"Increased vulnerability to depressive-like behaviour of mice with decreased expression of VGLUT1"

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Abstract

Background: Many studies have linked depression to an increase in the excitatory-inhibitory ratio in the forebrain. Presynaptic alterations in a shared pathway of the glutamate/GABA cycle may account for this imbalance. Recent evidence suggests that decreased vesicular glutamate transporter 1 (VGLUT1) levels in the forebrain affects the glutamate/GABA cycle and induces helpless behaviour. Here we studied decreased VGLUT1 as a potencial factor enhancing a depressive-like phenotype in an animal model.

Methods: Glutamate and GABA synthesis as well as oxidative metabolism were studied in heterozygous mice for the vesicular glutamate transporter 1 (VGLUT1+/-) and WT. Subsequently, the regulation of neurotransmitter levels, proteins involved in the glutamate/GABA cycle and behaviour by both genotype and chronic mild stress (CMS) was studied. Finally, the effect of chronic imipramine on VGLUT1 control and CMS mice was also studied.

Results: VGLUT1+/- mice showed increased neuronal synthesis of glutamate, decreased cortical and hippocampal GABA, VGLUT1 and EAAT1, as well as helplessness and anhedonia. CMS induced an increase of glutamate and a decrease of GABA, VGAT and GAD65 in both areas and led to upregulation EAAT1 in the hippocampus. Moreover, CMS induced anhedonia, helplessness, anxiety and impaired recognition memory. VGLUT1+/- CMS mice showed a combined phenotype (genotype plus stress) and specific alterations, such as an upregulation of VGLUT2 and hyperlocomotion. Moreover, an increased vulnerability to anhedonia and helplessness reversible by chronic imipramine was shown.

Conclusions: These studies highlight a crucial role for decreased VGLUT1 in the forebrain as a biological mediator of increased vulnerability to chronic mild stress.

Introduction

Abnormalities in glutamate and GABA signal transmission have been postulated to play a role in depression, but little is known about the underlying molecular mechanisms (1, 2). Using magnetic resonance spectroscopy, increased glutamate and reduced GABA levels have been observed in the cortex of depressed patients leading to an enhanced excitatory-inhibitory ratio (3-7). Interestingly, this imbalance is particularly associated with melancholic features in the patients (5) and is reverted by chronic treatment with antidepressants (8). Since the presynaptic pathways regulating the synthesis and cycling of glutamate and GABA are tightly coupled, it has been suggested that alterations in a shared pathway may account for the observed amino acid abnormalities. For instance, microarray analysis of specific areas of cerebral cortex from individuals who had suffered from major depression disorder have demonstrated significant down-regulation of the glial EAAT1 and EAAT2, key members of the glutamate/neutral amino acid transporter protein family (9). Other post-mortem studies have shown decreased expression of GAD 65 and 67, the enzymes that convert glutamate to GABA, in mood disorders (10-11). Altogether, these changes could limit GABA synthesis and elevate levels of extracellular glutamate, which in turn, could affect the efficiency of signaling by both neurotransmitters.

At the experimental level, the vesicular glutamate transporter 1 (VGLUT1) has been reported to play a key role in the synaptic release (12) and the efficacy of glutamatergic synaptic transmission (13-16). A recent study carried out in our laboratory reports that mice heterozygous for VGLUT1 (VGLUT1+/-), exhibit decreased cortical and hippocampal levels (35-45 %) of the inhibitory neurotransmitter GABA as well as helplessness in the forced swimming test (17). Here, we asked whether decreased VGLUT1 levels could be considered as a potential biological risk factor of major depression, alone and in combination with adverse environmental factors. Firstly, neuronal versus glial glutamate and GABA synthesis as well as oxidative metabolism,

which is highly regulated by glutamatergic neurotransmission (18,19) was studied by ^{13}C magnetic resonance spectroscopy (^{13}C-MRS) and cytochrome oxidase histochemistry respectively. Secondly, we studied how neurotransmitter levels, the expression of presynaptic proteins involved in the glutamate/GABA cycle as well as behaviour could be influenced by genotype (VGLUT1+/- mice), the environmental model of depression chronic mild stress (CMS) and by the interaction between both. Finally, we asked whether repeated treatment with the antidepressant imipramine was able to revert the behavioural deficit induced by CMS in the VGLUT1+/- mice.

Methods and Materials

Animals

Heterozygous VGLUT1 male mice (VGLUT1+/-) C57BL⁄6N were bred from heterozygous fathers (Dr S. Wojcik, Gottingen, Germany) and WT mothers (Harlan, France). The VGLUT1-/- knock-out allele was generated by truncation of the coding region of the VGLUT1 gene between the start codon and a *Bgl*II site in the fifth coding exon through homologous recombination in embryonic stem cells (129/ola background) (13). These mice show a progressive neuropathological phenotype and increased lethality rate at 2–3 weeks after birth.

Experimental procedures and animal husbandry were conducted according to principles of laboratory animal care as detailed in the European Communities Council Directive (2003/65/EC), Spanish legislation (Real decreto 1201/2005) and approved by our Ethical Committee (University of Navarra).

^{13}C-MRS Spectroscopy

A previously protocol described (20) was used. Briefly, VGLUT1+/- and WT mice (n= 8/group) received [1-^{13}C]glucose (543 mg/kg i.p.) and [1,2-^{13}C]acetate (504 mg/kg i.p.). Twenty minutes later, mice were decapitated, the heads snap frozen in liquid nitrogen and kept at –80 ºC. Brains were removed from the skull while frozen and, after discarding the cerebellum, homogenized with 600 µL of perchloric acid (7% vol/vol). Protein was removed by centrifugation (7,500 rpm; 6 min). Aliquot (5 µl) from the supernatant were reserved for HPLC measurement of the concentration of metabolites, and the rest neutralized with 1 M KOH followed by lyophilisation. Lyophilizates were dissolved in 400 µL D_2O containing 0.1% of ethylene glycol (vol/vol) as an internal standard. Proton decoupled ^{13}C-MRS spectra were obtained using a Bruker DRX-400 spectrometer (Bruker Analytik GmbH, Germany) and the number of scans was 25,000.

Metabolites derived from [1-^{13}C]glucose and [1,2-^{13}C]acetate represent the contribution from neurons and astrocytes to glutamate, glutamine and GABA formation respectively (20).

Cytochrome C oxydase histochemistry

WT and VGLUT1 mice (n=10/group) were decapitated, brains removed, immediately frozen and stored (−80 °C). Coronal sections (50 µm) were used for the staining procedure, as described previously (21) with minor modifications (22). Stained sections were photographed with a Nikon Coolpix 5400 digital camera mounted on a Nikon SMZ 1000 stereomicroscope. Image analysis of one hundred regions of interest (ROI) was conducted using the Image J1.39 freeware.

Neurotransmitter brain levels

GABA and glutamate concetrations in frontal cortex and hippocampus from CMS and control mice (WT and VGLUT1+/-) (n=15 mice/group) were determined by HPLC with electrochemical detection (DECADE, Antec-Leyden). A high sensitivity analytical flowcell (VT-03) was used and the working electrode was set at 0.7 V. A column (Biophase ODS 5 µm, 4.6mm×150 mm) including precolumn derivatization with *o*-phthaldehyde and β-mercapthoethanol (Sigma–Aldrich Ltd., Germany) was used. Results were expressed in ng per mg of wet tissue.

Western blotting

Cortical and hippocampal expression of different proteins involved in the glutamate/GABA cycle were studied by Western blotting using the following primary antibodies: rabbitt anti-VGLUT1 (1:2,000) (donated by Dr S. El Mestikawy, Paris, France), mouse anti-VGLUT2 (1:1,000) and rabbit anti-VGAT (1:1,000) (Chemicon Int., CA, USA), mouse anti-GAD65 (1:5,000) (Abcam, Cambridge, UK) and rabbitt anti-EAAT1 and EAAT2 (1:2500) (Santa Cruz, UK). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse

secondary antibodies (DAKO, U.K. 1:10,000) were used followed by visualization through chemiluminescence using SuperSignal West Pico (Pierce Biotechnology). Films were scanned and quantified using the ImageMaster 1D (Pharmacia Biotech, Sweden) software and normalised to β-actine.

Chronic mild stress (CMS) procedure

Unpredictable repeated mild stressors were applied for six weeks (23). In the first experiment CMS was applied to WT and VGLUT+/- mice (n=15 mice/group) and both control and stressed mice were sacrificed 24 h after the termination of CMS.

In the second experiment, the effect of repeated imipramine treatment on different behavioural paradigms was studied in control and CMS VGLUT1+/- mice. Imipramine HCl (10 mg/kg i.p. Sigma-Aldrich) or saline were administered daily the last 3 weeks of the CMS and continued for one week thereafter.

Over the last week of CMS (first experiment) and over the week immediately after the CMS (second experiment) a battery of behavioural tests was performed.

Behavioural tests

Sucrose intake test

Anhedonic-like behaviour was evaluated during the CMS procedure by weekly monitoring of sucrose intake (24). Mice were firstly trained to drink the sucrose solution during one week and during the procedure were given once a week a 15-h exposure to the sucrose solution and tap water. Body weight measurements were taken weekly and relative sucrose intake and sucrose preference (sucrose intake/total intake) was calculated as absolute intake (g) per weight.

Spontaneous locomotor activity

Locomotor activity was measured in an open field consisting of 9 black arenas (43 x 50 x 45) using a video tracking system (Ethovision XT, Noldus Information Technology B. V., Wageningen, The Netherlands) in a softly illuminated room. One mouse was placed in each cage and distance travelled (cm) was recorded during a 30 min period.

Forced swimming test

The forced swimming test is the most widely used test of depression in rodents. Mice were individually placed into glass cylinders (height 24 cm, diameter 13 cm) containing water (14 cm, 22–23°C). Immobility, indicative of helpless behaviour, was recorded during the last 4 min of the 6 min testing period and is considered a measure of helpless behaviour.

Elevated-plus maze (EPM)

This test was applied to measure unconditioned anxiety-like behaviour. EPM consisted of two open arms (30 x 5 cm), two enclosed arms (30 x 5 cm) and a central platform (5 x 5 cm) elevated 38.5 cm above the ground. Mice were placed in the central zone, facing one of the close arms. Percentage time in the open and the number of transitions were recorded during 5 min. An arm entry was defined as a mouse having entered an arm of the maze with all four legs.

Marble Burying test

Compulsive-anxiety behaviour in mice was assessed by this test (25). Eight marbles (1.5 cm diameter) were placed uniformly in a cage (45 × 28 × 20 cm) containing a constant amount of sawdust (3 cm deep). Mice were placed in the center of the cage and left for 30 minutes. The number of marbles buried was recorded.

Novel object recognition

Visual recognition memory was assessed by this test (23). The apparatus consisted of a black square arena (43 x 50 x 45 cm). On day 1, mice were placed for 30 min in the arena to habituate them. On day 2, mice were placed in the box at equal distance from two identical objects (A1 and A2; two prisms 7 x 3 x 3 cm) for 5 min (sample phase). One hour after, mice were placed back in the box and exposed to a familiar object (A3) and to a novel object (B; a ball of 3.5 cm of diameter mounted on a cube of 3 cm^3) and video recorded (Pinnacle Studio 9.0, Pinnacle systems Inc, Pittsburgh US) for a further 5 min (retention test). Discrimination index (DI) was calculated as the difference between times spent exploring the new (N) and familiar object (F) divided by the total time exploring the objects (N-F/F+N) in the retention test.

Statistical analysis

The effect of genotype in the spectroscopy and histochemistry studies was analyzed by using Student's *t*-test.

Neurotransmitter levels and protein expressions were analyzed with two-way ANOVA (stress × genotype). Different parameters of the behavioral test were analyzed using two-way ANOVA (stress × genotype for the first experiment and stress × treatment for the second). Significant main effects or interactions were analyzed using Student *t*-test.

Sucrose intake was analyzed with two-way ANOVA with repeated measures followed by two way ANOVA for each week.

Results

Neuronal *versus* glial GABA and glutamate synthesis

Injection of [1-13C] glucose and [1,2-13C] acetate lead to efficient labelling of the different metabolites studied (**Figure 1**). A significant increase of [4-13C] glutamate was shown in whole brain extracts of VGLUT1+/- mice compared to their WT littermates (**Table 1A**). However, no changes in the levels of the other metabolites were detected between the two groups. Similarly, acetate/glucose utilization ratios for glutamate, glutamine and GABA were not altered (**Table 1B**).

Cytochrome C oxydase activity

Pair-wise comparison of optical density values in VGLUT1+/- and WT mice for each of the 100 ROIs produced no significant differences. Interestingly, in almost all brain regions the VGLUT+/- mice showed a slight non-significant increase of activity.

Neurotransmitter brain levels

There was no interaction between genotype and stress in glutamate levels either in frontal cortex or in hippocampus. However, a significant effect of stress in both brain regions [$F_{1,56}$= 5.42 and 7.36; $p<0.05$, for frontal cortex and hippocampus respectively] was shown. CMS induced a significant increase in cortical and hippocampal glutamate levels in both WT and VGLUT1+/- mice (**Figure 2A**).

Both genotype and stress affected GABA levels in the frontal cortex [$F_{1, 56}$= 6.32; $p<0.05$] and in the hippocampus [$F_{1, 56}$= 9.26; $p<0.01$]. CMS induced a significant decrease of GABA levels in WT mice. Moreover, VGLUT1+/- mice, control and exposed to CMS, showed a significant decrease of GABA in both brain regions compared to WT controls (**Figure 2B**).

Regulation of presynaptic proteins

No interaction between stress and genotype was shown in VGLUT1 protein levels neither in the frontal cortex [$F_{1,28}$ =0.037 p>0.05] nor in the hippocampus [$F_{1,28}$ =1.37 p>0.05]. As expected VGLUT1 genotype correlated with VGLUT1 expression levels in both brain regions [$F_{1,28}$ = 160.3 and 116.9; p<0.001, for frontal cortex and hippocampus respectively]. Cortical and hippocampal VGLUT1 expression for the control heterozygote was 59% and 65% and for the VGLUT1+/- CMS mice was 52% and 54% respectively of control WT mice (**Figure 3A**).

A significant interaction between genotype and stress was shown for VGLUT2 levels in both the frontal cortex [$F_{1,28}$ = 10.36; p<0.01] and the hippocampus [$F_{1,28}$ = 5.22; p<0.05]. CMS significantly increased VGLUT2 expression in VGLUT1+/- mice, but not in WT, both in the frontal cortex (156 $\pm$ 18 %) and in the hippocampus (123 $\pm$ 14% of WT control) (**Figure 3B**).

No interactions between stress and genotype were found for VGAT [$F_{1,28}$ = 0.01 and 0.89; p>0.05] and GAD65 expression. However, CMS induced a significant decrease of VGAT [$F_{1,28}$ = 8.75 and 37.72 p<0.01] and GAD65 [$F_{1,28}$ = 12.07 and 7.86 p<0.01 for WT and VGLUT1] in frontal cortex and hippocampus respectively (**Figures 3C-D**).

A significant effect of genotype on the glial excitatory transporter EAAT1 was seen in the frontal cortex [$F_{1,28}$ = 3.9, p<0.05] and an interaction between stress and genotype was seen in the hippocampus [$F_{1,28}$ = 4.15, p<0.05]. VGLUT1+/- mice showed a downregulation of cortical (79 % and 60% of WT, for control and CMS VGLUT1+/- mice) and hippocampal (74 % of WT for control VGLUT1+/- mice) EAAT1 expression. CMS induced a significant increase of EAAT1 in the WT mice (~159 %) in the hippocampus compared to WT. (**Figure 3E**).

The expression of EAAT2 in the cortex and hippocampus was not altered neither by stress nor genotype (**Figure 3F**).

Sucrose intake

Body weight was not affected by CMS. Prior to onset of CMS, sucrose and water intake was similar in all the groups. No interaction between sucrose intake, stress and VGLUT1 genotype [$F_{1,56}$= 0.031; p<0.01] was found. However, two-way ANOVA for each week showed a significant effect of genotype on sucrose intake from the fourth week until the end of the procedure [$F_{1,56}$ = 5.34, 3.95, 4.24 p< 0.05 for weeks 4, 5 and 6 respectively]. Similarly, CMS induced a significant decrease in sucrose intake compared to control conditions on weeks 5 and 6 [$F_{1,56}$ = 4.97, p<0.05 and 18.417, p< 0.01 respectively] **(Figure 4A-B).** On the sixth week, CMS induced a higher decrease in sucrose intake in VGLUT1+/- than in WT mice.

Secondly, the effect of repeated treatment of imipramine on the decrease of sucrose intake induced by CMS in the VGLUT1+/- mice was studied. We found no interaction between sucrose intake, stress and treatment [$F_{1,56}$ = 0.628; p>0.05]. Two-way ANOVA of the individual measures showed a significant effect of stress on the fourth week [$F_{1,56}$ =4.107, p<0.05] and a significant interaction between stress and treatment on weeks 5 and 6 [$F_{1,56}$= 4.07, p<0.05; 3.97, p<0.05]. Interestingly, imipramine treatment reverted the decrease of sucrose intake induced by CMS in VGLUT1+/- mice both in the fifth and in the sixth week (**Figure 5A**).

On the other hand, no major differences were detected in sucrose preference (sucrose intake/total intake) among the groups (data not shown).

Forced swimming test

CMS induced helpless-like behaviour in the FST. Over the last week of CMS, there was no interaction between genotype and stress on immobility time. However, a significant effect of stress [$F_{1,56}$ = 8.501 p<0.01] and genotype [$F_{1,56}$ = 28.882 p<0.01] was shown. In

addition, CMS induced a higher increase in immobility time in VGLUT1+/- mice than in WT (**Figure 4C**).

In the second experiment, a significant effect of both stress [$F_{1,56}$ = 4.46 p<0.05], and treatment was shown [$F_{1,56}$ = 9.77 p<0.01]. CMS induced a decrease in immobility time that was reverted by imipramine treatment (**Figure 5B**).

Elevated-plus maze

Stressed mice were more anxious than controls. There was no interaction between stress and genotype in the percentage time spent in the open arms but a significant effect of stress [$F_{1,56}$= 5.11, p<0.05] was shown (**Figure 4D**).

In the second experiment, we found no interaction between stress and imipramine treatment. In addition, no effects either of stress or treatment were shown (**Figure 5C**).

Marble burying

Marble burying behaviour was not affected either by CMS or by genotype. Similarly,

Neither stress nor imipramine treatment altered this behaviour. (Data not shown).

Novel object recognition

Recognition memory was impaired by CMS. No interaction between genotype and stress on discriminating new and familiar objects was shown. CMS induced a decrease in discrimination index one hour after the sample phase [$F_{1,56}$ = 16.596, p<0.01] in both WT and VGLUT1+/- mice. (**Figure 4E).**

In the second experiment, a significant interaction between imipramine treatment and stress [$F_{1,56}$ = 4.601, p<0.05] was shown. VGLUT1+/- mice exposed to CMS and treated with saline showed a significant decrease in the discrimination index which was reverted by chronic imipramine treatment (**Figure 5D**).

Spontaneous motor activity

A significant interaction between genotype and stress [$F_{1,56}$ = 5.18, $p<0.05$] on distance travelled during 30 minutes was shown. CMS induced a significant increase on the distance travelled in VGLUT1+/- mice compared to the control groups (**Figure 6A**).

In addition, no interaction was shown between stress and imipramine treatment. Interestingly, CMS mice showed a significant increase in motor activity [$F_{1,56}$ =7.07, $p<0.05$]. (**Figure 6B**).

Discussion

This study shows that neurotransmitter levels, the expression of presynaptic proteins involved in the glutamate/GABA cycle and different behavioural paradigms related to clinical depression were differentially influenced by both decreased VGLUT1 levels and the chronic mild stress model of depression. Moreover, in VGLUT1+/- mice exposed to chronic mild stress neurochemical and behavioural alterations were found as a result of the interaction between stress and decreased VGLUT1 levels. Specifically, an upregulation of the VGLUT2 transporter in cortical and hippocampal areas was observed, as well as hyperlocomotion, which could be indicative of a glutamatergic overactivation. In addition, these mice manifested an increased vulnerability to anhedonia and helpless behaviour that was fully reverted by chronic imipramine treatment. Summing up, this study shows that reduced levels of VGLUT1 negatively affect behavioural outcome in the presence of adverse environmental conditions and could thus be a potential biological risk factor for major depression.

Regulation of glutamate/GABA cycle and behaviour by reduced VGLUT1

In agreement with our previous study, VGLUT1+/- mice showed normal levels of glutamate but decreased cortical and hippocampal levels of GABA and VGLUT1 as well as helpless behaviour compared to WT (17). In addition, we found here an increased synthesis of neuronal glutamate (^{13}C-4-glutamate) in whole brain extracts and a downregulation of the glial excitatory amino acid transporter 1 (EAAT1) in the frontal cortex and hippocampus. Moreover, an anhedonic behaviour was revealed, measured as a decrease of sucrose intake four weeks after individual housing. However, no changes in oxidative metabolism and in the expression of the rest of the presynaptic proteins studied were found.

VGLUT1+/- mice show a neurochemical and behavioural phenotype that could be relevant for clinical depression. Both anhedonia and helplessness are considered core and common symptoms of depression respectively (26). Several spectroscopy studies have shown decreased GABA levels in the cerebral cortex of depressed patients (3-7) associated with the mood state (5). Moreover, decreased glial EAAT1 has been observed in cortical postmortem tissue of patients who had suffered major depression (9) and preclinical studies have also linked decreased GABA levels to helplessness in the forced swimming test (27). Since glutamate is required for the major neuronal GABA synthesis pathway (28)**,** alterations in glutamate release and/or glial reuptake would be expected to affect GABAergic neurotransmission. Decreased glutamate release could limit the uptake of glutamate by the GABAergic terminal via the neuronal EAAT3-4 transporters (28). Another possibility is that the downregulation of EAAT1 detected by Western blotting could limit the uptake of glutamate and therefore the synthesis and transport of glutamine from the glial cells to the GABAergic terminal, compromising the synthesis of GABA as well (29).

Previous studies show that VGLUT1 is the major isoform in both the frontal cortex and the hippocampus (30, 31), where it plays a key role in the vesicular uptake and synaptic transmission of glutamate (13-16). The downregulation of cortical and hippocampal EAAT1 supports the idea of decreased glutamate release in the VGLUT1 dependent synapses because this glial transporter is regulated by the amount of glutamate released into the synaptic cleft (32, 33). In disagreement with this, in the initial characterization of the VGLUT1 knock-out mice, no apparent differences in the evoked excitatory postsynaptic current (EPSC) in VGLUT1+/- hippocampal cultured neurons were shown (13) suggesting that a 50% reduction in the vesicular VGLUT content does not affect the quantal size. However, these studies were carried out in individual cultured neurons, and we currently do not know whether the same is true for the intact brain of adult mice, where minor changes could potentially be physiologically relevant. The behavioural

phenotype would argue for a change in EPSC size in vivo. However, the behavioural phenotype could also be due to compensatory changes that occur in the VGLUT1+/- animals that leave the EPSC size unchanged. More specifically, the increase of the neuronal *de novo* synthesis of glutamate, derived from [1-13C]glucose, suggests a compensatory effect for the decreased vesicular VGLUT1 content and, perhaps, for the down-regulation of EAAT1 (29,34-35). This increase could contribute to the maintenance of both the cytoplasmic and the vesicular pools (36) of glutamate in the VGLUT1+/- neurons, which would agree with the normal tissue levels detected by HPLC and may be sufficient to normalize EPSC size. However, other studies suggest that the contribution of glutamate synthesis via the tricarboxylic acid (TCA) cycle to the vesicle pool of glutamate is limited (37) supporting the idea of decreased vesicular pool and synaptic release of glutamate in the VGLUT1+/- terminals. Due to the low limit of detection of some metabolites, the ^{13}C-MRS studies were carried out in whole brain extracts. Although the values therefore do not specifically represent only those areas in which VGLUT1 is the major isoform, they nonetheless should give a proportional representation of any change in the VGLUT1 expressing regions.

Regulation of glutamate/GABA cycle and behaviour by CMS in WT and VGLUT1+/- mice

In addition to core symptoms of depression, our CMS model (23) also addressed other clinical aspects highly comorbid with major depression such as anxiety (38) and impaired cognition (39,40). On the other hand, both depression (3-7) and anxiety (41,42) have been linked to impaired GABAergic function and overactive glutamatergic function has been shown in different anxiety disorders (43,44). Other studies have also shown decreased prefrontal cortex and cerebellar GAD65 and GAD67 levels in depressed patients (10, 11) and a link between anxiety and a polymorphism in the GAD65 (45) gene. Moreover, mice lacking an isoform of GAD65 have shown reduced GABA levels and increased anxiety (46,47) while rats exposed to CMS show reduced GABA levels,

anhedonia and helplessness (48, 49). We suggest that the downregulation of VGAT and its coupled GABA synthesising enzyme GAD65 in response to CMS could explain the decreased GABA levels observed in the frontal cortex and in the hippocampus of the CMS mice. In addition, a recent study addressing the effects of CMS on glial metabolism, has shown decreased glial GABA formation (49) which is consistent with our results. The upregulation of hippocampal EAAT1 may occur as a neuroprotective response to stress-induced elevations in glutamate in the synaptic cleft (32, 33) as well as in response to increased corticosterone levels (50).

VGLUT1+/- mice exposed to chronic mild stress showed a combined phenotype (stress plus genotype) but also specific neurochemical and behavioural alterations as a result of the interaction between stress and decreased VGLUT1 levels (Table 2). Interestingly, the striking upregulation of VGLUT2, along with the lack of upregulation of EAAT1 and decreased GABA formation, suggests a glutamatergic overactivation that could be linked to the increased locomotion (Figure 7). Given the segregated distribution of VGLUT1 and VGLUT2 to different synapses and pathways (31) long-loop feedback mechanisms of control could be underlying this interaction. Furthermore, VGLUT1+/- CMS mice showed increased anhedonia and helpless behaviour when compared to WT-CMS and these effects were reverted by repeated imipramine treatment, which further supports the idea that VGLUT1+/- mice are more vulnerable than WT mice to a stress-induced depressive-like phenotype.

This study shows the functional implications of decreased VGLUT1 levels for glutamatergic and GABAergic neurotransmission in the context of behavioural paradigms that model major depression. We propose that decreased VGLUT1 levels may be a biological factor that negatively interacts with well known environmental risk factors of major depression.

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Table 1. Amounts (nmol/g brain tissue) and percentage of enrichment of metabolites (A), and Acetate/Glucose utilization ratio (B), derived from [1-^{13}C]glucose and [1,2-^{13}C]acetate in whole brain extracts from WT and VGLUT1+/- mice.

A) Metabolite	**WT**		**VGLUT1**	
	nmol/g	**% enrichment**	**nmol/g**	**% enrichment**
[1-^{13}C]glucose				
[4-^{13}C]Glutamate	357.47 ± 46.2	5,97 ± 0,38	494.97 ± 58.7*	8,46 ± 0.50*
[2-^{13}C]GABA	88.10 ± 9.6	7,02 ± 0,85	92.56 ± 13.8	6,23 ± 0,35
[4-^{13}C]Glutamine	69.23 ± 9.5	2,87 ± 0,18	77.24 ± 14.3	3,19 ± 0,25
[1,2-^{13}C]acetate				
[4,5-^{13}C]Glutamate	60.53 ± 5.8	1,01 ± 0,08	72.10 ± 6.8	1,20 ± 0,09
[1,2-^{13}C]GABA	16.05 ± 1.5	1,26 ± 0,07	18.81 ± 2.8	1,27 ± 0,12
[4,5-^{13}C]Glutamine	80.06 ± 7.4	3,32 ± 0,20	89.6 ± 9.8	3,70 ± 0,26

B) Acetate/Glucose utilization ratio	**WT**	**VGLUT1**
[4,5-^{13}C]Glutamate/[4-^{13}C]Glutamate	0.173 ± 0.007	0.178 ± 0.011
[4,5-^{13}C]Glutamine/[4-^{13}C]Glutamine	1.2 ± 0.078	1.152 ± 0.058
[1,2-^{13}C]GABA/[2-^{13}C]GABA	0.193 ± 0.045	0.184 ± 0.019

All results were obtained by ^{13}C NMR spectroscopy. Metabolites derived from [1-^{13}C]glucose and [1,2-^{13}C]acetate represent the contribution from neurons and astrocytes to glutamate, glutamine and GABA formation respectively (20). Percentage enrichment correspond to the ratio between the concentration of the labelled metabolite ([^{13}C]metabolite-natural abundance (1.1%) of [^{13}C]metabolite) and the pool size of the metabolite in the brain. $^{*}p<0.05$ *versus* WT group (Student *t*-test).

Figure legends

Figure 1. Representative ^{13}C NMR spectrum of whole brain extracts from (A) a WT mice and (B) a VGLUT1+/- mice, injected with [1-^{13}C]glucose and [1,2-^{13}C]acetate. Peak assignments: 1, glutamine C-4; 2, glutamine C-4,5; 3, glutamate C-4; 4, glutamate C-4,5; 5, GABA C-2; 6, GABA C-1,2. Labelling of [4-^{13}C] glutamate, [4-^{13}C] glutamine and [2-13C] GABA formed singlets in the spectrum and derived from [1-^{13}C]glucose (neuronal metabolism). Labelling from [4,5-13C] glutamate, [4,5-13C] glutamine and [1,2-13C] GABA formed doublets in the spectrum and derived from [1,2-13C]acetate (astrocytic metabolism).

Figure 2. Effect of chronic mild stress on (A) glutamate and (B) GABA tissue levels in the frontal cortex and the hippocampus of VGLUT1+/- and WT mice. Values show the mean ± SEM (n= 15 mice per group). **p<0.01; *p< 0.05 *versus* WT controls; ^p<0.05 *versus* respective VGLUT1 control (Two-way Anova followed by Student t-test).

Figure 3. Effect of chronic mild stress on (A) VGLUT1, (B) VGLUT2, (C) VGAT, (D) GAD65, (E) EAAT1 and (F) EAAT2 expression levels in frontal cortex and hippocampal brain extracts of VGLUT1+/- and WT mice. Values show the mean ± SEM (n= 6-7 mice per group). **p<0.01; *p< 0.05 *versus* respective WT controls; ^p<0.05 *versus* respective VGLUT1 controls; $^{\#\#}$p<0.01 *versus* respective WT-CMS (Two-way Anova followed by Student t-test).

Figure 4. Performance of VGLUT1+/- and WT mice on chronic mild stress-induced (A) anhedonia in the sucrose intake test (C) helpless behavior in the forced swimming test (D) anxiety in the elevated plus maze and (E) impaired recognition memory in the novel object recognition test. In (B) the cumulative sucrose intake in weeks 4,5 and 6 for each

group is represented. Values show the mean ± SEM (n= 15 mice per group). **p<0.01; *p< 0.05 versus corresponding WT controls; ^^p<0.01; ^p< 0.05 *versus* corresponding VGLUT1 control; ##p<0.01 #p<0.05 *versus* respective WT-CMS. (Two-way Anova followed by Student t-test).

Figure 5. Effect of repeated administration of imipramine (at 10 mg/kg/day ,ip) on chronic mild stress-induced (A) anhedonia in the sucrose intake test, (B) helpless behaviour in the forced swimming test, (C) anxiety in the elevated plus maze and (D) impaired recognition memory in the novel object recognition test of VGLUT1+/- mice. Values show the mean ± SEM (n= 15 mice per group).**p<0.01; *p< 0.05 *versus* corresponding control saline; ^^p<0.01; ^p< 0.05 *versus* corresponding CMS saline. (Two-way Anova followed by Student t-test).

Figure 6. Effect of chronic mild stress on the spontaneous motor activities of (A) VGLUT1+/- and WT mice and (B) VGLUT1+/- mice chronically treated with saline or imipramine (10 mg/kg, i.p.). Values show the mean ± SEM (n= 15 mice per group).
**p<0.01; *p< 0,05 *versus* WT control in (A) and VGLUT1+/- control saline in (B) (Two-way Anova followed by Student t-test).

Figure 7. Proposed model for the interaction between decreased VGLUT1 levels and chronic mild stress. Decreased VGLUT1 induces a downregulation of the glial EAAT1 transporter. CMS induces a downregulation of GAD65 and VGAT that could affect the synthesis and release of GABA in the synaptic terminal. The interaction between CMS and decreased VGLUT1 induces an upregulation of VGLUT2, which along with the lack of upregulation of EAAT1, and decreased inhibitory GABA levels could lead to an increase in the glutamate/GABA ratio and a subsequent increase in motor activity.