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PARADOXICAL WIDESPREAD c-FOS EXPRESSION INDUCED BY A GABA AGONIST IN THE FOREBRAIN OF TRANSGENIC MICE WITH ECTOPIC EXPRESSION OF THE $GABA_A$ α6 SUBUNIT

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Abstract—A GABA-site agonist gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) at 3 mg/kg induces strong anxiolytic response in a transgenic Thy1α6 mouse line ectopically expressing the $GABA_A$ receptor α6 subunit gene under the Thy-1.2 promoter. Now, we compared brain activation patterns between Thy1α6 and wild-type mice to identify brain structures potentially mediating this anxiolytic response. Acutely efficient anxiolytics such as benzodiazepines typically depress most brain regions while activating specifically neurons within the central extended amygdala. Gaboxadol treatment (3 mg/kg, i.p., 2 h) induced a significant increase in c-Fos expression selectively in many Thy1α6 brain regions including the limbic cortex, anterior olfactory nucleus, septal area and central and basolateral nuclei of amygdala. It failed to activate the lateral part of mediodorsal thalamic nucleus (MDL) in the Thy1α6 mice that was activated in the wild-type mice. Detailed mapping of the α6 subunit mRNA by *in situ* hybridization revealed expression in the middle layers of the isocortex, olfactory areas, hippocampal formation and basolateral nucleus of amygdala (BLA) in the Thy1α6 forebrain. The ligand autoradiographies (*t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) and [^{3}H]Ro 15-4513) revealed high levels of pharmacologically active extrasynaptic α6β and α6βγ2 $GABA_A$ receptors in these same areas. However, c-Fos induction by gaboxadol treatment in Thy1α6 brain was not restricted to areas highly expressing the α6-containing $GABA_A$ receptors suggesting that indirect pathways lead to the paradoxically widespread activation. Interestingly, the activation pattern by gaboxadol at the dose that is anxiolytic in Thy1α6 mice resembled closely that observed after various fear- and stress-provoking challenges. However, our results are consistent with a recent observation that optogenetic activation of specific neuronal pathways in the extended amygdala mediates anxiolytic responses. Our results suggest that the widespread neuronal inhibition as typically associated with benzodiazepines is not the exclusive mechanism of anxiolysis.

Key words: $GABA_A$ receptor, extrasynaptic, gaboxadol, THIP, transgenic mouse, c-Fos.

INTRODUCTION

GABAergic inhibition is crucial for regulation of neuronal excitability and hence for optimal function of the nervous system. $GABA_A$ receptor-mediated inhibition can be divided to fast and transient synaptic inhibition and to background (tonic) inhibition that is mediated by extrasynaptic $GABA_A$ receptors. $GABA_A$ receptor subtypes producing tonic inhibition contain usually α4, α5, α6 or δ subunits, which render them highly sensitive to GABA and thus suitable for detection of low GABA concentrations (Brickley et al., 1996; Farrant and Nusser, 2005; Lee and Maguire, 2014). Although not yet well understood, tonic $GABA_A$ receptor-mediated inhibition may play an important role in physiological functions as well as in psychiatric and neurological disorders (Belelli et al., 2009; Egawa and Fukuda, 2013).

To study the function of extrasynaptic inhibition *in vivo*, we have utilized a transgenic mouse line (Thy1α6) with chronically enhanced extrasynaptic inhibition in the forebrain by ectopic expression of the $GABA_A$ receptor α6 subunit under the Thy-1.2 promoter (Wisden et al., 2002). Previously, we have shown that a synthetic GABA site agonist gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, also known as THIP) preferring extrasynaptic $GABA_A$ receptors (Chandra et al., 2006, 2010; Storustovu and Ebert, 2006) induces strong anxiolytic-like response in transgenic Thy1α6 mouse line in light:dark exploration and elevated plus-maze tests, while being inactive at that low dose (3 mg/kg, i.p.) in wild-type control animals (Saarelainen et al., 2008). Also other studies have failed to show clear anxiolytic responses by gaboxadol in wild-type rodents (Elfline et al., 2004; Vinkers et al., 2009). Gaboxadol acts as a full

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Abbreviations: BSA, bovine serum albumin; BLA, basolateral nucleus of amygdala; BNST, bed nucleus of stria terminalis; CA1 and CA3, CA1 and CA3 areas of hippocampus; CeA, central nucleus of amygdala; Cg1, cingulate cortex; DG, dentate gyrus; GABA, γ-aminobutyric acid; gaboxadol, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, also known as THIP; MDL, lateral part of mediodorsal thalamic nucleus; PBS, phosphate-buffered saline; PBST20, PBS containing 0.05% Tween 20; [^{35}S]TBPS, *t*-butylbicyclophosphoro[^{35}S]thionate; TBPS, *t*-butylbicyclophosphorothionate.

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agonist at recombinant $GABA_A$ α6β, α6βδ and α4βδ receptors, in comparison to the partial agonist GABA (Brown et al., 2002; Saarelainen et al., 2008). We have shown a similar efficacy difference between gaboxadol and GABA also in native receptors of the Thy1α6 mice (Saarelainen et al., 2008), revealing the presence of highly gaboxadol-sensitive receptors in these mice. The mechanisms of the robust anxiolytic response by gaboxadol in these transgenic mice are unknown.

In the Thy1α6 forebrain, ectopic α6 subunits form predominantly extrasynaptic, functional α6β and α6βγ2 $GABA_A$ receptors, at least in the hippocampus and in hippocampal principal neurons that have been studied in detail (Wisden et al., 2002; Sinkkonen et al., 2004b; Saarelainen et al., 2008). While tonic inhibition was 5-fold larger in CA1 pyramidal cells in the Thy1α6 hippocampus, the synaptic inhibition was reduced by 30% in these cells (Wisden et al., 2002). Thy1α6 mice also had decreased amplitudes of the AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in patch-clamp recordings of CA1 pyramidal neurons in hippocampal slices (Moykkynen et al., 2007), suggesting synaptic scaling to reduced synaptic inhibition. *In vivo*, the Thy1α6 mice were slightly more reactive to handling and acoustic stimulus and more sensitive to convulsions induced by $GABA_A$ receptor antagonists but less sensitive to convulsions induced by electroshock (Sinkkonen et al., 2004b). These findings indicate that neuronal excitability, also outside the hippocampus, might be altered in the Thy1α6 brain.

In addition to the hippocampal CA1 pyramidal neurons, other stress/anxiety-related brain regions might be targeted by gaboxadol in the Thy1α6 mice and involved in anxiolytic processes. For example, clinically used anxiolytics, benzodiazepines, produce widespread neuronal inhibition detected by reduced expression of activity-associated genes, such as c-Fos immediate early gene, in the cortical, hippocampal and many subcortical stress-associated regions (Beck and Fibiger, 1995; Hitzemann and Hitzemann, 1999; Panhelainen and Korpi, 2012; Lkhagvasuren et al., 2014). However, they do increase the number of c-Fos expressing neurons in the central extended amygdala areas, including the bed nucleus of stria terminalis (BNST) and central nucleus of amygdala (CeA), indicating that, in addition to general inhibition, anxiolytic processes involve also neuronal activation in specific brain pathways (Beck and Fibiger, 1995; Hitzemann and Hitzemann, 1999; Panhelainen and Korpi, 2012; Lkhagvasuren et al., 2014). In the Thy1α6 mice, in addition to hippocampal CA1 pyramidal cells, high α6 immunoreactivity has been observed in somato-dendritic surface of most of the largest pyramidal cells in the layer V of the isocortex, some of CA3 pyramidal cells and most dentate granule cells. In general, but not always, large cell types strongly express the α6 protein. Strong α6 expression has also been observed in the amygdala, globus pallidus, substantia nigra (zona reticulata) and other brain stem nuclei (Wisden et al., 2002). The widespread expression reflects both the distribution of the pan-neuronal promoter Thy-1.2 (Caroni, 1997) and the selection of the founder mice (Wisden et al., 2002). In wild-type rodents, gaboxadol enhances tonic inhibition and hence inhibits activation in various brain regions (Kelly and McCulloch, 1982; Wafford and Ebert, 2006), but, interestingly, increasing tonic inhibition by gaboxadol preferentially in interneurons can lead to increased cortical network excitability (Krook-Magnuson and Huntsman, 2005; Drasbek and Jensen, 2006). Therefore, it was of great interest to analyze the effects of systemic gaboxadol on neuronal excitability, since theoretically both neuronal inhibition and activation might take place in the Thy1α6 brain.

The main objective of the current study was to analyze the alterations in neuronal excitability by gaboxadol in the transgenic mouse brain as compared to wild-type mouse brain to add knowledge about brain structures that may have a critical role in mediating acute anxiolytic responses. c-Fos immunohistochemistry was used to analyze changes in neuronal excitability. c-Fos protein is coded by an immediate-early gene and used as a marker of neuronal activation and plasticity (reviewed in Kovacs, 1998). In this study, we also mapped the α6 subunit mRNA expression by *in situ* hybridization and the pharmacologically defined subunit combinations of α6-containing $GABA_A$ receptors by using selective autoradiography with the convulsant/ionophore site ligand *t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) and the benzodiazepine site ligand [^{3}H]Ro 15-4513 in order to correlate specific localized pharmacology with patterns of c-Fos expression.

EXPERIMENTAL PROCEDURES

Experimental animals

Thy1α6 mice were generated by microinjecting a transgene consisting of the Thy-1.2 pan-neuronal promoter followed by the mouse $GABA_A$ receptor α6 subunit cDNA and Thy1 polyadenylation site into the genome of two-cell embryos, strain CBA/cba × C57BL/6 (Caroni, 1997; Wisden et al., 2002), and by at least 10 backcrosses to C57BL/6 background. The Thy1α6 mice were produced for experiments by mating within a homozygous line, and compared with C57BL/6NHsd controls (Harlan Netherland, Horst, Netherlands), which were purchased at the age of 5 weeks and housed in the same facility until experiments took place at the age of 3–6 months as in our previous study on behavioral comparisons of transgenic and wild-type mice (Saarelainen et al., 2008). The mice were housed in groups of 2–6 in Makrolon cages (37 × 21 × 15 cm; Tecniplast, Buguggiate, Italy), under 12:12-h light:dark cycles (lights on 7 am–7 pm), at 21–23 °C and a humidity of 50–60%. The mice received standard rodent pellets (Harlan BV, Horst, Netherlands) and tap water *ad libitum*. All mice were prehandled to avoid injection stress-induced c-Fos expression in stress-sensitive brain regions (Ryabinin et al., 1999) before gaboxadol had reached the brain at effective concentrations (maximal effect approximately 30 min after administration, i.p.). The mice were prehandled and accustomed to the test room and to injections by gently picking them up and touching their stomach with a syringe