No adaptative changes of 5-HTergic and GABAergic markers in the brain of Vglut3−/− animals.

The 5-HT theory is one of the major postulation about the etiology of mood disorder and anxiety related behavior (for review see (Slattery et al., 2004)). In order to initiate an exploration of the role of VGLUT3 in 5-HT signaling and mood regulation, we examined whether VGLUT3 disruption led to major compensatory neuroanatomical changes in raphe nuclei as well as in limbic projection structures (i.e. prelimbic cortex and hippocampus). Using in situ hybridization, immunoautoradiography or ligand-based autoradiography, we quantitatively assessed expression level of major enzymes, transporters and receptors involved in serotonergic and GABAergic neurotransmission as well as of two other vesicular glutamate transporters VGLUT1 and VGLUT2 (Fig. S1).

Since VGLUT3 is expressed within 5-HT neurons, we first wondered whether its ablation could result in compensatory change of key serotonergic markers. Tryptophan hydroxylase (TPOH2) is the rate limiting step enzyme for the biosynthesis of 5-HT and its transcript is concentrated in serotonergic soma from raphe nuclei. The 5-HT plasma membrane transporter (SERT) is the primary target of commonly used antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs: fluoxetine, paroxetine or citalopram). Genetic disruption of the receptor (5HT2AR) and its restoration in the cortex have established that this receptor is involved in the modulation of anxiety (Weisstaub et al., 2006). Inhibition of the vesicular monoamine transporter type 2 (VMAT2) by reserpine precipitates depression (Slattery et al., 2004). We thus compared amounts of these markers in wildtype and Vglut3−/− mice and found that expression level of TPOH2 transcript in raphe nuclei as well SERT, 5-HT2A receptors and VMAT2 in projection areas were unaffected by VGLUT3 deletion (Fig. S1a-d). In addition, we assessed the 5-HT1A and 5-HT1B receptor functional coupling to G protein using [35S]GTP-γ-S binding. The functionality of these receptors was similar in both genotypes in the hippocampus (5-HT1A) and substantia nigra (5-HT1B) (data not shown). In the DRN, a difference in the 5-HT1AR coupling to G proteins was found in Vglut3−/− mice when compared to wild-type littermates (see Fig. 3c).

Because of their efficacy, rapid onset of action and favorable side effect profile benzodiazepines are often prescribed for the treatment of anxiety disorders. To assess the status of the GABAergic transmission in the absence of VGLUT3, we quantified three neurochemical markers (Fig. S1e-g). Immunomaterial of both isoforms of glutamic acid decarboxylase (GAD65 and GAD67) were found in similar amounts in the dorsal raphe, hippocampus and prelimbic cortex, but were slightly, although significantly augmented in the MRN of Vglut3−/− mice (+15%, P = 0.009, Fig. S1e). Neither the vesicular inhibitory amino acid transporter (VIAAT) immunopositive material nor GABA_A binding sites were altered by the ablation of VGLUT3 (Fig. S1 f,g).
We further inspected whether, the absence of VGLUT3 was compensated by the two other subtypes of vesicular glutamate transporter (VGLUT1 and VGLUT2). VGLUT3 ablation did not resulted into an increased or decreased compensatory expression of VGLUT1&2.

Brain-derived neurotrophic factor (BDNF) plays a pivotal role in hippocampal plasticity and in antidepressant treatment (Wang et al., 2008). As shown in Figure S1j, in the hippocampus and in the dentate gyrus, BDNF mRNA is unaffected by VGLUT3 deletion.

We have previously shown that VGLUT3 deletion had only a limited impact on the expression of major neurotransmitter receptors, transporter and synthetic enzyme in the caudate putamen (Gras et al., 2008). In conclusion, VGLUT3 ablation did not led to major compensatory adaptation of neurochemical markers in limbic circuits.
Supplementary Figure S1

**Figure S1.** VGLUT3 loss does not induce major neuroanatomical changes. Expression of serotonergic (a-d), GABAergic (e-g) and glutamatergic (h,i) anatomical markers, as well as BDNF mRNA (j). Autoradiograms shown in this figure represent coronal sections of wild-type mice taken at the level of the raphe, the dorsal hippocampus and the prelimbic cortex. Quantifications were performed by densitometry on sections from \textit{Vglut3}\(^{+/+}\) (white bars) and \textit{Vglut3}\(^{-/-}\) (black bars) animals. Autoradiograms of type 2 tryptophan hydroxylase (TPOH2) and BDNF were obtained by \textit{in situ} hybridization (mRNA). 5HT\(_{2A}\) receptor, vesicular monoamine transporter (VMAT2), glutamic acid decarboxylase (GAD), vesicular inhibitory amino acid transporter (VIAAT), vesicular glutamate transporters type 1 and 2 (VGLUT1 and VGLUT2) were detected by immunoautoradiography (IAR). The 5-HT transporter (SERT) and GABA\(_A\) receptors were visualized with \(^{3}\text{H}\)-Citalopram and \(^{3}\text{H}\)-Flunitrazepam (respectively). TPOH2 mRNA was quantified by densitometry in the dorsal and median raphe nuclei (DRN and MRN respectively) where 5-HT neurons are localized. In line with data from the literature suggesting a positive regulation of 5-HT on the expression of the gene coding for BDNF, we quantified its transcript in the total hippocampus (Hi) and in the dentate gyrus of the hippocampus (DG). Other markers were quantified in the DRN, MRN and the prelimbic cortex (Prl Cx). Comparison between \textit{Vglut3}\(^{+/+}\) and \textit{Vglut3}\(^{-/-}\) revealed no major difference following the loss of VGLUT3 in mutant animals (Mann-Whitney test). Only the levels of GAD in the MRN appeared slightly augmented in \textit{Vglut3}\(^{-/-}\) mice (+15%, Mann-Whitney test, \(P = 0.009\)). All experiments were performed on \(n = 5\) animals per genotype.
Supplementary Material and Methods

**In situ hybridization.** Mouse brains of wild-type or mutant mice were frozen in isopentane at -30°C. Sections were prepared with a cryostat at -20°C, thaw-mounted on glass slides and stored at -80°C until usage. Regional mRNA hybridization in situ was performed by Helios Biosciences (http://www.heliosbioscience.com) as already described (Gras et al., 2008) for the following Mus musculus sequences and accession numbers: Tph2 (NM_173391.2) and BDNF (recognition by the probe of the 4 BDNF mRNA variants NM_007540.4; NM_001048139.1; NM_001048141.1; NM_001048142.1) using Helios oligo design software (Helios Biosciences, France). Negative controls were performed using labeled sense oligonucleotides.

**Immunoautoradiography.** Immunoautoradiography was performed as described (Gras et al., 2008). Briefly, coronal sections were fixed with 4% paraformaldehyde for 15 min, washed three times for 10 min in PBS and pre-incubated in PBS containing 3% bovine serum albumine (BSA), 1% goat serum and 1 mM NaI (buffer A) for 60 min at room temperature (RT). Sections were incubated in buffer A supplemented with antibodies against VGLUT1, VGLUT2, GAD (1:10,000, Chemicon), VIAAT (1:10,000, Dumoulin et al., 1999), VMAT2 (1:1000, Gras et al., 2008) and 5-HT2A receptor (1:1,000, Immunostar), washed three times for 10 min in PBS before incubation with antirabbit [125I]-IgG (0.25 μCi mL⁻¹; GE Healthcare). Sections were then washed and dried.

**Autoradiography.** For both GABA_A receptors and SERT autoradiography, sections were prepared as described for *in situ* hybridization. Autoradiographic detection of SERT was performed as described previously with the following modifications (Fabre et al., 2000). Briefly, sections were dried and pre-incubated for 15 min in Tris buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl) at room temperature. Sections were then incubated 2 hr at room temperature in Tris buffer containing [³H]citalopram (1 nM) with or without 10 μM Fluoxetine to determine non-specific binding. Sections were washed and dried. Autoradiographic detection of GABA_A receptors was performed as described (Gras et al., 2008). Briefly, sections were dried, pre-incubated in Tris buffer (50 mM Tris-HCl, 120 mM NaCl). They were then incubated 1 hr at 4 °C in Tris buffer, in presence of 5 nM [³H]flunitrazepam (Amersham Biosciences) with or without 100 μM diazepam (Sigma Aldrich) to determine non-specific binding. Sections were then washed and dried.

References


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