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**Regionally-Selective Knockdown of Astroglial Glutamate Transporters
in Infralimbic Cortex Induces a Depressive Phenotype in Mice**

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Interest (63)
Figures 7, Table 1

Main Points (2 to 3 short sentences and should not exceed 250 characters (including spaces))

- 1- Selective knockdown of GLAST/GLT-1 expression in mouse infralimbic cortex and reduced astrocyte density
- 2- Downstream attenuations of serotonergic function and BDNF expression
- 3- Depressive-like phenotype reversed by citalopram and ketamine

Abstract

Elevation of energy metabolism and disturbance of astrocyte number/function in the ventral anterior cingulate cortex (vACC) contributes to the pathophysiology of major depressive disorder (MDD). Functional hyperactivity of vACC may result from reduced astrocytic glutamate uptake and increased neuronal excitation. Here we tested this hypothesis by knocking-down astrocytic glutamate transporter GLAST/GLT-1 expression in mouse infralimbic (IL, rodent equivalent of vACC) or prelimbic (PrL) cortices using RNAi strategies. Unilateral siRNA (small interfering RNA) microinfusion targeting GLAST or GLT-1 in mouse IL induced a moderate (20-30%) and long-lasting (7 days) decrease in their expression. Intra-IL GLAST/GLT-1 siRNA microinfusion reduced the number of glial fibrillary acidic protein (GFAP)-positive and glutamine synthetase (GS)-positive astrocytes and evoked a depressive-like phenotype reversed by citalopram and ketamine. Intra-IL GLAST or GLT-1 knockdown markedly reduced serotonin (5-HT) release in the dorsal raphe nucleus (DR) and induced an overall reduction of brain derived neurotrophic factor (BDNF) expression in ipsilateral and contralateral hemispheres. Egr-1 (early growth response protein-1) labelling suggests that both siRNAs enhance the GABAergic tone onto DR 5-HT neurons, leading to an overall decrease of 5-HT function, likely related to the widespread reduction on BDNF expression. Conversely, similar reductions of GLAST and GLT-1 expression in PrL did not induce a depressive-like phenotype. These results suggest that a *focal* glial change in IL translates into *global* change of brain activity by virtue of the descending projections from IL to DR and the subsequent attenuation of serotonergic function in forebrain, an effect perhaps related to the varied symptomatology of MDD.

KEYWORDS: astrocytes, MDD, glutamate, infralimbic cortex, mouse model, serotonin

1 | INTRODUCTION

Major depressive disorder (MDD) is a severe life threatening, recurrent psychiatric disorder with a very large socioeconomic impact worldwide (Murray et al., 2012; Whiteford et al., 2013; Global Burden of Disease Study, 2015). This situation is mainly attributable to three reasons: i) the high prevalence of MDD (Kessler et al., 2007; Phillips et al., 2009; Baxter et al., 2013); ii) the emergence of depressive episodes during active periods of adult life (Ferrari et al., 2013); and iii) the slow action and limited efficacy of monoamine-based antidepressant treatments (Rush et al., 2006; Trivedi et al., 2006). Therefore, there is an urgent need to understand the pathophysiology of MDD and to develop better therapeutic strategies.

Clinical and experimental evidence accumulated in recent years have identified key brain areas involved in the pathophysiology of MDD, including the prefrontal cortex (PFC), hippocampus (HPC), cingulate cortex, amygdala and basal ganglia (Mayberg, 2009; Savitz & Drevets, 2009). Particularly, neuroimaging studies have identified alterations of activity as well as in functional connectivity between the aforementioned brain areas (Mayberg, 2009; Savitz & Drevets, 2009). Interestingly, a functional hyperactivity of the ventral anterior cingulate cortex (vAAC; Brodman area 25; Cg25) has been reported in MDD patients (Seminowicz et al., 2004; Mayberg et al., 2005), which normalizes after pharmacological, psychological and electrical (deep brain stimulation, DBS) treatments. However, early studies indicated a decreased energy metabolism in the vACC (subgenual ACC) of MDD patients (Drevets et al., 1997; Ongür et al., 1998), which disappeared when the original data were adjusted by the reduction of the grey matter volume (Drevets et al., 2015).

Astrocytes play a major role in excitatory neurotransmission by controlling the glutamate-glutamine cycle, which is fundamental for the synaptic plasticity associated

with cognitive processes (Araque & Perea, 2004; Perea & Araque 2007; Oliveira et al., 2008; Perea et al., 2014). Moreover, they have been proposed as an early contributor to the underlying pathogenesis of MDD (Sanacora & Banasr 2013; Haroon et al., 2017; Liu et al., 2017; Wang et al., 2017). Reductions of astrocyte number and alterations of astroglial function in fronto-limbic structures have been found in MDD patients and suicide victims (Banasr & Duman, 2008; Rajkowska & Stockmeier, 2013; Peng et al., 2015). Therefore, the functional hyperactivity observed in vACC of MDD patients may result from a disturbance of astrocytic-based glutamate homeostasis, with reduced glutamate uptake and increased neuronal excitation (Ressler & Mayberg, 2007; Berlim et al., 2014).

Based on the above evidence, here we tested the hypothesis that a hyperactive of infralimbic cortex in rodents (IL, equivalent to human VACC) may evoke adaptive changes in cortico-subcortical circuits leading to the emergence of a depressive-like phenotype. To mimic astroglial dysfunction in MDD patients, we developed two acute knockdown models of astrocytic glutamate transporters, GLAST (excitatory amino acid transporter -EAAT1- in humans) or GLT-1 (EAAT2 in humans), in mouse IL using RNAi strategies. Given that both GLAST and GLT-1 remove most of synaptic glutamate, small interfering RNA (siRNA) microinfusion in IL was expected to evoke a functional alteration of the descending excitatory input to subcortical structures (in particular to the brainstem monoamine nuclei, directly innervated by IL and PrL subdivisions; Gabbott et al., 2005). We paid a special attention to the effect of IL manipulations on serotonergic markers, given the key role of serotonin (5-hydroxytryptamine, 5-HT) in controlling resilience to stress and mediating clinical antidepressant actions. Similar procedures were conducted in the adjacent prelimbic cortex (PrL) to examine the regional selectivity of the effect. Finally, to evaluate the validity of the model, we attempted to reverse the

evoked behavioral changes with classical (citalopram) and fast-acting (ketamine) antidepressant treatments.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6J mice (10–14 weeks; Charles River, Lyon, France) were housed under controlled conditions (22±1°C; 12 h light/dark cycle) with food and water available *ad libitum*. All experimental procedures were conducted in accordance with National (Royal Decree 53/2013) and European legislation (Directive 2010/63/EU, on the protection of animals used for scientific purposes, 22 September 2010), and were approved by the Institutional Animal Care and Use Committee of the University of Barcelona.

2.2 | siRNA synthesis

Synthesis and purification of siRNA targeting GLAST and GLT-1 (GLAST siRNA: GenBank accession NM_148938 and GLT-1 siRNA: GenBank accession NM_001077514) and nonsense siRNA (NS siRNA) were performed by nLife Therapeutics S.L. (Granada, Spain). An equimolar pool of three different siRNA sequences (**Table 1, Supporting Information Figure S1**) targeting GLAST and GLT-1 mRNAs was infused as a mixture (4.2 nmol in total). A scrambled nonsense sequence (NS siRNA) was used as control.

NS siRNA was additionally labeled with Alexa488 or Alexa555 in the antisense strand to examine the extent of the diffusion after the local application in the infralimbic (IL) and prelimbic (PrL) cortices. Stock solutions were prepared in RNase-free water and stored at -20°C until use.

2.3 | Treatments

Mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and were placed in the stereotaxic frame. Mice received unilaterally 1 µl of 1) artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1.26 mM CaCl₂ and 1.18 mM MgCl₂ with 5% glucose), 2) NS siRNA or 3) a pool of three different siRNAs targeting GLAST or GLT-1 into IL (AP+2, ML-0.2, DV-3.3 in mm) or PrL (AP+2, ML-0.2, DV-2.2 in mm) (Franklin & Paxinos, 2008).

siRNA microinfusion was performed using a glass capillary (240 µm-outer diameter, 110 µm-inner diameter) fitted to a 10 µl Hamilton syringe connected to a precision minipump (KDS 310 plus nano legacy syringe pump, Kd Scientific) at a rate of 0.5 µl/min. GLAST or GLT-1 siRNA pool was prepared in RNase-free aCSF and infused intra-IL or intra-PrL at the single dose of 60 µg/µl (20 µg of each sequence; 4.2 nmol/dose). Mice were sacrificed at 1, 3 or 7 days after siRNA infusion.

To examine the siRNA diffusion in both cortices, mice were locally administered into IL or PrL with Alexa488 or Alexa555 labeled NS-siRNA (60 µg/µl) and sacrificed 6 h later.

2.4 | *In situ* hybridization

Mice were killed by pentobarbital overdose and brains were rapidly removed, frozen on dry ice and stored at -80°C. Coronal tissue sections (14 µm-thick) were cut using a microtome-cryostat (HM500-OM, Microm, Walldorf, Germany), thaw-mounted onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA)-coated slides and kept at -20°C until use. Antisense oligoprobes were complementary to bases: GLAST/3332-3382 (GenBank accession NM_148938), GLT-1/666-716 (NM_011393), neuronal excitatory amino-acid carrier 1-EAAC1/3064-3108 (NM_009199.2, referred as EAAT3),

vesicular glutamate transporter-vGLUT1/884-934 (NM_182993) and brain derived neurotrophic factor-BDNF/1188-1238 (NM_007540), respectively (Göttingen, Germany). Oligonucleotides were individually labeled (2 pmol) at the 3'-end with [³³P]-dATP (>2500 Ci/mmol; DuPont-NEN, Boston, MA) using terminal deoxynucleotidyltransferase (TdT, Calbiochem, La Jolla, CA). Details of ISH procedures are shown in the Supporting Information.

Films were analyzed and relative optical densities (ROD) were obtained using a computer assisted image analyzer (MCID, Mering, Germany) as previously described (Bortolozzi et al., 2012; Ferrés-Coy et al., 2016). The slide background and non-specific densities were subtracted. ROD was evaluated in 2-3 duplicate adjacent sections from each mouse and averaged to obtain individual values along the anteroposterior axis (three consecutive and equidistant measures between +1.98 to +1.54 mm from Bregma). MCID system was also used to acquire black and white images. Figures were prepared for publication using Adobe Photoshop software (Adobe Software, San Jose, CA, USA). Contrast and brightness of images were the only variables we adjusted digitally.

2.5 | Immunohistochemistry

Mice were anesthetized with pentobarbital and transcardially perfused with 4% paraformaldehyde in sodium-phosphate buffer (pH 7.4). Brains were collected, post-fixed 24 h at 4°C in the same solution, and then placed in gradient sucrose 10–30% for 3 days at 4°C. After cryopreservation, serial sections (30 µm-thick) were cut through the PFC and midbrain raphe nuclei. Immunohistochemical procedures were performed for GLAST (rabbit anti-GLAST; 1:200; ab416, Abcam, Cambridge, UK), GLT-1 (rabbit anti-GLT-1; 1:500; ab41621, Abcam), glial fibrillary acidic protein - GFAP (rabbit anti-GFAP; 1:1000; Z0334, Dako, Santa Clara, USA) and glutamine synthetase - GS (rabbit

anti-GS; 1:5000; ab49873, Abcam) using staining biotin-labeled antibody procedure. Details are shown in the Supporting Information. The intensity of the labelling was quantified using ImageJ (v1.49g) software in three equidistantly sections between +1.98 to +1,54 mm from Bregma for each mouse.

2.6 | Confocal fluorescence microscopy

Alexa488- and Alexa555-NS siRNA localization in IL and PrL was examined by confocal microscopy using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Two different immediate-early genes (IEG) markers were used to visualize neuronal activity at physiological levels (Egr-1, early growth response protein-1, also known as Zif268) or after high induction threshold (c-Fos) (Worley, et al., 1991; Okuno, 2011). 5-HT neuronal activity in the dorsal raphe nucleus (DR) was evaluated by immunofluorescence using primary antibodies against tryptophan hydroxylase2 (TPH₂) and Egr-1, while neuronal activation in the IL was examined using primary antibodies against NeuN and c-Fos. Adjacent free-floating midbrain sections (30-µm) were rinsed with PBS/Triton 0.2%, incubated with normal serum from secondary antibody host and treated with primary antibodies: TPH₂ (sheep anti-TPH₂; 1:2500; ref.: AB1541, Merck Millipore, Madrid, Spain), Egr-1 (rabbit anti-Egr-1; 1:200; ref.: ab208780, Abcam), NeuN (mouse anti-NeuN; 1:500; ref: MAB 377, Merck Millipore) and c-Fos (rabbit anti-c-fos; 1:500; ref: ab102699, Abcam). Sections were then incubated overnight at 4°C, rinsed and treated with their respective secondary Alexa488 and Alexa555-conjugated antibody (donkey anti-rabbit and donkey anti-sheep; 1:500; ref.: A-31572/A-21436, Life Technologies, Carlsbad, CA, USA) for 120 min. Details are shown in the Supporting Information.

Images were obtained at 400Hz in a 1024 x 1024 pixels format and were composed using ImageJ (1.49g) software. Confocal analysis was performed by measuring Alexa555-Egr-1 intensity labelling in TPH₂⁺ cells and non-TPH₂⁺ in sections at three different AP coordinates per duplicate for each mouse. Alexa488-TPH₂ staining was used as a mask to quantify specifically Egr-1 intensity in TPH₂⁺ cells. Non-TPH₂⁺ cells labelling Egr1 was determined by extracting TPH₂ staining to the original image and measuring Alexa555 intensity in 7-μm radial distance from Hoescht-labelled nucleus into DR.

2.7 | Drugs

All reagents used were of analytical grade and were obtained from Merck (Germany). Veratridine and bicuculline were from Sigma-Aldrich-RBI (Madrid, Spain), citalopram from Tocris (Madrid, Spain) and ketamine from Hospital Clinic Pharmacy Service (Barcelona, Spain). Concentrated solutions (1 mM; pH adjusted to 6.5–7 with NaHCO₃ when necessary) were stored at -80°C and working solutions were prepared daily by dilution in aCSF. For microdialysis experiments, drugs were dissolved in aCSF (except for veratridine dissolved in aCSF + 1% of dimethyl sulfoxide - DMSO), and administered by reverse dialysis at the stated concentrations. For behavioral studies, drugs were dissolved in saline solution.

2.8 | Intracerebral microdialysis

Extracellular 5-HT concentrations in DR were measured by *in vivo* microdialysis as previously described (Bortolozzi et al., 2012; Ferrés-Coy et al., 2016). Briefly, one concentric dialysis probe (Cuprophane; 1.5mm-long) was implanted in the DR (coordinates in mm: AP-4.5; ML+1; DV-4.2 with a lateral angle of 20°) of anaesthetized

1 mice. Experiments were performed 24-48 h after surgery. The probes were continuously
2 perfused (WPI model, SP220i) with aCSF containing 1 μ M citalopram at 1.64 μ l/min and
3 20-min fractions were collected. 5-HT concentrations were analyzed by high performance
4 liquid chromatography (HPLC) with electrochemical detection (Waters 2465) at +0.75V
5 with detection limit of 1-2 fmol/sample. Baseline 5-HT levels were calculated as the
6 average of six pre-drug samples.

7 8 **2.9 | Behavioral studies**

9 All behavioral assessments were performed between 9:00-15:00 h, 24 h after the local
10 microinfusion of aCSF or siRNA targeting GLAST or GLT-1. Mice were evaluated in
11 different behavioral paradigms: i) open field test (OFT), ii) tail suspension test (TST), iii)
12 forced swim test (FST) and, iv) sucrose preference test (SPT) as previously reported
13 (Bortolozzi et al., 2012; Ferrés-Coy et al., 2016). On test day, animals were moved to a
14 dimly illuminated room and were left undisturbed for at least 1 h before testing. An
15 experimenter blinds to treatments performed all behavioral tests. Details are shown in the
16 Supporting Information.

17 18 **2.10 | Statistical analyses**

19 Results are given as mean $\pm$ SEM. Statistical analyses were performed by two-tailed
20 Student's *t*-test and one-way or two-way ANOVA followed by Tukey's *post-hoc* test as
21 appropriate (GraphPad Prism 7.01; San Diego, CA). Statistical significance has been set
22 at the 95% confidence level.

23 24 25 **3 | RESULTS**

3.1 | Selective downregulation of GLAST and GLT-1 expression in IL and PrL

We investigated the feasibility of selectively knocking-down GLAST and GLT-1 expression in IL and PrL using a mixture of three siRNA sequences targeting GLAST (GLAST siRNA) or GLT-1 (GLT-1 siRNA) (**Table 1, Supporting Information Figure S1**). To examine the extent of siRNA diffusion after its local administration, we applied a nonsense siRNA sequence (NS-siRNA, 60 µg/µl) labeled with a fluorophore (A488 or A555). Mice were sacrificed 6 h later. Confocal microscopy analysis revealed that A488- and A555-NS-siRNA were specifically localized in the application site (IL or PrL) reaching a radial siRNA distribution of about 90 µm/h (**Figure 1a**).

Next, we examined the temporal pattern of GLAST and GLT1 expression in IL and PrL (from AP +1.98 to + 1.54 mm) after acute treatment with GLAST or GLT-1 siRNAs. Mice were unilaterally microinjected into IL or PrL with: (1) vehicle (aCSF), (1) NS-siRNA or (3) GLAST or GLT-1 siRNAs (60 µg/µl, 20 µg of each siRNA sequence; total amount 4.2 nmol) and euthanized at different times after the last administration. GLAST/GLT-1 siRNAs reduced GLAST and GLT-1 mRNA levels along the anteroposterior (AP) axis of IL at 1, 3 and 7 days post-administration, as assessed by *in situ* hybridization (**Figures 1b,c**). Maximal reduction of GLAST mRNA level in IL was ~20% compared to contralateral side, while the corresponding value for GLT-1 mRNA level was ~31% at 1 and 7 days post-infusion, respectively (**Figure 1c**). Similar reductions of GLAST and GLT-1 mRNA expression were found after GLAST or GLT-1 siRNA infusion into PrL (**Supporting Information Figure S2**). Maximal reductions of GLAST and GLT-1 mRNA levels were ~23% and ~30% in ipsilateral PrL compared to contralateral hemisphere, respectively (**Supporting Information Figure S2b**).

There was no cross-silencing between astroglial glutamate transporters in any of the different AP coordinates examined in IL (**Supporting Information Figures S3a,b**).

Likewise, siRNA effects were confined to the application site, and no reductions in the expression of astrocytic transporters in PrL were found after the siRNA infused into IL, and vice versa (**Supporting Information Figures S3c,d**). Additionally, neither EAAC1 (EAAT3) nor vGLUT1 mRNAs expressed by neurons were affected by intra-IL GLAST or GLT-1 infusion (**Supporting Information Figure S4**). Finally, GLAST and GLT-1 mRNA expression in IL and PrL was unmodified by the local infusion of a NS siRNA sequence (**Supporting Information Figure S5**). Overall, these data support the specificity of siRNA sequences for each target gene as well as the restriction of the effects of siRNA sequences to the application site.

The reduced GLAST and GLT-1 mRNA expression in IL was further confirmed by immunohistochemistry procedures showing also reductions of GLAST and GLT-1 proteins (**Figures 1d,e**). GLAST siRNA (60 µg/µl) decreased GLAST protein level in the ipsilateral IL to $85.2 \pm 2.1\%$ of aCSF-treated mice (average value between APs from +1.98 to + 1.54 mm). Likewise, GLT-1 siRNA reduced GLT-1 protein level in the ipsilateral IL to $84.5 \pm 2.8\%$ of aCSF-treated mice (average value between APs from +1.98 to + 1.54 mm).

3.2 | Local GLAST or GLT-1 knockdown reduces astrocyte number in IL

Afterwards, we evaluated whether the downregulation of astroglial glutamate transporters triggered by a single dose of siRNAs was also affecting the number and morphology of astrocytes. Immunohistochemistry analysis of GFAP⁺ cells of mice treated with GLAST or GLT-1 siRNAs into IL showed significant changes in different astroglial markers in the ipsilateral IL compared to the control group treated with aCSF. We found significant reductions of 1) total GFAP integrated density (vehicle: $5.8 \times 10^8 \pm 60235$, GLAST-siRNA: $4.9 \times 10^8 \pm 72489$, GLT-1-siRNA: $4.7 \times 10^8 \pm 54924$); 2) intracellular GFAP density

(vehicle: 37289 ± 2547 , GLAST-siRNA: 31178 ± 1985 , GLT-1-siRNA: 28839 ± 3012); and 3) the number of GFAP⁺ cells (aCSF: 530.7 ± 20.33 , GLAST-siRNA: 423.1 ± 17.66 , GLT-1-siRNA: 405.2 ± 60.2) in the ipsilateral IL of siRNA treated mice (**Figures 2a,b**). However, no changes were detected in these astrocyte markers in the contralateral IL, which were used as negative controls (**Figures 2a,b**).

Similarly, the reduction of GS⁺ astrocyte number and of intracellular GS⁺ density levels in siRNA treated mice compared to aCSF group confirmed the loss of astrocytes in IL after GLAST or GLT-1 knockdown (**Figures 2c,d**). Significant differences were found in 1) total GS integrated density (vehicle: $3.6 \times 10^8 \pm 840401$, GLAST-siRNA: $3.0 \times 10^8 \pm 382645$, GLT-1-siRNA: $3.0 \times 10^8 \pm 788840$); 2) intracellular GS density (vehicle: $8.0 \times 10^8 \pm 692063$, GLAST-siRNA: $4.0 \times 10^8 \pm 558870$, GLT-1-siRNA: $3.7 \times 10^8 \pm 221512$); and 3) the number of GS⁺ cells (aCSF: 531.2 ± 19.14 , GLAST-siRNA: 312.6 ± 21.7 , GLT-1-siRNA: 326.5 ± 12.5) in the ipsilateral IL of siRNA treated mice. No such changes were observed in the contralateral IL (**Figures 2c,d**). Taken together, these data indicate that the diminished levels of astroglial glutamate transporters, GLAST and GLT-1 proteins, adversely affects other proteins linked to astrocyte function and survival.

3.3 | Focal reduction of astroglial GLAST or GLT-1 expression in IL induces a depressive-like state in mice

In order to examine whether GLAST and GLT-1 knockdown in IL could evoke a depressive-like phenotype, we conducted several behavioral paradigms routinely used to assess depressive-like behaviors in rodents. Mice received intra-IL: 1) aCSF, 2) NS siRNA or 3) GLAST or GLT-1 siRNAs (60 µg/µl) and were evaluated 24 h after administration. Downregulation of GLAST or GLT-1 in IL significantly increased the immobility time of mice in the TST, as compared to control groups (aCSF- and NS

siRNA- treated mice) (**Figure 3a**). In contrast, despite showing similar reductions of GLAST and GLT-1 expression (**Supporting Information Figure S1**), mice treated with GLAST or GLT-1 siRNAs (60 $\mu\text{g}/\mu\text{l}$) into PrL did not exhibit differences in TST performance compared to control group (aCSF group: 178.9 ± 5.1 ; GLAST siRNA group: 183.9 ± 7.8 ; GLT-1 siRNA group: 179.4 ± 3.2 expressed as seconds).

We also examined the emergence of depressive-like responses in IL GLAST or GLT1 knockdown mice using the FST and SFT. Mice microinfused in IL with GLAST or GLT-1 siRNAs showed an increased immobility time in the FST compared to control groups (**Figure 3b**). Furthermore, GLAST or GLT1 siRNA-treated mice showed a marked reduction of sucrose preference and sucrose intake when compared with control mice (**Figure 3c**). Neither of these behavioral changes was driven by changes in locomotor activity, as assessed by the OFT (**Figure 3d**).

Next, we evaluated whether the acute administration of the SSRI citalopram (10 mg/kg, i.p.) or the non-competitive NMDA receptor antagonist ketamine (10 mg/kg, i.p.) was able to attenuate the increased TST immobility induced by GLT-1 knockdown. Thirty min before TST, citalopram or ketamine were systemically administered. Interestingly, both drugs completely reversed the depressive-like phenotype evoked by GLT-1 siRNA infusion in IL cortex (**Figure 3e**). Altogether, these data support the astrocytic glutamate transporter knockdown of expression/function selectively in IL as a new mouse model of MDD.

3.4 | Focal knockdown of GLAST or GLT-1 increases neuronal activity in IL and reduces the activity of TPH₂⁺ cells in the DR

Morphological and functional interactions between astrocytes and neurons, such as the homeostatic control of surrounding synapses (energy supply, clearance of potassium and

glutamate, among others) have been well described (Perea et al., 2014). Therefore, to explore the influence of the knockdown of astroglial transporters on the local neuronal activation, we examined the effect of GLAST and GLT-1 siRNA application on c-Fos immunoreactivity in the medial prefrontal cortex (mPFC) sections containing the IL. Confocal microscopy analysis revealed a marked elevation of c-Fos protein levels in NeuN⁺ cells of IL neurons (layers I to VI) of GLAST and GLT-1 siRNA-treated mice compared to control group (**Figures 4b,c and Supporting Information Figure S6**).

Given i) the existence of an anatomical/functional reciprocal control between mPFC and midbrain 5-HT neurons (Hajós et al., 1998; Celada et al., 2001, 2013; Vertes, 2004, Gabbott et al., 2005), ii) the involvement of this pathway in coping with challenging situations (Warden et al., 2012), and iii) the key role played by 5-HT neurotransmission in resilience to stress and antidepressant effects (Artigas, 2015); we examined whether the activated neurons in IL could evoke downstream changes on DR 5-HT function (**Figure 4a**).

We used Egr-1 labelling as a surrogate marker of physiological neuronal activity associated to the elevation of cytosolic Ca²⁺ (Knapska & Kaczmarek 2004) in order to examine the distal effects of intra-IL GLAST or GLT-1 siRNAs on the activity of 5-HT and non-5-HT neuronal populations in the DR. To this end, midbrain sections containing the DR were immunelabelled with TPH₂ and Egr-1 antibodies. Confocal analysis of Egr-1 intensity labelling in TPH₂⁺ and non-TPH₂⁺ cells showed that GLAST and GLT-1 siRNA-treated mice exhibited significantly higher levels of Egr-1 in both cellular components of the DR, as compared to the control group (**Figures 4d,e and Supporting Information Figure S7**). Remarkably, this increase was higher in non-TPH₂⁺ than in TPH₂⁺ cells, indicating a larger activation of local non-5-HT neurons (putative

GABAergic interneurons) in mice treated with GLAST or GLT-1 siRNA in IL (**Figure 4f**).

3.5 | GLAST or GLT-1 knockdown in IL reduces serotonergic function

Using microdialysis procedures, we detected a significant reduction of baseline 5-HT levels in the DR of mice treated with GLAST or GLT-1 siRNAs in IL, compared with their respective control groups (aCSF: 10.7 ± 0.7 , GLAST siRNA: 3.9 ± 0.2 ; aCSF: 12.7 ± 1.0 , GLT-1 siRNA: 6.4 ± 0.9 expressed as fmol/20-min fraction) (**Figures 5a,b**). However, no differences in the extracellular 5-HT concentration in DR emerged after GLT-1 siRNA microinjection in PrL (vehicle: 12.6 ± 1.0 , GLT-1 siRNA: 12.2 ± 0.5 expressed as fmol/20-min fraction) (**Supporting Information Figure S8a**).

Local infusion of the depolarizing agent veratridine ($50 \mu\text{M}$) by reverse dialysis increased similarly ~6-8-fold 5-HT release in the DR of GLAST or GLT-1 knockdown and control mice (**Figures 5c,d and Supporting Information Figure S8b**), suggesting that the baseline 5-HT differences in intra-IL siRNA- and aCSF-treated mice were not due to differences in intracellular 5-HT stores.

Descending afferents from the mPFC exert a tight inhibitory control of DR 5-HT neurons via local GABA interneurons (Hajós et al. 1998; Celada et al. 2001, Varga et al., 2001), suggesting that the above differences in basal extracellular 5-HT in DR might be mediated by a distinct GABA input on both experimental groups. Therefore, we examined the effects of local GABA_A receptor blockade on extracellular 5-HT concentrations. Local bicuculline infusion ($30 \mu\text{M}$) enhanced 5-HT release in the DR, with a significantly greater effect in mice treated with intra-IL GLAST and GLT-1 siRNAs compared to control group (**Figures 5e,f**). In contrast, local bicuculline infusion evoked a similar increase of DR 5-HT release in mice treated with GLT-1 siRNA or aCSF into the PrL

(**Supporting Information Figure S8c**). Overall, these results support the view that GLAST and GLT-1 knockdown in IL enhances GABA_A-mediated inhibition of DR 5-HT neurons, likely through the activation of descending pathways from mPFC (Hajós et al. 1998; Celada et al. 2001; Varga et al. 2001). On the other hand, the different effects on 5-HT release in mice treated with GLT-1 siRNA in IL or PrL suggest a distinct role of each mPFC subdivisions in the control of DR 5-HT neuron activity.

3.6 | GLAST or GLT-1 knockdown in IL decreases BDNF mRNA expression in mPFC and HPC

Neurotrophic (growth) factors, specifically BDNF, have been strongly implicated in the pathophysiology of MDD (Duman & Monteggia 2006; Krishnan & Nestler 2008). In particular, reduced BDNF levels are relevant to the structural alterations caused by stress and MDD, as this neurotrophic factor is required for the activity-dependent synapse formation and its maintenance (Duman et al., 2016). We therefore examined whether the siRNA-induced knockdown of GLAST or GLT-1 in IL was associated to a reduction of BDNF expression in two key brain areas involved in MDD such as the mPFC and HPC.

Acute GLAST or GLT-1 knockdown in IL evoked an average ~25% reduction of the BDNF expression in both ipsilateral subdivisions (IL and PrL) of the mPFC. Interestingly, similar reductions of BDNF expression were also observed in the contralateral IL and PrL of intra-IL GLAST knockdown mice (**Figures 6a,b**).

Likewise, intra-IL GLAST or GLT1 siRNA infusion decreased BDNF expression in all -ipsilateral and contralateral- HPC subdivisions examined (CA1, CA2, CA3 and DG) compared to aCSF treated mice (**Figures 6a,b**).

Remarkably, BDNF mRNA levels in all brain areas examined of the ipsilateral and contralateral hemispheres were positively and significantly correlated with GLAST

and GLT-1 mRNA levels in the ipsilateral IL (**Figures 7a,b**). Overall, these data indicate that the selective reduction of both glial glutamate transporter expression in IL cortex evoked a widespread alteration of BDNF expression in mPFC and HPC, an effect likely associated to the depressive-like phenotype.

4 | DISCUSSION

Here, we show that a regionally selective knockdown of GLAST or GLT-1 in IL evokes widespread behavioral, cellular and neurochemical changes in mice, reminiscent of those observed in MDD patients. A moderate decrease of GLAST or GLT-1 expression in IL was enough to induce local astroglial disturbances and marked downstream attenuations of serotonergic function and of BDNF expression in mPFC and HPC, changes being classically associated to a depressive-like behavior. None of these changes happened when similar siRNA-induced reductions of GLAST and GLT-1 expression occurred in the adjacent PrL, which supports a crucial role of IL (rodent equivalent of vACC) in the emergence of an MDD-like phenotype. Interestingly, behavioral changes were reversed by classical (citalopram) and fast-acting (ketamine) antidepressant treatments, which supports the validity of the model. Overall, the present observations give rise to a highly translational model, mimicking pathophysiological changes in MDD, including alterations in glial number/function, reduction of 5-HT markers and of BDNF expression in cortico-limbic areas. On the other hand, the present data add to previous studies showing the suitability of RNAi strategies to modulate genes relevant for the pathophysiology and treatment of MDD in rodent brain (Artigas & Bortolozzi, 2017; Artigas et al., 2018).

Methodological considerations. Given the anatomical proximity between IL and PrL in mouse brain, we paid a particular attention to confirm that siRNA-evoked changes were confined to the area of interest. First, we used a microinjection procedure allowing the delivery of small amounts of siRNA to discrete nuclei in mouse brain, such as the DR (Ferrés-Coy et al., 2013a,b). Second, we used histological procedures (*in situ* hybridization, immunohistochemistry and confocal microscopy) to evaluate the changes of different mRNAs and proteins in tissue sections at several AP levels in the structures examined, thus providing a detailed anatomical specificity. Notably, the present results support selective effects of the siRNAs on mRNA targets were restricted to the application site (IL or PrL). Hence, GLAST or GLT-1 expression in PrL was unaffected by the intra-IL GLAST or GLT-1 siRNA application and vice versa. Likewise, despite siRNAs may have off-target effects (Artigas et al., 2018), GLAST and GLT-1 siRNAs did not affect the expression of each other mRNA nor that of neuronal glutamate transporters (EAAC1 and vGLUT1), showing an excellent target specificity. Moreover, the same amount of nonsense siRNA infused into IL or PrL did not change GLAST or GLT-1 expression. The regional and target selectivity of the present procedure is also due to the use of low siRNA concentrations, consistent with previous observations, which allowed the knockdown of genes expressed by 5-HT neurons in mouse DR, without affecting the neighboring median raphe nucleus (Ferrés-Coy et al., 2013a,b). Third, while siRNAs are become powerful tools for silencing brain genes, an important issue concerns the effective delivery of siRNA to their respective sites of action in the cytoplasm. In cell cultures, delivery agents (such as cationic lipids or polymers) are required to achieve significant siRNA effects (Juliano et al., 2008). However, small amounts of naked non-viral siRNAs efficiently knockdown gene expression *in vivo* (Cryan et al., 2007; Juliano et al., 2008; Ferrés-Coy et al., 2013a,b, Alarcón-Arís et al., 2018; present study). Overall,

our results demonstrate a selective and sustained (at least for 7 days) reduction of GLAST or GLT-1 mRNA expression (20-30%) in IL or PrL of mice that mimics a comparable decrease of EAAT1 (GLAST) and EAAT2 (GLT-1) mRNA levels (30-50%) in vACC and dorsolateral prefrontal cortex of MDD patients (Zhao et al., 2016).

Alterations of astroglial number/function in fronto-limbic structures have been associated with MDD (Altshuler et al., 2010; Rajkowska & Stockmeier, 2013; Peng et al., 2015). Studies in post-mortem brain samples from MDD patients and suicide victims revealed reductions in the number and changes in the morphology of astrocytes (Rajkowska et al., 1999; Nagy et al., 2015; Torres-Plata et al., 2016). Preclinical and clinical studies reported decreases in mRNA and protein expression for astrocytic markers such as GFAP, GS, gap junction proteins (connexin 40 and 43), water channel aquaporin-4 (AQP4), EAAT1/GLAST and EAAT2/GLT-1 (Miguel-Hidalgo et al., 2000, 2010, 2014; Choudary et al., 2005; Sequeira et al., 2009; Gittins & Harrison, 2011; Bernstein et al., 2015; Zhao et al., 2016). The tight –perhaps causal- relationship between astrocyte dysfunction and depressive-like behavior is also supported by several experimental approaches using glial toxins (Banasr & Duman, 2008), blunting astrocyte vesicular release (Cao et al., 2013), disrupting connexin-mediated gap-junctions (Sun et al., 2012), or inhibiting astrocyte GLT-1 glutamate transporter using dihydrokainic acid-DHK (Bechtholt-Gompf et al., 2010; John et al., 2012; but not Gasull-Camós et al., 2017). In the present mouse model, a single unilateral GLAST or GLT-1 siRNA dose into IL was sufficient to decrease GLAST and GLT-1 expression for at least 7 days, ultimately inducing significant reductions of GFAP⁺ and GS⁺ immunoreactive astrocytes (reductions of ~20-28% comparable with 24% described in MDD patients) (Ongur et al., 1998; Sanacora & Banasr, 2013; Bernstein et al., 2015). The sequence of events leading from reduced GLAST/GLT-1 expression to astrocyte loss is not fully understood. Based on the

1 abovementioned studies, one possibility is that decreased levels of GLAST/GLT-1 and
2 GS could cause a glutamate-mediated astroglial toxicity. Likewise, it was reported that
3 the reduced GFAP density decreases the traffic of astroglial glutamate transporters to cell
4 surface, which would cause an additional increase in excitotoxicity and loss in astrocyte
5 number, as shown in our model (Hughes et al., 2004; Sullivan et al., 2007).

6 The present observations add to the increasing body of evidence linking MDD to
7 an abnormal astrocyte function and support the IL as a crucial brain area for its
8 pathophysiology. Early hypothesis on MDD focused on a reduced activity of brainstem
9 monoaminergic systems (mainly 5-HT and norepinephrine, NE). One commonality of
10 these ascending monoamine systems is that they are densely innervated by descending
11 afferents from the PFC (Groenewegen & Uylings, 2000; Vertes, 2004; Gabbott et al.,
12 2005), which tightly control their activity via direct (e.g., monosynaptic, excitatory) and
13 indirect inputs (e.g., local GABA interneurons and self-inhibitory responses) (Hajós et
14 al., 1998; Jodo et al., 1998; Celada et al., 2001; Martín-Ruiz et al., 2001). In particular,
15 the mPFC-raphé projection plays a major role in coping with behavioral challenges
16 (Warden et al., 2012). Therefore, given the top-down control exerted by the PFC on 5-
17 HT/NE activity and the widespread brain innervation by these monoamine systems, a
18 *focal* alteration of neuronal activity in a restricted PFC area (e.g., IL) may translate into a
19 *global* change in brain activity resulting from monoamine hypofunction, and leading to
20 the multiple and varied depressive symptomatology (Artigas, 2015). Supporting this
21 hypothesis, at least regarding 5-HT, the selective reduction of GLAST and GLT-1
22 expression in IL evoked a very marked reduction of 5-HT release in the DR, and a
23 widespread fall in BDNF expression, not only in IL, but also in subcortical areas such as
24 the ipsilateral and contralateral hippocampal formation. Since the HPC does not receive
25 direct projections from the PFC in rodent brain (Gabbott et al., 2005), the significant

1 decrease in hippocampal BDNF expression induced by GLAST and GLT-1 knockdown
2 in IL cannot be explained by a direct effect of IL on HPC activity. More convincingly and
3 given the relevance of the 5-HT system for BDNF expression (Nibuya et al., 1996; Ivy et
4 al., 2003; Martinowich & Lu, 2008), the reduced serotonergic function may drive the
5 changes in BDNF expression. Indeed, decreased BDNF mRNA levels were found in both
6 hemispheres, an observation consistent with the involvement of 5-HT neurons, located in
7 the brain midline and projecting to both hemispheres.

8 The absence of behavioral effects in the TST and of 5-HT changes after
9 GLAST/GLT-1 knockdown in PrL indicates that both mPFC subdivisions control 5-HT
10 neuron activity in a different manner. In the rat, IL and PrL show differential projections
11 to cortical and subcortical areas, including the raphe nuclei (Vertes, 2004; Gabbott et al.,
12 2005, Ji & Neugebauer, 2012). Given the role of GLAST/GLT-1 on synaptic glutamate
13 level control, their siRNA-induced knockdown leaves a marked neuronal activation, as
14 indicated by increased c-Fos expression and, by the more depolarized membrane potential
15 and increased firing activity observed in layer V pyramidal neurons of siRNA-treated
16 mice (Fullana et al., in preparation). Hence, the activation of DR-projecting pyramidal
17 neurons would subsequently attenuate serotonergic activity via raphe GABA interneurons
18 (Hajós et al., 1998; Celada et al., 2001; Varga et al., 2001) and self-inhibition through 5-
19 HT_{1A} receptors (Celada et al., 2001), thus evoking a reduction of firing-dependent 5-HT
20 release. Of note, this is in line with the observations that GLAST and GLT-1 knockdown
21 increased the number of Egr-1⁺ cells in the DR compared to controls, yet, with a relatively
22 greater proportion of non-TPH₂⁺ Egr-1-labelled cells than of TPH₂⁺ cells, suggesting a
23 greater activation of putative GABA interneurons than of 5-HT neurons.

24 Furthermore, this view is supported by the greater bicuculline-mediated increase
25 of 5-HT release in the DR of GLAST and GLT-1 knockdown mice, as compared to

control mice, confirming a greater local GABAergic tone in the former mice. The marked neurobiological and behavioral differences evoked by GLAST and GLT-1 knockdown in IL and PrL warrant further investigation on the role of both mPFC subdivisions in the control of 5-HT neuronal activity, an aspect not appropriately examined in previous electrophysiological studies on the mPFC control of serotonergic activity (Hajós et al., 1998; Celada et al., 2001).

The relevance of IL, but not PrL, in mood control is also supported by recent studies in rats using pharmacological approaches (Fuchikami et al., 2015; Gasull-Camós et al., 2017). Direct application of S-AMPA, ketamine or DHK (GLT-1 inhibitor) into rat IL evoked acute antidepressant-like effects. In particular, those involved the attenuation of IL GLT-1 expression (present study) or function (Gasull-Camós et al., 2017) may appear contradictory, since both depressive-like and antidepressant-like effects were observed. However, these differences may be partly explained by the different stimulation of glutamate receptors in IL resulting from: 1) a single-point, selective activation of AMPA receptors (Gasull-Camós et al., 2017) or 2) a long-lasting activation (1 to 7 days) of ionotropic and metabotropic glutamate receptors after genetic regulation of GLAST and GLT-1 induced by siRNA strategies (present study). Interestingly, blockade of astrocytic glutamate uptake with DHK (i.c.v.) induced signs of anhedonia and impaired spatial memory in rats (Bechtholt-Gompf et al., 2010) and, depressed excitatory synaptic transmission in hippocampal neuron culture (Maki et al., 1994), suggesting that species differences may also contribute. Moreover, selective optogenetic stimulation of astrocytes induced synaptic long-term depression in the hippocampal CA1 region through activation of P2Y receptors by ATP released from astrocytes (Chen et al., 2013). Despite the presence of such inhibitory mechanisms has not been established in cortical areas yet,

1 they indicate a complex regulation of excitatory and inhibitory synaptic transmission
2 exerted by astrocytes.

3 Interestingly, a classical (citalopram) and a fast-acting antidepressant (ketamine)
4 reversed the behavioral effect of GLT-1 knockdown in IL, thus supporting the validity of
5 the model. The mechanisms involved are not known, but they may involve an attenuation
6 of the increased neuronal activation evoked by GLT-1 knockdown. On the one hand, the
7 augmented 5-HT evoked by citalopram in IL would preferentially activate inhibitory 5-
8 HT_{1A} receptors, which are expressed by a twice as much neuronal proportion than
9 excitatory 5-HT_{2A} receptors in mouse IL (Amargós-Bosch et al, 2004), thus reducing the
10 activity of pyramidal neurons induced by GLT-1 knockdown. On the other hand, the
11 blockade of NMDA receptors in overactive neurons by ketamine may also reduce their
12 neuronal discharge and their downstream effects on DR neurons. Additionally, ketamine
13 increases 5-HT release in mPFC (Amargós-Bosch et al., 2007), an effect that may also
14 contribute to the attenuation of pyramidal neuron hyperactivity in IL through the same
15 mechanisms than citalopram.

16 In summary, the present study shows that the regionally-selective knockdown of
17 glutamate uptake by astrocytes in IL evokes a depressive-like phenotype in mice. To our
18 knowledge, this is the first study showing that a *focal* alteration in a cortical area can
19 evoke *global* changes in brain activity, associated to the behavioral MDD-like phenotype
20 and to a serotonergic hypoactivity. In addition, given the dense projections from IL to the
21 ventral striatum (Gabbott et al., 2005) and the key role of the latter area in reward
22 processing, its additional involvement cannot be discarded. Further work will help to
23 clarify the precise brain circuits involved.

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CONFLICT OF INTEREST

F.A. has received consulting honoraria on antidepressant drugs from Lundbeck and he has been PI of grants from Lundbeck. A.B. has been PI of grants from Nlife Therapeutics S.L. F.A. and A.B. are co-authors of the patent WO/2011/131693 for the siRNA and ASO (antisense oligonucleotides) molecules and the targeting approach related to this work. The rest of authors declare no competing financial interest.

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REFERENCES

- Alarcón-Arís, D., Recasens, A., Galofré, M., Carballo-Carbajal, I., Zacchi, N., Ruiz-Bronchal, E.,...Bortolozzi, A. (2018). Selective α -synuclein knockdown in monoamine neurons by intranasal oligonucleotide delivery: potential therapy for Parkinson's disease. *Molecular Therapy*, 26, 550-567. doi: 10.1016/j.ymthe.2017.11.015
- Altshuler, L. L., Abulseoud, O. A., Foland-Ross, L., Bartzokis, G., Chang, S., Mintz, J.,...Vinters, H. V. (2010). Amygdala astrocyte reduction in subjects with major depressive disorder but not bipolar disorder. *Bipolar Disorder*, 12, 541-549. doi: 10.1111/j.1399-5618.2010.00838
- Amargós-Bosch, M., Bortolozzi, A., Puig, M. V., Serrats, J., Adell, A., Celada, P.,... Artigas, F. (2004). Co-expression and in vivo interaction of serotonin1A and serotonin2A receptors in pyramidal neurons of prefrontal cortex. *Cerebral Cortex*, 14, 281-299.
- Amargós-Bosch, M., Adell, A., & Artigas, F. (2007). Antipsychotic drugs reverse the AMPA receptor-stimulated release of 5-HT in the medial prefrontal cortex. *Journal of Neurochemistry*, 102, 550-561. doi: 10.1111/j.1471-4159.2007.04532.x
- Araque, A., & Perea, G. (2004). Glial modulation of synaptic transmission in culture. *Glia*, 47, 241-248
- Artigas, F. (2015). Developments in the field of antidepressants, where do we go now? *European Neuropsychopharmacology*, 25, 657-670. doi:10.1016/j.euroneuro.2013.04.013
- Artigas, F., & Bortolozzi, A. (2017). Therapeutic potential of conjugated siRNAs for the treatment of major depressive disorder. *Neuropsychopharmacology*, 42, 317. doi: 10.1038/npp.2016.182

- 1 Artigas, F., Celada, P., & Bortolozzi, A. (2018). Can we increase the speed and efficacy
2 of antidepressant treatments? Part II. Glutamatergic and RNA interference
3 strategies. *European Neuropsychopharmacology*, 28, 457-482. doi:
4 10.1016/j.euroneuro.2018.01.005
- 5 Banasr, M., & Duman, R. S. (2008). Glial loss in the prefrontal cortex is sufficient to
6 induce depressive-like behaviors. *Biological Psychiatry*, 64, 863-870. doi:
7 10.1016/j.biopsych.2008.06.008
- 8 Baxter, A. J., Patton, G., Scott, K. M., Degenhardt, L., & Whiteford, H. A. (2013). Global
9 epidemiology of mental disorders: what are we missing? *PLoSOne*, 8, e65514. doi:
10 10.1371/journal.pone.0065514
- 11 Bechtholt-Gompf, A. J., Walther, H. V., Adams, M. A., Carlezon, W. A. Jr., Ongür, D.,
12 & Cohen, B. M. (2010). Blockade of astrocytic glutamate uptake in rats induces
13 signs of anhedonia and impaired spatial memory. *Neuropsychopharmacology*, 35,
14 2049-2059. doi: 10.1038/npp.2010.74
- 15 Berlim, M. T., McGirr, A., Van den Eynde, F., Fleck, M. P., & Giacobbe, P. (2014).
16 Effectiveness and acceptability of deep brain stimulation (DBS) of the subgenual
17 cingulate cortex for treatment-resistant depression: a systematic review and
18 exploratory meta-analysis. *Journal of Affective Disorders*, 159, 31-38. doi:
19 10.1016/j.jad.2014.02.016
- 20 Bernstein, H. G., Meyer-Lotz, G., Dobrowolny, H., Bannier, J., Steiner, J., Walter, M., &
21 Bogerts, B. (2015). Reduced density of glutamine synthetase immunoreactive
22 astrocytes in different cortical areas in major depression but not in bipolar I
23 disorder. *Frontiers Cellular Neuroscience*, 9, 273. doi: 10.3389/fncel.2015.00273
- 24 Bortolozzi, A., Castañé, A., Semakova, J., Santana, N., Alvarado, G., Cortés, R.,...
25 Artigas, F. (2012). Selective siRNA-mediatedpression of 5-HT1A autoreceptors

1 evokes strong anti-depressant-like effects. *Molecular Psychiatry*, 17, 612-623. doi:
2 10.1038/mp.2011.92

3 Cao, X., Li, L. P., Wang, Q., Wu, Q., Hu, H. H., Zhang, M.,...Gao, T. M. (2013).
4 Astrocyte-derived ATP modulates depressive-like behaviors. *Nature Medicine*, 19,
5 773-777. doi: 10.1038/nm.3162

6 Celada, P., Puig, M. V., Casanovas, J.M., Guillazo, G., & Artigas, F. (2001). Control of
7 dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of
8 serotonin-1A, GABA(A), and glutamate receptors. *Journal of Neuroscience*, 21,
9 9917-9929

10 Celada, P., Puig, M. V., & Artigas, F. (2013). Serotonin modulation of cortical neurons
11 and networks. *Frontiers in Integrative Neuroscience*, 7, 25. doi:
12 10.3389/fnint.2013.00025

13 Chen, J., Tanm Z., Zengm L., Zhang, X., He, Y., Gao, W.,... Duan, S. (2013).
14 Heterosynaptic long-term depression mediated by ATP released from astrocytes.
15 *Glia*, 61, 178-191. doi: 10.1002/glia.22425

16 Choudary, P. V., Molnar, M., Evans, S. J., Tomita, H., Li, J. Z., Vawter, M. P.,...Jones,
17 E. G. (2005). Altered cortical glutamatergic and GABAergic signal transmission
18 with glial involvement in depression. *Proceedings of the National Academy*
19 *Sciences of the United States of America*, 102, 15653-15658

20 Cryan, J. F., Thakker, D. R., & Hoyer, D. (2007). Emerging use of non-viral RNA
21 interference in the brain. *Biochemical Society Transactions*, 35, 411-415

22 Drevets, W. C., Price, J. L., Simpson, J. R. Jr, Todd, R. D., Reich, T., Vannier, M., &
23 Richle, M. E. (1997). Subgenual prefrontal cortex abnormalities in mood disorders.
24 *Nature*, 386, 824-827

- 1 Drevets, W. C., Savitz, J., & Trimble, M. (2015). The subgenual anterior cingulate cortex
2 in mood disorders. *CNS Spectrums*, 13, 663-681
- 3 Duman, R. S., & Monteggia, L. M. (2006). A neurotrophic model for stress-related mood
4 disorders. *Biological Psychiatry*, 59, 1116-1127
- 5 Duman, R. S., Aghajanian, G. K., Sanacora, G., & Krystal, J. H. (2016). Synaptic
6 plasticity and depression: new insights from stress and rapid-acting antidepressants.
7 *Nature Medicine*, 22, 238-249. doi: 10.1038/nm.4050
- 8 Ferrari, A. J., Charlson, F. J., Norman, R. E., Patten, S. B., Freedman, G., Murray, C.
9 J.,... Whiteford, H. A. (2013). Burden of depressive disorders by country, sex, age,
10 and year: findings from the global burden of disease study 2010. *PLoS Medicine*,
11 10, e1001547. doi: 10.1371/journal.pmed.1001547
- 12 Ferrés-Coy, A., Santana, N., Castañé, A., Cortés, R., Carmona, M. C., Toth, M.,...
13 Bortolozzi, A. (2013a). Acute 5-HT_{1A} autoreceptor knockdown increases
14 antidepressant responses and serotonin release in stressful conditions.
15 *Psychopharmacology (Berl)*, 225, 61-74. doi: 10.1007/s00213-012-2795-9
- 16 Ferrés-Coy, A., Pilar-Cuellar, F., Vidal, R., Paz, V., Masana, M., Cortés, R., ...
17 Bortolozzi, A. (2013b). RNAi-mediated serotonin transporter suppression rapidly
18 increases serotonergic neurotransmission and hippocampal neurogenesis.
19 *Translational Psychiatry*, 15, 3:e211. doi: 10.1038/tp.2012.135
- 20 Ferrés-Coy, A., Galofré, M., Pilar-Cuellar, F., Vidal, R., Paz, V., Ruiz-Bronchal,
21 E.,... Bortolozzi, A. (2016). Therapeutic antidepressant potential of a conjugated
22 siRNA silencing the serotonin transporter after intranasal administration.
23 *Molecular Psychiatry*, 21, 328-338. doi: 10.1038/mp.2015.80.
- 24 Franklin, K. B. J., & Paxinos, G. (2008). *The Mouse Brain in Stereotaxic Coordinates*
25 (3rd ed.). New York, NY: Academic Press

- 1 Fuchikami, M., Thomas, A., Liu, R., Wohleb, E. S., Land, B. B., DiLeone, R. J.,
2 ...Duman, R. S. (2015). Optogenetic stimulation of infralimbic PFC reproduces
3 ketamine's rapid and sustained antidepressant actions. *Proceedings of the National*
4 *Academy Sciences of the United States of America*, 112, 8106-8111. doi:
5 10.1073/pnas.1414728112
- 6 Gabbott, P. L., Warner, T. A., Jays, P. R., Salway, P., & Busby, S. J. (2005). Prefrontal
7 cortex in the rat: projections to subcortical autonomic, motor, and limbic centers.
8 *Journal of Comparative Neurology*, 492, 145-177
- 9 Gasull-Camós, J., Tarrés-Gatius, M., Artigas, F., & Castañé, A. (2017). Glial GLT-1
10 blockade in infralimbic cortex as a new strategy to evoke rapid antidepressant-like
11 effects in rats. *Translational Psychiatry*, 7, e1038. doi: 10.1038/tp.2017.7
- 12 Gittins, R. A., & Harrison, P. J. (2011). A morphometric study of glia and neurons in the
13 anterior cingulate cortex in mood disorder. *Journal of Affective Disorders*, 133,
14 328-332. doi: 10.1016/j.jad.2011.03.042
- 15 Global Burden of Disease Study 2013 Collaborators. (2015). Global, regional, and
16 national incidence, prevalence, and years lived with disability for 301 acute and
17 chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for
18 the Global Burden of Disease Study 2013. *Lancet*, 386, 743-800. doi:
19 10.1016/S0140-6736(15)60692-4
- 20 Groenewegen, H. J., & Uylings, H. B. (2000). The prefrontal cortex and the integration
21 of sensory, limbic and autonomic information. *Progress in Brain Research*, 126, 3-
22 28
- 23 Hajós, M., Richards, C. D., Székely, A. D., & Sharp, T. (1998). An electrophysiological
24 and neuroanatomical study of the medial prefrontal cortical projection to the
25 midbrain raphe nuclei in the rat. *Neuroscience*, 87, 95-108

- 1 Haroon, E., Miller, A. H., & Sanacora, G. (2017). Inflammation, glutamate, and glia: A
2 trio of trouble in mood disorders. *Neuropsychopharmacology*, 42, 193-215. doi:
3 10.1038/npp.2016.199
- 4 Hughes, E. G., Maguire, J. L., McMinn, M. T., Scholz, R. E., & Sutherland, M. L. (2004).
5 Loss of glial fibrillary acidic protein results in decreased glutamate transport and
6 inhibition of PKA-induced EAAT2 cell surface trafficking. *Brain Res Molecular*
7 *Brain Research*, 124, 114-123
- 8 Ivy, A. S., Rodriguez, F. G., Garcia, C., Chen, M. J., & Russo-Neustadt, A. A. (2003).
9 Noradrenergic and serotonergic blockade inhibits BDNF mRNA activation
10 following exercise and antidepressant. *Pharmacology, Biochemistry and Behavior*,
11 75, 81-88
- 12 Ji, G., & Neugebauer, V. (2012). Modulation of medial prefrontal cortical activity using
13 in vivo recordings and optogenetics. *Molecular Brain*, 5, 36. doi: 10.1186/1756-
14 6606-5-36
- 15 Jodo, E., Chiang, C., & Aston-Jones, G. (1998). Potent excitatory influence of prefrontal
16 cortex activity on noradrenergic locus coeruleus neurons. *Neuroscience*, 83, 63-79.
- 17 John, C. S., Smith, K. L., Van't Veer, A., Gompf, H. S., Carlezon, W. A. Jr., Cohen, B.
18 M., ... Bechtholt-Gompf, A. J. (2012). Blockade of astrocytic glutamate uptake in
19 the prefrontal cortex induces anhedonia. *Neuropsychopharmacology*, 37, 2467-
20 2475. doi: 10.1038/npp.2012.105
- 21 Juliano, R., Alam, M. R., Dixit, V., & Kang, H. (2008). Mechanisms and strategies for
22 effective delivery of antisense and siRNA oligonucleotides. *Nucleic Acids*
23 *Research*, 36, 4158-5171. doi: 10.1093/nar/gkn342

- 1 Kessler, R. C., Merikangas, K. R., & Wang, P. S. (2007). Prevalence, comorbidity, and
2 service utilization for mood disorders in the United States at the beginning of the
3 twenty-first century. *Annual Review of Clinical Psychology*, 3, 137-158.
- 4 Knapska, E., & Kaczmarek, L. (2004). A gene for neuronal plasticity in the mammalian
5 brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Progress in Neurobiology*, 74,
6 183-211
- 7 Krishnan, V., & Nestler, E. J. (2008). The molecular neurobiology of depression. *Nature*,
8 455, 894-902. doi: 10.1038/nature07455
- 9 Liu, B., Teschemacher, A. G., & Kasparov, S. (2017). Astroglia as a cellular target for
10 neuroprotection and treatment of neuro-psychiatric disorders. *Glia*, 65, 1205-1226.
11 doi: 10.1002/glia.23136
- 12 Maki, R., Robinson, M. B., & Dichter, M. A. (1994). The glutamate uptake inhibitor L-
13 trans-pyrrolidine-2,4-dicarboxylate depresses excitatory synaptic transmission via
14 a presynaptic mechanism in cultured hippocampal neurons. *Journal of*
15 *Neuroscience*, 14, 6754-6762
- 16 Martín-Ruiz, R., Puig, M. V., Celada, P., Shapiro, D.A., Roth, B. L., Mengod G, &
17 Artigas, F. (2001). Control of serotonergic function in medial prefrontal cortex by
18 serotonin-2A receptors through a glutamate-dependent mechanism. *Journal of*
19 *Neuroscience*, 21, 9856-9866
- 20 Martinowich, K., & Lu, B. (2008). Interaction between BDNF and serotonin: role in mood
21 disorders. *Neuropsychopharmacology*, 33, 73-83
- 22 Mayberg, H. S., Lozano, A. M., Voon, V., McNeely, H. E., Seminowicz, D., Hamani,
23 C.,...Kennedy, S. H. (2005). Deep brain stimulation for treatment-resistant
24 depression. *Neuron*, 45, 651-660

- Mayberg, H. S. (2009). Targeted electrode-based modulation of neural circuits for depression. *The Journal of Clinical Investigation*, 119, 717-725. doi: 10.1172/JCI38454
- Miguel-Hidalgo, J. J., Baucom, C., Dilley, G., Overholser, J. C., Meltzer, H. Y., Stockmeier, C. A., & Rajkowska, G. (2000). Glial fibrillary acidic protein immunoreactivity in the prefrontal cortex distinguishes younger from older adults in major depressive disorder. *Biological Psychiatry*, 48, 861-873
- Miguel-Hidalgo, J. J., Waltzer, R., Whittom, A. A., Austin, M. C., Rajkowska, G., & Stockmeier, C. A. (2010). Glial and glutamatergic markers in depression, alcoholism, and their comorbidity. *Journal of Affective Disorders*, 127, 230-240. doi: 10.1016/j.jad.2010.06.003
- Miguel-Hidalgo, J. J., Wilson, B. A., Hussain, S., Meshram, A., Rajkowska, G., & Stockmeier, C. A. (2014). Apoptosis-related proteins and proliferation markers in the orbitofrontal cortex in major depressive disorder. *Journal of Affective Disorders*, 158, 62-70. doi: 10.1016/j.jad.2014.02.010
- Murray, C. J., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C., ... Memish, Z. A. (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, 380, 2197-2223. doi: 10.1016/S0140-6736(12)61689-4
- Nagy, C., Suderman, M., Yang, J., Szyf, M., Mechawar, N., Ernst, C., & Turecki, G. (2015). Astrocytic abnormalities and global DNA methylation patterns in depression and suicide. *Molecular Psychiatry*, 20, 320-328. doi: 10.1038/mp.2014.21

- 1 Nibuya, M., Nestler, E. J., & Duman, R. S. (1996). Chronic antidepressant administration
2 increases the expression of cAMP response element binding protein (CREB) in rat
3 hippocampus. *Journal of Neuroscience*, 16, 2365-2372
- 4 Okuno, H. (2011). Regulation and function of immediate-early genes in the brain: Beyond
5 neuronal activity markers. *Neuroscience Research*, 69, 175-186. doi:
6 10.1016/j.neures.2010.12.007
- 7 Ongür, D., Drevets, W. C., & Price, J. L. (1998). Glial reduction in the subgenual
8 prefrontal cortex in mood disorders. *Proceedings of the National Academy Sciences*
9 *of the United States of America*, 95, 13290-13295
- 10 Oliveira, J. F., Krügel, U., Köles, L., Illes, P., & Wirkner, K. (2008). Blockade of
11 glutamate transporters leads to potentiation of NMDA receptor current in layer V
12 pyramidal neurons of the rat prefrontal cortex via group II metabotropic glutamate
13 receptor activation. *Neuropharmacology*, 55, 447-453. doi:
14 10.1016/j.neuropharm.2008.07.032
- 15 Peng, L., Verkhratsky, A., Gu, L., & Li, B. (2015). Targeting astrocytes in major
16 depression. *Expert Review of Neurotherapeutics*, 15, 1299-1306. doi:
17 10.1586/14737175.2015.1095094
- 18 Perea, G., & Araque, A. (2007). Astrocytes potentiate transmitter release at single
19 hippocampal synapses. *Science*, 317, 1083-1086
- 20 Perea, G., Sur, M., & Araque, A. (2014). Neuron-glia networks: integral gear of brain
21 function. *Frontiers in Cellular Neuroscience*, 8, 378. doi:
22 10.3389/fncel.2014.00378
- 23 Phillips, M. R., Zhang, J., Shi, Q., Song, Z., Ding, Z., Pang, S.,...Wang, Z. (2009).
24 Prevalence, treatment, and associated disability of mental disorders in four

provinces in China during 2001-05: an epidemiological survey. *Lancet*, 373, 2041-2053. doi: 10.1016/S0140-6736(09)60660-7

Puig, M. V., Artigas, F., & Celada, P. (2005). Modulation of the activity of pyramidal neurons in rat prefrontal cortex by raphe stimulation in vivo: involvement of serotonin and GABA. *Cerebral Cortex*, 15, 1-14. doi: 10.1093/cercor/bhh104

Rajkowska, G., Miguel-Hidalgo, J. J., Wei, J., Dilley, G., Pittman, S. D., Meltzer, H. Y., ...Stockmeier, C. A. (1999). Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. *Biological Psychiatry*, 45, 1085-1098.

Rajkowska, G., & Stockmeier, C. A. (2013). Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue. *Current Drug Targets*, 14, 1225-1236

Ressler, K. J., & Mayberg, H. S. (2007). Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. *Nature Neuroscience*, 10, 1116-1124

Rush, A. J., Trivedi, M. H., Wisniewski, S. R., Nierenberg, A. A., Stewart, J. W., Warden, D.,...Fava, M. (2006). Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *American Journal of Psychiatry*, 163, 1905-1917.

Sanacora, G., & Banasr, M. (2013). From pathophysiology to novel antidepressant drugs: glial contributions to the pathology and treatment of mood disorders. *Biological Psychiatry*, 73, 1172-1179. doi: 10.1016/j.biopsych.2013.03.032

Savitz, J. B., & Drevets, W. C. (2009). Imaging phenotypes of major depressive disorder: genetic correlates. *Neuroscience*, 164, 300-330. doi: 10.1016/j.neuroscience.2009.03.082

1 Seminowicz, D. A., Mayberg, H. S., McIntosh, A. R., Goldapple, K., Kennedy, S., Segal,
2 Z., & Rafi-Tari, S. (2004). Limbic-frontal circuitry in major depression: a path
3 modeling metanalysis. *Neuroimage*, 22, 409-418.

4 Sequeira, A., Mamdani, F., Ernst, C., Vawter, M. P., Bunney, W. E., Lebel, V.,...Turecki,
5 G. (2009). Global brain gene expression analysis links glutamatergic and
6 GABAergic alterations to suicide and major depression. *PLoS One*, 4, e6585. doi:
7 10.1371/journal.pone.0006585

8 Sullivan, S. M., Lee, A., Björkman, S. T., Miller, S. M., Sullivan, R. K., Poronnik,
9 P.,...Pow, D.V. (2007). Cytoskeletal anchoring of GLAST determines
10 susceptibility to brain damage: an identified role for GFAP. *Journal of Biological*
11 *Chemistry*, 282, 29414-29423

12 Sun, J. D., Liu, Y., Yuan, Y. H., Li, J., & Chen, N. H. (2012). Gap junction dysfunction
13 in the prefrontal cortex induces depressive-like behaviors in rats.
14 *Neuropsychopharmacology*, 37, 1305-1320. doi: 10.1038/npp.2011.319

15 Torres-Platas, S. G., Nagy, C., Wakid, M., Turecki, G., & Mechawar, N. (2016). Glial
16 fibrillary acidic protein is differentially expressed across cortical and subcortical
17 regions in healthy brains and downregulated in the thalamus and caudate nucleus
18 of depressed suicides. *Molecular Psychiatry*, 21, 509-515. doi:
19 10.1038/mp.2015.65

20 Trivedi, M. H., Fava, M., Wisniewski, S. R., Thase, M. E., Quitkin, F., Warden, D.,
21 ...Rush, A.J.; STAR*D Study Team. (2006). Medication augmentation after the
22 failure of SSRIs for depression. *The New England Journal of Medicine*, 354, 1243-
23 1252

- 1 Varga, V., Székely, A. D., Csillag, A., Sharp, T., & Hajós, M. (2001). Evidence for a role
2 of GABA interneurons in the cortical modulation of midbrain 5-hydroxytryptamine
3 neurons. *Neuroscience*, 106, 783-792.
- 4 Vertes, R. P. (2004). Differential projections of the infralimbic and prelimbic cortex in
5 the rat. *Synapse*, 51, 32-58.
- 6 Wang, Q., Jie, W., Liu, J. H., Yang, J. M., & Gao, T. M. (2017). An astroglial basis of
7 major depressive disorder? An overview. *Glia*, 65, 1227-1250. doi:
8 10.1002/glia.23143
- 9 Warden, M. R., Selimbeyoglu, A., Mirzabekov, J. J., Lo, M., Thompson, K. R., Kim, S.
10 Y.,... Deisseroth, K. (2012). A prefrontal cortex-brainstem neuronal projection that
11 controls response to behavioural challenge. *Nature*, 492, 428-432. doi:
12 10.1038/nature11617
- 13 Whiteford, H. A., Degenhardt, L., Rehm, J., Baxter, A. J., Ferrari, A. J., Erskine, H.
14 E.,... Vos, T. (2013). Global burden of disease attributable to mental and substance
15 use disorders: findings from the Global Burden of Disease Study 2010. *Lancet*, 382,
16 1575-1586. doi: 10.1016/S0140-6736(13)61611-6
- 17 Worley, P. F., Christy, B. A., Nakabeppu, Y., Bhat, R. V., Cole, A. J., & Baraban, J. M.
18 (1991). Constitutive expression of zif268 in neocortex is regulated by synaptic
19 activity. *Proceedings National Academic of Science U S A*, 88, 5106–5110.
- 20 Zhao, J., Verwer, R. W., van Wamelen, D. J., Qi, X. R., Gao, S. F., Lucassen, P. J., &
21 Swaab, D. F. (2016). Prefrontal changes in the glutamate-glutamine cycle and
22 neuronal/glial glutamate transporters in depression with and without suicide.
23 *Journal of Psychiatry Research*, 82, 8-15. doi: 10.1016/j.jpsychires.2016.06.017

Figure Legends

Figure 1. Local siRNA microinfusion down-regulates glial glutamate transporter GLAST and GLT-1 expression in IL. **(a)** Confocal images showing siRNA diffusion in IL and PrL cortices. Mice were infused with Alexa488-labeled or Alexa555-labeled nonsense siRNA (NS siRNA) at 60 µg/µl and sacrificed 6 h later. Scale bar: 90 µm. **(b)** Coronal brain sections showing GLAST and GLT-1 mRNA expression in medial prefrontal cortex sub-divisions (Cg1: cingulate cortex area1, PrL and IL) of mice assessed by *in situ* hybridization. Mice were locally infused with: (1) artificial cerebro-spinal fluid (aCSF), (2) GLAST siRNA, and (3) GLT-1 siRNA into IL at 60 µg/µl and sacrificed at 1, 3 and 7 days post-infusion. Red arrowheads show the reduced GLAST or GLT-1 mRNA density in IL of siRNA-treated mice. Scale bar: 1 mm. **(c)** Time course of GLAST and GLT-1 mRNA suppression in IL after local siRNA microinfusion. Bar graphics showing a significant reduction of GLAST and GLT-1 mRNA levels compared with their respective control groups ($n = 5$ mice per group). Two-way ANOVA followed by Tuckey test revealed an effect of time $F_{(5,72)}=13.62$ ($p < .0001$), but not antero-posterior (AP) coordinate nor interaction day-by-AP coordinate for GLAST mRNA and, an effect of time $F_{(5,72)}=32.58$ ($p < .0001$), but not AP coordinate nor interaction day-by-coordinate for GLT-1 mRNA. **(d)** Representative images of GLAST- and GLT-1-immunoreactive (GLAST-ir or GLT-1-ir) fibers in the contralateral and ipsilateral IL. Acute siRNA treatment significantly reduced the target GLAST or GLT-1 protein density in the ipsilateral IL as compared with the vehicle-treated mice. Scale bar: 100 µm **(e)** Quantitative analysis of GLAST and GLT-1 protein levels in the IL at different AP coordinates assessed by immunohistochemistry. Bar graphics showing reduced GLAST and GLT-1 protein densities in siRNA-treated mice compared to control aCSF-treated mice ($n = 6$ mice per group). Two-way ANOVA followed by Tuckey tests revealed an effect of group $F_{(1,30)}=153.3$ ($p < .0001$), but not AP coordinate nor interaction group-by-coordinate for GLAST protein and, an effect of group $F_{(1,30)}=61.79$ ($p < .0001$), but not AP coordinate nor interaction group-by-brain for GLT-1 protein. Data are presented as the mean $\pm$ SEM. * $p < .05$, ** $p < .01$ and *** $p < .001$ compared to respective aCSF-treated mice.

Figure 2. Reduced number of astrocytes and, GFAP and GS protein levels in IL after glial glutamate transporter GLAST and GLT-1 knockdown. Mice were locally infused with: (1) artificial cerebro-spinal fluid (aCSF), (2) GLAST siRNA, and (3) GLT-1 siRNA into IL at 60 µg/µl and sacrificed at 1 day post-administration. **(a)** Immunohistochemistry images showing GFAP staining in ipsilateral and contralateral IL. Right columns are high-magnification photomicrographs of the frames in left columns. Scale bars: low = 100 µm, high = 20 µm. **(b)** Bar graphics showing decreased total GFAP protein density in the ipsilateral IL of siRNA-treated mice compared to aCSF-treated mice ($n = 5-6$ mice per group). Two-way ANOVA revealed an effect of group $F_{(2,26)}=22.73$ ($p < .0001$), brain side $F_{(1,26)}=119.9$ ($p < .0001$) and interaction group-by-brain side $F_{(2,26)}=7.779$ ($p = 0.0023$). In addition, GLAST and GLT-1 siRNAs also decreased intracellular GFAP protein density ($n = 5-6$ mice per group). Two-way ANOVA revealed effect of group $F_{(2,26)}=20.66$ ($p < .0001$), brain side $F_{(1,26)}=164.6$ ($p < .0001$) and interaction group-by-brain side $F_{(2,26)}=17.96$ ($p < .0001$). Reduced number of GFAP⁺ cells were detected in the ipsilateral IL of GLAST or GLT-1 siRNA-treated mice compared to control group ($n = 5-6$ mice per group). Two-way ANOVA revealed an effect of group $F_{(2,26)}=21.72$ ($p < .0001$), brain side $F_{(1,26)}=55.73$ ($p < .0001$) and interaction group-by-brain side $F_{(2,26)}=13.1$ ($p = .0001$). **(c)** Immunohistochemistry images showing GS staining in ipsilateral and contralateral IL. White arrows indicated GS⁺ cells. Scale bar: 20 µm. **(d)** Bar graphics showing decreased total GS protein density in the ipsilateral IL compared to contralateral side of the same mice and with ipsilateral IL of aCSF-treated mice ($n = 5$ mice per group). Two-way ANOVA revealed an effect of brain side $F_{(1,24)}=43.39$ ($p < .0001$) and interaction group-by-brain side $F_{(2,24)}=7.771$ ($p = .0025$), but not effect of group. In addition, GLAST and GLT-1 siRNAs also decreased intracellular GS protein levels in the ipsilateral IL ($n = 5$ mice per group). Two-way ANOVA revealed effect of group $F_{(2,24)}=8.48$ ($p = .0016$), brain side $F_{(1,24)}=22.13$ ($p < .0001$) and interaction group-by-brain side $F_{(2,24)}=11.43$ ($p = .0003$). Reduced number of GS⁺ cell numbers were detected in the ipsilateral IL of GLAST or GLT-1 siRNA-treated mice compared to control groups ($n = 5$ mice per group). Two-way ANOVA revealed an effect of group $F_{(2,24)}=55.63$ ($p < .0001$), brain side $F_{(1,24)}=55.79$ ($p < .0001$) and interaction group-by-brain side $F_{(2,24)}=19.28$ ($p < .0001$). Data are presented as the mean $\pm$ SEM. ** $p < .01$ and **** $p < .0001$ compared to ipsilateral IL of aCSF-treated mice; + $p < .05$, ++ $p < .01$, +++ $p < .001$ and ++++ $p < .0001$ compared to contralateral IL of siRNA-treated mice.

Figure 3. GLAST or GLT-1 knockdown in IL evokes a mouse depressive-like phenotype. Mice were unilaterally infused with: (1) artificial cerebro-spinal fluid (aCSF), (2) nonsense siRNA (NS siRNA), (3) GLAST siRNA, and (4) GLT-1 siRNA into IL at 60 µg/µl and behavioral paradigms were assessed at 1 day post-administration. **(a-b)** GLAST and GLT-1 siRNAs in IL increased the immobility time in the tail suspension test (TST, $n = 10-14$ mice per group) and forced swim test (FST, $n = 6-8$ mice per group) compared to control groups treated with aCSF or NS siRNA. One-way ANOVA followed by Tuckey test revealed an effect of group $F_{(3,34)}=12.53$ ($p = .0003$) for TST and $F_{(3,23)}=9.809$ ($p = .0002$) for FST. **(c)** GLAST- and GLT-1 siRNAs in IL induced anhedonia compared to aCSF-treated mice assessed by a reduction in the sucrose preference and intake ($n = 10$ mice per group). One-way ANOVA followed by Tuckey test revealed an effect of group $F_{(2,34)}=18.54$ ($p < .0001$) for sucrose preference and $F_{(2,37)}=9.766$ ($p = .0004$) for sucrose intake. **(d)** GLAST or GLT-1 knockdown in IL unchanged the motor activity in the open field test ($n = 10$ mice per group). **(e)** Acute treatment with citalopram (Cit, 10 mg/kg, i.p.) or ketamine (Ket, 10 mg/kg, i.p.) administered 30 min before TST reversed the increased immobility time in the TST of GLAST or GLT-1 knockdown mice compared to respective control groups treated with aCSF or saline (Sal) ($n = 5-8$ mice per group). One-way ANOVA followed by Tuckey test revealed an effect of group $F_{(7,42)}=9.639$ ($p < .0001$). Data are presented as the mean $\pm$ SEM. $**p < .01$, $***p < .001$ and $****p < .0001$ compared to aCSF- and NS siRNA-treated mice; $+p < .05$, $+++p < .001$ compared to respective Sal-treated mice; $\&p < .01$ compared to aCSF-treated or WT mice.

Figure 4. Local GLAST or GLT-1 knockdown increases neuronal activity in IL leading to a lower activation of TPH₂⁺ cells in the dorsal raphe nucleus (DR). Mice were locally infused with: (1) artificial cerebro-spinal fluid (aCSF), (2) GLAST siRNA, and (3) GLT-1 siRNA into IL at 60 µg/µl and sacrificed at 1 day post-administration. **(a)** Schematic representation of the reciprocal connectivity between the medial prefrontal cortex (mPFC) and the dorsal raphe nucleus (DR), showing also the localization of main 5-HT receptors: 5-HT_{1A} (1A), 5-HT_{1B} (1B) and 5-HT_{2A} (2A) receptors. Axons from pyramidal neurons in the mPFC innervate the DR and make excitatory synapses mediated by AMPA and NMDA receptors on 5-HT neurons and GABAergic interneurons. In rats, the

physiological stimulations of the mPFC results mainly in inhibitory responses of 5-HT neurons due to 1) the excitatory effect on the raphe GABAergic interneurons and, 2) self-inhibitory responses in 5-HT neurons mediated by 5-HT_{1A} autoreceptors, following excitatory inputs that release 5-HT (Celada et al., 2001). **(b)** Confocal images showing the co-localization of c-Fos (red) with NeuN⁺ neurons (green) in different IL layers. Cell nuclei were stained with Hoechst (blue). Scale bars: 100 μ m. **(c)** Bar graphics showing increased number of c-Fos⁺ cells, but not NeuN⁺ cells, in the IL at different anteroposterior coordinates (AP) of GLAST- and GLT-1 siRNA-treated mice compared to control mice ($n = 5$ mice per group). Two-way ANOVA revealed an effect of group $F_{(2,36)}=62.69$ ($p < .0001$), but not AP nor interaction group-by-AP. In addition, increased c-Fos protein intensity labeling in NeuN⁺ cells was found in the same siRNA-treated mice compared to aCSF-treated mice. Two-way ANOVA revealed an effect of group $F_{(2,36)}=64.6$ ($p < .0001$), but not AP nor interaction group-by-AP. **(d)** Confocal images showing the co-localization of Egr-1 (red) with TPH₂⁺ neurons (green) in the DR identified with white arrowheads. Cell nuclei were stained with Hoechst (blue). Scale bars: low = 100 μ m, high = 20 μ m. **(e)** Bar graphics showing increased Egr-1 protein intensity labelling in TPH₂⁺ and non-TPH₂⁺ cells in the DR at different AP of siRNA-treated mice compared to aCSF-treated mice ($n = 4-6$ mice per group). Two-way ANOVA revealed effect of group $F_{(2,13)}=83.95$ ($p < .0001$), but not AP nor interaction group-by-AP by TPH₂⁺ cells and, effect of group $F_{(2,12)}=28.27$ ($p < .0001$), but not AP nor interaction group-by-AP by non-TPH₂⁺ cells. **(f)** Bar graphics showing total Egr-1 protein density labelling in TPH₂⁺ and non-TPH₂⁺ cells in the DR of siRNA- or aCSF-treated mice ($n = 5$ mice per group). Two-way ANOVA revealed effect of group $F_{(2,24)}=74.52$ ($p < .0001$), effect of cell type $F_{(1,24)}=14.38$ ($p = .0009$) and group-by-cell type $F_{(2,24)}=3.52$ ($p = .0456$). Data are presented as the mean $\pm$ SEM. *** $p < .001$ and **** $p < .0001$ compared to aCSF-treated mice; + $p < .05$ and ++ $p < .01$ compared to TPH₂⁺ cells.

Figure 5. GLAST or GLT-1 knockdown in IL decreases extracellular 5-HT concentration in the dorsal raphe nucleus (DR). Mice were locally infused with: (1) artificial cerebro-spinal fluid (aCSF), (2) GLAST siRNA, and (3) GLT-1 siRNA into IL at 60 μ g/ μ l and intracerebral microdialysis were performed 1-2 days post-administration. **(a-b)** Intra-IL GLAST (a) or GLT-1 (b) siRNA infusion reduced extracellular 5-HT

baseline concentration in the DR of mice compared to control group ($n = 6-8$ mice per group). t -Student test showed significant differences for: intra-IL GLAST siRNA ($t = 8.917$, $df = 12$, $p < .001$) and intra-IL GLT-1 siRNA ($t = 4.661$, $df = 22$, $p < .0001$). **(c-d)** Local infusion of veratridine (depolarizing agent, $50 \mu\text{M}$) by reverse dialysis increased 5-HT release in the DR of mice ($n = 6-8$ mice per group). This effect was similar in all experimental groups. Two-way ANOVA followed by Tuckey test showed no significant differences between groups. **(e-f)** Local infusion of bicuculline (GABA_A receptor antagonist, $30 \mu\text{M}$) increased 5-HT release in the DR of mice, but this effect was greater in intra-IL GLAST or GLT-1 siRNA-treated mice compared with aCSF-treated mice ($n = 6-8$ mice per group). Two-way ANOVA followed by Tuckey test showed an effect of group $F_{(1,144)}=31.12$ ($p < .0001$), time $F_{(11,144)}=16.88$ ($p < .0001$) and interaction group-by-time $F_{(11,144)}=3.553$ ($p = .0002$) for intra-IL GLAST siRNA and, an effect of group $F_{(1,96)}=32.48$ ($p < .0001$), time $F_{(11,96)}=10.96$ ($p < .0001$) and interaction group-by-time $F_{(11,96)}=2.644$ ($p = .0054$) for intra-IL GLT-1 siRNA. Data are presented as the mean $\pm$ SEM. $**p < .01$ and $****p < .0001$ compared to aCSF-treated mice.

Figure 6. GLAST or GLT-1 knockdown in IL reduces BDNF mRNA expression in cortical and sub-cortical areas. Mice were unilaterally infused with: (1) artificial cerebrospinal fluid (aCSF), (2) GLAST siRNA, and (3) GLT-1 siRNA into IL at $60 \mu\text{g}/\mu\text{l}$ and sacrificed at 1 day post-administration. **(a)** Coronal brain sections showing BDNF mRNA expression in different sub-divisions of medial prefrontal cortex and hippocampus of mice assessed by *in situ* hybridization. The boxes delimit areas of medial prefrontal cortex and hippocampus shown at higher magnification. Cg1: cingulate cortex area 1, PrL: prelimbic cortex, IL: infralimbic cortex, DP: dorsal peduncular cortex, CA1-CA3: CA1-CA3 hippocampus fields, DG: dentate gyrus. Scale bars: low = 1 mm, high = $500 \mu\text{m}$. **(b)** Bar graphics showing reduced BDNF mRNA levels in both ipsilateral and contralateral IL and PrL cortices as well as the hippocampal formation including CA1, CA2-CA3 and DG of siRNA-treated mice compared to aCSF-treated mice ($n = 5$ mice per group). Two-way ANOVA followed Tuckey test revealed for: **IL** [effect of group $F_{(2,34)}=122.7$ ($p < .0001$), brain side $F_{(1,34)}=96.45$ ($p < .0001$) and interaction $F_{(2,34)}=34.1$ ($p < .0001$)]; **PrL cortex** [effect of group $F_{(2,34)}=101.5$ ($p < .0001$), brain side $F_{(1,34)}=131.4$ ($p < .0001$) and interaction $F_{(2,34)}=43.03$ ($p < .0001$)]; **CA1** [effect of group $F_{(2,34)}=97.8$ ($p < .0001$), but not brain side nor interaction group-by-brain side]; **CA2-CA3** [effect of group

1 $F_{(2,33)}=110.9$ ($p < .0001$), but not brain side nor interaction group-by-brain side]; and **DG**
2 [effect of group $F_{(2,34)}=503.77$ ($p < .0001$), but not brain side nor interaction group-by-
3 brain side]. Data are presented as the mean $\pm$ SEM. *** $p < .001$ compared to aCSF-treated
4 mice.

5
6 **Figure 7.** Correlation analysis between BDNF and GLAST or GLT-1 mRNA levels in
7 cortical and hippocampal areas of mouse depressive-like phenotype. **(a-b)** Graphics show
8 the existence of a highly significant linear relationship between BDNF mRNA levels in
9 IL and PrL cortices as well as in the hippocampal formation (CA1-CA3 and dentate girus
10 – DG) and GLAST (a) or GLT-1 (b) mRNA levels in IL ($n = 10$ mice per group). R
11 coefficients and P values are inserted to each graph. Data are presented as the mean $\pm$
12 SEM.

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