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Effects of GABA agonists on body temperature regulation in $GABA^{-/-}_{B(1)}$ mice

[1,5]Christophe Quéva, [2]Marianne Bremner-Danielsen, [3,6]Anders Edlund, [3,7]A. Jonas Ekstrand, [4]Susanne Elg, [1]Sven Erickson, [3,8]Thore Johansson, *[,2]Anders Lehmann & [4]Jan P. Mattsson

[1]Transgenic Research Laboratory, AstraZeneca R&D Mölndal, S-431 83 Mölndal, Sweden; [2]Gastrointestinal Biology, Integrative Pharmacology, AstraZeneca R&D Mölndal, S-431 83 Mölndal, Sweden; [3]Molecular Biology, AstraZeneca R&D Mölndal, S-431 83 Mölndal, Sweden and [4]Cell Biology and Biochemistry, AstraZeneca R&D Mölndal, S-431 83 Mölndal, Sweden

1 Activation of $GABA_B$ receptors evokes hypothermia in wildtype ($GABA^{+/+}_{B(1)}$) but not in $GABA_B$ receptor knockout ($GABA^{-/-}_{B(1)}$) mice. The aim of the present study was to determine the hypothermic and behavioural effects of the putative $GABA_B$ receptor agonist γ-hydroxybutyrate (GHB), and of the $GABA_A$ receptor agonist muscimol. In addition, basal body temperature was determined in $GABA^{+/+}_{B(1)}$, $GABA^{+/-}_{B(1)}$ and $GABA^{-/-}_{B(1)}$ mice.

2 $GABA^{-/-}_{B(1)}$ mice were generated by homologous recombination in embryonic stem cells. Correct gene targeting was assessed by Southern blotting, PCR and Western blotting. $GABA_B$ receptor-binding sites were quantified with radioligand binding. Measurement of body temperature was done using subcutaneous temperature-sensitive chips, and behavioural changes after drug administration were scored according to a semiquantitative scale.

3 $GABA^{-/-}_{B(1)}$ mice had a short lifespan, probably caused by generalised seizure activity. No histopathological or blood chemistry changes were seen, but the expression of $GABA_{B(2)}$ receptor protein was below the detection limit in brains from $GABA^{-/-}_{B(1)}$ mice, in the absence of changes in mRNA levels.

4 $GABA_B$ receptor-binding sites were absent in brain membranes from $GABA^{-/-}_{B(1)}$ mice.

5 $GABA^{-/-}_{B(1)}$ mice were hypothermic by approximately 1°C compared to $GABA^{+/+}_{B(1)}$ and $GABA^{+/-}_{B(1)}$ mice.

6 Injection of baclofen (9.6 mg kg^{-1}) produced a large reduction in body temperature and behavioural effects in $GABA^{+/+}_{B(1)}$ and in $GABA^{+/-}_{B(1)}$ mice, but $GABA^{-/-}_{B(1)}$ mice were unaffected. The same pattern was seen after administration of GHB (400 mg kg^{-1}). The $GABA_A$ receptor agonist muscimol (2 mg kg^{-1}), on the other hand, produced a more pronounced hypothermia in $GABA^{-/-}_{B(1)}$ mice. In $GABA^{+/+}_{B(1)}$ and $GABA^{+/-}_{B(1)}$ mice, muscimol induced sedation and reduced locomotor activity. However, when given to $GABA^{-/-}_{B(1)}$ mice, muscimol triggered periods of intense jumping and wild running.

7 It is concluded that hypothermia should be added to the characteristics of the $GABA^{-/-}_{B(1)}$ phenotype. Using this model, GHB was shown to be a selective $GABA_B$ receptor agonist. In addition, $GABA^{-/-}_{B(1)}$ mice are hypersensitive to $GABA_A$ receptor stimulation, indicating that $GABA_B$ tone normally balances $GABA_A$-mediated effects.

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; ES, embryonic stem; GABA, γ-aminobutyric acid; GHB, gammahydroxybutyrate; PBST, phosphate-buffered saline/0.1%Tween 20

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory transmitter in the mammalian brain. In the adult CNS, it mediates both fast and slow neuronal inhibition by activating the ligand-gated ionotropic $GABA_A$ and the G-protein-coupled metabotropic $GABA_B$ receptors, respectively. $GABA_B$ receptors inhibit adenylate cyclase through $G_{\alpha i}$ subunits, and activate the inwardly rectifying K^+ channels, to mediate hyperpolarisation of postsynaptic membranes. Stimulation of presynaptic $GABA_B$ receptors suppresses neurotransmitter release by inhibition of voltage-sensitive P, N, and L types of Ca^{2+} channels (for review, see Couve *et al.*, 2000).

Two $GABA_B$ receptor genes have been identified, $GABA_{B(1)}$ and $GABA_{B(2)}$ (Marshall *et al.*, 1999; Bowery & Enna, 2000). In addition, $GABA_{B(1)}$ encodes several splice variants and the more abundant ones, $GABA_{B(1a)}$ and $GABA_{B(1b)}$, are detected

*Author for correspondence;
E-mail: anders.lehmann@astrazeneca.com
[5]Current address: AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, MA 02451, U.S.A.
[6]Current address: Department of Public Health and Clinical Medicine, Umeå University, S-901 87 Umeå, Sweden
[7]Current address: Medivir AB, Department of Biology and Molecular Sciences, Lunastigen 7, S-141 44 Huddinge, Sweden
[8]Current address: UmanGenomics AB, Umestan, S-903 47 Umeå, Sweden
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in the mouse, rat and human, and differ in their amino-terminal ligand-binding domain, where the first 147 amino acids of $GABA_{B(1a)}$ are replaced by a sequence of 18 amino acids in $GABA_{B(1b)}$ (Kaupmann *et al*., 1997). When expressed alone in heterologous systems, $GABA_{B(1a)}$, $GABA_{B(1b)}$ and $GABA_{B(2)}$ display a low affinity for GABA, and a poor coupling to adenylate cyclase and inwardly rectifying K^+ channels (see Marshall *et al*., 1999; Bowery & Enna, 2000). In addition, $GABA_{B(1a,b)}$ receptors are prevented from reaching the cell surface, due to the presence of an endoplasmic reticulum retention motif in the C-terminus (Couve *et al*., 1998). However, coexpression of $GABA_{B(1a,b)}$ and $GABA_{B(2)}$ allows trafficking of $GABA_{B(1a,b)}$ to the cell membrane and efficient $GABA_B$ coupling to ion channels (Marshall *et al*., 1999; Bowery & Enna, 2000). $GABA_{B(1a,b)}$ and $GABA_{B(2)}$ mRNA were also found to be coexpressed in the brain and coimmunoprecipitated *in vitro* and *in vivo* (Marshall *et al*., 1999; Bowery & Enna, 2000). The pharmacology and the affinity for agonist of the $GABA_{B(1a,b)}$–$GABA_{B(2)}$ combination is comparable to the ones reported for the native receptors (Marshall *et al*., 1999; Bowery & Enna, 2000). Together, these results suggest that the heterodimer $GABA_{B(1a,b)}$–$GABA_{B(2)}$ is the functional metabotropic $GABA_B$ receptor.

Among other effects *in vivo*, stimulation of the $GABA_B$ receptor is known to produce hypothermia which parallels behavioural changes (Gray *et al*., 1987). This effect is considered to be of central origin, since intracerebroventricular injection of the prototypic $GABA_B$ receptor agonist baclofen lowers body temperature (Gray *et al*., 1987). The specific site at which $GABA_B$ receptor agonists act is unknown, but it probably resides within the hypothalamic thermoregulation centre (Yakimova *et al*., 1996).

The aims of the current study were three-fold. Effects of baclofen on body temperature have been reported previously in $GABA_{B(1)}^{-/-}$ mice (Schuler *et al*., 2001), but no alteration in basal temperature has been demonstrated. Here, we report on basal body temperature in $GABA_{B(1)}^{-/-}$ mice. Also, gamma-hydroxybutyrate (GHB), a drug recently approved by the US Food and Drug Administration for treatment of narcolepsy with episodes of cataplexy but also a common drug of abuse, may produce CNS effects through stimulation of $GABA_B$ receptors (Carai *et al*., 2001), but the issue is controversial (Feigenbaum & Howard, 1996). It has been shown that GHB may be converted to GABA (Hechler *et al*., 1997), which acts on both $GABA_B$ and $GABA_A$ receptors. We used $GABA_{B(1)}^{-/-}$ mice to resolve this issue as far as hypothermia and behavioural changes are concerned. Finally, similar to baclofen, the selective $GABA_A$ receptor agonist muscimol can induce hypothermia and sedation (Zarrindast & Oveissi, 1988). Given the close inter-relationship between $GABA_A$ and $GABA_B$ receptors, the effects of muscimol on body temperature and behaviour were studied in $GABA_{B(1)}^{-/-}$ mice.

Methods

Generation of $GABA_{B(1)}$ mutant mice

The murine genomic $GABA_{B(1)}$ locus (17 kb) encompassing exons 1a1 to 5 (numbered according to Lamp *et al*., 2001) was isolated from a 129Sv/J genomic library (Stratagene, La Jolla, CA, U.S.A.; Figure 1, Ekstrand, 1999). The gene-targeting vector was prepared as follows: a 4126 bp *Eco*R1–*Not*1 fragment upstream of exon E1a1 was subcloned in the Sac2 *Not*1 sites of pSAβGeo(LoxP)$_2$PGKDTA (obtained from Philippe Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.). The 5304 bp *Hpa*1–*Sal*1 fragment encompassing the 3′ end of exon E1a/b to exon 5 was subcloned into the *Nhe*1–*Sal*1 of the same vector. The expression of the positive selection cassette SAβGeo is under the control of the $GABA_{B(1)}$ promoter, and replaces a genomic region encompassing the exon E1a1 (start of the $GABA_{B(1a)}$ form) to the translation initiation of the $GABA_{B(1b)}$ form located in exon E1a/b (Figure 1a). A mock PCR-positive control for the targeted $GABA_{B(1)}$ locus was made by cloning a 4776 bp *Not*1 fragment corresponding to the 5′ part of the $GABA_{B(1)}$ locus into the *Not*1 site of pSAβGeo(LoxP)$_2$PGKDTA.

An *Xho*1 linearised targeting vector was electroporated into R1 embryonic stem (ES) cells and placed under G418 selection (300 mg l^{-1}). Surviving clones were first selected by PCR on purified DNA, with the primers CQ113 5′-TATTGCCTTGGTTACTCTAAA-3′ and CQ 114 5′-CCCTGGACTACTGCGCCCTAC-3′. Positive clones were expanded and frozen. Correct targeting was ascertained by Southern blotting of *Sca*1-digested genomic DNA, using a 253 bp probe located outside the targeting construct, and generated by PCR amplification using the primers 5′-ACTGAGCCTGGTCAAGGTCAG-3′ and 5′-CAACGCCACCGTGAAACCCT-3′. The frequency of homologous recombination with the gene trap vector was 16%. Two ES cell clones were injected into C57Bl/6 blastocysts and then transplanted into pseudopregnant females according to standard procedures. They gave rise to chimeric founders that transmitted the targeted allele through the germ line. Heterozygote progeny was interbred to produce homozygous mice. Genotyping was performed by Southern blotting or by PCR from tail DNA with the primers CQ170 5′-TCGCATTGTCTGAGTAGGTGT-3′, CQ171 5′-GCCCTCTTGCCTCTCTAAATC-3′ and CQ172 5′-CCTCCTCAGAACGGCGTGCAG-3′. The wild-type and mutant PCR products are 552 and 692 bp, respectively (data not shown).

RNA preparation and analysis

Total RNA was prepared from cerebellum with Trizol reagent (Life Technologies, Carlsbad, CA, U.S.A.), according to the manufacturer's recommendation. The S16 control was amplified with the sense primer 5′-AGGAGCGATTTGCTGGTGTGGA-3′ and the antisense primers 5′-GCTACCAGGCCTTTGAGATGGA-3′ (20 cycles, primers generate a 102 bp fragment). $GABA_{B(1)}$ cDNA was amplified with the primer pair 5′-CTGCCCGGATGTGGAACCTTA-3′ and 5′-TCAGCATACCACCCGATGAGA-3′ and $GABA_{B(2)}$ cDNA with the primer pair 5′-ATCGAGCAGATCCGCAACGAG-3′ and 5′-ACACAACTTGACCCGTGACCC-3′, yielding respective fragments of 427 and 970 bp after 25 cycles.

Western blot analysis

Total protein extracts were prepared by lysis and sonication in a radioimmunoprecipitation buffer (10 mM Tris 7.4, 0.15 M NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS, 0.5% aprotinin), in the presence of proteinase inhibitor cocktail

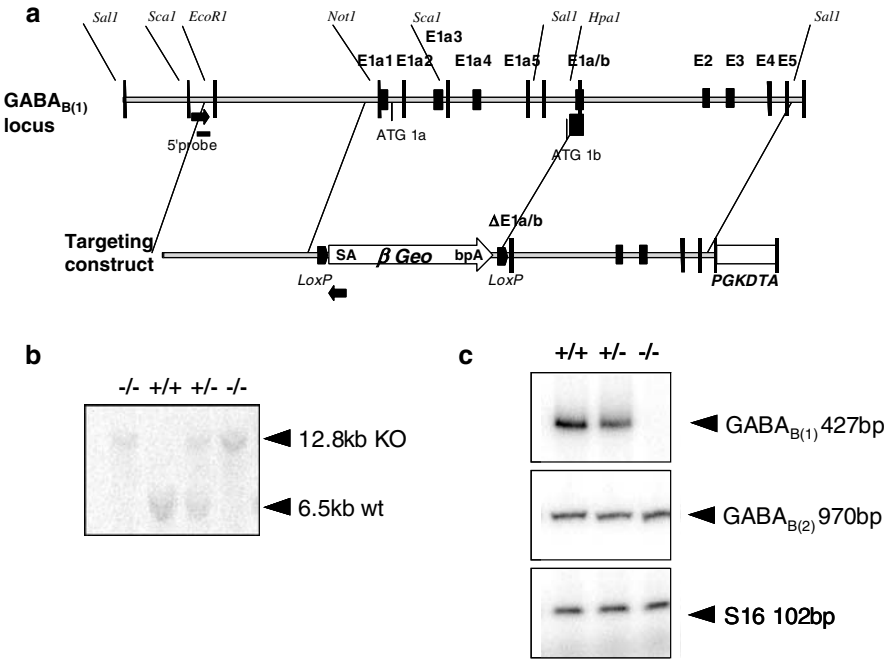

Figure 1 Strategy for deletion of the $GABA_{B(1)}$ gene. (a) Map of the murine $GABA_{B(1)}$ locus, showing the exon–intron structure of the gene. Exon nomenclature is according to Lamp *et al*. (2001). The targeting construct comprised the SAβGeo(LoxP)$_2$, flanked by a 4126 bp *Eco*R1–*Not*1 5′ fragment and a 5304 bp *Hpa*1–*Sal*1 3′ fragment. Recombination between the $GABA_{B(1)}$ locus and the targeting construct resulted in the replacement of the genomic sequence from exon E1a1 to the 5′ part of exon E1a/b. Homologous recombination was selected by PCR, using the primer CQ113 and CQ114 (arrows), and later confirmed by Southern blotting. (b) Southern blot analysis of *Sca*1-digested genomic DNA confirming the correct junction. 5′ probe label indicates the 253 bp probe generated by PCR amplification using the primers 5′-ACTGAGCCTGGTCAAGGTCAG-3′ and 5′-CAACGCCACCGT-GAAACCCT-3′. (c) Detection of $GABA_{B(1)}$ mRNA by RT–PCR gave rise to the expected 427 bp fragment, confirming the absence of $GABA_{B(1)}$ transcript in $GABA_{B(1)}$ mutant mice. $GABA_{B(2)}$ (970 bp RT–PCR product) transcript levels are normal in $GABA_{B(1)}$ mutant mice, despite the finding that the protein product could not be detected by Western blot. Amplification of the S16 sRNA was used as a control for efficient cDNA synthesis, and generated a 102 bp PCR product.

(Boehringer Mannheim, Indianapolis, IN, U.S.A.). Brain protein extract (30 μg) was resolved on a 4–12% Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane (Bis-Tris) gel in 3-(*N*-morpholino)propanesulphonic acid sodium dodecyl-sulphate running buffer, and electroblotted to polyvinylidene fluoride membranes by semidry transfer. Filters were blocked in phosphate-buffered saline containing 0.1%Tween 20 (PBST) and 5% milk over night in +4°C. The primary antibody was diluted in PBST with 5% nonfat dry milk, and incubated for 1 h in room temperature. Filters were washed three times for 15 min in PBST in room temperature, and subsequently, a secondary horseradish peroxidase-linked antibody (Amersham, Aylesbury, U.K.) was added, diluted in PBST with 5% nonfat dry milk for 45 min in room temperature. Before the detection, reaction filters were washed as above. Antibody–antigen interaction was visualised by enhanced chemiluminescence (Amersham, Aylesbury, U.K.).

$GABA_{B(1)}$ receptor protein expression was detected with two different polyclonal antibodies, Grim raised in rabbits (epitope: CEDVNSRRDILPDYELKLIHH) at AstraZeneca (4 μg ml^{-1} dilution) and AB1531 (Chemicon International, Temecula, CA, U.S.A.) (1 : 5000 dilution). $GABA_{B(2)}$ receptor protein was analysed with three different antibodies: two polyclonal, Gunlög raised (epitope: TEPSRTCKDPIEDIN-SPEHI) at AstraZeneca (4 μg ml^{-1} dilution), AB5394 (Chemicon International, Temecula, CA, U.S.A.) (1 : 5000 dilution) and one monoclonal G12020 (Transduction Laboratories, Lexington, KY, U.S.A.) (1 : 2500 dilution).

Evaluation of phenotype

Routine histological, haematological and clinical chemistry analysis was made on the mice (Safety Assessment, AstraZeneca R&D Södertälje, Sweden). The histopathological evaluation included all major tissues which were fixed in 10% formalin, embedded after dehydration in Histowax, cut at 4–6 μm and stained with haematoxylin/eosin. Apart from careful observation of the behaviour, some mice were videofilmed for 24 h.

Preparation of synaptic membranes from mouse brain

Mouse brain synaptic membranes were prepared from the whole brain of $GABA_{B(1)}^{+/+}$ or $GABA_{B(1)}^{-/-}$ mice, using the method described by Zukin *et al*. (1974) with some modifications. In brief, mouse whole brain was homogenised in 10 volumes of ice-cold buffer containing 0.32 M sucrose (Sigma-Aldrich, Steinheim, Germany), 10 mM Tris(hydroxymethyl)amino-methane (Tris; Sigma-Aldrich, Steinheim, Germany), 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF; Roche Diagnostics, Mannheim, Germany) and 20 μg ml^{-1} bacitracin, pH 7.4 (Sigma-Aldrich, Steinheim, Germany). The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was then centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended (by vortex) in ice-cold distilled water containing 0.1 mM AEBSF and 20 μg ml^{-1} bacitracin (pH set to 7.0) and centrifuged at 8000 × *g* for 20 min. The supernatant

and the upper layer of the pellet were centrifuged at 33,000 × *g* for 20 min. The pellet was resuspended in 50 mM Tris, pH 7.4, containing 0.1 mM AEBSF and 20 μg ml^{-1} bacitracin, and centrifuged at 33,000 × *g* for 20 min two times before it was snap frozen in methanol (Rathburn Chemicals, Walkerburn, Scotland)/dry ice and stored overnight at −70°C. Up to this point, the membranes were kept on ice at all times, and all centrifugation steps were performed at 4°C. The frozen pellet was thawed and washed six times in 50 mM Tris, pH 7.4, by centrifugation at 8000 × *g* for 10 min at 18°C. The resulting pellet was resuspended in TC buffer (50 mM Tris, 2.5 mM $CaCl_2$, pH 7.4), snap frozen in methanol/dry ice and stored at −70°C. Protein concentration was determined according using Bio-Rad's protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine gamma globulin as a standard.

[^{3}H]CGP54626 saturation-binding analysis

$GABA_B$ receptor sites in mice brain membranes were studied by saturation-binding analysis using the $GABA_B$ receptor antagonist [^{3}H]CGP54626A (Tocris, Bristol, U.K.). [^{3}H]CGP54626A saturation binding was measured by incubation of [^{3}H]CGP54626A (0.0051–100 nM, final concentrations) in 200 μl TC buffer containing 0.75% ethanol (Rathburn Chemicals, Walkerburn, Scotland) and 80 μg membrane protein. Nonspecific binding was determined in the presence of 1 mM GABA (Sigma-Aldrich, Steinheim, Germany). After incubation for 20 min at room temperature, incubations were terminated by rapid filtration through a glass fibre filter (Printed filtermat B filters, Wallac, Turku, Finland), which had been presoaked in 0.3% polyethyleneimine (Sigma-Aldrich, Steinheim, Germany), using a TOMTEC cell harvester (Orange, CT, U.S.A.). The filters were washed with 5 ml of 50 mM Tris (pH 7.4) at 4°C and dried for 1.5 min in a microwave followed by incubation at 55°C for 30 min. MeltiLex B/HS scintillator sheet was melted onto the filter, and radioactivity was determined in a Microbeta scintillation counter (Wallac, Turku, Finland). K_D and B_{max} values were calculated by fitting binding data to the equation $B = B_{max}/(1 + K_D/[L])$, using Xlfit for Microsoft Excel.

Measurement of body temperature and behaviour

Age-matched, C57Bl6/129Sv F1 hybrid $GABA_{B(1)}^{+/+}$, $GABA_{B(1)}^{+/-}$ and $GABA_{B(1)}^{-/-}$ mice were used. They were kept in Perspex cages at an ambient temperature between 21 and 23°C and a relative humidity between 52 and 56%. The lights were on between 0700 and 1800, and there was a gradual dimming of the lights to complete darkness between 1800 and 1900, and a gradual increase in illumination back to normal daylight between 0600 and 0700. The distribution of sexes was relatively even across the groups (see the legend of Figure 4), and there was no tendency for any difference between males and females with respect to basal temperature or drug-induced changes. A thermosensitive chip (BioMedic Data Systems, Maywood, NJ, U.S.A.) was implanted in the interscapular region under brief isoflurane anaesthesia, and the animals were allowed to recover for at least 1 day. The animals had free access to food and water, except for during the experiment. On the experimental day, the mice were placed in individual cages between 0900 and 1000, and the ambient temperature was 20.5 ± 1.0°C. After 30 min, three basal temperature recordings were made using a transponder (IPTT-100, BioMedic Data Systems, Maywood, NJ, U.S.A.) communicating with a PC for data acquisition. In preliminary experiments, the system was evaluated in mice by measuring the interscapular temperature and rectal temperature at the same time. There was a strong correlation between the two ($R^2 = 0.86$, $P < 0.0001$, $n = 23$). The thermosensitive chips were calibrated by the producer in the range from 32 to 43°C, and they were calibrated against a thermistor in a water bath before implantation. The resolution of the chips was 0.1°C. Compounds (baclofen 9.6 mg kg^{-1}, RBI, Natick, MA, U.S.A.; GHB 400 mg kg^{-1}, Sigma-Aldrich, Steinheim, Germany, and muscimol 2 mg kg^{-1}, Tocris Cookson, Bristol, U.K.) were injected subcutaneously at 5 ml kg^{-1} after the last measurement. The doses were chosen based on pilot experiments in which they were found to produce a significant hypothermia. Measurements were then made at regular intervals (see Figure 4). Behavioural scoring was made at each time point, and the behavioural data are presented as the maximal effect. The following definitions were used for behavioural effects:

0, no effect;
1, exophthalmus, slight motor impairment;
2, more pronounced motor impairment;
3, immobile with intact righting reflex;
4, no righting reflex, disturbed respiration, occasional seizures, detectable but very low muscle tonus;
5, paralysed, no muscle tonus, moribund (killed for ethical reasons).

The behaviour was scored by the same experienced observer (MBD) in all experiments. The doses used were obtained from pilot dose–response experiments. All animal experiments were approved by the Animal Ethics Committee of the Göteborg region.

The Student's *t*-test or Mann–Whitney *U*-test was used to compare the groups. A *P*-value below 0.05 was considered to be statistically significant. In the experiments on drug-induced changes in body temperature, only nadir temperatures were compared.

Results

Cloning and characterisation of the 5′-end of the mouse $GABA_{B(1)}$ gene

A mouse genomic library was screened and a 17 kb of the $GABA_{B(1)}$ gene was cloned and characterised. The cloned fragment encompassed exons E1a1 to 5. The exon/intron organisation was found to be similar to the human $GABA_{B(1)}$ gene (Ekstrand, 1999), and predicts the expression of the two receptor isoforms, 1a and 1b, as previously described for rats (Kaupmann *et al*., 1997) and for humans (Ekstrand, 1999). The ATG initiating the translation of the mouse $GABA_{B(1a)}$ isoform is located in exon E1a2, and the ATG for the $GABA_{B(1b)}$ isoform in exon E1a/b (Figure 1). The predicted partial mouse $GABA_{B(1a)}$ isoform amino-acid sequence encoded by exons E1a2 to 5 is 99.5% homologous to the human (Ekstrand, 1999) and 99.2% homologous to the rat sequence (Kaupmann *et al*., 1997), and the predicted partial mouse $GABA_{B(1b)}$ isoform amino-acid sequence encoded by exons

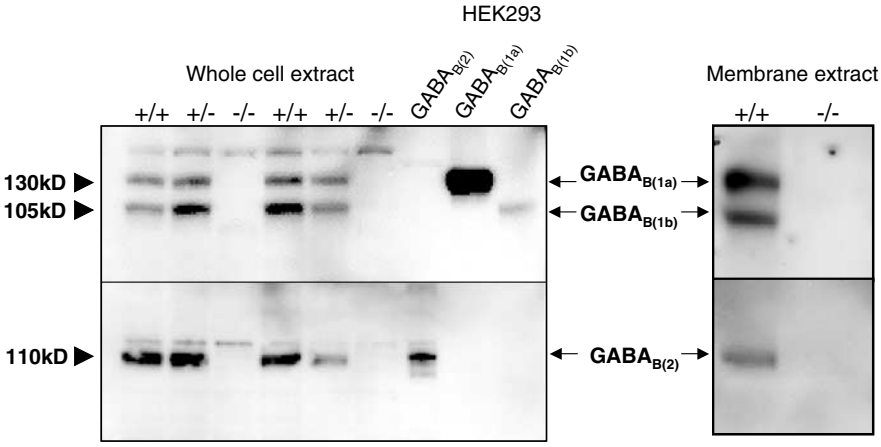

Figure 2 Western blot analysis showing the expression of $GABA_B$ receptor isoforms in whole brain and brain membrane extracts from $GABA_{B(1)}^{+/+}$, $GABA_{B(1)}^{+/-}$ and $GABA_{B(1)}^{-/-}$ mice. Equal amounts of proteins were separated by a 4–12% gradient Bis-Tris gel, and transferred on polyvinylidene difluoride membranes. Two bands with the apparent molecular weights of 130 and 105 kDa were detected when probing the Western blot with the polyclonal antibody Grim. These bands, corresponding to $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptor proteins, were identified in the brain extract and membrane extract of wild-type, heterozygote, but not mutant $GABA_{B(1)}$ mice, and in HEK293 cell lysate transfected with $GABA_{B(1a)}$ and $GABA_{B(1b)}$ expression vector. Similarly, $GABA_{B(2)}$, 110 kDa, was detected by the Gunlög polyclonal antibody in HEK293 cell transfected with a $GABA_{B(2)}$ expression vector, and in $GABA_{B(1)}^{+/+}$ and $GABA_{B(1)}^{+/-}$ mice, but not in $GABA_{B(1)}^{-/-}$.

E1a/b to 5 is 97.7% homologous to the human and 100% homologous to the rat sequence.

Phenotype of $GABA_{B(1)}^{-/-}$ *mice*

Homologous recombination between the targeting vector and the $GABA_{B(1)}$ locus resulted in the deletion of the exons E1a1 to E1a/b and their replacement by the βGeo gene. Breeding of heterozygous mice resulted in the generation of $GABA_{B(1)}^{-/-}$ mice at the expected Mendelian ratios. Southern blot analysis, RT–PCR and Western blot confirmed the absence of $GABA_{B(1)}$ receptor transcript and protein (Figures 1,2).

The lifespan of $GABA_{B(1)}^{-/-}$ mice was greatly reduced so that only few $GABA_{B(1)}^{-/-}$ lived longer than 8 weeks in the C57Bl6/129Sv F1 hybrid background used in this study. The exact cause of the premature death was not formally determined, but it is reasonable to assume that it was secondary to spontaneous seizures occurring intermittently. Apart from the epileptic behaviour, no overt behavioural changes were seen. In addition, no abnormalities were observed with respect to histological, clinical chemistry and haematological parameters. The latter two included the following: glucose, bilirubin (total), urea, creatinine, total protein, albumin, albumin/globulin ratio, cholesterol, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, sodium, potassium, calcium, haematocrit, haemoglobin, erythrocytes, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, leucocytes, platelets, neutrophils, eosinophils, basophils, lymphocytes and monocytes. There were no differences in body weight. For instance, male $GABA_{B(1)}^{(+/+)}$ mice weighed 28.5 ± 2.1 g ($n=5$) 60–69 days after birth. The corresponding figure for male $GABA_{B(1)}^{(-/-)}$ mice was 26.1 ± 1.0 g ($n=8$).

Expression of $GABA_B$ *subunits*

Successful targeting of the $GABA_{B(1)}$ gene was confirmed by complete absence of $GABA_{B(1)}$ mRNA and protein (Figures 1, 2). Furthermore, the expression of $GABA_{B(2)}$ receptor was abolished in whole-brain homogenates and in brain membranes (Figure 2).

Comparison of the receptor binding of CGP54626A in brain synaptic membranes from $GABA_{B(1)}^{(+/+)}$ *and* $GABA_{B(1)}^{(-/-)}$ *mice*

Ligand binding at $GABA_B$ receptor sites in whole-brain synaptic membranes from $GABA_{B(1)}^{(+/+)}$ and $GABA_{B(1)}^{(-/-)}$ mice was studied using [^{3}H]CGP54626A. In membranes from $GABA_{B(1)}^{(+/+)}$ mice, [^{3}H]CGP54626 bound with an apparent K_D of 4.3 ± 0.65 nM (mean $\pm$ s.e.m., $n=3$) and a B_{max} of 4.6 ± 3.0 pmol mg^{-1} (mean $\pm$ s.e.m., $n=3$). In contrast, no specific [^{3}H]CGP54626 binding could be detected in membranes from $GABA_{B(1)}^{(-/-)}$ mice.

Hypothermia and behaviour

Analysis on basal body temperature was done on pooled data from all experiments before drug administration. Unexpectedly, the basal temperature of $GABA_{B(1)}^{-/-}$ mice was significantly lower than that of the two other groups, the difference being about 1°C ($P<0.001$; Figure 3). There was no difference between males and females in this regard (data not shown).

Baclofen (9.6 mg kg^{-1}) produced a marked hypothermia, which reached its nadir at 60–80 min after administration, and subsequently returned towards baseline levels (Figure 4). This effect was accompanied by behavioural alterations (Table 1). The hypothermic and behavioural effects of baclofen in $GABA_{B(1)}^{+/-}$ mice were identical to those seen in $GABA_{B(1)}^{+/+}$ mice, but the $GABA_{B(1)}^{-/-}$ mice did not respond to baclofen at all.

A high dose of GHB (400 mg kg^{-1}) was required to elicit hypothermia and behavioural changes in wild-type and heterozygote mice (Figure 4, Table 1). As was the case after baclofen administration, GHB did not produce any hypother-

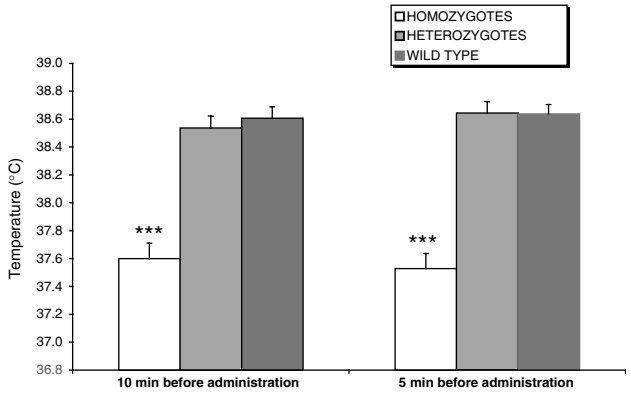

Figure 3 Basal temperature in $GABA_B^{+/+}$, $GABA_B^{+/-}$ and $GABA_B^{-/-}$ mice. Temperature was measured using temperature-sensitive subcutaneous chips, which allowed measurement without restraining the animals. ***$P<0.001$, Student's two-tailed, unpaired t-test, $n=43$ (28 males, $GABA_{B(1)}^{+/+}$), 49 (26 males, $GABA_{B(1)}^{+/-}$), 40 (23 males, $GABA_{B(1)}^{-/-}$). Predrug measurements from other studies were also included in the material.

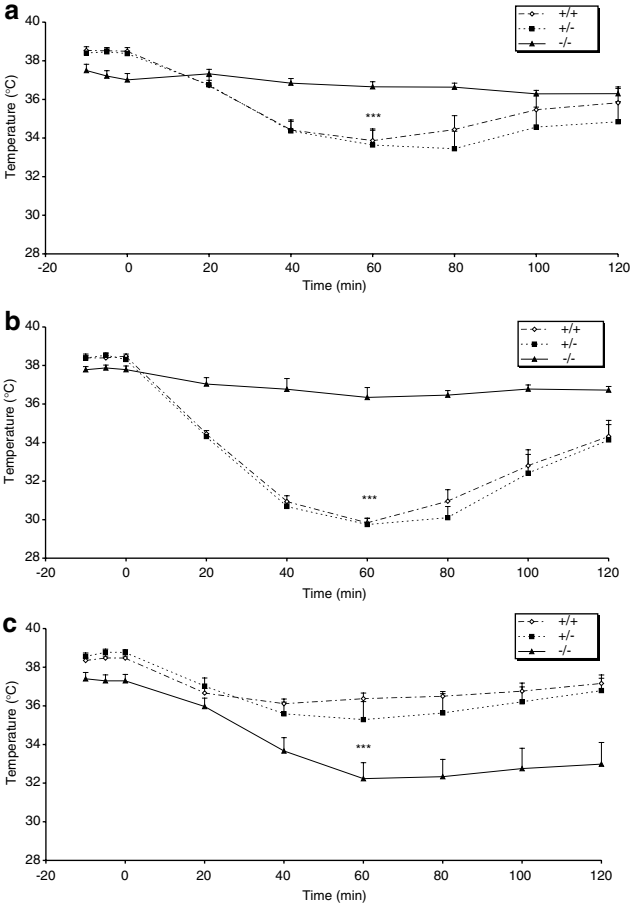

Figure 4 Effects of baclofen (a), GHB (b) and muscimol (c) on body temperature in $GABA_B^{+/+}$, $GABA_B^{+/-}$ and $GABA_B^{-/-}$ mice. Drugs were administered at time 0; $n=11$ (eight males), 12 (seven males) and 9 (five males) ($GABA_B^{+/+}$, $GABA_B^{+/-}$ and $GABA_B^{-/-}$ baclofen groups, respectively); $n=9$ (five males), 11 (three males) and 14 (eight males) ($GABA_B^{+/+}$, $GABA_B^{+/-}$ and $GABA_B^{-/-}$ GHB groups, respectively); $n=8$ (six males), 9 (five males) and 6 (four males) ($GABA_B^{+/+}$, $GABA_B^{+/-}$ and $GABA_B^{-/-}$ muscimol groups, respectively); ***$P<0.001$, Student's t-test.

Table 1 Effects of baclofen, GHB and muscimol on the behaviour in $GABA_{B(1)}^{+/+}$, $GABA_{B(1)}^{+/-}$ and $GABA_{B(1)}^{-/-}$ mice

Genotype	*Behavioural effect (mean±s.e.m., n)*		
	Baclofen	*GHB*	*Muscimol*
$GABA_{B(1)}^{+/+}$	2.0±0.1(11)	4.5±0.0(9)	1.8±0.4(8)
$GABA_{B(1)}^{+/-}$	1.7±0.3(12)	4.3±0.2(11)	1.8±0.5(9)
$GABA_{B(1)}^{-/-}$	0.0±0.0(9)*	0.0±0.0(14)*	Not assessed[a]

Values are means+s.e.m. (n). *$P<0.01$, $GABA_{B(1)}^{+/+}$ vs $GABA_{B(1)}^{-/-}$ (Mann–Whitney U-test). [a]Could not be assessed due to a qualitative change in behaviour.

mic or behavioural effects in $GABA_{B(1)}^{-/-}$ mice. The response to GHB was not sex-dependent in any of the groups.

Even if it can be assumed that the spectrum of behavioural changes would differ between muscimol and $GABA_B$ receptor agonists using more detailed evaluation, the behavioural scale used for mice given baclofen and GHB could also be applied to $GABA_{B(1)}^{+/+}$ and $GABA_{B(1)}^{+/-}$ mice. These two groups did not differ in this regard (Table 1). However, the scale could not be used on $GABA_{B(1)}^{-/-}$ mice, since they showed a completely different pattern of behaviour after muscimol treatment ($2\,mg\,kg^{-1}$) in that they were extremely sensitive to, for example, handling which induced wild running and jumping ('popcorn') behaviour.

Discussion

The phenotype of the $GABA_{B(1)}^{-/-}$ mice generated in our laboratory is similar to that described previously (Prosser *et al*., 2001; Schuler *et al*., 2001). In particular, the observations confirm the finding that the lifespan is short (a few weeks) in mice with a C57Bl6/129SvJ background (Prosser *et al*., 2001), and that this is most likely due to generalised epileptic seizures. In contrast, $GABA_{B(1)}$ null mutants with a Balb/*c* background also have seizures, but their lifespan seems to be normal (Schuler *et al*., 2001). We also confirm that there are no histopathological alterations nor any routine clinical chemistry changes associated with deletion of the $GABA_{B(1)}$ gene. Similar to the findings reported previously, $GABA_{B(2)}$ protein was undetectable in whole-brain extracts and in membrane preparations (Prosser *et al*., 2001; Schuler *et al*., 2001). Since the expression of $GABA_{B(2)}$ mRNA is normal in $GABA_{B(1)}^{-/-}$ mice (present results and Schuler *et al*., 2001), this suggests that the absence of $GABA_{B(2)}$ protein is due to a translational or stability defect.

In agreement with the study of Schuler *et al*. (2001), baclofen neither affected temperature regulation nor behaviour in $GABA_{B(1)}^{-/-}$ mice, which confirms the notion that the $GABA_{B(1)}$ subunit is necessary for these effects of baclofen. Since temperature was measured in a region where brown adipose tissue can be found, it may be argued that rectal measurement might provide a better reflection of core body temperature. However, baclofen has been shown in a number of studies to produce hypothermia, as measured with a rectal probe in awake rats and mice (e.g. Gray *et al*., 1987; Zarrindast & Oveissi, 1988; Schuler *et al*., 2001). Hyperthermia has been reported after systemic administration of baclofen to anaesthetised rats, but the temperature changes interscapularly and rectally parallel each other well (Addae *et al*., 1986). The

hyperthermic response is only seen under anaesthesia, or after very high doses of baclofen given to conscious rats (Zarrindast & Oveissi, 1988).

An unexpected finding was that $GABA_{B(1)}^{-/-}$ mice had a basal hypothermia which is particularly surprising in view of the hyperlocomotor activity observed in $GABA_{B(1)}^{-/-}$ mice (data not shown; Schuler *et al*., 2001). Telemetric studies on mice have clearly demonstrated that locomotor activity and body temperature are closely correlated (Johansson *et al*., 1999). Although the hypothermic effects of $GABA_B$ receptor agonists have been known for a long time (e.g. Gray *et al*., 1987), antagonist experiments have to our knowledge not suggested that there would be any endogenous $GABA_B$ergic tone in this system. In a previous study on $GABA_{B(1)}^{-/-}$ mice, lack of effect of baclofen on body temperature was established, but no data on basal body temperature were presented (Schuler *et al*., 2001). While an earlier study revealed that $GABA_{B(1)}^{-/-}$ mice weighed significantly less than wild-type mice by 3 weeks of age (Prosser *et al*., 2001), this could not be verified in our colony and therefore, differences in body weight cannot explain the disparities in body temperature. Whether the basal hypothermia reflects direct involvement of $GABA_B$ receptors in thermoregulatory circuits or if this depends on secondary alterations remains to be studied. At any rate, the effects of $GABA_{B(1)}$ deletion on body temperature warrant further studies on general energy metabolism in these mice. In addition, body temperature was measured at room temperature in the present experiments. Since this is well below the thermoneutral zone for the mouse, future studies should include body temperature measurements at thermoneutrality.

Several mechanisms for the actions of GHB in the CNS have been proposed. One hypothesis is that GHB directly stimulates $GABA_B$ receptors. However, even if very high doses of GHB are required to produce CNS effects, the affinity of GHB for $GABA_B$ receptors is very low (Lingenhoehl *et al*., 1999), or almost undetectable (unpublished own publications). There seem to exist specific GHB-binding sites distinct from $GABA_B$ receptor-binding sites, through which GHB can exert its effects (Snead, 2000). Further, GHB activates G-protein-coupled receptors separate from those stimulated by baclofen. However, it is also possible that administration of GHB elevates the cerebral levels of GABA acting on $GABA_B$ receptors (see Maitre (1997) for review). While the present findings do not rule out the existence of GHB receptors, they unambiguously show that the $GABA_{B(1)}$ receptor is necessary for the effects of GHB on body temperature and behaviour. Consistent with this is the report that selective $GABA_B$ receptor antagonists abolish the sedative/hypnotic effects of GHB, while a putative GHB antagonist has no inhibitory effect (Carai *et al*., 2001). Only one dose of GHB was used, and it caused profound effects on body temperature and behaviour. Therefore, although unlikely, it cannot be excluded that effects of lower doses of GHB may not be exclusively mediated by $GABA_B$ receptors.

Muscimol-induced hypothermia was significantly enhanced in $GABA_{B(1)}^{-/-}$ mice. This finding indicates that there are tonically active $GABA_{B(1)}$ receptors which counteract the hypothermic effects of muscimol, and it reaffirms the notion that discovery of phenotypic changes in mice carrying gene deletions sometimes depends on the application of external challenges. The mechanism behind this effect is presently unknown. It may be due to global or region-specific changes in the expression of $GABA_A$ receptors. Alternatively, the absence of $GABA_B$ receptors may alter the characteristics of the circuitry controlling body temperature after $GABA_A$ receptor stimulation. Acute blockade of $GABA_B$ receptors with CGP62349 failed to affect muscimol-induced hypothermia in wild-type mice (unpublished own observations), which indicates that complete loss of the $GABA_B$ response is required to enhance the muscimol-induced hypothermia, or that absence of $GABA_B$ from the embryonic stage and onwards underlies the hypersensitivity to muscimol. The effect was not generalised for all hypothermia-inducing drugs in that preliminary experiments failed to reveal differences between $GABA_{B(1)}^{+/+}$ and $GABA_{B(1)}^{-/-}$ mice in terms of hypothermia induced by the cholinomimetic oxotremorine. In addition, the markedly altered behaviour seen in $GABA_{B(1)}^{-/-}$ mice after muscimol administration may reflect the inhibitory effects of the $GABA_B$ system on $GABA_A$-ergic mechanisms. The 'popcorn' behaviour observed in $GABA_{B(1)}^{-/-}$ mice has been reported earlier after pharmacological treatment with cannabinoid receptor agonists (Patel & Hillard, 2001), and has been interpreted as hyper-reflexia. At the cellular level, inhibitory effects of $GABA_B$ receptor stimulation on $GABA_A$-mediated currents (Obrietan & van den Pol, 1998) may underlie both enhanced hypothermic effects as well as changes in behavioural response recorded after muscimol administration. Additional studies are needed to further elucidate the effects of muscimol in $GABA_{B(1)}^{-/-}$ mice. However, the present results clearly suggest that in contrast to some claims (Yamauchi *et al*., 2000), muscimol does not stimulate $GABA_B$ receptors *in vivo*.

In summary, the present study demonstrates that deletion of the $GABA_{B(1)}$ receptor gene is associated with basal hypothermia, abolition of responsiveness to GHB and hypersensitivity to $GABA_A$ receptor stimulation.

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