Regulation of markers of synaptic function in mouse models of depression: chronic mild stress and decreased expression of VGLUT1

Elizalde N., Pastor P.M*., Garcia-García A.L.*, Serres F., Venzala E., Huarte J., Ramírez M.J., Del Rio J., Sharp T and Tordera R.M.

1. Department of Pharmacology, University of Navarra, 31080 Pamplona, Spain

2. Department of Pharmacology, University of Oxford, OX1 3QT Oxford, United Kingdom.

* These authors contributed equally to this work

Corresponding author:

Dr. R. M. Tordera,

Dept. Pharmacology

University of Navarra

31080 Pamplona,

SPAIN

Email: rtordera@unav.es Tel: 0034 948 425600 Fax: 0034 948 425649

Abbreviations:

CMS, Chronic mild stress; SVP, synaptic vesicle protein; VGLUT1, vesicular glutamate transporter 1; Arc, activity regulated cytoskeletal protein; BDNF, brain derived neurotrophic factor; IEG, immediate early gene; EAAT1, excitatory aminoacid transpoter 1; mPFC , medial prefrontal cortex; DG, dentate gyrus.

Abstract

Depression has been linked to failure in synaptic plasticity originating from environmental and/or genetic risk factors. The chronic mild stress (CMS) model regulates the expression of synaptic markers of neurotransmitter function and associated depressive-like behaviour. Moreover, mice heterozygous for the synaptic vesicle protein (SVP) vesicular glutamate transporter 1 (VGLUT1), have been proposed as a genetic model of deficient glutamate function linked to depressive-like behaviour. Here, we aimed to identify, in these two experimental models, mechanisms of failure in synaptic plasticity, common to stress and impaired glutamate function.

First, we show that CMS induced a transient decrease of different plasticity markers (VGLUT1, synapsin 1, sinaptophysin, rab3A and activity regulated cytoskeletal protein Arc) but a long-lasting decrease of the brain derived neurotrophic factor (BDNF) as well as depressive-like behaviour. The immediate early gene (IEG) Arc was also downregulated in VGLUT1+/- heterozygous mice. In contrast, an opposite regulation of synapsin 1 was observed. Finally, both models showed a marked increase of cortical Arc response to novelty.

Increased Arc response to novelty could be suggested as a molecular mechanism underlying failure to adapt to environmental changes, common to chronic stress and altered glutamate function. Further studies should investigate whether these changes are associated to depressive-like behaviour both in animal models and in depressed patients.

Keywords: Arc, synapsin 1, BDNF, major depression, synaptic vesicle protein

Running title: Common markers of plasticity failure in depression

Introduction

Increasing evidence suggests that depression is characterized by impaired brain plasticity that might originate from both environmental and genetic risk factors (Molteni, 2009a). In order to test this hypothesis, the regulation of different plasticity markers in experimental models based on stress paradigms or in genetic manipulations has been studied. For instance, the environmental chronic mild stress (CMS) model regulates the expression of synaptic proteins such as synaptic vesicle proteins (SVP), immediate early genes (IEG) and/or neurotrophins, critical for neuroplasticity as well as associated depressive-like behaviors. Specifically, CMS induces a downregulation of the SVP synapsin 1 (Silva et al., 2008; Bessa et al., 2009) and the brain-derived neurotrophic factor (BDNF) (Duman et al.,2006; Dang et al.,2009; Li et al.,2008), that might be linked to anhedonia and helplessness. Moreover, other models based on stress paradigms have shown decreased levels of synaptophysin together with other SVPs (Thome et al., 2001). However, these studies were carried out shortly after the termination of the stress procedure while CMS has shown to induce a long-lasting depressive-like behaviour (Elizalde et al., 2008). Thus, whether these synaptic proteins are involved in the long-term CMS induced behavioural alterations remains unknown.

On the other hand, recent clinical (Uezato et al., 2009) and preclinical studies (Tordera et al., 2007; García-García et al., 2009) have linked decreased levels of the synaptic vesicle protein (SVP) vesicular glutamate transporter 1 (VGLUT1), with a key role in glutamate release in the forebrain (Fremeau et al., 2004; Wojcik et al., 2004), to depressive like behaviour. Specifically, decreased VGLUT1 levels in the frontal cortex of depressed subjects have been reported (Uezato et al., 2009) in postmortem studies. In addition, recent studies with the VGLUT1 heterozygous mice suggest that decreased VGLUT1 levels affects glutamate transmission (Balschun et al., 2009) and induces depressive-like behaviour comorbid with anxiety and impaired recognition memory

(Tordera et al., 2007). However, the regulation of other stress and/or glutamate sensitive plasticity markers has not been studied in these mice.

Here, we aimed to identify in one environmental (chronic mild stress) and one genetic model (VGLUT1+/- mice) showing depressive-like behaviours, changes in the expression of genes critical for neuroplasticity, common to stress and impaired glutamate function. Firstly, we studied comparatively, how CMS and decreased VGLUT1 levels (VGLUT1 heterozygous mice), affects the expression of selected plasticity markers already suggested to be regulated by different forms of stress and/or glutamate function. Among them, the SVPs (VGLUT1, synapsin 1, sinaptophysin and rab3A), neurotrophins (BDNF) and the activity regulated cytoskeletal protein (Arc) were included. Moreover, in order to study the persistence of stress-induced changes, the expression of these plasticity markers was measured at short and long-term after the CMS procedure. Subsequently, some relevant mRNA changes detected by *in situ* hybridization were selected for protein expression studies by western-blot. Secondly, we aimed to study cortical and hippocampal Arc protein expression induced by exposure to a novel environment. Arc has been suggested to play a major role in brain areas displaying a prominent emotion-related synaptic plasticity (Ons et al., 2004; Vazdarjanova et al., 2002). Specifically, a striking Arc induction in telencephalic regions after exposure to novel environment, a mild emotional stressor that involves learning processes, has been reported (Ons et al., 2004; Vazdarjanova et al., 2002).

Methods and Materials

Animals

For the CMS procedure, male mice C57BL/6 mice (8-10 weeks of age) were housed in individual cages and allowed for 2 weeks to habituate before beginning experimentation. Food and water were available *ad libitum* for the duration of the experiments unless otherwise specified.

Heterozygous VGLUT1 male mice (VGLUT1+/-; C57BL/6) (8-10 weeks of age) obtained from Dr S. Wojcik (Gottingen, Germany) were used. 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 and compared to their WT littermates. Heterozygous mice exhibited no apparent phenotypic abnormalities during development and adulthood.

All the animals were maintained in a temperature (21 ± 1 ºC) and humidity-controlled room (55 ± 2%) on a 12-h light-dark cycle (lights on 08.00 h) with food and water provided *ad libitum* unless otherwise specified. Experimental procedures and animal husbandry were conducted according to standard ethical guidelines of the European Communities Council Directive (2003/65/EC) Spanish legislation (Real decreto 1201/2005) and approved by the Ethical Committee of University of Navarra.

Experimental design

Chronic mild stress. Mice were divided into control and CMS groups (n=64 mice/group). Chronic mild stress (CMS) procedure was applied for six weeks (Elizalde et al., 2008). Anhedonic-like behavior was evaluated by weekly monitoring of sucrose intake in both control and CMS mice during the 6 weeks of the stress procedure and

one month after. One day (24 h) after the stress procedure a group of control (n=12) and CMS mice (n=12) were exposed to the forced swimming test. Similarly, one month after the stress procedure another group of control and CMS mice were tested in this paradigm. Mice exposed to this test were excluded from posterior neurochemical studies (Figure 1A). To study the short and long-term effects of CMS on the expression of different plasticity markers animals were killed by cervical dislocation 24 hours (short-term effects) or one month after CMS (long-term effects) (Figure 1B). For *in situ* hybridization studies (n=6 mice/group), after sacrifice, brains were rapidly removed and frozen in isopentane (-40 ºC) and for western blot (n=6 mice/group), the hippocampus and the frontal cortex was rapidly dissected and frozen in dry ice. In addition, short and long-term effects of CMS on cortical and hippocampal Arc expression induced by exposure to novel environment, was studied in a separate group of animals (n=6-8 mice/group). Novel environment consisted on the placement of mice inside a cardboard box (15 × 28 × 20 cm) softly illuminated during 1 h before sacrifice (Figure 1B). Prefrontal cortex was rapidly dissected and frozen in dry ice. Moreover, as a control experiment, a separate group of control mice (n= 6, C57BL/6, 8-10 weeks of age) were placed in individual cages for two weeks and cortical and hippocampal Arc expression induced by exposure to novel environment was studied.

VGLUT1+/- mice. Sucrose intake and forced swimming tests were applied to a group of VGLUT1+/- mice and WT littermates (n=12 mice/group), and these mice were excluded from posterior neurochemical studies. The expression of different plasticity markers in VGLUT1+/- and WT littermates was studied by *in situ* hybridisaton (n=6 mice/group) and western-blot studies (n=6 mice/group). In addition a separate group of animals (n=6-8 mice/group) were used to study Arc response to novelty.

Chronic mild stress procedure

The following unpredictable mild stressors (2-3 in any 24 h period) were randomly applied for 6 weeks (Elizalde et al., 2008): stroboscopic illumination (8h), intermittent bell (10 db, 1s/10s) or white noise (4 h), rat odour (8h), cage tilt 45º (8 h), soiled bedding (6 h), paired housing (2h) overnight illumination, removal of nesting material (12 h) and confinement (1 h). Once a week, during the 15 h of duration of the sucrose intake test (from 6 p.m to 9 a.m.) no stressors were applied.

Sucrose intake test

Anhedonic-like behaviour was evaluated by weekly monitoring of sucrose intake (Elizalde et al., 2008). Mice were first trained to drink a sucrose solution by exposing them to two standard drinking bottles, one containing 2.5 % sucrose and the other tap water, for every other night during one week. After this preliminary phase, mice were food deprived and exposed to the sucrose solution and water from 6:00 p.m. until 09:00 h in the morning. The intake baseline for the sucrose solution was established, which corresponded to the average of three consecutive measurements.

In the CMS procedure, mice were divided into two groups (CMS and non-stressed controls) matched for sucrose consumption and body weight. Mice were weekly given a 15-h exposure to the sucrose solution and tap water as described above and during this test no stressors were applied. In VGLUT 1+/- mice (n=12 mice/group), sucrose intake was evaluated in individualized mice during three consecutive weeks and the average of the sucrose intake of these three weeks was calculated. The position of the 2 bottles (right/left) was varied randomly from trial to trial. Body weight measurements were taken weekly and relative sucrose intake was calculated as absolute intake (g) per body weight.

Forced swimming test

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. 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. Immobility times were scored by Ethovision XT 5.0 (Noldus, Netherland).

In situ hybridization studies

Coronal brain sections (14 µm thick) were serially cut with a cryostat. Slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 5.8). The oligonucleotides (Sigma Genosis, UK) complementary to the following mouse genes were used:

VGLUT1: 5´CGGGGGCCTCGGAGGCTGCACTGTGCTGTGTGTGGCCCCGTAGGA3´

Synapsin 1: 5´-TGGCCTGAGAGGCAGACTGCTGAGGTGCTGCGGTGGGAC-3´

Synaptophisin: 5´-TGGATCCAACCCAAACCTGCCCACTCCGGAACCCACCATG-3´

Rab3A: 5´-TCTTCTCACAGATCACGTCCACCAGACGTTCAAACGTC-3´

Arc: 5´-CTTGGTTGCCCATCCTCACCTGGCACCCAAGACTGGTATTGCTGA-3´

BDNF: 5´-GGTCTCGTAGAAATATTGCTTCAGTTGGCCTTTTGATACCGGGAC-3´

Oligonucleotides were 3´-tail labeled with [35S]dATP, specific activity >1,000 Ci/mmol (GE Healthcare Biosciences, Europe), using terminal deoxynucleotide transferase (Roche, Diagnostics, USA). Negative controls including sense oligonucleotides showed minimal background signals. Labeled probes were purified by chromatographyon Sephadex G-25 columns (Nick column, GEHealthcare Biosciences, Europe). The sections were incubated overnight in humid chambers (containing 50% formamide in

4x SSC) at 35-37 ºC, with 200 µl hybridization buffer (50% deionized formamide, 20% 20xSSC, 10% dextran sulfate, 10% Denhardt's solution; 500 lg/ml salmon sperm DNA; 20 mM sodium phosphate; 100 mM DTT; 250 lg/ml yeast tRNA; and 1% N-laurosylsarcosine) containing $1x10^6$ cpm of the ^{35}S-ATP labeled antisense. After incubation, slices were washed three times in SSC buffer at 55ºC for 20 min, followed by washing twice in SSC at room temperature for 60 min. Sections were then air-dried, and exposed to Biomax MR film (Kodak) for 5-7 days except for VGLUT1 (3 days).

The relative abundance of mRNA in each region was determined by densitometric quantification of autoradiograms using an image analysis system (Scion Image, Scion Corporation, USA) correcting for nonspecific signals. Optical density values were calibrated to ^{35}S tissue equivalents using ^{14}C microscales (Amersham, UK) and the appropriate conversion factor. Densitometric values from three sections of each animal were averaged and expressed as nCi/g tissue. Percentage changes versus control or WT animals were calculated.

Western-blotting studies

Preparation of the protein extracts. Frontal cortex and hippocampus from CMS and control 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 mg/ml aprotinine and 5 mg/ml leupeptine to a final protein concentration of 0.8-1 mg/ml and stored at –80 °C.

Western-blot. Protein extracts from only one individual were loaded in each lane of the blot. Thus, in each blot protein extracts from 4-5 animals from two different groups (control and CMS or WT and VGLUT1+/-) were loaded. Equal amounts of protein (10-50 µg) per lane were separated by SDS-PAGE (NuPage Bis-Tris 10%, InVitrogen) and

transferred onto a PVDF 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 VGLUT1 (1:2.000;donated by Dr S. El Mestikawy, Paris, France and already tested in mice (Tordera et al., 2007)), Synapsin I (1:5000; Chemicon International) , Synaptophysin (1:5000; Chemicon International), Arc (1:1000; Santa Cruz Biotechnology) and proBDNF (1:1000; Chemicon International) antibodies overnight at 4°C. Membranes were washed 3 times (PBS-Tween buffer, 10 min) and incubated for 2 h with respective secondary horseradish peroxidase-conjugated goat anti-rabbit for VGLUT1, synapsin 1, proBDNF and Arc and goat anti-mouse antibodies for synaptophysisne (DAKO, U.K. 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 and normalised to β-actin.

Statistical analysis

Both behavioural and neurochemical studies were analyzed using Student t-test analysis with the exception of sucrose intake test for the CMS procedure in which one way ANOVA repeated measures followed by Student t-test for individual weeks was applied.

Results

Forced swimming and sucrose intake tests

Mice body weight was not affected by CMS. Similarly, no differences in body weight were observed between VGLUT1+/- mice and WT littermates. CMS mice decreased sucrose intake (solution (g)/body weight) from the fourth week of CMS until the end of this procedure (Fig. 2A) ($F_{1,118}$ = 3,14 $p<0.05$). In addition, a decrease in sucrose intake was also observed one month thereafter (Fig. 2B) ($t_{(22)}$=2.13, p=0.04). VGLUT1+/- mice decreased also sucrose intake measured as the average of sucrose intake on three consecutive weeks (Fig. 2C) ($t_{(22)}$=2.3, p=0.03). In addition, during the 15 h exposure to the test, water intake did not vary between the groups, and due to the small amount taken (around 0.3 ml) and the relatively big volume of sucrose solution intake (10-20 ml), all the groups showed a preference for sucrose higher than 97%. Therefore, no major differences were detected in sucrose preference (sucrose intake/total intake) among the groups (data not shown).

In the forced swimming test (FST), increased immobility times were observed in CMS exposed mice both short (Fig. 2D) ($t_{(22)}$=2.5, p=0.02) and long-term after CMS (Fig.2E) ($t_{(22)}$=2.24, p=0.03). On the other hand, VGLUT1+/- heterozygous mice showed a significant increase in the immobility time in the FST compared to their WT littermates (Fig. 2F) ($t_{(22)}$=2.3, p=0.03).

Regulation of synaptic vesicle proteins (SVPs) by chronic mild stress and decreased VGLUT1 levels

CMS induced a short-term decrease of VGLUT1 mRNA levels in the frontal cortex (83.52 ± 5.8% *vs* control, $t_{(10)}$=3.46, p=0.02) and in the CA1 region (71.24 ± 8.8% *vs*

control, $t_{(10)}$=3.22, p=0.01) of the hippocampus (Fig. 3A). Similarly, 24 hours after the CMS, synapsin I mRNA was decreased in the cingulated cortex (83.23 ± 5.8% *vs* control, $t_{(10)}$=2.33, p=0.04) and in the different hippocampal regions (84-88% *vs* control, $t_{(10)}$=3.0, 5.0, 6.2, p=0.02, 0.001, 0.001 for CA1, CA3 and DG respectively) (Fig. 3B). In addition, a significant decrease in synaptophysin ($t_{(8)}$=3.1, p=0.02) and Rab3A ($t_{(8)}$=2.9, p=0.02) mRNA (Fig. 3C,D) levels in the cingulate cortex was observed. However, one month after the chronic stress procedure, a complete recovery of mRNA alterations induced by CMS over these SVPs was shown (Fig. 3 E-H).

VGLUT1 heterozygous genotype was confirmed since a significant decrease in mRNA in cortical (~75% *vs* WT, $t_{(10)}$=3.2, 2.8, p=0.01, 0.02 for frontal and cingulate cortex respectively) and hippocampal areas (~63-75% *vs* WT, $t_{(10)}$=6.1, 4.9, 3.7 p=0.001, 0.001, 0.004 for CA1, CA3 and DG respectively) (Fig. 4A) was shown. With regard to the rest of SVP, a significant increase (~140-145% *vs* WT) in synapsin I mRNA expression was found in the CA1 ($t_{(10)}$=3.9, p=0.005), CA3 ($t_{(10)}$=4.0, p=0.004) and dentate gyrus (DG) ($t_{(10)}$=4.0, p=0.003) regions of the hippocampus (Fig.4B). Synaptophysin mRNA levels were also increased, specifically in the CA1 region of the hippocampus ($t_{(10)}$=3.4, p=0.01) (Fig. 4C). Finally, no changes were observed in Rab3A mRNA levels (Fig.4D). Autoradiograms showing the expression of VGLUT1 and synapsin 1 mRNA in the dorsal hippocampus of both short-term CMS and VGLUT1+/- mice with their corresponding controls can be observed in Figures 5A and B respectively.

Western studies on hippocampal protein extracts showed a significant decrease of VGLUT1 (79.5 ± 12.1% *vs* control, $t_{(10)}$=2.5, p=0.04) and of synapsin 1 (67.29 ± 16.1% *vs* control, $t_{(10)}$=2.8, p=0.02) in the group of mice exposed to CMS, when measured 24 after the termination of the stress procedure (Fig 6A). In addition, a significant decrease of VGLUT1 in the frontal cortex (59.3 ± 5.2% *vs* WT, $t_{(13)}$=3.49, p=0.004) and the hippocampus (54.7 ± 5.5% *vs* WT, $t_{(13)}$=9.9, p=0.000) and an increase of synapsin 1

(129.69± 6.6% *vs* WT, $t_{(10)}$=2.23, p=0.049) in VGLUT1+/- mice could be observed (Fig 6B). However, no changes in hippocampal synaptophysin protein levels were detected.

Regulation of BDNF by chronic mild stress

A persistent decrease in BDNF mRNA levels in the CA1 region of the hippocampus was shown both short and long-term after CMS (78.28 ± 8.8%) and 81.16± 4.5%), *vs* control respectively) (Fig. 7A,B,D). Western-blot analysis of the immature form of BDNF (proBDNF) protein levels in the hippocampus, revealed a significant decrease of this protein in CMS mice when measured (71.60 ± 11.2% *vs* control) one month after this procedure (Fig. 8B). However, no alterations on BDNF mRNA levels were found in VGLUT1 deficient mice (Fig. 7C).

Regulation of Arc by chronic mild stress and decreased VGLUT1 levels

A short-term decrease of Arc mRNA levels was observed in the frontal and cingulated cortex and in the CA1 region of the hippocampus of mice exposed to CMS (68-76% *vs* control, $t_{(10)}$=2.6, 3.6, 2.8, p=0.03, 0.007, 0.03) (Fig.9 A,D). No changes in basal Arc mRNA were observed in any brain region of CMS mice in the long-term (Fig 9B). However, a positive correlation between Arc and VGLUT1 mRNA levels was detected in the frontal cortex of both short-term (r=0.82, p<0.05) and long-term CMS (r=0.84, p<0.05) mice.

VGLUT 1 +/- mice showed a decrease in Arc mRNA levels in the frontal and cingulated cortex and in the CA1 region of the hippocampus (76-82% *vs* WT, $t_{(10)}$=4.6,5.2,2.3, p=0.002, 0.001, 0.04 for frontal and cingulate cortex and CA1 respectively) (Fig 9 C,D).

No changes in Arc protein expression in the frontal cortex or in the hippocampus of mice exposed to CMS (24 h) or in the VGLUT1+/- mice were detected (data not shown).

Arc protein expression was significantly increased in both the prefrontal cortex (151.8 ± 13.3% *vs* control, $t_{(10)}$=3.4, p=0.007) and in the hippocampus (140.2 ± 13.4% *vs* control, $t_{(10)}$=2.3, p=0.049) of control mice when exposed to novel environment (Fig 10 A). Similarly, novelty induced Arc expression was higher in the prefrontal cortex of mice exposed to CMS when measured one day (142.9 ± 11.5% *vs* control, $t_{(10)}$=2.85, p=0.02) (Fig. 10B) and one month (169.1± 22.1% *vs* control, $t_{(10)}$=3.7, p=0.002) (Fig. 10C) after the stress procedure as well as in VGLUT1 heterozygous mice (144.5 ± 13.8% *vs* WT, $t_{(10)}$=2.8, p=0.02) (Fig. 10D). However, in the hippocampus no significant differences between controls and CMS mice or WT and VGLUT1+/- mice were observed (Fig 10B-10D).

Discussion

Here, we firstly show that in agreement with previous studies carried out in our laboratory (García-Gacía et al., 2009; Elizalde et al., 2008) both the genetic (VGLUT1+/- mice) and the environmental (chronic mild stress) model of depression showed helpless and anhedonic-like behavior. Unlike our previous studies, mice were free of a course of saline injections, which made the results more comparable to the neurochemistry studies carried out here. Subsequently, we aimed to identify mechanisms of failure in synaptic plasticity, common to stress and impaired glutamate function. We observed that CMS induced a transient decrease of different plasticity markers (VGLUT1, synapsin 1, sinaptophysin, rab3A and the activity regulated cytoskeletal protein; Arc) but a long-lasting decrease of the brain derived neurotrophic factor (BDNF). Among these, Arc mRNA expression was also downregulated in VGLUT1+/- heteozygous mice. In contrast, an opposite regulation of synapsin 1 was observed. Moreover, both models showed a marked increase of cortical Arc response to novelty.

Regulation of synaptic vesicle proteins by chronic mild stress and decreased VGLUT1 levels

Synaptic vesicle proteins (SVPs) have been identified as possible factors involved in the pathophysiology of psychiatric disorders (Uezato et al., 2009; Eastwood and Harrison, 2001; Vawter et al., 2002). Importantly, alterations in the expression pattern of these proteins have shown to affect neural plasticity and connectivity as well as associated behaviour (Silva *et al.,* 2008; Reinés et al., 2008; Tordera et al., 2007). Here, we studied the regulation of four SVPs (VGLUT1, synapsin 1, synaptophysin and Rab3A) critical for neurotransmitter release.

The environmental chronic mild stress (CMS) model induced here a short-term decrease in the mRNA expression of the four SVPs in cortical regions, a specific downregulation of VGLUT1 in frontal cortex and CA1 and a downregulation of synapsin 1 in all the hippocampal subfields. Moreover, both synapsin 1 and VGLUT1 proteins were decreased in the hippocampus. These changes, in brain areas specifically vulnerable to stress, have been suggested to contribute to the molecular effects of CMS on synaptic plasticity and connectivity (Thome et al., 2001; Silva et al., 2008; Bessa et al., 2009). In particular, an important role of synapsin 1 in synaptic remodelling of the cortex and hippocampus of stressed animals linked to depressive-like behaviour has been suggested (Silva et al*.,* 2008; Bessa et al., 2009). Moreover, other models based on stress paradigms have shown decreased levels of synaptophysin together with other SVPs (Thome et al., 2001). However, it is important to note that these studies and ours were carried out shortly after the termination of the stress procedure. In addition, we show here for the first time, no changes in any of the four SVP studied, either in cortical or hippocampal brain regions of CMS mice, when analyzed one month after the termination of the stress procedure. These novel results suggest that CMS triggers a transient decrease in the four SVPs that is not associated with either the development of anhedonic-like behaviour or with other long-lasting behavioral alterations described in our previous study (Elizalde et al*.,* 2008). Yet they might still contribute to the initiation of acute depressive episodes. Importantly, distinct neurobiological mechanisms are suggested to be responsible for first depressive episodes, very often preceded by exposure to stressful life events, and subsequent, more autonomous, recurrences (Stroud et al., 2008).

It should be noted however that previous observations in models based on early life stress (Aisa et al., 2009; Burton et al., 2007; Law et al., 2009) suggest that when adverse events are applied during brain development, the regulation of these SVPs are

long-lasting and therefore, negatively affect synaptic function and behavioral outcome later in life. The clinical relevance of these studies for depressive disorders specifically related to adverse events at developmental stages warrants further investigation. On the other hand, several post-mortem studies suggest a key role for these proteins in more severe psychiatric disorders such as schizophrenia or bipolar disorders, which, are believed to be related to impaired synaptic plasticity and connectivity during developmental stages (Vawter et al., 2002; Eastwood et al., 2000; Honer et al., 1999; Uezato et al., 2009).

The vesicular glutamate transporter 1 (VGLUT1) is the major isoform in cortical and hippocampal regions (Takamori et al., 2000; Fremeau et al., 2001), where it plays a key role in the vesicular uptake and synaptic transmission of glutamate (Wojcik et al., 2004: Fremeau et al., 2004). The functional significance of decreased VGLUT1 levels in the VGLUT1+/- mice for the synaptic transmission has been recently investigated. A recent study, using a different strain of VGLUT1 heterozygous mice, shows impaired hippocampal long-term potentiation suggesting reduced glutamate release under high frequency stimulation (Balschun et al., 2009). In agreement with this, we recently showed in these mice decreased cortical and hippocampal levels of the excitatory aminoacid transpoter 1 (EAAT1) (García-García et al., 2009), a glial transporter that can be regulated by the amount of glutamate released into the synaptic cleft (Duan et al., 1999; Liang et al., 2008). Thus, the anhedonic and helpless behaviour shown by these mice could be linked to decreased glutamate transmission in those areas in which VGLUT1 is the major isoform. In keeping with this, recent post mortem studies showing decreased cortical VGLUT1 in depressed subjects (Uezato et al., 2009) together with clinical findings of an excitatory inhibitory imbalance in the cortex of depressed patients (Sanacora et al., 2004; Bhagwagar et al., 2007) suggest that decreased VGLUT1 levels has functional and clinical implications. Yet, other postmortem studies suggest that variations in VGLUT1 in different cortical areas could

be also involved in bipolar disorder or schizophrenia (Oni-Orisan et al., 2008; Uezato et al., 2009; Eastwood and Harrison 2010).

Decreased VGLUT1 levels affected also the expression of other SVPs. A striking upregulation of both mRNA and protein hippocampal levels of synapsin 1 were shown in VGLUT1+/- mice. It is noteworthy that VGLUT1 colocalizes fully with synapsin 1 in the hippocampus (Bogen et al., 2009) and that development of glutamatergic synaptic vesicles and synapses appears to be synapsin 1 dependent (Bogen et al., 2009; Fremeau et al., 2004; Wojcik et al., 2004). We suggest that, synapsin 1 upregulation could respond to a need of maintaining the vesicle pool integrity in the reserve pool (Bogen et al., 2006), in where, it preferentially localizes and has a specific role tethering synaptic vesicles to each other and maintaining them in the vicinity of the active zone (Fdez et al., 2006). In line with this, only a small reduction (~15 %) in the reserve pool of synaptic vesicles of hippocampal excitatory terminals was shown in VGLUT1+/- mice (Tordera et al., 2007) compared to the around 50% reduction in both synaptic vesicles and synapsin 1 found in the VGLUT1 knockouts (Fremeau et al., 2004). Yet, further studies would be needed to explore the molecular mechanism underlying synapsin 1 upregulation in these mice. Finally, despite the upregulation of synaptophysin mRNA in CA1 of VGLUT1+/- mice, that could be linked to synapsin 1 (Lietz et al., 2003), protein levels of this integral membrane SVP were not changed. These results suggest that genetic ablation of one allele of VGLUT1 might induce compensatory mechanisms that alter the vesicle protein composition.

Taken together, our results show dissociation between synapsin 1 expression levels and depressive-like behaviours. Firstly, development of long-lasting anhedonia or helplessness (Elizalde et al., 2008) can occur despite a full recovery of synapsin 1 expression levels after CMS. Secondly, synapsin 1 is regulated in an opposite direction in short-term CMS and VGLUT1+/- mice while, both models show anhedonia and

helpless behaviour. Yet, dissociations reported here do not fully exclude the possibility of a direct involvement of SVPs in depressive like behaviour. Further studies should investigate the regulation of these proteins in other brain regions as well as possible changes in their functional synaptic/neuronal properties.

On the other hand, our results suggest that decreased VGLUT1 in both short-term CMS and VGLUT1+/- mice could lead to an altered glutamate function that could be associated to depressive-like behavior.

Increased cortical Arc response to novel environment in both models

Arc (activity regulated cytoskeletal protein) is an effector immediate early gene rapidly activated by synaptic activity (Guzowski et al., 2006; Tzingounis and Nicoll, 2006) that might bridge neuronal activity with structural and functional changes (Lyford et al., 1995). Importantly, a causative role for Arc in memory formation is quite established (Bramham et al., 2008) and a possible role in both synaptogenesis (Ujike et al., 2002) and neurogenesis (Bramham et al., 2008; Larsen et al., 2007) has been also suggested. Hypothesis linking depression to impaired synaptic plasticity and connectivity in the forebrain suggests that Arc, together with other activity regulated synaptic proteins, could form part of the altered synaptic “molecular context” of this illness. Yet, no evidence causally linking Arc to any aspect of depression has been established.

Constitutive Arc mRNA expression was downregulated in cortical and hippocampal areas (CA1) by both stress and decreased VGLUT1 levels. Given that Arc expression is highly regulated by glutamate synaptic release (Steward et al.,2001; Bramham et al., 2009) these findings would agree with the idea of deficient glutamate transmission in both short-term CMS and VGLUT1+/- models, in those areas mainly VGLUT1

dependent. Moreover, a positive correlation between Arc and VGLUT1 mRNA cortical levels was found in CMS mice. However, western studies showed no changes in Arc protein expression suggesting that the functional relevance of mRNA alterations might be limited.

Most importantly, we found an increased cortical expression of Arc after exposure to novel environment in CMS and VGLUT1+/- mice. Previous studies have shown that novel experience activates a cell-specific genomic program in which *Arc* function seems to play an important role (Pinaud et al., 2001; Vazdarjanova et al., 2002). Novel environment is considered an emotional "processing" stressor in that it requires cognitive processing but does not represent an immediate physical threat (Herman et al., 1997). Importantly, the medial prefrontal cortex (mPFC) is a key component of the neuronal circuitry mediating responses to novelty including those directed to emotional control and memory formation (Roozendaal, 2002). Thus, altered inducible Arc levels in the prefrontal cortex of both models, could be reflecting either poor behavioral control of stress or a compensation for a deficient working memory. Indeed, both models have previously shown impaired recognition memory (Tordera et al., 2007; Elizalde et al., 2008), which, is highly dependent of the prefrontal cortex. Interestingly, increased cortical Arc response to mild stress has been also observed in other genetic models of depression (Molteni et al., 2009). Moreover, matching with these experimental studies, various clinical studies have shown cortical hyperactivity in depressed patients following exposure to different processing tasks (Wagner et al., 2006; Siegle et al., 2006; Konarski et al., 2009; Brody et al., 2001; Yoshimura et al., 2009). Thus, this IEG could form part of the "dysfunctional synaptic molecular context" of major depression and perhaps, underlie either behavioural disinhibitions or an altered cognitive function such as attention to environmental events in depressed patients.

Yet, the molecular consequences of an increased Arc expression after exposure to novel environment should be further explored. For instance, it could be studied whether increased expression of Arc induces an increase in AMPA remodelling in the synapsis, which might affect to the synaptic strength (Braham et al., 2008).

Long lasting decreased BDNF after CMS

Brain derived neurotrophic factor (BDNF) was affected by stress but not by decreased VGLUT1 levels. BDNF is a neurotrophin with a key role on synaptic transmission in addition to the stimulation of growth, differentiation and survival of neurones. The neurotrophin hypothesis of depression is based largely on observations that decreases in hippocampal BDNF levels are correlated with stress-induced depressive behaviours and that antidepressant treatment enhances the expression of BDNF (Duman and Monteggia 2006). Further low levels of brain and serum BDNF is suggested to be associated with major depression (Sen et al., 2008; Nestler, 2002; Altar et al., 2009).

The long-lasting helpless and anhedonic like behaviour induced by CMS could be associated to the long-lasting decrease in BDNF mRNA as well as proBDNF protein that we found in the hippocampus. Further, long-lasting downregulations of hippocampal BDNF have been found in previous mice studies, using the chronic social defeat stress (Tsankova et al., 2006) and CMS (Song et al., 2006). Thus, unlike the SVPs studied here, decreased hippocampal BDNF seems to form part of the long-lasting molecular effects of stress. Moreover, it could address together with the increased cortical Arc response to novelty, molecular mechanism involved in major depression disorders triggered by exposure to stressful life events in adult life, even, on a longitudinal perspective.

Conclusions

Among the different synaptic plasticity markers studied here, increased cortical Arc response to novelty could be a marker of increased neuronal activity, common to chronic mild stress and an altered glutamate function as well as associated depressive-like behaviour. Moreover, it could be suggested as a molecular mechanism underlying failure to adapt to environmental changes both in animal models and in depressed patients.

Acknowledgements

We are very grateful to Ms Sandra Lizaso for her excellent technical assistance and to Dr Sonja Wojcik for the generous donation of the VGLUT1+/- mice. This work was supported by the EU Framework 6 Integrated Project NEWMOOD (LSHM-CT-2004-503474), the Ministry of Science and Innovation (SAF2008-02217 Spanish Government) and a fellowship from the Spanish Government (Department of Education) to N. Elizalde.

Tha authors declare no conflict of interest.

References

Aisa B., Elizalde N., Tordera R., Lasheras B., Del Rio J. and Ramirez M. J. (2009) Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: Implications for spatial memory. *Hippocampus* **19**, 1222-31.

Altar C. A., Vawter M. P. and Ginsberg S. D. (2009) Target identification for CNS diseases by transcriptional profiling. *Neuropsychopharmacology* **34,** 18-54.

Balschun D., Moechars D., Callaerts-Vegh Z., Vermaercke B., Van Acker N., Andries L. and D'Hooge R. (2009) Vesicular Glutamate Transporter VGLUT1 Has a Role in Hippocampal Long-Term Potentiation and Spatial Reversal Learning. *Cereb Cortex*.(ahead of print)

Bessa J. M., Ferreira D., Melo I., Marques F., Cerqueira J. J., Palha J. A., Almeida O. F. and Sousa N. (2009) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* **14,** 764-773, 739.

Bhagwagar Z., Wylezinska M., Jezzard P., Evans J., Ashworth F., Sule A., Matthews P. M. and Cowen P. J. (2007) Reduction in occipital cortex gamma-aminobutyric acid concentrations in medication-free recovered unipolar depressed and bipolar subjects. *Biol Psychiatry* **61,** 806-812.

Bogen I. L., Boulland J. L., Mariussen E., Wright M. S., Fonnum F., Kao H. T. and Walaas S. I. (2006) Absence of synapsin I and II is accompanied by decreases in vesicular transport of specific neurotransmitters. *J Neurochem* **96,** 1458-1466.

Bogen I. L., Jensen V., Hvalby O. and Walaas S. I. (2009) Synapsin-dependent development of glutamatergic synaptic vesicles and presynaptic plasticity in postnatal mouse brain. *Neuroscience* **158,** 231-241.

Bramham C. R., Worley P. F., Moore M. J. and Guzowski J. F. (2008) The immediate early gene arc/arg3.1: regulation, mechanisms, and function. *J Neurosci* **28,** 11760-11767.

Bramham C. R., Alme M. N., Bittins M., Kuipers S. D., Nair R. R., Pai B., Panja D., Schubert M., Soule J., Tiron A. and Wibrand K. (2009) The Arc of synaptic memory. *Exp Brain Res*. (ahead of print)

Brody A. L., Saxena S., Mandelkern M. A., Fairbanks L. A., Ho M. L. and Baxter L. R. (2001) Brain metabolic changes associated with symptom factor improvement in major depressive disorder. *Biol Psychiatry* **50,** 171-178.

Burton C. L., Chatterjee D., Chatterjee-Chakraborty M., Lovic V., Grella S. L., Steiner M. and Fleming A. S. (2007) Prenatal restraint stress and motherless rearing disrupts expression of plasticity markers and stress-induced corticosterone release in adult female Sprague-Dawley rats. *Brain Res* **1158,** 28-38.

Dang H., Chen Y., Liu X., Wang Q., Wang L., Jia W. and Wang Y. (2009) Antidepressant effects of ginseng total saponins in the forced swimming test and chronic mild stress models of depression. *Prog Neuropsychopharmacol Biol Psychiatry* **33,** 1417-1424.

Duan S., Anderson C. M., Stein B. A. and Swanson R. A. (1999) Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. *J Neurosci* **19,** 10193-10200.

Duman R. S. and Monteggia L. M. (2006) A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* **59,** 1116-1127.

Eastwood S. L., Cairns N. J. and Harrison P. J. (2000) Synaptophysin gene expression in schizophrenia. Investigation of synaptic pathology in the cerebral cortex. *Br J Psychiatry* **176,** 236-242.

Eastwood S. L. and Harrison P. J. (2001) Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a Western blot study of synaptophysin, GAP-43 and the complexins. *Brain Res Bull* **55,** 569-578.

Eastwood S.L. and Harrison P.J. (2010) Markers of Glutamate Synaptic Transmission and Plasticity Are Increased in the Anterior Cingulate Cortex in Bipolar Disorder. *Biol Psychiatry.* Epub ahead of print.

Elizalde N., Gil-Bea F. J., Ramirez M. J., Aisa B., Lasheras B., Del Rio J. and Tordera R. M. (2008) Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. *Psychopharmacology (Berl)* **199,** 1-14.

Fdez E. and Hilfiker S. (2006) Vesicle pools and synapsins: new insights into old enigmas. *Brain Cell Biol* **35,** 107-115.

Fremeau R. T., Jr., Troyer M. D., Pahner I., Nygaard G. O., Tran C. H., Reimer R. J., Bellocchio E. E., Fortin D., Storm-Mathisen J. and Edwards R. H. (2001) The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* **31,** 247-260.

Fremeau R. T., Jr., Kam K., Qureshi T., Johnson J., Copenhagen D. R., Storm-Mathisen J., Chaudhry F. A., Nicoll R. A. and Edwards R. H. (2004) Vesicular glutamate transporters 1 and 2 target to functionally distinct synaptic release sites. *Science* **304,** 1815-1819.

Garcia-Garcia A. L., Elizalde N., Matrov D., Harro J., Wojcik S. M., Venzala E., Ramirez M. J., Del Rio J. and Tordera R. M. (2009) Increased vulnerability to depressive-like behavior of mice with decreased expression of VGLUT1. *Biol Psychiatry* **66,** 275-282.

Guzowski J. F., Miyashita T., Chawla M. K., Sanderson J., Maes L. I., Houston F. P., Lipa P., McNaughton B. L., Worley P. F. and Barnes C. A. (2006) Recent behavioral history modifies coupling between cell activity and Arc gene transcription in hippocampal CA1 neurons. *Proc Natl Acad Sci U S A* **103,** 1077-1082.

Herman J. P. and Cullinan W. E. (1997) Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* **20,** 78-84.

Honer W. G., Falkai P., Chen C., Arango V., Mann J. J. and Dwork A. J. (1999) Synaptic and plasticity-associated proteins in anterior frontal cortex in severe mental illness. *Neuroscience* **91,** 1247-1255.

Konarski J. Z., Kennedy S. H., Segal Z. V., Lau M. A., Bieling P. J., McIntyre R. S. and Mayberg H. S. (2009) Predictors of nonresponse to cognitive behavioural therapy or venlafaxine using glucose metabolism in major depressive disorder. *J Psychiatry Neurosci* **34,** 175-180.

Larsen M. H., Rosenbrock H., Sams-Dodd F. and Mikkelsen J. D. (2007) Expression of brain derived neurotrophic factor, activity-regulated cytoskeleton protein mRNA, and enhancement of adult hippocampal neurogenesis in rats after sub-chronic and chronic treatment with the triple monoamine re-uptake inhibitor tesofensine. *Eur J Pharmacol* **555,** 115-121.

Law A. J., Pei Q., Feldon J., Pryce C. R. and Harrison P. J. (2009) Gene expression in the anterior cingulate cortex and amygdala of adolescent marmoset monkeys following parental separations in infancy. *Int J Neuropsychopharmacol* **12,** 761-772.

Li S., Wang C., Wang W., Dong H., Hou P. and Tang Y. (2008) Chronic mild stress impairs cognition in mice: from brain homeostasis to behavior. *Life Sci* **82,** 934-942.

Liang J., Takeuchi H., Doi Y., Kawanokuchi J., Sonobe Y., Jin S., Yawata I., Li H., Yasuoka S., Mizuno T. and Suzumura A. (2008) Excitatory amino acid transporter expression by astrocytes is neuroprotective against microglial excitotoxicity. *Brain Res* **1210,** 11-19.

Lietz M., Hohl M. and Thiel G. (2003) RE-1 silencing transcription factor (REST) regulates human synaptophysin gene transcription through an intronic sequence-specific DNA-binding site. *Eur J Biochem* **270,** 2-9.

Lyford G. L., Yamagata K., Kaufmann W. E., Barnes C. A., Sanders L. K., Copeland N. G., Gilbert D. J., Jenkins N. A., Lanahan A. A. and Worley P. F. (1995) Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* **14,** 433-445.

Molteni R., Calabrese F., Chourbaji S., Brandwein C., Racagni G., Gass P. and Riva M. (2009) Depression-prone mice with reduced glucocorticoid receptor expression display an altered stress-dependent regulation of brain-derived neurotrophic factor and activity-regulated cytoskeleton-associated protein. *J Psychopharmacol*. *Epub ahead of print.*

Molteni R., Calabrese F., Maj P. F., Olivier J. D., Racagni G., Ellenbroek B. A. and Riva M. A. (2009) Altered expression and modulation of activity-regulated cytoskeletal associated protein (Arc) in serotonin transporter knockout rats. *Eur Neuropsychopharmacol* **19,** 898-904.

Nestler E. J., Barrot M., DiLeone R. J., Eisch A. J., Gold S. J. and Monteggia L. M. (2002) Neurobiology of depression. *Neuron* **34,** 13-25.

Oni-Orisan A**.**, Kristiansen L.V., Haroutunian V., Meador-Woodruff J.H. and McCullumsmith R.E. (2008) Altered vesicular glutamate transporter expression in the anterior cingulate cortex in schizophrenia. *Biol Psychiatry* **63,** 766-75.

Ons S., Martí O. and Armario A. (2004). Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA*. J Neurochem.* **89,** 1111-8.

Pinaud R., Penner M. R., Robertson H. A. and Currie R. W. (2001) Upregulation of the immediate early gene arc in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. *Brain Res Mol Brain Res* **91,** 50-56.

Reines A., Cereseto M., Ferrero A., Sifonios L., Podesta M. F. and Wikinski S. (2008) Maintenance treatment with fluoxetine is necessary to sustain normal levels of synaptic markers in an experimental model of depression: correlation with behavioral response. *Neuropsychopharmacology* **33,** 1896-1908.

Roozendaal B. (2002) Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. *Neurobiol Learn Mem* **78,** 578-595.

Sanacora G., Gueorguieva R., Epperson C. N., Wu Y. T., Appel M., Rothman D. L., Krystal J. H. and Mason G. F. (2004) Subtype-specific alterations of gamma-aminobutyric acid and glutamate in patients with major depression. *Arch Gen Psychiatry* **61,** 705-713.

Sen S., Duman R. and Sanacora G. (2008) Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications. *Biol Psychiatry* **64,** 527-532.

Siegle G. J., Carter C. S. and Thase M. E. (2006) Use of FMRI to predict recovery from unipolar depression with cognitive behavior therapy. *Am J Psychiatry* **163,** 735-738.

Silva R., Mesquita A. R., Bessa J., Sousa J. C., Sotiropoulos I., Leao P., Almeida O. F. and Sousa N. (2008) Lithium blocks stress-induced changes in depressive-like

behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3beta. *Neuroscience* **152,** 656-669.

Song L., Che W., Min-Wei W., Murakami Y. and Matsumoto K. (2006) Impairment of the spatial learning and memory induced by learned helplessness and chronic mild stress. *Pharmacol Biochem Behav* **83,** 186-193.

Steward O. and Worley P. F. (2001) Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation. *Neuron* **30,** 227-240.

Stroud CB, Davila J, Moyer A (2008) The relationship between stress and depression in first onsets versus recurrences: a meta-analytic review. *J Abnorm Psychol* **117,** 206-13

Takamori S., Rhee J. S., Rosenmund C. and Jahn R. (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* **407,** 189-194.

Thome J., Pesold B., Baader M., Hu M., Gewirtz J. C., Duman R. S. and Henn F. A. (2001) Stress differentially regulates synaptophysin and synaptotagmin expression in hippocampus. *Biol Psychiatry* **50,** 809-812.

Tordera R. M., Totterdell S., Wojcik S. M., Brose N., Elizalde N., Lasheras B. and Del Rio J. (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). *Eur J Neurosci* **25,** 281-290.

Tsankova N. M., Berton O., Renthal W., Kumar A., Neve R. L. and Nestler E. J. (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* **9,** 519-525.

Tzingounis A. V. and Nicoll R. A. (2006) Arc/Arg3.1: linking gene expression to synaptic plasticity and memory. *Neuron* **52,** 403-407.

Uezato A., Meador-Woodruff J. H. and McCullumsmith R. E. (2009) Vesicular glutamate transporter mRNA expression in the medial temporal lobe in major depressive disorder, bipolar disorder, and schizophrenia. *Bipolar Disord* **11,** 711-725.

Ujike H., Takaki M., Kodama M. and Kuroda S. (2002) Gene expression related to synaptogenesis, neuritogenesis, and MAP kinase in behavioral sensitization to psychostimulants. *Ann N Y Acad Sci* **965,** 55-67.

Vawter M. P., Thatcher L., Usen N., Hyde T. M., Kleinman J. E. and Freed W. J. (2002) Reduction of synapsin in the hippocampus of patients with bipolar disorder and schizophrenia. *Mol Psychiatry* **7,** 571-578.

Vazdarjanova A., McNaughton B. L., Barnes C. A., Worley P. F. and Guzowski J. F. (2002) Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks. *J Neurosci* **22,** 10067-10071.

Wagner G., Sinsel E., Sobanski T., Kohler S., Marinou V., Mentzel H. J., Sauer H. and Schlosser R. G. (2006) Cortical inefficiency in patients with unipolar depression: an event-related FMRI study with the Stroop task. *Biol Psychiatry* **59,** 958-965.

Wojcik S. M., Rhee J. S., Herzog E., Sigler A., Jahn R., Takamori S., Brose N. and Rosenmund C. (2004) An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. *Proc Natl Acad Sci U S A* **101,** 7158-7163.

Yoshimura S., Okamoto Y., Onoda K., Matsunaga M., Ueda K., Suzuki S. I. and Shigetoyamawaki (2009) Rostral anterior cingulate cortex activity mediates the relationship between the depressive symptoms and the medial prefrontal cortex activity. *J Affect Disord*. (ahead of print)

Figure legends

Figure1. Time course of the CMS procedure. (A) Short and long-term effects of CMS on sucrose intake and forced swimming test. (B) Short and long-term effects of CMS on the expression of synaptic plasticity proteins (neurochemistry) and on induced cortical Arc protein expression after novelty exposure. Abbreviations: CMS, chronic mild stress; FS, forced swimming test.

Figure 2. Performance of CMS and control mice in the sucrose intake and in the forced swimming test both at short (A,D) and long-term (B,E). In addition, performance of VGLUT1 heterozygous and wild type (WT) mice on the sucrose intake test (C) and on the forced swimming test (F). Values show the mean±SEM (n=12 mice/group for FST and test carried out in the VGLUT1+/- model, n=60 mice/group for sucrose intake test during the CMS procedure and n=48 mice/group for the sucrose intake one month after). *p<0.05 *vs* corresponding control or WT mice (One-way ANOVA repeated measures for the sucrose intake test during CMS, and Student *t*-test for the rest of the experiments).

Figure 3. Short and long-term effects of chronic mild stress (CMS) on VGLUT1 (A, E), synapsin 1 (B, F), synaptophysin (C, G) and Rab3A (D, H) mRNA in different forebrain regions. Each column represents mean ± SEM (n= 6 mice/group). **p< 0.01, *p <0.05 *versus* controls (Student *t*-test). Abbreviations: Fr, frontal cortex: Cg, cingulated cortex.

Figure 4. Expression of VGLUT1 (A), synapsin 1 (B), synaptophysin (C) and Rab3A (D) mRNA in different forebrain regions of VGLUT1+/- mice compared to WT littermates. Values show the mean ± SEM (n= 6 mice/group). **p< 0.01; *p <0.05

versus WT mice (Student *t*-test). Abbreviations: WT, Wild Type; Fr, frontal cortex; Cg, cingulated cortex; DG, dentate gyrus.

Figure 5. Representative autoradiograms showing the regulation of hippocampal VGLUT1 and synapsin 1 mRNA levels by short-term CMS (A) and by VGLUT1+/- mice (B).

Figure 6. Protein expression of hippocampal synapsin 1 (Syn1) and of VGLUT1 (Syp) in the hippocampus and frontal cortex of short-term CMS (A) and VGLUT1+/- (B) mice. Values show the mean ± SEM (n= 6 mice per group). ** p< 0.01, *p<0.05, *versus* corresponding control or WT mice respectively (Student *t*-test).

Figure 7. Effect of chronic mild stress, at short (A) and long-term (B) and of decreased VGLUT1 levels (VGLUT1+/- mice) (C) on BDNF mRNA in different forebrain regions. In (D), representative autoradiogram showing the decreased of BDNF mRNA expression in CA1 by long-term CMS. Values show the mean ± SEM (n= 5/6 mice/group). * P <0.05 versus WT mice (Student t-test).

Figure 8. Short (B) and long-term (B) effects of CMS on proBDNF protein levels in the hippocampus. Values show the mean ± SEM (n= 6 mice per group). *p<0.05 *versus* corresponding control mice (Student *t*-test).

Figure 9. Effect of chronic mild stress (CMS), at short (A) and long-term (B) and of decreased VGLUT1 levels (VGLUT1+/- mice) (C) on Arc mRNA expression in different forebrain regions. In (D) representative autoradiograms showing decreased Arc mRNA in the frontal cortex of VGLUT1+/- and short-term CMS mice. Values show the mean ± SEM (n= 5/6 mice/group). **p< 0.01, *p <0.05 *versus* WT mice (Student *t*-test).

Figure 10. Effect of exposure to novel environment on cortical and hippocampal Arc expression in control mice (A). In B-D, effect of exposure to novel environment on cortical and hippocampal Arc expression in mice exposed to CMS, at short (B) and long-term (C) compared to control mice and in VGLUT1+/- mice (D) compared to WT. Values show the mean ± SEM (n= 6 mice per group). **p<0.01, *p<0.05, *versus* corresponding WT or control mice respectively (Student *t*-test).

.