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Cortical glutamatergic neurons mediate the motor sedative action of diazepam

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Running title: Cortical neurons mediate diazepam sedation

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Abstract

The neuronal circuits mediating the sedative action of diazepam are unknown. While the motor depressant action of diazepam is suppressed in α1(H101R) homozygous knock-in mice expressing diazepam-insensitive α1-$GABA_A$ receptors, global α1-knock-out mice show greater motor sedation with diazepam. To clarify this paradox, attributed to compensatory upregulation of the α2 and α3 subunits, and further identify the neuronal circuits supporting diazepam-induced sedation, we generated Emx1-cre-recombinase-mediated conditional mutant mice, selectively lacking the α1 subunit (forebrain-specific $\alpha1^{-/-}$) or expressing either a single wildtype (H) or a single point-mutated (R) α1 allele (forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice, respectively) in forebrain glutamatergic neurons. In the rest of the brain, $\alpha1^{-/R}$ mutants are heterozygous α1(H101R) mice. Forebrain-specific $\alpha1^{-/-}$ mice showed enhanced diazepam-induced motor depression and increased expression of the α2 and α3 subunits in the neocortex and hippocampus, in comparison to their pseudo-wildtype littermates. Forebrain-specific $\alpha1^{-/R}$ mice were less sensitive than $\alpha1^{-/H}$ mice to the motor depressing action of diazepam, but each of these conditional mutants had a similar behavioral response as their corresponding control littermates. Unexpectedly, expression of the α1 subunit was reduced in forebrain, notably in $\alpha1^{-/R}$ mice, and the α3 subunit was upregulated in neocortex, indicating that proper α1 subunit expression requires both alleles. In conclusion, conditional manipulation of $GABA_A$ receptor α1 subunit expression can induce compensatory changes in the affected areas. Specifically, alterations in $GABA_A$ receptor expression restricted to forebrain glutamatergic neurons reproduce the behavioral effects seen after a global alteration, thereby implicating these neurons in the motor sedative effect of diazepam.

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$GABA_A$ receptors mediate fast GABAergic inhibition in the adult mammalian central nervous system. $GABA_A$ receptors are pentameric ligand-gated ion channels, the majority of them containing two α, two β and one γ subunit (Barnard et al., 1998; Sieghart and Ernst, 2005). These receptors are the targets of many clinically important drugs (Rudolph and Mohler, 2006), including benzodiazepines (Rudolph and Mohler, 2004), barbiturates, neurosteroids (Belelli and Lambert, 2005) and general anesthetics (Rudolph and Antkowiak, 2004). Benzodiazepine binding to $GABA_A$ receptors modulates vigilance and anxiety states as well as a wide range of sensorimotor and cognitive functions. Notably, diazepam through α1-$GABA_A$ receptor activation can promote sedation, as measured by its motor depressant action, (Rudolph et al., 1999; McKernan et al., 2000), anterograde amnesia, and display anticonvulsant properties (Rudolph et al., 1999). This spectrum of effects has been shown genetically by introducing a histidine-to-arginine point mutation at position 101 of the murine $GABA_A$ receptor α1 subunit gene. The α1(H101R)-$GABA_A$ receptor is insensitive to allosteric modulation by benzodiazepine-site ligands, including zolpidem, both *in vitro* and *in vivo*, while regulation by the physiological neurotransmitter GABA is preserved (Benson et al., 1998; Rudolph et al., 1999; Crestani et al., 2000b; Marowsky et al., 2004). The corresponding α1(H101R) mice fail to show the motor depressant and anterograde amnesic effect of diazepam and are partly resistant to its anticonvulsant action (Rudolph et al., 1999; McKernan et al., 2000). In contrast, the effects of diazepam on sleep EEG are not affected in these mice (Tobler et al., 2001) and rather depend on α2-$GABA_A$ receptors (Kopp et al., 2004). The role of α1-$GABA_A$ receptors in mediating the sedative action of benzodiazepine-site ligands was further supported by pharmacological studies using L838-417. This substance, which acts as a partial agonist at α2-, α3- and α5-$GABA_A$ receptors and as an antagonist at α1-$GABA_A$ receptors, displays no sedative properties in rodents (McKernan et al., 2000). However, ocinaplon, a partial agonist at all diazepam-sensitive $GABA_A$ receptors, has been reported to produce selective anxiolysis (Lippa et al., 2005) and to depress motor activity at high doses only. The mechanisms underlying this different profile of action are not known. Furthermore, the global α1 subunit knock-out mice treated with diazepam display enhanced motor sedation compared to wildtype littermates (Kralic et al., 2002a; 2002b), indicating that α1-$GABA_A$ receptors can be substituted. These mutants also show increased expression of the $GABA_A$ receptor α2 and α3 subunits notably in cerebral cortex (Kralic et al., 2002a, 2006). The compensatory upregulation of other α subunits might underlie the pharmacological phenotype of α1 subunit knock-out mice.

To further clarify the molecular mechanisms and neural circuits mediating the motor sedative action of diazepam, we focused the current study on the pharmacological significance of α1-$GABA_A$ receptors expressed in the forebrain. To achieve this goal, we investigated genetically engineered mice with either a constitutive deficit in α1-$GABA_A$ receptors, or carrying a single diazepam-insensitive α1(H101R) allele, restricted to forebrain glutamatergic neurons for their responsiveness to the motor sedative action of diazepam. In behavioral pharmacology, the term sedation refers to a drug-induced diminution in spontaneous activity of experimental animals (Trevor and Way, 1995). Conditional gene deletion was obtained by combining a wildtype α1 subunit allele flanked by loxP sites (floxed) with a cre transgene expressed from an Emx1 promoter or by combining a H101R point-mutated α1 subunit allele with a floxed wildtype α1 subunit allele and the Emx1-cre transgene. These forebrain-specific mutants were analyzed immunohistochemically for possible changes in α1, α2 and α3 subunit expression patterns and were tested behaviorally for diazepam-induced changes in spontaneous locomotor activity.

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Material and Methods

Animals. Forebrain-specific deletion of the α1 subunit was achieved upon excision of alleles with an exon flanked by loxP sites (floxed) by cre recombinase driven by the Emx1 promoter. To obtain these mice, Emx1-cre Tg3 PAC transgenic mice [B6-Tg(Emx-cre) described in (Iwasato et al., 2004), maintained in Zurich onto the C57BL/6JOlaHsd background] were crossed initially with mice homozygous for the floxed α1 subunit allele [B6.129(FVB)-Gabra1^{tm1Geh}/J, at least six backcrosses onto C57BL/6J, Jackson Laboratory, Bar Harbor, ME; firstly described in (Vicini et al., 2001)] (Fig. 1A, C). Offspring, heterozygous for the floxed α1 subunit allele and carrying the Emx1-cre transgene, were crossed again with mice homozygous for the floxed α1 subunit allele to obtain the desired genotype (Fig. 1A, C). Because two generations were necessary to obtain the mutant mice for analysis, the genetic background of the experimental animals was approximately 75% C57BL/6J and 25% C57BL/6JOlaHsd. The Emx1-cre transgene is expressed principally in glutamatergic cells (but not interneurons) of the neocortex and hippocampal formation, and to a lower extent in septum, amygdala, allocortex and olfactory bulb (Iwasato et al., 2004). Homozygous deletion of the α1 subunit floxed alleles was expected to result in a region-specific disappearance of the α1 subunit during late prenatal and early postnatal development.

To obtain mice with a forebrain-specific α1(H101R) point mutation (forebrain-specific heterozygous knock-in, $\alpha1^{-/R}$), we first crossed Emx1-cre transgenic mice with homozygous α1(H101R) mice. All offspring had one wildtype (H) and one point mutated (R) α1 allele; those carrying the Emx1-cre transgene were then crossed with mice homozygous for the wildtype floxed α1 subunit allele (Fig. 1B, C) to obtain four genotypes of experimental animals, including pseudo-wildtype ($\alpha1^{H/H}$), forebrain-specific heterozygous knock-out ($\alpha1^{-/H}$) , global heterozygous knock-in mice ($\alpha1^{H/R}$) and forebrain-specific heterozygous knock-in mice ($\alpha1^{-/R}$) (Fig. 1B, C). The forebrain-specific $\alpha1^{-/R}$ mice carried a single diazepam-insensitive α1(H101R) allele in forebrain glutamatergic cells and both a wildtype floxed α1 allele and a point-mutated diazepam-insensitive α1(H101R) allele in all other cells. In these mice, diazepam was therefore expected to have no effect on α1-$GABA_A$ receptors in forebrain glutamatergic cells, but to activate these receptors in the rest of the brain. The other heterozygous mice were $\alpha1^{-/H}$ mice, which had a single wildtype floxed allele in forebrain glutamatergic neurons, and two floxed alleles in the rest of the brain (Fig. 1C), and were therefore expected to display diazepam sensitivity throughout the brain. The nomenclature used to distinguish the six genotypes generated in this study denotes the presence or absence

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of the Emx1-driven cre recombinase, the floxed wildtype α1 subunit allele and the point mutated α1(H101R) subunit allele (Fig. 1C). In all cases, H denotes an α1 subunit allele with a histidine in position 101 and R a point-mutated α1(H101R) subunit.

In some animals, the Emx1-cre transgene can be present in the germline and induce recombination at this stage. Such recombination can be detected in the liver of the offspring because of the lack of somatic cre expression in this organ (Iwasato et al., 2004). To identify mice with germ line recombination, we therefore genotyped liver biopsies from all mice used in behavioral and immunohistochemical experiments from breeding pairs carrying both the Emx1-cre transgene and the wildtype α1 floxed allele. The frequency of germline cre recombination was not dependent on the gender of the parents. Mice showing germline cre recombination (36%) were excluded from the study.

The following PCR primers were used to identify the cre transgene (5'-TGA CAG CAA TGC TGT TTC ACT GG-3' and 5'-GCA TGA TCT CCG GTA TTG AAA CTC C-3', product size 570 bp; germline recombination 5'-CTG TAC TGT GTA TAT TAG GAT AAA GTA-3' and 5'-TTC TGC ATG TGG GAC AAA GAC TAT T-3', providing a product size 1476 bp when no recombination occurred, and a product size of 296 bp when cre-mediated recombination had occurred and exon 8 was excised) and the point mutated α1(H101R) allele (5'-CAA TGG TAG GCT CAC TCT GGG AGA TGA TA-3' and 5'-AAC ACA CAC TGG CAG GAC TGG CTA GG-3', product size ca. 300 bp for the wildtype (H) allele, and ca. 350 bp for the (R) allele, the size difference being due to the presence of a loxP site in the R allele). The PCR reaction used for the detection of the wildtype α1 floxed allele is described at http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=584).

Immunohistochemistry. Adult mice were deeply anesthetised with pentobarbital (50 mg/kg, i.p.) and perfused through the aorta with 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4). Brains were postfixed for 3 hours, cryoprotected in sucrose, frozen and cut parasagitally at 40 μm with a sliding microtome. Sections were collected in phosphate buffered saline and stored in an antifreeze solution. Immunoperoxidase staining was performed to visualize and quantify the distribution of $GABA_A$ receptor α1, α2 or α3 subunits in forebrain-specific mutant mice and their corresponding controls (Fig. 1). Free-floating sections were incubated overnight at 4°C with subunit-specific primary antibodies diluted in Tris buffer containing 2% normal goat serum and 0.2% Triton X-100; see Kralic et al. (2006) for details on the characterization of these primary antibodies. Sections were

washed and incubated for 30 min at room temperature in biotinylated secondary antibodies (1:300, Jackson Immunoresearch, West Grove, PA) in the same buffer as the primary antibodies. After washing, sections were incubated in the avidin-biotin-peroxidase complex (1:100 in Tris buffer, Vectastatin Elite Kit, Vector Laboratories, Burlinghame, CA) and after another wash finally reacted with diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in Tris buffer (pH 7.7) containing 0.015% hydrogen peroxide. The color reaction was stopped after 5-20 min with ice-cold buffer. Sections were then mounted on gelatin-coated slides and air-dried. Finally, they were dehydrated with an ascending series of ethanol, cleared in xylene, and coverslipped with Eukitt (Erne Chemie, Dällikon, Switzerland). In separate experiments, double immunofluorescence staining for the α1 subunit along with markers of cortical interneurons (parvalbumin, calbindin, calretinin) was performed in forebrain-specific $\alpha1^{-/-}$ mice and their pseudo-wildtype littermates (see Fig. 1). Sections were incubated in a mixture of primary antibodies (mouse anti-parvalbumin, mouse anti-calbindin, rabbit anti-calretinin; Swant, Bellinzona, Switzerland) and guinea pig anti-α1 subunit as above. After washing, sections were incubated in a mixture of secondary antibodies coupled to Alexa 488 (Molecular Probes, Eugene, OR) or Cy3 (Jackson Immunoresearch). After mounting, sections were air-dried and coverslipped with aqueous mounting medium (Dako, Glostrup, Denmark). In all experiments, sections from wildtype and mutant mice were processed in parallel under identical conditions to minimize variability in staining intensity.

The densitometric analysis was carried out with the MCID M5 imaging system (Imaging Research, St. Catherines, ON, Canada) on sections from 4 animals per genotype processed for immunoperoxidase staining. Images were digitized with a high resolution black-and-white camera. Optical density values were calibrated with grey scale standards, arbitrary ranging from 0 (white) to 100 (black). Background was measured in the cerebellar granule cell layer for the α3 subunit and in the inferior colliculus for the α2 subunit and subtracted from the optical density values measured in the regions of interest. Results, expressed as mean ± S.D., were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney tests.

Behavioral testing. The effect of diazepam on motor activity was measured as a determinant of its sedative action (Trevor and Way, 1995) in the different mutant mouse lines. Mice were adapted to a reversed 12-h day-night cycle (lights off at 8 a.m.) for at least two weeks before testing (between 9 and 12 a.m.). Motor activity was measured during the active phase in automated individual circular runways equipped with photocells (Imetronic, Pessac, France)

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for an hour, starting 30 min after oral administration of either 10 $mg.kg^{-1}$ diazepam or vehicle (0.3% Tween 80 in saline). The dose of diazepam was chosen based on previous dose-response experiments showing a marked reduction in motor activity in wildtype C57BL/6J mice, but not in α1(H101R) mutants. Because of absence of difference, data from male and female mice were pooled and analyzed using two-way (genotype x treatment) repeated measures ANOVA followed by *post hoc* Scheffe tests. Results are expressed as mean ± S.E.

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Results

Expression of $GABA_A$ receptor subunits in mice lacking the α1 subunit in forebrain glutamatergic neurons

The immunohistochemical analysis of the regional distribution and relative immunoreactivity (IR) levels for the α1, α2, and α3 subunit revealed differences between forebrain-specific $\alpha1^{-/-}$ mice and their corresponding pseudo-wildtype littermates ($H^{flox}H^{flox}$/Emx1-cre^{tg-}). In wildtype brain sections, α1 subunit-IR was prominent and nearly evenly distributed across all cortical areas (Fig. 2A). The α1 subunit staining was most pronounced in layers I and III-IV, as shown in the parietal cortex (Fig. 2B). α1 subunit-IR was also intense and diffuse in all subregions of the hippocampal formation, except in the pyramidal and the granule cell layers (Fig. 2C). No structure or single neuron could be distinguished at low magnification, except in the CA3 stratum lucidum where interneurons and their dendrites were visible (Fig. 2C, arrowhead). In brain sections from forebrain-specific $\alpha1^{-/-}$ mice, a marked decrease in α1 subunit-IR was apparent and restricted to the neocortex and hippocampus (Fig. 2D). In these mice, no change in α1 subunit-IR could be detected in brain regions in which Emx1-cre is not expressed, confirming the specificity of the cre recombination driven by the Emx1 promoter. Remarkably, the α1 subunit staining was absent from all cortical glutamatergic cells while being retained in interneurons, as seen at high magnification (Fig. 2E, arrowhead). This finding was even more evident in hippocampal sections, where a large population of interneurons selectively showed an intense α1 subunit-IR against a white background (Fig. 2F). Thus, as expected, forebrain-specific $\alpha1^{-/-}$ mice displayed a deficit in α1 subunit restricted to glutamatergic neurons. The interneuronal nature of α1 subunit-positive cells in the neocortex and hippocampus was verified by double immunofluorescence staining with parvalbumin (Fig. 2G-J), calbindin (Fig. 2K), and calretinin (not shown), three calcium-binding proteins that label largely non-overlapping subpopulations of GABAergic interneurons (Freund and Buzsaki, 1996).

Forebrain-specific $\alpha1^{-/-}$ mice showed a regional expression pattern for the α2 and α3 subunit comparable to that of the pseudo-wildtype controls, but the IR of both subunits was stronger. In control mice, α2 subunit-IR was confined to the outer layers of the neocortex, while being virtually absent in layers V and VI. In the hippocampal formation, it was most prominent in the dentate gyrus, followed by CA3 and CA1 (Fig. 3A). A significant increase in α2 subunit-IR was apparent in the neocortex of mutants, but not in the hippocampal formation (Fig. 3B

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and Table 1). The α3 subunit-IR in the neocortex of pseudo-wildtype mice was most intense in V and VI, particularly in frontal cortex. In the hippocampal formation, it predominated in the CA1 area similarly in pseudo-wildtype and forebrain-specific $\alpha1^{-/-}$ mice (Fig. 3C, D) while being almost absent in the dentate gyrus. Mutant mice showed enhanced levels of α3 subunit IR in neocortex, comparable to the increase in α2 subunit-IR (Table 1). Notably, the α3 subunit, almost absent in layer IV of parietal cortex in wildtype animals, could be detected in the mutants (Fig. 3D). As in forebrain-specific $\alpha1^{-/-}$ mice, no change in subunit expression was seen in regions where Emx1-cre was not expressed, such as striatum, thalamus and cerebellum (Table 1). Thus, a deficit of α1 subunit in cortical glutamatergic neurons was accompanied by an increased expression of the α2 and α3 subunits in the corresponding regions.

Sedative action of diazepam in forebrain-specific $\alpha1^{-/-}$ mice

Forebrain-specific $\alpha1^{-/-}$ mice displayed heightened sensitivity to the sedative action of diazepam, as indicated by the greater drug-induced decrease in motor activity in the mutants as compared to the pseudo-wildtype mice [$p < 0.05$ after $F_{(1,36)} = 13.09$, $p < 0.01$] (Fig. 4A). No genotype difference was observed with the vehicle treatment.

Expression of $GABA_A$ receptor subunits in mice carrying a single α1(H101R) allele in forebrain glutamatergic neurons

A second series of experiments was carried out to obtain mice in which diazepam-sensitivity, but not expression of α1-$GABA_A$ receptors, would be selectively suppressed in forebrain neurons expressing Emx1. The breeding scheme adopted (Fig. 1) resulted in four genotypes, including pseudo-wildtype mice ($H^{flox}H$/Emx1-cre^{tg-}), global heterozygous knock-in $\alpha1^{H/R}$ mice, forebrain-specific $\alpha1^{-/H}$ mice (carrying a single floxed α1 subunit allele in forebrain), and forebrain-specific $\alpha1^{-/R}$ (carrying a single point-mutated α1(H101R) subunit allele in forebrain). The pseudo-wildtype and $\alpha1^{H/R}$ mice showed an expression pattern for the $GABA_A$ receptor α1, α2 and α3 subunits similar to that seen in pseudo-wildtype $H^{flox}H^{flox}$/Emx1-cre^{tg-} (see Figs. 2A, 3A, C). Unexpectedly, in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice, α1 subunit-IR was decreased in cortical and hippocampal principal cells (Fig. 5). At high magnification, individual interneurons and their dendrites could be easily visualized at low magnification in the neocortex (Figs. 5A and B, arrowhead) and the hippocampus

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(Figs. 5C and D, arrowhead) in sections from both mutants. The α1 subunit deficit in parietal cortex was more pronounced in $\alpha1^{-/R}$ mice (Fig. 5B). However, the prominent labeling of interneurons largely masked the decrease in pyramidal cells, so that no selective densitometric quantification was feasible. Nevertheless, these results indicate that a single, either wildtype or point-mutated, α1 subunit allele in cortical glutamatergic neurons was insufficient to provide normal expression of the α1 subunit.

No consistent alteration in α2 subunit expression pattern and IR levels was detected in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice (Table 2). A difference in α3 subunit-IR was observed specifically in the cerebral cortex of $\alpha1^{-/R}$ mice (Table 2). A trend was seen in $\alpha1^{-/H}$ mice, but the changes were significant only in layers V-VI of frontal cortex (Table 2). In these mice, weakly stained areas (CA3, DG, striatum) exhibited increased staining compared to control (Table 2). However, since these changes were not seen in other mutants and included regions where Emx1-cre is not expressed, their significance is uncertain. Overall, we conclude that expression of a single α1(H101R) allele in forebrain glutamatergic neurons is associated with a selective upregulation of the α3 subunit in the neocortex.

Sedative action of diazepam in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice

Diazepam treatment decreased motor activity levels in all four mouse lines, but to a lesser degree in $\alpha1^{H/R}$ and forebrain-specific $\alpha1^{-/R}$ mice [$p < 0.01$ as compared to $\alpha1^{H/H}$ and forebrain-specific $\alpha1^{-/H}$ mice, after $F_{(3,80)} = 4.68$, $p < 0.01$] (Fig. 4B). Vehicle-treated animals did not differ from each other, irrespective of the genotype.

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Discussion

The present report provides evidence for a major contribution of cortical glutamatergic neurons in diazepam-induced motor sedation. First, we show that a constitutive deficit in α1 subunit restricted to the forebrain glutamatergic cells was sufficient to reproduce the enhanced sensitivity to the motor depressant action of diazepam, as reported in the global $\alpha1^{-/-}$ mice (Kralic et al., 2002a, 2002b). Second, forebrain-specific $\alpha1^{-/R}$ mice were less sensitive than $\alpha1^{-/H}$ mice to the motor depressing action of diazepam, but each of these conditional mutants had a similar behavioral response than their corresponding control littermates ($\alpha1^{H/R}$ and $\alpha1^{H/H}$, respectively), underscoring the involvement of forebrain $GABA_A$ receptors in mediating the residual drug effect. Third, also reminiscent of the global $\alpha1^{-/-}$ phenotype, is the upregulation of the α3 subunit in the neocortex of both forebrain-specific $\alpha1^{-/-}$ and $\alpha1^{-/R}$ mutants. An overexpression of the α2 subunit could be detected only in the forebrain-specific $\alpha1^{-/-}$ mice. These results strongly suggest that $GABA_A$ receptors overexpressed in cortical glutamatergic neurons lacking of α1 subunit substitute pharmacologically for α1-$GABA_A$ receptors. Therefore, modulation of the activity of neuronal circuits in the neocortex is a major determinant of diazepam-induced motor sedation in mice. Finally, because forebrain-specific $\alpha1^{-/-}$ mice have the same pharmacological phenotype as global α1 subunit knockout mice despite retaining a prominent α1 subunit expression in interneurons, enhancing $GABA_A$ receptor function in these cells is unlikely to be required for the sedative action of diazepam. A dose of 10 mg/kg diazepam was selected for our experiments since it has a robust sedative action reducing motor activity by approximately two thirds, while still allowing to detect a further decrease in motor activity caused by individual genotypes.

Global deletion of the α1 subunit gene results in a marked compensatory overexpression of the $GABA_A$ receptor α2, α3 and α4 subunit, selectively in those brain regions where the α1 subunit is absent (Kralic et al., 2002a, 2006; Schneider Gasser et al., 2007). Upregulation likely takes place at the level of translation, without increase in subunit gene transcription, as shown by several studies (Bosman et al., 2005b). These compensatory changes do not fully restore the function of the missing α1 subunit, as evidenced for instance by the decrease of GABAergic currents in cerebellar slices (Vicini et al., 2001) or the complete loss of $GABA_A$ receptors in Purkinje cells in these mutants (Sur et al., 2001; Kralic et al., 2005; Fritschy et al., 2006). We opted for a conditional mutation strategy, expecting no compensatory α subunit upregulation. Nevertheless, deletion of the α1 subunit restricted to forebrain principal

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cells leads to overexpression of the α2 and α3 subunit, underscoring the need for homeostatic compensation to retain normal brain function in the absence of a major $GABA_A$ receptor subtype. The change in subunit expression was restricted to regions where Emx1-cre-induced recombination had occurred, further indicating that $GABA_A$ receptors were likely unaffected in other brain areas of conditional mutant mice.

The decreased α1 subunit-IR in the forebrain of $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice, which both carry a single α1 subunit allele in forebrain glutamatergic neurons, is reminiscent of the decreased expression of the γ2 subunit occurring mostly in neocortex and hippocampus in mice heterozygous for the γ2 subunit deletion ($\gamma2^{+/-}$) (Crestani et al., 1999). These findings reveal that certain major $GABA_A$ receptor subunits are available in limited amounts whenever expressed by a single allele. In $\gamma2^{+/-}$ mice no compensatory upregulation of other $GABA_A$ receptor subunits could be detected, presumably because the remaining α/β subunit variants could form functional $GABA_A$ receptors in these mutants (Lorez et al., 2000). The partial deficit in α1-$GABA_A$ receptors in $\alpha1^{-/R}$ mice appears to be sufficient to induce compensatory changes, likely because α subunits are required for receptor assembly (Kralic et al., 2006; Rudolph and Mohler, 2006; Studer et al., 2006).

In line with the loss of diazepam binding to $GABA_A$ receptors containing the α1(H101R) point-mutation, forebrain-specific $\alpha1^{-/R}$ mice were less sensitive than $\alpha1^{-/H}$ mice to the motor sedative action of diazepam, underscoring again the contribution of cortical circuits to this pharmacological effect. However, these two mutants show diazepam responsiveness similar to that of their respective global heterozygote or pseudo-wildtype control ($\alpha1^{H/R}$ and $\alpha1^{H/H}$). In the case of $\alpha1^{-/H}$ mice, one might argue that the remaining pool of diazepam-sensitive α1-$GABA_A$ receptors in the cerebral cortex is sufficient for the full manifestation of the sedative drug action. In $\alpha1^{-/R}$ mice, the mild reduction in motor activity produced by diazepam is best explained by the upregulation of the α3 subunit, which might restore a complement of diazepam-sensitive α3-$GABA_A$ receptors selectively in neocortical regions.

The consequences of the upregulation of α2 and α3 subunit in forebrain-specific mutant mice for the function of cortical circuits remain to be established. Cortical pyramidal cells express multiple $GABA_A$ receptor α subunits with a differential subcellular distribution. In particular, α1-$GABA_A$ receptors predominate on distal dendrites, whereas α2-$GABA_A$ receptors mediate most of the perisomatic GABAergic inputs (Prenosil et al., 2006). In addition, the α1- and α2 subunits are located in the synapses of separate subpopulations of basket cells (distinguished

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by expression of parvalbumin and cholecystokinin, respectively) (Nyiri et al., 2001). These differences likely underlie the contribution of these $GABA_A$ receptor subtypes to distinct neuronal circuits. Although the upregulation of the α3 subunit suggests that this subunit could replace the α1 subunit at its original location, a reorganization of GABAergic circuits within the cortex cannot be excluded.

In addition to the circuit-specific localization of $GABA_A$ receptor subtypes, their functional properties are determined by their subunit composition. Thus, $GABA_A$ receptors expressed in the neocortex and hippocampus of global $\alpha1^{-/-}$ mice have longer decay kinetics (Goldstein et al., 2002; Bosman et al., 2005b; Schneider Gasser et al., 2007), characteristic of α2- and α3-$GABA_A$ receptors expressed early during development (Hutcheon et al., 2000). The number of functional GABAergic synapses is not changed in the neocortex (Bosman et al., 2005b), but the longer kinetics influences γ oscillations (Bosman et al., 2005a). Taken together, upregulation of the α2 and α3 subunit in forebrain-specific $\alpha1^{-/-}$ mice might functionally compensate for the loss of the α1 subunit when no substance challenges the system, resulting in a normal behavioral response, as seen in vehicle-treated mice. However, because of the slow kinetics of α3-$GABA_A$ receptors, the effects induced by diazepam in cortical neurons lacking α1-$GABA_A$ receptors might be more pronounced than those observed in wildtype. This difference might be manifested behaviorally by the enhanced sensitivity of forebrain-specific $\alpha1^{-/-}$ mutants to the motor sedative effect of diazepam compared to pseudo-wildype mice (Fig. 4A). The importance of $GABA_A$ receptor kinetics for normal brain function has been underscored by introducing a (S270H) point mutation in the α1 subunit gene that causes a marked slowing of $GABA_A$ receptor deactivation (Homanics et al., 2005). The corresponding point-mutated mice exhibit major physiological, behavioral and pharmacological impairments, e.g. loss of sensitivity to the volatile anesthetic isoflurane, likely due to functional abnormalities in neuronal circuits expressing α1(S270H)-$GABA_A$ receptors (Homanics et al., 2005).

Our results strongly implicate neocortical circuits in the mediation of diazepam-induced motor sedation. The sedative effect of benzodiazepines is often assessed using tests of motor coordination (e.g. rotarod), which likely engage additional brain circuits, notably the cerebellum (Lalonde and Strazielle, 2001; Levin et al., 2006). However, while such behavioral paradigms arguably provide a more complete readout of the drug effect as a reduction in motor activity, their validity for predicting sedative effects in human has been questioned (Stanley et al., 2005). A reduction in muscle tone in diazepam-treated mice might

possibly affect motor activity. However, this effect is unlikely to confound the present results, since the myorelaxant effect of diazepam is mediated by α2 and α5-$GABA_A$ receptors and cannot be attributed selectively to cortical circuits (Crestani et al., 2000a). Rather, in support for a major involvement of cortical networks in mediating the motor depressant effects of diazepam, it has been shown in rats *in vivo* that sedative doses of the volatile anesthetics isoflurane and enflurane reduce cortical firing rate by 65% due to increased $GABA_A$ receptor-mediated inhibition (Hentschke et al., 2005). This correlation between behavioral sedation and depression of cortical firing rate is consistent with the assumption that low doses of volatile anesthetics mediate sedation via modulation of cortical circuits. Likewise, fMRI experiments in humans show that low, sedative doses of the $GABA_A$ receptor specific general anesthetic propofol reduce neuronal activity prominently in cortical networks (Heinke et al., 2004; Heinke and Koelsch, 2005). Only when higher, hypnotic doses of propofol are administered to the subjects, neuronal activity also decreases in subcortical structures, including the thalamus and midbrain reticular formation.

In conclusion, the present study demonstrates that even conditional, temporally and spatially restricted manipulation of $GABA_A$ receptor α1 subunit expression can induce compensatory changes selectively in the affected areas. Alterations of $GABA_A$ receptor expression or pharmacology restricted to forebrain glutamatergic neurons produce the same behavioral effects as seen after a global alteration, thereby implicating these neurons in the motor sedative effect of diazepam.

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Footnotes

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Legends for figures

Figure 1

Breeding schemes and description of the genotypes of mice used in the present study. A. Breeding scheme to obtain forebrain-specific $\alpha1^{-/-}$ ($H^{flox}H^{flox}$/Emx1-cre^{tg+}) mice and their pseudo-wildtype littermates. B. Breeding scheme to obtain forebrain-specific heterozygous $\alpha1^{-/R}$ (H^{flox}R/Emx1-cre^{tg+}), $\alpha1^{-/H}$ (H^{flox}H/Emx1-cre^{tg+}), and corresponding control mice (global heterozygous and pseudo-wildtype). H^{flox} = α1 floxed allele, H = α1 wildtype allele with a codon for histidine at amino acid position 101, R = α1(H101R) point-mutated allele with a codon for arginine at amino acid position 101, Emx1-cre^{tg} = absence (–) or presence (+) of cre transgene. C. The left panel lists the genotypes of all mouse lines and the right panel the functional genotype resulting from Emx1-cre-mediated excision of the floxed allele(s) selectively in forebrain principal neurons. For the description of the phenotypes, the floxed alleles are not indicated separately because the loxP sites present in introns did not have an appreciable effect on gene expression.

Figure 2

Region- and cell type-specific loss of α1 subunit-IR in forebrain-specific $\alpha1^{-/-}$ mice. A, D. Pseudo-colored photomicrographs of parasagittal brain sections processed for α1 subunit immunoperoxidase staining from pseudo-wildtype mice and forebrain-specific $\alpha1^{-/-}$ mutants. Yellow-white indicates a strong labeling and blue, background level. Note the selective reduction of IR in neocortex and hippocampus. B, E and C, F: Color photomicrographs of parietal cortex and hippocampal formation illustrating the reduction of α1 subunit-IR in the neuropil of forebrain-specific $\alpha1^{-/-}$ mice, reflecting loss of expression in pyramidal cells and retention of the α1 subunit in a large subset of interneurons (arrowheads), which are not visible in wildtype due to the diffuse staining of pyramidal cell dendrites in the neuropil. G-K: Double immunofluorescence staining for the α1 subunit (green) and parvalbumin (PV; red; G-J) or calbindin (CB; red; K) in parietal cortex layer III (G-H) and CA1 (I-K) from pseudo-wildtype mice (G, I) and forebrain-specific $\alpha1^{-/-}$ mutants (H, J, K). In wildtype, the α1 subunit staining is prominent in the neuropil (green) and in PV-positive interneurons (yellow); in mutants, the α1 subunit staining is present in the soma and dendrites of interneurons, most of which are double labeled for PV (yellow; H, J); only few α1 subunit-positive interneurons

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also contain CB immunoreactivity (K); the single-labeled cells in panels H, J, K (green) represent other subtypes of interneurons. Abbreviations: s. gran., stratum granulosum; s. lm, stratum lacunosum-moleculare; s. luc, stratum lucidum; s. mol, stratum moleculare; s. or, stratum oriens; s pyr, stratum pyramidale; s rad, stratum radiatum; Scale bars in A, D, 2 mm; B, C, E, F, 200 μm; insets in B, E, 50 μm; G-H, 30 μm; I-K, 20 μm.

Figure 3

Region-specific increase of α2 and α3 subunit-IR in forebrain-specific $\alpha1^{-/-}$ mice. Pseudo-colored photomicrographs of parasagittal sections processed for immunoperoxidase staining. A. α2 subunit-IR in pseudo-wildtype mice. B. Increased α2 subunit-IR in the neocortex in forebrain-specific $\alpha1^{-/-}$ mice. C. α3 subunit-IR in pseudo-wildtype mice. D. Increased α3 subunit-IR in the neocortex in forebrain-specific $\alpha1^{-/-}$ mice. Scale bar 2 mm.

Figure 4

Motor sedative effect of diazepam. Motor activity was measured for 1 hr, starting 30 min after oral administration of either vehicle or 10 $mg.kg^{-1}$ diazepam. A. Forebrain-specific $\alpha1^{-/-}$ mice: Note the greater reduction in mean activity counts in diazepam-treated forebrain-specific $\alpha1^{-/-}$ mice compared to pseudo-wildtype $\alpha1^{H/H}$ mice (n = 19 mice per group; # $p < 0.05$ as compared to wildtype mice; *** $p < 0.001$ as compared to vehicle). B. Forebrain-specific heterozygous $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice. Overall, this series of animals displayed lower levels of motor activity, as seen in vehicle-treated $\alpha1^{H/H}$ mice in comparison to the corresponding pseudo-wildtype $\alpha1^{H/H}$ animals in panel A, but this experimental variability did not change the vehicle-diazepam ratio. Diazepam treatment induced a decrease in motor activity in all four groups, but the drug effect was smaller in $\alpha1^{-/R}$ than $\alpha1^{-/H}$ mice and the effect in each of these forebrain-specific mutants was not different from that in their corresponding $\alpha1^{H/R}$ and $\alpha1^{H/H}$ control littermates (n = 21 mice per group; # $p < 0.05$ as compared to $\alpha1^{H/H}$ and $\alpha1^{-/H}$ mice; ** $p < 0.01$ and *** $p < 0.001$ as compared to vehicle, Scheffe tests).

Figure 5

Reduction of α1 subunit-IR in forebrain-specific heterozygous $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice. Photomicrographs of parasagittal sections through the parietal cortex (A, B) and hippocampus (C, D) processed for immunoperoxidase staining. A, C. Slight reduction of α1 subunit-IR in

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the neuropil of $\alpha1^{-/H}$ mice as compared to wildtype mice in Figs. 2B, C. Individual interneurons and their dendrites become visible (arrowheads). B, D. More pronounced reduction of α1 subunit-IR in the neuropil of $\alpha1^{-/R}$ mice. In all hippocampal regions and in the neocortex, individual interneurons and their dendrites become visible (arrowheads). Scale bars in A, B, 200 μm, insets in A, B 50 μm.

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Table 1
Quantification of $GABA_A$ receptor α2 and α3 subunit immunoreactivity in forebrain-specific $\alpha1^{-/-}$ mice compared to wildtype littermates ($H^{flox}H^{flox}$/ Emx1-Cre^{tg-})

Region	Wildtype OD α2	Forebrain-specific $\alpha1^{-/-}$ % WT OD α2	Wildtype OD α3	Forebrain-specific $\alpha1^{-/-}$ % WT OD α3
Parietal cortex, layer I-III	12±5.8	**146**	33±2.4	**142**
Parietal cortex, layer IV	11±5.3	**172**	23±1.8	**169**
Parietal cortex, layers V-VI	5±4.8	**185**	35±2.3	122
Frontal cortex, layers I-III	12±4.4	**143**	39±1.9	**136**
Frontal cortex, layers V-VI	9±5.0	**162**	41±1.7	**131**
CA1	34±6.1	110	22±3.9	109
CA3	37±5.8	101	20±5	102
Dentate gyrus	43±7.7	117	16±4.0	125
Subiculum	30±5.2	118	33±1.6	**135**
Striatum	39±4.2	93	21±3.0	119
Thalamic reticular nucleus	-	-	30±3.1	114
Cerebellum, molecular layer	21±3.1	101	8±1.6	118

Optical density values (OD) were measured in sections processed for immunoperoxidase staining (adult mice, n = 4 per genotype) using gray scale standards for calibration. Background was measured in a region of gray matter lacking the expression of these subunits (inferior colliculus for the α2 subunit and cerebellum for the α3 subunit) and subtracted from the measured values. Values in mutants are expressed as % of wildtype control. Statistically significant differences in absolute values are indicated in bold (p<0.05, Mann-Whitney test).

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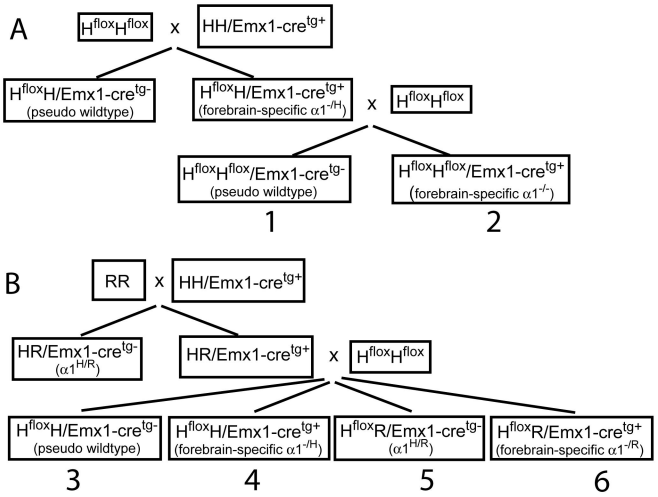
Table 2

Quantification of the $GABA_A$ receptor α2 and α3 subunit immunoreactivity in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice compared to wildtype littermates ($H^{flox}H$/ Emx1-Cre^{tg-} and $H^{flox}R$/Emx1-Cre^{tg-})

Region	Forebrain-specific $\alpha1^{-/H}$ % OD α2	Forebrain-specific $\alpha1^{-/R}$ % OD α2	Forebrain-specific $\alpha1^{-/H}$ % OD α3	Forebrain-specific $\alpha1^{-/R}$ % OD α3
Parietal cortex, layer I-III	101	118	123	127
Parietal cortex, layer IV	129	129	115	**142**
Parietal cortex, layers V-VI	120	109	105	**130**
Frontal cortex, layers I-III	72	112	124	118
Frontal cortex, layers V-VI	**56**	112	**140**	**134**
CA1	80	97	122	106
CA3	84	88	**148**	109
Dentate gyrus	76	109	**160**	118
Subiculum	88	103	124	126
Striatum	95	114	**156**	120
Thalamic reticular nucleus	-	-	128	126
Cerebellum, molecular layer	127	126	126	125

Densitometry was performed in sections processed for immunoperoxidase staining (see Table 1). Values are expressed as % of wildtype control. Statistically significant differences in absolute values are indicated in boldface ($p<0.05$, Mann-Whitney test).



C

	Genotype				Phenotype		
	Floxed allele of α1	$\alpha1^{H101R}$	Emx1-Cre^{tg}	Combined genotype	Forebrain principal cells	Rest of brain	Short notation
1	$H^{flox}H^{flox}$		-	$H^{flox}H^{flox}$/ Emx1-Cre^{tg-}	H/H	H/H	Pseudo wt
2	$H^{flox}H^{flox}$		+	$H^{flox}H^{flox}$/ Emx1-Cre^{tg+}	-/-	H/H	Forebrain-specific $\alpha1^{-/-}$
3	H^{flox}	H	-	H^{flox}H/ Emx1-Cre^{tg-}	H/H	H/H	Pseudo wt
4	H^{flox}	H	+	H^{flox}H/ Emx1-Cre^{tg+}	-/H	H/H	Forebrain-specific $\alpha1^{-/H}$
5	H^{flox}	R	-	H^{flox}R/ Emx1-Cre^{tg-}	H/R	H/R	$\alpha1^{H/R}$
6	H^{flox}	R	+	H^{flox}R/ Emx1-Cre^{tg+}	-/R	H/R	Forebrain-specific $\alpha1^{-/R}$

Figure 1

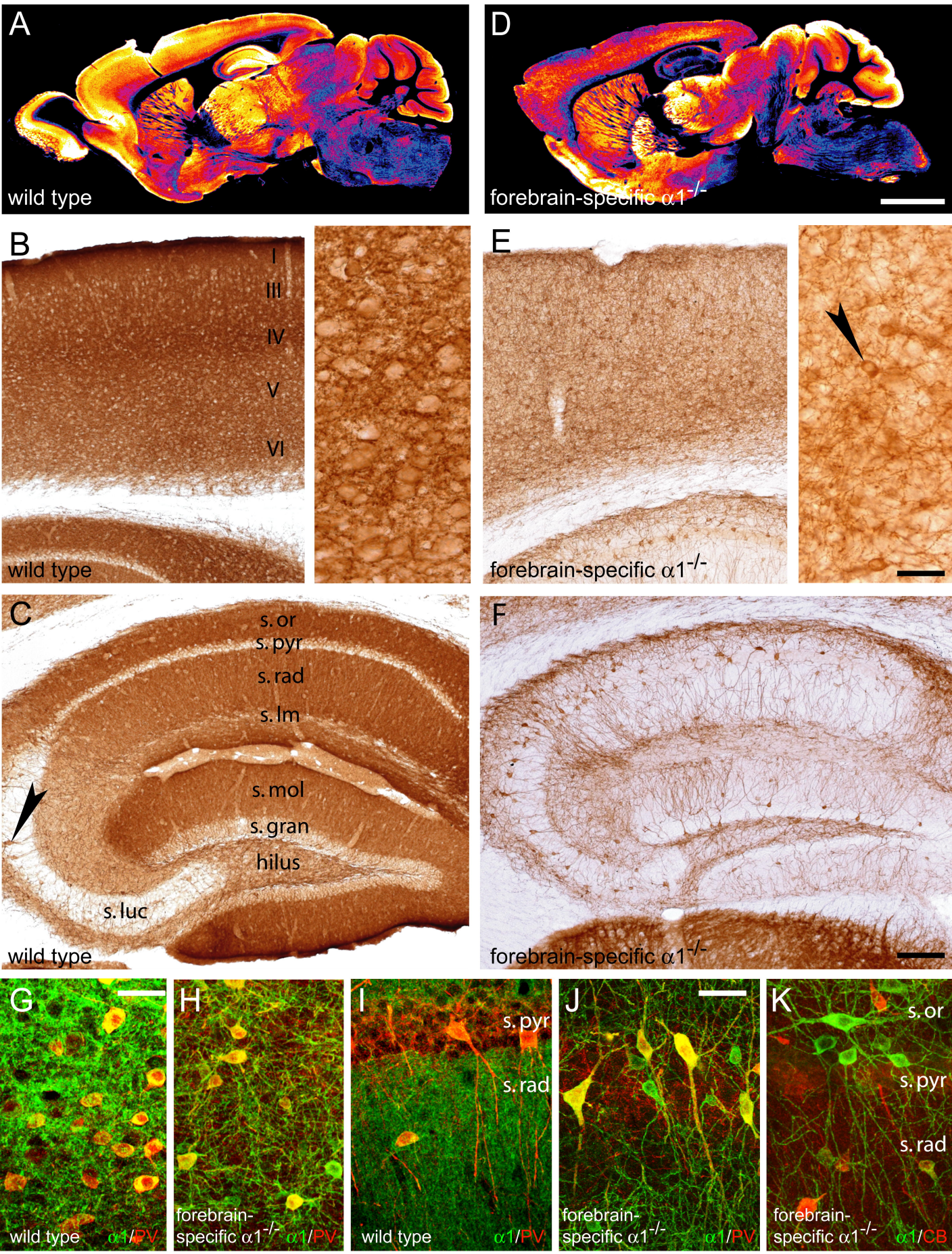

A
wild type
D
forebrain-specific α1-/-
B
I
III
IV
V
VI
wild type
E
forebrain-specific α1-/-
C
s. or
s. pyr
s. rad
s. lm
s. mol
s. gran
hilus
s. luc
wild type
F
forebrain-specific α1-/-
G
wild type α1/PV
H
forebrain-specific α1-/- α1/PV
I
s. pyr
s. rad
wild type α1/PV
J
forebrain-specific α1-/- α1/PV
K
s. or
s. pyr
s. rad
forebrain-specific α1-/- α1/CB

Figure 2

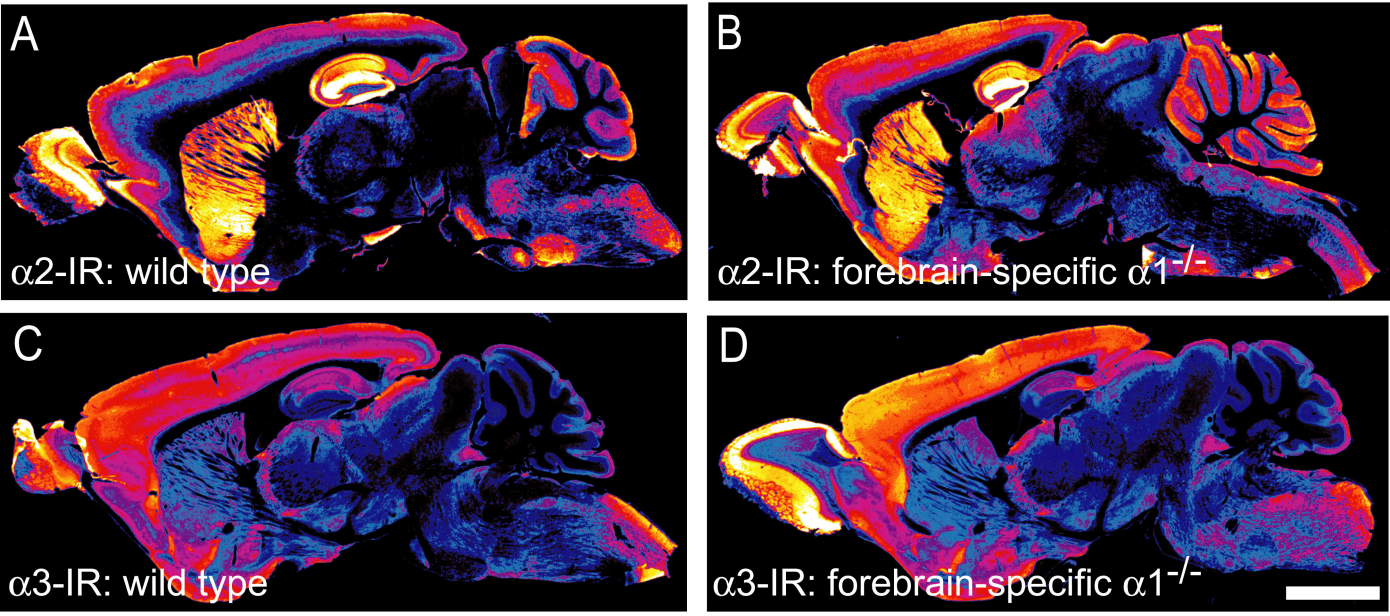

A
α2-IR: wild type
B
α2-IR: forebrain-specific α1-/-
C
α3-IR: wild type
D
α3-IR: forebrain-specific α1-/-

Figure 3

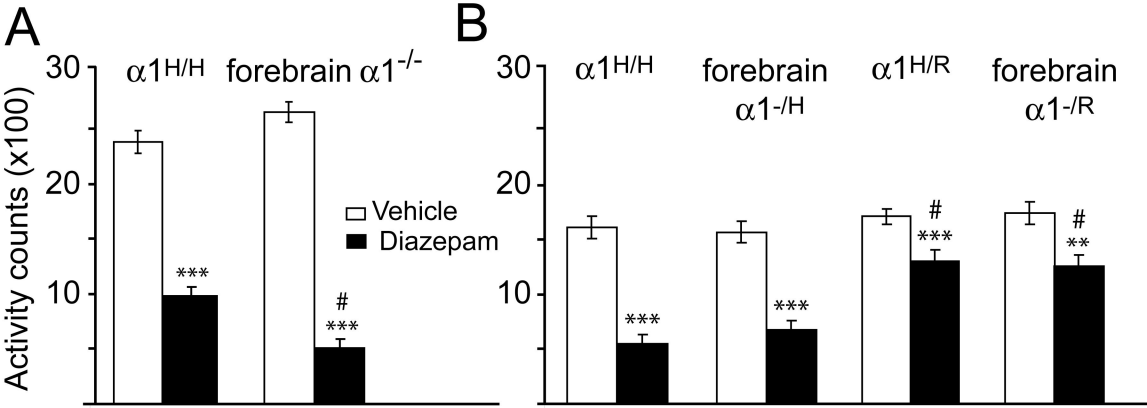

A
B
30
20
10
Activity counts (x100)
α1H/H
forebrain α1-/-
α1H/H
forebrain α1-/H
α1H/R
forebrain α1-/R
Vehicle
Diazepam
***
#
**

Figure 4

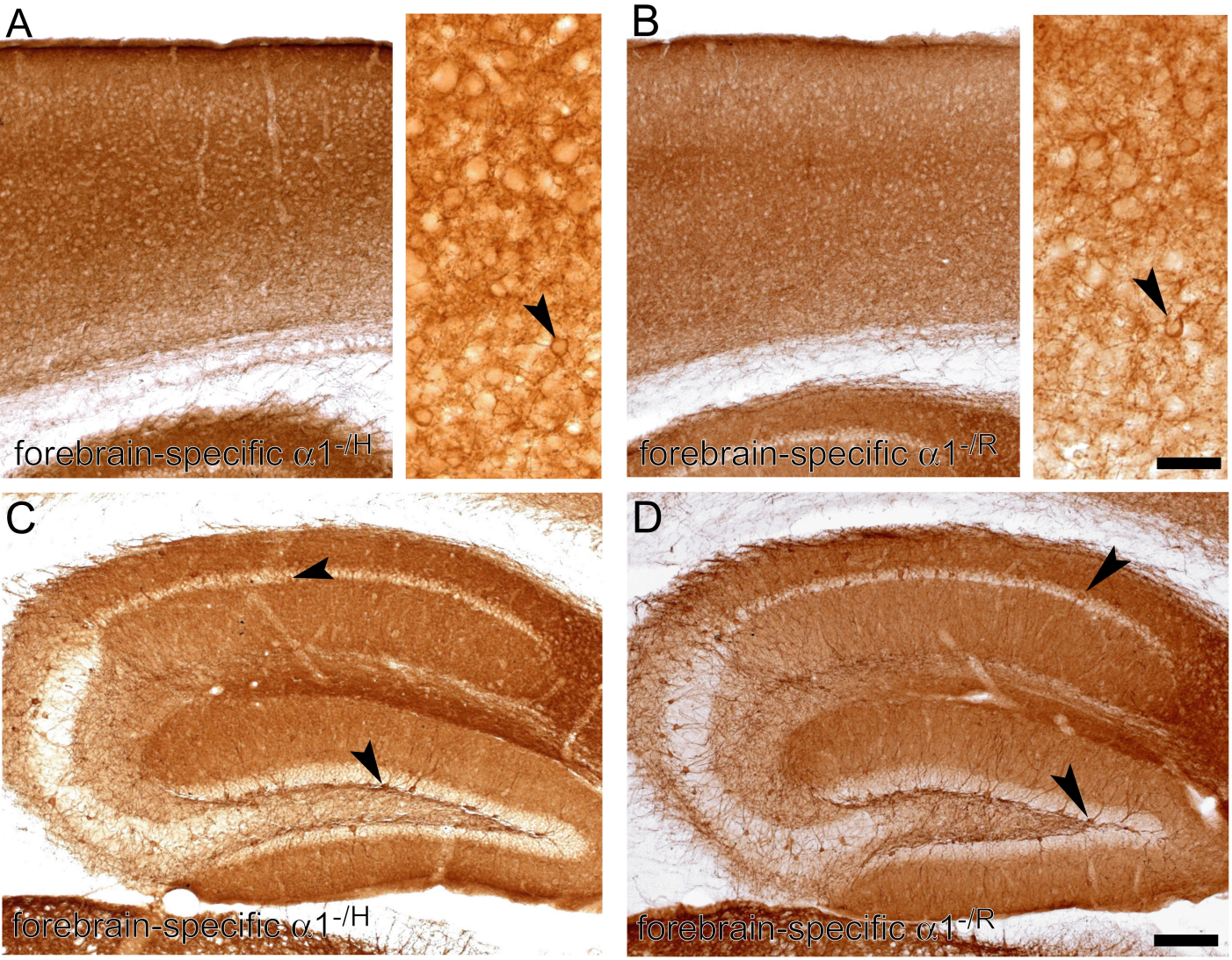

A
forebrain-specific α1-/H
B
forebrain-specific α1-/R
C
forebrain-specific α1-/H
D
forebrain-specific α1-/R

Figure 5