real-time PCR

Prepared by the SMRI.

Immunohistochemical staining

Antibodies used in this study, their manufacturers, specificities, and staining protocols are presented in **Table S2**. In brief, after deparaffinization and rehydration, the sections were washed, antigen retrieved by microwave, and/or pre-incubated to block background staining. Subsequently, the sections were incubated with primary antibody at 4 °C, then with biotinylated secondary antibody (1:400; Vector Labs, Inc. Burlingame, USA), followed by Avidin-Biotin-peroxidase Complex (1:800; Vector Labs, Inc. Burlingame, USA). Finally, sections for bright field imaging were incubated in a solution of 0.5 mg/mL 3,3-diaminobenzidine (Sigma) in a total volume of 15 mL TBS containing 5 μL H_2O_2 30% (Merck) and 0.035 g ammonium Nickel sulfate (DAB-Ni), at room temperature for color development. For double labeling (DAB-Ni vs DAB), ammonium nickel sulfate was not included in the second DAB development. The enzyme reaction was stopped in aqua dest. Subsequently, the sections were dehydrated, cleared, and coverslipped in Entellan (Merck). After the incubation of primary antibodies, sections for confocal imaging were incubated with fluorescent-conjugated antibodies to visualize the immunoreactions. Finally, sections were incubated with Hoechst to visualize the nuclei and coverslipped with antifade mounting medium (VECTASHIELD) and sealed.

Table S2a. Specification of the antibodies used.

Antibody	Species	Manufacturer	Catalog number
P2RX7	Goat polyclonal	Novus Biologicals	NBP1-37775
P2RY12	Rabbit polyclonal	Sigma-Aldrich	HPA014518
GFAP	Rabbit polyclonal	DAKO	GA524
CD68	Mouse monoclonal	DAKO	M0814

Abbreviations: CD68, the cluster of differentiation 68; GFAP, glial fibrillary acidic protein; P2RX7, P2X purinoceptor 7; P2RY12; P2Y purinoceptor 12.

Note: 1 Specificity of commercial antibodies was provided by the manufacturers.

Table S2b. Immunohistochemistry for single staining.

Antibody	Antigen retrieval	Blocking buffer	Dilution
P2RX7	0.01M citrate buffer (pH 6.0) microwave 800 w 10 min	–	1:800
P2RY12	0.01M citrate buffer (pH 6.0) microwave 800 w 10 min	–	1:2000
GFAP	–	1×TBS–4% milk	1:100000
CD68	0.05M Tris–HCl buffer (pH 9.0) microwave 800 w 20 min	–	1:400

Note: SUMI: supermix, 0.25 g gelatin in 100 ml 1×TBS, pH 7.6 heat the gelatin, until it dissolves, and add Triton-X 0.5 ml. Store in fridge.

Table S2c. Immunohistochemistry for confocal staining.

Antibody	Antigen retrieval	Primary antibodies dilutions	Secondary antibodies	Nuclear staining
P2RY12	0.05M Tris-HCl buffer (pH 9.0) microwave 800 w 20 min	1:500	Alexa 594-conjugated goat anti-rabbit (1:200)	Hoechst 1:1200 in SUMI 10 min
CD68		1:100	Alexa 488-conjugated goat anti-mouse (1:200)	

Image analysis

Cell counting

To determine the total nuclei/cells in the target areas, a tissue scanner (ZEISS Axio Scan.Z1 (Oberkochen, Germany)) was used to collect images with hematoxylin/ immunohistochemical staining at a Plan_Apochromat 20×/0.8 objective. Analysis was done with the open software QuPath 0.3.0². For the nuclei and astrocytes counting, 20% of the manually outlined areas of interest (AOI) were systematic random sampled. Following P2RX7 immunohistochemical staining, target cells (nuclei or astrocyte cell bodies with positive staining) within the AOI were quantified. Subjects with over 5% of total nuclei with P2RX7 nuclear staining were recorded, in which P2RX7⁺ nuclei were at detectable levels in microscopy practice. In the DG, the ratio (%) of P2RX7⁺ nuclei to total nuclei in one subject was calculated, because the granule cells were tightly packed together in a laminated and homogeneous manner. To determine the density of P2RX7⁺ nuclei in the DG, CA1, CA2-4, Sub, and Ent, we divided the P2RX7⁺ nuclei by the volumes of interest.

Quantification of protein expression

Quantitative image analyses were carried out by one investigator who was blind to the experimental conditions. The setup consisted of an image analysis system (Image-Pro version 6.3, Media Cybernetics, Rockville, USA) connected to a black and white camera (SONY XC-77E) mounted on microscopes (ZEISS Axioskop with Plan-NEOFLUAR ZEISS objectives, Carl Zeiss GmbH, Jena, Germany) or Zen system. The part of the hypothalamic nuclei and hippocampal subfields containing P2RX7, P2RY12, GFAP, or CD68 immunoreactive cells was delineated manually at a 20×/0.5 objective, according to the thionine staining. A computer program developed in-house was used to mask and extract the P2RX7, P2RY12, GFAP, or CD68 immunoreactive positive signal structures. The threshold for a positive signal was set at 0.1. The computer determined the optical density (OD) of the pixels and the percentage of surface area covered by the signal (area mask). In the hippocampal subregions, the integrated optical density (IOD) of every AOI was then calculated by multiplying the OD by the area mask corrected for background and total area. Regarding the thionine staining throughout the hypothalamic nuclei, 5 sections, including the first, first quarter, middle, last quarter, and last sections that contained PVN/SON neurons, were mounted for immunohistochemical staining and IOD quantification. The IOD of each section was calculated by multiplying the OD by the area of the mask. The IOD of each subject was calculated by adding up the IODs of five sections, then dividing by the added total AOIs of five sections. The volume of PVN/SON-containing neurons shown by thionine staining was calculated based on the Cavalieri principle³. Of note, in the imaging processing for SON volume quantification, AOIs from images that showed both medial and lateral SONs were delineated and added to the area of the corresponding level.

Confocal microscopy and imaging processing

A confocal laser-scanning microscope SP8 (DM6000 CFS; acquisition software, Leica Application Suite AF v3.2.1.9702, RRID: SCR_013673) was used for image capture. Z-stack images were collected with a 63×/1.4 oil objective at 3.5× zoom and z-step of 0.5 μm . To validate the integrity of microglial debris, which was structurally disconnected by processes, the entire P2RY12⁺ microglia debris was captured within the z-stack for image processing. Three-dimensional reconstruction of P2RY12⁺ microglia was performed using Imaris software (version 8.3, Bitplane AG, Zurich, Switzerland).

Morphological parameters of P2RY12⁺ microglia

Using ImageJ, the total number of P2RY12⁺ microglia in the mEnt was manually counted within the outlined area when the cell nucleoli were exposed. Results were shown in cell body number/mm³. For cell size measurement, positive particles larger than 50 μm^2 and smaller than 400 μm^2 (size determined in the pilot study) were considered microglial cell body + processes. The radius of a microglia cell body was the semidiameter that could draw a max circle within the particles, resulting in pixels. For fragment measurement, positive particles smaller than 50 μm^2 (size determined in the pilot study) were considered microglial fragments. The size was presented as the average value per subject, resulting in average size (μm^2). The total number of microglial fragments was recorded within the outlined area. Fragment density was shown in fragment number/mm³.

Quantitative real-time PCR

RNA isolation and cDNA synthesis were performed as described previously⁴. RNA integrity value (RIN), an indicator of human post-mortem tissue RNA quality (Stan et al., 2006), did not show any significant difference between the diagnostic groups in the SMRI material (RIN value of the ACC/DLPFC from MD: 8.06 ± 0.79 and the control group: 8.09 ± 0.74 , mean $\pm$ SEM). A cDNA template (equivalent to 5 ng of total RNA) was amplified in a final volume of 10 μl using SYBR Green PCR master mix (Applied Biosystems, CA, USA) and a mixture of forward and reverse primers (each 2 pmol/ μl).

Data were acquired and processed automatically by the Applied Biosystems 7300 Real-Time PCR System. The specificity of amplification was checked by melting curve analysis. Sterile water and RNA samples without the addition of reverse transcriptase during cDNA synthesis served as negative controls. The linearity of each qPCR assay was tested by preparing a series of dilutions of the same stock cDNA in multiple plates. To reduce the effect of sample variability, reference genes were selected based on expression stability measurement⁵ and the proportion of explained variability regarding all target genes. The higher stability of a reference gene means that its variance was relatively lower than the other considered reference genes. Further, if the expression of a reference gene reflects the variability of the samples well, it is expected

that its application should reduce the overall variability of the target genes. Briefly, we used the following reference genes: actin beta (ACT β), glyceraldehyde-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), tubulin alpha (TUB α), tubulin beta (TUB β), and ubiquitin C (UBC). For primer information see **Table S3**.

Table S3. Reference gene and target gene sequence.

Gene	Full name	Accession Code	Forward Primer	Reverse Primer
Reference Gene				
ACT β	Actin beta	NM_001101	CCCAGCCATGT ACGTTGCTA	TCACCGGAGTCC ATCACGAT
GAPDH	Glyceraldehyde-phosphate dehydrogenase	NM_002046	CAAATTCCAT GGCACCGTC	TCTCGCTCCT GGAAGATGGT
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	NM_000194	GGACAGGACTG AACGTCTTGC	ATAGCCCCCCT TGAGCACAC
TUB α	Tubulin alpha	NM_006082	CTTTGAGCCA GCCAACCAGA	GTACAACAGGCA GCAAGCCAT
TUB β	Tubulin beta	NM_006087	GGGCCAAGTTT TGGGAGGT	CACTGTCCCCATG GTATGTGC
UBC	Ubiquitin C	NM_021009	GCTGCTCAT AAGACTCGGCC	GTCACCCAAGT CCCGTCCTA
Target Gene				
P2RX7	P2X purinoceptor 7	NM_002562	CGTGGAGAAT GGAGTGAAGA	GGGATACTCGGG ACACAA

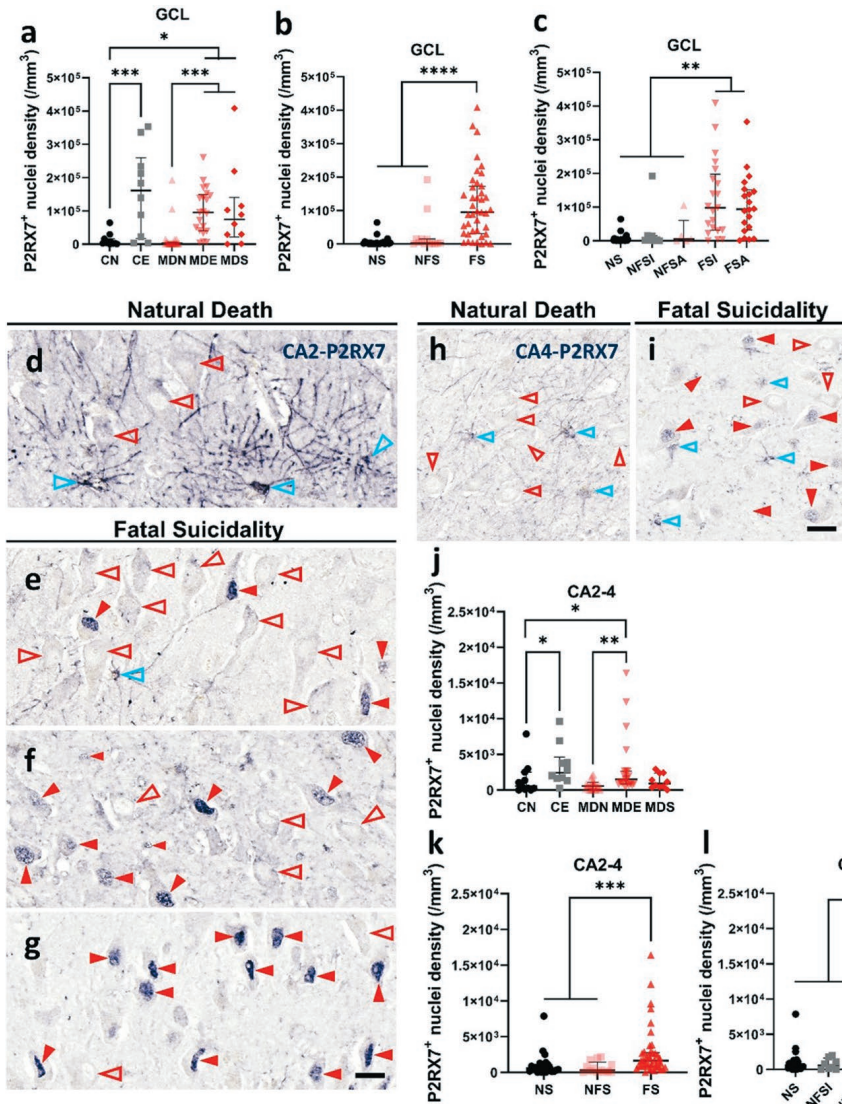


Figure S1. P2RX7⁺ nuclei in the DG and CA2-4 mark fatal suicidality, irrespective of MD. (a-c) show the P2RX7⁺ nuclei densities in the GCL in accordance with P2RX7⁺ nuclei ratios presented in **Figure 1i-k**. **d-l**) Nuclear P2RX7 expression in the CA2 and CA4 of individuals without (**d** and **h**) and with fatal suicidality (**e-g** and **i**). Red solid arrowheads point to neurons with P2RX7⁺ nuclei. Red empty arrowheads point to neurons without nuclear P2RX7 staining. Blue empty arrowheads point to astrocytes. (**j-l**) More nuclei in the CA2-4 expressed P2RX7 in both Ctr and MD in relation to fatal suicidality. Scale bars, 20 μ m.

Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, *** indicates $0.0001 \leq p < 0.001$.

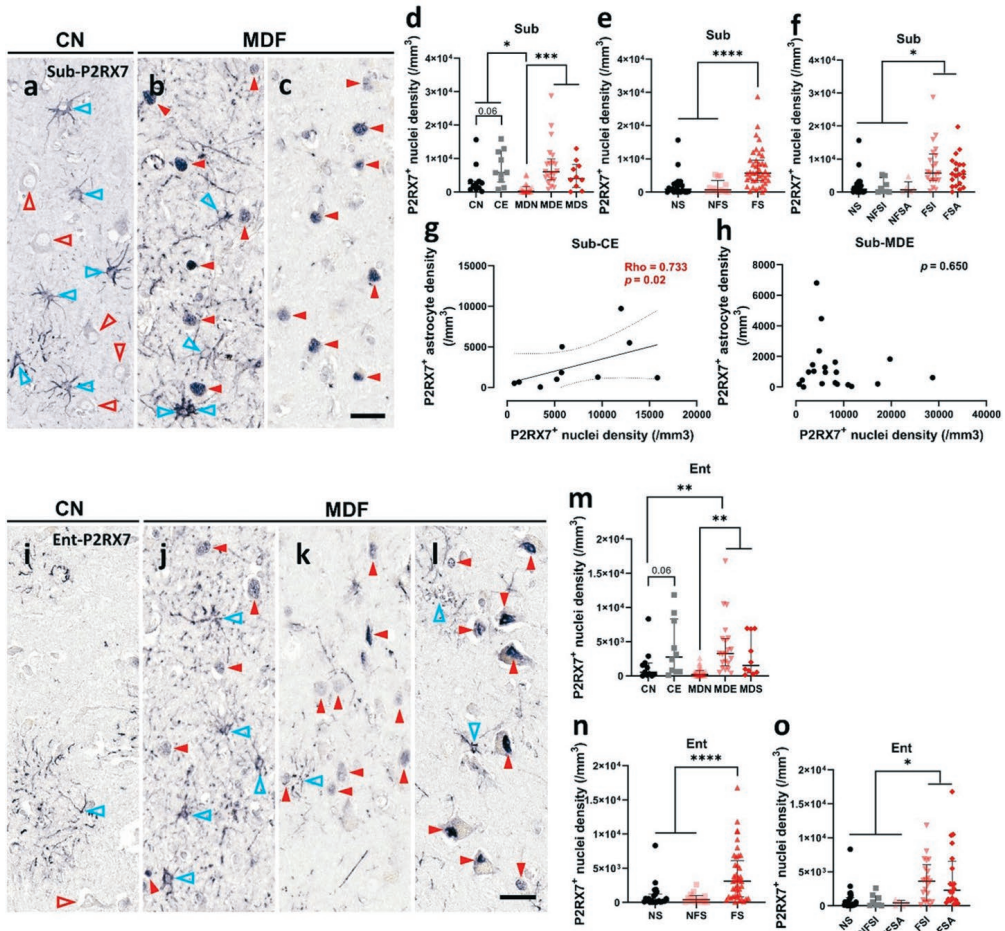


Figure S2. P2RX7⁺ nuclei in the Sub and Ent mark fatal suicidality in MD. P2RX7 expression in the Sub (a-h) and Ent (i-o) of individuals without (a and i) and with fatal suicidality (b and c, j-l). Red solid arrowheads point to neurons with P2RX7⁺ nuclei. Red empty arrowheads point to neurons without nuclear P2RX7 staining. Blue empty arrowheads point to astrocytes. (d-f) More nuclei in the Sub express P2RX7 in MD in relation to fatal suicidality, which is a trend of increase within the control subsets. (g) shows a positive correlation between P2RX7⁺ nuclei and P2RX7⁺ astrocytes in the Sub of control subjects who died by legal euthanasia, which is absent in the MD subjects who died of the same cause (h). (m-o) More nuclei in the Ent express P2RX7 in MD in relation to fatal suicidality, which is an increasing trend in CE versus CN. Scale bars, 20 µm.

Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, *** indicates $0.0001 \leq p < 0.001$.

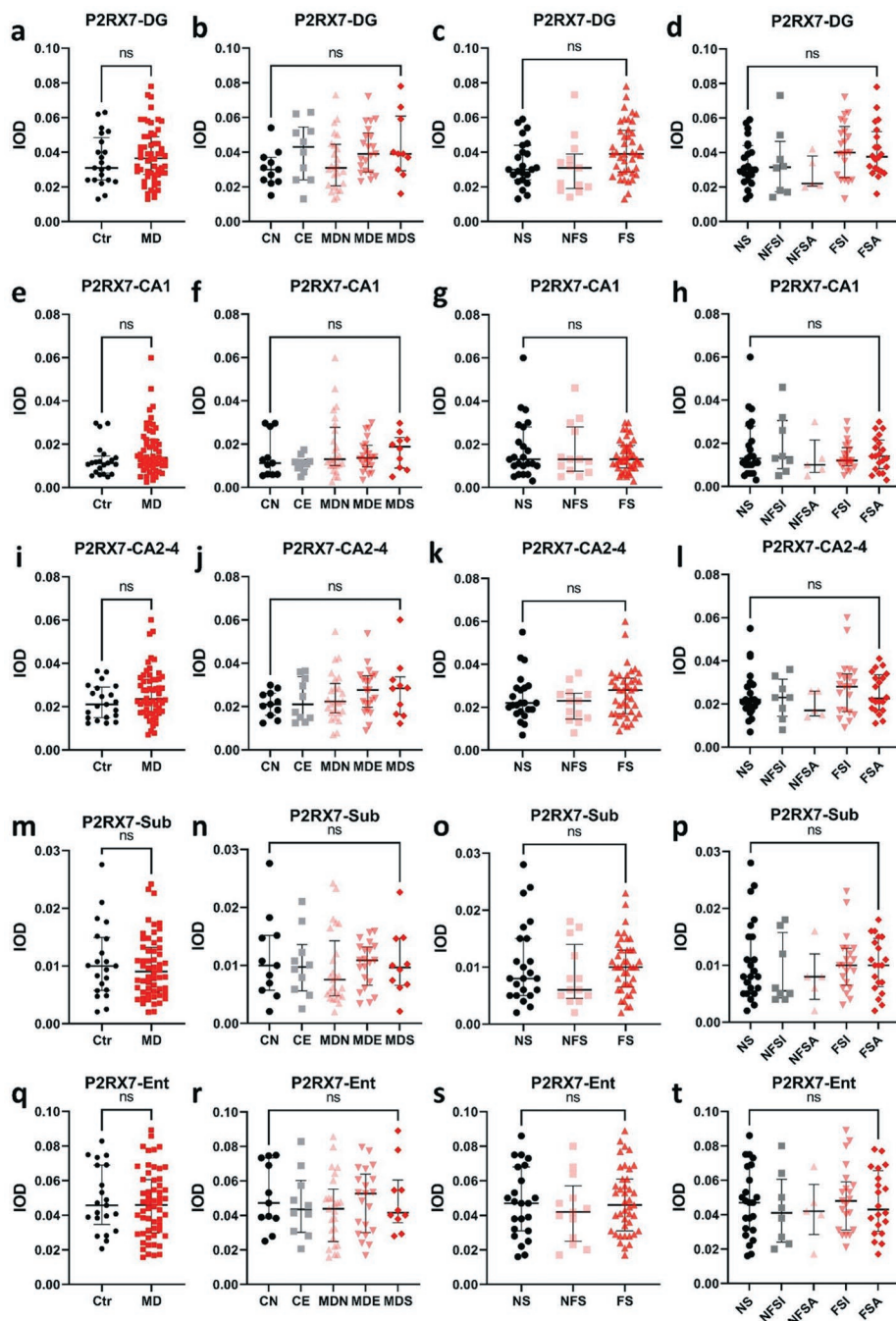


Figure S3. Unchanged P2RX7 total expression throughout the hippocampal subfields in relation to suicide or MD.

Data are presented as medians with interquartile ranges. ns, not significant.

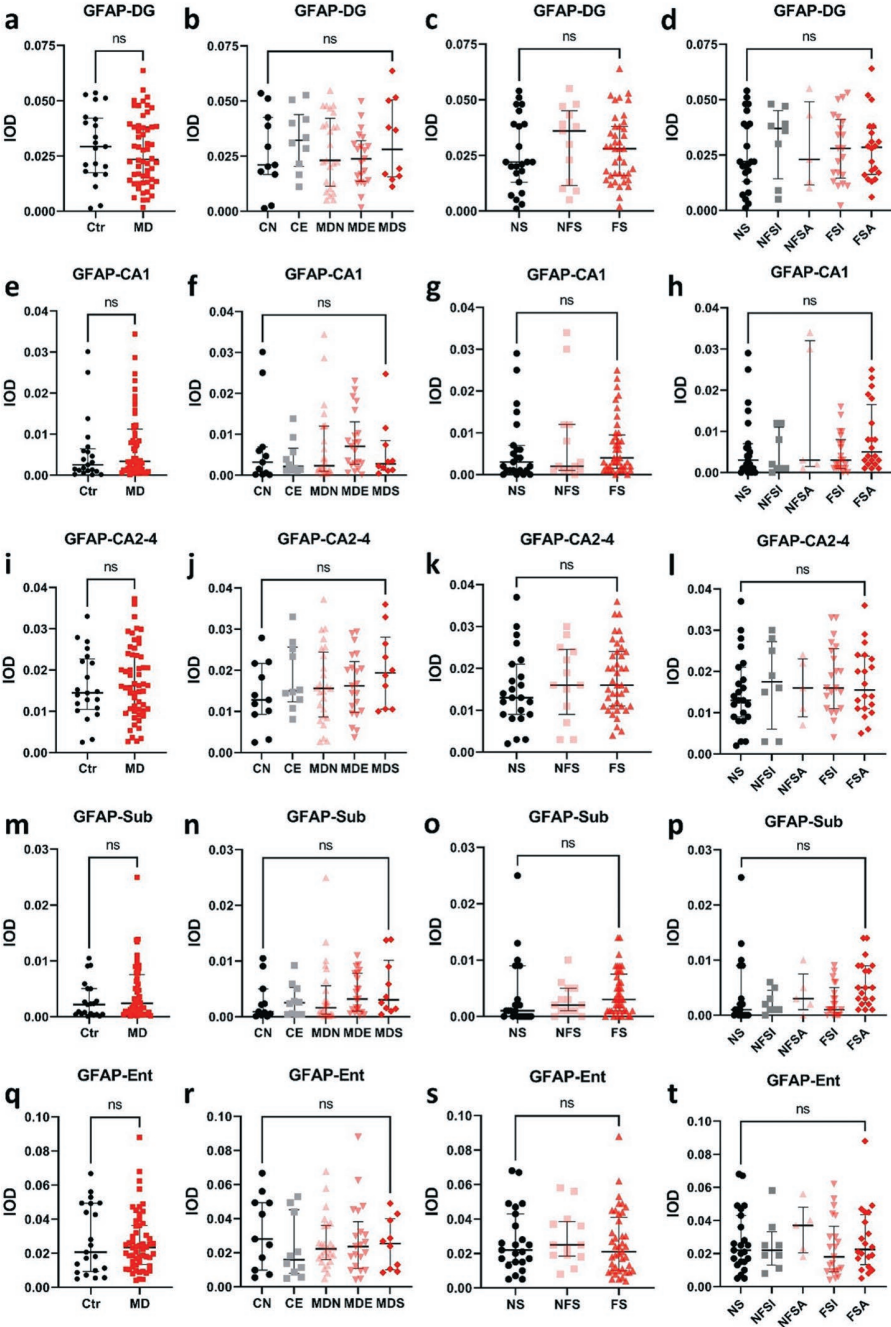


Figure S4. Unchanged GFAP total expression throughout the hippocampal subfields in relation to suicide or MD.

Data are presented as medians with interquartile ranges.

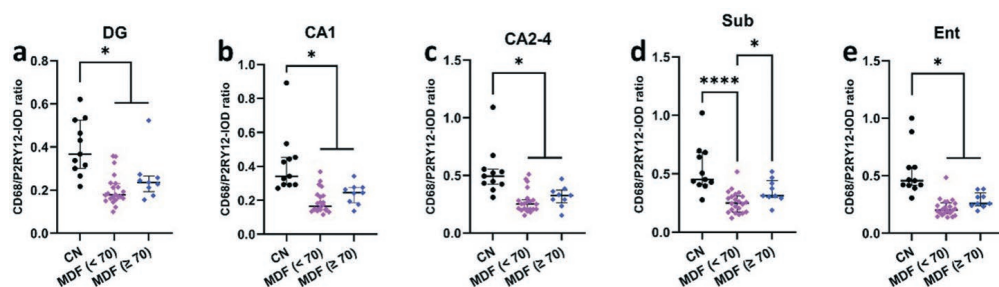


Figure S5. Age did not confound the P2RY12⁺ microglial priming in the hippocampal subareas except for the Sub. (a-e) The ratios of CD68 to P2RY12 from depressed patients who had fatal suicidality (MDF) are subdivided by the age of 70 and compared with non-suicidal controls.

Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, **** indicates $p < 0.0001$.

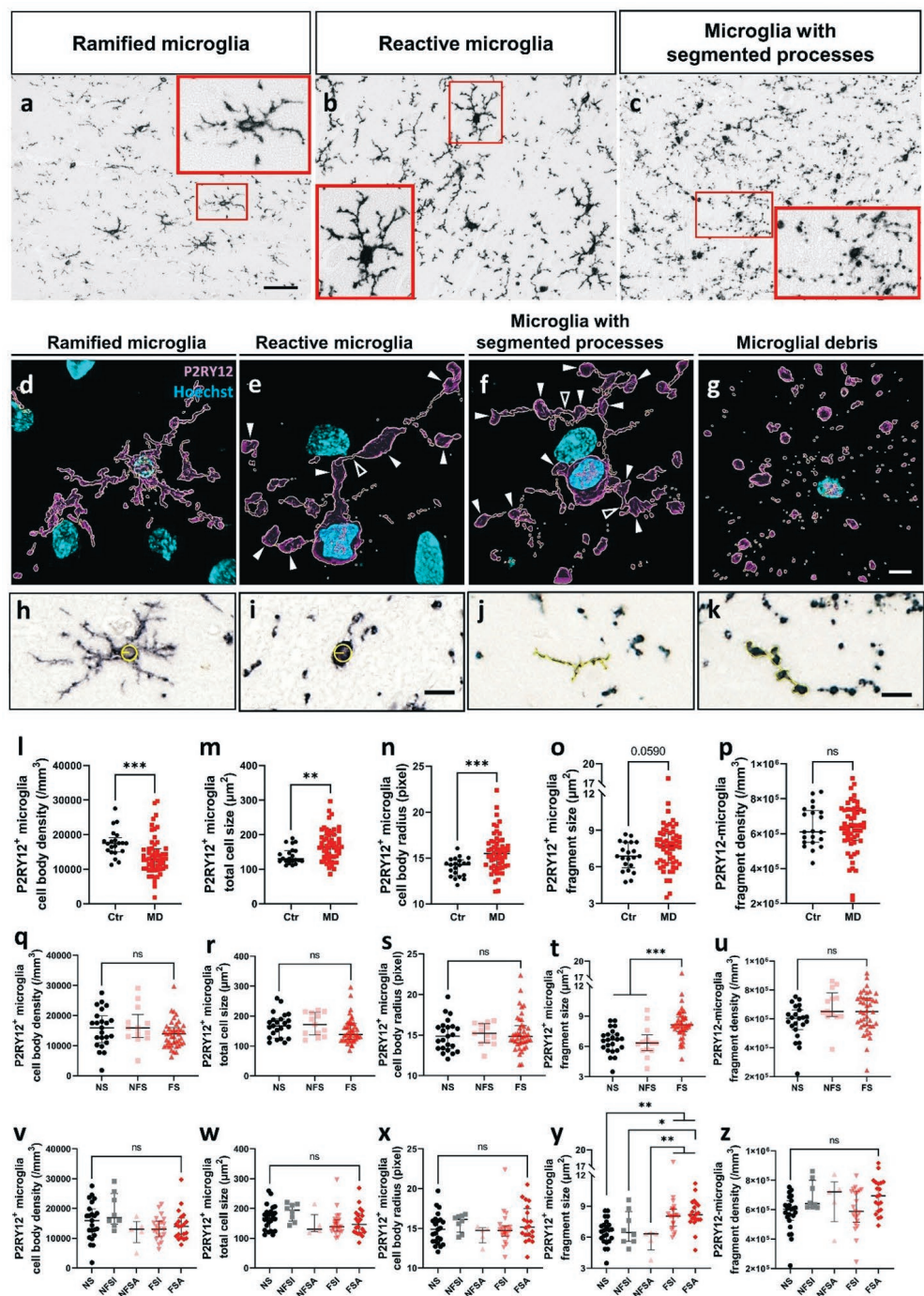


Figure S6. P2RY12⁺ microglial deformation in the medial entorhinal cortex in MD and suicide. (a-c show ramified microglia (a) in a control subject who died of natural causes, reactive microglia (b) in a depressed patient who died of natural causes, and microglia with segmented processes (c) in a subject with fatal suicidality. The red boxes magnify their typical morphology. (d-g) 3D reconstruction validates process connection/disconnection of microglia phenotypes: (d) ramified microglia; e reactive microglia; (f) microglia with segmented processes and disconnected debris; (g) microglial microspheres and debris. White solid arrows point to the microglial microspheres. White empty arrows point to the connected processes. (h-k) graphically illustrate the measurement of microglial cell body radius and fragment size in a control case (h and j) and a suicidal case (i and k). Quantitative analyses reveal that the density of P2RY12⁺ microglia cell bodies in subjects with MD is lower than in non-psychiatric controls (l). (m and n) P2RY12⁺ microglia in the depressed patients have larger total cell size and cell body radius than the controls, which is a trend of increase in measuring the microglial fragments (o). (q-s and v-x) show that the sole presence of suicidality does not confound the morphological profile of the microglial cell body. (t and y) indicate that microglial fragments in the mEnt are larger in individuals who had fatal suicidality compared to subjects without suicidality or nonfatal suicidality. However, the density of microglial fragments in this region does not change in relation to MD (p), the presence of suicidality (u), or suicidal classifications (z). Scale bar, a 20 μ m; g 5 μ m; i and k 10 μ m.

Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$, *** indicates $0.0001 \leq p < 0.001$.

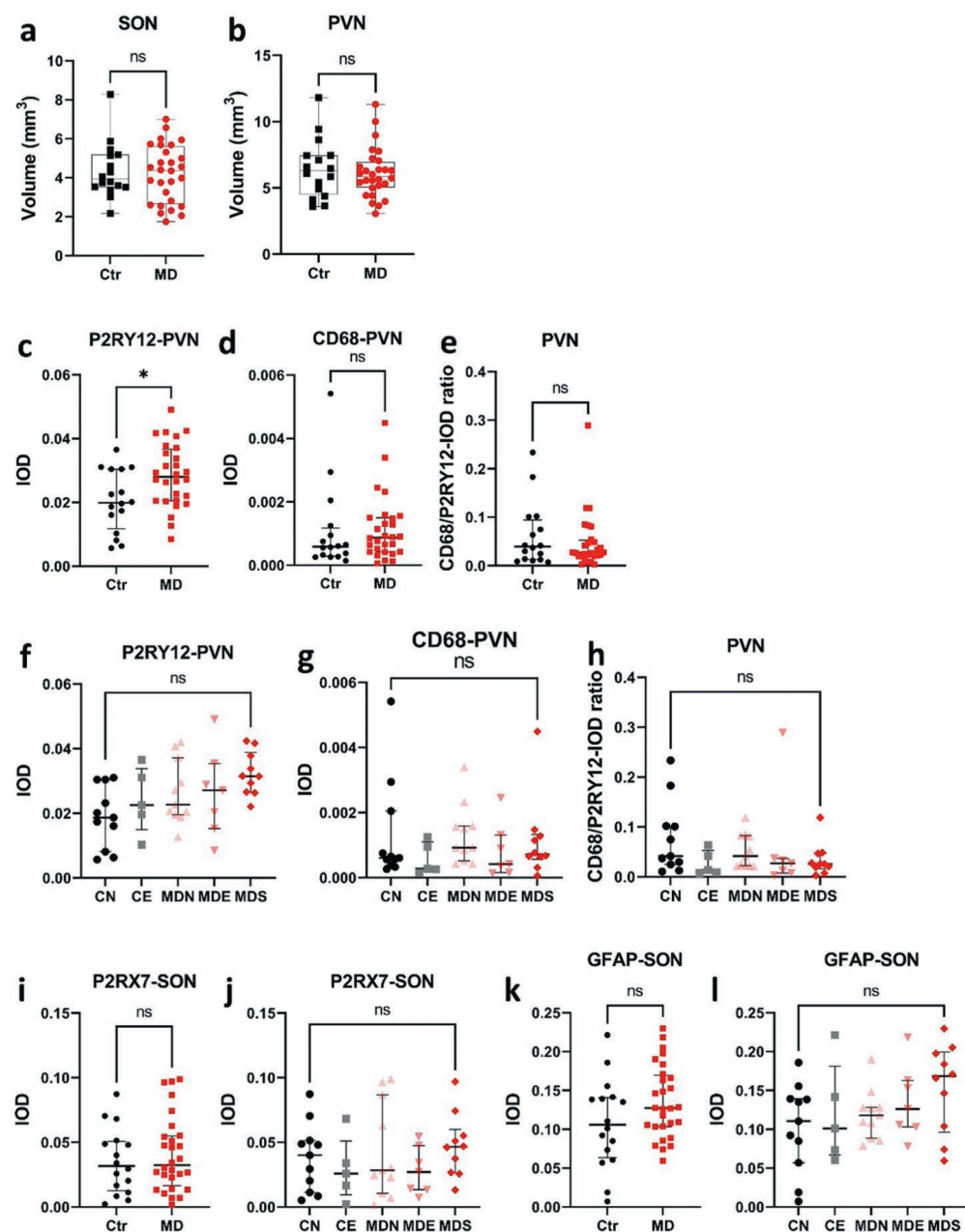


Figure S7. Purinergic receptors and glial expression in stress-reactive hypothalamic nuclei. No difference in the volume of SON (a) or PVN (b) is found between Ctr and MD. (c-h) P2RY12 and CD68 expression in the PVN. (i-l) P2RX7 and GFAP expression in the SON.

Data are presented as medians with interquartile ranges. * indicates $0.01 \leq p < 0.05$.

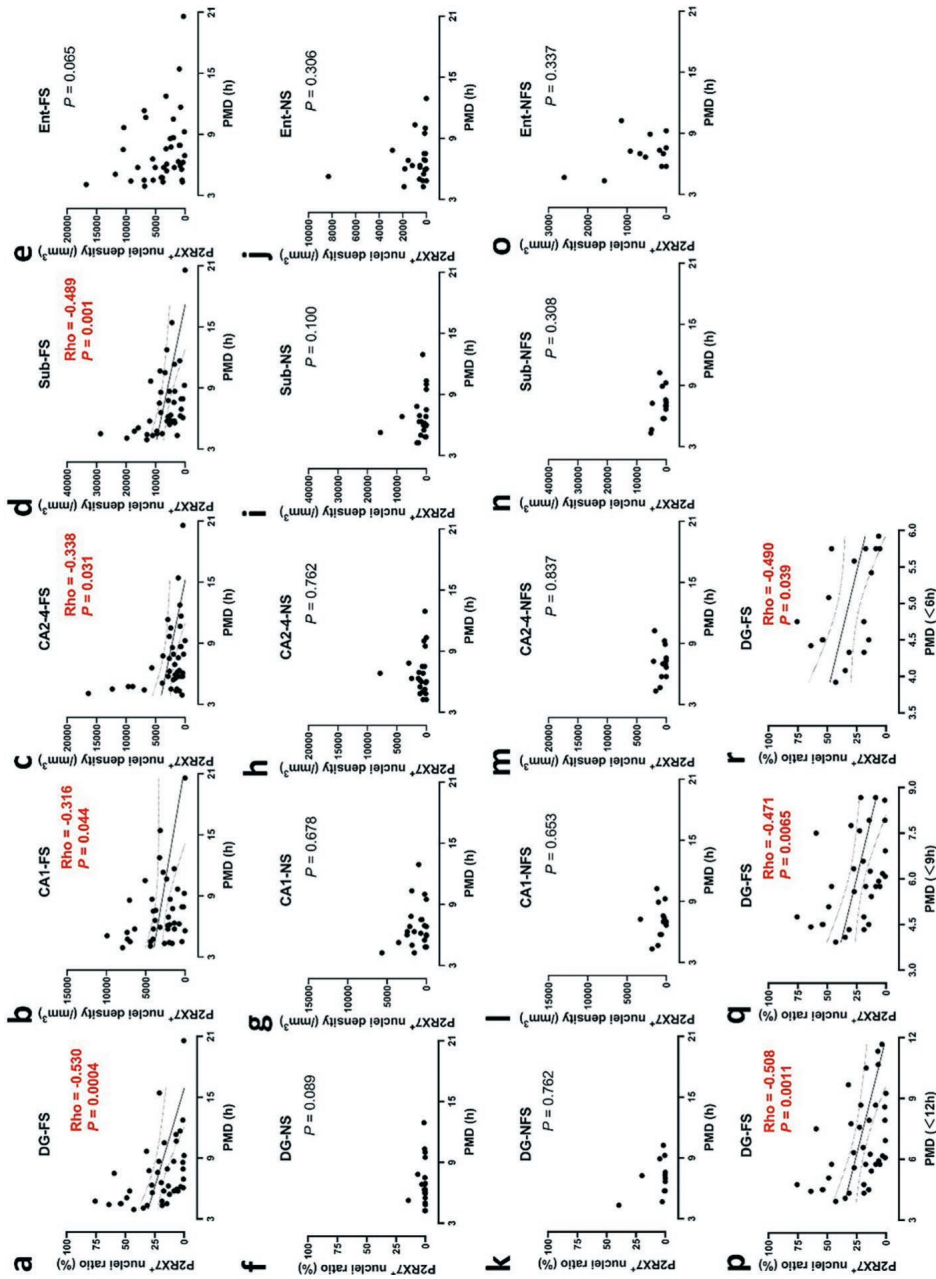


Figure S8. Confounder analysis of PMD. The ratios and densities of P2RX7⁺ nuclei in the hippocampal subareas (DG, CA1, CA2-4 and Sub) of individuals who had fatal suicidality is negatively associated with the PMDs (a-e), which is absent in individuals who did not have suicidality or nonfatal suicidality (f-o). (p-r) In the DG, the negative correlations remain significant when the PMDs are shortened to 12h, 9h, and 6h, respectively.

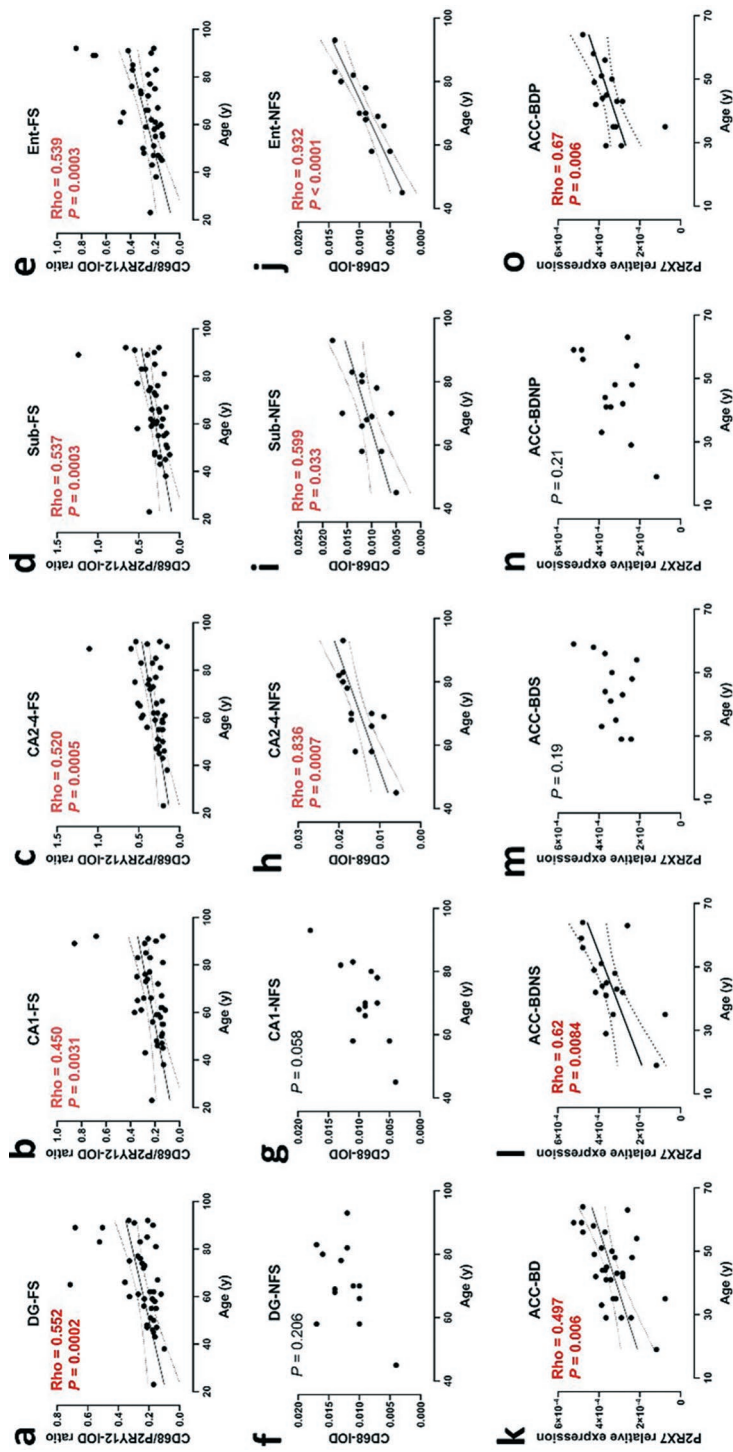


Figure S9. Confounder analysis of age. (a-e) In individuals with fatal suicidality, the ratio of CD68 to P2RY12 throughout the hippocampal formation positively correlates with age. (f-j) In individuals with non-fatal suicidality, CD68 expression in the CA2-4, Sub and Ent shows positive correlations with age. (k-o) P2RX7 mRNA expressed through the ACC of BD positively correlates with age, which is contributed by patients with BD who developed psychotic features.

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CHAPTER 9

LEGAL EUTHANASIA OPENS DOOR TO NEUROBIOLOGICAL DISCOVERIES: ONE STEP CLOSER TO THE SILENCED MAJORITY OF SUICIDE

Zhang L, Denys D, Swaab DF.

Under review.

Suicide is a major global concern causing an estimated 703,000 deaths each year (WHO, 2021), with an additional 1.5 million people attempting suicides (Hoven et al., 2010). Every one in five people has experienced suicidal thoughts during their lifetime (Mind, 2017). In highly developed countries, over 90% of suicide completers had a psychiatric diagnosis during their lifetime (Bertolote and Fleischmann, 2002), which has been ranked the major risk factor for suicide. Clinical practice generally complies with the rule that the effects of remitted psychiatric disorders on suicide risks are largely determined by the co-existing mental disorders (Hoertel et al., 2015). However, diagnostic variability and self-concealment of suicide attempts have largely disrupted suicide prevention through mental care, and thus urge the development of novel therapeutic strategies grounded by suicide neuropathology, which is largely independent of the underlying mental disorders (Hogge and Blankenship, 2020; Jørgensen et al., 2023; Zhao et al., 2019).

Although suicide is a serious mental health issue, a large proportion of total suicides that take place in developing countries are psychiatrically silenced, as the availability of, and access to mental health services in these regions are difficult to reach or religiously, economically, culturally or legally disadvantaged. Compared to high-income countries, risk factors for suicide in many low- or middle-income countries are generally related to a breakdown in the ability to address life stressors, instead of to psychiatric diagnosis. Insufficient psychiatric interventions have attributed to the high rate of suicide deaths in underdeveloped countries. This status quo will probably continue for a long period. Therefore, the key to reducing global suicides, eventually, is to focus research on suicidal individuals who are beyond the reach of psychiatric interventions.

Pathological evidence concerns the study of the nature and therapeutics of suicide. The biological underpinnings of suicide, which are considered to be unaffected by the underlying mental disorders, may provide the opportunity to develop therapies, expanding the scope of anti-suicidal effects. In the studies to date, suicide research is hindered by three factors. Firstly, the attempt of suicide has been molecularly connected to two categories of genes, risk genes that increase the vulnerability to suicidal behaviors, and deterministic genes (pathogenic genes) that are unique for suicidality (**Figure**). So far, suicide has not been widely accepted as an independent disorder. In contrast to non-psychiatric suicide victims, a research cohort for a collection of samples from donors who had psychiatric disorders and died of suicide will probably result in a mixture of risk genes related to pre-existing psychiatric disorders, as well as deterministic genes unique to suicide. The status quo is that the former has received much more attention from researchers than the latter. Secondly, the molecular features underlying suicidal ideations and behaviors are included in samples obtained from suicide completers, ruling out the possibility of addressing the biological characteristics of suicidal ideations solely. Thirdly, it has been reported that some protein and messenger RNA rapidly degrade after death (Nagy et al., 2015; Zhang et al., 2022). In suicide research, particularly in omic studies, the postmortem

delay (PMD), defined as the interval between the time of death and autopsy, may thus be a decisive confounder. In forensic investigations, the PMDs in suicide cases are generally estimated values, concealing two shortcomings in human sample research: first, differentially expressed molecules having existed may have decomposed with prolonged PMDs. Second, correlation and regression analyses between key molecules and estimated PMDs can then not produce accurate information.

In these circumstances, donors who died by legal euthanasia are helping to close the gap between low-income and high-income countries by providing identical biological basis of death ideations. In the Netherlands, legal euthanasia is the practice of intentionally ending a life to eliminate hopeless pain and suffering, which is performed by a doctor at the request of a patient according to a careful and well-controlled procedure. People who died by legal euthanasia or completed suicide achieved the final goal by different approaches but had strong and intense death ideations in common. Bio-samples from legal euthanasia donors have five main strengths for research. Firstly, some donors did not have a mental disorder throughout their lifetime but died by legal euthanasia, which can provide a specific molecular fingerprint of death ideations in non-psychiatric subjects. By comparing such a cohort with those who had a certain psychiatric disorder and died by legal euthanasia, researchers would obtain a molecular map of fatal suicidality exclusively linked to the underlying disorder. Secondly, by integrating these molecular signatures of suicide between different mental disorders, a “commonly shared” suicide targetome can be identified, independent of the psychiatric background (**Figure**). Such a targetome can cover the entire spectrum of molecules unique to suicide and can be used to design drugs for suicidal individuals, especially for those who lack access to, or are unwilling to receive mental health service. Thirdly, by measuring the overlap between the suicide targetome and risk genes in relation to certain psychiatric disorders, or other predisposing conditions – such as family history, early life adversity, personality traits or cognitive impairments, researchers may assess the risk factor for suicide under particular psychiatric circumstances. Fourthly, since the final and fatal self-harm acts belonging to accomplished suicide are absent, legal euthanasia specimens can provide an opportunity to address the neurobiological clues that are merely associated with death ideations, and therefore, may help unravel the therapeutic targets for relieving suicidality before suicidal acts take place. Fifthly, in legal euthanasia cases, the clinically documented time of death reveals a short and precise postmortem delay, which maximizes the detection of suicide biomarkers. By regression analyses, neurobiologists may calculate the gene expression as present *in vivo*.

The participation of legal euthanasia applicants in suicide research has another profound impact. So far, neuroimaging studies are striving to construct the neurocircuits of suicidal ideations (Schmaal et al., 2020), but no research has yet shown a longitudinal follow-up in relation to the consequences of suicidality. By collecting neuroimaging data of subjects who subsequently died by legal euthanasia, one can possibly identify sensitive/responsive brain areas

and their structural and functional connectivity related to fatal suicidal intentions. Molecular studies that follow in these hub areas can provide subsequent evidence-based targets for anti-suicidal therapies.

In combination with well-stratified clinical data and neuroimaging output, neurobiological evidence commonly identified between non-psychiatric euthanasia, psychiatric euthanasia and suicide seem to be a way out to generalize the neuropathology of suicide. Accumulating suicide-specific molecular targets may result in effective and affordable treatments for suicidal individuals in dilemma (Gibbons et al., 2022).

Acknowledgements

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Conflicts of interest

We declare no conflicts of interest.

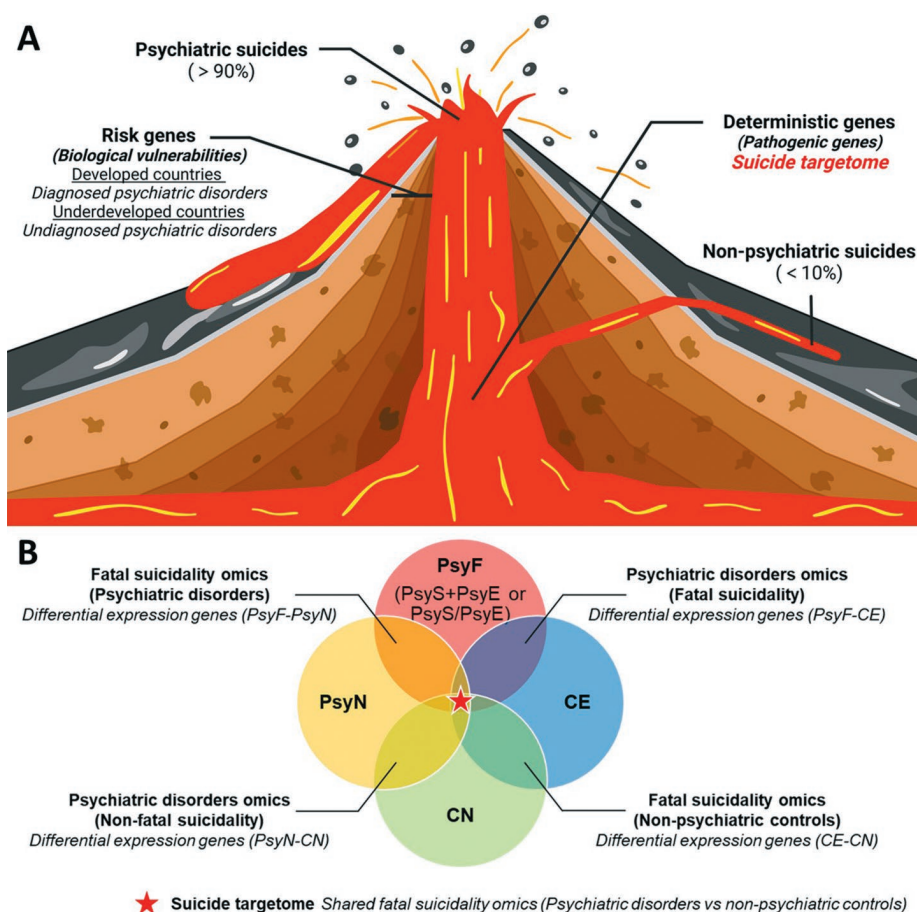


Figure. Graphical metaphor and practical procedures of the suicide targetome. (A) Global suicides are divided into suicides with comorbid psychiatric disorders (over 90%) and non-psychiatric suicides (less than 10%). They have overlapping molecular traits, termed the *suicide targetome*. Although developed and underdeveloped countries have claimed clear differences in suicide risk factors, the lack of mental care in developing countries has probably lowered the detection of psychiatric illnesses. Varied risk factors form the biological vulnerability of suicide, which, however, may not be the key molecules that represent suicidal behaviors but only increase the likelihood of suicide development. (B) By addressing the molecular basis of non-psychiatric suicides and psychiatric suicides respectively, one may obtain suicide-specific gene expression patterns (*suicide targetome*) shared by psychiatric and control cohorts, excluding the genetic vulnerabilities resulting from suicide risk factors.

Abbreviations: CE, control subjects who died of legal euthanasia; CN, control subjects who died of natural causes; PsyE, patients with psychiatric disorders who died of legal euthanasia; PsyF, patients with psychiatric disorders who had fatal suicidality; PsyN, patients with psychiatric disorders who died of natural causes; PsyS, patients with psychiatric disorders who died by suicide.

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CHAPTER 10

GENERAL DISCUSSION

Suicide is conceptualized as having three stages with increasing graveness: suicidal ideations, suicide attempts, and completed suicide. On a range from moderate to severe, the former two can be further divided into a temporary ideation/single attempt and intense ideations/repeated attempts (Klonsky et al., 2016). Suicide and its catalysts, i.e., neuropsychiatric disorders, have a series of symptoms that affect people socially, occupationally, educationally and interpersonally. Recently, pharmacological and physiological studies found that the anti-suicidal effects of ketamine on patients with treatment-resistant depression were associated with acute reductions in the functional connectivity of their prefrontal cortex-related circuits, involving the ACC, DLPFC and superior parietal regions (Chen et al., 2019). Therefore, one of the main goals of neurobiological research in this field is to understand the molecular, cellular, and neurocircuitry alterations that underlie and distinguish the transitions from occasional to relapsing and intense suicidal ideations, and from single to control-losing suicidal behaviors. For many, treatment with medications for opioid use disorder is associated with a substantial reduction in suicide mortality (Watts et al., 2022). The increasing stages of suicidal severity, together with these opioid-based, anti-suicidal effects have prompted the hypothesis that the molecular background of suicide may at least to some extent, overlap with addictive disorders (Williams et al., 2019).

Studies to clarify the neurobiology of suicide have progressed through the research of the human brain, both by antemortem neuroimaging and postmortem neuropathological studies. More specifically, neuroimaging is highly valuable to identify brain regions responsive to stress stimuli, and allow subsequent neurobiological investigations including genetic, epigenetic, transcriptional, translational approaches, and neural networking reconstructions. Understanding the neurobiological mechanisms underlying the two stages of suicidality, suicidal ideations and suicide attempts, can respectively reveal the specific neurocircuits involved in either suicidal thoughts or suicide acts, and can, in turn, provide novel ideas for suicide prevention. Therefore, we first summarize here the neuroimaging data in relation to suicidal ideations and attempts separately, which lays the basis for the rest of our General Discussion.

Neurocircuitry of suicidal ideations

The range of suicidal ideations varies from temporary thoughts to detailed planning. The major risk factors for suicidal ideations consist of several elements, such as psychiatric disorders, adverse life events and family history (Barry, 2019). We focus here on the findings in the human brains specifically associated with suicidal thoughts, irrespective of these underlying factors.

Volumetric measurement is the first step to study the association between the onset of suicide tendencies and vulnerable brain domains. There is a large amount of evidence showing that the cortical circuits are critically involved in suicidal ideations. Compared to non-psychiatric controls, patients with suicidal thoughts display decreased gray matter volumes in the orbital-frontal-precentral-parietal circuits and the insula (Drachman et al., 2022; Segreti et al., 2019; Taylor et al., 2015). Correspondingly, functional constructs implicated in these brain regions,

such as executive control, stress regulation, and emotional processing were also affected. Studies have subsequently shown that the white matter integrity in cortical regions and their connections with subcortical limbic structures, such as the basal ganglia and thalamus, was also decreased in relation to the intensity of suicidal ideations (Davey et al., 2021; Fan et al., 2019; Myung et al., 2016; Taylor et al., 2015), suggesting that the cognitive control over incentive salience and habits might have been compromised (Upadhyay et al., 2022). These findings were specific to the presence of suicidal thoughts, independent of the psychiatric status of the subjects.

In the second place, resting-state functional connectivity (rsFC), in individuals with suicidal ideations, shows decreases in global connectivity across the cortico-striato-thalamic loops (Du et al., 2017; Kim et al., 2017). Of note, although volumetric and structural alterations associated with suicidal ideations have not been reported in the habenula, its connectivity to emotion- and reward-processing circuits (e.g., habenula-precuneus and -inferior frontal gyrus) were found to decrease in patients with suicidal ideations, compared to those without suicidal thoughts (Qiao et al., 2020), with notably, the reduction being positively correlated with the frequency of suicidal thoughts (Wills et al., 2020). However, a significant increase in resting-state involving the connection between the precuneus and amygdala, was found only in females with first-episode MDD and suicidal ideation, suggesting that the gender-biased neural correlates of self-centered mental dominance and aggression might be enhanced in the early stage of suicidality (Cavanna and Trimble, 2006; Wei et al., 2018).

Alterations in task-based functional connectivity of suicide ideators are task-dependent. Under conflict- and self-monitoring conditions, a higher activation occurred in the anterior cingulate cortex (ACC) and related circuitry in individuals at risk for suicidal thoughts, which was strongly associated with their suicidal intensity (Matthews et al., 2012; Minzenberg et al., 2015a; Minzenberg et al., 2016; Minzenberg et al., 2015c). On the other hand, past suicidal ideations were associated with a lower activation in the prefrontal cortex-based circuit under goal-representation demands (Minzenberg et al., 2014).

Neurocircuitry of suicide attempts

Compared to suicidal ideations, volumetric reductions have been found in subcortical regions in individuals with suicidal behaviors. For example, in addition to cortical areas, decreased gray matter thickness has been shown in their functionally connected subcortical areas, such as the amygdala, hippocampus, basal ganglia, and thalamus in people with previous suicide attempts (Giakoumatos et al., 2013; Kang et al., 2020; Wang et al., 2020). Among suicide attempters, a history of high lethality behaviors was associated with extensively diminished gray matter in the major components of the temporal lobe, such as the temporal gyrus, parahippocampal gyrus, and fusiform gyrus, together with adjacent regions, e.g., the inferior frontal orbital gyrus, insula, caudate, lingual gyrus and cuneus (Giakoumatos et al., 2013; Huber et al., 2019; Soloff et al., 2012; Yang et al., 2020).

In patients with suicide attempts, increases in functional connectivity, as determined by resting-state fMRI, involves the activation of a multi-centered orbitofrontal cortex-precuneus-paracingulate reward circuit (Dimick et al., 2022). This psychobiological framework is of special relevance for the suicide-addiction comorbid nature. In non-drug addictions, such as pathological gambling, the activation of the orbitofrontal cortex was involved in craving-induced dopamine release and its D2 receptor-associated reductions in glucose metabolism (Koob and Volkow, 2016), and similar increases hold for the precuneus and paracingulate gyrus (Liu, 2010; Tarumov et al., 2019). Among these three hubs, the orbitofrontal cortex of suicide attempters had a higher connectivity with the dorsomedial prefrontal cortex and middle frontal gyrus compared to non-suicidal individuals (Chen et al., 2021). The connections between the precuneus and the supraorbital middle frontal gyrus and inferior parietal lobe were higher in suicide attempters than non-attempters among non-psychiatric youths (Cao et al., 2015). Moreover, its functional connectivity to the amygdala was higher in mood disorder patients with suicidal behaviors than in those with suicidal thoughts only (Zhang et al., 2021b). In patients with post-traumatic stress disorder, functional connectivity of the paracingulate gyrus to the pars orbitalis positively correlated with suicidality (Barredo et al., 2019). Of note, the neural connectivity of the habenula-parahippocampus circuit in individuals with suicidal behaviors was elevated compared to the healthy controls (Ambrosi et al., 2019). Additionally, the strength of the network between the abovementioned sensory cortex and striatum has been suggested to predict suicide-related behaviors (Marchand et al., 2012).

On the other hand, a prefrontal cortex-centered reduction in connectivity, suggestive of a loss of cognitive control, occurs in emotion-encoding circuits in suicide. For example, MDD patients with a history of suicidal behaviors had diminished functional connectivity between the ventral and medial prefrontal cortex and amygdala, compared to the controls and patients without attempts (Wang et al., 2020). Young suicide attempters showed decreased connectivity of the middle frontal gyrus with the superior parietal gyrus and anterior cingulate cortex compared to non-suicide attempters (Jun et al., 2021).

Task-based functional connectivity studies have demonstrated increases in decision-making and executive functions but decreases in reward-processing and cognitive control in association with suicide acts. During the Iowa Gambling Task that assesses decision-making, adolescents with suicide attempts and depression had a better performance than non-attempters with depression and the controls, involving a neural activation of the hippocampus, frontal and temporal cortical, striatal and thalamic regions (Pan et al., 2013). During Stroop task performance, higher activity was found in the frontal motor cortex to be associated with past suicidal behaviors (Minzenberg et al., 2015b). The activity of the motor cortex with its connection to the striatum is regarded a trait marker of suicide-related behaviors (Marchand et al., 2012).

On the other hand, suicide-attempting adolescents could be distinguished from their non-attempting counterparts by a blunted reward circuitry responsivity in the bilateral caudate

nucleus during negatively valenced self-processing (Quevedo et al., 2022). In contrast, in cognitive control task performance, a history of suicidal behavior was related to lower activity in midline parietal regions, including the precuneus and cuneus (Minzenberg et al., 2015c). During conflict-monitoring tasks, past suicidal behavior was associated with lower conflict-related anterior cingulate cortex, and in connectivity with multiple lateral and medial prefrontal, parietal and temporal cortical regions (Minzenberg et al., 2015a). However, in the ventrolateral and rostrolateral prefrontal cortex, frontal operculum, and insula, past suicidal behavior was associated with higher control-related activation (Minzenberg et al., 2015c). In individuals with long-term suicide risk who performed a goal-representation task, reported suicidal behavior was associated with lower control-related activation in the premotor cortex ipsilateral to the active primary motor cortex (Minzenberg et al., 2014).

Based on the above findings, structural and functional profiles of suicidal ideations and suicide attempts show considerable variance, suggesting that molecular fingerprints of these two processes may also differ. As a result, we carefully grouped the data for analysis in this thesis, with each category having a distinct relevance.

The originality of legal euthanasia brain samples in suicide research

As reviewed above, it is necessary to study suicide through every stage of the entire process. Therefore, a novelty of our current research is the introduction of brain samples from donors who died by legal euthanasia. There are three points of innovation: First, the applicants were driven by intense and persistent suicidal thoughts derived from incurable physical or/and psychiatric diseases. Second, studying the neuroimaging abnormalities involved in the neurocircuits associated with these strong and intense suicidal thoughts can discover hub brain regions with considerable relevance for the vulnerability, maintenance, and relapse associated with fatal suicidality. Therefore, in contrast to non-psychiatric or non-suicidal controls, we propose to investigate the neuroimaging profiles that map the neurocircuits in people with or without a psychiatric disease, who ultimately died by legal euthanasia. With these neural networks, one can observe the hubs and their functional connectivities in relation to strong and intense suicidal tendencies. Third, neuropathological alterations underlying legal euthanasia can imitate completed suicide but provide a short and precise postmortem interval against the postmortem decay of key molecules. In previous chapters, we demonstrated unique molecular features that were related to fatal suicidality by studying hypothalamic and hippocampal samples of individuals who died by legal euthanasia.

Neurobiological mechanisms of suicide

Neuroimaging studies have displayed differences in the pattern of dysfunctional features over the course of suicide processes. The divergent manifestations in structural and functional connectivity were shown in particular brain regions, especially the DLPFC, ACC and hippocampus. Although

these results reflect the differences between suicidal thoughts and suicidal acts, these studies did not provide information concerning the follow-up or prognosis in their analysis. On the other hand, neuropathological studies have so far mostly reported molecular or cellular findings from postmortem tissue of completed suicide cases. These changes can (partly) be independent of the underlying psychiatric disorders and take place in various anatomical structures, as illustrated in this thesis.

First of all, we found that completed suicide went together with distinct transcriptional alterations in the PFC relative to psychiatric disorders. Previous studies in our group have proposed two patterns of suicide-associated alterations in MDD (Zhao et al., 2015; Zhao et al., 2018; Zhao et al., 2016). One showed that suicide-associated alterations were presented while MDD-related changes were absent, compared to non-psychiatric controls. This was for example the case with an increased expression of glutamate- and gamma-aminobutyric acid (GABA)-related genes in the ACC. The other pattern was that alterations of the same genes in association with MDD and suicide, were both present, but they changed in opposite ways, especially in the dorsolateral prefrontal cortex (DLPFC). Interestingly, our former data did not report stress-related genes that showed elevations in suicide in addition to an increase in MDD. This supported the view that the molecular fingerprints of suicide might be distinct from MDD in the prefrontal areas.

In this thesis, and in addition to MDD, bipolar disorder (BD) and schizophrenia (SCZ) were added as conditions to our research cohorts (Zhang et al., 2020a, b; Zhang et al., 2021a). We addressed the expression of glial genes in the PFC and found that the majority of detected genes were not activated in suicide, except for the genes that reflected microglial phagocytosis (*CD68*) and neuronal monitoring (*P2RY12*). In addition, the altered microglia expression of different phenotypes has demonstrated that psychiatric disorders share specific pathogenic mechanisms in the prefrontal cortex, such as dopaminergic activation (*ALDH1L1*) and metabolic dysregulation of glutamate (*GS*) in MDD and SCZ, and synaptic pruning deficiency (*TREM2*) in BD and SCZ.

Second, the human brain samples from the NBB (hypothalami and hippocampi) have allowed us to further analyze the concept of “fatal suicidality” in non-psychiatric controls and MD patients. We, surprisingly, found that the infundibular nucleus (INF) was a hypothalamic nucleus sensitive to suicide and MD in terms of different molecular patterns. Another important finding in these materials was that we confirmed the participation of the endogenous opioid system in suicide. This was shown by the dramatic increase of POMC neurons in the INF of MD patients who had completed suicide. Large sample epidemiological studies have emphasized that repeated suicide attempts, underlying psychiatric disorders and substance use disorders are the highest risk factors for attempting suicide (Fazel and Runeson, 2020). Repeated attempts are critically connected to suicidal fatality. Among the first-time suicide attempters, approximately 30% repeat suicidal behaviors. The proportion of completed suicides in individuals with repeated attempts is 500 to 1000 times higher than in the general public (Harvard, 2021). This repeatability

of suicidal behaviors is the basis for the hypothesis, that a similar mechanism may be involved as present in addictive disorders. Also, medications for opioid use disorder were found associated with a substantial reduction in suicide mortality (Watts et al., 2022). The relationship between fatal suicidality and an activation of the opioid system activation in MD has additionally been confirmed by the increase of a subgroup of POMC-neurons that co-expressed progesterone receptors in the legal euthanasia subset. Epidemiological studies have further shown that the influence of progesterone on neural systems is related to the risk for suicide (Ho et al., 2022). Our data indicate that the POMC neurons may be involved in this mechanism.

Third, with increased sample sizes and detailed psychiatric records, more observations can be performed on the molecular differences between suicidal ideations and suicide attempts, and on suicidal severity (single/repeated attempts). By discussing the hippocampal results in this thesis, we conclude that from non-psychiatric controls to MD and then suicide, there may be a process of cellular homeostasis (in non-psychiatric controls) that changes into an activation (in MD) and subsequently into an overactivation-apoptosis (in suicide). In the actual practice of human postmortem studies in literature, however, results were often derived only from brain samples from donors with MD who died by suicide versus non-psychiatric controls, and the conclusions often seemingly based on the interest of the investigator as being related either to suicide or MD (Zhao et al., 2019). Therefore, our grouping aims to include only one suicide-related variable in every comparison.

In the second review of this thesis, we propose that suicide can be regarded as a cognitive disorder, and suicide-associated cognitive impairments have indeed been reported to be persistent across ages (Zhang et al., 2022). It has been found that the volumetric alteration of the entorhinal cortex was linked to the development of mild cognitive impairment (López et al., 2014). Our current study showed that in the entorhinal cortex of individuals with non-fatal suicidality, a strongly positive correlation was present between age and CD68 expression, indicative of a microglia subset that typically function to clear cellular debris and promote microglial phagocytosis. We, therefore, consider the possibility that compared to individuals without suicidal thoughts, those who had pre-existing suicidality, even though it was not fatal, might present with specific cellular changes in certain brain regions that maintain throughout the lifetime.

In recent years, investigations associated with molecular pathology in suicide mostly focused on the prefrontal cortex, including the DLPFC and ACC, which no doubt are partly because of their involvement in executive functions, especially decision-making and impulse control (Bush et al., 2002; Liu et al., 2012; Philiastides et al., 2011). However, studies aimed to discover the brain areas that respond to the onset and development of suicidal thoughts, especially the thoughts that led to fatal outcomes, have not yet been published. The completion of suicide has a certain degree of randomness, which does not accurately reflect the severity of suicidality when the completers were still alive. Therefore, studies that address biomarkers

in key brain regions of suicidal ideations may identify therapeutic targets for future treatment strategies for suicide individuals before they attempt.

FUTURE RESEARCH

On the basis of the results obtained in this thesis, three projects are formulated for future research.

Endogenous opioid system and suicide

A previous history of suicide attempts, underlying psychiatric disorders, and substance use disorders represent the highest risk factors for subsequent suicide attempts (Fazel and Runeson, 2020). Repeated suicidal behaviors are critically connected to suicidal fatality. The repetition of suicidal behaviors is the basis for the hypothesis, that a similar mechanism may be involved as in relapses of addictive disorders. In addition, treatment with medications for opioid use disorder has been found associated with a substantial reduction in suicide mortality (Watts et al., 2022). Thus, addressing the molecular features of neural networks that specifically respond to suicidality, mental disorders and addiction is imperative for an early prevention of suicide acts. Our next step of work is to profile the expression of key molecules in the endogenous opioid system in suicide-related brain areas and to observe whether the opioid activity is associated with MD, suicide tendencies and accomplished suicide.

The neurobiological independence of suicide

Neurobiological alterations in key brain areas are decisive features that distinguish suicide from comorbid psychiatric disorders. Comparing the molecular fingerprints of suicide with or without underlying psychiatric disorder may therefore provide specific or/and shared treatments for these two psychiatric aspects. Our current findings have identified brain regions that are sensitive to fatal suicidality in both depression patients and non-psychiatric controls, as well as regions that are only sensitive in psychiatric patients. Therefore, the next stage of our research will be to find differences or similarities in suicide-specific changes in these brain regions between patients with psychiatric disorder and controls. We hope that by understanding the neurobiological independence of suicide, we may be able to provide new generation treatments for the suicidal populations with or without psychiatric disorders, and for the suicidal populations with or without psychiatric diagnosis.

Sex differences in bipolar disorder

Gender differences penetrate every aspect of BD, from epidemiology, onset and development, clinical symptoms and cognitive flexibility to neuroimaging and biological data, comorbidity,

treatments, and prognosis. It is a clinical consensus that sex is a major factor in influencing treatment outcomes for BD (Altshuler et al., 2010). However, it is poorly understood which key brain areas show neurobiological evidence of sex differences. To the best of our knowledge, the gender differences we reported in glial expression in the DLPFC disclosed the first human brain region that had clear sexual dimorphisms in BD (Zhang et al., 2020b). In the first review of this thesis, the complex interactions are summarized between gender and clinical manifestations in BD, in as much as they are implicated in the prefrontal regions, as well as in cognitive functions integrated in emotion- and reward-related regulations. In addition, there is substantial overlap in gender-biased alterations of the primary and secondary symptoms of BD, which may share neurobiological portraits and even magnify the differences. As a next step, we will focus on the biological hallmarks that display sex-specific changes in the DLPFC of BD and try to discover new therapeutic targets for BD treatments.

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CHAPTER 11

SUMMARY (SAMENVATTING)

SUMMARY

Suicide is a major health problem as every year, it terminates approximately 700,000 documented human lives, while worldwide, many more people commit suicide. Individuals with a suicidal tendency have a 6 times higher chance to have a psychiatric illness compared to control subjects. The research in this thesis describes molecular alterations in the human brain in relation to suicide and we follow four novel points of view. First, we investigated the molecular signature of suicide, independent of the underlying psychiatric disorder. Second, we demonstrated that suicidality had distinct molecular features from completed suicide. Third, the classifications of suicide (i.e., suicidal ideations, suicide attempts and completed suicide), as well as the consequences of suicidality (non-fatal and fatal), are key topics to consider when studying the pathophysiology of suicide. Fourth, since brain samples with a short postmortem delay enable a better preservation of suicide-specific molecular fingerprints, we studied for the first time the molecular alterations in the brains of legal euthanasia donors from the Netherlands Brain Bank.

In **Chapter 1**, we provide a brief introduction to suicide, and its relation to underlying psychiatric disorders, age, gender, and the current limited efficacy of available treatments. Since the majority of antidepressants are not able to provide sound evidence in reversing suicidal thinking or behaviors, or even may trigger them under some specific circumstances, it is concluded that novel anti-suicide treatments are needed, preferably on the basis of the molecular mechanisms that are specific for suicide rather than for the pre-existing psychiatric disorders. We focused in our research on brain networks that are important for suicide, consisting of the prefrontal cortex (PFC), hypothalamus and hippocampus. Recent neuroimaging evidence has demonstrated that these three regions are involved in mood, impulsivity and predicting the subjective sensation of stress in humans. In the present thesis, we focused on the molecular changes in this potential circuit in relation to suicide. We further highlight the value of introducing brain samples from donors who died by legal euthanasia and review existing knowledge on the brain regions investigated in our studies. Individuals who died of legal euthanasia were subdivided into patients with mood disorders (MD) (to compare differences between suicide behaviors and suicidal thoughts) and controls (without neurological or psychiatric disorders, but who died of non-neuronal diseases such as cancer).

When studying the neurobiological underpinning of major depressive disorder (MDD) and suicide, our previous research had found aberrant expression of genes involved in neuron-glia interactions in the dorsolateral prefrontal cortex (DLPFC) and anterior cingulate cortex (ACC). In **Chapter 2**, we investigated, therefore, the expression of glia-related genes in the DLPFC and ACC in MDD patients in relation to, suicide, and psychosis. We found that changes in gene expression in astrocytes, microglia, and oligodendrocytes in MDD were related to specific brain areas, suicide or psychotic features. It was proposed that these changed glial transcripts may relate to altered neuronal functions, including dopamine and gamma-aminobutyric acid

signaling, blood-brain barrier permeability, innate immune response and neuroinflammation, platelet coagulation, and myelin stability. Of interest, we found that *CD68*, a microglial gene that was functionally related to phagocytosis, was specifically elevated in the ACC of MDD patients who completed suicide.

Patients with bipolar disorder (BD) have higher risks of committing suicide than patients with other psychiatric disorders or physical diseases. Adequate treatments for BD are key to reduce the prevalence of suicide in this disorder. It has been clinical consensus that sex is a non-negligible confounder in designing BD therapies. However, the molecular basis of sex differences in BD was poorly understood. In **Chapter 3**, we compared suicidal and non-suicidal patients with BD and found differences in gene expression in the DLPFC, involving microglial phagocytosis and purinergic homeostasis. In addition, we demonstrated that the DLPFC is, so far, the only area that presented overall sex-biased characteristics in glial gene expression in BD patients. There were 14 glia-related genes that were expressed at significantly higher levels in males versus females, in all three glial cell types, particularly in patients with psychotic features. Therefore, in **Chapter 4**, we reviewed the broader literature for evidence of sex differences of BD in epidemiology, cognition, clinical manifestation, neuroimaging, neurobiological mechanisms, and highlight the DLPFC as a key niche in providing the molecular basis for these differences.

Schizophrenia (SCZ) patients have a higher suicide rate compared to the general population, leading to an average decrease in life expectancy by at least 20 years. In **Chapter 5**, we studied glia-related gene expression in the DLPFC and ACC in relation to SCZ and suicide. We found differential changes in astrocyte and microglia genes in the prefrontal subregions in relation to SCZ and suicide, indicating possible disturbances of glia homeostasis in these conditions. The changes were related to glutamate-dopamine interaction, microglia phagocytosis, and purinergic metabolism. The expression of the purinergic receptor gene *P2RY12* was specifically increased in suicidal versus non-suicidal patients and controls.

Recent epidemiological studies have associated peripheral progesterone levels with the risk of suicide. In **Chapter 6**, we mapped the distribution of progesterone receptors (PR) in the human hypothalamus in relation to age and gender and characterized its (co-)expression in specific cell types. The infundibular nucleus (INF) appeared to be the primary hypothalamic structure via which progesterone modulates stress-related neural circuitry. Subsequently, we determined the relationship between PR co-labeled neurons in the INF and suicide, and we found that a high proportion (approximately 40%) of pro-opiomelanocortin (POMC) neurons that co-expressed PR, was related to accomplished suicide in patients with MD. There was a reciprocal relationship in control subjects between the numbers of PR co-expressing neurons in the INF, and peptidergic neurons (corticotropin-releasing hormone neurons and thyrotropin-releasing hormone neurons) in the paraventricular nucleus. In this study, brain samples from donors who died of legal euthanasia were for the first time embedded in a postmortem study to investigate the molecular signature related to suicidal ideations. The observations in this study indicate

the involvement of the sex hormones and endogenous opioid system in the pathophysiology of suicide.

In **Chapter 7**, we reviewed the current knowledge of molecular neuropathology in the hippocampus in suicide. We discussed the cognitive dysfunctions associated with suicide risk and several leading theories of suicide, including the involvement of the serotonergic system, Wnt pathway and brain-derived neurotrophic factor/tropomyosin receptor kinase B signaling, and we showed evidence for their roles in suicide and treatment. The neurobiological changes exhibited by subjects who completed suicide are generally considered as strong molecular features of their underlying mental illnesses. To further investigate the molecular independence of suicide, we proposed to apply in the future advanced techniques, i.e., single-nucleus RNA sequencing and mass spectrometry histochemistry on hippocampal samples from donors who died by suicide or legal euthanasia. This may lead to further clarifying the etiology of suicide and develop the novel therapeutic strategies.

In **Chapter 8**, we demonstrated that the hippocampus encoded fatal suicidal ideations in the dentate gyrus (DG) by showing a large number of granule cell nuclei with a purinergic receptor gene *P2RX7* expression, notably independent of an underlying MD, i.e., MDD or BD. In comparison to the controls, patients with MD further showed a general microglial dyshomeostasis throughout their hippocampal formation. In addition, *P2RY12*⁺ microglia with segmented processes were remarkably present in the superficial layers of the medial entorhinal cortex, specifically in individuals with fatal suicidality. In the hypothalamic stress-related supraoptic nucleus, *P2RY12*⁺ microglia expression was elevated in MD and even higher when MD was accompanied by fatal suicidality. In the prefrontal cortex, *P2RX7* transcripts sharply dropped in suicidal individuals, which may represent a removal of the prefrontal inhibition on the hippocampus and hypothalamus. Another novel finding was that suicide-specific molecular features faded as the postmortem interval prolonged.

When comparing suicide with its comorbid psychiatric disorders, we could distinguish four types of neurobiological features: First, fatal suicidality had distinct molecular characteristics, which were independent of the psychiatric background. Second, when a neuropsychiatric disorder is comorbid, the suicide/suicidality-associated molecular alterations occurred as signs of disease progression. Third, specific sets of molecules were sensitive to both suicide and psychiatric disorders. Fourth, completed suicide, or the development of suicidality, does not confound the molecular changes linked to comorbid psychiatric disorders. We highlight the significance of the hippocampal dentate gyrus and entorhinal cortex in this respect for future research.

We realize that genetic and epigenic risk factors that provide a vulnerability for specific psychiatric disorders cannot all be seamlessly integrated as therapeutic targets for suicide treatment. Exploring the specific molecular basis of suicide remains the most urgent topic for

research. In **Chapter 9**, we have disclosed the significance of using brain samples from donors who died by legal euthanasia as novel potent research materials in suicide research. These samples can provide the biological signatures of suicide without psychiatric disturbance. Their short and precise PMDs allow the preservation of molecular clues that were ignored in previous studies. Importantly, the major population of suicide, worldwide, often lack mental health care, which has impeded anti-suicidal treatments. Unraveling the molecular basis specific to suicide, therefore, can enable a better therapeutic design for suicidal individuals with or without psychiatric diagnosis.

In conclusion (**Chapter 10**), we propose that specific biological indicators in key brain areas, as described in this thesis, appear strongly involved in, and likely decisive for fatal suicidality. The molecular alterations related to the different stages of suicide may be either independent or (partially) overlapping with the alterations observed in the underlying psychiatric disorders. Following this line of research, we will further dissect the neuropathology of suicide on the basis of its severity and outcome in key regions, and thereby for the first time profile a comprehensive neurobiological landscape that could help provide a basis for novel therapies.

SAMENVATTING

Zelfdoding (suicide) is een wereldwijd gezondheidsprobleem. Het maakt per jaar een einde aan ongeveer 700.000 registreerde mensenlevens, terwijl een veelvoud hiervan een poging tot zelfdoding doen en/of niet geregistreerd worden. Suïcidale neiging komen 6 maal vaker voor bij mensen met een psychiatrische ziekte dan bij controle personen. Het onderzoek in dit proefschrift betreft moleculaire veranderingen bij zelfdoding vanuit vier nieuwe gezichtspunten. Ten eerste onderzochten wij of de moleculaire veranderingen bij suïcide onafhankelijkheid waren van de onderliggende psychiatrische stoornis. Ten tweede toonden wij aan dat suïcidaliteit andere moleculaire kenmerken had dan een voltooide suïcide. Ten derde werd de pathofysiologie van suïcide bestudeerd in relatie tot de verschillende classificaties/fases; suïcidale gedachten, suïcidepogingen en voltooide suïcide, en de gevolgen van suïcidaliteit (niet-dodelijk en dodelijk). Ten vierde bestudeerden wij voor het eerst de moleculaire veranderingen in de hersenen van donoren van de Nederlandse Hersenbank die overleden na legale euthanasie. Hersenmateriaal met een korte postmortem tijd maakt het beter mogelijk om suïcide-specifieke moleculaire veranderingen te kunnen bestuderen.

In **Hoofdstuk 1** hebben we een korte inleiding gegeven over zelfdoding en de relatie met onderliggende psychiatrische stoornissen, leeftijd, geslacht en de beperkte effectiviteit van huidige behandelingsopties. Aangezien de meeste antidepressiva niet in staat zijn om de suïcidale gedachten of gedragingen te keren, en deze in sommige omstandigheden suïcide zelfs kunnen uitlokken, wordt geconcludeerd dat nieuwe behandelingen van suïcide moeten worden gevonden. Idealiter gebeurt dit op basis van de moleculaire mechanismen die specifiek zijn voor suïcide in plaats van voor de onderliggende psychiatrische stoornissen. Wij hebben ons in ons onderzoek gericht op een hersen-netwerk dat belangrijk is voor suïcide en dat bestaat uit de prefrontale cortex (PFC), de hypothalamus en de hippocampus. Recente neuroimaging studies tonen aan dat het neurale netwerk van deze drie regio's een rol spelen bij stemming, impuls controle en de subjectieve sensatie van stress bij mensen voorspelt. In dit proefschrift bestudeerden we de moleculaire veranderingen bij zelfdoding in dit circuit. We benadrukken het belang van hersenweefsel van donoren die stierven door legale euthanasie en bespreken de beschikbare kennis over de hersengebieden die werden onderzocht in onze studies. Personen die stierven door legale euthanasie werden onderverdeeld in patiënten met stemmingsstoornissen (MD) (om de verschillen tussen zelfdodingsgedrag en suïcidale gedachten te vergelijken) en controles (patiënten zonder neurologische of psychiatrische stoornissen die stierven aan lichamelijke ziekten zoals kanker).

Ons eerdere onderzoek heeft zich gericht op de dorsolaterale prefrontale cortex (DLPFC) en de anterieure cingulaire cortex (ACC), hierin vonden we dat de genexpressie van moleculen betrokken bij neuron-glia interacties veranderd was specifiek in relatie tot depressie (MDD) en suïcide. In **Hoofdstuk 2** onderzochten wij daarom de expressie van glia-gerelateerde genen in

de DLPFC en ACC bij MDD-patiënten maar nu in relatie tot suïcide en psychose. Wij vonden veranderingen in de genexpressie van astrocyten, microglia en oligodendrocyten bij MDD, die gerelateerd waren aan specifieke hersengebieden, suïcide en psychotische kenmerken. We stellen voor dat deze veranderde glia transcriptwaarden gerelateerd zijn aan veranderde neuronale functies zoals dopamine en gamma-aminoboterzuur neurotransmissie, doorlaatbaarheid van de bloed-hersenbarrière, schade aan het centrale zenuwstelsel, de immuunrespons en neuro-inflammatie, stolling door bloedplaatjes en myeline stabiliteit. Interessant is dat *CD68*, een microglia-gen betrokken bij fagocytose, specifiek verhoogd was in de ACC van MDD-patiënten na zelfdoding.

Patiënten met een bipolaire stoornis (BD) lopen een groter risico op zelfdoding dan patiënten met andere psychiatrische stoornissen of lichamelijke medische aandoeningen. Adequate behandeling van BD en de suicidaliteit is essentieel om de prevalentie van suïcide bij deze stoornis te verminderen. Er bestaat klinische consensus dat het geslacht van de patiënt een niet te verwaarlozen confounder is bij het zoeken naar BD-therapieën. De moleculaire basis van sekseverschillen bij BD wordt echter slecht begrepen. In **Hoofdstuk 3** vonden wij in de DLPFC verschillen in genexpressie tussen suïcidale en niet-suïcidale patiënten met BD die gerelateerd waren aan microgliale fagocytose en purinerge homeostase. Bovendien toonden wij aan dat de DLPFC tot dusver het enige gebied is dat algemene geslachts-gebonden kenmerken vertoont in glia genexpressie bij BD-patiënten. Veertien glia-gerelateerde genen kwamen hoger tot expressie bij mannen versus vrouwen, en in alle drie de glia celtypes, in het bijzonder bij patiënten met psychotische kenmerken. In **Hoofdstuk 4** geven we een breder overzicht van de sekseverschillen bij BD mbt epidemiologie, cognitie, klinische manifestaties, neuroimaging en neurobiologische mechanismen, en belichten we de rol van moleculaire veranderingen in de DLPFC als belangrijke hub bij de mogelijke basis voor deze verschillen.

In vergelijking met de algemene bevolking, plegen naast BD, ook schizofrenie patiënten (SCZ) vaak suïcide, en hun levensverwachting is dientengevolge minstens 20 jaar lager. In **Hoofdstuk 5** bestudeerden wij glia-gerelateerde genexpressie in de DLPFC en ACC in relatie tot SCZ en suïcide. Wij vonden differentiële veranderingen in astrocyt- en microglia-genen in de prefrontale subregio's in relatie tot SCZ en suïcide, wat wijst op mogelijke verstoringen van de glia homeostase in deze condities. Deze veranderingen waren gerelateerd aan glutamaat-dopamine interactie, microglia fagocytose, en purinerge stofwisseling. Ook hier was de expressie van het purinerge receptorgen *P2RY12* specifiek verhoogd bij suïcidale patiënten in vergelijking met niet-suïcidale patiënten en controles.

Recente epidemiologische studies hebben perifere progesteron niveaus in verband gebracht met het risico op zelfdoding. In **Hoofdstuk 6** bestudeerden we daarom de distributie van progesteron-receptoren (PR) in de menselijke hypothalamus in relatie tot leeftijd en geslacht, en karakteriseerden wij de (co-)expressie ervan in specifieke celtypes. De infundibulaire kern (INF) bleek de primaire hypothalamische structuur te zijn die rijk is aan PR en via welke progesteron

stress-gerelateerde neurale circuits kan moduleren. Vervolgens bepaalden wij de relatie tussen neuronen in de INF die PR tot co-expressie brachten en zelfdoding, en we vonden dat een hoog aandeel (ongeveer 40%) van de pro-opiomelanocortine (POMC) neuronen de PR tot co-expressie brachten bij patiënten met MD na een voltooide zelfdoding. Bij controlepersonen was er een reciproke omgekeerd evenredige relatie tussen neuronen die PR tot co-expressie brachten in de INF en peptiderge neuronen in de paraventriculaire kern. In deze studie werd voor het eerst post-mortem hersenweefsel opgenomen van donoren die door legale euthanasie overleden waren, om de moleculaire veranderingen bij suïcidale gedachten te onderzoeken. De waarnemingen in deze studie wezen op de betrokkenheid van geslachtshormonen en het endogene opioïde systeem bij de pathofysiologie van zelfdoding.

Hoofdstuk 7 geeft een overzicht van de huidige kennis van de moleculaire neuropathologie van de hippocampus bij suïcide. We bespreken de cognitieve stoornissen die in verband worden gebracht met het risico op zelfdoding en verschillende toonaangevende theorieën over zelfdoding, waaronder het serotoninerge systeem, Wnt-signalering en de brain-derived neurotrophic factor/tropomyosin receptor kinase B signalering, en bespreken hun rol bij zelfdoding en behandeling. De neurobiologische veranderingen die zich voordoen bij personen na zelfdoding, worden over het algemeen beschouwd als sterke moleculaire kenmerken van de onderliggende psychiatrische ziekte. Om de moleculaire onafhankelijkheid van zelfdoding verder te onderzoeken stelden wij voor om in de toekomst geavanceerde technieken toe te passen, zoals single-nucleus RNA sequencing en massaspectrometrie histochemie op hippocampaal weefsel van donoren die stierven door zelfdoding of na legale euthanasie, om zo de etiologie en mechanismes van zelfdoding verder te verduidelijken en op termijn nieuwe therapeutische strategieën te kunnen ontwikkelen.

In **Hoofdstuk 8** ontdekten wij dat in de hippocampus van mensen met fatale suïcidale gedachten, de granulaire celkernen van de gyrus dentatus (DG) een sterke toename laten zien van de expressie van het purinerge receptor gen *P2RX7*, onafhankelijk van de onderliggende MD, d.w.z. MDD of BD. In vergelijking met de controles vertoonden patiënten met MD een algemene verstoorde homeostase van microglia genen in de gehele hippocampus. Bovendien waren *P2RY12*⁺ microglia met gesegmenteerde processen opvallend veel aanwezig in de oppervlakkige lagen van de mediale entorhinale cortex bij personen met fatale suïcidaliteit. In de hypothalamie, stressgerelateerde supraoptische kern kwamen *P2RY12*⁺ microglia meer tot expressie bij MD en de expressie werd zelfs hoger wanneer MD gepaard ging met fatale suïcidaliteit. In de prefrontale cortex daalden *P2RX7* transcripten sterk bij suïcidale personen, wat zou kunnen wijzen op een mogelijke remming van de prefrontale inhibitie op de hippocampus en hypothalamus. Een andere nieuwe bevinding was dat suïcide specifieke moleculaire kenmerken minder groot werden naarmate het postmortem interval langer werd.

Er waren verder vier soorten neurobiologische kenmerken als we zelfdoding en co-morbide psychiatrische stoornissen vergelijken: ten eerste heeft fatale suïcidaliteit aparte moleculaire

kenmerken, onafhankelijk van de psychiatrische achtergrond. Ten tweede, wanneer er sprake is van een co-morbide neuropsychiatrische stoornis, worden de suïcide/suïcidaleiteit-geassocieerde moleculaire veranderingen vooral gevonden in relatie tot ziekteprogressie. Ten derde waren er moleculen die veranderd waren voor bij zowel suïcide als bij een psychiatrische stoornis. Ten vierde, voltooide zelfdoding of de ont-wikkeling van suïcidaleiteit zijn geen confounder voor de moleculaire veranderingen bij de co-morbide psychiatrische stoornissen. Wij benadrukken dan ook het belang van de gyrus dentatus van de hippocampus en de entorhinale cortex voor toekomstig onderzoek naar suïcide.

Wij beseffen dat genetische en epigenetische kwetsbaarheden die het risico op psychiatrische stoornissen beïnvloeden, niet direct kunnen worden gebruikt als mogelijke therapeutische doelwitten voor de behandeling van suïcide, omdat dit complex is, en bv ook vroege levenservaringen een rol spelen. Echter, een beter begrip van de moleculaire basis van zelfdoding blijft van urgent belang voor onderzoek. In **Hoofdstuk 9** hebben we de betekenis besproken van het gebruik van hersenmonsters van donoren na legale euthanasie als nieuw, waardevol onderzoeksmateriaal voor onderzoek naar zelfdoding. Dit weefsel kan helpen een moleculaire ‘signatuur’ van zelfdoding per se op te leveren, zonder een verstoring door bv de postmortem tijd of de onderliggende psychiatrische aandoening. Hun korte en nauwkeurige postmortem tijd maakt het mogelijk moleculaire veranderingen te zien die in eerdere studies over het hoofd werden gezien. Nog belangrijker is dat de grote populatie suïcidale mensen wereldwijd vaak geen toegang heeft tot de geestelijke gezondheidszorg, wat anti-suïcidale behandelingen of therapie belemmert. Beter begrip van de specifieke moleculaire basis voor zelfdoding zou op termijn nieuwe therapie mogelijk kunnen maken voor suïcidale personen met of zonder psychiatrische diagnose.

Als conclusie (**Hoofdstuk 10**) stellen wij dat biologische indicatoren in de belang-rijkste hersengebieden die in dit proefschrift zijn beschreven, bepalend lijken te zijn voor het in gang zetten van suïcide. De moleculaire veranderingen, gerelateerd aan de verschillende stadia van suïcide, kunnen onafhankelijk of (gedeeltelijk) overlappend zijn met de veranderingen die veroorzaakt worden door de onderliggende psychiatrische stoornissen. Volgens deze lijn van onderzoek, waarbij de specifieke moleculaire signatuur voor suïcide in kaart gebracht wordt, zullen wij in de toekomst de neuropathologie van suïcide verder kunnen ontleden op basis van de ernst en het resultaat in belangrijke hersengebieden, en zo mogelijk een meer compleet neurobiologisch ‘landschap’ kunnen verkrijgen wat gebruikt kan worden voor het ontwikkelen van nieuwe therapieën om suïcide te voorkomen.

APPENDICES

CURRICULUM VITAE

Lin Zhang has been associated with the neuropathology of suicide since 2010. After she obtained her Bachelor's degree of Medicine in Xiangya School of Medicine (established by Yale Mission in China in 1913), Central South University (China), she was recommended to the Department of Forensic Science for a master study with full scholarship (2010). In 2013, she obtained her Master's degree of Forensic Science and gained her National Scholarship from the Ministry



of Education of China and full scholarship from the Department of Forensic Science, Tongji Medical College (founded by Dr. Erich Paulun as German Medical School in 1907), Huazhong University of Science and Technology (China), where she completed her three-year training as a forensic pathologist and obtained her Doctoral degree of Medicine. From 2010 to 2016, Lin participated in over 500 forensic autopsies, which has inspired her interest to study the pathology of suicide as objective evidence in forensic investigation. At the end of 2016, she moved to Netherlands for her PhD studies in the Netherlands Institute for Neuroscience and Netherlands Brain Bank, worldwide the first human brain bank founded by Prof. dr. Dick F. Swaab, under the supervision of Prof. dr. Dick F. Swaab and Prof. Paul J. Lucassen. Lin is worldwide the first person who clarified the significance of using human bio-samples from donors who died by legal euthanasia in suicide research.

Lin is now living in Amsterdam as a neuroscientist.

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