**Serotonergic mechanisms involved in antidepressant-like responses evoked by GLT-1
blockade in rat infralimbic cortex**

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Abstract

Novel fast-acting antidepressant strategies, such as ketamine and deep brain stimulation, enhance glutamatergic neurotransmission in medial prefrontal cortex (mPFC) regions via AMPA receptor (AMPA-R) activation. We recently reported that the regionally-selective blockade of the glial glutamate transporter-1 (GLT-1) by dihydrokainic acid (DHK) microinfusion in rat infralimbic cortex (IL), the most ventral part of the mPFC, evoked immediate (10 min) antidepressant-like responses, which involved AMPA-R activation and were associated to increased serotonin (5-hydroxytryptamine, 5-HT) release. Given the reciprocal connectivity between the mPFC and the serotonergic dorsal raphe nucleus (DR), here we examined the serotonergic mechanisms involved in the reported antidepressant-like responses of DHK infusion. First, we show that antidepressant-like responses evoked by IL application of DHK and citalopram are mediated by local 5-HT_{1A} receptors (5-HT_{1A}-R), since they are cancelled by previous IL WAY100635 microinfusion. Second, IL DHK microinfusion increases excitatory inputs onto DR, as shown by an increased glutamate and 5-HT release in DR and by a selective increase of c-Fos expression in DR 5-HT neurons, not occurring in putative GABAergic neurons. This view is also supported by an increased 5-HT release in ventral hippocampus following IL DHK microinfusion. Interestingly, antidepressant-like responses evoked by IL DHK lasted for 2 h and could be prolonged for up to 24 h by attenuating self-inhibitory effects via 5-HT_{1A} autoreceptors. In contrast, the antidepressant-like effects of S-AMPA microinfusion in IL were short-lasting. Together, our results further support a prominent role of the IL–DR pathway and of ascending 5-HT pathways in mediating antidepressant-like responses evoked by glutamatergic mechanisms.

Keywords: antidepressant effects, dihydrokainic acid, dorsal raphe, GLT-1, infralimbic cortex, serotonin.

Highlights

- IL 5-HT_{1A}-R mediate the antidepressant responses evoked by enhanced IL glutamate
- Enhancing glutamate transmission in IL activates excitatory inputs onto 5-HT neurons
- This effect enhances forebrain 5-HT release via IL-DR reciprocal connectivity
- GLT-1 blockade in IL induces parallel changes in behavior and 5-HT neurochemistry

1. Introduction

Major depressive disorder (MDD) is a severe psychiatric syndrome with very high socioeconomic impact worldwide (Global Burden of Disease Study 2013 Collaborators, 2015). This is attributable to the elevated prevalence of MDD, the emergence of depressive episodes during active periods of life and the slow clinical action and limited efficacy of monoamine antidepressants (Rush et al., 2006). During the last decades, the development of novel antidepressant strategies based on the modulation of glutamatergic neurotransmission has opened new venues for the improvement of MDD treatment. Hence, the non-competitive N-methyl-D-aspartate receptor (NMDA-R) antagonist ketamine and deep brain stimulation (DBS) overcome some of these limitations and evoke rapid and long-lasting clinical improvements in MDD patients, including those presenting treatment resistant depression (Mayberg et al., 2005; Zarate et al., 2012, 2006). Thus, research efforts in the field are focused on the identification of the neurobiological mechanisms underlying these rapid antidepressant actions.

MDD has been associated to astrocyte dysfunction in frontostriatal structures (Peng et al., 2015) and to alterations in the number, morphology and function of astrocytes (Rajkowska and Stockmeier, 2013; Sanacora and Banasr, 2013). Astrocytes play a key role in the regulation of glutamatergic neurotransmission (Perea et al., 2014; Perea and Araque, 2007) and in modulating complex behaviors (Halassa and Haydon, 2010; Oliveira et al., 2015). Upon release, glutamate is cleared from the synaptic cleft by excitatory amino acid transporters (EAATs), from which EAAT1 and EAAT2 are located in astrocytes and show altered expression in MDD patients (Choudary et al., 2005). In the rat brain, the glutamate transporter-1 (GLT-1) is the homologous of the human EAAT2 and is the main responsible for cortical glutamate reuptake (Danbolt et al., 1992; Rothstein et al., 1994). We recently reported that GLT-1 blockade in the infralimbic area (IL) of the medial prefrontal cortex (mPFC) evoked fast (10 min) and robust antidepressant-like responses in rats, which depended on AMPA receptor (AMPA-R) activation

and were associated to an increased serotonin (5-hydroxytryptamine, 5-HT) release in IL (Gasull-Camós et al., 2017a). None of these effects occurred when GLT-1 was blocked in the adjacent prelimbic cortex (PrL), indicating a key role of IL in mediating fast antidepressant-like effects, as also observed with ketamine and DBS (Fuchikami et al., 2015; Jiménez-Sánchez et al., 2015). Likewise, in common with ketamine (Amargós-Bosch et al., 2006; du Jardin et al., 2016; Pham et al., 2017), antidepressant responses evoked by GLT-1 blockade are associated to an increase in 5-HT release (Gasull-Camós et al., 2017a).

Behavioral differences evoked by GLT-1 blockade in IL and PrL likely involve the recruitment of different brain circuits, as assessed with microPET scan in rats (Gasull-Camós et al., 2017b). The mPFC and the serotonergic dorsal raphe nucleus (DR) are reciprocally connected (Azmitia and Segal, 1978; Gabbott et al., 2005; Groenewegen and Uylings, 2000; Vertes, 2004) and exert mutual control of their activity (Celada et al., 2013, 2001; Hajós et al., 1998; Puig et al., 2005). In view of this reciprocal interaction and given the relevance of the mPFC-DR pathway in mediating responses to behavioral challenges (Warden et al., 2012), here we confirm and extend our previous observations (Gasull-Camós et al., 2017a,b) to examine the potential serotonergic mechanisms involved in the antidepressant-like responses induced by GLT-1 blockade in IL.

2. Materials and Methods

2.1. Subjects

Male Wistar rats (Charles River, Lyon, France) weighing between 275 and 325 g at the time of surgery were used. Rats had free access to food and water and were maintained in a controlled environment (22 ± 1 °C; 12 h light/dark, lights on at 7 am). Rats were acclimatized to the housing conditions for at least 7 days and were daily handled. After surgery, all rats were singly housed and randomly assigned to treatment. Animal care followed the national (Royal Decree 53/2013) and European Union regulation (Directive 2010/63/EU, on the

protection of animals used for scientific purposes, 22 September 2010), and was approved by the Institutional Animal Care and Use Committee of the University of Barcelona.

2.2. Drugs

Dihydrokainic acid (DHK), (S)- α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (S-AMPA) and citalopram hydrobromide were purchased from Tocris (Bristol, UK). WAY100635 maleate was purchased from Sigma-Aldrich (Tres Cantos, Spain).

For intracerebral microinfusion, DHK (5 nmoles) and S-AMPA (50 pmoles) were dissolved in PBS 10X as previously reported (Gasull-Camós et al., 2017a; Lee et al., 2007); citalopram (15 pmoles) and WAY100635 (50 pmoles) were dissolved in artificial cerebrospinal fluid (aCSF). The microinfusion volume was 0.5 μ l per side in all instances. For intraperitoneal (i.p.) systemic administration, WAY100635 (0.3 mg/kg) maleate was dissolved in saline and injected at 1 ml/kg.

2.3. Behavioral studies

2.3.1. Surgery

Rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and implanted with stainless steel 22-gauge bilateral guide cannulae (Plastics One, Roanoke, VA, USA) in the cingulate cortex: AP +3.2; ML $\pm$ 0.75; DV -2.4 (Paxinos and Watson, 2005). The coordinates -in mm- were taken from bregma and the skull surface. Guide cannulae were fixed with stainless steel screws using dental acrylic. To prevent occlusion, a dummy cannula was inserted into the guide cannula and removed and reinserted daily. This procedure reduced stress associated with the microinfusion on the testing day (Slattery et al., 2011). Rats were allowed 7 days to recover after surgery.

2.3.2. Microinfusions

Microinfusions were performed as described (Gasull-Camós et al., 2017a). The microinfusion cannulae (28 gauges) extended 3 mm beyond the guide cannulae aiming the IL cortex (DV -5.4). Prior to the tests, three mock infusions were performed. Drug or vehicle solutions were

bilaterally administered over 1 min through two 50 µl Hamilton syringes connected to the microinfusion cannulae via 0.28 mm ID polyethylene tubing, using an infusion/withdrawal pump (Harvard Apparatus, Holliston, MA, USA). Microinfusion cannulae were left in place for 3 min to allow drug diffusion. Behavioral tests were performed 10 min, 2 h or 24 h after drug local administration. In double microinfusion procedures (WAY100635/DHK or WAY100635/citalopram), the second administration was performed 5 min after the first one. For 24 h measurements, microinfusions of the drug took place 15 min after the pretest session.

2.3.3. Forced swimming test (FST)

We conducted the modified FST protocol as previously described (Slattery and Cryan, 2012). Briefly, rats were individually placed into a clear methacrylate cylinder (46 cm x 20 cm) filled with water (24 ± 1 °C) to a depth of 30 cm, in a 15 min pretest session. Then, rats were dried and returned to their home cages. The swim cylinders were filled with fresh water for every single rat. No behavioral score was performed during the pretest session. 24 h later (testing day), rats were exposed to the same conditions during a 5 min test and behavior was video recorded. Subsequently, the 5 min test was divided into 5 sec periods where the predominant behavior was rated as immobility, swimming or climbing by an experimenter blind to the treatment.

2.3.4. Locomotor activity test

The effect of local drug administration on locomotor activity was assessed during a 15 min trial. Briefly, under the same microinfusion timings, rats were placed in the corner of a dimly lighted black open field (35 cm x 35 cm) and movement was monitored with a video camera. The distance moved (in cm) was calculated for each rat using the VideoTrack View Point software (Lyon, France).

2.4. Microdialysis studies

Microdialysis experiments were conducted as previously described (Jiménez-Sánchez et al., 2015). Concentric dialysis probes with 2 mm or 4 mm membrane length were implanted under sodium pentobarbital anesthesia (60 mg/kg, i.p.) in the DR (AP -7.8; ML -3.1; DV -7.5; angle 30°) or in the ventral hippocampus (vHPC; AP -5.3; ML +4.8; DV -8.8), respectively. Likewise, bilateral guide cannulae were implanted in the cingulate cortex, as described above (AP +3.2; ML $\pm$ 0.75; DV -2.4). Coordinates were taken from bregma and the skull surface (Paxinos and Watson, 2005). Freely moving rats were continuously perfused with aCSF (125 mM NaCl, 2.5 mM KCl, 1.26 mM CaCl₂, 1.18 mM MgCl₂) containing 1 μ M citalopram at a rate of 1.65 μ l/min. A period of stabilization of 3 h was used to ensure stable 5-HT and glutamate levels. Four dialysate basal fractions were collected (25 min/each) before the microinfusion of the drug or vehicle into the IL (see microinfusions section for details). After infusion, six fractions (25 min/each) were collected. Neurotransmitter concentrations in the DR or vHPC were determined by high performance liquid chromatography with electrochemical (5-HT) or fluorimetric (glutamate) detection.

2.5. Immunohistochemistry

2.5.1. Tissue processing

Rats received a bilateral microinfusion of vehicle or DHK (5 nmoles/0.5 μ l/side) into the IL 90 min before perfusion. Administration time was selected based on findings of previous studies (Fukumoto et al., 2016). Rats were anesthetized with sodium pentobarbital and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Then, brains were removed, post-fixed during 24 h, cryoprotected in a 30% sucrose solution (in PB 0.1M) and stored at 4 °C until they sank. Coronal frozen sections (30 μ m) throughout the DR were obtained on a freezing microtome and stored in a 5% sucrose solution until used.

2.5.2. Immunofluorescence

The expression of c-Fos in DR 5-HT neurons was studied by triple immunofluorescence. Free-floating brain sections were washed thrice with PB 0.1M and blocked with 3% normal donkey

serum, 0.3% Triton X-100 in PB (NDS-T-PB) at room temperature for 2 h. Slices were incubated in the NDS-T-PB with primary antibodies to c-Fos (rabbit, 1:500, sc-72, Santa Cruz Biotechnology), anti-neuronal nuclei (NeuN) (mouse, 1:500, MAB-377, Chemicon) and tryptophan hydroxylase 2 (TPH2) (sheep, 1:2500, AB-1541, Millipore). The next day, sections were rinsed three times in PB 0.1M and incubated at room temperature with the secondary antibody to rabbit (Alexa 488, 1:500, Jackson ImmunoResearch, West Grove, USA), mouse (Alexa 647 IR, 1:500, Life Technologies, Carlsbad, CA, USA) or sheep (Alexa 555, 1:500, Jackson ImmunoResearch) in NDS-T-PB for 2 h. After incubation, sections were rinsed and mounted onto glass slides coated with gelatin. Mowiol was used as mounting medium.

2.5.2. Image processing

DR sections were assessed for the number of single NeuN-positive and TPH2-negative cells (putative GABAergic neurons), NeuN-positive and TPH2-positive cells (5-HT neurons), double-labeled cells for NeuN and c-Fos (putative GABAergic neurons expressing c-Fos) and triple-labeled cells for NeuN, TPH2 and c-Fos (5-HT neurons expressing c-Fos). Images were collected using a confocal microscope at a magnification of 40X (Leica TCS SP5, Leica Microsystems Heidelberg GmbH, Mannheim, Germany) and cells were counted using the ImageJ software (National Institutes of Health, Bethesda, MD).

2.6. Histological verification

At the completion of experiments, rats were killed by sodium pentobarbital overdose. Brains were rapidly removed and stored at -80°C. For histological verification of the cannulae and probes placement, brain sections (30 µm) were mounted onto slides and posteriorly stained with neutral red (supplementary figure 1). Rats infused outside target region (IL, vHPC or DR) were excluded from all analyses.

2.7. Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Statistical analysis was carried out using unpaired Student's t-test, one- or two-way analysis of variance (ANOVA), as appropriate, followed by Bonferroni *post hoc*. The level of significance was set at $p < 0.05$, in all cases.

3. Results

3.1. 5-HT_{1A}-R in IL mediate DHK- and citalopram-induced immediate antidepressant-like responses

Post-synaptic 5-HT_{1A}-R activation has been associated to antidepressant responses (reviewed in Celada et al., 2013). Given the expression of 5-HT_{1A}-R in a large proportion of IL neurons (Santana et al., 2004), we examined their putative involvement in mediating antidepressant-like responses evoked by IL DHK microinfusion. In agreement with our previous observations (Gasull-Camós et al., 2017a) the bilateral microinfusion of DHK (5 nmoles/0.5 μ l/side) into IL evoked an immediate (10 min) antidepressant-like response in the FST. This effect was prevented by the previous local microinfusion of WAY100635 (50 pmoles/0.5 μ l/side) into the IL (Figure 1a). One-way ANOVA showed a significant effect of the treatment on immobility ($F_{3,33} = 4.656$; $p < 0.01$) and swimming behaviors ($F_{3,33} = 6.769$; $p < 0.01$) (Figure 1a). *Post hoc* comparisons showed that WAY100635 prevented the effect of DHK on immobility ($p < 0.05$) and swimming behaviors ($p < 0.01$).

Likewise, the bilateral microinfusion of citalopram (15 pmoles/0.5 μ l/side) into the IL induced an antidepressant-like response, as previously reported (Gasull-Camós et al., 2017a), which was also prevented by the prior microinfusion of WAY100635 (50 pmoles/0.5 μ l/side) in IL. One-way ANOVA showed a significant effect of treatment on immobility ($F_{3,28} = 5.175$; $p < 0.01$) and swimming ($F_{3,28} = 6.185$; $p < 0.01$) behavior (Figure 1b). *Post hoc* comparisons showed that WAY100635 prevented the reduced immobility ($p < 0.05$) and the increased swimming behavior ($p < 0.05$) induced by citalopram.

3.2. DHK infusion in IL stimulates glutamate and 5-HT release in the DR

The extracellular concentrations of glutamate and 5-HT in the DR were used as surrogate neurochemical markers of the activity of IL-descending excitatory inputs, and of 5-HT neuron activity, respectively. The bilateral microinfusion of DHK (5 nmoles/0.5 µl/side) in IL increased glutamate and 5-HT output in the DR, as assessed by *in vivo* microdialysis. Baseline extracellular concentrations of glutamate and 5-HT in DR were 18 ± 3 pmol/fraction ($n = 15$) and 27 ± 5 fmol/fraction ($n = 15$), respectively. Two-way repeated measures ANOVA showed that DHK microinfusion in IL enhanced extracellular glutamate concentration in the DR, with a significant effect of treatment ($F_{1,17} = 6.730$; $p < 0.05$), fraction ($F_{9,153} = 2.718$; $p < 0.001$) and treatment $\times$ fraction interaction ($F_{9,153} = 2.370$; $p < 0.05$) (Figure 2a). Likewise, DHK microinfusion in IL increased 5-HT concentration in the DR, with a significant effect of treatment ($F_{1,17} = 8.871$; $p < 0.01$), fraction ($F_{9,153} = 8.402$; $p < 0.0001$) and treatment $\times$ fraction interaction ($F_{9,153} = 5.277$; $p < 0.0001$) (Figure 2b). Bonferroni *post hoc* analysis revealed that glutamate concentration was increased in fractions 6 and 8 ($p < 0.05$, DHK vs. vehicle) and 5-HT concentration remained significantly elevated for up to 2 h after IL DHK microinfusion ($p < 0.01$ in fraction 5, $p < 0.05$ for fractions 6 to 9; DHK vs. vehicle).

On the following day (24 h after IL DHK microinfusion) the basal 5-HT concentration in the DR remained significantly elevated. Two-way repeated measures ANOVA revealed a significant effect of time ($F_{1,13} = 9.780$; $p < 0.01$) and Bonferroni *post hoc* showed a significant difference between pre- and post-treatment measures in the DHK group ($p < 0.05$) (Figure 2c).

3.3. DHK microinfusion in IL stimulates 5-HT release in vHPC

We next examined whether DHK microinfusion in IL increased 5-HT release in the vHPC, a forebrain area involved in MDD treatment, innervated by the DR and devoid of descending inputs from mPFC. Baseline extracellular concentration of 5-HT in vHPC was 47 ± 4 fmol/fraction ($n = 18$). DHK (5 nmoles/0.5 µl/side) microinfusion in IL elevated the 5-HT output in vHPC to a maximum of $166 \pm 13\%$ of baseline, an effect that was followed by a faster decline

than that observed in DR (Figure 2d). Two-way repeated measures ANOVA revealed a significant effect of treatment ($F_{1,16} = 8.838$; $p < 0.01$), fraction ($F_{9,144} = 8.047$; $p < 0.0001$) and treatment x fraction interaction ($F_{9,144} = 5.145$; $p < 0.0001$). Bonferroni *post hoc* analysis revealed that 5-HT was significantly increased in dialysate fractions 5 to 7 ($p < 0.01$, fractions 5 and 6; $p < 0.05$ fraction 7) in the DHK group vs. the vehicle group.

3.4. DHK microinfusion in IL stimulates c-Fos expression in DR 5-HT neurons

The bilateral microinfusion of DHK (5 nmoles/0.5 μ l/side) into IL stimulated c-Fos expression specifically in DR 5-HT neurons. Unpaired *t*-test showed that NeuN-positive/TPH2-positive cells (serotonergic) presented a significant increase in c-Fos immunoreactivity in DHK-treated rats compared to vehicle ($t_{(11)} = 2.927$, $p < 0.05$), while NeuN-positive/TPH2-negative cells -mostly GABAergic neurons (Day et al., 2004)- presented similar expression of c-Fos in the two treatment groups ($t_{(11)} = 0.773$; n.s.) (Figure 3). The total number of 5-HT and putative GABAergic cells were similar in the DR sections studied (5-HT: 346.15 ± 33.08 , putative GABA: 317.69 ± 18.33 ; $t_{(24)} = 0.752$; n.s.). As expected, the number of 5-HT (vehicle: 382.83 ± 47.79 , DHK: 314.71 ± 45.55 ; $t_{(11)} = 0.325$; n.s.) and putative GABAergic cells (vehicle: 341.83 ± 33.98 , DHK: 297.00 ± 16.39 ; $t_{(11)} = 0.238$; n.s.) did not differ between groups.

3.5. IL DHK evoked antidepressant responses are crucially modulated by 5-HT_{1A} autoreceptors

The antidepressant-like response evoked by DHK microinfusion into the IL (5 nmoles/0.5 μ l/side) was still present 2 h after drug microinfusion (Figure 4a), with a significant decrease in immobility ($t_{(14)} = 3.630$; $p < 0.001$) and increase in swimming behaviors ($t_{(14)} = 3.011$; $p < 0.001$; unpaired *t*-test). These changes were not associated to changes in locomotor activity as assessed by the distance traveled (cm) in an open field (mean $\pm$ S.E.M in vehicle: 1831 ± 172 , DHK: 1979 ± 266 ; $t_{(10)} = 0.4671$, n.s.; $n = 6$ rats/group). However, DHK microinfusion in IL did not evoke any antidepressant-like response in the FST 24 h after drug application (Figure 4b).

The lack of an antidepressant-like response 24 h after DHK microinfusion was somehow discordant with the elevated serotonergic activity at this time, as suggested by the increase in DR 5-HT output (Figure 2c). Given the crucial role of somatodendritic 5-HT_{1A} autoreceptors in controlling ascending serotonergic activity and in presenting antidepressant-like responses (Artigas, 2013), we then examined whether 5-HT_{1A} autoreceptor blockade could disinhibit serotonergic activity and bring about an antidepressant-like response at 24 h.

Thus, the systemic administration of WAY100635 (0.3 mg/kg, i.p.) 40 min before FST reduced the immobility time in rats microinfused with DHK in IL 24 h before (15 min after the pretest session), but not in control rats microinfused with vehicle. One-way ANOVA showed a significant effect of treatment on immobility ($F_{(3,27)} = 3.419$; $p < 0.05$) and swimming behaviors ($F_{(3,27)} = 3.680$; $p < 0.05$) (Figure 4c). Bonferroni *post hoc* comparisons indicated a significant decrease in immobility and increase in swimming behavior ($p < 0.05$, in both cases) in the DHK/WAY100635 group compared to vehicle/saline. WAY100635 alone was ineffective in rats treated with vehicle on the preceding day.

The antidepressant-like behavior seen in the FST was not associated to changes in locomotor activity, as assessed by the similar distance travelled (cm) in the open field by both experimental groups (mean $\pm$ S.E.M in DHK/WAY100635: 6300 ± 408 , vehicle/WAY100635: 7302 ± 303 ; $t_{(10)} = 1.973$, n.s.; $n = 6$ rats/group).

3.6. S-AMPA microinfusion in IL produces a different temporal response pattern

We previously showed that the fast (10 min) antidepressant-like effects of GLT-1 blockade in IL were prevented by AMPA-R blockade and mimicked by S-AMPA microinfusion (Gasull-Camós et al., 2017a). We therefore examined the effect of the bilateral microinfusion of S-AMPA (50 pmoles/0.5 μ l/side) into IL on glutamate and 5-HT output in the DR. Baseline extracellular glutamate and 5-HT concentrations in DR were 15 ± 1 pmol/fraction ($n = 16$) and 64 ± 13 fmol/fraction ($n = 17$), respectively. Two-way repeated measures ANOVA showed that IL S-AMPA microinfusion significantly heightened DR extracellular glutamate concentration, with a

significant effect of treatment ($F_{1,18} = 7.055$; $p < 0.05$), fraction ($F_{9,162} = 4.485$; $p < 0.0001$) and treatment $\times$ fraction interaction ($F_{9,162} = 4.588$; $p < 0.0001$) (Figure 5a). Subsequent *post hoc* comparisons showed that glutamate was significantly enhanced during the first and second fractions collected after S-AMPA microinfusion (fraction 5, $p < 0.05$; and fraction 6, $p < 0.01$). IL S-AMPA increased DR 5-HT concentration, as shown by significant effect of treatment ($F_{1,18} = 6.428$; $p < 0.05$), fraction ($F_{9,162} = 3.480$; $p < 0.001$) and treatment $\times$ fraction interaction ($F_{9,162} = 3.288$; $p < 0.001$) (Figure 5b), and *post hoc* comparisons showed that 5-HT was significantly elevated only during the second fraction collected after S-AMPA microinfusion (fraction 6; $p < 0.01$).

S-AMPA did not evoke any antidepressant-like response 2 h after drug microinfusion in IL (Figure 5c). Unpaired *t*-test showed no significant difference in immobility ($t_{(16)} = 0.954$; n.s.), swimming ($t_{(16)} = 0.352$; n.s.) nor climbing behaviors ($t_{(16)} = 1.070$; n.s.).

4. Discussion

The present study shows that antidepressant-like responses evoked by the enhancement of glutamatergic neurotransmission in IL are associated to an activation of descending excitatory pathways to the DR and the subsequent enhancement of serotonergic neurotransmission. Thus, a single bilateral microinfusion of DHK into IL activates DR 5-HT neurons, as observed by an increased 5-HT release in DR, with a time course parallel to that of antidepressant-like responses; and by an increased c-Fos expression in DR 5-HT neurons, but not in putative GABAergic neurons. Moreover pre- and post-synaptic 5-HT_{1A}-R appear to play opposite roles in modulating these antidepressant-like responses, as post-synaptic 5-HT_{1A}-R in IL are required for the immediate (10 min) antidepressant-like responses (Gasull-Camós et al., 2017a, present study), while pre-synaptic 5-HT_{1A}-R prevent a larger duration of the antidepressant response (24 h), possibly due to self-inhibitory actions on 5-HT neurons.

Post-synaptic 5-HT_{1A}-R located in IL are necessary for the immediate antidepressant-like responses of DHK and citalopram

We previously reported that the enhancement of glutamatergic neurotransmission in IL following GLT-1 blockade with DHK evoked antidepressant-like responses 10 min after drug microinfusion, which were associated to an enhancement of 5-HT release in IL and were prevented by prior 5-HT depletion with pCPA (Gasull-Camós et al., 2017a). Given the role of post-synaptic 5-HT_{1A}-R in mediating antidepressant responses (Celada et al., 2013), we first investigated whether they were involved in this immediate antidepressant response. The mPFC is enriched in 5-HT_{1A}-R and 5-HT_{2A}-R (Pompeiano et al., 1994, 1992), with varying proportions of neurons expressing both receptors in PrL and IL. The former subdivision contains a roughly similar proportion of pyramidal neurons expressing the mRNAs encoding inhibitory 5-HT_{1A}-R and excitatory 5-HT_{2A}-R (61 ± 2 vs. 51 ± 3 %, respectively) (Santana et al., 2004). In contrast, the IL subdivision contains a significantly greater proportion of pyramidal neurons expressing 5-HT_{1A}-R vs 5-HT_{2A}-R mRNAs (40 ± 4 vs. 12 ± 1 %, respectively). The proportion of GABA interneurons expressing one or other receptor in both subdivisions varies in a non-significant manner, between 20 ± 1 and 32 ± 1 %. This suggests that the antidepressant-like effects resulting from an enhanced serotonergic neurotransmission in IL would be **most likely** mediated by the activation of post-synaptic 5-HT_{1A}-R. In agreement, here we show that the local application of the selective 5-HT_{1A}-R antagonist WAY100635 in IL prevented the antidepressant-like responses induced by DHK and citalopram. Remarkably, the similarity of the antidepressant-like effect of local (in IL) and systemic citalopram administrations supports a key role of IL in SSRI-induced antidepressant-like responses.

Since the activation of 5-HT_{1A}-R inhibits the activity of mPFC pyramidal neurons (Amargós-Bosch et al., 2004; Puig et al., 2003), the present results seem in apparent controversy with the previously reported role of IL AMPA-R in DHK-evoked antidepressant-like responses (Gasull-Camós et al., 2017a) and the increased metabolic activity in IL after DHK

microinfusion (Gasull-Camós et al., 2017b). One possible explanation is that 5-HT_{1A}-R mediating immediate antidepressant-like responses may be expressed by pyramidal neuron populations different from those projecting to the DR. In this regard, the IL subdivision contains a large number of pyramidal neurons projecting to subcortical structures involved in MDD, such as the amygdala, the lateral hypothalamus, the septum or the periaqueductal gray matter (Gabbott et al., 2005). Hence, activation of AMPA-R in midbrain-projecting pyramidal neurons would enhance the activity of ascending 5-HT neurons, leading to an increased 5-HT release in IL which would then inhibit pyramidal neurons projecting to MDD-related areas, thus evoking an antidepressant-like phenotype. An alternative –yet less likely– explanation relies on the presence of 5-HT_{1A}-R in GABAergic interneurons. The exogenous administration of selective 5-HT_{1A}-R agonists increases pyramidal neuron activity and c-fos expression in PFC (Hajós et al., 1998; Lladó-Pelfort et al., 2010). This effect is mediated by the preferential activation of 5-HT_{1A}-R in fast-spiking GABAergic interneurons, resulting in the disinhibition and subsequent excitation of pyramidal neurons (Llado-Pelfort et al., 2012). The reasons for the preferential action on fast-spiking GABA interneurons remain unknown.

DHK infusion in IL stimulates the IL-DR pathway

Given that the increased 5-HT in the IL was apparently due to enhanced activity of the mPFC–DR pathway (Gasull-Camós et al., 2017b), we then studied the consequences of IL GLT-1 blockade on DR neurochemistry. *In vivo* microdialysis studies revealed that IL DHK microinfusion increased glutamate and 5-HT release in the DR. The increased glutamate release in DR likely results from the increased activity of pyramidal axons reaching the DR. Similarly, IL DBS also increases glutamate release in DR (Jiménez-Sánchez et al., 2015). Moreover, optogenetic activation of the mPFC–raphe evokes fast antidepressant-like responses, which depend on the glutamatergic synaptic input onto the DR, as microinfusion of the AMPA-R antagonist NBQX into the DR blocks the stimulation driven antidepressant-like response (Warden et al., 2012).

Glutamate release by pyramidal axons in the DR can activate or inhibit 5-HT neurons, depending on the final cellular target in the midbrain. Hence, mPFC-driven excitations of 5-HT neurons are monosynaptic and involve the activation of local AMPA-R and NMDA-R, whereas inhibitions are driven by (i) local negative feedback through 5-HT_{1A} autoreceptors and/or (ii) activation of local GABA interneurons and the subsequent inhibition of 5-HT neurons (Celada et al., 2001; Varga et al., 2001). Our results show a simultaneous increase of glutamate and 5-HT release in the DR and a significant increase in c-Fos expression specifically in DR 5-HT neurons, but not in putative GABAergic neurons (TPH2- neurons) after IL DHK microinfusion. This suggests that –despite the simultaneous innervation of 5-HT and GABA neurons– activation of IL descending pathways by DHK enhances direct glutamatergic inputs onto 5-HT neurons more markedly than those on GABA neurons. Moreover, our results indicate a predominant activation of DR GABAergic neurons under basal conditions, as c-Fos expression was greater in TPH2- than in TPH2+ neurons in control rats. In agreement, others have reported that both systemic and intra-mPFC ketamine administration increase c-Fos expression in DR 5-HT neurons (Fukumoto et al., 2016), yet they did not examine the effect on TPH2- neurons.

The activation of DR 5-HT neurons induced by DHK microinfusion in IL is also supported by increased 5-HT release in IL (Gasull-Camós et al., 2017a) and in vHPC (present study). The latter effect cannot be mediated by a direct descending mPFC-vHPC pathway, since the PFC does not project to the hippocampal formation in rats (Gabbott et al., 2005). More convincingly, and given the increased c-Fos expression in DR after DHK microinfusion in IL, this effect possibly results from the excitation of raphe 5-HT neurons, leading to an increased 5-HT release in vHPC, an area innervated by both DR and median raphe nuclei (McQuade and Sharp, 1997). In line with the present results, DBS of the mPFC also increased 5-HT release in the HPC (Hamani et al., 2010) and hippocampal plasticity related to DBS depends on 5-HT release into the HPC (Etiévant et al., 2015).

It is important to note that 5-HT release in DR remained stable for 2 h and slightly elevated until 24 h later, suggesting that plastic changes may occur in the DR after the stimulation of descending IL afferents. In line with the former observation, a 20 min electrical stimulation of mPFC neurons at moderate –yet physiological– frequencies, such as 20 Hz, increased 5-HT release in the DR for at least 1 h after the cessation of the stimulation (Celada et al., 2001). Moreover, it has also been reported that DBS of the vmPFC induces neuroplastic changes in DR 5-HT neurons, associated to increased 5-HT excitability and antidepressant-like responses (Etiévant et al., 2015; Veerakumar et al., 2014).

Sustained antidepressant-like responses after IL stimulation

We then studied the time course of the IL DHK-evoked antidepressant-like responses by testing the effects of IL DHK in the FST at 2 h and 24 h after drug microinfusion. The antidepressant-like responses found at 2 h after IL DHK are consistent with the DR 5-HT elevation observed. However, behavioral responses disappeared 24 h after IL DHK microinfusion despite the presence of a moderate (yet statistically significant) elevation in DR 5-HT release. In an attempt to potentiate subthreshold responses, we systemically administered the 5-HT_{1A}-R antagonist WAY100635, under the hypothesis that this receptor was inducing a 5-HT-mediated negative feedback on 5-HT neurons, and consequently preventing the emergence of the antidepressant response. WAY100635 on its own did not affect behavior, probably due to the lack of a tonic activation of 5-HT_{1A} autoreceptors in control rats. However, systemic administration of WAY100635 allowed the appearance of an antidepressant effect 24 h after IL DHK infusion. This suggests that 24 h after IL DHK a moderate enhancement of serotonergic neurotransmission is still present, yet attenuated by self-inhibition through 5-HT_{1A} autoreceptors activation. Unlike the blockade of post-synaptic 5-HT_{1A}-R in IL by a higher local dose (see above), the systemic dose of WAY100635 used did not prevent the antidepressant-like effects of boosting serotonergic activity via 5-HT_{1A} autoreceptor blockade, suggesting a different contribution of pre- and post-synaptic 5-HT_{1A}-R

in both experimental situations. These results are consistent with the key role of pre-synaptic 5-HT_{1A}-R in the control of serotonergic activity and in antidepressant responses (Albert and Le François, 2010; Artigas et al., 2001, 1996; Blier and de Montigny, 1994). However, a limitation of the present experiments lies in the systemic administration of WAY100635. Hence, unlike local (IL) WAY100635 administration, which enabled to clearly identify the population of post-synaptic receptors involved in antidepressant-like effects of DHK and citalopram, the present experiments cannot discard the involvement of 5-HT_{1A}-R receptor populations other than somatodendritic autoreceptors. Figure 6 shows a scheme of DHK effects on the IL-DR pathway at the different times examined.

Finally DHK and S-AMPA microinfusion in IL evoked similar antidepressant-like effects in the FST at 10 min post-administration (Gasull-Camós et al., 2017a), thus adding further evidence to the role of AMPA-R activation in fast-acting antidepressant responses (Jiménez-Sánchez et al., 2015; Maeng et al., 2008). However, unlike DHK, S-AMPA did not evoke a behavioral response nor enhanced glutamate and 5-HT release in the DR 2 h after administration, suggesting that other ionotropic or metabotropic glutamate receptors may contribute to sustained DHK-induced antidepressant-like effects.

5. Conclusions

In summary, the present study shows that antidepressant-like responses induced by enhancing glutamatergic neurotransmission in IL are mediated essentially by serotonergic mechanisms (Figure 6). The blockade of the glial glutamate transporter GLT-1 with DHK evoked antidepressant-like responses at 10 min and 2 h post-administration, which were paralleled by an increased 5-HT release in the IL-DR pathway. Moreover, post-synaptic 5-HT_{1A}-R in IL appear to be relevant for the immediate antidepressant-like effects. The antidepressant-like responses could also be evoked 24 h post-administration by removing pre-synaptic 5-HT_{1A} autoreceptors-mediated self-inhibition of serotonergic activity. The temporal differences

between the effects of DHK and S-AMPA suggest the involvement of glutamatergic receptors other than AMPA-R in the persistence of antidepressant-like responses. Overall, the present results indicate that boosting serotonergic neurotransmission by enhancing glutamatergic inputs onto 5-HT neurons may be a more efficient way than blocking SERT, a process triggering a series of pre- and post-synaptic negative feed-back mechanisms (Artigas et al., 1996, 2001; Riga et al., 2016). This view is also supported by the efficacy of a single DHK administration in IL to reduce the latency to feed in the novelty-suppressed feeding test, which is typically sensitive to chronic –but not acute– SSRI treatment. In addition, the present study contributes to a better understanding of neural mechanisms involved in fast-acting antidepressant responses and may help to identify new antidepressant targets.

Conflict of interests or Disclosure

FA has received consulting honoraria from Lundbeck A/S and has been PI of a grant from Lundbeck A/S. He is also a member of the scientific advisory board of Neurolix and a co-inventor of two patents on conjugated oligonucleotide sequences. The remaining authors declare no conflict of interest.

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Figure 1. IL 5-HT_{1A}-R are necessary for the DHK and citalopram-induced antidepressant-like actions. (a) Effects of WAY100635 (50 pmoles/0.5 µl/side) and DHK (5 nmoles/0.5 µl/side) double microinfusion into the IL cortex on the FST. The reduction in immobility and increase in swimming counts observed in vehicle/DHK group (n = 8) compared to control (vehicle/vehicle; n = 9) was prevented by previous local microinfusion of the 5-HT_{1A}-R antagonist WAY100635 (WAY100635/DHK; n = 9). WAY100635/vehicle group (n = 8) was similar to controls. (b) Effects of WAY100635 (50 pmoles/0.5 µl/side) and citalopram (15 pmoles/0.5 µl/side) double microinfusion into IL. Reduced immobility and increased swimming counts observed in vehicle/citalopram group (n = 7) compared to control (vehicle/vehicle; n = 7) was also prevented by previous IL microinfusion of 5-HT_{1A} antagonist (WAY100635/citalopram; n = 7). WAY100635/vehicle group (n = 8) did not differ from control group. The values are expressed as mean ± S.E.M. **p* < 0.05, ***p* < 0.01 vs. vehicle/vehicle; #*p* < 0.05, ##*p* < 0.01 vs. vehicle/DHK or vehicle/citalopram (Bonferroni *post hoc* test).

Figure 2. IL blockade of GLT-1 transporter activates serotonergic neurotransmission in DR and evokes 5-HT release in vHPC. The bilateral microinfusion of DHK (5 nmoles/0.5 μ l/side) into the IL cortex **(a)** increases glutamate (Bonferroni *post hoc* analysis revealed that glutamate concentration was increased in fractions 6 and 8; $p < 0.05$ in both cases, DHK vs. vehicle; $n=10$ /group) and **(b)** serotonin (5-HT) release (Bonferroni *post hoc* analysis revealed that 5-HT concentration remained significantly elevated for up to 2 h after IL DHK microinfusion; $p < 0.01$ in fraction 5, $p < 0.05$ for fractions 6 to 9; DHK vs. vehicle; $n = 10$ /group) in the DR. **(c)** DR 5-HT increased extracellular concentration remains increased 24 h after IL DHK microinfusion (pre-treatment vehicle, $n = 7$; pre-treatment DHK, $n = 8$; post-treatment vehicle, $n = 7$; post-treatment DHK, $n = 8$). **(d)** IL DHK microinfusion transiently increases 5-HT release in the vHPC (Bonferroni *post hoc* analysis revealed that 5-HT was significantly increased in dialysate fractions 5 to 7 ($p < 0.01$ in fractions 5 and 6, $p < 0.05$ in fraction 7; DHK vs. vehicle; $n = 9$ /group), which receives important afferents from DR. Microdialysis data are expressed as percentages of four basal values. The values are expressed as mean $\pm$ S.E.M. * $p < 0.05$ and ** $p < 0.01$ (significant main effect of treatment in the two-way ANOVA; pre-treatment vs. post-treatment Student's *t*-test). The arrow represents the start of the IL microinfusion with vehicle or DHK. Dialysate fractions were 25 min each.

Figure 3. IL blockade of GLT-1 increases c-Fos expression specifically in DR serotonergic neurons.

(a) Confocal images of c-Fos expressing cells (green), 5-HT neurons (NeuN-positive/TPH2-positive cells; red) and putative GABA interneurons (NeuN-positive/TPH2-negative cells; blue) in vehicle- and DHK-treated rats. Arrowheads show 5-HT neurons expressing c-Fos (triple c-Fos, NeuN and TPH2 positive), tailed arrows show putative GABAergic neurons expressing c-Fos (double c-Fos and NeuN positive). **(b)** Percentages of colocalization 90 minutes after IL microinfusion of vehicle ($n = 6$) or DHK (5 nmoles/0.5 μ l/side; $n = 7$) in 5-HT or GABA neurons. No differences in the number of 5-HT or GABA neurons in the DR were observed between groups. Values indicate the mean $\pm$ S.E.M. * $p < 0.05$ vs. vehicle (Student's t -test).

Figure 4. IL blockade of GLT-1 evokes fast antidepressant-like responses that can be prolonged by blockade of pre-synaptic 5-HT_{1A}-R.

(a) DHK (5 nmoles/0.5 μ L/side) produced antidepressant-like responses 2 h after IL bilateral microinfusion, as shown by reduced immobility and increased swimming counts (vehicle; DHK, $n = 8$). **(b)** 24 h after IL DHK microinfusion there was no behavioral response on the FST (vehicle; DHK, $n = 7$). **(c)** Systemic administration of WAY100635 (0,3 mg/kg, i.p.) 40 minutes before the FST in combination with IL DHK microinfusion on the previous day evoked antidepressant-like responses (vehicle/saline, $n = 6$; DHK/saline, $n = 6$; vehicle/WAY100635, $n = 9$; DHK/WAY100635, $n = 7$). Values are expressed as mean $\pm$ S.E.M. ** $p < 0.01$ compared to vehicle (Student's t -test); # $p < 0.05$ compared to vehicle/WAY100635 (Bonferroni *post hoc* test).

Figure 5. Direct activation of AMPA-R in IL cortex evokes a transient increase in DR 5-HT and glutamate release and does not maintain its antidepressant effect 2 h after microinfusion. The bilateral microinfusion of S-AMPA (50 pmoles/0.5 μ l/side) into the IL cortex transiently increases **(a)** glutamate (*Post hoc* comparisons showed that glutamate was significantly enhanced during the first and second fractions collected after S-AMPA microinfusion; $p < 0.05$ in fraction 5; and $p < 0.01$ in fraction 6; S-AMPA vs. vehicle, $n=10$ /group) and **(b)** serotonin (5-HT) release (*Post hoc* comparisons showed that 5-HT was significantly enhanced only during the second fraction collected after S-AMPA microinfusion; $p < 0.01$ in fraction 6; S-AMPA vs. vehicle; $n = 10$ /group) in the DR. **(c)** IL S-AMPA produced no behavioral response on the FST 2 h after drug microinfusion (vehicle; S-AMPA, $n = 9$ /group). The values are expressed as mean $\pm$ S.E.M. * $p < 0.05$ (significant main effect of treatment in the two-way ANOVA). The arrow represents the start of the IL microinfusion with vehicle or S-AMPA. Dialysate fractions were 25 min each.

a. Immediate antidepressant-like effects

b. Sustained antidepressant-like effects

Figure 6. Schematic diagram of serotonergic mechanism involved in the antidepressant-like responses evoked by IL DHK application. (a) Immediate antidepressant-like effects. Blockade of the glial GLT-1 transporter in the infralimbic cortex (IL) evokes an immediate local increase in glutamate, which then activates pyramidal neurons projecting –among other areas– to midbrain, thus increasing extracellular glutamate and 5-HT concentrations in the DR. The increased activity of serotonergic neurons is then translated into an increased 5-HT release in forebrain, including the IL and vHPC (not shown here, see Figure 2). The activation of 5-HT neurons bypasses self-inhibitory actions of 5-HT_{1A} autoreceptors, this allowing the appearance of the antidepressant response. Thick lines indicate enhanced activity of glutamatergic or serotonergic axons. **(b) Sustained antidepressant-like effects.** 24 h after IL DHK infusion, 5-HT remains elevated in the DR. In this condition, 5-HT may activate 5-HT_{1A} pre-synaptic receptors and attenuates forebrain 5-HT release. Removal of the self-inhibitory action through the administration of the 5-HT_{1A}-R antagonist WAY100635 allows the activation of the ascending 5-HT pathways and the emergence of the antidepressant-like response.

Supplementary Figure 1. Representative coronal sections of the rat brain (30 μ m) stained with neutral red showing the location of microinfusion sites into the infralimbic cortex (IL, AP: +4.2) and the microdialysate probes placement into the ventral hippocampus (vHPC, AP: -5.0) and the dorsal raphe nucleus (DR, AP: -7.2). Scale bar (1 mm).

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a. Immediate antidepressant-like effects

b. Sustained antidepressant-like effects

Supplementary figure 1

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