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Effects of vortioxetine on biomarkers associated with glutamatergic activity in an SSRI insensitive model of depression in female rats

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ABSTRACT

The aim of this study was to investigate the antidepressant activity of vortioxetine in a tryptophan (TRP) depletion female rat model of depression and compare it to that of paroxetine using doses that fully occupy the serotonin transporter (SERT). We evaluated the effects of vortioxetine on potential biomarkers associated with TRP depletion including serum aldosterone, corticosterone and IL-6 levels together with indirect indicators of glutamate neurotransmission. Female Sprague-Dawley rats were randomized to control, low TRP, low TRP/paroxetine or low TRP/vortioxetine groups. Vortioxetine and paroxetine were administered via diet (10 mg/kg/day) and drinking water (10 mg/kg/day) respectively for 14 days. Vortioxetine but not paroxetine reversed TRP depletion-induced depressive-like behavior. Vortioxetine reduced TRP depletion-induced increases of serum corticosterone, aldosterone, IL-6 and *N*-methyl-D-aspartate and α7-nicotinic acetylcholine receptor expression in the amygdala and hippocampus, respectively. Paroxetine demonstrated little effect except a reduction of aldosterone. Vortioxetine but not paroxetine reversed TRP depletion-induced reductions of serum and brain kynurenic acid. In conclusion, vortioxetine, but not paroxetine, enabled reversals of TRP depletion-induced changes of depression-like behavior and markers of glutamatergic activity. These observations support the hypothesis that vortioxetine's antidepressant activity may involve mechanisms beyond SERT inhibition.

1. Introduction

Major depressive disorder occurs two-threefold more frequently in women than men and non-responsiveness to treatment presents a huge problem for its management. Clinical data suggests that 50% of depressed patients respond to initial treatment with serotonin (5-HT) specific reuptake inhibitors (SSRIs) and only around 30% achieve remission (Gaynes, 2009). Thus, new treatments with novel modes of action are required. To this point, animal models of depression that address SSRI insensitivity would be of interest to study new compounds. The development of these models is in itself problematic because the effectiveness of antidepressants constitutes an important component of the model predictive validation process. We have validated a model of depression based on diet-induced tryptophan (TRP) depletion in male rats (Franklin et al., 2012a) and thereafter developed a similar model in female rats (Franklin et al., 2015). Diet-induced TRP depletion changes in female rats were generally larger and occurred earlier than in the males. Numerous defining time-course studies in these models demonstrated that 14 days of TRP depletion produced optimal effects on both biochemical and behavioral indices. Beyond this time point the model fails to demonstrate good construct validity due largely to the occurrence of adaptive changes in animals following extended TRP depletion (Franklin et al., 1996).

Diet-induced TRP depletion in female rats resulted in a significant reduction of brain 5-HT and induction of depression-like behavior (Franklin et al., 2015). The depression-like state was associated with an increase in the stress hormone aldosterone, which has a potential relationship to depression (Hlavacova et al., 2012; Häfner et al., 2013; Buttner et al., 2015). In the female rat model of depression (Franklin et al., 2015), aldosterone secretion increased after just 4 days of TRP depletion, prior to the rise in serum corticosterone. TRP depletion led to cortical 5-HT_{2A} receptor upregulation and paroxetine treatment normalized this effect. However, the TRP depletion-induced behavioral changes were not reversed by paroxetine thus suggesting a non-5-HT mediated resistance (Franklin et al., 2015). Changes in the kynurenine (K)/kynurenic acid (KA) ratio, magnesium and *N*-methyl-D-aspartate

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(NMDA) receptor expression were not reversed by paroxetine treatment and therefore the K pathway and glutamate neurotransmission might be involved in the mechanisms of the development of non-responsiveness to SSRIs in this model (Franklin et al., 2015). It should be noted that similar effects in male rats were reversed by paroxetine treatment (Franklin et al. 2012a).

The involvement of the glutamate system in mood disorders was first proposed based on preclinical studies of NMDA receptor antagonists (Skolnick et al., 1996). In addition, early clinical studies showed altered glutamate levels in serum and cerebrospinal fluid from patients with mood disorders (Kim et al., 1982; Sanacora et al., 2004). There is also growing evidence that inflammation is associated with dysregulation of the kynurenine pathway in suicide patients which may be the result of an imbalance of neuroactive metabolites such as quinolinic acid and KA found in these patients (Bryleva and Brundin, 2017).

Pro-inflammatory cytokines, such as interleukin-6 (IL-6), were described as predictive biomarkers of treatment resistant depression (Yang et al., 2015) and depressed patients often demonstrate increased levels of pro-inflammatory cytokines (Howren et al., 2009). In the TRP/K pathway, K is the product of the oxidative opening of the TRP indole ring by 2,3-dioxygenase (IDO) and its hepatic component tryptophan 2,3-dioxygenase (TDO) (Schwarcz and Pellicciari, 2002; Dantzer and Walker, 2014). There is a degree of acceptance that the K/TRP ratio may be a proxy measure of in vivo IDO activity (Christmas et al., 2011). Hence, increases in the ratio are reflective of enzyme induction and a decrease is indicative of inhibition.

Vortioxetine is a new antidepressant that inhibits the 5-HT transporter (SERT) and is a 5-HT_{1A} receptor agonist, 5-HT_{1B} receptor partial agonist and a 5-HT_{1D}, 5-HT_3 and 5-HT_7 receptor antagonist and demonstrates cognition enhancing properties (Mork et al., 2013; Sanchez et al., 2015). The aim of the present studies was to investigate the antidepressant activity of vortioxetine in female rats depleted of TRP and compare it to that of paroxetine using doses that fully occupy the serotonin transporter (SERT) (Li et al., 2013; Leiser et al., 2015). Moreover, we evaluated the effects of vortioxetine on potential biomarkers associated with TRP depletion including serum aldosterone, corticosterone and IL-6 levels, and indirect indicators of abnormal glutamate neurotransmission, e.g. serum and brain levels of KA, an antagonist at both NMDA and α7-nicotinic acetylcholine receptors (α7-nAchR).

2. Methods

2.1. Animals

Forty-eight female Sprague-Dawley rats aged 10 weeks (200–225 g) on arrival were used in this study (VELAZ s.r.o., Brno, Czech Republic). Animals were acclimatized to housing facilities for 8 days prior to testing. They were kept in a temperature controlled room (22 °C ± 2 °C) under a 12:12 h light/dark cycle (lights on at 06:00 a.m.). Animals were grouped 2 per cage with free access to food and water and were weighed daily. All animal procedures were performed in the morning and were approved by the Animal Health and Animal Welfare Division of State Veterinary and Food Administration of the Slovak Republic and conformed to the NIH Guidelines for Care and Use of Laboratory Animals as for previous studies with vortioxetine.

2.2. Model of sub-chronic TRP depletion

Rats were fed a low TRP (0.04%) containing diet or a control diet (0.2% TRP) (Scientific Diet Supplies Ltd., Witham, Essex, UK) for 14 days (Franklin et al. 2015). The experimental diets were in powder form rather than the more standard pellet form. We have used this diet form in all of our previous studies. Therefore, for habituation purposes all animals were put on the control diet for at least 5 days prior to commencement of experimental procedures. Food intake was measured daily for the individual cages and the average mean intake/rat was calculated. The body weights were measured weekly.

2.3. Drugs

For 5 days before drug treatments began, we monitored daily food and water consumption and determined that rats consumed, on average, 22 g of food and 30 ml of water per day. Based on this estimation, paroxetine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was dissolved in drinking water at a concentration of 1 mg/10 ml to approach the target dose of 10 mg/kg/day (Franklin et al., 2012a, 2015). The paroxetine solutions were renewed every 2 days. Vortioxetine hydrobromide was supplied by Lundbeck Research USA (Paramus, NJ, USA). Vortioxetine was mixed into the low TRP diet at a concentration of 0.76 g of vortioxetine hydrobromide (corresponding to 0.6 of vortioxetine base per 1 kg of powdered low TRP diet to approach the target dose of 10 mg/kg/day. This dose was shown to fully occupy SERT (Li et al., 2013).

2.4. Study design

Animals were fed a control or low TRP diet and simultaneously treated with vortioxetine or paroxetine for 14 days. According to diet and treatment, animals were randomly assigned into the following groups (n = 12) and fed: 1) control diet, 2) low TRP diet, 3) low TRP diet plus paroxetine or 4) low TRP diet plus vortioxetine. Effects on procedures carried out in TRP depleted female rats may be influenced by the time of their estrous cycle (Jans et al., 2007). Pro-estrus/estrus events tend to be dictated by lighting times, but under normal lighting schedules (as in the present study) tend to occur during the late afternoon to early hours of the morning (Witcher and Freeman, 1985). Hence, all animal procedures were performed in the morning (usually 08.00–11.00 h) to reduce estrous cycle effects. Serum estradiol concentrations were measured to take account of possible differences in the estrous cycle.

On treatment days 13 and 14 rats were tested in the forced swim test (FST), as described previously (Franklin et al., 2015). After the first 15-min session (pre-test, day 13) rats were removed from water, dried with towels and returned to their home cages. Twenty-four hours later rats were subjected to the 5-min test session (test day 14), dried with towels and returned to their home cages. Both swimming sessions were conducted during the light phase i.e. between 09:30 and 10:30 a.m., and were videotaped by a camera positioned in front of the water tanks. To avoid any possible effects of the FST on stress hormone measures, animals were sacrificed 24 h after the FST. The experimenters were blind with respect to the treatment conditions of the animals tested.

Measures of brain SERT occupancy for both paroxetine and vortioxetine treatments were included in the study design since it is a more direct measure of dose equivalence between drugs than serum concentrations, which is the measure we had used in our previous studies (Franklin et al., 2012a, 2015).

2.5. Blood and organ collection

Following the end of procedures rats were quickly moved to an adjacent room and immediately killed by decapitation. The 2 rats from the same cage were killed within 30 s. Trunk blood was collected into polyethylene tubes without anticoagulant. The clotted blood was spun at 3000 rpm for 15 min at 4 °C and the serum was separated. The brain was quickly removed from the skull. The prefrontal cortex, hippocampus and amygdala were dissected out on an ice-cold plate and frozen in liquid nitrogen. Tissues were weighed and placed into plastic cryo-tubes. Serum aliquots and brain tissue samples were placed in storage at − 25 °C and − 80 °C respectively until required for analysis.

2.6. Biochemical measures

Serum and prefrontal cortex samples for TRP determination were initially deproteinised with 8% trichloroacetic acid and then measured by high performance liquid chromatography (HPLC) with fluorescence (excitation and emission wavelengths at 285 and 335 nm respectively). Both inter- and intra-assay coefficients of variation (CV) were < 8% (Franklin and Odontiadis, 2003). Prefrontal cortex 5-HT levels were measured by HPLC with amperometric detection following homogenization of brain tissue and deproteinization via filtration (molecular weight cut-off 8000 Da) (Chi et al., 1999). Serum large neutral amino acids (LNAAs) were measured by gradient HPLC via an adaptation of a method described by Fekkes et al. (1995), which utilized fluorescence end-point detection. Samples were initially deproteinised by filtration similar to that described for 5-HT. The intra- and inter-assay CVs were maximally 4.7% and 8.9%, respectively, across the range of amino acids measured. Serum K and KA, as well as KA in the prefrontal cortex were measured by HPLC with UV (wavelength 200 nm) and fluorescence (excitation and emission wavelengths were 365 and 480 nm, respectively) end-point detection after deproteinization by filtration (as for 5-HT).

Serum corticosterone was measured by double-antibody radioimmunoassay (MP Biomedicals, Orangeburg, USA). Normal range was 20–70 ng/ml serum. Both inter- and intra-assay CVs were < 5%. Serum aldosterone was analyzed by a coated-tube radioimmunoassay (Riazenco, Angleu, Belgium). The intra- and inter-assay CVs were 3.8% and 6.2%, respectively and the detection limit was 1.4 pg/ml. Serum estradiol was measured by an in-house highly specific double antibody radioimmunoassay which utilized ^{125}I-estradiol. The limit of detection was 2.3 pg/ml when 50 µl of serum was dispensed and the CVs for both intra- and inter-assay were < 5%, respectively.

SERT occupancy was determined by ex vivo brain slice autoradiography using [^{3}H]-escitalopram (Jensen et al., 2014). ^{3}H-MK-801, NMDA receptor binding parameters (β_{max} and K_d (affinity) were measured by an adaptation of a method previously described (Franklin et al., 2012a). α7-nAchR content in the hippocampus was measured by an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., Houston, Texas, USA). The assay sensitivity was 0.13 ng/ml and the intra- and inter-assay CVs were 10 and 12%, respectively.

2.7. Statistics

Data were checked for normality of distribution by the Shapiro-Wilk test. Non-normally distributed data were subjected to natural log (Ln) transformation prior to statistical analyses. Data were analyzed by one-way Analysis of Variance (ANOVA) and followed by the Tukey post hoc test when appropriate. Results are expressed as untransformed mean ± SEM values. Overall level of significance was defined as $p < 0.05$.

3. Results

A significant main effect was observed for serum total TRP ($F = 52.4$; df = 3,36; $p < 0.001$), serum free TRP ($F = 33$; df = 3,36; $p < 0.001$), serum total TRP/LNAA ratio ($F = 32.7$; df = 3,36; $p < 0.001$), prefrontal cortex TRP ($F = 65.9$; df = 3,36; $p < 0.001$) and 5-HT ($F = 40.9$; df = 3,36; $p < 0.001$) respectively. Post hoc analysis showed that these parameters were significantly reduced in all groups of TRP-depleted animals without effect of drug treatment (Table 1).

One-way ANOVA showed a significant difference between groups in serum corticosterone ($F = 5.7$; df = 3,38; $p = 0.002$) and aldosterone ($F = 3.6$; df = 3,37; $p = 0.02$) concentrations. Tukey post hoc comparisons showed that serum corticosterone concentrations were significantly increased in TRP-depleted rats compared to controls ($p = 0.01$). Vortioxetine ($p = 0.05$) but not paroxetine significantly reduced the TRP depletion-induced rise in corticosterone (Fig. 1A). Serum aldosterone concentrations were significantly increased in TRP depleted rats compared to controls. Both vortioxetine ($p = 0.01$) and paroxetine ($p = 0.03$) significantly decreased the rise in aldosterone concentrations induced by TRP depletion (Fig. 1B). No significant changes were observed in serum estradiol concentrations ($F = 0.96$; df = 3,37; $p = 0.42$).

Serum K was similar across groups (Fig. 2A). Statistical analysis of serum KA data showed a significant main effect of group ($F = 7.2$; df = 3,38; $p < 0.001$). TRP depletion resulted in decreased KA concentrations compared to controls ($p < 0.001$). Vortioxetine significantly ($p = 0.03$) increased TRP depletion-induced KA reductions (Fig. 2B) whereas paroxetine showed no effect. The K/TRP ratio (Fig. 2C) showed a significant effect of group ($F = 12.9$; df = 3,37; $p < 0.0001$). TRP depletion resulted in a significantly enhanced K/TRP ratio ($p < 0.01$). Neither vortioxetine nor paroxetine affected the TRP depletion-induced increase of the K/TRP ratio to a statistically significant degree. The K/KA ratio showed a significant effect of group ($F = 3.0$; df = 3,38; $p = 0.04$). Post hoc comparisons showed that TRP depletion ($p = 0.03$) and TRP depletion plus paroxetine groups ($p = 0.007$) showed a significant increase in the K/KA ratio compared to controls, whereas those from the TRP depletion plus vortioxetine group were similar to controls (Fig. 2D). One-way ANOVA showed a significant difference between groups (Fig. 2E) in prefrontal cortex KA concentrations ($F = 10.2$; df = 3,36; $p = 0.0001$). Prefrontal cortex KA concentrations were significantly decreased both in the low TRP ($p < 0.0001$) and low TRP + paroxetine ($p < 0.001$) groups compared to control. Vortioxetine reversed the TRP depletion-induced reduction of KA ($p = 0.02$ vs. TRP depleted group).

The serum concentrations of the inflammatory cytokine, IL-6, were significantly different between groups ($F = 21.8$; df = 3,37; $p = 0.005$). Vortioxetine ($p = 0.01$) significantly reduced the rise of IL-6 levels induced by TRP depletion ($p < 0.001$) whereas paroxetine did not (Fig. 2F).

Analysis of NMDA receptor [3H-MK-801] binding B_{max} data in the amygdala (Fig. 3A) showed significant differences ($F = 12.6$; df = 3,37; $p < 0.01$). Treatment with vortioxetine significantly reduced the TRP depletion-induced increase of NMDA receptor B_{max} ($p = 0.02$) whilst paroxetine showed no effect. α7-nAchR content in the hippocampus demonstrated a significant effect ($F = 7.1$; df = 3,28; $p < 0.001$) of group (Fig. 3B). Vortioxetine significantly lowered the TRP depletion-induced increase in α7-nAchR content ($p = 0.01$) whereas paroxetine showed no effect.

Statistical analysis of data from the FST showed a significant effect of group on immobility time ($F = 5.2$; df = 3,36; $p = 0.004$). TRP-depleted rats spent significantly longer time immobile compared to controls ($p = 0.04$). Paroxetine reduced immobility to control levels although this was not significant and may be due to a greater variance in this data group. Vortioxetine ($p = 0.004$) significantly reversed these changes (Fig. 4A). The latency to immobility was numerically increased by vortioxetine treatment (Fig. 4B), however the difference was not statistically significant ($F = 2.6$; df = 3,35; $p = 0.07$). There were no differences in climbing and swimming across the groups (data not shown).

SERT was fully occupied (> 90%) following both paroxetine and vortioxetine treatments (Table 1). Daily food intake was similar across the groups (control = 23.04 ± 0.86, low-TRP = 22.45 ± 0.42, low TRP + parox = 21.42 ± 0.84 and low TRP + vortiox = 22.69 ± 0.42 g/rat/day). There were no differences in daily water intake across the groups (control = 26.74 ± 0.73, low-TRP = 29.16 ± 1.17, low TRP + parox = 30.3 ± 0.1.36 and low TRP + vortiox = 27.75 ± 1.42 ml/rat/day). Body weight gain did not differ among the groups (control = 32 ± 1.81, low-TRP = 32.71 ± 2.79, low TRP + parox = 26.83 ± 1.87 and low TRP + vortiox = 30.41 ± 3.53 g).

Table 1
Effect of vortioxetine and paroxetine on serum total TRP, free TRP, LNAA, TRP/LNAA ratio, brain pre-frontal cortex TRP, brain pre-frontal cortex 5-HT and % SERT occupancy in TRP depleted female rats versus control. Results are expressed as means ± SEM (n = 10–12).

	Control	Low TRP	Low TRP + parox	Low TRP + vortiox
Serum total TRP (μg/ml)	26.2 ± 2.9	13.3 ± 3.2***	12.5 ± 3.1***	15.7 ± 3.7***
Serum free TRP (μg/ml)	3.7 ± 0.5	2.1 ± 0.5***	2.1 ± 0.5***	1.8 ± 0.4***
Serum LNAA (nmol/ml)	378 ± 38	362 ± 43	364 ± 32	376 ± 63
Serum TRP/LNAA ratio	0.035 ± 0.005	0.018 ± 0.005***	0.016 ± 0.005***	0.016 ± 0.006***
Prefrontal cortex TRP (μg/wet wt)	4.9 ± 0.6	2.3 ± 0.3***	2.2 ± 0.5***	2.5 ± 0.4***
Prefrontal cortex 5-HT (μg/wet wt)	0.8 ± 0.09	0.47 ± 0.14***	0.41 ± 0.07***	0.45 ± 0.08***
% SERT occupancy	2.7 ± 2.9	1.4 ± 2.0	90.0 ± 1.1***	95.1 ± 0.8***

Statistics: ***$p < 0.001$, **$p < 0.01$ versus control, *$p < 0.05$ versus control.

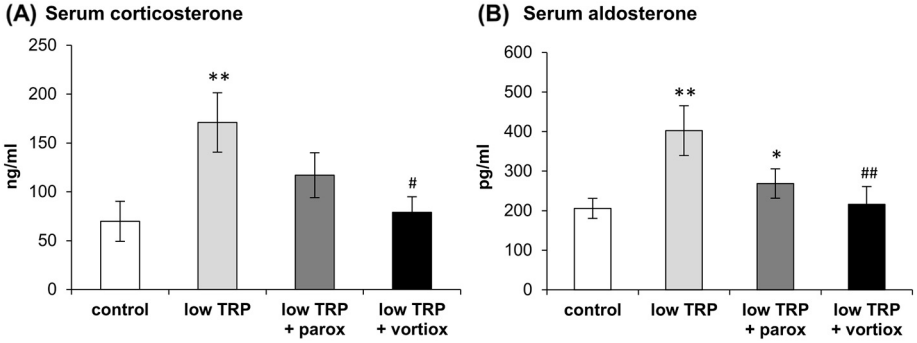


Fig. 1. Effect of vortioxetine and paroxetine on serum corticosterone (A) and aldosterone (B) in TRP depleted female rats versus control. Results are expressed as means ± SEM (n = 10–12). Statistics: **$p < 0.01$ versus control, *$p < 0.05$ versus low TRP, ##$p < 0.01$ versus low TRP.

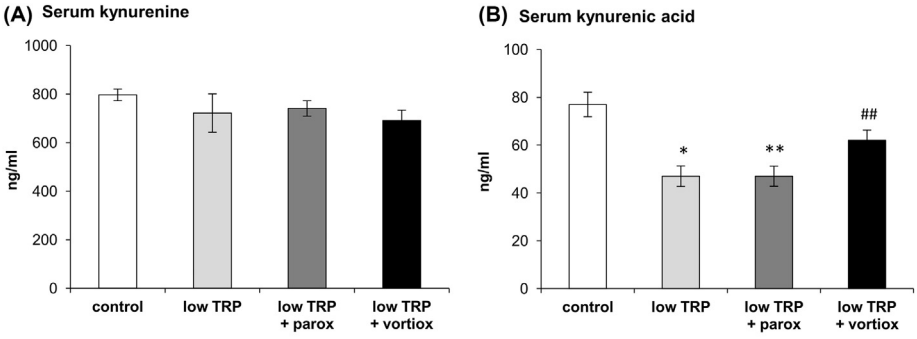
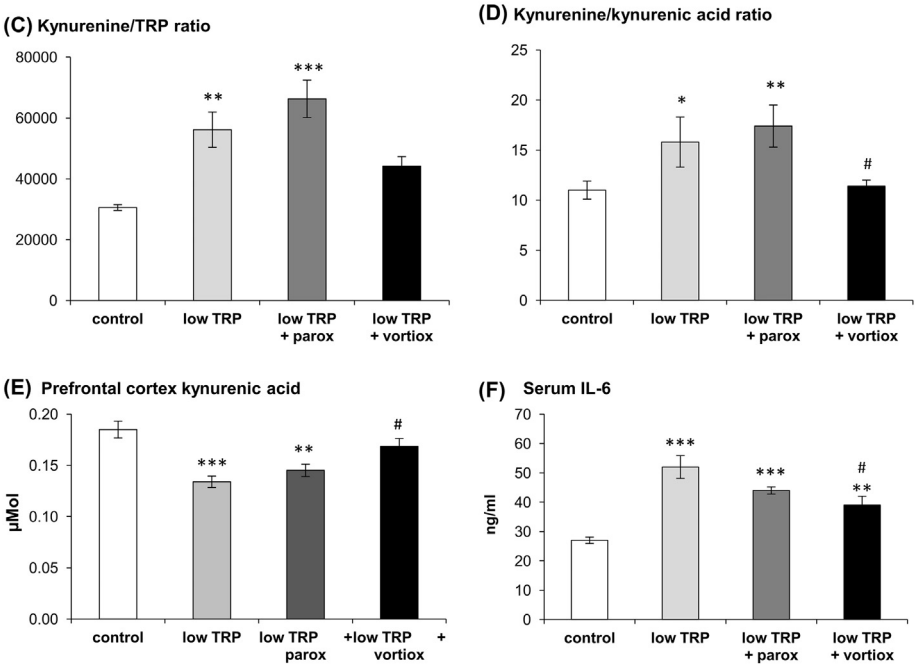


Fig. 2. Effect of vortioxetine and paroxetine on serum K (A), serum KA (B), serum K/TRP ratio (C), serum K/KA ratio (D), prefrontal cortex KA (E) and serum IL-6 (F) in TRP depleted female rats versus control. Results are expressed as means ± SEM (n = 10–12). Statistics: **$p < 0.01$, *$p < 0.05$ versus control, ##$p < 0.01$, #$p < 0.05$ versus low TRP.



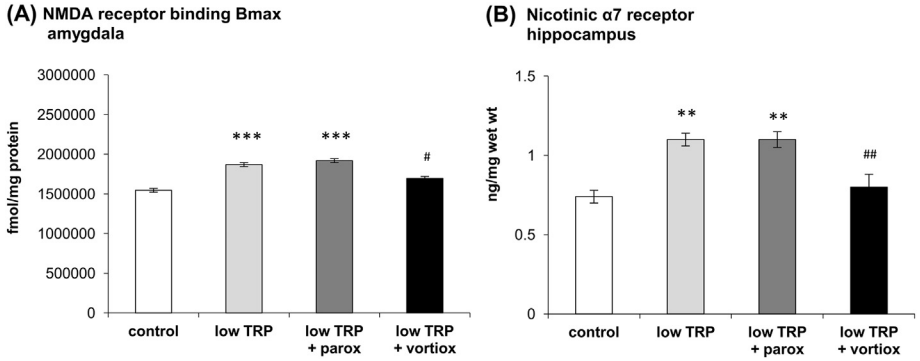


Fig. 3. Effect of vortioxetine and paroxetine on NMDA receptor B_{max} in the amygdala (A) and α7-nAchR content in the hippocampus (B) in TRP depleted female rats versus control. Results are expressed as means ± SEM (n = 10–12). Statistics: $^{***}p < 0.001$ versus control, $^{**}p < 0.01$ versus low TRP, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ versus low TRP.

4. Discussion

In the present study vortioxetine demonstrated antidepressant-like effects in a female rat TRP depletion model of depression. Vortioxetine generally reversed biochemical changes induced by TRP depletion, whereas paroxetine did not.

The SERT occupancy was > 90% for both vortioxetine and paroxetine. This confirms a very adequate take up of both treatments through their various intake routes (Li et al., 2013; Leiser et al., 2015). At this level of SERT occupancy vortioxetine is predicted to fully occupy 5-HT_3 and 5-HT_{1B} receptors and to occupy 5-HT_7 and 5-HT_{1A} receptors at functionally relevant levels (i.e. approximately 25–35% receptor occupancy; Leiser et al., 2015). Thus all vortioxetine's therapeutic targets may potentially contribute to the net effects observed in the present study.

The rise in serum concentrations of aldosterone observed in TRP-depleted animals is consistent with our previous findings (Franklin et al., 2012a,b, 2015). Both vortioxetine and paroxetine treatments significantly reduced serum aldosterone concentrations in TRP-depleted animals.

Serum corticosterone was increased in TRP-depleted animals as compared to controls in agreement with previous findings (Franklin et al., 2012a, 2015). Further, in the current study, paroxetine treatment reduced corticosterone by around 25% whereas vortioxetine almost reduced it to control levels. In our previous studies paroxetine reduced corticosterone by approximately 30%, however these corticosterone levels were still more than two-fold greater than those found in controls (Franklin et al., 2015), thus suggesting that it may be necessary to near normalize corticosterone levels in order to achieve reversal of depression-like behavior in this model. Interestingly, mirtazapine was found to reduce plasma cortisol in both healthy subjects and patients, possibly through blockade of 5-HT_3 receptors and α_2 adrenoceptors. Vortioxetine itself is a potent 5-HT_3 receptor antagonist (Sanchez et al., 2015). Furthermore, the selective 5-HT_3 receptor antagonist, ondansetron reduced hyperactivity in obese mice by reducing corticosterone (Kurhe and Mahesh, 2015). Hence, it is possible that vortioxetine's 5-HT_3 receptor antagonistic properties may contribute to reducing corticosterone levels in the present study. Further studies would be required to investigate this hypothesis.

Prefrontal cortex KA content decreased in a similar manner as serum KA levels in TRP depleted animals compared to control animals. Additionally, vortioxetine treatment significantly enhanced TRP-depletion mediated reductions of KA whereas paroxetine treatment did not. Interestingly, TRP depletion increased serum levels of IL-6 and this was subsequently significantly reduced by vortioxetine but not by paroxetine treatment. It has been hypothesized that chronic stress-induced activation of the immune system may lead to increased levels of pro-inflammatory cytokines such as IL-6 and subsequent development of major depressive disorder (Won and Kim, 2016). Pro-inflammatory cytokines provoke activation of the HPA axis to increase the synthesis of corticosteroids (Franklin et al., 2015) and also activate the TRP/K pathway enzymes, TDO and IDO (Leonard, 2017; Savitz, 2017; Ball et al., 2014). This has been suggested to play an important role in the etiology of depression (Christmas et al., 2011; Myint and Kim, 2014). Over activity of these enzymes can lead to an imbalance of downstream pathway metabolites such as KA, which is neuroprotective in nature (Erhardt et al., 2009).

In our studies the K/KA ratio has consistently proved to be a more sensitive measure than the more regularly used K/TRP ratio in the kynurenine pathway as a measure of neurotoxicity flux in terms of downstream effects on glutamate transmission change. Our results (from the present and in previously published and unpublished studies) demonstrate only small changes in serum K concentrations and hence smaller changes in the K/TRP ratio may only truly reflect changes in total TRP. Whereas, KA is very consistently reduced by TRP depletion. Therefore, this may suggest that the enzymes which convert K to KA, i.e. the aminotransferases may be in themselves more susceptible to induction than TDO. This hypothesis requires further investigation. In the present study K/TRP ratio was enhanced in TRP depleted animals and may be indicative of TDO induction. The peripheral K/KA ratio was decreased by vortioxetine treatment to control levels but this was again not the case with paroxetine. Therefore, we suggest that via the outlined inhibition processes on TDO activity, the neuroprotective potential of KA was increased by vortioxetine in this animal model of depression. Whether this was achieved by a direct effect of vortioxetine on IL-6 itself or on TDO is debatable. Since PFC KA content reflect on

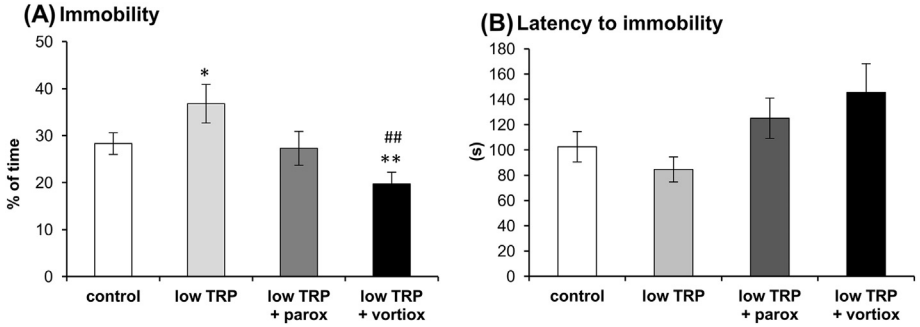


Fig. 4. Effect of vortioxetine and paroxetine on immobility (A) and the latency to immobility (B) in the FST in TRP depleted female rats versus control. Results are expressed as means ± SEM (n = 10). Statistics: $^{**}p < 0.01$, $^{*}p < 0.05$ versus control, $^{\#\#}p < 0.01$ versus low TRP.

similar changes of those in serum, we assume that such effects may also be true for IDO centrally. However, fluoxetine treatment in patients with depression has been shown to reduce IL-6 (Sluzewska et al., 1995). In addition, non-responders to SSRIs continue to exhibit raised IL-6 levels, suggesting the possibility that response to treatment may be linked to reductions in IL-6 (O'Brien et al., 2007). The mechanism through which vortioxetine exerts its effects on IL-6 levels and the K pathway remain to be elucidated but certainly appears to involve mechanisms that go beyond SERT inhibition.

Further, corticosterone has also been shown to induce the same TDO enzyme in the kynurenine pathway (Young, 1981; Christmas et al., 2011). Corticosterone was reduced by vortioxetine treatment, to near control levels and both the K/TRP and K/KA ratios were also decreased, although in the case of the K/TRP this was not significant. Hence, it is reasonable to hypothesize that reduction of corticosterone by vortioxetine may have decreased hepatic TDO and perhaps also IDO activity in addition to changes via previously discussed IL-6 mediations.

NMDA receptor and α7-nAchR parameters were measured in different brain regions. This was mainly for technical reasons which were due to the different preparation procedures involved and in some instances due to insufficient available tissue volume. NMDA (B_{max}) receptor binding in the amygdala was significantly upregulated following TRP depletion and reversed to control levels by vortioxetine but not by paroxetine. Observations such as significantly higher levels of the NR_{2A} sub-unit of NMDA receptors in post mortem brains of depressed patients vs. healthy subjects (Karolewicz et al., 2009) are supportive of the construct validity of the TRP depletion model. In addition, in support of the model's predictive validity, there is compelling evidence for antidepressant activity of glutamatergic modulators (Zarate et al. 2010) and evidence that vortioxetine, unlike an SSRI, enhances glutamatergic signaling in depression relevant brain regions such as the prefrontal cortex and hippocampus (Riga et al., 2016). Vortioxetine does not have notable affinity for glutamate receptors (Sanchez et al., 2015), however, it has been shown to attenuate the inhibitory control that GABAergic interneurons exert on glutamate signaling through its potent $5\text{-}HT_3$ receptor antagonism (Riga et al., 2016). The α7-nAchR content in the hippocampus was also significantly upregulated following TRP depletion and reversed to control levels by vortioxetine but not by paroxetine. Pharmacological experiments using selective α7-nAchR agonists and antagonists may suggest that α7-nAchRs, rather than NMDA receptors, may be the primary target of endogenous KA in the brain on neurotransmitter levels (Elgamal and MacQueen 2008; Andreasen et al., 2009). Interestingly, other drugs active on neuronal α7-nAchRs including cytosine and varenicline have demonstrated antidepressant-like effects in preclinical studies (Mineur et al. 2007; Rollema et al. 2009). However, a putative therapeutic benefit of α7-nAchR stimulation in major depressive disorder remains to be studied. Vortioxetine's effect on α7-nAchR expression is indirect since vortioxetine is devoid of notable affinity for this receptor (Sanchez et al., 2015). The mechanism through which vortioxetine modulates α7-nAchR expression remains to be elucidated, but may involve its ability to modulate glutamate signaling through inhibition of GABA interneurons.

All taken together, we hypothesize that vortioxetine's reversal of TRP depletion-induced elevation of corticosterone and/or pro-inflammatory cytokines (here IL-6) leads to deactivation of the TRP/K pathways (via TDO and IDO enzymes) and a subsequent increase of KA, and decrease of α7-nAchR and NMDA receptor numbers, which results in a normalized glutamate transmission and antidepressant-like activity. This hypothesis will need to be substantiated in additional studies. Based on the limited effects of the SSRI paroxetine in the model, we suggest that vortioxetine's actions in the TRP depletion model were likely due to its 5-HT receptor actions rather than SERT inhibition.

Unlike in our previous studies (Franklin et al., 2015; other presently unpublished data), in the present experiment, paroxetine treatment reduced immobility in the FST to control levels, although this was without reaching statistical significance. We suggest that greater data set variance in the present study than that found in our previous ones may be the cause of this result. Vortioxetine reduced immobility to levels significantly below those found in both control and TRP-depleted animals. The antidepressant effect of vortioxetine is not likely to be associated with increased locomotion, as vortioxetine was previously shown not to have any influence on locomotor activity (Guilloux et al., 2013; Jensen et al., 2014; du Jardin et al., 2016).

5. Study limitations

It is possible that some physiological functions such as pro-estrus hormonal surges (Marvan et al., 1997) could confound the present findings. However, we found no differences in serum estradiol concentrations across experimental groups. Other recent similar studies have also indicated that the estrous cycle neither impacts behavior nor TRP metabolite levels (Eskelund et al., 2016). Finally, another study limitation relates to the fact that not all the peripheral measures were studied in the brain.

6. Conclusions

In conclusion, we have shown that vortioxetine, but not paroxetine, enabled a reversal of TRP depletion-induced changes of depression-like behavior and biomarkers of glutamatergic activity in a female rat model of SSRI-insensitive depression. These preclinical observations support the hypothesis that vortioxetine's antidepressant activity may involve mechanisms beyond SERT inhibition. A more detailed understanding of the mechanisms remains to be elucidated.

Ethical statement

All animal procedures were performed in the morning and were approved by the Animal Health and Animal Welfare Division of State Veterinary and Food Administration of the Slovak Republic and conformed to the NIH Guidelines for Care and Use of Laboratory Animals as for previous studies with vortioxetine.

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