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Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1)

Abbreviatted Title: Behaviour of VGLUT1$^{+/-}$ mice

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Abstract

Three isoforms of a vesicular glutamate transporter (VGLUT1-3) have been identified. Of these, VGLUT1 is the major isoform of the cerebral cortex and hippocampus where it is selectively located on synaptic vesicles of excitatory glutamatergic terminals. Variations in VGLUT1 expression levels have a major impact on the efficacy of glutamate synaptic transmission. Given evidence linking alterations in glutamate neurotransmission to various neuropsychiatric disorders, we investigated the possible influence of a down-regulation of VGLUT1 transporter on anxiety, depressive-like behaviour and learning. The behavioural phenotype of VGLUT1 heterozygous mice (C57BL/6) was compared to WT littermates. Moreover, VGLUT1-3 expression, hippocampal excitatory terminal ultrastructure and neurochemical phenotype were analysed. VGLUT1 heterozygous mice displayed normal spontaneous locomotor activity, increased anxiety in the light-dark exploration test and depressive-like behaviour in the forced swimming test: no differences were shown in the elevated plus-maze model of anxiety. In the novel object recognition test, VGLUT1$^{+/-}$ mice showed normal short-term but impaired long-term memory. Spatial memory in the Morris water maze was unaffected. Western blot analysis confirmed that VGLUT1 heterozygotes expressed half the amount of transporter compared to WT. In addition, a reduction of the reserve pool of synaptic vesicles of hippocampal excitatory terminals and a 35-45 % reduction of GABA in the frontal cortex and the hippocampus were observed in the mutant mice. These observations suggest that a VGLUT1-mediated presynaptic alteration of the glutamatergic synapses, in specific brain regions, leads to a behavioural phenotype resembling certain aspects of psychiatric and cognitive disorders.

Introduction

The loading of glutamate into the synaptic vesicles via the vesicular glutamate transporter (VGLUT) is an essential step in glutamatergic synaptic transmission. Three vesicular glutamate transporters have been identified (VGLUT1, VGLUT2 and VGLUT3). Although highly homologous with similar characteristics when loading vesicles with glutamate (Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Gras et al., 2002; Varoqui et al., 2002), they have different CNS distributions. VGLUT1 and VGLUT2 are the predominant isoforms, accounting for most of the presumed excitatory glutamatergic terminals in the CNS. Interestingly, VGLUT1 and VGLUT2 display complementary expression patterns. VGLUT1 predominates in the cerebral and cerebellar cortices and hippocampus, whereas VGLUT2 is widely expressed in the diencephalon, brainstem and spinal cord (Fremeau et al., 2001; Herzog et al., 2001). However, none of these brain regions exclusively expresses one isoform and, although most of glutamatergic neurons express either VGLUT1 or VGLUT2, co-expression of both has been reported (Hisano et al., 2002; Herzog et al., 2006). Furthermore, there is a developmental switch from VGLUT2 to VGLUT1 in the hippocampus and cerebellum (Miyazaki et al., 2003; Fremeau et al., 2004). The third isoform, VGLUT3, defines a discrete subpopulation of neurons and is co-expressed with cholinergic, serotonergic or GABAergic markers (Schafer et al., 2002; Herzog et al., 2004).

Variations in VGLUT1 levels have a major impact on efficacy of glutamate synaptic transmission (Wojcik et al., 2004; Fremeau et al., 2004; Wilson et al., 2005). Genetic inactivation of VGLUT1 drastically reduces glutamatergic neurotransmission in cortical and hippocampal neurons (Wojcik et al., 2004; Fremeau et al., 2004) with a specific

reduction in quantal size seen in cultured hippocampal neurons from VGLUT1 knock-out (VGLUT1$^{-/-}$) mice (Wojcik et al., 2004). Meanwhile, over-expression of VGLUT1 boosts presynaptic quantal size to levels that exceed WT values (Wojcik et al., 2004, Daniels et al., 2004, Wilson et al., 2005). Interestingly, VGLUT1$^{-/-}$ mice show a progressive neurological phenotype including blindness, uncoordination, enhanced startle response and lethality rate that starts 2-3 weeks after birth, coincident with the developmental switch from VGLUT2 to VGLUT1 in the hippocampus (Fremeau et al., 2004; Wojcik et al., 2004).

Clinical and preclinical studies suggest a key role of the glutamatergic system and a therapeutic value for glutamatergic targets in psychiatric and cognitive disorders (reviewed in Javitt, 2004; Kugaya and Sanacora 2005; Robbins and Murphy, 2006). The segregated distribution of VGLUT1 and VGLUT2 provides an opportunity to distinguish between cortical and subcortical glutamatergic neurons and to specifically study their pathophysiology. Cortical, frontal and hippocampal circuits, in which VGLUT1 positive excitatory neurons are central, play a key operational executive role in integrating affective imprints and cognitive processes.

Here we investigate how a down-regulation of VGLUT1 transporter might influence anxiety, depressive behaviour and learning. The behavioural phenotype of male and female VGLUT1$^{+/-}$ mice (C57BL/6) was compared to WT in a battery of tests, including motor activity, models for anxiety, depression and learning tasks. Furthermore, we also investigated how reduced VGLUT1 levels correlated with biochemical and anatomical changes. Specifically, VGLUT1-3 and other synaptic proteins expression levels, excitatory terminal ultrastructure and neurochemical phenotype were analysed.

Materials and methods

Animals

Heterozygous VGLUT1 mice (VGLUT1$^{+/-}$), (C57BL/6N) were obtained from Dr. S. Wojcik (Göttingen, Germany). A colony of wild type (WT) and VGLUT1$^{+/-}$ mice were bred from heterozygous fathers and WT mothers (Harlan, France). Mice were weaned and genotyped at the age of 3 weeks. VGLUT1$^{+/-}$ mice were studied comparatively to their WT littermates. Heterozygous mice exhibited no apparent phenotypic abnormalities during development and adulthood.

Animals were maintained in a temperature (21 ± 1 C) and humidity-controlled room (55 ± 2%) on a 12 h light-dark cycle (lights on 07:00 h) with food and water provided *ad libitum.* Experimental procedures and animal husbandry were conducted according to standard ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the Ethical Committee of University of Navarra.

Spontaneous locomotor activity

Locomotor activity was measured in an open field consisting of 9 black square arenas (43 x 50 x 45) using a video tracking system (Ethovision 3.0, Noldus Information Technology B. V., Wageningen, The Netherlands) in a softly illuminated experimental room. One mouse was placed in each box and spontaneous locomotor activity was analyzed in the novel cage. Distance traveled (cm) and speed were recorded at 15 min intervals during a 1 hour period.

Dark–light exploration test

This is an ethological model of anxiety-like behaviour designed for mice (Crawley & Goodwin 1980). The apparatus is an open-topped rectangular box (45×27×27 cm high) divided into a small (2/5) black area and a large (3/5) white area illuminated at about 500 lx. The floor of the white compartment was marked into 9-cm squares. Each mouse was placed individually in the centre of the white area and behaviour was recorded over a 5 min-period, recording the percentage of time spent in the white area and the number of line crossings. On the first day of testing there was a high variability in the data and this was reduced by testing animals in the box for two consecutive days. The time spent and the number of line crossings in the white compartment as well as the number of transitions between dark and light compartments, were recorded for each mouse.

Elevated plus maze

Elevated plus maze (EPM) assesses unconditioned anxiety-like behaviour in rats and mice (Lister 1987). EPM consisted of two open arms (30 x 5 cm), two enclosed arms (30 x 5 cm) and a connecting central platform (5 x 5 cm). The maze was raised to 38.5 cm above the floor. The mouse was placed in the center of the maze facing one of the enclosed arms and observed for 5 min. The following parameters were recorded by the experimenter: latency to the first open arm entry, number of open and closed arm entries and the time spent in different parts of the maze (open and closed arms, central platform). An arm entry was defined as a mouse having entered an arm of the maze with all four legs. In addition, the number of head dipping over the edge of open arms was recorded.

Forced swimming test

The forced swimming test is the most widely used model of depression in rodents. Mice were placed individually for 6 min into glass cylinders (height 24 cm, diameter 13 cm) containing 14 cm of water, maintained at 22–23°C. This procedure was repeated for 2 consecutive days. On the second day, the duration of immobility was recorded during the last 4 min of the 6 min testing period. A mouse was considered to be immobile when it floated in an upright position, and made only small movements to keep its head above water.

Novel object recognition

Visual recognition memory was assessed using the novel object recognition test. The apparatus consisted of a black square arena (43 x 50 x 45 cm). During two consecutive days, mice were placed for 15 min in the empty box to habituate them to the apparatus and test room. On day 3, two identical objects (A1 and A2; two prisms 7 x 3 x 3 cm) were placed symmetrically 11 cm away from the wall and separated 22 cm from each other. The mouse was placed in the box at equal distance from both objects and videorecorded for 5 min (sample phase). Then the mouse was returned to its cage. After a delay of 1 or 24 h the mouse was placed back in the box and exposed to the familiar object (A3) and to a novel object (B or C for 1 or 24 h retention interval respectively) for a further 5 min (retention tests). The novel object B consisted of a ball (3,5 cm diameter) mounted on a cube (3 cm) and C was a cylindrical plastic bottle (7 x 3 cm). The positions of the familiar and the new object alternated between the 1 h and the 24 h retention test. The box and objects were cleaned between mice and trials to stop the build-up of olfactory cues.

Mice were video recorded (Pinnacle Studio 9.0, Pinnacle systems Inc, Pittsburgh US) and the total time spent exploring each of the two objects in the sample phase and retention tests

was measured by a blind experimenter. Object exploration was defined as the orientation of the nose to the object at a distance <2 cm. Turning around, climbing over, or sitting on the object were not included. Mice that explored <1 s both new and familiar objects were excluded. A discrimination index (DI) was calculated as the difference between the time spent exploring the new (N) and familiar object (F) divided by the total time exploring the objects (N-F/F+N). Higher DI is considered to reflect greater memory retention for the familiar object.

Morris Water Maze

The Morris Water maze assesses spatial memory. A black circular pool (diameter: 120 cm; height, 40 cm) filled to a depth of 25 cm with water (22 ºC) and located in a lit room with visual cues was used. Four positions around the edge of the tank were arbitrarily designated north (N), south (S), east (E), and west (W), which provided four alternative positions and also defined the division of the tank into four quadrants: NE, SE, SW, and NW. A circular black escape platform (diameter: 9.5 cm) was submerged 0.5 cm below the water surface and placed at the center of NW quadrant throughout the training period. Mice were trained for four trials per day over four days (with and inter-trial interval of 10-12 minutes). The start position (N, S, E, or W) was pseudo-randomized across trials. Mice were allowed up to 60 s to locate the escape platform. Whether or not a mouse found the platform within 60 s, it was maintained on the platform for 15 s. The escape latency and the distance traveled were recorded. A probe trial was performed (24 h after the last training day) during which the escape platform was removed from the pool and the swimming path of each mouse was video-recorded (Ethovision 3.0, Noldus Information Technology B. V., Wageningen, The Netherlands) over 60 s while it searched for the missing platform.

Western blotting

Frontal cortex and hippocampus from pups (post-natal days 7 and 14) and adult mice were rapidly dissected and homogenized in 50 mM Tris-HCl-sucrose buffer (pH 7.4, 4 ºC) and centrifuged at 900 g for 10 min. The resultant post-nuclear supernatant was centrifuged at 12800 g for 10 min and the pellet was suspended in Tris-HCl-sucrose buffer containing 0.32 M EDTA, 1 mM PMSF, 5 μg/ml aprotinine and 5 μg/ml leupeptine to a final protein concentration of 0.8-1 μg/μl and stored at –80 °C.

Equal amounts of protein (10 μg per lane) were separated by SDS-PAGE (NuPage Bis-Tris 10%, InVitrogen) and transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Bioscience), in Tris 50 mM, borate 50 mM buffer. The trans-blots were blocked for 1 h with 5 % not-fat milk in buffer PBS containing 0.1 % Tween 20 and then probed with one of the following primary antibodies overnight at 4 °C: VGLUT1 (1:2,000), VGLUT2 (1:1,000) or VGLUT3 (1:500) rabbit antibodies (donated by Dr S. El Mestikawy, Paris, France); vesicular inhibitory amino acid transporter (VGAT) rabbit antibody (1:1,000), glutamic acid decarboxylase (GAD67) (1:2,000) and synaptophysin mouse antibodies (1:2,000) (by Chemicon International, USA). Membranes were washed 3 times (5 min) with the same buffer and incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Denmark. 1:10,000). Peroxidase activity was detected by chemiluminescence using SuperSignal West Pico (Pierce Biotechnology). Films were scanned and quantified using the ImageMaster 1D (Pharmacia Biotech, Sweden) software.

Electron microscopy

Ten adult mice, 6 heterozygotes and 4 WT, were anaesthetized and then perfused through the heart with saline to flush out blood from the circulation followed by 50 ml of a fixative containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) over a period of 20-25 min. The brains were removed from the skulls, post-fixed in the same solution for 2-3 h at 4 ºC and then stored in PBS at 4ºC until required. Free-floating sections 50 microns thick were cut horizontally through the hippocampus using a vibrating microtome and collected in vials containing 0.1 M PB. After washing, the sections were flattened onto watch glasses and floated in 1 % osmium tetroxide in 0.1M PB for 30 minutes. After a brief wash in water, the sections were dehydrated through an ascending series of ethanol concentrations, containing 1% uranyl acetate to provide additional contrast. After a final dehydration stage in propylene oxide the sections were placed in Durcupan resin (ACM Fluka, UK) overnight. The resin was then heated gently and the sections transferred to cleaned slides and a coverslip applied. The resin was cured in an oven at 60 ºC for 48 hours.

The resin-embedded sections were examined in the light microscope. To ensure comparability between electron microscope sample blocks, these were prepared from sections containing the decussation of the fimbria fornix. The coverslip was removed from the slide and a small piece of section (about 1mm x 1mm) was cut out from the mid level of the stratum radiatum of the CA1 region and glued onto a pre-formed resin block. The blocks were then coded and serial ultrathin sections were cut using an Ultracut E ultramicrotome (Leica, Milton Keynes, UK). Sections were collected onto copper mesh grids, stained with lead citrate (Reynolds, 1963) and examined in a Philips 410 electron microscope.

Using a systematic sampling schedule, boutons forming asymmetrical synapses were photographed at a final magnification of x62,000 using a digital camera (Multiscan, Gatan, UK) and Digital Micrograph software. At least 30 boutons were identified for each sample block. Only one sample block was examined per mouse. A scaled overlay 200 nm wide was aligned with the synaptic specialization and vesicles falling within this width were recorded according to whether they were within 100 nm of the synapse, measured at right angles to the synapse, or further away. All boutons forming asymmetrical specializations within one photographic field of view were assessed, accounting for the different numbers of boutons sampled per animal.

Neurotransmitter brain levels

The concentrations of GABA, glutamate, 5-HT and dopamine in frontal cortex and hippocampus were determined by HPLC with electrochemical detection (DECADE, Antec-Leyden) with a high sensitivity analytical flowcell (VT-03). Glutamate and GABA levels were detected as previously described (Garcia-Alloza et al., 2005). The working electrode was set at 0.7 V. A column (Biophase ODS 5μm, 4.6mm×150 mm) including precolumn derivatization with *o*-phthaldehyde and β mercapthoethanol (Sigma–Aldrich Ltd., Germany) was used. For 5-HT and dopamine detection, homogenates were prepared following the procedure described by Perez-Otaño et al., (1991). The working electrode was set at 0.8 V and a column (Spherisorb ODS2 5 μm, 15 × 0.46 mm; Teknokroma, San Cugat del Valles, Spain) was used. All samples were assayed in duplicate and results were expressed in ng per mg of wet tissue.

Stastistical analysis

Data from the Western blot, neurochemical and electron microscopy experiments were analyzed statistically across genotype using Student's *t*-test.

Immobility time (FST), percentage change (time and line crossings for the light-dark exploration test) and different behavioural items of the elevated plus maze test were compared for each sex across genotype using Student´s *t*-test.

Novel object recognition test: during the sample phase, the total time spent exploring each object (two identical objects) was recorded and compared for each sex across genotype using Student´s *t*-test. During the retention test, comparisons between time spent exploring the new and old objects were performed within groups using paired *t*-test. DIs were compared for each sex and time interval across genotype with Student´s *t*-test.

For MWM, performance of each group in the learning phase was compared with two-way ANOVA (time and genotype, repeated measures). Performance of each group in the probe tests was compared with one-way ANOVA using *post hoc* Dunnett's *t*-test.

Finally, spontaneous locomotor activity in mice was analyzed using a two way ANOVA (time and genotype, repeated measures).

Results

Spontaneous locomotor activity is unaffected in VGLUT1$^{+/-}$ animals

VGLUT1$^{+/-}$ mice and their WT littermates did not differ in their spontaneous locomotor activity (Table 1). Two-way ANOVA comparison with repeated measures revealed no interaction genotype × time. However, distance travelled decreased gradually across the 15 min time blocks in all groups.

Increased anxiety in VGLUT1$^{+/-}$ animals in the light-dark exploration test

In the light-dark exploration test, VGLUT1$^{+/-}$ mice were more anxious than their WT littermates (Figure 1A). The time spent in white area (second exposure day) was significantly lower for the male and female heterozygotes than for their WT littermates. The number of line crossings (Figure 1B) was significantly lower for the male heterozygous group compared to WT. Similarly, the number of transitions was significantly lower, being 9.45 ± 0.6 and 5.8 ± 0.8 transitions for WT and VGLUT1+/- mice, respectively ($p < 0.01$, Student´s *t*-test). However, no major differences were detected between female groups both in the line crossings (Figure 1B) and in the number of transitions (5.1 ± 1 and 4.8 ± 0.7 transitions for WT and VGLUT1+/- mice respectively). In addition, female (heterozygotes and WT) groups were more anxious (lower time and line crossings in white compartment and less transitions) than male groups.

Anxiety levels in the elevated plus maze are indistinguishable between VGLUT1$^{+/-}$ and WT animals

In another model of anxiety, the elevated plus maze, no significant differences were found

between VGLUT1$^{+/-}$ mice and their WT littermates in any of the behavioural items recorded (Table 2). Females from both groups (heterozygotes and WT) spent less time in open arms, indicating higher anxiety levels than the corresponding age matched male groups.

Depressive like behaviour of VGLUT1$^{+/-}$ animals in the forced swimming test

Both male and female VGLUT1$^{+/-}$ mice showed a significant increase in the immobility time in the forced swimming test (FST) compared to their WT littermates (Figure 2). The magnitude of increase was 60% and 47% for VGLUT1$^{+/-}$ males and females respectively. No significant differences in the immobility time were detected between sexes.

VGLUT1$^{+/-}$ animals show impaired long-term memory in the object recognition test

During the sample phase, the total time spent in exploration did not differ significantly between groups (data not shown).

With a one hour interval between the sample and the test phase, VGLUT1$^{+/-}$ and WT groups spent significantly more time exploring the new object than the familiar one (Fig. 3A). The discrimination index (DIs) did not differ significantly, indicating that both groups had similar memory retention for the familiar object (Fig. 3C). Twenty-four hours after the sample phase, both male and female VGLUT1$^{+/-}$ mice failed to discriminate the novel object whereas the WT mice were still able to discriminate between the two objects (Fig. 3B). At this time interval, heterozygous mice showed a deficit in recognition memory compared to their corresponding WT littermates (significantly lower DIs) (Fig. 3C).

Normal spatial memory in VGLUT1$^{+/-}$ animals in the Morris Water Maze test

VGLUT1$^{+/-}$ mice as well as their WT littermates were able to learn the location of the platform. Learning curves for WT and VGLUT1$^{+/-}$ mice did not differ significantly (two way repeated measures ANOVA, time and genotype, Fig. 4A).

In the probe test (24 hour after the last training day), all groups spent significantly longer time in the target quadrant compared to the rest of the quadrants (Fig. 4B). No significant differences were found between males and females.

Quantification of VGLUT1-3, VGAT and GAD67 protein levels

VGLUT1 and VGLUT2 protein levels in frontal cortex brain extracts were quantified on post-natal days (P) 7, P14 and compared to the protein levels of adult mice (P90) (Figure 5). VGLUT3 isoform was measured only in adult mice. In both WT and heterozygous mice VGLUT1 levels increased progressively during post-natal development (Figure 5A). One week after birth (P7), VGLUT1 levels in WT mice had reached approximately 10 % of adult levels and then increased up to 75 % two weeks after birth (P14) (Figure 5B). VGLUT1$^{+/-}$ mice showed a similar developmental increase of VGLUT1 levels. However, VGLUT1 protein levels in heterozygotes at the respective ages were only 40% (P7), 49% (P14) and 59% (adults) of WT VGLUT1 levels (Fig. 5B). The expression of VGLUT2 also increases during development and at one and two weeks was approximately 50 % of adult levels (Figure 5C). However, we detected no differences in the VGLUT2 protein levels between WT and heterozygous animals (Fig. 5C). Similarly, VGLUT3 levels in the frontal cortex of VGLUT1 heterozygotes were not changed compared to WT (data not shown).

In the hippocampus of adult mice, VGLUT1$^{+/-}$ mice had about two-thirds the level of VGLUT1 protein found in WT mice (Figure 6B). VGLUT2 expression was not altered in the hippocampus. However, VGLUT3 was slightly increased (15 %) in the VGLUT1

heterozygotes compared to WT (Figure 6B).

We also quantified VGAT, GAD67 and synaptophysin protein levels. No changes in any of these proteins were found neither in the hippocampus (Figure 6) nor in the frontal cortex (data not shown) of adult VGLUT1$^{+/-}$ mice compared to WT.

Electron microscopy

On initial examination it was found that, due to poor ultrastructural preservation, three mice (one WT and two heterozygotes) had to be eliminated from the study. Boutons forming asymmetric synaptic specializations were identified in all sections examined from the remaining blocks. In general the boutons contained numerous small round vesicles and the majority of post-synaptic targets had the morphological characteristics of spines although some were consistent with being small dendrites (Figure 7A). These features are characteristic of glutamatergic terminals. Within 100 nm of the active zone of the synapse, there was no difference between VGLUT1$^{+/-}$ and WT mice in the number of synaptic vesicles (258 ± 9 and 263 ± 24 vesicles respectively). However, the heterozygotes had significantly fewer vesicles at a distance > 100 nm from the active zone (324 ± 24) compared to the WT (388 ± 11), which is thought to correspond to the reserve pool (Figure 7B).

Neurotransmitter brain levels

VGLUT1$^{+/-}$ mice showed a significant reduction of GABA levels in frontal cortex and dorsal hippocampus compared to WT animals. However 5-HT and dopamine levels were not changed in VGLUT1$^{+/-}$ mice compared to WT, in any of the brain regions studied (Table 3).

Discussion

The present study shows that VGLUT1 heterozygous mice (C57/BL/6) display behavioural and learning deficits. In particular, VGLUT1$^{+/-}$ mice showed increased anxiety in the light-dark exploration test and depressive-like behaviour in the forced swimming test. In the novel object recognition test, VGLUT1$^{+/-}$ mice showed normal short-term but impaired long-term memory. In contrast, spatial memory in the Morris water maze test was not affected. Western-blot studies confirmed a reduction of VGLUT1 levels in VGLUT1$^{+/-}$ mice at all stages of development. Furthermore, a moderate reduction in the reserve pool of synaptic vesicles in excitatory terminals was found, suggesting that a 50% reduction in VGLUT1 levels is sufficient to affect vesicle recycling. Finally, GABA levels were significantly reduced in frontal cortex and hippocampus. Our study suggests that reduced VGLUT1 levels affect behaviour and learning. This behavioural phenotype might be linked to a presynaptic alteration of the VGLUT1-dependent glutamate synaptic transmission.

VGLUT1$^{+/-}$ mice show enhanced anxiety, depressive-like behaviour and impaired recognition memory

In the light–dark box test, VGLUT1$^{+/-}$ mice showed increased anxiety compared to WT littermates but did not differ in the elevated plus-maze, another model of anxiety. Although both models are based on a similar conflict between the tendency of mice to explore an open and illuminated novel environment and its aversive properties, the elevated plus-maze includes two additional anxiety-provoking environmental parameters (height and a totally open area) (Crawley et al. 1997). Previous studies have shown that C57BL/6 mice exhibit higher levels of anxiety in the elevated plus-maze than in the light-dark test (Griebel et al. 2000). Thus, similar levels of anxiety between WT and heterozygous animals in the

elevated plus maze may be due to a ceiling effect. Interestingly, the light-dark test is considered a good model to measure anxiogenic behaviour in C57BL/6 transgenic mice (Crawley et al. 1997).

The forced swimming test is the most widely used pharmacological model to asses antidepressant activity in rodents. This test is also used to detect depression and antidepressant-related phenotypes in genetically altered mice (Porsolt, 2000; Seong et al., 2002; Cryan et al., 2002; 2003). VGLUT1$^{+/-}$ mice showed increased immobility time in this test compared to WT littermates and the increase was similar to that reported in a variety of animal models of depression, including chronic mild stress and transgenic mice models (Solberg et al, 1999; Griebel et al., 2002; Tannenbaum et al, 2002; Strekalova et al., 2004; Svenningsson et al., 2006). Interestingly, the depressive-like behaviour of mice with reduced VGLUT1 levels, matches other studies showing an increase of this transporter in rat cortical and hippocampal regions following a course of antidepressant drug or ECS treatment (Tordera et al., 2005; Moutsimilli et al., 2005). Taken together, these results suggest that alterations in VGLUT1-dependent glutamate neurotransmission might have a pivotal role in both the cause of depression and its successful treatment.

Learning and memory were studied using object recognition and spatial maze learning tasks. The object recognition test relies strongly on visual recognition memory and is based on rodents' exploratory behaviour and spontaneous preference for novel objects (Ennaceur and Delacour 1988). Interestingly, this test is an incidental rather than aversive learning paradigm and overcomes the disadvantages of lengthy training procedures. On the other hand, the Morris water maze (Morris, 1984), one of the most widely used paradigms for testing spatial memory in transgenic mice, involves training over several days.

In the novel object recognition test, VGLUT1$^{+/-}$ mice showed impaired long-term

(24 h) but not short-term (1 h) recognition memory. Several lines of evidence suggest a role for the prefrontal cortex (PFC) in discrimination of object familiarity (Meunier et al., 1997; Ragozzino et al., 2002; Akirav et al., 2006). Moreover, local injection of an NMDA receptor antagonist in the medio-ventral prefrontal cortex (mvPFC), shows that long-term recognition memory depends on normal function of the NMDA receptor-mediated glutamatergic transmission in this brain region (Akirav et al., 2006). The memory impairment shown by VGLUT1$^{+/-}$ mice could relate to a down-regulation of VGLUT1-dependent glutamatergic transmission in those cortical brain regions where VGLUT1 is the predominant isoform. However further studies would be needed to explore the hypothesis that NMDA receptor-dependent glutamatergic transmission is altered in these mice.

Surprisingly, spatial memory in the Morris water maze was unaffected in VGLUT1$^{+/-}$ mice. This task depends on proper functioning of the hippocampus (Moser et al., 1993; Moser et al., 1995; Duva et al., 1997; Broadbent et al., 2004), while the fimbria fornix and the perforant pathways, the routes for extrinsic glutamatergic input to the hippocampus, are implicated in spatial memory (Galani et al., 2002). A possible mechanism is that the VGLUT2-dependent transmission in the hippocampus could be playing a leading role in spatial memory. In support of this hypothesis, a rich network of VGLUT2-immunoreactive afferent fibers in the rat hippocampus (Kaneko and Fujiyama, 2002) originating in the fimbria fornix (Halasy et al., 2004) has been described. Interestingly, since both isoforms localize to different glutamatergic terminals in the adult hippocampus the decrease of VGLUT1 would not necessarily affect VGLUT2-dependent transmission. On the other hand, the fact that VGLUT1 is the majority isoform in the hippocampus also suggests that important compensatory changes might be taking place here. For instance, post-synaptic glutamate receptors could have adapted to maintain the synaptic strength in the VGLUT1

dependent synapses in this brain region and others. The apparent normal locomotor activity of these mutants would also support this hypothesis. Further studies will be carried out to explore both hypotheses.

VGLUT1$^{+/-}$ mice show a reduction in VGLUT1, in the reserve pool of synaptic vesicles and in GABA levels.

Western blot analysis from frontal cortex extracts confirmed that the VGLUT1 heterozygotes expressed half the amount of transporter compared to WT, both in the developing (post-natal days P7, P14) and in the adult (P90) mouse brain. The progressive increase of VGLUT1 immunoreactivity in the WT mice (expressed as percentage of adult levels) resembled previous studies carried out in mouse brain (Gras et al., 2005) and rat cerebral cortex extracts (Minelli et al., 2003).

We also addressed the possibility that alterations in VGLUT2 and/or VGLUT3 isoforms might compensate for reduced VGLUT1 levels in the heterozygotes. Previous studies using whole brain extracts of VGLUT1$^{-/-}$ knockout mice (Wojcik et al., 2004; Fremeau et al., 2004) found no changes in VGLUT2 and VGLUT3 expression. Similarly, and consistent with the complementary distribution of VGLUT1, no changes were found in VGLUT2 in the cortex and hippocampus of the VGLUT1$^{+/-}$ mice. In contrast, the VGLUT3 isoform was slightly up-regulated (15 %) in the hippocampus. Unlike the other two isoforms, VGLUT3 is diffusely distributed in the brain, defining a discrete subpopulation of non-glutamatergic neurones. Specifically in the hippocampus, VGLUT3 is expressed in a subpopulation of GABA interneurons in the dentate gyrus and CA2-3 fields (Schafer et al., 2002; Gras et al., 2002; Herzog et al., 2004). These hippocampal VGLUT3-positive neurons and their relatively sparse innervations may exert a feed-forward control of the

excitability of projection neurons that would have significant impact on modulation of hippocampal functions such as learning and memory (Schafer et al., 2002; Herzog et al., 2004).

At the ultra-structural level, the reserve pool of synaptic vesicles in hippocampal excitatory terminals of the heterozygous mice was significantly reduced. This agrees with a previous study where homozygous VGLUT1$^{-/-}$ mice displayed a severe reduction in the reserve pool of synaptic vesicles, suggesting a specific role for VGLUT1 in synaptic vesicle recycling (Fremeau et al., 2004). A similar function remains to be demonstrated for other vesicular transporters (Zhou et al., 2000; Parsons et al., 1999). However, our results show that, for VGLUT1, even a 50% reduction in protein levels is sufficient to affect the vesicle pool.

VGLUT1$^{+/-}$ mice showed a significant reduction of GABA in frontal cortex and hippocampus. Although a compensatory mechanism involving the balance between inhibitory and glutamatergic excitatory neurotransmission might be predicted, in these brain regions, glutamate levels were unchanged, and the ratio of excitatory-inhibitory neurotransmitter levels actually increased. Subsequently, we addressed the possibility that either a down-regulation of the vesicular inhibitory aminoacid transporter (VGAT) or the GABA-synthesizing enzyme GAD67 might explain these results. However, neither of these proteins was altered in either the cortex or in the hippocampus of the VGLUT1$^{+/-}$ mice. It is still possible, although not likely, that the other GAD isoform, GAD65, which synthesizes GABA for secretory vesicles (Olsen and Betz, 2006) would be affected. Importantly, given evidence linking low levels of GABA in prefrontal cortex to depression (reviewed in Kedell et al., 2004), it would be interesting to further explore other possible mechanisms involved in the neurochemical phenotype of these mutants.

Functional implications

Although the present study did not address the effect of reduced VGLUT1 levels on the efficacy of glutamatergic neurotransmission directly, there is some evidence that this would result in a less efficient accumulation of glutamate in the synaptic vesicles and a decreased synaptic availability of glutamate during neurotransmission. Firstly, despite a 50% reduction in protein levels as measured by Western blots, only a minor reduction in the reserve pool of synaptic vesicles were found in the heterozygotes. In addition, the synaptic vesicle protein synaptophysin was unaltered. Taken together, these results suggests that the number of VGLUT1 molecules per synaptic vesicle might be reduced in the heterozygotes. Secondly, the quantal size of glutamatergic neurons is reduced by knocking-out VGLUT1 (Wojcik et al., 2004) and enhanced with over-expression of VGLUT1 (Wojcik et al., 2004; Daniels et al., 2004; Wilson et al., 2005). Finnally, decreased expression of other vesicular transporters (vesicular acetylcholine transporter and vesicular monoamine transporter 2) reduces the vesicular contents and the amount of transmitter released per quanta (Song et al., 1997; Pothos, 2002). However, these observations do not exclude the possibility that important compensatory mechanisms could take place at the postsynaptic level, to maintain the synaptic streght in the VGLUT1-dependent synapses.

Pre-clinical and clinical studies have linked alterations in glutamate neurotransmission to affective disorders (Javitt, 2004; Kugaya and Sanacora 2005) and impaired cognition (Robbins and Murphy, 2006). In line with these studies, we show that reduced VGLUT1 levels has significant effects on behaviour and learning. Hence, the modulation of this transporter may be both an endogenous mechanism of more subtle behavioural adjustments and a potential target of therapeutic intervention.

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Abbreviations:

EPM: Elevated plus maze

FST: Forced swimming test

GAD67: Glutamic acid decarboxilase

VGLUT: Vesicular glutamate transporter:

VGAT: Vesicular inhibitory amino acid transporter

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Figure 1. Performance of VGLUT1$^{+/-}$ and wild type (WT) mice in the light-dark exploration test. Values show the mean ± SEM (n= 18-20 mice per group) of (A) time spent in the white area and (B) number of line crossings, recorded in the same animals 24 h before. $^{**}P < 0.01$; $^{*}P < 0.05$ *versus* corresponding WT mice (Student *t*-test).

Figure 2. Immobility time (sec) of VGLUT1$^{+/-}$ and WT mice in the forced swimming test. Values show the mean ± SEM (n= 18-20 mice per group). $^{**}P < 0.01$; $^{*}P < 0.05$ *versus* corresponding WT mice (Student´s *t*-test).

Figure 3. Performance of VGLUT1$^{+/-}$ and wild type (WT) mice in the novel-object recognition test. Time engaged in exploring each object type (new and familiar) during the test phase performed (A) 1 h and (B) 24 h after the end of the sample phase. $^{**}P < 0.01$; $^{*}P < 0.05$ *versus* familiar object (Student´s paired *t*-test). (*C*) Discrimination index. DI = $(N - F)/(N + F)$, where *N* is time spent in exploring new object and *F* is time spent in exploring familiar object. $^{**}P < 0.01$; $^{*}P < 0.05$ *versus* corresponding WT mice (Student´s *t*-test). Values show the mean ± SEM (n= 18-20 mice per group).

Figure 4. Performance of VGLUT1$^{+/-}$ and WT mice in the Morris-Water maze. (A) Learning curves for WT and VGLUT1$^{+/-}$ mice showing latencies (sec) to platform during four training days. Each mouse was subjected to four trials per day. (B) Results of the probe trial. Time spent in the four quadrants. Both WT and VGLUT1$^{+/-}$ mice spent significantly more time in the NE quadrant. $^{**}P < 0.01$;$^{*}P < 0.05$ *versus* NE quadrant (one-way ANOVA followed by Dunnett *t*-test). Values are expressed as the mean ± SEM (n = 12

mice per group).

Figure 5. Abundance of VGLUT1 and VGLUT2 protein expression in frontal cortex extracts as measured by Western blotting. (A) Representative immunoblots of VGLUT1 and VGLUT2 from three WT mice and three VGLUT1$^{+/-}$ mice of each age (P7, P14 and P90; adults). Densitometric analysis of (B) VGLUT1 and (C) VGLUT2 protein expression. Optical density values were normalised to β-actin. Results are expressed as the percentage of adult WT values (means ± SEM of 4–8 animals, performed in duplicates). ***P*<0.05 *versus* corresponding age-matched WT mice (Student's *t*-test).

Figure 6. Abundance of VGLUT1-3 protein expression in hippocampal extracts as measured by Western blotting. (A) Representative immunoblots of VGLUT1-3, VGAT, GAD67 and synaptophysin from four WT and four VGLUT1$^{+/-}$ mice (P90; adults), (B) Densitometric analysis of VGLUT1-3 protein expression in the hippocampus. Optical density values were normalised to β-actin. Results are expressed as the percentage of WT values (means ± SEM of 8 animals, performed in duplicates). ***P*<0.01; **P*<0.05 *versus* corresponding WT mice (Student's *t*-test).

Figure 7. Morphological changes in the VGLUT1$^{+/-}$ mice. (A) Representative micrographs from WT and VGLUT1$^{+/-}$ mice showing boutons in the CA1 region of the hippocampus that were evaluated in this study. All boutons form asymmetrical synaptic contacts. (B) Synaptic vesicles in the reserve pool (> 100nm from the active zone of the synapse) are reduced in VGLUT1$^{+/-}$ mice. Mean ± SEM: n= 3 WT and n= 5 VGLUT1$^{+/-}$, *p=0.01

(Student´s *t*-test). Scale bar 0.5 μm.

Table 1. Performance of VGLUT1$^{+/-}$ mice and wild type (WT) on motor activity.

Time	Males		Females	
	WT	**VGLUT1+/-**	**WT**	**VGLUT1+/-**
0 - 15 min	5562 ± 314	6221 ± 338	6085 ± 503	5931 ± 258
15 - 30 min	4231 ± 379	4681 ± 288	5024 ± 443	4511 ± 359
30 - 45 min	3724 ± 364	4105 ± 254	4153 ± 493	3846 ± 330
45 - 60 min	3057 ± 340	3447 ± 313	3924 ± 343	3127 ± 317

Data show the time course of distance travelled (cm) in 15-min time blocks for 60 min. The data were statistically evaluated using a two-way ANOVA with repeated measures over time. Data represent mean ± SEM of 18-20 mice. (Genotype and sex $P > 0.05$; time $P < 0.001$; interaction $P > 0.05$).

Table 2. Performance of VGLUT1$^{+/-}$ and WT mice in the elevated plus-maze.

Parameter	Males		Females	
	WT	**VGLUT1+/-**	**WT**	**VGLUT1+/-**
Open arms (time, sec)	5.2 ± 2.1	6.7 ± 2.1	1.5 ± 0.9	1.1 ± 0.8
Center arms (time, sec)	34.7 ± 6.3	32.1 ± 4.7	45.9 ± 8.2	43.5 ± 7.0
Closed arms (nº entries)	9.1 ± 1.3	9.6 ± 1.3	8.6 ± 1.6	7.4 ± 1.1
Attempts (nº)	7.1± 1.5	7.1 ± 1.1	13.2 ± 1.0	11.3 ± 1.2

Data represent mean ± SEM of 18-20 mice. The mouse was placed in the center of the maze facing one of the enclosed arms and observed for 5 min.

Table 3. Glutamate, GABA, 5-HT and dopamine levels (expressed as ng/mg wet tissue) in frontal cortex and hippocampal tissue from VGLUT1$^{+/-}$ and wild type (WT) mice.

Brain region	Neurotransmitter	WT	VGLUT1+/-
Frontal Cortex	Glutamate	5231 ± 259	5184 ± 337
	GABA	1726 ± 294	930 ± 45*
	5-HT	0.555 ± 0.023	0.630 ± 0.025
	Dopamine	0.260 ± 0.068	0.243 ± 0.099
Hippocampus	Glutamate	5194 ± 248	4708 ± 94
	GABA	1473 ± 109	959 ± 55**
	5-HT	0.743 ± 0.047	0.760 ± 0.067
	Dopamine	0.033 ± 0.003	0.032 ± 0.005

Data represent means ± SEM of 8-12 mice per group. *P<0.05; **P<0.01 *versus* corresponding WT (Student´s *t*-test).