Cortical Glutamatergic Neurons Mediate the Motor Sedative Action of Diazepam

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ABSTRACT

The neuronal circuits mediating the sedative action of diazepam are unknown. Although the motor-depressant action of diazepam is suppressed in $\alpha1$(H101R) homozygous knockin mice expressing diazepam-insensitive $\alpha1$-GABA$_A$ receptors, global $\alpha1$-knockout mice show greater motor sedation with diazepam. To clarify this paradox, attributed to compensatory up-regulation of the $\alpha2$ and $\alpha3$ subunits, and to further identify the neuronal circuits supporting diazepam-induced sedation, we generated Emx1-cre-recombinase-mediated conditional mutant mice, selectively lacking the $\alpha1$ subunit (forebrain-specific $\alpha1^{-/-}$) or expressing either a single wild-type (H) or a single point-mutated (R) $\alpha1$ 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 $\alpha1$(H101R) mice. Forebrain-specific $\alpha1^{-/-}$ mice showed enhanced diazepam-induced motor depression and increased expression of the $\alpha2$ and $\alpha3$ subunits in the neocortex and hippocampus, in comparison with their pseudo-wild-type 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 $\alpha1$ subunit was reduced in forebrain, notably in $\alpha1^{-/R}$ mice, and the $\alpha3$ subunit was up-regulated in neocortex, indicating that proper $\alpha1$ subunit expression requires both alleles. In conclusion, conditional manipulation of GABA$_A$ receptor $\alpha1$ 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.

GABA$_A$ receptors mediate fast GABAergic inhibition in the adult mammalian central nervous system. GABA$_A$ receptors are pentameric ligand-gated ion channels, with 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 Möhler, 2006), including benzodiazepines (Rudolph and Möhler, 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 and a wide range of sensorimotor and cognitive functions. It is noteworthy that diazepam, through $\alpha1$-GABA$_A$ receptor activation, can promote sedation, as measured by its motor-depressant action (Rudolph et al., 1999; McKernan et al., 2000), and antero-

grade amnesia, and it displays 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 $\alpha1$ subunit gene. The $\alpha1$(H101R)-GABA$_A$ receptor is insensitive to allosteric modulation by benzodiazepine-site ligands, including zolpidem, both in vitro and in vivo, whereas regulation by the physiological neurotransmitter GABA is preserved (Benson et al., 1998; Rudolph et al., 1999; Crestani et al., 2000; Marowsky et al., 2004). The corresponding $\alpha1$(H101R) mice fail to show the motor-depressant and anterograde amnesic effect of diazepam, and they 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); rather, they depend on $\alpha2$-GABA$_A$ receptors (Kopp et al., 2004). The role of $\alpha1$-GABA$_A$ receptors in mediating the sedative action of benzodiazepine-site ligands was further supported by pharmacological studies using L838-417 (Scott-Stevens et al., 2005). This substance, which acts as a partial

ABBREVIATIONS: bp, base pair(s); OD, optical density; IR, immunoreactivity; PV, parvalbumin; CB, calbindin; cre, cre recombinase.

agonist at $\alpha 2$-, $\alpha 3$- and $\alpha 5$-GABA$_A$ receptors and as an antagonist at $\alpha 1$-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 $\alpha 1$ subunit knockout mice treated with diazepam display enhanced motor sedation compared with wild-type littermates (Kralic et al., 2002a,b), indicating that $\alpha 1$-GABA$_A$ receptors can be substituted. These mutants also show increased expression of the GABA$_A$ receptor $\alpha 2$ and $\alpha 3$ subunits notably in cerebral cortex (Kralic et al., 2002a, 2006). The compensatory up-regulation of other α subunits might underlie the pharmacological phenotype of $\alpha 1$ subunit knockout 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 $\alpha 1$-GABA$_A$ receptors expressed in the forebrain. To achieve this goal, we investigated genetically engineered mice with either a constitutive deficit in $\alpha 1$-GABA$_A$ receptors, or carrying a single diazepam-insensitive $\alpha 1$(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 wild-type $\alpha 1$ subunit allele flanked by loxP sites (floxed) with a cre transgene expressed from an Emx1 promoter or by combining a H101R point-mutated $\alpha 1$ subunit allele with a floxed wild-type $\alpha 1$ subunit allele and the Emx1-cre transgene. These forebrain-specific mutants were analyzed immunohistochemically for possible changes in $\alpha 1$, $\alpha 2$, and $\alpha 3$ subunit expression patterns, and they were tested behaviorally for diazepam-induced changes in spontaneous locomotor activity.

Materials and Methods

Animals. Forebrain-specific deletion of the $\alpha 1$ 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 Zürich onto the C57BL/6JOlaHsd background] were crossed initially with mice homozygous for the floxed $\alpha 1$ subunit allele [B6.129(FVB)-Gabra1^{tm1Geh}/J, at least six backcrosses onto C57BL/6J (The Jackson Laboratory, Bar Harbor, ME), first described in Vicini et al. (2001)] (Fig. 1, A and C). Offspring, heterozygous for the floxed $\alpha 1$ subunit allele and carrying the Emx1-cre transgene, were crossed again with mice homozygous for the floxed $\alpha 1$ subunit allele to obtain the desired genotype (Fig. 1, A and 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 lesser extent in septum, amygdala, allocortex, and olfactory bulb (Iwasato et al., 2004). Homozygous deletion of the $\alpha 1$ subunit floxed alleles was expected to result in a region-specific disappearance of the $\alpha 1$ subunit during late prenatal and early postnatal development.

To obtain mice with a forebrain-specific $\alpha 1$(H101R) point mutation (forebrain-specific heterozygous knockin, $\alpha 1^{-/R}$), we first crossed Emx1-cre transgenic mice with homozygous $\alpha 1$(H101R) mice. All offspring had one wild-type (H) and one point-mutated (R) $\alpha 1$ allele; those carrying the Emx1-cre transgene were then crossed with mice homozygous for the wild-type floxed $\alpha 1$ subunit allele (Fig. 1, B and C) to obtain four genotypes of experimental animals, including pseudo-wild-type ($\alpha 1^{H/H}$), forebrain-specific heterozygous knockout ($\alpha 1^{-/H}$), global heterozygous knockin mice ($\alpha 1^{H/R}$), and forebrain-specific heterozygous knockin mice ($\alpha 1^{-/R}$) (Fig. 1, B and C). The forebrain-specific $\alpha 1^{-/R}$ mice carried a single diazepam-insensitive $\alpha 1$(H101R) allele in forebrain glutamatergic cells and both a wild-type floxed $\alpha 1$ allele and a point-mutated diazepam-insensitive $\alpha 1$(H101R) allele in all other cells. In these mice, diazepam was therefore expected to have no effect on $\alpha 1$-GABA$_A$ receptors in forebrain glutamatergic cells, but it was expected to activate these receptors in the rest of the brain. The other heterozygous mice were $\alpha 1^{-/H}$ mice, which had a single wild-type floxed allele in forebrain glutamatergic neurons and two floxed alleles in the rest of the brain (Fig. 1C); therefore, they were 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 of the Emx1-driven cre recombinase, the floxed wild-type $\alpha 1$ subunit allele, and the point-mutated $\alpha 1$(H101R) subunit allele (Fig. 1C). In all cases, H denotes an $\alpha 1$ subunit allele with a histidine in position 101, and R denotes a point-mutated $\alpha 1$(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). Therefore, to identify mice with germline recombination, we genotyped liver biopsies from all mice used in behavioral and immunohistochemical experiments from breeding pairs carrying both the Emx1-cre transgene and the wild-type $\alpha 1$ 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 polymerase chain reaction 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′, providing a product size of 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 of 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 $\alpha 1$(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 of approximately 300 bp for the wild-type (H) allele and approximately 350 bp for the (R) allele; the size difference was due to the presence of a loxP site in the R allele]. The polymerase chain reaction used for the detection of the wild-type $\alpha 1$ floxed allele is described at http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=584.

Immunohistochemistry. Adult mice were deeply anesthetized with pentobarbital (50 mg/kg i.p.), and then they were perfused through the aorta with 4% paraformaldehyde in 0.15 M phosphate buffer, pH 7.4. Brains were postfixed for 3 h, cryoprotected in sucrose, frozen, and then cut parasagittally at 40 μm with a sliding microtome. Sections were collected in phosphate-buffered saline, and they were stored in an antifreeze solution. Immunoperoxidase staining was performed to visualize and quantify the distribution of GABA$_A$ receptor $\alpha 1$, $\alpha 2$, or $\alpha 3$ 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 Laboratories Inc., 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, Burlingame, CA), and after another wash, they were finally reacted with diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) in Tris buffer, pH 7.7, containing 0.015% hydrogen peroxide. The color reaction was stopped after 5 to 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 $\alpha 1$ subunit along with markers of cortical interneurons (parvalbumin, calbindin, and calretinin) was performed in forebrain-specific $\alpha 1^{-/-}$ mice and their pseudo-wild-type littermates (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-$\alpha 1$ subunit as described above. After washing,

sections were incubated in a mixture of secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes/Invitrogen, Carlsbad, CA) or Cy3 (Jackson ImmunoResearch Laboratories Inc.). After mounting, sections were air-dried and coverslipped with aqueous mounting medium (Dako Denmark A/S, Glostrup, Denmark). In all experiments, sections from wild-type 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. Catharines, ON, Canada) on sections from four animals per genotype processed for immunoperoxidase staining. Images were digitized with a high-resolution black-and-white camera. Optical density values were calibrated with gray-scale standards, arbitrarily ranging from 0 (white) to 100 (black). Background was measured in the cerebellar granule cell layer for the $\alpha 3$ subunit and in the inferior colliculus for the $\alpha 2$ subunit and subtracted from the optical density values measured in the regions of interest. Results, expressed as mean $\pm$
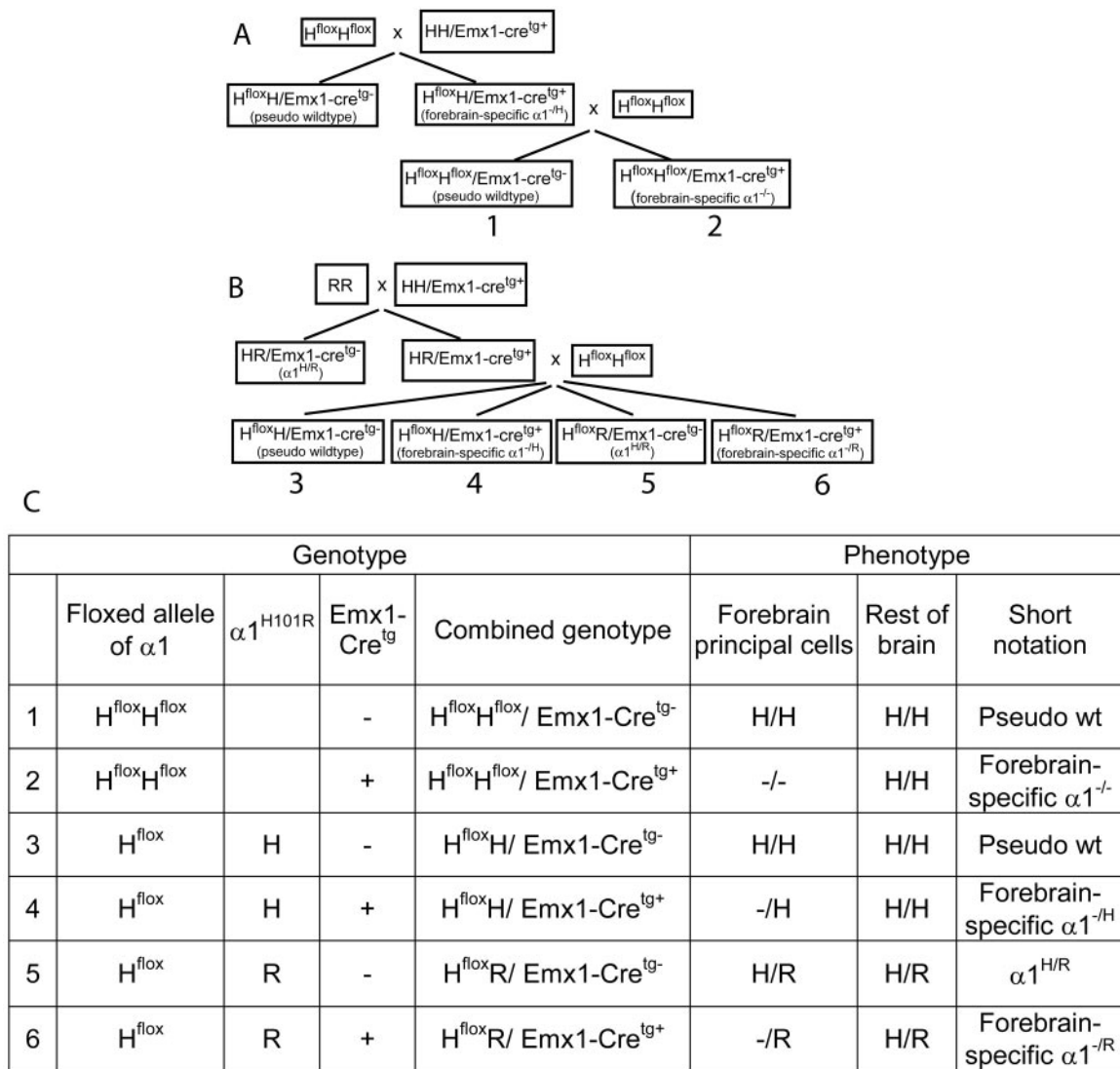
	Genotype				Phenotype		
	Floxed allele of $\alpha 1$	$\alpha 1^{H101R}$	Emx1-Cre^{tg}	Combined genotype	Forebrain principal cells	Rest of brain	Short notation
1	$H^{flox}H^{flox}$		-	$H^{flox}H^{flox}$/ Emx1-Cre^{tg-}	H/H	H/H	Pseudo wt
2	$H^{flox}H^{flox}$		+	$H^{flox}H^{flox}$/ Emx1-Cre^{tg+}	-/-	H/H	Forebrain-specific $\alpha 1^{-/-}$
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 $\alpha 1^{-/H}$
5	H^{flox}	R	-	$H^{flox}R$/ Emx1-Cre^{tg-}	H/R	H/R	$\alpha 1^{H/R}$
6	H^{flox}	R	+	$H^{flox}R$/ Emx1-Cre^{tg+}	-/R	H/R	Forebrain-specific $\alpha 1^{-/R}$

Fig. 1. Breeding schemes and description of the genotypes of mice used in the present study. A, breeding scheme to obtain forebrain-specific $\alpha 1^{-/-}$ ($H^{flox}H^{flox}$/Emx1-cre^{tg+}) mice and their pseudo-wild-type littermates. B, breeding scheme to obtain forebrain-specific heterozygous $\alpha 1^{-/R}$ ($H^{flox}R$/Emx1-cre^{tg+}), $\alpha 1^{-/H}$ ($H^{flox}H$/Emx1-cre^{tg+}), and corresponding control mice (global heterozygous and pseudo-wild type). H^{flox}, $\alpha 1$ floxed allele; H, $\alpha 1$ wild-type allele with a codon for histidine at amino acid position 101; R, $\alpha 1$(H101R) point-mutated allele with a codon for arginine at amino acid position 101; Emx1-cre^{tg}, absence (−) or presence (+) of cre transgene. C, left, genotypes of all mouse lines right, 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.
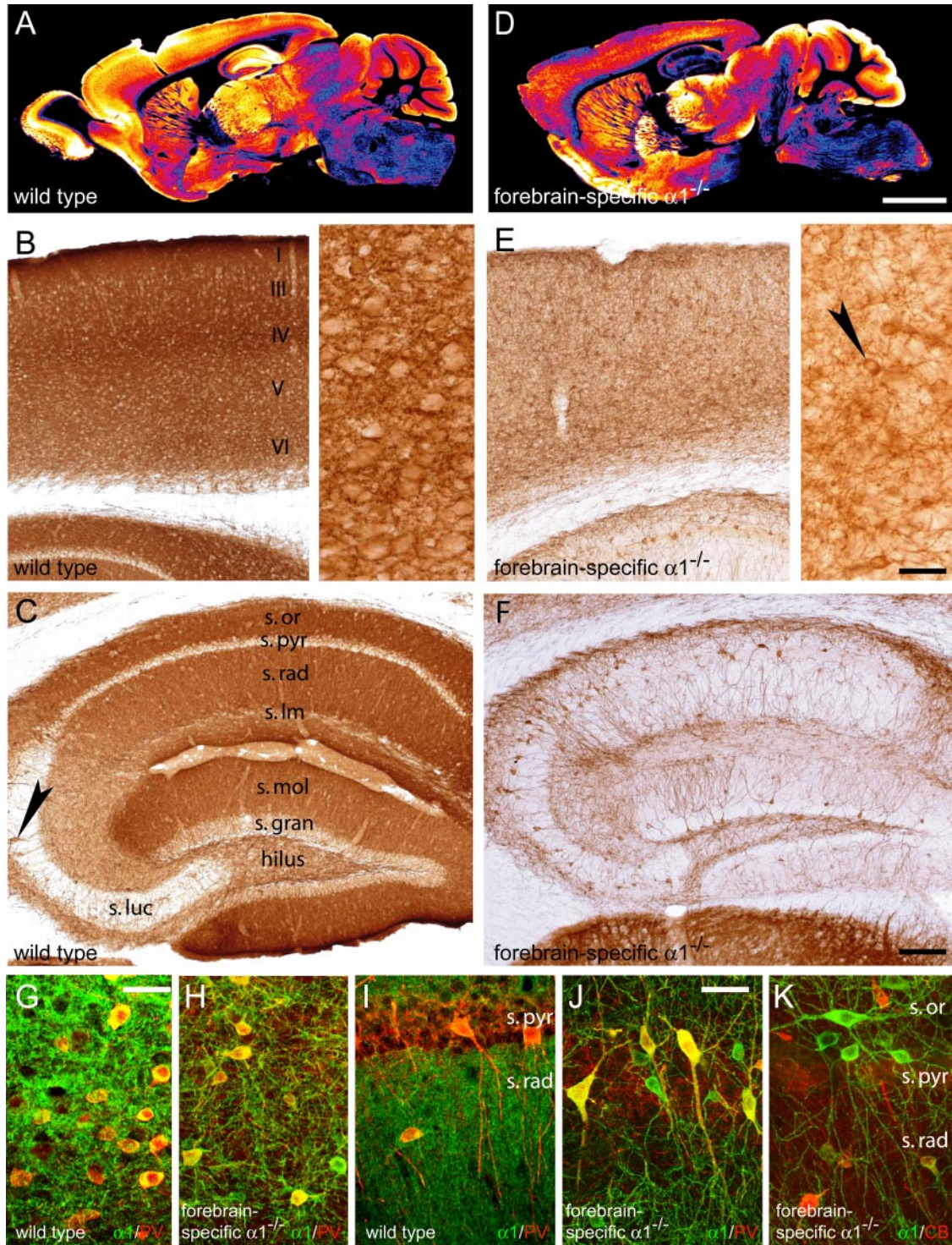
Fig. 2. Region- and cell type-specific loss of $\alpha 1$ subunit IR in forebrain-specific $\alpha 1^{-/-}$ mice. A and D, pseudo-colored photomicrographs of parasagittal brain sections processed for $\alpha 1$ subunit immunoperoxidase staining from pseudo-wild-type mice and forebrain-specific $\alpha 1^{-/-}$ mutants. Yellow-white indicates a strong labeling and blue, background level. Note the selective reduction of IR in neocortex and hippocampus. B and E and C and F, color photomicrographs of parietal cortex and hippocampal formation illustrating the reduction of $\alpha 1$ subunit IR in the neuropil of forebrain-specific $\alpha 1^{-/-}$ mice, reflecting loss of expression in pyramidal cells and retention of the $\alpha 1$ subunit in a large subset of interneurons (arrowheads), which are not visible in wild type because of the diffuse staining of pyramidal cell dendrites in the neuropil. G to K, double immunofluorescence staining for the $\alpha 1$ subunit (green) and parvalbumin (PV; red; G–J) or calbindin (CB; red; K) in parietal cortex layer III (G and H) and CA1 (I–K) from pseudo-wild-type mice (G, I) and forebrain-specific $\alpha 1^{-/-}$ mutants (H, J, and K). In wild type, the $\alpha 1$ subunit staining is prominent in the neuropil (green) and in PV-positive interneurons (yellow); in mutants, the $\alpha 1$ subunit staining is present in the soma and dendrites of interneurons, most of which are double labeled for PV (yellow; H, J); only few $\alpha 1$ subunit-positive interneurons also contain CB immunoreactivity (K); the single-labeled cells in H, J, and K (green) represent other subtypes of interneurons. 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, 2 mm (A and D), 200 μm (B, C, E, and F), 50 μm (insets in B and E), 30 μm (G and H), and 20 μm (I–K).

S.D., were analyzed using nonparametric Kruskal-Wallis and Mann-Whitney U 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 AM) for at least 2 weeks before testing (between 9 AM and 12 PM). Motor activity was measured during the active phase in automated individual circular runways equipped with photocells (Imetronic, Pessac, France) for an hour, starting 30 min after oral administration of either 10 mg/kg 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 wild-type C57BL/6J mice, but not in $\alpha1$(H101R) mutants. Because of the absence of a difference, data from male and female mice were pooled and analyzed using two-way (genotype × treatment) repeated measures analysis of variance followed by post hoc Scheffé's test. Results are expressed as mean ± S.E.

Results

Expression of GABA$_A$ Receptor Subunits in Mice Lacking the $\alpha1$ Subunit in Forebrain Glutamatergic Neurons. The immunohistochemical analysis of the regional distribution and relative immunoreactivity (IR) levels for the $\alpha1$, $\alpha2$, and $\alpha3$ subunit revealed differences between forebrain-specific $\alpha1^{-/-}$ mice and their corresponding pseudo-wild-type littermates (H^{flox}H^{flox}/Emx1-cre^{tg-}). In wild-type brain sections, $\alpha1$ subunit IR was prominent and nearly evenly distributed across all cortical areas (Fig. 2A). The $\alpha1$ subunit staining was most pronounced in layers I, III, and IV, as shown in the parietal cortex (Fig. 2B). $\alpha1$ 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 stra-
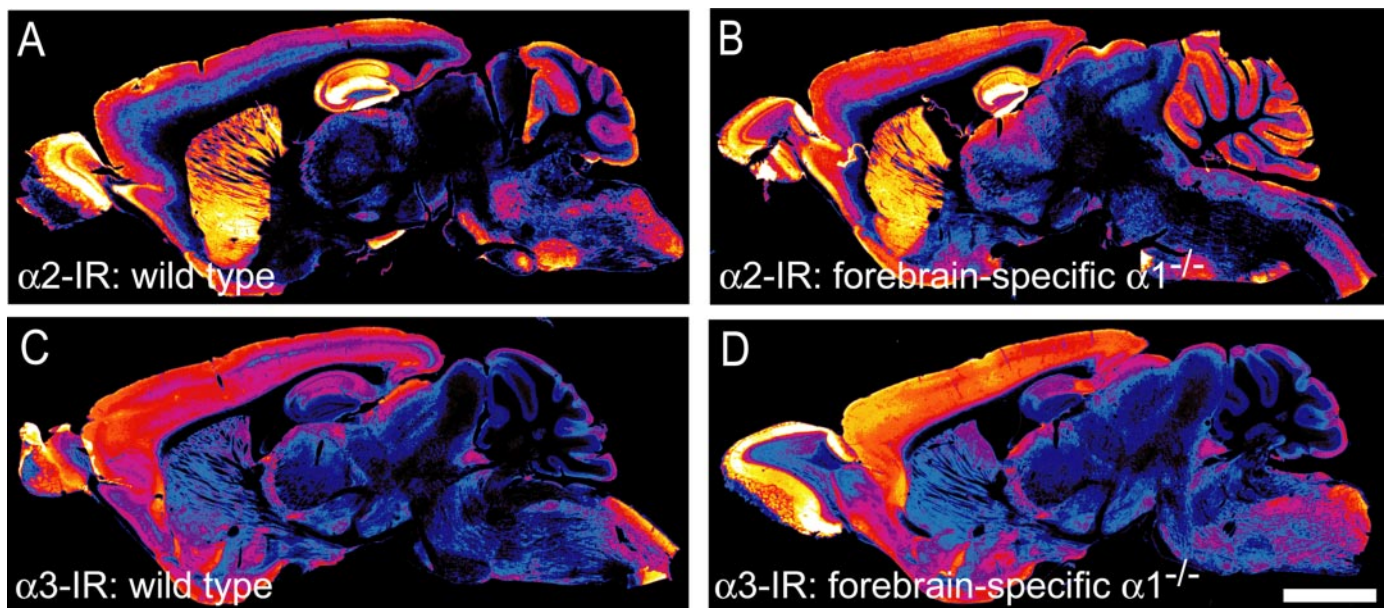

Fig. 3. Region-specific increase of $\alpha2$ and $\alpha3$ subunit IR in forebrain-specific $\alpha1^{-/-}$ mice. Pseudo-colored photomicrographs of parasagittal sections processed for immunoperoxidase staining. A, $\alpha2$ subunit IR in pseudo-wild-type mice. B, increased $\alpha2$ subunit IR in the neocortex in forebrain-specific $\alpha1^{-/-}$ mice. C. $\alpha3$ subunit IR in pseudo-wild-type mice. D, increased $\alpha3$ subunit IR in the neocortex in forebrain-specific $\alpha1^{-/-}$ mice. Scale bar, 2 mm.

TABLE 1

Quantification of GABA$_A$ receptor $\alpha2$ and $\alpha3$ subunit immunoreactivity in forebrain-specific $\alpha1^{-/-}$ mice compared with wild-type littermates (H^{flox}H^{flox}/Emx1-cre^{tg-})

Optical density (OD) values were measured in sections processed for immunoperoxidase staining (adult mice; n = 4/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 $\alpha2$ subunit and cerebellum for the $\alpha3$ subunit) and subtracted from the measured values. Values in mutants are expressed as percentage of wild-type control. Statistically significant differences in absolute values are indicated in bold ($p < 0.05$; Mann-Whitney U test).

Region	Wild-Type OD $\alpha2$	Forebrain-Specific $\alpha1^{-/-}$ as a Proportion of WT OD $\alpha2$	Wild-Type OD $\alpha3$	Forebrain-Specific $\alpha1^{-/-}$ as a Proportion of WT OD $\alpha3$
		%		%
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 and 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 and 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

tum lucidum where interneurons and their dendrites were visible (Fig. 2C, arrowhead). In brain sections from forebrain-specific $\alpha1^{-/-}$ mice, a marked decrease in $\alpha1$ subunit IR was apparent, and it was restricted to the neocortex and hippocampus (Fig. 2D). In these mice, no change in $\alpha1$ 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 $\alpha1$ subunit staining was absent from all cortical glutamatergic cells, whereas it was 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 $\alpha1$ subunit IR against a white background (Fig. 2F). Thus, as expected, forebrain-specific $\alpha1^{-/-}$ mice displayed a deficit in $\alpha1$ subunit restricted to glutamatergic neurons. The interneuronal nature of $\alpha1$ subunit-positive cells in the neocortex and hippocampus was verified by double immunofluorescence staining with parvalbumin (Fig. 2, G–J), calbindin (Fig. 2K), and calretinin (data not shown), three calcium-binding proteins that label largely nonoverlapping subpopulations of GABAergic interneurons (Freund and Buzsaki, 1996).

Forebrain-specific $\alpha1^{-/-}$ mice showed a regional expression pattern for the $\alpha2$ and $\alpha3$ subunit comparable with that of the pseudo-wild-type controls, but the IR of both subunits was stronger. In control mice, $\alpha2$ subunit IR was confined to the outer layers of the neocortex, whereas it was 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 $\alpha2$ subunit IR was apparent in the neocortex of mutants, but not in the hippocampal formation (Fig. 3B; Table 1). The $\alpha3$ subunit IR in the neocortex of pseudo-wild-type 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-wild-type and forebrain-specific $\alpha1^{-/-}$ mice (Fig. 3, C and D), and it was almost absent in the dentate gyrus. Mutant mice showed enhanced levels of $\alpha3$ subunit IR in neocortex, comparable with the increase in $\alpha2$ subunit IR (Table 1). It is noteworthy that the $\alpha3$ subunit, almost absent in layer IV of parietal cortex in wild-type 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 $\alpha1$ subunit in cortical glutamatergic neurons was accompanied by an increased expression of the $\alpha2$ and $\alpha3$ 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 compared with the pseudo-wild-type 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 $\alpha1$(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 $\alpha1$-GABA$_A$ receptors, would be selectively suppressed in forebrain neurons expressing Emx1. The

breeding scheme adopted (Fig. 1) resulted in four genotypes, including pseudo-wild-type mice (H^{flox}H/Emx1-cre^{tg-}), global heterozygous knock-in $\alpha1^{H/R}$ mice, forebrain-specific $\alpha1^{-/H}$ mice (carrying a single floxed $\alpha1$ subunit allele in forebrain), and forebrain-specific $\alpha1^{-/R}$ [carrying a single point-mutated $\alpha1$(H101R) subunit allele in forebrain]. The pseudo-wild-type and $\alpha1^{H/R}$ mice showed an expression pattern for the GABA$_A$ receptor $\alpha1$, $\alpha2$, and $\alpha3$ subunits similar to that seen in pseudo-wild-type H^{flox}H^{flox}/Emx1-cre^{tg-} (Figs. 2A and 3, A and C). Unexpectedly, in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice, $\alpha1$ 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 (Fig. 5, A and B, arrowhead) and the hippocampus (Fig. 5, C and D, arrowhead) in sections from both mutants. The $\alpha1$ 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 wild-type or point-mutated, $\alpha1$ subunit allele in cortical glutamatergic neurons was insufficient to provide normal expression of the $\alpha1$ subunit.

No consistent alteration in $\alpha2$ subunit expression pattern and IR levels was detected in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice (Table 2). A difference in $\alpha3$ 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 and VI of frontal cortex (Table 2). In these mice, weakly stained areas (CA3, dentate gyrus, and striatum) exhibited increased staining compared with control (Table 2). However, because these changes were not seen in other mutants and they included regions where Emx1-cre is not expressed, their significance is uncertain. Overall, we conclude that expression of a single $\alpha1$(H101R) allele in forebrain glutamatergic neurons is associated with a selective up-regulation of the $\alpha3$ subunit in the neocortex.
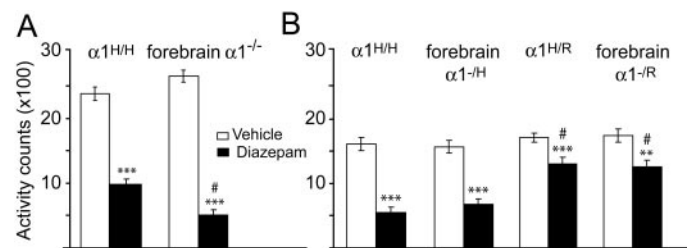

Fig. 4. Motor-sedative effect of diazepam. Motor activity was measured for 1 h, starting 30 min after oral administration of either vehicle or 10 mg/kg diazepam. A, forebrain-specific $\alpha1^{-/-}$ mice. Note the greater reduction in mean activity counts in diazepam-treated forebrain-specific $\alpha1^{-/-}$ mice compared with pseudo-wild-type $\alpha1^{H/H}$ mice ($n = 19$ mice/group; #, $p < 0.05$ compared with wild-type mice; and ***, $p < 0.001$ compared with 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 with the corresponding pseudo-wild-type $\alpha1^{H/H}$ animals in 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/group; #, $p < 0.05$ compared with $\alpha1^{H/H}$ and $\alpha1^{-/H}$ mice; and **, $p < 0.01$ and ***, $p < 0.001$ compared with vehicle; Scheffé's test).

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 it did so to a lesser degree in $\alpha1^{H/R}$ and forebrain-specific $\alpha1^{-/R}$ mice [$p <$ 0.01 compared with $\alpha1^{H/H}$ 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.

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 $\alpha1$ 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,b). 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 up-regulation of the $\alpha3$ subunit in the neocortex of both forebrain-specific $\alpha1^{-/-}$ and $\alpha1^{-/R}$ mutants. An overexpression of the $\alpha2$ 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 $\alpha1$ subunit substitute pharmacologically for $\alpha1$-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 $\alpha1$ subunit knockout mice despite retaining a prominent $\alpha1$ 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 because it
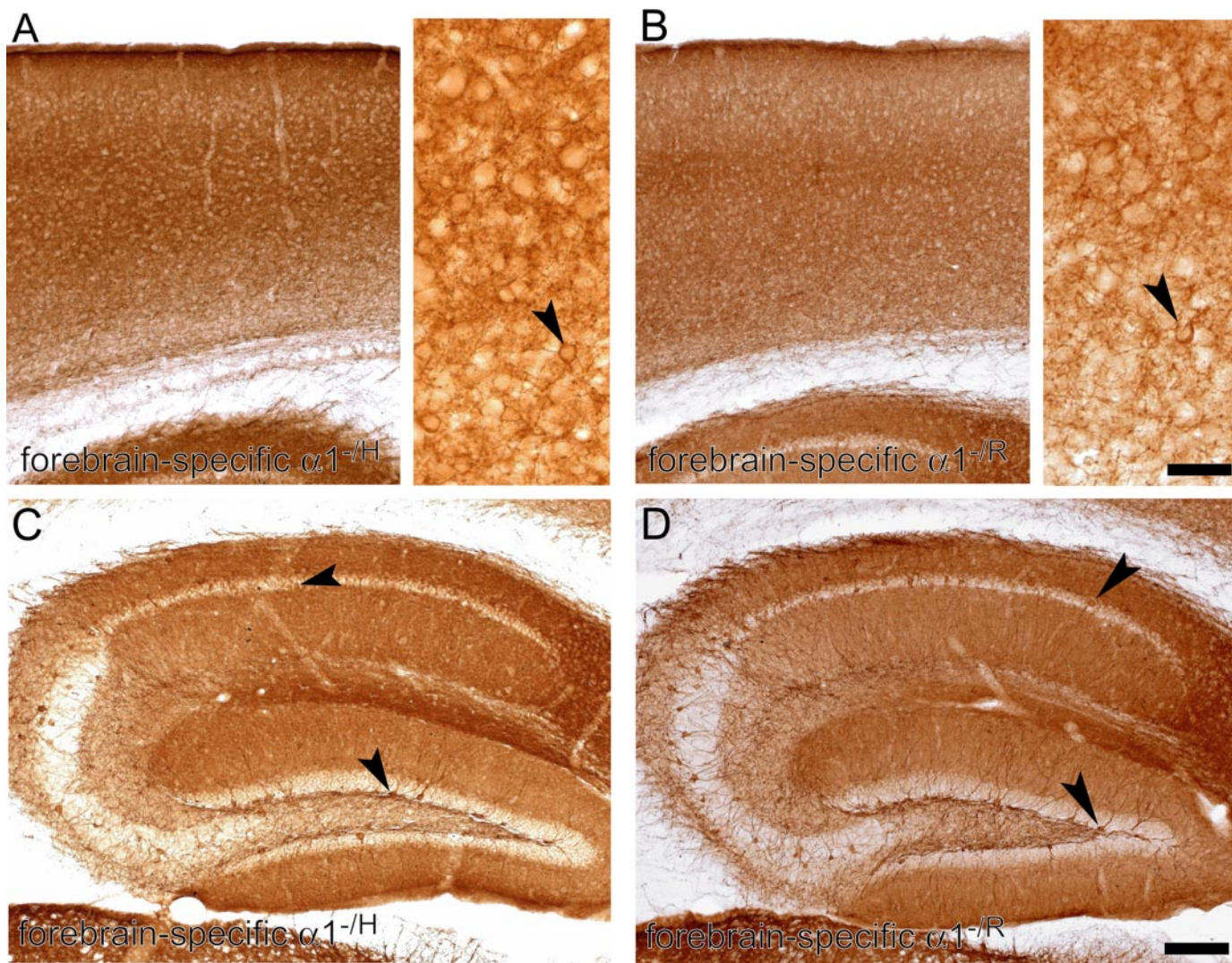

Fig. 5. Reduction of $\alpha1$ subunit IR in forebrain-specific heterozygous $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice. Photomicrographs of parasagittal sections through the parietal cortex (A and B) and hippocampus (C and D) processed for immunoperoxidase staining. A and C, slight reduction of $\alpha1$ subunit IR in the neuropil of $\alpha1^{-/H}$ mice compared with wild-type mice in Fig. 2, B and C. Individual interneurons and their dendrites become visible (arrowheads). B and D, more pronounced reduction of $\alpha1$ 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, 200 μm (A and B) and 50 μm (insets in A and B).

has a robust sedative action, reducing motor activity by approximately two thirds, but still allows us to detect a further decrease in motor activity caused by individual genotypes.

Global deletion of the $\alpha1$ subunit gene results in a marked compensatory overexpression of the GABA$_A$ receptor $\alpha2$, $\alpha3$, and $\alpha4$ subunits, selectively in those brain regions where the $\alpha1$ subunit is absent (Kralic et al., 2002a, 2006; Schneider Gasser et al., 2007). Up-regulation probably 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 $\alpha1$ subunit, as evidenced, for example, 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 up-regulation. Nevertheless, deletion of the $\alpha1$ subunit restricted to forebrain principal cells leads to overexpression of the $\alpha2$ and $\alpha3$ 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 probably unaffected in other brain areas of conditional mutant mice.

The decreased $\alpha1$ subunit IR in the forebrain of $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice, which both carry a single $\alpha1$ subunit allele in forebrain glutamatergic neurons, is reminiscent of the decreased expression of the $\gamma2$ subunit occurring mostly in neocortex and hippocampus in mice heterozygous for the $\gamma2$ 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 up-regulation 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 $\alpha1$-GABA$_A$ receptors in $\alpha1^{-/R}$ mice

TABLE 2
Quantification of the GABA$_A$ receptor $\alpha2$ and $\alpha3$ subunit immunoreactivity in forebrain-specific $\alpha1^{-/H}$ and $\alpha1^{-/R}$ mice compared with wild-type littermates (H^{flox}H/ Emx1-cre^{tg-} and H^{flox}R/Emx1-cre^{tg-})
Densitometry was performed in sections processed for immunoperoxidase staining (see Table 1). Values are expressed as percentage of wild-type control. Statistically significant differences in absolute values are indicated in bold ($p < 0.05$; Mann-Whitney U test).

Region	Forebrain-Specific, as a Proportion of OD $\alpha2$		Forebrain-Specific, as a Proportion of OD $\alpha3$	
	$\alpha1^{-/H}$	$\alpha1^{-/R}$	$\alpha1^{-/H}$	$\alpha1^{-/R}$
	%		%	
Parietal cortex, layer I–III	101	118	123	127
Parietal cortex, layer IV	129	129	115	**142**
Parietal cortex, layers V and VI	120	109	105	**130**
Frontal cortex, layers I–III	72	112	124	118
Frontal cortex, layers V and 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

seems to be sufficient to induce compensatory changes, probably because α subunits are required for receptor assembly (Kralic et al., 2006; Rudolph and Möhler, 2006; Studer et al., 2006).

In line with the loss of diazepam binding to GABA$_A$ receptors containing the $\alpha1$(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-wild-type control ($\alpha1^{H/R}$ and $\alpha1^{H/H}$). In $\alpha1^{-/H}$ mice, one might argue that the remaining pool of diazepam-sensitive $\alpha1$-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 up-regulation of the $\alpha3$ subunit, which might restore a complement of diazepam-sensitive $\alpha3$-GABA$_A$ receptors selectively in neocortical regions.

The consequences of the up-regulation of $\alpha2$ and $\alpha3$ 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, $\alpha1$-GABA$_A$ receptors predominate on distal dendrites, whereas $\alpha2$-GABA$_A$ receptors mediate most of the perisomatic GABAergic inputs (Prenosil et al., 2006). In addition, the $\alpha1$- and $\alpha2$ subunits are located in the synapses of separate subpopulations of basket cells (distinguished by expression of parvalbumin and cholecystokinin, respectively) (Nyíri et al., 2001). These differences probably underlie the contribution of these GABA$_A$ receptor subtypes to distinct neuronal circuits. Although the up-regulation of the $\alpha3$ subunit suggests that this subunit could replace the $\alpha1$ 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 $\alpha2$- and $\alpha3$-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, up-regulation of the $\alpha2$ and $\alpha3$ subunit in forebrain-specific $\alpha1^{-/-}$ mice might functionally compensate for the loss of the $\alpha1$ 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 $\alpha3$-GABA$_A$ receptors, the effects induced by diazepam in cortical neurons lacking $\alpha1$-GABA$_A$ receptors might be more pronounced than those observed in wild type. This difference might be manifested behaviorally by the enhanced sensitivity of forebrain-specific $\alpha1^{-/-}$ mutants to the motor-sedative effect of diazepam compared with pseudo-wild-type 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 $\alpha1$ 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) probably 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 probably engage additional brain circuits, notably the cerebellum (Lalonde and Strazielle, 2001; Levin et al., 2006). However, although such behavioral paradigms arguably provide a more complete measure 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, because the myorelaxant effect of diazepam is mediated by α2- and α5-GABA$_A$ receptors, and it cannot be attributed selectively to cortical circuits (Crestani et al., 2001). 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% as a result of 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, functional magnetic resonance imaging 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.

Acknowledgments

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