

Transcutaneous auricular vagal nerve stimulation inhibits P2X7R expression in limbic brain regions and reverses depression-like behavior in Zucker diabetic fatty rats

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Abstract

Background

Previous studies confirmed that Zucker diabetic fatty rats (ZDF, fa/fa) develop type 2 diabetes (T2D) with depression-like behavior innately, and transcutaneous auricular vagal nerve stimulation (taVNS) was found to have anti-diabetic and anti-depressive effect in ZDF rats. However, there is still a lack of molecular-biological evidence that ZDF rats are a good rodent model of depression, and how does taVNS take the anti-depressive effect to the ZDF rats. P2 × 7R, a purinergic receptor most-related to inflammation and depression, is found to be elevated in depressed brains and is gradually considered as a potential therapeutic target for depression.

Methods

We deployed taVNS and transcutaneous none vagal nerve stimulation (tnVNS) to ZDF rats. We applied forced swimming test (FST) to evaluate to the depression-like behavior of the rats. We used Western blot to test the P2 × 7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex of the rats. Furthermore, we used immunohistochemical staining to colocalize the P2 × 7R expressing cells in the ZDF rats' brains.

Results

We found that compared with their lean littermates (ZL rats), naïve ZDF rats developed depression-like behavior innately with elevated P2 × 7R expression in their limbic brain regions (hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex); and taVNS but not tnVNS inhibited the P2 × 7R expression in their limbic brain regions and reversed the depression-like behavior. Moreover, P2 × 7R was found majorly expressing in astrocytes and microglia of ZDF rats.

Conclusions

ZDF rats are a good rodent model of depression, and taVNS plays an anti-depressive effect in ZDF rats by inhibiting glial P2 × 7R expression in their limbic brain regions.

Background

Major depressive disorders (MDD) and type 2 diabetes (T2D) are prevalent comorbid diseases with a high prevalence in the clinical setting [1–3]. An epidemiological article suggests a bi-directional relationship between these two common disorders [4]. Patients with MDD are more likely to have comorbid T2D than the general population [1]. In the meantime, comorbid MDD affects approximately 20–25% of patients with T2D, which is crucially higher than the general population affected by MDD [5–7]. The underlying

mechanism of why T2D induces MDD remains poorly understood. Their possible risk gene pathway and co-shared genetics partially explain the comorbidity [2]. Previous studies confirmed that Zucker diabetic fatty rats (ZDF, fa/fa) develop T2D (hyperinsulinemia and hyperglycemia) innately [8] with depression-like behavior [9–11], which might be a good rodent model of T2D with MDD.

Recent findings gradually revealed that ATP-mediated signaling via the $P2 \times 7$ receptors plays a crucial role in regulating depressive pathologies, such as alterations in cognitive and behavioral functions, neuronal degeneration, and synaptic plasticity [12]. There is a lot of evidence that $P2 \times 7R$ modulates 5-hydroxytryptamine, noradrenaline, glutamate, gamma-aminobutyric acid, and nitric oxide release, which are mechanisms consistently related to depression [13–15]. Also, $P2 \times 7R$ is an essential target in facilitating stress adaptation and supporting potential antidepressant effects through its capability to regulate inflammasome activation [12, 16–18]. The results acquired from animal models have provided valuable information regarding the role of $P2 \times 7R$ in depressive disorders and stress responses. For example, mice exposed to chronic unpredictable mild stress (CUMS) have increased $P2 \times 7R$ expression in the medial prefrontal cortex and the hippocampus [19], and increased hippocampal $P2 \times 7R$ levels of mice disclosed to chronic restraint stress (CRS) were also recorded [20]. Moreover, clemastine [19] and ketamine [20] induces antidepressant-like effect, which was associated with downregulated hippocampal $P2 \times 7R$ expression in the in stressed mice. Thus, it is worth investigating whether the depression-like behavior in ZDF rats is related to increased $P2 \times 7R$ expression in the central nervous system (CNS), which can further prove that whether ZDF rats are an excellent rodent model of depression.

A systematic review stated that antidepressants, such as tricyclic antidepressants and selective serotonin reuptake inhibitors, adversely affect glucose metabolism, which is a risk factor for T2D and impaired glucose regulation [21]. Therefore, it is essential to develop a novel method that can regulate both glucose metabolism and depression safely. Vagal nerve stimulation (VNS) was approved by the FDA for the treatment of chronic or recurring depression in 2005 [22]. And VNS was also thought to be a potential application for diabetes [23]. A recent study found that VNS reduces blood glucose in diabetic rats [24]. VNS is expensive because of the surgical procedure and the implanted regulators. To cut the costs, we developed a new method, called transcutaneous auricular vagal nerve stimulation (taVNS), taking the advantage that the vagal nerve has an afferent branch of projections in the auricular concha and external ear channels [25]. And taVNS deploys comparable efficacy with classic VNS in epilepsy and depression [26, 27].

Either via the nucleus of the solitary tract (NTS) or monosynaptically, the vagal nerve carries visceral and somatic efferents and afferents disseminated throughout the whole brain [28]. There are ascending projections from NTS to the hypothalamus [29], amygdala [30], hippocampus [31], prefrontal cortex [32], and cingulate cortex [32, 33], etc., which are limbic structures deal with emotions [34–36]. It has been proven that VNS or taVNS, which regulates the limbic system, benefit to depression [27, 37].

Diabetes is an inflammatory-related disorder [23]. Meanwhile, inflammation is thought to be one of the significant courses of MDD [4]. Molecular-biological evidence supports a role for inflammation in the

pathogenesis and pathophysiology of each disorder individually [4]. Hence, inflammation is an overlapping course of both diseases [4]. No matter T2D leading to depression or depression leading to T2D [4], VNS or taVNS is beneficial for that [23, 38]. Previously, taVNS was found to have anti-diabetic and anti-depressive effect in ZDF rats, possibly through upregulating the insulin receptor (IR) expression and triggering the melatonin secretion [9, 10]. Since $P2 \times 7R$ has become a potential target in mental disorders, because of its activity in neuroinflammatory procedures, which are remarkably involved in MDD [39–41], it is crucial to investigate whether $P2 \times 7R$ expression in the CNS, especially the limbic brain regions, is included in the anti-depressive effect of taVNS in ZDF rats.

Furthermore, it is still unclear in which kinds of cells $P2 \times 7R$ is expressed. $P2 \times 7R$ was hardly found in neurons [42] and was majorly found in glial cells [43]. It is essential to clear it out in ZDF rats.

In this study, we examined whether ZDF rats are a good model of depression through behavior tests and testing the $P2 \times 7R$ levels in the limbic brain regions, compared with their lean littermates, ZL rats [ZDF (fa/+)]. Meanwhile, we applied taVNS and transcutaneous none vagal nerve stimulation (tnVNS) to the ZDF rats, in order to find whether taVNS can effectively reverse the depression-like behavior of the ZDF rats and whether the altered $P2 \times 7R$ levels in the limbic brain regions play a crucial role in the reversal. Also, we colocalized the $P2 \times 7R$ expressing cells in the brains of ZDF rats.

Methods

Animals

Male ZL [lean littermates, ZDF (fa/+)] rats (n=8), and ZDF [ZDF (fa/fa)] rats (n=30) were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd, which is a joint venture of Charles River Laboratories in China. The rats were all transported to our facility at 5 weeks of age. After arrival, they were all housed in standard conditions (large cages, 4 rats per cage) under artificial 12 h light/dark cycle and at an ambient temperature of $22 \pm 1^\circ\text{C}$, and they were free to get food (Purina #5008) and water and habituated to the experimental environment for 1 week. Every other day, the beddings and cages were changed. The person who conducted the experiments was also the same one taking care of the animal's welfare. There was no prior handling of the rats during the first week after arrival. And the rats got into the experimental process at their 6 weeks of age and were divided into ZL group (n=8), ZDF group (n=8), ZDF + taVNS group (n=8), and ZDF + tnVNS group (n=8) according to their genotype and further handlings. The left ZDF rats (n=6) were raised without handling for the further immunohistochemical staining study to colocalize the expressing cells. The experimental protocol was approved by the Ethics Committee of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China. Animal assays were carried out according to the *Guideline on the Humane Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of the People's Republic of China in 2006.

taVNS and tnVNS administration

Throughout the whole experimental procedure, rats of the ZL group and the ZDF group received no intervention. ZDF + taVNS group received taVNS administration, and ZDF + tnVNS group received tnVNS administration. For taVNS, under 2% isoflurane inhaling anesthesia, we placed two magnetic electrodes (+/-) over the auricular concha of both left ear and right ear, inside and outside of the rats individually so that the electric current can transmit through the skin, including the auricular vagal nerve fibers. For tnVNS, under 2% isoflurane inhaling anesthesia, we placed the two magnetic electrodes (+/-) over the auricular margin of both sides, where no vagal nerve fibers were distributed. To improve electronic conduction, we applied saline between an electrode and the skin. We administered a 30 min taVNS or tnVNS process at an intensity of 2 mA and a frequency of 15 Hz once a day using an electric stimulator (HANS-100). We deployed the procedure in the afternoon between 2-5 pm for consecutive 4 weeks.

Forced swimming test (FST)

Because the pre-tested rats prone to remain still in the official examination, and it will tremendously prolong the immobility time, which blurs the difference among the animal groups, the FST was performed once in this study. It was carried out between 8-11 am on day 36, based on the methods we used in our previous research [9, 10]. Briefly speaking, a rat was put in a transparent plastic tank (35×45×60 cm) for 5 min, which was containing 30-35 cm of water (24±1°C). Videos were recorded during the procedure. We used a stopwatch to record the entire duration of the non-swimming time of the rats in the tank within the 5-min session as an immobility score. And we compared the scores among groups. When no essay was made to flee the tank, the rats were judged to be non-swimming, and the rats were at a floating position (bent forward). After the FST session, the rat was moved out of the tank, dried with a towel, and sent back to their home cage. The videos were watched, and the immobility score was determined by the experimenter who was blind to the group assignation to minimize between-session and between-experimenter divergences.

Western blot

We used Western blot to test the expression of P2X7R in the hypothalamus, amygdala, hippocampus, prefrontal cortex, cingulate cortex. Under 5% isoflurane inhaling anesthesia, rats were decapitated, and the hypothalamus, amygdala, hippocampus, prefrontal cortex, cingulate cortex samples were collected. The tissue was homogenized by a high-speed tissue homogenate at 15000 rpm on ice and then incubated on ice for 20 min. We separated the protein samples on some SDS-PAGE gel, and then we transferred them to polyvinylidene difluoride (PVDF) filters (wet transfer method). We blocked the membranes with 5% milk, and we incubated them at 4°C overnight with a P2X7R antibody [rat monoclonal, 1:100, Santa (Hano43) /Sc-134224]. Then, we incubated the membranes for 1 hr at room temperature with an HRP-conjugated secondary antibody (Santa/Sc-2005, 1:5000). We visualized the blots in ECL solution (Thermo/34080), and we exposed the blots onto hyperfilms (Amersham Biosciences). After that, we incubated the blots in a stripping buffer, and we reprobed the blots with a mouse monoclonal beta Actin antibody (Abcam/ab8226, 1:3000) as the loading control. We used Image

J software (NIH) to measure the gray value, and we normalized them against loading controls. We used one-way ANOVA to compare the differences.

Immunohistochemical staining

We anesthetized the left ZDF rats (n=6) with sodium pentobarbital, and then we perfused transcardially with saline (200 ml) followed by 4% paraformaldehyde in 0.1 M PB (300ml, cold). We dissected the brain sections from Bregma -1.4 to -4 [44]. And then we posteriorly fixed them for 2 hours. We kept them in 30% sucrose in 0.1 M PB till they reached the bottom. Subsequently, we the tissues in an OCT compound, and we froze them on dry ice.

We cut the brain sections (30 μ m) on a cryostat and mounted them on microscope slides. And then, we stored them at -80 °C. We used immunohistochemical staining to detect P2X7R (rabbit polyclonal, 1:1000; Abcam, Cambridge, MA), GFAP (astrocyte marker, chicken polyclonal, 1:1000; Abcam, Cambridge, MA), IBA-1 (microglia marker, goats polyclonal, 1:1000; Abcam, Cambridge, MA) and NeuN (neuronal marker, rabbit monoclonal, 1:1000; Abcam, Cambridge, MA). We blocked the sections at room temperature with 1% goat serum in 0.3% Triton for 1 hour, and then we incubated them with the primary antibody overnight at 4 °C. For controls, a primary antibody was omitted. We incubated the sections at room temperature with corresponding FITC- or CY3-conjugated secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 hr. We randomly selected 4-6 non-adjacent brain sections. We used a LEXT OLS4000 3D laser measuring microscope (Olympus) to analyze, a digital camera to record, and the Adobe Photoshop software to process.

Timeline

All the time points of all the above experimental procedures are uniformly shown in Figure 1.

Statistical analysis

GraphPad Prism 6 was used to analyze the data and presented as mean \pm SD. One-way ANOVA was used to judge the differences. $P<0.05$ was identified as statistical significance between groups.

Results

Naïve ZDF rats develop depression-like behavior innately, and taVNS is anti-depressive to ZDF rats.

The FST carried out on day 36 in this study is for judging the depression-like behavior and the anti-depressive effect of taVNS. As shown in Figure 2, the naïve ZDF rats performed badly in the plastic tank with an immobility time of 90.38 \pm 9.96s (mean \pm SD, n=8), which was much longer than the immobility time of the ZL rats (41.00 \pm 12.08s, n=8, $P<0.05$). The result is consistent with the previous studies [9, 10], which shows that naïve ZDF rats develop depression-like behavior innately compared with their lean littermates (ZL rats).

Compared with that of the naïve ZDF rats, ZDF rats treated with taVNS displayed significantly shorter immobility time ($P<0.05$); however, ZDF rats treated with tnVNS display no statistical significance ($P>0.05$) compared with the naïve ZDF rats. This result demonstrated that taVNS but not tnVNS has an anti-depressive effect on the ZDF rats.

P2X7R expression in limbic brain regions of naïve ZDF rats and the effect of taVNS on the expression.

P2X7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex was detected by Western blots (Figure 3). Compared with those of the ZL rats, P2X7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex of the naïve ZDF rats were much higher ($P<0.05$). Compared with those of the naïve ZDF rats, taVNS significantly inhibited the P2X7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex ($P<0.05$). However, the P2X7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex of tnVNS treated ZDF rats displayed no statistical significance compared with those of the naïve ZDF rats ($P>0.05$).

P2X7R colocalized with GFAP and IBA-1

In double-labeling immunofluorescence brain sections, the P2X7R was colocalized with majorly GFAP (Figure 4, a-d) and slightly IBA-1 (Figure 4, e-h), but not NeuN (Figure 4, i-l), indicating that P2X7R-immunopositive cells in the brains of ZDF rats are glial in characteristics.

Discussion

In the previous studies, ZDF rats were reported to develop not merely diabetes but also depression-like behavior innately and that taVNS simultaneously prevent progression of hyperglycemia and depression-like behavior in ZDF rats [9, 10]. It is confirmed that ZDF rats are an excellent rodent model of T2D. However, there is still a lack of molecular-biological evidence of whether ZDF rats are a good rodent model of depression.

Mounting evidence indicates that depression is associated with inflammatory alteration in emotion-related brain-subregions, such as the hippocampus and prefrontal cortex [19, 45, 46], and inhibition of neuroinflammation deploys anti-depressant effect [47, 48]. Both human and preclinical studies support P2 × 7R-mediated effects, which primarily through activation of neuroinflammatory responses, and have been remarkably involved in depression [12]. And P2 × 7R was reported as new signaling of depression intervention in mice [49]. The characteristic of T2D, an inflammation-related disease that quickly leads to neuroinflammation, inspired us to hypothesize that the depression-like behavior of ZDF rats is related to the elevated P2 × 7R expression in the limbic system, which is the part of the brain involved in behavioral and emotional responses. And we majorly focus on the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex this time. The results of this study demonstrated that the hypothesis is correct. Compared with the ZL rats, the naïve ZDF rats have longer immobility time in FST,

and much higher P2 × 7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex, which further proved that ZDF rats are a characteristic rodent model of depression.

Comorbid diabetes and depression are a significant challenge clinically, as the outcomes of both diseases are worsened by the other [50]. The more substantial problem is that most antidepressants affect metabolism seriously, which will exacerbate the diabetes condition [51, 52]. Novel treatments, which can be beneficial to both diseases, is urgently needed to develop. The previous studies show the potential of taVNS [9, 10, 24, 27]. By reducing sympathetic tone or increasing parasympathetic activity, taVNS elicits the anti-depressive effect; in turn, the anti-depressive efficacy of taVNS helps to evoke the anti-diabetic impact [10]. A more in-depth insight into the anti-depressive effect of taVNS might be accounted to its anti-inflammatory effect [53]. P2 × 7R, a purinergic receptor, which marks the degree of neuroinflammation, is crucial to the depression-like behavior in rodents and thought to be a new therapeutic target of treating depression [45, 54, 55]. The results of this study demonstrated that taVNS inhibited P2 × 7R expression in the hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex of the ZDF rats significantly; as a result, the neuroinflammation in these brain regions was suppressed, which leads to the anti-depressive effect in ZDF rats. On the other hand, tnVNS, which no vagal nerve was stimulated, failed to deploy such efficacy in ZDF rats. It should be mentioned that taVNS is multi-targeting, and P2 × 7R should not be the only target of its anti-depressive effect. Nevertheless, decreased P2 × 7R expression in the limbic brain regions is crucial to the anti-depressive effect of taVNS in ZDF rats.

Some studies questioned the expression of P2 × 7R in neurons [42, 56]. And it has been confirmed that P2 × 7R expressed in glial cells [43]. Also, it has been found that functional P2 × 7R expressed in astrocytes but not neurons [57]. In this study, we detected P2 × 7R majorly showed in astrocytes and slightly showed in microglial cells, but not in neurons of ZDF rats. This indicated that P2 × 7R expressing cells are glial in characteristics in ZDF rats. Other types of glial cells, such as ependymal cells and oligodendrocytes, might be considered in the future.

We tried to deploy taVNS to conscious rats in our pre-test. However, it failed. It seems that only big animals [58] or human [27] can take the taVNS without anesthesia for now. A more advanced rodent taVNS equipment need to be developed to elucidate the influence of anesthesia in the future. Another limitation of this study is that we only focused on the limbic brain regions (hypothalamus, amygdala, hippocampus, prefrontal cortex, cingulate cortex). The P2 × 7R is distributed in multiple brain regions [43, 59, 60]. Other emotion-related brain-subregions, such as orbitofrontal cortex, insula, serotonergic dorsal raphe, and dopaminergic ventral tegmentum, etc., need to be considered in the future. Moreover, we only tested one frequency (15 Hz) this time. More frequencies and intensities need to be tested in further studies to confirm that whether the anti-depressive effect of taVNS is frequency-dependent or intensity-dependent. Also, due to the original design, we did not test whether the blockade of P2 × 7R signaling prevents the anti-depressive effect of taVNS. Future studies need to discriminate against them.

Taken together, taVNS inhibits P2 × 7R expression in limbic brain regions of ZDF rats, which in turn will suppress the neuroinflammation and play an anti-depressive effect to ZDF rats. And P2 × 7R was found majorly in the glial cells of ZDF rats.

Conclusion

In conclusion, ZDF rats are a good rodent model of depression with elevated P2 × 7R expression in the limbic brain regions, and that taVNS plays an anti-depressive effect crucially by inhibiting P2 × 7R expression in limbic brain regions of ZDF rats. Moreover, P2 × 7R majorly expressed in the glial cells of ZDF rats.

Abbreviations

ZDF rats: Zucker diabetic fatty rats; ZL rats: lean littermates of ZDF rats; T2D: type 2 diabetes; MDD: major depressive disorders; taVNS: transcutaneous auricular vagal nerve stimulation; tnVNS: transcutaneous none vagal nerve stimulation; CUMS: chronic unpredictable mild stress; CRS: chronic restraint stress; CNS: the central nervous system; VNS: vagal nerve stimulation; NTS: the nucleus of the solitary tract;

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China. Animal assays were carried out according to the *Guideline on the Humane Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of the People's Republic of China in 2006.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author's Contribution

YY and PR designed the experiments. YY, XH, and YW performed the experiments. YY, CT, and JZ analyzed the data. YY drafted the manuscript. All the authors discussed the results, reviewed the final manuscript, and approved it for the publication.

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Figures

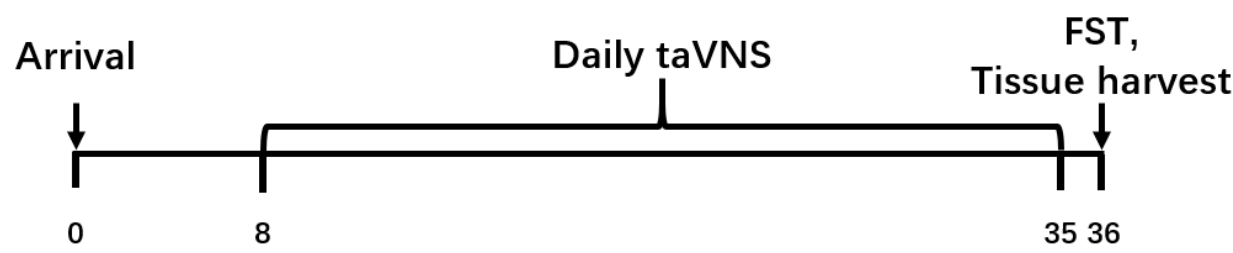


Figure 1

Illustration of experimental time in days. Showing time points for animal arrival, daily taVNS, FST, and tissue harvest. The daily taVNS administration began one week after arrival (on day 8) and continued for 4 weeks (to day 35). On day 36, FST were done in the morning (8-11 am), and tissue harvest were done in the afternoon (2-5 pm).

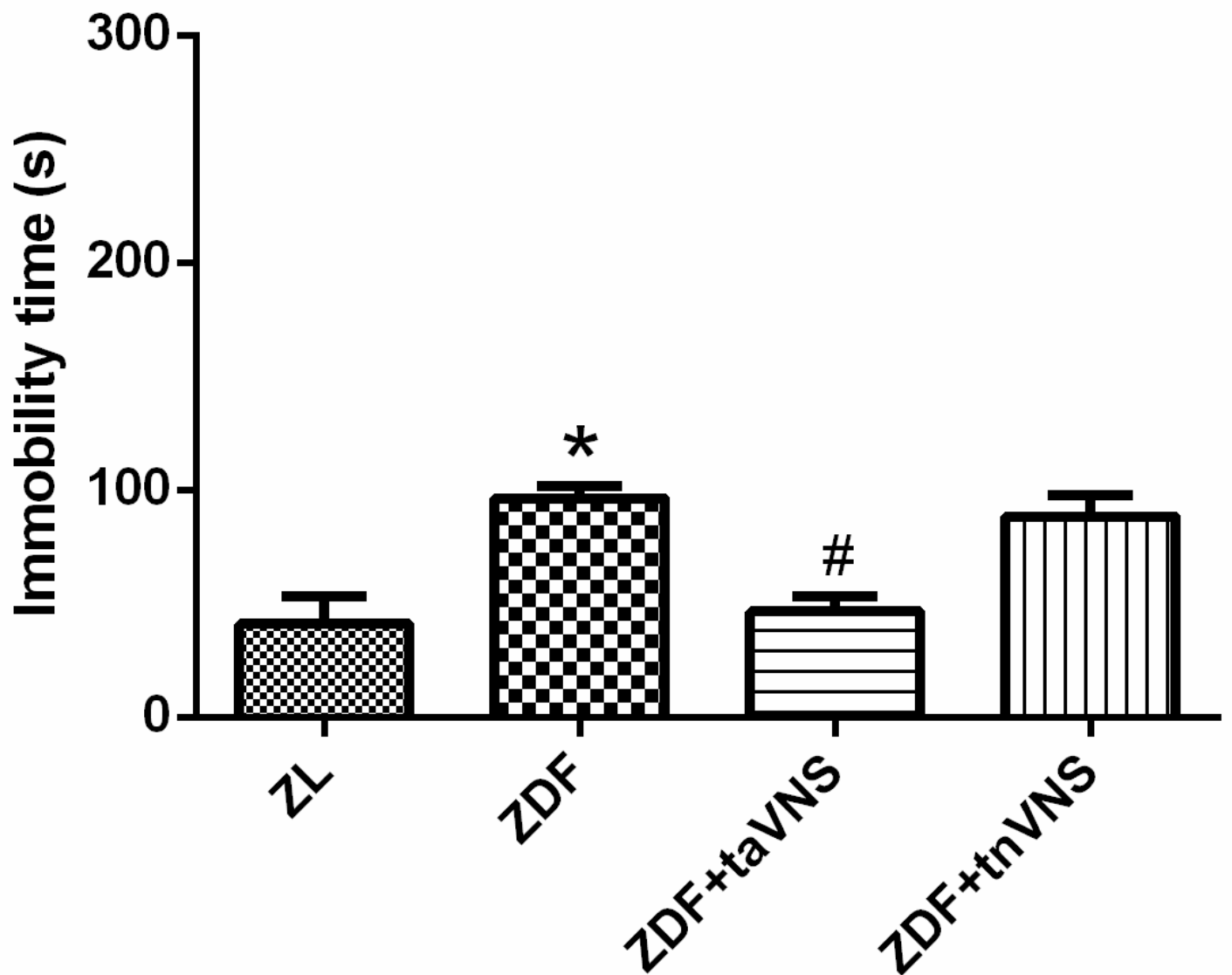


Figure 2

Immobility time in FST on day 36. In the forced swimming session, compared with the ZL rats, a longer immobility time exists in naïve ZDF rats. Compared with the naïve ZDF rats, the taVNS treated ZDF rats display significantly shorter immobility time. *, $P < 0.05$ ZDF vs. ZL; #, $P < 0.05$ ZDF+taVNS or ZDF+tnVNS vs. naïve ZDF.

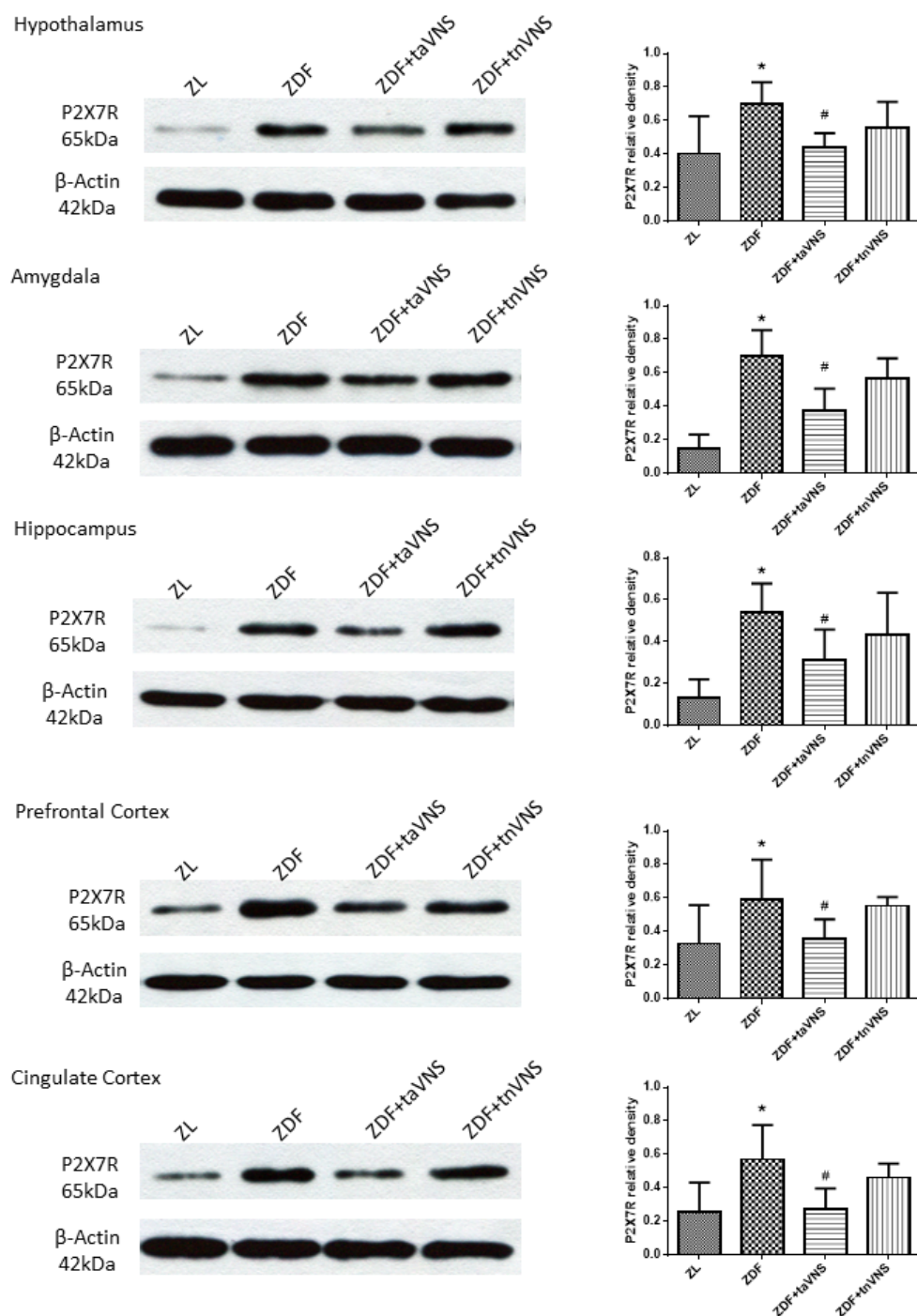


Figure 3

Expression of P2X7R in limbic brain regions of ZDF rats. Western blot results showing the expression of P2X7R in limbic brain regions (hypothalamus, amygdala, hippocampus, prefrontal cortex, and cingulate cortex) of ZL rats, naïve ZDF rats, ZDF rats treated with taVNS or ZDF rats treated with tnVNS for 4 consecutive weeks. The ZL group, the ZDF group, the ZDF+taVNS group, the ZDF+tnVNS group, n=8 each group, *, $P < 0.05$, ZDF vs. ZL; #, $P < 0.05$ ZDF+taVNS or ZDF+tnVNS vs. naïve ZDF.

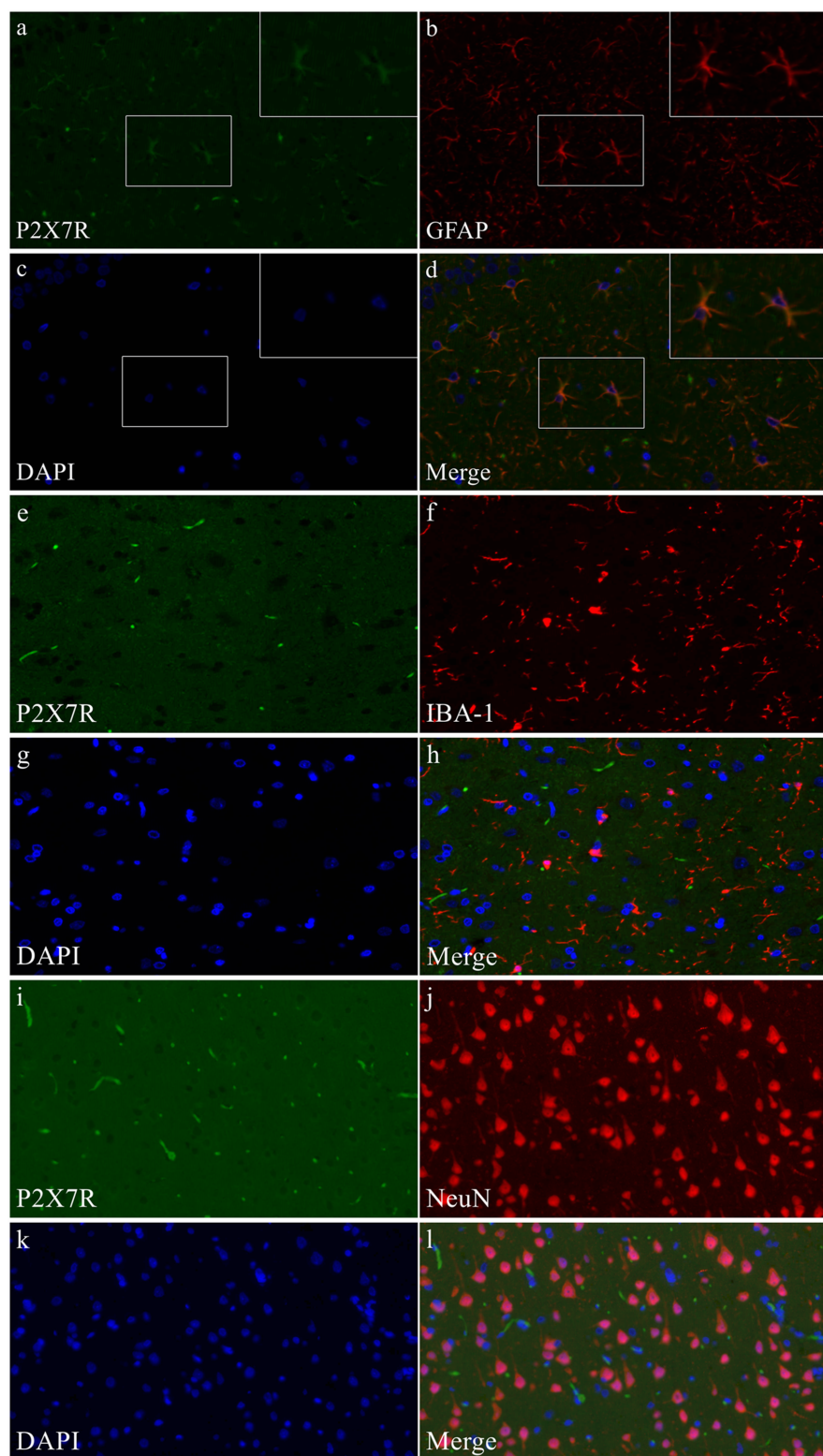


Figure 4

Neurochemical characteristics of P2X7R positive cells in the brain. Double-labeling immunofluorescence results showing that P2X7R is colocalized with majorly GFAP (a-d) and slightly IBA-1 (e-h) but not NeuN (i-l).

Supplementary Files

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