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Alpha 1 subunit-containing γ -aminobutyrate type A receptors in forebrain
contribute to the effect of inhaled anesthetics on conditioned fear

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α 1 GABA_A-R and Anesthetic Responses

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Abbreviations: GABA, γ -aminobutyric acid; GABA_A-R, GABA type A receptor; MAC, minimum alveolar concentration; LORR, loss of the righting reflex; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]benzodiazepine-3-carboxylate;

TBPS, *t*-butylbicyclophosphorothionate.

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Abstract

Inhaled anesthetics are thought to produce anesthesia by their actions on ion channels. Because inhaled anesthetics robustly enhance γ -aminobutyric acid type A receptor (GABA_A-R) responses to GABA, these receptors are considered prime targets of anesthetic action. However, the importance of GABA_A-Rs and individual GABA_A-R subunits to specific anesthetic-induced behavioral effects in the intact animal is unknown. We hypothesized that inhaled anesthetics produce amnesia, as assessed by loss of fear conditioning, by acting on forebrain GABA_A-Rs that harbor the α 1 subunit. To test this, we used global knockout mice that completely lack the α 1 subunit, and forebrain-specific, conditional knockout mice that lack the α 1 subunit only in the hippocampus, cortex, and amygdala. Both knockouts were 75-145% less sensitive to the amnestic effects of the inhaled anesthetic isoflurane. These results indicate that α 1-containing GABA_A-Rs in the hippocampus, amygdala, and/or cortex influence the amnestic effects of inhaled anesthetics, and may be an important molecular target of the drug isoflurane.

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Inhaled anesthetics produce anesthesia by unknown mechanisms. The prevailing theory, initially proposed by Franks and Lieb (1984), posits that specific protein targets in the nervous system are the molecular sites of action of inhaled anesthetics. Numerous putative protein targets have been identified, including a wide variety of ion channels (for review, see: Campagna et al., 2003). However, the contribution that each of these targets make to whole animal behavioral responses to inhaled anesthetics is not clear.

A plausible target that has received considerable attention is the γ -aminobutyric acid (GABA) type A receptor (GABA_A-R). The GABA_A-R is a five-subunit chloride channel activated by GABA and muscimol, and blocked competitively by bicuculline and non-competitively by picrotoxin (Olsen, 1982). Eccles et al. (1963) noted that many general anesthetics prolong inhibition of spinal motoneurons, an effect mediated by GABA. Nicoll (1972) suggested that GABA-mediated enhancement of synaptic inhibition might underlie anesthetic actions. Consistent with this suggestion, Pearce et al. (1989) reported that anesthetics greatly prolong the time-course of recurrent inhibition in the rat hippocampus. However, our knowledge of the importance of GABA_A-Rs and individual GABA_A-R subunits to anesthetic-induced behavioral effects remains incomplete.

Clinically, inhaled anesthetics produce two universal effects, amnesia for events during surgery and immobility in response to noxious stimulation (e.g., surgical incision). Although the primary neuroanatomic site at which inhaled anesthetics act to produce immobility is the spinal cord (Antognini and Schwartz, 1993; Rampil et al., 1993),

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supraspinal structures probably mediate amnesic effects. A plausible site of action by which inhaled anesthetics interfere with memory is the hippocampus where GABA_A-Rs participate in memory formation (Bailey et al., 2002; Collinson et al., 2002). The α 1 subunit appears to be of particular importance in this process. The α 1 subunit of the GABA_A-R is the most abundant α subunit in adult brain (McKernan and Whiting, 1996) and is expressed at high levels in many brain regions, including hippocampus (Sperk et al., 1997). Benzodiazepines, which only act by allosterically enhancing the action of GABA, specifically cause amnesia by an action on the α 1 GABA_A-R (Rudolph et al., 1999).

The study investigated the hypothesis that α 1 subunit-containing hippocampal GABA_A-Rs in part mediate the amnesia caused by inhaled anesthetics. We used genetically engineered mice that completely lack the α 1 subunit (Vicini et al., 2001) in all cells of the body (i.e., a global knockout) and mice that conditionally lack the α 1 subunit in restricted neuronal populations (i.e., forebrain-specific knockout) to address this hypothesis. We assessed amnesia by Pavlovian fear conditioning. For comparison, we tested whether either knockout would influence the capacity of an inhaled anesthetic to produce loss of the righting reflex or suppression of nociceptive reflexes. We predicted that these effects would not be influenced by the α 1 subunit.

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Methods

Mouse Production

Global $\alpha 1$ knockout mice were produced as previously described (Vicini et al., 2001). Mice heterozygous for a floxed $\alpha 1$ allele (exon 8 flanked by loxP sites) and a cre-recombined, inactive $\alpha 1$ allele that lacks exon 8 were interbred to produce homozygous floxed controls, and heterozygous and homozygous global knockouts. Expression of the unrecombined floxed allele does not differ from wild type $\alpha 1$ expression; the recombined $\alpha 1$ allele is a true null allele (Kralic et al., 2002; Vicini et al., 2001). Global knockouts and controls were of a mixed C57BL/6J X Strain 129S1/X1 x FVB/N hybrid background (Vicini et al., 2001) of the F₆₊ generation.

α CamKII-cre transgenic mice, line T29-1 (Tsien et al., 1996), were crossed with B6;129S4-*Gt(ROSA)26Sor^{tm1Sor}/J* (Soriano, 1999) or B6.Cg-*Tg(xstpx-lacZ)32^{And}/J* (Zinyk et al., 1998) reporter mice obtained from The Jackson Laboratory (stock# 003309 and 002982, respectively). Adult (56 days of age) F₁ mice from these crosses were analyzed for functional β -galactosidase activity to reveal tissue-specific patterns of cre-mediated recombination as described below.

Crossing the α CamKII-cre transgene (Tsien et al., 1996) onto the $\alpha 1$ floxed background (Vicini et al., 2001) produced forebrain-specific $\alpha 1$ knockouts. Breeding pairs were used in which the male lacked the α CamKII-cre transgene (Cre-) but was homozygous floxed $\alpha 1$ and the female was hemizygous for the α CamKII-cre transgene (Cre+) and homozygous for the floxed $\alpha 1$ gene. Conditional knockouts and controls were of a mixed C57BL/6J X Strain 129Sv/SvJ hybrid background of the F₆₋₉ generation.

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All mice were maintained under a 12-h light dark schedule with lights on at 7am. Mice were group housed, provided ad libitum access to food and water, and genotyped by Southern blot analysis. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the NIH and were approved by the Institutional Animal Care and Use Committees at UCSF and Pittsburgh.

Histology and Immunohistochemistry

Tissue sections derived from mice at 8 weeks of age were analyzed for cre activated β -galactosidase staining to determine the extent of recombination throughout the brain using standard techniques (Tsien et al., 1996). Slides were counterstained with eosin and examined by light microscopy.

For GABA_A-R α 1 immunostaining, animals (56 days of age) were deeply anesthetized with pentobarbital (Nembutal, 40 mg/kg) and perfused transcardially (Fritschy and Mohler, 1995). The GABA_A-R α 1 subunit was visualized in 40- μ m sections processed for immunoperoxidase staining with subunit-specific antisera raised against amino acids 1-16 of the α 1 subunit (Gao et al., 1993). Free-floating sections were washed 3 x 10 min in Tris buffer (Tris saline, pH 7.4, 0.05% Triton X-100) and incubated at 4°C overnight in primary antibody solution (1:20000) diluted in Tris buffer containing 2% normal goat serum. Sections were then washed 3 x 10 min in Tris buffer and incubated in biotinylated secondary antibody solution (Jackson Immunoresearch) diluted 1:300 in Tris buffer containing 2% normal goat serum for 30 min at room temperature. After additional washing, sections were transferred to the avidin-peroxidase solution (Vectastain Elite Kit, Vector Laboratories) for 20 min, washed and

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processed using diaminobenzidine hydrochloride (Sigma) as the chromogen. Slides were air-dried, dehydrated with ascending series of ethanol, cleared with xylene and coverslipped with Eukitt. Changes in regional distribution of GABA_A-R α 1 subunits were analysed by light microscopy (Zeiss Axioplan microscope).

Ligand Autoradiography

Whole brains from 5 adult (11.6-12.7 weeks of age) male mice of each genotype were used. The autoradiographic procedures for regional localization of the benzodiazepine (labeled by [³H]ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]benzodiazepine-3-carboxylate: [³H]Ro 15-4513; NEN Life Science Products, Boston, MA), GABA ([³H]muscimol; NEN) and convulsant ionophoric (*t*-butylbicyclophosphoro[³⁵S]thionate: [³⁵S]TBPS; NEN) binding sites were as described (Mäkelä et al., 1997). Nonspecific binding was determined with 10 μ M flumazenil (donated by F. Hoffmann-La Roche, Basel, Switzerland), 100 μ M picrotoxinin (Sigma Chemical Co., St. Louis, MO) and 100 μ M GABA (Sigma) in [³H]Ro 15-4513, [³⁵S]TBPS and [³H]muscimol assays, respectively.

Autoradiography films were quantified using AIS image analysis system (Imaging Research, St. Catharines, Canada) as described (Mäkelä et al., 1997). Binding densities for each brain area were averaged from measurements of one to three sections/brain. The standards exposed simultaneously with brain sections were used as reference with the resulting binding values given as radioactivity levels estimated for gray matter areas (nCi/mg for ³H and nCi/g for ¹⁴C).

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Significance of the differences between the mouse lines in each brain region was assessed with ANOVA followed by Tukey-Kramer post-test.

Fear Conditioning and MAC Determinations

At 8-12 weeks of age, 99 control, 154 heterozygous and 100 homozygous GABA_A-R α 1 global knockout mice, and 40 control and 37 conditional GABA_A-R α 1 knockout mice were studied.

Fear conditioning was performed as previously described (Eger et al., 2003). Briefly, animals were exposed to target concentrations of isoflurane in oxygen or oxygen alone (control) for 30 min, then placed in a gas tight training chamber containing the same concentration of isoflurane. After allowing 3 min for exploration of the training chamber, a 90-dB, 2-kHz tone sounded, co-terminating with a 2 sec footshock. This was repeated twice, with one min between tones. Footshock intensity varied between 1 and 3 mA as a function of anesthetic concentration to equalize the response of mice to the footshock. Animals were observed by closed circuit television.

The following day, fear to tone was tested by placing animals in a different context. After allowing 3 min for exploration, the animals were exposed to a 90-dB, 2-kHz tone for eight min and then immediately returned to the animal's home container. Fear to context was tested later that day by placing the mice in the original training chamber for 8 min, with no tone imposed. Animals were observed by closed circuit television. Fear was assessed by behavioral freezing (i.e., immobility except for respiration) and was measured every 8 sec for 8 min per mouse by a blinded observer. The number of freezes out of the 60 measurements gave the probability of freezing ("freeze score") for

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each animal.

The minimum alveolar concentration (MAC) of anesthetic preventing movement in 50% of animals in response to a noxious stimulus (a tail clamp) is a standard EC₅₀ measure of anesthetic potency. MAC values for desflurane, halothane, and isoflurane were measured as previously described (Sonner et al., 2000).

For each anesthetic, MAC values and regression parameters estimated in calculating amnestic EC₅₀s for different genotypes were compared using either an analysis of variance (ANOVA) with a Student-Newman-Keuls test for post-hoc multiple comparison testing, or Student's t-test. Nonlinear regression was performed to calculate an EC₅₀ and the maximum value of the dose-response curve (A) for fear conditioning according to the equation:

$$\text{Equation\#1: } \textit{FreezeScore} = A * \left(1 - \frac{\textit{isoflurane}^n}{\textit{isoflurane}^n + \textit{ED}_{50}^n} \right)$$

Means ± SE are reported except where otherwise noted. P ≤ 0.05 was taken as statistically significant.

Loss-of-Righting Reflex

Groups of 6-8 mice (8-19 weeks old; 15.9-33.1 g) were tested for loss of righting reflex (LORR) in individual wire mesh cages in a rotating carousel enclosed in a sealed acrylic chamber as previously described (Homanics et al., 1997; Quinlan et al., 1998). Halothane (Halocarbon Laboratories, River Edge, NJ) and isoflurane (Halocarbon Laboratories, River Edge, NJ) mixed with oxygen were monitored with an infrared anesthesia analyzer (Datex-Ohmeda Inc., Andover, MA). Constant anesthetic concentrations were supplied for 15 min before testing. A blinded observer scored the

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mice positive for LORR if they passively rolled twice in a 75 sec time period while the carousel rotated at 4 RPM. Mice tested with both volatile drugs were given at least 7 days to recover between anesthetics.

Sleeptime.

The sleeptime (duration of the loss of the righting reflex) was used to assess the sedative/hypnotic effects of pentobarbital (45 mg/kg, i.p.) and zolpidem (60 mg/kg, i.p.). Normothermia was maintained with a heat lamp.

Results

Production and Characterization of Genetically Engineered Mice. To test the role of $\alpha 1$ GABA_A-Rs in specific brain regions in anesthetic mechanisms, we set out to create conditional $\alpha 1$ knockouts in which the $\alpha 1$ gene was inactivated only in hippocampal pyramidal cells of the CA1 region. We obtained α CamKII-cre mice purported to induce postnatal hippocampal CA1 pyramidal cell-specific recombination (Tsien et al., 1996). However, when we crossed this mouse line with the *Gt(ROSA)26^{Sor}* floxed β -galactosidase reporter mouse line (Soriano, 1999), we observed high levels of recombination-induced β -galactosidase expression in many areas of the hippocampus in addition to CA1, and a lower level of staining in cortex and amygdala, but no staining in other areas of the brain (Fig. 1A-C, F, & I). We also tested the α CamKII-cre mice by crossing to the *Tg(xstpx-lacZ)32^{And}/J* floxed β -galactosidase reporter mouse line (Zinyk et al., 1998). This reporter mouse line revealed hippocampal CA1-specific staining

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(data not shown) as originally reported by Tsien et al. (1996). Thus, the pattern of recombination appears to depend upon the reporter mouse line used.

We next created tissue-specific conditional $\alpha 1$ knockouts by crossing α CamKII-cre transgenic mice (Tsien et al., 1996) with floxed $\alpha 1$ mice (Vicini et al., 2001). Immunohistochemistry with an $\alpha 1$ -specific antibody showed a pattern of reduction of $\alpha 1$ staining in the conditional knockout mice that paralleled that observed in the *Gt(ROSA)26^{Sor}* β -galactosidase reporter mouse study. This indicated successful creation of a forebrain-specific $\alpha 1$ knockout mouse line in which $\alpha 1$ is selectively inactivated in hippocampus, cortex, and amygdala. The pattern of staining was as follows. In control mice, $\alpha 1$ immunoreactivity was found on cell bodies and densely packed dendritic processes in all layers of the cerebral cortex (Fig 1D). Staining for $\alpha 1$ was decreased in the outer cortical layers (Fig 1E) of the mutant in a similar pattern to that shown in Fig 1C. A loss of $\alpha 1$ immunoreactivity was observed in the CA1 region and subiculum of the mutant hippocampus and to a lesser extent in the CA3 and dentate gyrus (Fig 1H) compared to control (Fig 1G). The staining pattern follows the expected loss of $\alpha 1$ on pyramidal cells since $\alpha 1$ on this cell type is localized mostly to dendritic processes (Fritschy and Mohler, 1995; Sperk et al., 1997). Note that staining does not completely disappear in these areas since the $\alpha 1$ gene was not inactivated in hippocampal interneurons as observed, for example, by the presence of $\alpha 1$ -stained interneurons in the CA3 region of both control and mutant mice. A reduction in $\alpha 1$ staining was observed in the basomedial (Fig 1K) and basolateral amygdala (not shown) of mutant mice compared to control (Fig 1J). Staining for $\alpha 1$ in other brain regions of mutant mice did not differ from control.

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Ligand autoradiography was used to visualize the brain regional distribution of binding sites. We used three different ligands, each of them known to have heterogenous brain regional distribution (Korpi et al., 2002a). Flumazenil-sensitive benzodiazepine sites labeled by [³H]Ro 15-4513 were rather evenly distributed throughout the brain, with the lowest binding densities being observed in the thalamus (Fig. 2A). The conditional knockout mice had significantly lower binding only in the cerebral cortex of the brain horizontal sections compared to controls (Fig. 2A; Table 1). This ligand detects primarily the $\gamma 2$ subunit-containing GABA_A-Rs, irrespective of the α subunits in the receptor subunit complex (Lüddens and Korpi, 1996; Pritchett et al., 1989). GABA-sensitive [³H]muscimol binding resulted in a different pattern, labeling strongly the cerebellar granule cell layer and more faintly the thalamus, cerebral cortex, caudate-putamen and the olfactory bulbs in both mouse lines (Fig. 2B). This binding is largely due to the high-affinity binding to δ subunit-containing receptors, which are assembled in the forebrain mostly with $\alpha 4$ subunits and in the cerebellum with the $\alpha 6$ subunits, since [³H]muscimol binding signal is abolished in $\alpha 6$ and δ deficient mouse brains (Korpi et al., 2002b). Quantitation of this signal in the conditional knockouts and controls failed to reveal any differences in the brain regions analyzed (Table 1). Lastly, we applied picrotoxin-sensitive [³⁵S]TBPS binding to label the GABA_A receptor associated ionophores. The labeling pattern of [³⁵S]TBPS again differed from the previous ligands (Fig. 2C). With this ligand there were several brain regions that were less labeled in the conditional knockouts than controls. These regions included the cerebral cortex, caudate-putamen and hippocampus (Table 1). There were no

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differences in the thalamus and cerebellum (Table 1), nor in the strongly labeled inferior colliculus (Fig. 2C).

Global deletion of $\alpha 1$ subunits reduced the amnestic effects of isoflurane. Homozygous global knockout mice were ~2.3 fold more resistant to the amnestic effects of isoflurane, as assessed by fear to context, compared to control littermates ($p < 0.05$; Fig. 3A). Mice heterozygous for the global knockout were also more resistant to the amnestic effects of isoflurane compared to controls ($p < 0.05$; Fig. 3A). Baseline freeze scores in the absence of anesthetic did not differ by genotype: wild type mice had a freeze score of $38.8 \pm 6.8\%$, heterozygous mice $45.3 \pm 5.5\%$, and knockout mice $40.7 \pm 11.9\%$ without anesthetic.

Genotype influenced the baseline responses for tone conditioning in the absence of anesthetic, making comparison of EC_{50} s problematic. Wild type animals had a starting freeze score of $49.4 \pm 9.6\%$, heterozygous mice $62.5 \pm 4.8\%$, and knockout mice $77.8 \pm 9.2\%$. This problem was surmounted, as noted below, in the conditional knockout of the $\alpha 1$ GABA_A-R, where the different genotypes showed similar baseline responses.

Forebrain-specific deletion of $\alpha 1$ also reduced amnestic effects of isoflurane. For contextual fear conditioning, conditional deletion of the $\alpha 1$ GABA_A-R increased the isoflurane amnestic EC_{50} compared to controls by 145% (Fig. 3B; $p < 0.001$). The baseline responses in the absence of anesthetic did not differ between genotypes (freeze scores were $58.1 \pm 11.0\%$ and $76.8 \pm 6.7\%$ for knockout and wild type mice, respectively; $p = 0.18$).

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For tone fear conditioning, the conditional deletion increased the isoflurane amnestic EC_{50} compared to controls by 75% (Fig. 3C; $p < 0.001$). The baseline responses in the absence of anesthetic ($82.2 \pm 5.3\%$ and $82.7 \pm 8.7\%$ for knockout and wild type mice, respectively) did not differ for the two genotypes.

Deletion of $\alpha 1$ minimally affected other anesthetic-induced behaviors. Isoflurane MAC did not differ between control, heterozygous, and global $\alpha 1$ knockout littermates (Table 2). As would be expected from the importance of the spinal cord for the capacity of inhaled anesthetics to produce immobility, MAC did not differ between control and littermate forebrain-specific knockout mice for halothane or desflurane (Table 2). There was a small (11%) increase in MAC for isoflurane ($p < 0.05$; Table 2).

Global knockout mice were less sensitive to the effects of halothane when compared to controls for LORR, but by only 19% (Table 3; $P < 0.0001$). Global knockout mice had comparable EC_{50} values for isoflurane-induced LORR. LORR EC_{50} values were similar for conditional knockout mice and controls for both isoflurane and halothane.

Sleeptime of forebrain-specific $\alpha 1$ knockouts (23.2 ± 2.4 min, mean \pm SEM; $n = 19$) in response to zolpidem did not differ from controls (19.6 ± 3.1 ; $n = 15$). Furthermore, sleeptime induced by pentobarbital in forebrain-specific $\alpha 1$ knockouts (46.8 ± 2.9 ; $n = 19$) also did not differ from controls (50.5 ± 3.1 ; $n = 13$).

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Discussion

These studies reveal two novel findings about the mechanism of action of inhaled anesthetics. First, we demonstrated that $\alpha 1$ -containing GABA_A-Rs contribute to the amnestic effects of isoflurane, as assessed by fear conditioning in two different mouse lines lacking these receptors. The more than two-fold increase in amnestic EC₅₀s we observed is among the largest relative increase in anesthetic effect ever observed. Secondly, these studies provide novel insight into the brain regions that are responsible for the amnestic effects of inhaled anesthetics. GABA_A-Rs containing the $\alpha 1$ subunit in hippocampus, amygdala, and/or cortex appear to be critically important in the amnestic effects of isoflurane. This is the first demonstration of specific brain regions involved in this universal effect of inhaled anesthetics.

We found that mice that completely lacked the $\alpha 1$ subunit, i.e., global $\alpha 1$ knockouts, were 139% less sensitive to the amnestic effects of isoflurane as assessed by fear conditioning to context compared to control littermates (Fig. 3A). These results parallel results from other studies that demonstrate that benzodiazepines cause amnesia by interacting primarily with the $\alpha 1$ subunit (Rudolph et al., 1999).

We hypothesized that the hippocampus, because of its central role in learning and memory, mediates the amnestic effects of inhaled anesthetics. To test this hypothesis, we used conditional $\alpha 1$ knockout mice that selectively lacked the $\alpha 1$ GABA_A-R subunit in forebrain structures including the hippocampus, cortex and amygdala. Like the global knockouts, forebrain-specific $\alpha 1$ knockout mice also resisted the amnestic effects of isoflurane. The EC₅₀s for isoflurane increased 145 and 75% compared to control values for fear to context and tone, respectively (Fig. 3B, C). Both

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tone and context conditioning depend on the amygdala; in addition, context conditioning is hippocampus-dependent (Fendt and Fanselow, 1999). Because this conditional knockout affected $\alpha 1$ subunits in the amygdala, it is not surprising that the amnesic effect of isoflurane decreased for both tone and context conditioning. Furthermore, the greater decrease in context conditioning may be attributed to the additional dependence on the hippocampus for this behavior.

In contrast to an important role of $\alpha 1$ GABA_A-Rs in amnesia, these GABA_A-R isoforms appear to contribute little to MAC. Global knockout mice did not differ in response to isoflurane for MAC. Conditional knockouts were slightly less sensitive to isoflurane but were normally sensitive to halothane and desflurane for MAC. Thus, the $\alpha 1$ subunit of the GABA_A-R appears to contribute little to the mechanism by which these inhaled drugs produce immobility in response to a noxious stimulus. Studies of other GABA_A-R mutant mice have also failed to find an effect of subunit deletion on MAC (Homanics et al., 1997; Mihalek et al., 1999), although $\beta 3$ knockouts and point mutants are an exception as they are modestly less sensitive to inhaled anesthetics (MAC is increased by approximately 20%) (Jurd et al., 2002; Quinlan et al., 1998). These results from mutant mice are in accord with our pharmacologic studies demonstrating that GABA plays at most a minor role in the immobilizing effect of anesthetics (Sonner et al., 2003).

GABA_A-Rs containing the $\alpha 1$ subunit similarly do not appear to be key mediators of inhaled anesthetic action for the righting reflex behavioral endpoint. While global knockouts were slightly less sensitive to halothane for LORR, they did not differ in response to isoflurane. Conditional knockouts did not differ from controls in response to

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either halothane or isoflurane. Studies of other GABA_A-R mutant mice have also failed to find a robust effect of GABA_A-R manipulation on the righting reflex for inhaled anesthetics (Homanics et al., 1997; Jurd et al., 2002; Mihalek et al., 1999; Quinlan et al., 1998).

To produce the conditional knockout mouse line, we relied upon tissue-specific inactivation of the $\alpha 1$ gene using cre-lox technology. The mouse line used to direct tissue-specific expression of cre recombinase was a transgenic mouse line that had been reported to induce hippocampal CA1 pyramidal cell-specific recombination (Tsien et al., 1996). We did observe CA1-specific recombination when we tested this transgenic mouse line by crossing with the *Tg(xstpx-lacZ)32^{And}/J* reporter mouse line (Zinyk et al., 1998). However, we observed that this α CamKII-cre mouse line induced recombination in a much broader tissue-specific pattern when mated to the *Gt(ROSA)26^{Sor}* reporter line (Soriano, 1999) and to our floxed $\alpha 1$ mouse line (see Fig. 1). We found recombination in CA1, CA3 and dentate gyrus of the hippocampus, the amygdala, and the cortex.

Ligand autoradiographic analyses of brain GABA_A-R binding activities also documented forebrain, but not cerebellar, alterations in the conditional knockout animals. First, [³H]muscimol binding which depends on $\alpha 6$ subunits in the cerebellum and on δ subunits in the forebrain (Korpi et al., 2002b) was not altered. This excludes a general alteration in the transcription of GABA_A-R subunits genes located on chromosome 11 close to the $\alpha 1$ subunit, i.e. the $\alpha 6$ and $\gamma 2$ genes (Russek, 1999). This contrasts with targeting of the $\alpha 6$ subunit which resulted in attenuated transcription of $\alpha 1$ and $\beta 2$ genes (Uusi-Oukari et al., 2000). Second, [³H]Ro 15-4513 is a

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benzodiazepine site ligand that has little subtype selectivity between α subunits, but the $\gamma 2$ subunit is obligatory for its high-affinity binding (Lüddens and Korpi, 1996). The small alterations in its binding to the cortical and hippocampal region of the forebrain-specific $\alpha 1$ knockout mice may reflect the fact that $\alpha 1$ is not inactivated in all cells in these brain regions. Third, [^{35}S]TBPS autoradiography reflects most receptor subtypes, but much less is known about its subtype dependency compared to [^3H]muscimol and [^3H]Ro 15-4513. In recombinant receptor studies, [^{35}S]TBPS binding is not always formed by theoretically relevant subunit combinations (see: Lüddens and Korpi, 1995). However, it is clear that $\alpha 1$ subunit-containing receptors do bind [^{35}S]TBPS efficiently (Korpi and Lüddens, 1993). This is consistent with the present findings as we observed the greatest reductions in the cortical and hippocampal regions. We also had global $\alpha 1$ knockout brains in the same assay, and they showed widespread reduction in both [^{35}S]TBPS and [^3H]Ro 15-4513 autoradiographic signals (Kralic et al., 2002), but no alteration in [^3H]muscimol signal (data not shown). The reduction of [^{35}S]TBPS binding in the caudate-putamen is difficult to explain, as cre expression is low there as is the expression of $\alpha 1$ subunits (Korpi et al., 2002a). [^{35}S]TBPS binding was reduced in this brain region also in the global $\alpha 1$ knockouts (Kralic et al., 2002).

In summary, the immunohistochemical and autoradiographic analyses indicate that the conditional $\alpha 1$ knockout mice have regional alterations in GABA_A-R subtypes restricted to forebrain. The differences in recombination patterns observed in the present studies and those of Tsien et al. (1996) are most likely due to differences in accessibility of the floxed loci to the recombinase. This may be influenced by differences in genetic background between mouse lines. The differences are not due to

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age-dependent changes in the pattern of cre expression since recombination was studied in mice at 8 weeks of age in the present studies and in those of Tsien et al. (1996).

A caveat to these experiments is that global deficiency of a GABA_A-R gene may induce compensatory changes in other genes and/or gene products (e.g., Brickley et al., 2001; Korpi et al., 2002b; Peng et al., 2002). We have observed an increase in $\alpha 2$ and $\alpha 3$ subunits and a decrease in $\beta 2/3$ and $\gamma 2$ subunits in $\alpha 1$ global knockouts (Kralic et al., 2002). Such changes should be less widespread in the conditional knockout animals. Note that the magnitude of changes in amnestic and other effects did not differ between global and conditional knockout mice. However, it is possible that compensation occurred in cells of these conditional mice in which the $\alpha 1$ gene was inactivated. This caution must be considered when interpreting the results of our studies. Thus, compensatory changes, rather than a direct effect on $\alpha 1$ -containing receptors could mediate the phenotypic changes in anesthetic responsiveness.

The gene knockin approach has recently been employed to determine the contribution of GABA_A-R subunits to the behavioral effects of benzodiazepines and intravenous anesthetics (Cope et al., 2004; Jurd et al., 2002; Low et al., 2000; Reynolds et al., 2003; Rudolph et al., 1999). These studies created individual mouse lines with point mutations that reduced/eliminated sensitivity to these drugs. The results provide compelling evidence for subunit specificity in behavioral response to these drugs. Furthermore, these studies demonstrate that different anesthetics have different molecular mechanisms of action, and different mechanisms can mediate different behavioral responses to the same drug. The results presented here build upon these

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studies and suggest that inhaled anesthetics may also have distinct mechanisms of action for specific behavioral endpoints. These studies support the multisite agent-specific mechanism of anesthetic action (Pittson et al., 2004).

In summary, using mice harboring global and conditional knockouts of the $\alpha 1$ subunit of the GABA_A-R, we find that $\alpha 1$ -containing GABA-Rs in the hippocampus, amygdala, and/or cortex are important molecular targets influencing the amnestic effects of the inhaled anesthetic isoflurane. By contrast, these receptors appear to contribute little to suppression of pain-evoked movement and righting reflexes by this inhaled anesthetic.

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Footnotes

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Legends for Figures

Figure 1. (A) In β -galactosidase staining of a sagittal section from a mouse that harbors only the α CamKII-cre transgene, no cells are stained blue. (B) β -galactosidase staining (blue cells) in a sagittal section from a mouse that harbors the α CamKII-cre and ROSA26 reporter transgenes indicates regions of the hippocampus, cerebral cortex, and amygdala that have undergone cre-mediated recombination of the reporter. (C, F, I) Higher magnification view of areas boxed in panel B shows β -galactosidase staining in cortex, hippocampus, and amygdala, respectively. (D,E) Immunostaining for α 1 in cortex of control and conditional α 1 knockout, respectively shows decreased α 1 staining in outer cortical layers of mutant compared to controls. (G,H) Immunostaining for α 1 in hippocampus of control and conditional α 1 knockout, respectively shows decreased α 1 staining in CA1 and subiculum and to a lesser extent in CA3 and dentate gyrus, but not in the thalamus of the mutant compared to control. (J,K) Immunostaining for α 1 in amygdala of control and conditional α 1 knockout, respectively shows decreased α 1 staining in basomedial nucleus of amygdala of mutant compared to control. Scale bars: 1 mm (A,B) and 200 μ m (C-K). Amygdala, amg; basomedial nucleus of amygdala, bm; ce, central nucleus of amygdala; cerebral cortex, ctx; hippocampus, hi; thalamus, th.

Figure 2. Representative autoradiographic images show (A) the flumazenil-sensitive benzodiazepine site binding labeled by [3 H]Ro 15-4513, (B) the GABA-sensitive agonist site binding labeled by [3 H]muscimol and (C) the picrotoxin-sensitive ion channel site binding labeled by [35 S]TBPS for the Cre negative (controls) and Cre positive (conditional knockout) GABA_A-R α 1 mice. The images were scanned with identical

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brightness and contrast in both genotypes. Ctx, cerebral cortex; Th, thalamus; Hi, hippocampus; CPu, caudate-putamen; Cere, cerebellar cortex; IC, inferior colliculus.

Figure 3: (A) Global knockout of the of the GABA_A-R α 1 subunit significantly increases the EC₅₀ at which isoflurane interferes with fear conditioning to context in heterozygous and homozygous knockouts compared to controls. (B) Forebrain-specific knockout of the GABA_A-R α 1 subunit significantly increases the EC₅₀ at which isoflurane interferes with fear conditioning to context compared to controls. (C) Forebrain-specific knockout significantly increases the EC₅₀ at which isoflurane interferes with fear conditioning to tone compared to controls. Values presented are the mean \pm S.E.M.; * P<0.05; ** P<0.001

Table 1. Autoradiographic analysis of GABA_A-R binding sites in horizontal sections of control and conditional GABA_A-R α 1 subunit knockout mice.

Ligand	Brain region				
	Ctx	Th	Hi	CPu	Cere
[³H]Ro15-4513 binding to benzodiazepine sites					
Cre-, α 1F/F mice	121±10	50±3	115±5	51±4	97±5
Cre+, α 1F/F mice	90±13 [*]	47±7	108±6	44±6	103±9
[³H] Muscimol binding to GABA sites					
Cre-, α 1F/F mice	12.3±1.4	17.5±5.4	8.2±1.3	8.8±2.0	35.4±5.6
Cre+, α 1F/F mice	11.5±3.1	15.0±1.0	8.1±1.9	8.3±1.2	31.8±5.3
[³⁵S]TBPS binding to ionophore sites					
Cre-, α 1F/F mice	226±54	503±94	157±26	338±32	122±26
Cre+, α 1F/F mice	70±14 ^{**}	472±128	121±22 [*]	193±36 ^{**}	115±21

The data are means \pm SD for 5 mice in each genotype and are expressed as nCi/mg for ³H-ligands and as nCi/g for ³⁵S-ligand. Significance of the difference from control (Student's t-test): ^{*} P<0.01, ^{**} P<0.001. Ctx, cerebral cortex; Th, thalamus; Hi, hippocampus; CPu, caudate-putamen; Cere, cerebellar cortex.

Table 2. MAC values for global and conditional GABA_A-R α 1 subunit knockout mice.

Genotype	MAC		
	Isoflurane (n)	Halothane (n)	Desflurane (n)
Control	1.54±0.17 (6)	n.d.	n.d.
Heterozygous Global Knockout	1.59±0.11 (10)	n.d.	n.d.
Homozygous Global Knockout	1.55±0.11 (9)	n.d.	n.d.
Control	1.37±0.16 (10)	1.27±0.11 (8)	7.24±0.36 (9)
Conditional Knockout	1.52±0.08 (10)*	1.25±0.11 (8)	7.49±0.50 (10)

MAC is the minimum alveolar concentration of anesthetic which prevents movement in 50% of animals subjected to a noxious stimulus.

MAC values are reported as mean \pm SD (n = number of animals studied).

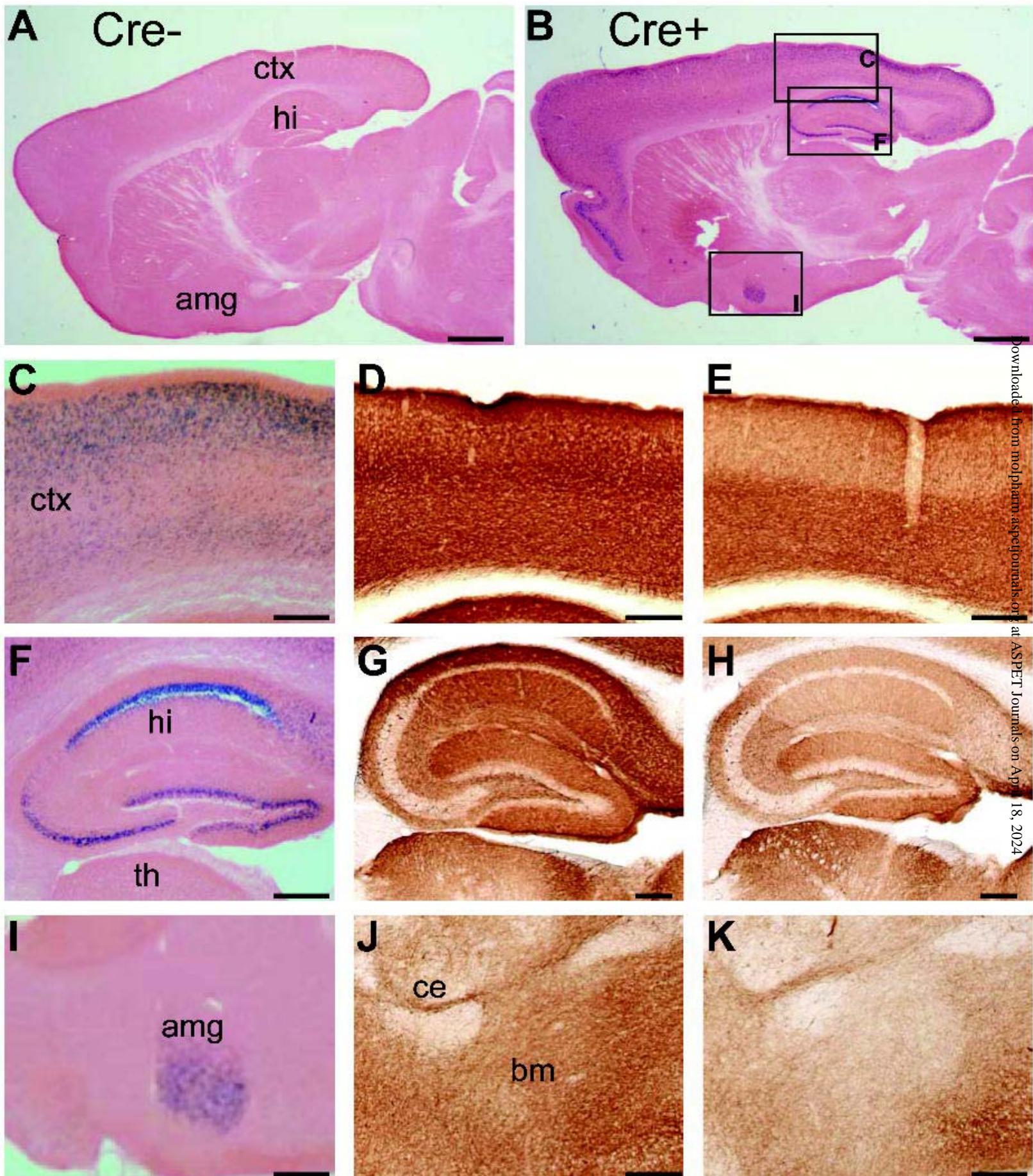
n.d.: not determined

* P<0.05

Table 3. Loss-of-righting reflex assay results for global and conditional $\alpha 1$ GABA_A-R subunit knockout mice.

Genotype	n(M/F)	Anesthetic	EC50
			(atm% \pm SE)
Control	17(9/7)	Halothane	0.82 \pm 0.02
Global KO	19(14/5)		0.98 \pm .019 [*]
Control	18(9/9)	Isoflurane	0.80 \pm 0.01
Global KO	18(14/4)		0.80 \pm 0.02
Control	12(6/6)		0.94 \pm 0.03
Conditional KO	10(5/5)	Halothane	0.92 \pm 0.03
Control	11(5/6)		0.79 \pm 0.02
Conditional KO	11(5/6)	Isoflurane	0.76 \pm 0.03

^{*}
 P<0.0001



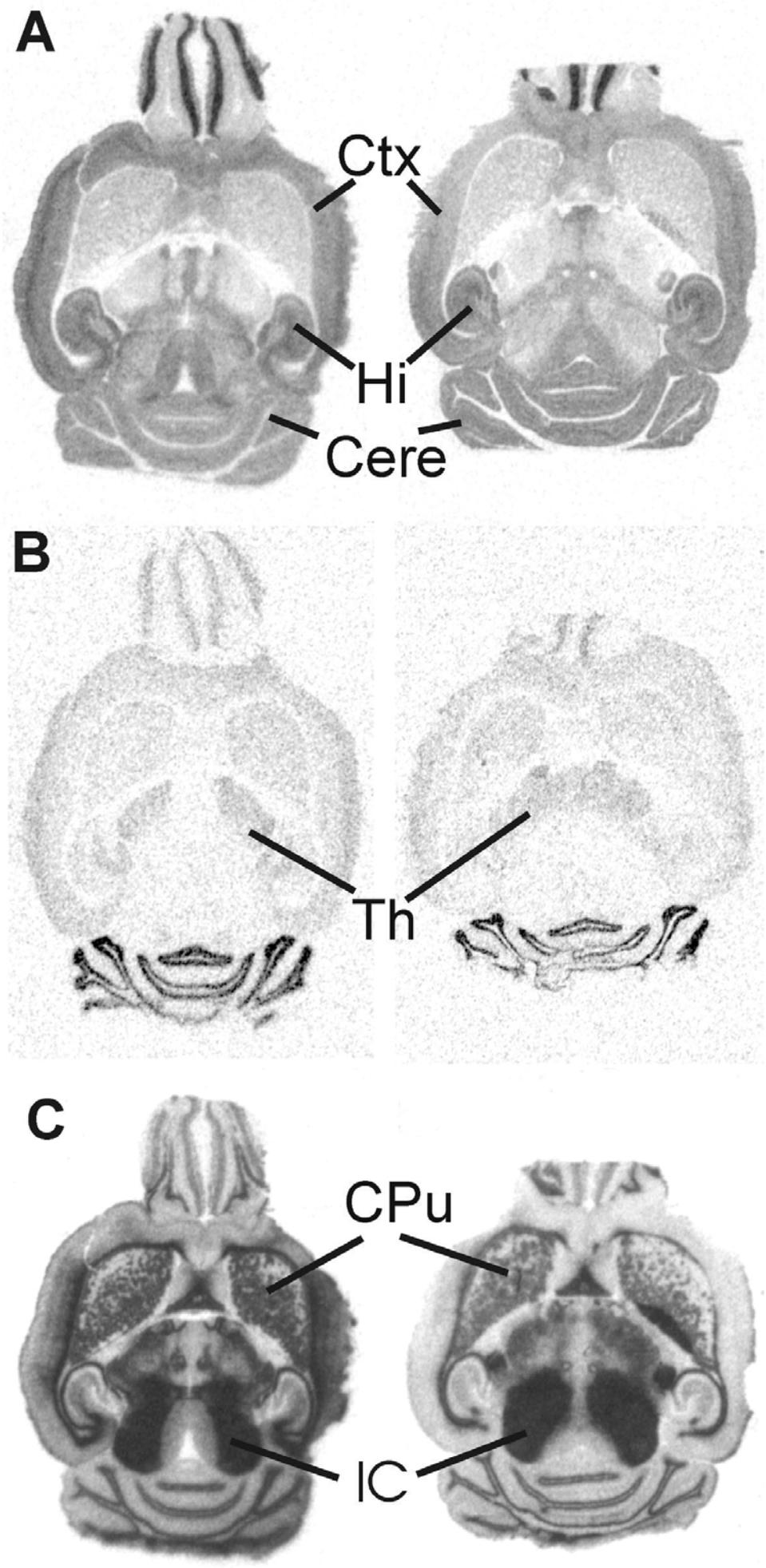
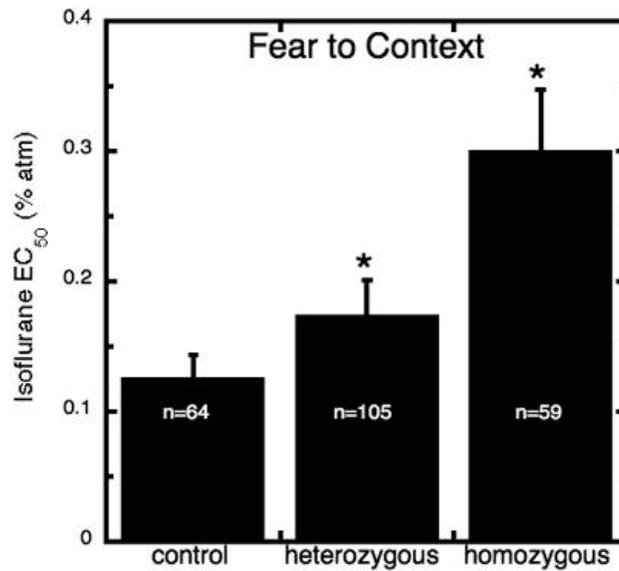
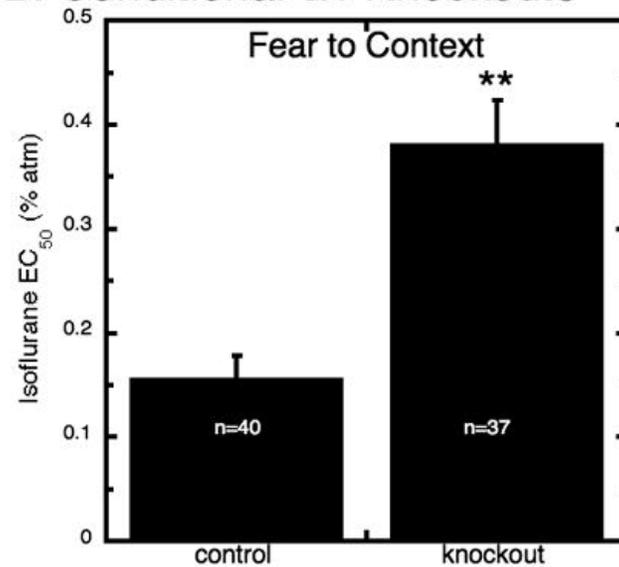


Figure 3

A. Global $\alpha 1$ Knockouts



B. Conditional $\alpha 1$ Knockouts



C. Conditional $\alpha 1$ Knockouts

