GABA_A Receptor Subtypes as Neuronal Substrates for Selective Actions of Benzodiazepines and General Anesthetics

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presented by
ANJA ZELLER
M.Sc., University of Berne
born 01.03.1978
citizen of Germany

accepted on the recommendation of
Prof. Isabelle Mansuy, examiner
Prof. Uwe Rudolph, co-examiner
Prof. Hanns Möhler, co-examiner

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Zwar weiss ich viel, doch will ich alles wissen.

Wagner, Faust, Goethe
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2. Synopsis

General anesthetics are widely used to induce a state conducive for surgery. Despite their importance and their use in clinical practice for more than 160 years, the mechanism and sites of action of general anesthetics are only now beginning to be understood. This study was designed to identify molecular targets and neuronal circuits mediating specific actions of general anesthetics by analyzing knock-in and knock-out mouse models.

Studies in recombinant systems revealed that the activity of neuronal ligand-gated and other ion channels is modulated by general anesthetics. The observation that most general anesthetics (with the exceptions of nitrous oxide and ketamine) modulate the activity of the GABA<sub>A</sub> receptor prompted us to evaluate the potential involvement of GABA<sub>A</sub> receptor subtypes in anesthetic actions. We investigated this in vivo by analyzing the effects of general anesthetics in mice with knock-in point mutations that render individual receptor subtypes insensitive to these drugs. The usefulness of this approach has been demonstrated previously in β3(N265M) knock-in mice by showing that the immobilizing action of etomidate and propofol and in part their hypnotic action are mediated by β3-containing GABA<sub>A</sub> receptors. We determined that the respiratory depressant action of etomidate and propofol is also mediated by β3-containing GABA<sub>A</sub> receptors, whereas the hypothermic, heart rate depressant, anterograde amnestic and sedative actions are mediated by other targets, presumably β2-containing GABA<sub>A</sub> receptors. The barbiturate pentobarbital showed a similar dependence of immobility and hypnosis on the β3-containing GABA<sub>A</sub> receptors; however, in contrast to the observations made for etomidate and propofol, the respiratory depressant action was β3-independent.

To identify the precise subunit composition of GABA<sub>A</sub> receptors mediating immobility and hypnosis, diazepam-induced immobilisation and hypnosis were examined in α1(H101R), α2(H101R), α3(H126R) and α5(H105R) knock-in mice in which GABA<sub>A</sub> receptors containing the respective mutated α subunit are diazepam-insensitive. The hypnotic action of diazepam was partly reduced in α5(H105R) knock-in mice, whereas the immobilizing action of diazepam was essentially absent in α3(H126R) and α5(H105R) mice, demonstrating that both α3- and α5-containing GABA<sub>A</sub> receptors are necessary for mediating this action.
To assess the involvement of cortical pyramidal neurons in sedation (measured as a reduction of motor activity) induced by CNS depressant drugs, mice with a knockout of the α1 subunit specifically in forebrain pyramidal neurons were generated. While it was known from studies with α1(H101R) mice that diazepam-induced sedation is mediated by α1-containing GABA_A receptors, the brain region or circuits harboring the relevant α1-containing GABA_A receptors were unknown. Our experiments suggest that diazepam-induced sedation is critically dependent on pyramidal neurons in neocortex or hippocampus.

Based on a variety of experiments both in animals and in humans, brain regions that mediate different effects of CNS depressants are being identified. fMRI experiments in humans suggested that the cortex is the site where small, sedative doses of general anesthetics first attenuate neuronal activity. Higher doses of general anesthetics decrease the blood flow in subcortical areas like thalamus and midbrain reticular formation. From spinal cord transection experiments in animals it is known that immobility is largely mediated by the spinal cord.

In summary, our studies in α and β subunit knock-in mice, taken together with previous studies by other groups, suggest that α3β3γ2 and α5β3γ2 GABA_A receptors in the spinal cord mediate the immobilizing action, that α5β2/3γ2 GABA_A receptors in subcortical structures like pons, midbrain and hypothalamus mediate the hypnotic action and that α1β2γ2 GABA_A receptors in cortical pyramidal cells mediate the sedative action of CNS depressant drugs.

Thus, we were able to identify molecular targets and neuronal circuits mediating specific actions of general anesthetics. These new insights provide novel opportunities for the development of more specific CNS depressant drugs.
3. Zusammenfassung


Um die genaue Zusammensetzung, d.h. Kombination von Untereinheiten des GABA_A-Rezeptors zu ermitteln, die die immobilisierende und hypnotische Wirkung von Allgemeinanästhetika vermitteln, untersuchten wir diazepaminduzierte Immobilisierung und Hypnose in α1(H101R), α2(H101R), α3(H126R) and α5(H105R) punktmutierten Mäuse, in denen GABA_A Rezeptoren mit der jeweiligen
punktmutierten α Untereinheit Diazepam-insensitiv sind. Die hypnotische Wirkung von Diazepam war teilweise abhängig von α5-enthaltenden GABAA-Rezeptoren, während die immobilisierende Wirkung von Diazepam in α3(H126R) und α5(H105R) punktmutierten Mäusen nicht mehr vorhanden war. Dies zeigt, dass sowohl α3- als auch α5-enthaltenden GABAA Rezeptoren notwendig sind für die Vermittlung dieses Diazepameffekts.

Um zu bestimmen, ob kortikale Pyramidenzellen an der durch ZNS-dämpfende Medikamente verursachten Sedation beteiligt sind, stellten wir Mäuse her, in denen die α1-Untereinheit spezifisch in den Pyramidenzellen des Vorderhirns nicht exprimiert wird. Durch Studien mit α1(H101R) Mäusen ist bereits bekannt, dass Sedation von α1-enthaltenden GABAA-Rezeptoren vermittelt wird; die Hirnregionen und neuronalen Schaltkreise, die Sedation vermitteln, waren aber noch unklar. Unsere Experimente zeigen, dass die von Diazepam induzierte Sedation von Pyramidenzellen im Neocortex oder Hippocampus abhängt.


Zusammengefasst deuten unsere Studien in α- und β-punktmutierten Mäusen, in Verbindung mit den Ergebnissen anderer Arbeitsgruppen darauf hin, dass α3β3γ2 und α5β3γ2 GABAA-Rezeptoren im Rückenmark die immobilisierende Wirkung, α5β2/3γ2 GABAA-Rezeptoren in subkortikalen Strukturen wie Pons, Mittelhirn und Hypothalamus die hypnotische Wirkung, und α1β2γ2 GABAA Rezeptoren in kortikalen Pyramidenzellen die sedative Wirkung von Allgemeinanästhetika vermitteln.

Folglich konnten wir sowohl molekulare Ziele als auch neuronale Schaltkreise, die spezifische Wirkungen von Allgemeinanästhetika vermitteln, identifizieren. Diese Erkenntnisse zeigen Möglichkeiten zur Entwicklung neuer anästhetisch wirkender Medikament mit einem spezifischere Wirkspektrum auf.
4. Introduction

General anesthetics are used to render patients unaware of and unresponsive to painful stimulation and are a prerequisite for many surgical procedures. Already in ancient times, ethanol, hashish and opium were utilized, since they produce some insensibility and obliviousness to pain, however, these agents lacked an essential requirement for a good general anesthetic - the controllability and thus therapeutic safety. The introduction of general anesthesia into clinical practice started in 1846 with the first public demonstration of an ether narcosis during an operation at the Massachusetts General Hospital in Boston, which was an essential milestone that opened the way for the development of modern surgical practice. At the beginning of the 20th century intravenous anesthetics became available.

4.1. General anesthesia

General anesthesia is commonly defined as a reversible depression of central nervous system functions sufficient to permit surgery to be performed without movement, obvious distress, or recall. It is a behavioural state easily recognized by clinical practitioners but difficult to describe precisely. Depending on the clinical procedure, effective anesthesia requires varying degrees of immobility, amnesia, unconsciousness/hypnosis, analgesia, muscle relaxation and depression of autonomic reflexes (Harrison and Flood, 1998). No general anesthetic provides all of these effects, although immobility, unconsciousness/hypnosis and amnesia are behavioural hallmarks of most general anesthetics (Marshall and Longnecker, 1996). In clinical use, several anesthetic agents with different properties are typically combined, also with other drugs such as muscle relaxants to achieve the desired clinical effects.

4.2. Classes of general anesthetics

General anesthetics are a quite heterogeneous group of compounds with a wide range of structurally diverse molecules. Based on the mode of application, they are generally divided into volatile anesthetics and intravenous anesthetics.
4.2.1. Volatile anesthetics

Volatile anesthetics were the first general anesthetics used. Even long before the first demonstrations of an ether narcosis, in 1800 Humphrey Davy noted that nitrous oxide causes euphoria, analgesia and loss of consciousness and suggested that it might be used to relieve pain during surgery. With the exception of N₂O (also called laughing gas) all volatile anesthetics introduced before 1950 have been abandoned in clinical practice mainly because of unfavourable pharmacokinetic properties, side effects like respiratory depression, and hepatic or renal toxicity due to metabolites. Therefore, modern volatile anesthetics are substances which are poorly metabolized. The most commonly used volatile anesthetics are the halogenated ethers isoflurane, desflurane, and sevoflurane. N₂O is commonly used in combination with other anesthetics because it provides only an analgesic component. Mixing of NO₂ and volatile anesthetics decreases the dose requirements for the volatile anesthetics by about 60%. Volatile anesthetics render a patient unconscious within a minute after start of application. The concentration necessary for induction of anesthesia is higher.
than the concentration required for maintenance. Recovery after termination of application is swift, especially with sevoflurane and desflurane.

4.2.2. Intravenous anesthetics

Clinically used intravenous anesthetics include etomidate, propofol and ketamine and barbiturates such as thiopental. An advantage of all intravenous anesthetics is their rapid onset of action. Whereas barbiturates, etomidate, and propofol have a hypnotic profile, ketamine is more analgesic than hypnotic.

Thiopental is structurally very similar to pentobarbital and is the most frequently used barbiturate in use as an induction anesthetic. It has no analgesic action and induces strong respiratory depression at high concentrations.

Etomidate was introduced in the 1970s. It has a wider therapeutic range compared to barbiturates, but essentially no analgesic effect, similar to barbiturates. It is rapidly metabolized and therefore produces a swift recovery. Etomidate has a minimal depressant effect on the cardiovascular system and may be used in hemodynamically unstable patients (Bergen and Smith, 1997). During induction, it can cause excitatory effects, such as involuntary movements and myoclonus (Doenicke et al., 1999; Krieger et al., 1985). Due to its inhibitory effect on adrenal steroidogenesis, etomidate is not used for longer anesthesia.

Propofol was introduced in the mid 1980s. Like most other intravenous anesthetics it has little analgesic effect. After bolus injection, a short apnoea frequently occurs. The duration of action is short. Propofol is rapidly metabolized in the liver to inactive metabolites. Today, it is the most commonly used intravenous anesthetic. It can be used for continuous infusion during prolonged surgery without any additional anesthetic being required. However, it is often used in combination with opioids which reduce the propofol dose required for induction of anesthesia (Lichtenbelt et al., 2004).

Ketamine has, compared with other intravenous anesthetics, a very different profile of action. Ketamine produces an atypical behavioural state comprising of sedation, immobility, amnesia, analgesia and a feeling of dissociation from the environment without true loss of consciousness (Adams et al., 1992).

The neurosteroidal anesthetic alphaxalone was clinically used in combination with alphadolone, with which it was combined to increase its solubility. Alphaxalone, when given alone, produces sedation, muscle relaxation and hypnosis. Alphadolone, when
given alone, produces only analgesia (Nadeson and Goodchild, 2000). The mixture first became available in 1970 under the name of Althesin®, but was withdrawn from clinical use in 1984 due to anaphylactic reactions caused by the vehicle, polyoxyethylated castor oil (Cremophor EL). A mixture with the same formulation, Saffan®, was used in veterinary practice in Switzerland until 2005.

Benzodiazepines are often used as a premedication to achieve anxiolysis and sedation before the actual anesthesia. They are also often used as an induction agent or in combination with opioids and neuroleptics for neuroleptanalgesia which consists only of analgesia, indifference and deep sedation, with patients still able to respond to verbal commands. This kind of anesthesia is used for neurosurgery and interventions where strong sedation but not complete anesthesia is desired. The fast-acting benzodiazepine midazolam is the most frequently used benzodiazepine in anesthesia.

### 4.3. Mechanisms of general anesthetic action

Based on experiments reported independently by Hans Meyer and Charles Ernest Overton at the end of the 19th century, a non-specific mechanism of action was postulated for general anesthetics. Meyer and Overton determined that the potency of general anesthetics correlated well with their water/oil partition coefficients (Meyer, 1899; Overton, 1901). The traditional view since then has been that general anesthetics exert their effects by dissolving in cell membranes, particularly in the CNS (Seeman, 1972). The presence of general anesthetics is thought to perturb the structural and dynamic properties of the lipid bilayer so that the function of crucial but unspecified membrane proteins is affected. Over time, numerous inconsistencies between experimental observations and non-specific theories of general anesthesia were found (Franks and Lieb, 1984). The main problems were that some molecules predicted to be anesthetics did not produce anesthesia and that anesthetic isomers show different potencies. Alterations in membrane bilayer fluidity as an effect of anesthetics on lipids is often negligible and easily reproduced by very small increases in ambient temperature. In the 1980s, general anesthetics, i.e. the volatile anesthetics halothane and ether, and alcohols and ketones were shown to bind to the soluble enzyme firefly luciferase and inhibit its activity (Franks and Lieb, 1984), questioning the necessity of the lipid bilayer as an essential “target” for general
What are the targets for general anesthetic drugs?

There are many molecular targets known whose activity is modulated by at least one general anesthetic. These include ligand-gated ion channels, such as GABA<sub>A</sub> receptors, glycine receptors, nicotinic acetylcholine receptors, 5-HT<sub>3</sub> (5-hydroxytryptamine, type 3) receptors, AMPA, kainate and NMDA receptors (Figure 2). Recently, the two-pore domain potassium channels (Franks and Honore, 2004) have also emerged as potential targets for general anesthetics. The ligand-gated ion channels are involved in both excitatory and inhibitory neurotransmission. Anesthetics can act either by enhancing inhibitory synaptic function through activation of inhibitory ion channels or by reducing excitatory synaptic function through inhibition of excitatory ion channels (Rudolph and Antkowiak, 2004). Ion channels thus have emerged as strong candidates for the role of molecular mediators of the CNS effects of general anesthetics (Franks and Lieb, 1996). Volatile anesthetics generally bind to a wider range of targets than intravenous anesthetics.

With the exception of ketamine and nitrous oxide, all general anesthetics show a prominent potentiation of GABA<sub>A</sub> and glycine receptor function, and a strong inhibition of neuronal nACh receptors. Barbiturates markedly potentiate and/or directly activate GABA<sub>A</sub> receptors and inhibit nACh, AMPA and kainate receptors (Krasowski and Harrison, 1999). Propofol and etomidate are quite selective for GABA<sub>A</sub> receptors, however, other targets include for etomidate the 11β-hydroxlyse, whose inhibition causes suppression of adrenal steroidogenesis (Preziosi and Vacca, 1988), and for propofol the strychnine-sensitive glycine receptor (Pistis et al., 1997) and, at high concentrations, which are not clinically relevant, the P2X receptors.
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Steroidal anesthetics like alphaxalone potentiate GABA\textsubscript{A} receptors and inhibit neuronal nACh receptors. Ketamine apparently does not affect GABA\textsubscript{A} receptors but inhibits NMDA receptors potently and neuronal nACh receptors with lower potency. Thus, only GABA\textsubscript{A} receptors are sensitive to almost every volatile and intravenous anesthetic at clinically relevant concentrations.

4.5. Ligand-gated ion channels

The ligand-gated ion channels include the \(\gamma\)-aminobutyric acid type A (GABA\textsubscript{A}), glycine, serotonin-3 (5-HT\textsubscript{3}) and nicotinic acetylcholine (ACh) receptors along with the alpha-amino-3-hydroxy-4-methyl-4-isoxazole propionic acid (AMPA)-, kainate- and N-methyl-D-aspartate (NMDA)-sensitive subtypes of ionotropic glutamate receptors. All members of the ligand-gated ion channel superfamily appear to have a

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**Figure 2. Effects of general anesthetics on ligand-gated ion channels.** A dark green or pink spot indicates significant potentiation or inhibition, respectively, of agonist actions at the receptor in vitro by the anesthetic with an EC\textsubscript{50} or IC\textsubscript{50}, that is no greater than 3 times higher than the EC\textsubscript{50} for producing immobility; a light green or pink spot indicates little potentiation or inhibition, respectively, at concentrations that were less than three times the EC\textsubscript{50} for immobility; an empty spot indicates no effect at any concentration measured; no spot indicates no available data. The data are from (Krasowski and Harrison, 1999; Yamakura and Harris, 2000), the graph is taken from (Rudolph and Antkowiak, 2004).
similar basic subunit topology with a large N-terminal extracellular domain, four putative transmembrane regions (TM1-4), a heterogeneous loop between TM3 and TM4, and a short extracellular C-terminal domain (see Figure 4, section 4.8.3.). Residues within the extracellular N-terminal domain form the agonist binding domains (Kuhse et al., 1995; Lindstrom et al., 1996), amino acids in TM2 line the ion channel pore (Xu and Akabas, 1993). Native receptors are composed of pentameric arrangements of individual receptor subunits (Langosch et al., 1988). GABA_A and glycine receptors are chloride-selective ion channels. They mediate fast synaptic inhibition, since opening of the chloride channel results in chloride influx and thus membrane hyperpolarisation and/or stabilization of the membrane potential away from the threshold for firing action potentials (McCormick, 1989). Glycine is abundant in the spinal cord and brain stem (Zafra et al., 1997), while GABA is present in the spinal cord and higher brain regions (McCormick, 1989). It has been estimated that one-third of all synapses in the CNS are GABAergic (Bloom and Iversen, 1971). GABA_A receptors therefore mediate the majority of inhibitory neurotransmission in the brain.

4.6. GABA_A receptors

The GABA receptor and specific binding sites for diazepam were identified in 1977 (Mohler and Okada, 1977a; Mohler and Okada, 1977b), and in 1985 it was shown that GABA_A and benzodiazepine receptors are identical (Schoch et al., 1985). GABA_A receptors are composed of a plethora of subunits, including α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3, resulting in a heterogeneous population of receptor subtypes (Barnard et al., 1998; Whiting, 2003). More than 90% of all GABA_A receptors are thought to be composed of 2 α, 2 β and 1 γ subunit. The remaining less than 10 % of all GABA_A receptors are thought to represent receptors, in which the γ subunit is replaced by an δ subunit or an ε subunit , the β subunit is replaced by a θ subunit, or, in case of ρ, the GABA_A receptor is assembled as homopentamer (Enz and Cutting, 1998). GABA_A receptors are distributed throughout the brain, with each subtype exhibiting a specific expression pattern at the cellular and subcellular level (Fritschy and Mohler, 1995). The most abundant GABA_A receptor subunit compositions are α1β2γ2, α2β3γ2, α3β3γ2 (Benke et al., 1994; Fritschy and Mohler, 1995; Mohler et al., 2002). The α1β2γ2 GABA_A receptor is the most prevalent of all,
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constituting 33% of all GABA<sub>A</sub> receptors (Mohler et al., 2002; Whiting, 2003)(Figure 3). The differential expression and specific subunit composition suggests that individual receptor subunit combinations might have specific functions in the central nervous system.

4.7. Structural determinants of general anesthetic actions on GABA<sub>A</sub> receptors

The activity of GABA<sub>A</sub> receptor subtypes has been found to be modulated by essentially all general anesthetics with the exception of ketamine and N<sub>2</sub>O. By constructing chimeras between the isoflurane-sensitive glycine receptor α<sub>1</sub>-subunit and the isoflurane-insensitive GABA<sub>A</sub> receptor ρ<sub>1</sub> subunit and site-directed mutagenesis of the glycine receptor α<sub>1</sub> subunit and the GABA<sub>A</sub> receptor α<sub>1</sub>, α<sub>2</sub>, β<sub>1</sub> and ρ<sub>1</sub> subunits specific amino acid positions in transmembrane regions 2 and 3 (TM2 and TM3) have been identified that are critical for potentiation of agonist-induced currents by general anesthetics in glycine and GABA<sub>A</sub> receptors (Krasowski et al., 1998a; Krasowski and Harrison, 1999; Krasowski et al., 1998b; Mihic et al., 1997a; Pistis et al., 1997)(reviewed by (Krasowski and Harrison, 1999)). Etomidate sensitivity of GABA<sub>A</sub> receptors is dependent on the type of β subunit. Receptors containing β<sub>2</sub> or β<sub>3</sub> are highly sensitive to etomidate, whereas β<sub>1</sub> renders GABA<sub>A</sub> receptors largely insensitive to etomidate (Belelli et al., 1997). This sensitivity to etomidate is determined by a single amino acid located within the TM2 domain, N265 in β<sub>2</sub> and β<sub>3</sub>, and S265 in β<sub>1</sub> (Belelli et al., 1997). This N265 amino acid residue also

Figure 3. Pie chart representing the approximate concentration of GABA<sub>A</sub> receptors in the rat brain. Subscript x indicates that the subtype of α, β or γ subunit is not known. Picture from (Whiting, 2003)
determines the sensitivity of the β2- or β3-containing GABA_A receptors to propofol, enflurane, and pentobarbital (Pistis et al., 1999). Although β1-containing GABA_A receptors are insensitive to etomidate, they appear to be sensitive to propofol (Hill-Venning et al., 1997). When the N265 residue in the β3 subunit is mutated to methionine, the residue found in the etomidate-insensitive Drosophila orthologue of the GABA_A receptor, the RDL receptor, the respective receptor is insensitive to etomidate and propofol and partially insensitive to pentobarbital, but still sensitive to alphaxalone, indicating that the putative binding site for alphaxalone is distinct from the putative binding site for etomidate and propofol. (Jurd et al., 2003; Siegwart et al., 2002; Siegwart et al., 2003). When the N265 residue is mutated to serine in the β2 subunit, which is the residue present in the naturally etomidate-insensitive β1 subunit, the β2(N265S) receptor is insensitive to etomidate, but still sensitive to propofol and pentobarbital (Reynolds et al., 2003b).

4.8. Analysis of GABA_A receptor functions using mouse genetics

Benzodiazepines have been in clinical used for more than 40 years for their anxiolytic, sedative, hypnotic, muscle relaxant and anticonvulsant action. They exert all of their effects by binding to the benzodiazepine site on the GABA_A receptor, which results in allostERIC enhancement of chloride influx. In the last decade, the specificity of different GABA_A receptor subunits in mediating the effects of benzodiazepines has been investigated using many lines of knock-out and knock-in mice with mutations in GABA_A receptor subunits (Rudolph and Mohler, 2004; Rudolph and Mohler, 2006). These mouse lines and relevant knowledge gained from them as well as the advantages and drawbacks of different mouse models will be summarized below.

4.8.1. GABA_A receptor knock-out mice

α1 knock-out mice have been independently generated in two laboratories (Sur et al., 2001; Vicini et al., 2001). Although this mouse lacks the major GABA_A receptor α subunit, mice are viable (although less knockout mice have been found than expected from Mendelian laws) and have mild behavioural changes like intention tremor (Kralic et al., 2005; Kralic et al., 2002a). Yet, α1 knock-out mice generated by Sur and collaborators (Sur et al., 2001) showed initially 50% prenatal or perinatal
mortality which decreased to 25% mortality in the F5 generation. These α1 knock-out mice show a generation-dependent upregulation of α2 and α3 subunits which probably accounts for the decrease in mortality. α1 knockout mice generated by Vicini and collaborators (Vicini et al., 2001) show massive compensatory alterations which are stable throughout many generations in all brain regions where the α1 subunit is expressed in wild type mice. The α2, α3, and α4 subunits were upregulated strongly, but only in those brain regions in which the respective α subunit is expressed in wild type mice. α1 knock-out mice show increased sensitivity to the sedative action of diazepam, measured as a greater decrease in locomotor activity (Kralic et al., 2002a; Kralic et al., 2002b; Reynolds et al., 2003a). Diazepam induced an anxiolytic-like effect in α1 knock-out mice at slightly lower doses compared to wild type mice (Kralic et al., 2002b). The duration of LORR (loss of the righting reflex) is increased after diazepam, but decreased after zolpidem application in α1 knock-out mice compared to wild type mice (Kralic et al., 2002b). The decrease in zolpidem response is likely due to the fact that zolpidem is relatively selective for the α1-containing GABA_A receptors, whereas diazepam displays no such selectivity and presumably also strongly modulates the upregulated α2 and α3 subunits in the α1 knock-out mice.

The α5 subunit is expressed quite specifically in the hippocampus, but also in olfactory bulb, deep layers of the cortex and in spinal cord. Knock-out of the α5 subunit led to no obvious compensations. These mice display a better performance in the Morris water maze test, a spatial learning task dependent on hippocampal function (Collinson et al., 2002).

The expression of the α6 subunit is restricted to the cerebellum. Knock-out of this subunit, however, did not lead to a reduction of normal exploratory activity in the open field or an impaired performance in the horizontal wire task (Jones et al., 1997). Although the β2 subunit is the most abundant of all β subunits, knock-out of this subunit had no obvious behavioural consequences (Sur et al., 2001). Although the GABA_A receptor expression is reduced by 50%, β2−/− mice display normal performance on the rotarod, but an increased locomotor activity.

On the other hand, knock-out of the β3 subunit led to cleft palate in 60% of the mice, and 90% of newborns die within the first 24 h after birth. Mice surviving until adulthood show features resembling the Angelman syndrome in humans, i.e.
hyperactivity, poor learning and memory, poor motor coordination, and seizures (Culiat et al., 1995; DeLorey et al., 1998; Homanics et al., 1997).

The knock-out of the γ2 subunit leads to death of all mice perinatally or a few days after birth (Gunther et al., 1995). Transgenic expression of the γ3 subunit on a γ2−/− background did not rescue the lethal phenotype (Baer et al., 1999). Mice heterozygous for the γ2 knock-out, γ2+−/− mice, were viable and fertile. These mice have a reduced expression of the γ2 subunit in the hippocampus and represent a model of chronic anxiety, suggesting that GABA\(_A\) receptor dysfunction may underlie anxiety disorders (Crestani et al., 1999).

The δ subunit is a minor subunit, expressed at highest levels in the cerebellum, and at lower levels in thalamus, striatum and hippocampus. In δ subunit knock-out mice, the duration of the righting reflex was decreased in response to neurosteroidal anesthetics, but not in response to other general anesthetics (Mihalek et al., 1999).

The ρ1 subunit is exclusively expressed in the terminals of retinal bipolar cells. Ablation of the ρ1 subunit leads to alteration of inner retinal function (McCall et al., 2002).

Knock-out mice have provided some significant insights into the importance of different GABA\(_A\) receptor subunits for proper development and function of the brain. However, many of these knock-out mice have very severe phenotypes, simply indicating that this specific subunit, i.e. the α1, β3 and γ2 in particular, are indispensable for proper brain function. Lethality at early stages of development precludes the study of the function of the knocked out gene product at later stages. Due to large compensatory changes in GABA\(_A\) receptor subunit expression or the complexity of the phenotype due to severe developmental deficits, these mice are frequently poor models for elucidating drug actions in a normal brain.

4.8.2. **GABA\(_A\) receptor α subunit knock-in mice**

To overcome the limitations of the knock-out method, mouse models were designed with more subtle alterations of the GABA\(_A\) receptor function in order to obtain more reliable evidence for drug actions mediated by specific GABA\(_A\) receptor subtypes (Rudolph and Mohler, 2004). Point mutations were introduced into GABA\(_A\) receptor subunit genes which would leave the physiological functions of the mutated receptor subunit and the whole receptor complex intact and thus largely avoid compensatory
alterations, while blocking drug action specifically at this receptor subtype. α subunits containing a conserved histidine (α1, 2, 3, 5 at position 101, 101, 126, and 105, respectively) bind diazepam, whereas α subunits containing an arginine at this position (α4, 6 at position 99, 100) are insensitive to diazepam. When the histidine in a diazepam-sensitive subunit is replaced by an arginine, the receptor is rendered diazepam-insensitive (Benson et al., 1998; Wieland et al., 1992). Knock-in mice containing these point mutations (α1(H101R), α2(H101R), α3(H126R), α5(H105R) (Crestani et al., 2002; Low et al., 2000; McKernan et al., 2000; Rudolph et al., 1999)) were subsequently generated to elucidate the involvement of defined GABA_A receptor subtypes in different actions of diazepam in vivo (Table 1). The mutant mice are expected to lack the action of diazepam that is mediated by the mutated subunit.

The α1 subunit of the GABA_A receptor is widely expressed across the brain, most pronounced in the cortex, hippocampus, thalamus, inferior colliculus and cerebellum (Fritschy and Mohler, 1995). In α1(H101R) mice it was demonstrated that the α1-containing GABA_A receptors mediate the sedative action of diazepam, determined by a lack of reduction in motor activity after application of diazepam in α1(H101R) mice (McKernan et al., 2000; Rudolph et al., 1999). The anterograde amnesic action of diazepam, determined in the passive avoidance test, is largely mediated by α1-containing GABA_A receptors and the anticonvulsant action of diazepam is partly mediated by α1-containing GABA_A receptors (Rudolph et al., 1999). α1(H101R) mice are not only resistant to the sedative effect of diazepam, but also to the sedative effect of the imidazo-pyridine zolpidem, a widely used hypnotic which has some selectivity for α1-containing GABA_A receptors (Crestani et al., 2000).

The α2 subunit of the GABA_A receptor is expressed in the cortex, striatum, and the limbic system (Fritschy and Mohler, 1995). α2-containing GABA_A receptors constitute approximately 15% of the diazepam-sensitive GABA_A receptors (Marksitzer et al., 1993). The anxiolytic-like effect of diazepam is absent in α2(H101R) mice in the light/dark choice test and the elevated plus maze test, indicating that α2-containing GABA_A receptors mediate the anxiolytic-like action of diazepam (Low et al., 2000). The myorelaxant action of diazepam, measured in the horizontal wire test, was also strongly reduced in α2(H101R) mice, indicating that this action of diazepam is, at least at lower doses, mediated by α2-containing GABA_A receptors (Crestani et al., 2001).
α3(H126R) mice respond to diazepam like wild type mice in most behavioural tests. The myorelaxant action of diazepam is slightly reduced at higher doses, indicating that α3-containing GABA<sub>A</sub> receptors partly mediate this effect of diazepam (Crestani et al., 2001; Low et al., 2000).

The introduction of the α5(H105R) point mutation into the receptor led to a 30% reduction of α5 immunoreactivity and thus presumably in the expression of the α5 subunit exclusively in the hippocampus, but to no obvious compensations. This is in contrast to the α1(H101R), α2(H101R) and α3(H126R) mice, in which the point mutant subunits are expressed at levels similar to those seen in wild type mice. In α5(H105R) mice, most effects of diazepam are comparable to diazepam effects in wild type mice. The myorelaxant action is slightly reduced (Crestani et al., 2002). The α5(H105R) mice perform better in a trace fear conditioning paradigm, i.e. they show increased freezing compared to wild type mice. This indicates that α5-containing GABA<sub>A</sub> receptors, which are expressed mostly extrasynaptically in the hippocampus, are control elements of the temporal association of threat cues in trace fear conditioning (Crestani et al., 2002).

With exception of the α5(H105R) mice, the α subunit knock-in mice (α1(H101R), α2(H101R) and α3(H126R) mice) did not show a decrease in the knocked-in mutant subunit or any compensatory changes of other GABA<sub>A</sub> receptor subunits. This shows that a strategy using knock-in mice to study molecular targets of drugs is much less prone to compensatory alterations and thus behavioural deficits often seen in knock-out mice.
Introduction

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Table 1. Dissection of Benzodiazepine Pharmacology. The analysis is largely based on knock-in mice ($\alpha_1$(H101R), $\alpha_2$(H101R), $\alpha_3$(H126R), $\alpha_5$(H105R) mice). (+) indicates that this effect of diazepam is mediated by this receptor in wild type mice and abolished in the respective knock-in mouse. (-) indicates that the effect is not mediated by this receptor subtype. ND, not determined. Data from (Rudolph et al., 1999; Low et al., 2000; Crestani et al., 2001; Tobler et al., 2001; Crestani et al., 2002; Kopp et al., 2003; Kopp et al., 2004; van Rijnsoever et al., 2004; Cheng et al., 2006)

4.8.3. GABA$_A$ receptor $\beta$ subunit knock-in mice

Knock-in mice have been generated which contain a N265M point mutation in the $\beta_3$ subunit (Jurd et al., 2003; Lambert et al., 2005) or a N265S point mutation in the $\beta_2$ subunit (Reynolds et al., 2003b) (Figure 5). In $\beta_3$(N265M) mice, the immobilizing action of etomidate and propofol is completely absent and the hypnotic action of etomidate and propofol is partially reduced (Figure 4). In $\beta_2$(N265S) mice, the immobilizing action of etomidate is still present, while the hypnotic action is partly reduced (Reynolds et al., 2003b). This indicates that immobility induced by etomidate and propofol is completely mediated by $\beta_3$-containing GABA$_A$ receptors, while the hypnotic action is mediated by both $\beta_2$- and $\beta_3$-containing GABA$_A$ receptors.

The expression of the $\beta_2$ and $\beta_3$ subunit of the GABA$_A$ receptor overlaps in cortex and cerebellum. $\beta_2$ is expressed in the thalamus, substantia nigra, globus pallidus and inferior colliculus, i.e. in subcortical and midbrain structures (Miralles et al., 1999). $\beta_3$ is expressed in the striatum, in the spinal cord (Persohn et al., 1991) and to a smaller extent in the hippocampus, where $\beta_2$ is expressed only in some interneuron subtypes (Miralles et al., 1999).
Figure 4. Behavioural responses to i.v. anesthetics in wild-type and β3(N265M) mice. Reduction in the duration (min) of the loss of righting reflex (LORR) induced by A) etomidate and B) propofol in β3(N265M) mice vs. wild type. Etomidate (15 mg/kg) and propofol (40 mg/kg) were lethal for 50% and 58% of the wild type, respectively, but none of the β3(N265M) mice. C) Alphaxalone induced a similar duration (min) of LORR in both genotypes. D) Etomidate and E) propofol failed to induce loss of the hind limb withdrawal reflex (LHWR) in β3(N265M) mice in contrast to wild type mice (P<0.01, Fischer’s exact test). F) Alphaxalone induced LHWR with similar duration in β3(N265M) and wild-type mice. All drugs were administered i.v.. Wild type mice (black shading), β3(N265M) mice (gray shading). **P < 0.01, ***P < 0.001 compared with wild type; median test (n=6–12 per group). Picture taken from (Jurd et al., 2003).
The differential expression of the two β subunits and the resistance of the two β subunit knock-in mice to different endpoints of anesthesia indicates that different actions of anesthetics are mediated by different neuronal circuits. Immobility is likely mediated largely by spinal cord circuits (Antognini and Schwartz, 1993; Antognini et al., 2000; Rampil, 1994; Rampil et al., 1993), and hypnosis by subcortical structures like thalamus and midbrain reticular formation (Rudolph and Antkowiak, 2004).
5. Aims

The molecular and neuronal targets mediating the diverse actions of general anesthetics *in vivo* are still largely unknown. The goal of this thesis was to characterize the contribution of different GABA<sub>A</sub> receptor subtypes to the action of a wide range of CNS depressant agents, in particular general anesthetics and benzodiazepines.

5.1. Investigation of the involvement of β3-containing GABA<sub>A</sub> receptors in the respiratory and cardiac actions of etomidate and propofol (Paper 1).

It was already known that the β3-containing GABA<sub>A</sub> receptor mediates the immobilizing actions of etomidate and propofol, and in part their hypnotic action. Here, we investigated the involvement of β3-containing GABA<sub>A</sub> receptors in respiratory depression, hypothermia, heart rate depression, and sedation in β3(N265M) and wild type mice was investigated.

5.2. Investigation of the involvement of β3-containing GABA<sub>A</sub> receptors in the pharmacological actions of pentobarbital (Paper 2).

Until now, no target has been shown to mediate any action of barbiturates *in vivo*. We studied the response of β3(N265M) and wild type mice to pentobarbital by measuring duration of the loss of righting reflex, duration of the loss of the hindlimb withdrawal reflex, respiratory depression, hypothermia, heart rate depression and effects on the ECG.

5.3. Characterization of changes in the mouse ECG induced by general anesthetics and the possible mediation of these effects by β3-containing GABA<sub>A</sub> receptors (Paper 3).

The anesthetic-induced ECG changes were determined for etomidate, propofol and isoflurane in β3(N265M) mice and wild type mice to evaluate a potential involvement of β3-containing GABA<sub>A</sub> receptors. In addition, we tested the potential involvement of β3-containing GABA<sub>A</sub> receptors in the anterograde amnesic action of propofol.
5.4. Identification of the GABA\textsubscript{A} receptor subtype mediating the immobilizing action of diazepam (Paper 4).

The precise composition of the GABA\textsubscript{A} receptors mediating immobility and hypnosis induced by CNS depressant drugs is unknown. Therefore, we determined diazepam-induced hypnosis and immobility in \(\alpha1(H101R), \alpha2(H101R), \alpha3(H126R),\) and \(\alpha5(H105R)\) mice carrying point mutations rendering the respective GABA\textsubscript{A} receptors insensitive for diazepam.

5.5. Identification of a neuronal population mediating benzodiazepine-induced sedation (Paper 5).

While it is known that diazepam-induced sedation is mediated by GABA\textsubscript{A} receptors containing the \(\alpha1\) subunit, the neuronal populations relevant for sedation are still unknown. Using a conditional knock-out/knock-in approach, we investigated whether diazepam-induced sedation is mediated by forebrain pyramidal cells.
6. Results

6.1. Distinct molecular targets for the central respiratory and cardiac actions of the general anesthetics etomidate and propofol (Paper 1)

Anja Zeller *, Margarete Arras #, Anelise Lazaris *, Rachel Jurd *, Uwe Rudolph*  
* Institute of Pharmacology and Toxicology and # Institute of Laboratory Animal Science, University of Zürich, Winterthurerstr. 190, CH-8057 Switzerland

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Abstract

General anesthetics are among the most widely used and important therapeutic agents. The molecular targets mediating different endpoints of the anesthetic state in vivo are currently largely unknown. The analysis of mice carrying point mutations in neurotransmitter receptor subunits is a powerful tool to assess the contribution of the respective receptor subtype to the pharmacological actions of clinically used general anesthetics. We examined the involvement of β3-containing GABA_A receptors in the respiratory, cardiovascular, hypothermic and sedative actions of etomidate and propofol using β3(N265M) knock-in mice carrying etomidate- and propofol-insensitive β3-containing GABA_A receptors. While the respiratory depressant action of etomidate and propofol, as determined by blood gas analysis, was almost absent in β3(N265M) mice, the cardiac depressant and hypothermic effects, as determined by radiotelemetry, and the sedative effect, as determined by decrease of motor activity, were still present. Taken together with previous findings, our results show that both immobilization and respiratory depression are mediated by β3-containing GABA_A receptors, hypnosis by both β3- and β2-containing GABA_A receptors, while the hypothermic, cardiac depressant and sedative actions are largely independent of β3-containing GABA_A receptors.

Key words: anesthesia, respiratory depression, animal model, GABA_A receptor
Introduction

The introduction of general anesthetics into medical practice more than 150 years ago has revolutionized surgical practice, however, the mechanisms of action of this class of drugs are still only poorly understood. Although general anesthetics have been shown to modulate the activity of a number of proteins, e.g. ligand-gated ion channels (Krasowski and Harrison, 1999) and two-pore domain potassium channels (Franks and Honore, 2004) in vitro, the targets mediating specific actions of general anesthetics in vivo are largely unknown.

GABA\textsubscript{A} receptors are pentameric ligand-gated ion channels, the majority of them apparently containing two \(\alpha\), two \(\beta\) and one \(\gamma\) subunit (Barnard et al., 1998). Mutagenesis studies have identified amino acid residues in GABA\textsubscript{A} receptor \(\beta\) subunits to be crucial for the actions of the general anesthetics propofol and etomidate in vitro (Belelli et al., 1997; Krasowski et al., 1998b; Mihic et al., 1997; Siegwart et al., 2002; Siegwart et al., 2003). Whereas in a recombinant system etomidate shows some selectivity for \(\beta\textsubscript{2}\) and \(\beta\textsubscript{3}\)-containing GABA\textsubscript{A} receptors, propofol acts on GABA\textsubscript{A} receptors containing any of the \(\beta\) subunits \(\beta\textsubscript{1}\), \(\beta\textsubscript{2}\) or \(\beta\textsubscript{3}\). In addition, both agents also interact with targets distinct from the GABA\textsubscript{A} receptor (for review see Rudolph and Antkowiak (Rudolph and Antkowiak, 2004)). Recombinant receptors containing the \(\beta\textsubscript{3}(N265M)\) subunits are insensitive to etomidate (Belelli et al., 1997; Siegwart et al., 2002; Siegwart et al., 2003), while receptors containing the \(\beta\textsubscript{2}(N265S)\) subunits have reduced sensitivity for etomidate but still are sensitive to propofol (Reynolds et al., 2003). It has recently been shown that \(\beta\textsubscript{3}(N265M)\) mice are insensitive for the immobilizing action of etomidate and propofol and have a reduced sensitivity for the hypnotic action of these drugs, suggesting that \(\beta\textsubscript{3}\)-containing GABA\textsubscript{A} receptors mediate these actions (Jurd et al., 2003). In line with these findings, \(\beta\textsubscript{2}(N265S)\) mice are still sensitive to the immobilizing and hypnotic actions of etomidate, but lack the sedative response to low doses of etomidate (Reynolds et al., 2003). Furthermore, the hypothermic response to etomidate in \(\beta\textsubscript{2}(N265S)\) mice is strongly decreased (Cirone et al., 2004).

Etomidate and propofol induce depression of respiratory and cardiovascular functions (Bergen and Smith, 1997; Keyl et al., 2000; Lichtenbelt et al., 2004), but it
is currently unknown by which targets these actions are mediated. Therefore we have examined the effects of etomidate and propofol anesthesia in β3(N265M) and wild type mice on respiration using blood gas analysis, on heart rate and core body temperature using radiotelemetry, and the effect of etomidate on motor activity. The identification of the targets relevant for individual actions of the pharmacological spectrum of general anesthetics is expected to provide important information for the development of novel general anesthetic agents with a significantly broader therapeutic range.

**Materials and Methods**

**Animals**

Generation, characterization and breeding of β3(N265M) mice has been described previously (Jurd et al., 2003). Female mice used for telemetry were 3 months old at the time of implantation. Before surgery mice were group housed, after implantation of the transmitter they had to be kept single housed. Female mice used for blood gas analysis were 5 to 8 weeks old and group housed. All animal experiments have been approved by the cantonal veterinary office in Zurich.

**Blood Gas Measurements**

10 naïve female mice aged 5 to 8 weeks from both genotypes were injected with 40 mg/kg propofol, 15 mg/kg etomidate or 30 mg/kg alphaxalone i.v., respectively. In previous experiments, at these doses approximately 50 % of the wild type mice die, but none of the β3(N265M) mice (Jurd et al., 2003). Arterial blood samples were taken from the carotid artery 120 seconds (105 to 170 seconds) after injection following the procedure described by Arras (Arras et al., 2001). Briefly, the ventral aspect of the neck was incised, the right common carotid artery was dissected, and a small hole was cut in the artery, using a fine-bladed pair of scissors. Arterial blood was collected in a heparinised syringe. Oxygen partial pressure (paO2, mmHg), carbon dioxide partial pressure (paCO2, mmHg), and the pH value were determined immediately by use of a blood gas analyser (AVL Compact 3, AVL List, Graz, Austria).

**Surgery**

16 female mice aged 3 months (8 GABA_A receptor β3(N265M) mice and 8 wild-type controls) were implanted under isoflurane anesthesia (3-5% isoflurane in oxygen) with intraperitoneal radiotelemetry transmitters for measuring core body temperature and ECG (model No. ETA-F20, Data Sciences International (DSI), St. Paul, MN). The transmitter body was implanted under sterile conditions in the abdominal cavity and the sensing leads were positioned as described previously (Spáni et al., 2003). Mice received postoperative antibiotics (20 mg/kg sulfadoxin, 5 mg/kg trimethoprim (Borgaf® 7.5 %), Hoechst Roussel vet, Provet AG, Lyssach, Switzerland) and postoperative pain treatment (2.5 mg/kg flunixin s.c., Finadyne, BERNA Veterinärprodukte AG, Berne, Switzerland). Mice were allowed to recover for 4 weeks before the first experiment.

**Experimental Conditions for Telemetry Measurements**

Mice were singly housed in standard laboratory conditions with a 12 h light: dark schedule (lights on 8:00 a.m., lights off 8:00 p.m.) and free access to food and water. Experiments were performed between 9 a.m. and 12 a.m.

**Effect of Anaesthetics on Core Body Temperature (CBT) and Heart Rate (HR)**

For drug and vehicle administration experiments, a one hour baseline was recorded in the first two hours after lights on and drugs were administered immediately afterwards. Mice were treated (in this chronological order) with 30 mg/kg propofol i.v. (Sigma-Aldrich Chemicals, Buchs, Switzerland) (group sizes: wt n=7, β3(N265M) n=5), 10 mg/ kg etomidate i.v. (Janssen-Cilag, Neuss, Germany) (group sizes: wt n=7, β3(N265M) n=7), 15 mg/kg alphaxalone i.v.(Saffan® (BERNA, Bern, Switzerland), alphaxalone/alphadolone 15/5 mg/kg, from now on referred to as “15 mg/kg alphaxalone”) (group
Results

s sizes: wt n=6, β3(N265M) n=6), 180 mg/kg propofol i.p. (group sizes: wt n=4, β3(N265M) n=6) and 20 mg/kg etomidate i.p. (group sizes: wt n=5, β3(N265M) n=5). Vehicle solutions were as follows: propofol, 14 % Cremophor EL (BASF, Ludwigshafen, Germany); etomidate, 35 % propylene glycol (Fluka, Buchs, Switzerland); Saffan®, 0.9% saline (Fresenius, Bad Homburg, Germany). The doses used for the i.v. route have been previously examined for their effects on loss of reflexes and their non-lethality (Jurd et al., 2003). The time interval between single injections was 7 days. Half of the mice in each group were injected first with vehicle and then with the corresponding anesthetic, the other half vice versa.

After turning on the transmitters with a magnet, a one hour baseline was measured with data sampling for 30 s every 3 minutes. Five minutes before injection the sampling schedule was switched to sampling every 30 s. Two hours after the return of righting reflex the continuous sampling was switched to a data sampling for 30 s every 3 minutes and then continued for another 15 hours.

Motor Activity

Naïve female mice (10 mice per genotype and drug dose) were placed in individual circular alleys (Imetronic, Pessac, France) for one hour and then injected i.p. with either vehicle or etomidate (2.5, 5 or 10 mg/kg) and their motor activity was measured as the number of photocell interruptions during a 15 min period.

Statistical Analysis

Results are expressed as mean±SEM. For analysis of telemetry data statistical differences were assessed by using the paired Student’s t-test for testing whether the effect of anesthetic is significant compared to the baseline and the unpaired Student’s t-test for determining potential differences between wild-type and mutant mice. The minimum CBT or HR after injection of anesthetic and the mean of vehicle values over a time period of two hours after injection were determined and compared to the mean of one hour baseline before injection. For analysis of blood gas data the unpaired Student’s t-test was used.

Motor activity data were analysed using parametric statistics (analysis of variance, ANOVA) with consideration of factors genotype and treatment. Post-hoc comparisons were used for analysis of motor activity after administration of etomidate.

Results

Intravenous anesthetics-induced respiratory depression is absent in β3(N265M) mice

To identify the targets mediating the respiratory depressant action of the general anesthetics etomidate and propofol, arterial blood gases and pH values were determined after intravenous injection of these agents in β3(N265M) and wild type mice (Fig. 1). After i.v. injection of 15 mg/kg etomidate or 40 mg/kg propofol the oxygen partial pressure (paO₂) was 53±5 mmHg and 48±4 mmHg, respectively, in wild-type mice, but 96±3 mmHg and 85±3 mmHg, respectively, in β3(N265M) mice. The normal range for paO₂ in mice is 101±3 mmHg (Arras et al., 2001). Thus, while the paO₂ is dramatically decreased in wild type mice, it is only slightly decreased in β3(N265M) mice. The difference between wild type and β3(N265M) mice is highly significant both for etomidate and propofol (p=0.001). In wild-type mice, the carbon dioxide partial pressure (paCO₂) was 70±2 mmHg (etomidate) and 67±2 mmHg (propofol), whereas in the β3(N265M) mice the paCO₂ was 32±2 mmHg (etomidate)
and 38±3 mmHg (propofol). The normal range for \( \text{paCO}_2 \) in wild-type mice is 25±1 mmHg (Arras et al., 2001). Thus, the \( \text{paCO}_2 \) is dramatically increased by these agents in wild-type mice but only moderately in \( \beta_3(\text{N265M}) \) mice. The difference between both genotypes is highly significant (\( p=0.001 \) for etomidate and for propofol).

In contrast, the actions of alphaxalone on the \( \text{paO}_2 \) and the \( \text{paCO}_2 \) are indistinguishable between genotypes (\( \text{paO}_2 \): WT 55±4 mm Hg, \( \beta_3(\text{N265M}) \) 59±5 mmHg, \( p=0.515 \), \( \text{paCO}_2 \): WT 61±3 mmHg, \( \beta_3(\text{N265M}) \) 55±3 mmHg, \( p=0.183 \)), demonstrating that the \( \beta_3(\text{N265M}) \) mice respond normally to general anesthetics whose action is not affected by the N265M point mutation \textit{in vitro}. In summary, blood gas analysis revealed that the respiratory depressant effects of etomidate and propofol are likely mediated by \( \beta_3 \)-containing GABA\( _A \) receptors.

Another hallmark of respiratory depression is respiratory acidosis. In the same samples used to determine the arterial blood gases we also measured the pH (normal value in mice: \( \text{pH}=7.44±0.01 \), (Arras et al., 2001)). In wild-type mice, the pH was 7.05±0.02 after etomidate and 7.09±0.01 after propofol, in \( \beta_3(\text{N265M}) \) mice the pH was 7.18±0.02 after etomidate and 7.25±0.02 after propofol. The difference between the genotypes was highly significant for both agents (\( p=0.001 \) for all comparisons). Thus, wild-type mice develop a much stronger acidosis than \( \beta_3(\text{N265M}) \) mice, consistent with the respiratory depression being largely mediated by \( \beta_3 \)-containing GABA\( _A \) receptors. The pH after administration of alphaxalone was indistinguishable between genotypes (pH: WT 7.14±0.09, \( \beta_3(\text{N265M}) \) 7.15±0.10, \( p=0.757 \)).
Intravenous anesthetics decrease heart rate in β3(N265M) and wild type mice

To evaluate whether the cardiac depressant effect of etomidate and propofol is mediated by β3-containing GABA<sub>A</sub> receptors, heart rate (HR) was monitored using a radiotelemetry system in unrestrained animals. Etomidate and propofol decreased the heart rate significantly in both genotypes (Fig. 2). After i.v. injection of 10 mg/kg etomidate the decrease in heart rate is marginally larger in wild-type compared to β3(N265M) mice (p=0.053), and after i.p. injection of 20 mg/kg etomidate there is a
significantly larger decrease in heart rate in wild-type compared to β3(N265M) mice (p=0.046). After i.v. injection of 15 mg/kg alphaxalone (p=0.14) and 30 mg/kg propofol (p=0.371) and also after i.p. injection of 180 mg/kg propofol (p=0.102) the decrease in heart rate is similar in wild-type and β3(N265M) mice. After injection of vehicle a transient increase in the HR is visible which we consider to be most likely due to handling stress associated with the injection. This increase is followed by a transient decrease in heart rate (Fig. 2b), which may be due at least in part to the volume load associated with the vehicle injections. Slight fluctuations in HR after injection of vehicle show that normal HR regulation is still operative.

Etomidate and propofol depress heart rate in both β3(N265M) and wild type mice, and the genotype difference for etomidate suggests that β3-containing GABA<sub>A</sub> receptors play a minor role in this action.

Figure 2. Anesthetic-induced heart rate depression. With injection of general anesthetics heart rate (HR) decreases in both wild-type and β3(N265M) mice. A) Maximum HR change after injection of anesthetic or vehicle compared to 1 hour baseline before injection. B) Time course of HR change after injection of etomidate i.v.. Group sizes: propofol i.v.: wt n=7, β3(N265M) n=5, etomidate i.v.: wt n=7, β3(N265M) n=7, alphaxalone i.v.: wt n=6, β3(N265M) n=6, propofol i.p.: wt n=4, β3(N265M) n=6, etomidate i.p.: wt n=5, β3(N265M) n=5. * p<0.05, ** p<0.01.
Intravenous Anesthetics induce Hypothermia in β3(N265M) and wild type mice

General anesthetics are known to cause hypothermia. To evaluate the role of β3-containing GABA<sub>A</sub> receptors for hypothermia, we monitored the core body temperature (CBT) changes in response to etomidate and propofol in β3(N265M) mice and wild type mice (Fig. 3). After injection of etomidate and propofol the CBT decreased significantly in both genotypes. In the drug-injected mice the temperature decreases steadily to a minimum and starts to increase at about the same time when mice regain the righting reflex (data not shown). After injection of 10 mg/kg etomidate i.v., CBT decreased significantly less in β3(N265M) compared to wild-type mice (p=0.010) (Fig. 3). After i.p. injection of 20 mg/kg etomidate hypothermia is marginally less pronounced in β3(N265M) mice compared to wild-type mice (p=0.058).

We believe that the initial small and transient decrease in CBT after injection of the vehicle (for etomidate) seen in Figure 3B may due to the volume load of the injections (7.5 μl/g body weight for i.v. injections and 10 μl/g body weight for i.p. injections) (Sessler, 1997). After i.v. injection of propofol (p=0.952) and also after i.p. injection of propofol (p=0.071) the decrease in body temperature is indistinguishable in wild type and β3(N265M) mice. As was previously observed for the respiratory depression and cardiac depression, the hypothermic action of alphaxalone is indistinguishable between both genotypes (p=0.296). Etomidate and propofol induce hypothermia in both β3(N265M) and wild type mice, and the difference between genotypes for etomidate indicates a minor role for β3-containing GABA<sub>A</sub> receptors in mediating etomidate-induced hypothermia.
Sedative Action of Etomidate is independent of β3-containing GABA<sub>A</sub> receptors

It has previously been shown that the immobilising and hypnotic actions of etomidate and propofol are mediated by β3-containing GABA<sub>A</sub> receptors (Jurd et al., 2003). In this study, we investigated whether the sedative action of etomidate is also mediated by β3-containing GABA<sub>A</sub> receptors. Mice from both genotypes display a decrease in motor activity after administration of 5 mg/kg (WT: p<0.05, β3(N265M): p<0.01) and 10 mg/kg (WT: p<0.01, β3(N265M) p<0.001) etomidate compared to vehicle. Furthermore, there is no significant difference between genotypes for either vehicle or any dose of etomidate (Fig. 4). Thus, the sedative action of etomidate is present in β3(N265M) mice, indicating that β3-containing GABA<sub>A</sub> receptors do not mediate this effect.
Results

Figure 4. Motor activity. Both genotypes show a decrease in motor activity after administration of 5 and 10 mg/kg etomidate compared to vehicle. n = 10. * p<0.05, ** p<0.01, *** p<0.001.

Discussion

In this report, we investigated the contribution of β3-containing GABA<sub>A</sub> receptors to various physiological and behavioural endpoints of the general anesthetics propofol and etomidate. We show that the respiratory depressant effect of propofol and etomidate is mediated by β3-containing GABA<sub>A</sub> receptors, whereas the cardiac depressant effect, hypothermia and sedation are largely mediated independently of β3-containing GABA<sub>A</sub> receptors.

We studied the effects of intravenous general anesthetics in mice harboring an asparagine to methionine point mutation in position 265 of the β3 subunit of the GABA<sub>A</sub> receptor. This point mutation renders β3-containing GABA<sub>A</sub> receptors insensitive to the actions of the general anesthetics propofol and etomidate, but not alphaxalone. In β3(N265M) mice the suppression of noxious-evoked movements in response to the anesthetics propofol and etomidate was completely abolished and the obtunding (hypnotic) response was also decreased significantly (Jurd et al., 2003). This suggested that the amino acid asparagine-265 is critically important for mediating behavioural responses to these general anesthetics and that β3-containing GABA<sub>A</sub> receptors are involved in these actions.
In addition to their immobilizing and hypnotic actions, general anesthetics also induce depression of respiratory functions, depression of cardiovascular functions and hypothermia. These actions may be fatal and define the upper limit of the very narrow therapeutic range of general anesthetics. It would therefore be desirable to develop agents with a more specific pharmacological profile and a broader therapeutic range, and thus improved clinical safety. This can only be done in a rational way when the targets mediating individual aspects of the pharmacological profile of general anesthetics are known. Thus, convincing proof that a specific target mediates a defined action of etomidate or propofol should be based on the observation that this action is absent in mice in which the target is rendered insensitive to the drug, e.g. by a knock-in point mutation.

The general anesthetics propofol and etomidate strongly reduce respiration in wild-type mice, whereas there is almost no such effect in β3(N265M) mice. The oxygen partial pressure in wild-type mice is reduced to almost 50% compared to β3(N265M) mice. The carbon dioxide partial pressure is increased in wild-type mice twice as much compared to β3(N265M) mice. We used the same doses of propofol and etomidate that in our previous study were lethal for approximately 50% of wild-type mice, but for none of the β3(N265M) mice. In contrast, after injection of 30 mg/kg alphaxalone the oxygen partial pressure is similarly reduced in both wild-type and β3(N265M) mice and the carbon dioxide partial pressure is similarly increased. This dose of alphaxalone was lethal for 50% of both wild-type and β3(N265M) mice in our previous study. Although we cannot formally rule out the possibility that the surgical preparation and puncture of the carotid artery might have induced hyperventilation in β3(N265M) mice potentially leading to an apparent “normalization” of blood gas values, this appears to be unlikely since upon clinical observation the β3(N265M) mice did not display evidence of hyperventilation. Moreover, our observation that etomidate- and propofol-induced cardiac depression and hypothermia are similar in β3(N265M) mice and wild-type mice suggest that these drug actions are unlikely to be the case of drug-induced lethality in wild-type mice, which is not seen in β3(N265M) mice. Thus, the likely cause of the lethality seen in wild type mice is respiratory depression.
The anesthetic-induced heart rate depression is quite substantial in both genotypes (e.g. 44\% after i.v. injection of etomidate), with minor but significant differences for etomidate but not for propofol. We do not know whether the decrease in heart rate is primarily due to an inhibition of sympathetic reflexes or due to an increase of parasympathetic activity. However, it is known that propofol in man strongly inhibits sympathetic reflexes (Lichtenbelt et al., 2004; Sellgren et al., 1994), and thus we consider it more likely that the decrease in the heart rate observed in the mice is due to inhibition of the sympathetic system. It is reasonable to predict that decreases in heart rate of this magnitude would have profound effects on blood pressure (Erhardt et al., 1984; Krassioukov et al., 1993; Paris et al., 2003). While propofol may cause severe cardiovascular depression in humans, the cardiovascular depressant effects of etomidate in humans are small. It is striking that in our experiments etomidate appears to have an apparently more pronounced heart rate depressant effect than propofol. The doses of anesthetics used in our experiments are higher than the doses used in humans. Both drugs have been employed at concentrations corresponding to ca. 70 \% of the dose lethal for approximately 50 \% of the wild-type mice (Jurd et al., 2003).

Generation of respiratory rhythms occurs in a network of neurons originating from the pre-Bötzinger complex (Richter et al., 2003). Synaptic interactions involving AMPA, NMDA, GABA\textsubscript{A}, GABA\textsubscript{B} and glycine receptors are thought to play a major role in regulating this network. Another class of drugs used in anesthesia, the opioids, also induce respiratory depression by direct inhibition of rhythm-generating neurons in the pre-Bötzinger complex, and activation of 5-HT4 receptors in pre-Bötzinger reverses opioid-induced breathing depression (Manzke et al., 2003). Although GABA\textsubscript{A} receptors are known to modulate the discharge frequency of respiratory neurons (Zuperku and McCrimmon, 2002), it is unknown whether general anesthetics would exert their respiratory depressant actions via GABA\textsubscript{A} receptors or via other targets. We now show that the respiratory depressant effect of etomidate and propofol is mediated by β3-containing GABA\textsubscript{A} receptors. It is currently unknown which neurons specifically mediate this effect and there are no useful data available on the common or differential expression of the GABA\textsubscript{A} receptor β2 and β3 subunits in neurons relevant for respiration. There is also a possibility that β3-containing GABA\textsubscript{A} receptors on spinal motoneurons innervating e.g. the phrenic muscle may play a role in mediating some of the respiratory effects of these agents.
After injection of etomidate hypothermia is less pronounced in β3(N265M) mice compared to wild-type mice. This result is consistent with the finding that in β2(N265S) mice, in which the sensitivity of the β2-containing GABA<sub>A</sub> receptors for etomidate is decreased by an order of magnitude, the hypothermic action of etomidate is attenuated compared to wild-type mice, but still present (Cirone et al., 2004). Thus, the hypothermic action of etomidate appears to be mediated by both β2- and β3-containing GABA<sub>A</sub> receptors, with the β2-containing GABA<sub>A</sub> receptors playing a dominant role. For propofol, the hypothermic response was indistinguishable between β3(N265M) mice and wild-type mice, indicating that it is mediated almost exclusively via targets distinct from β3-containing GABA<sub>A</sub> receptors, most likely β2-containing GABA<sub>A</sub> receptors.

To address the question of whether the response of the β3(N265M) mice to general anesthetics which are not affected by the point mutation in vitro is altered, the neurosteroidal anesthetic alphaxalone was used. For all parameters analysed so far, i.e. immobility, hypnosis (Jurd et al., 2003), hypothermia, cardiac depression and respiratory depression, the action of alphaxalone was indistinguishable in β3(N265M) mice and wild type mice. From this we conclude that β3(N265M) mice are specifically insensitive to etomidate and propofol, but still sensitive to alphaxalone.

As behavioural endpoints for the anesthetic state, we previously measured the loss of the hindlimb withdrawal reflex as a measure for immobility or surgical tolerance and the loss of the righting reflex as a measure for the hypnotic or obtunding action of general anesthetics (Jurd et al., 2003). At subanesthetic doses, e.g. during recovery from anesthesia, general anesthetics may have a sedative effect, measured in mice as a decrease of motor activity. Here we describe that etomidate decreases motor activity in both β3(N265M) mice and wild-type mice, indicating that this action is mediated by targets distinct from β3-containing GABA<sub>A</sub> receptors. This is consistent with a previous report by Reynolds and colleagues (Reynolds et al., 2003) describing that subanesthetic doses of etomidate do not decrease locomotor activity in β2(N265S) mice. Taken together, these results demonstrate clearly that the sedative action of etomidate is mediated by β2-containing but not by β3-containing GABA<sub>A</sub> receptors.
In summary, all data available to date for etomidate point to $\beta_3$-containing GABA$_A$ receptors mediating immobility and respiratory depression, in part hypnosis and to a minor degree hypothermia, while $\beta_2$-containing GABA$_A$ receptors mediate hypothermia (for the most part), in part hypnosis and sedation. Based on our results, we hypothesise that the cardiac depressant actions of etomidate are largely not mediated by $\beta_3$-containing GABA$_A$ receptors, but by other targets, possibly $\beta_2$-containing GABA$_A$ receptors (Table 1).

<table>
<thead>
<tr>
<th>Effects</th>
<th>$\beta_3$-containing GABA$_A$ receptors</th>
<th>$\beta_2$-containing GABA$_A$ receptors and other targets</th>
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<tr>
<td>Immobility</td>
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<td>Respiratory depression</td>
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<td>Hypnosis</td>
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<td>Cardiac depression</td>
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**Table 1. Proposed roles of etomidate and propofol on GABA$_A$ receptor subtypes.** These assignments are based on the following tests: immobility: loss of hind limb withdrawal reflex; respiratory depression: increase in $p$CO$_2$ and decrease in $p$O$_2$ and pH; hypnosis: loss of righting reflex; sedation: decrease in motor activity; hypothermia: decrease in core body temperature; cardiac depression: decreased heart rate. Data are based on this study and previous studies by Jurd (Jurd et al., 2003), Reynolds (Reynolds et al., 2003), Cirone (Cirone et al., 2004).
For propofol, we know that β3-containing GABA_A receptors mediate immobility, respiratory depression, and in part hypnosis, while distinct targets, potentially β2-containing GABA_A receptors mediate hypnosis (in part), cardiac depression and hypothermia.

With respect to future drug development, our data indicate that anesthetic agents with a high degree of selectivity for β3-containing GABA_A receptors over β2-containing GABA_A receptors provide immobility and respiratory depression but would not (or only to a limited degree) produce cardiovascular depression, hypothermia and sedation and the latter might be reflected in a decreased “hangover” after anesthesia. In the clinical setting, respiratory depression can easily be controlled by mechanical ventilation, however, cardiovascular depression represents a more serious problem, and it would be desirable to develop immobilizing agents without significant cardiovascular side effects. Our data demonstrate that immobilizing and cardiac depressant actions of intravenous general anesthetics are indeed mediated by different targets and thus can be separated pharmacologically, enabling the development of general anesthetic agents with an improved therapeutic range.

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References


6.2. Identification of a molecular target mediating the general anesthetic actions of barbiturates (Paper 2)

Anja Zeller ¹, Margarete Arras ², Rachel Jurd¹, Uwe Rudolph ¹,³

¹ Institute of Pharmacology and Toxicology, University of Zürich, Winterthurerstr. 190, Zürich, 8057 Switzerland, ² Institute of Laboratory Animal Science, University of Zürich, Winterthurerstr. 190, Zürich, 8057 Switzerland, ³ Laboratory of Genetic Neuropharmacology, McLean Hospital, Harvard Medical School, Belmont, MA 02478

Abstract

Barbiturates were introduced into medical practice in 1934. They are widely used today as general anesthetics. Although in vitro studies revealed that the activity of a variety of ligand-gated channels is modulated by barbiturates, the target(s) mediating the anesthetic actions of barbiturates in vivo are unknown. Studying pentobarbital action in β3(N265M) mice harboring β3-containing GABA_A receptors insensitive to a variety of general anesthetic agents, we found that the immobilizing action of pentobarbital is mediated fully and the hypnotic action is mediated in part by this receptor subtype. Surprisingly, the respiratory depressant action of pentobarbital is indistinguishable between β3(N265M) and wild type mice and thus is mediated by other as yet unidentified targets. While the target for the immobilizing and hypnotic actions of pentobarbital appears to be the same as for etomidate and propofol, these latter agents’ respiratory depressant actions are mediated by β3-containing GABA_A receptors. Thus, in contrast to etomidate and propofol, pentobarbital can elicit respiratory depression by a β3-independent pathway. Pentobarbital reduced heart rate and body temperature to a slightly smaller extent in β3(N265M) mice as compared to wild type mice, indicating that these actions are largely mediated by other targets. Pentobarbital-induced increase of heart rate variability and prolongation of ECG intervals are seen in both β3(N265M) mice and wild type mice, suggesting that they are not dependent on β3-containing GABA_A receptors. In summary, we show a clear pharmacological dissociation of the immobilizing/hypnotic and respiratory/cardiovascular actions of pentobarbital.

Keywords: anesthesia, immobility, hypnosis, animal model, GABA_A receptor
Introduction

The introduction of general anesthetics into medical practice 160 years ago has revolutionized surgery, however, the mechanisms of action of this class of drugs are still only poorly understood. Although general anesthetics have been shown to modulate the activity of a number of proteins, e.g. ligand-gated ion channels (Krasowski and Harrison, 1999) and two-pore domain potassium channels in vitro (Franks and Honore, 2004), the identification of targets mediating specific actions of general anesthetics in vivo has only just begun.

GABA<sub>A</sub> receptors are pentameric ligand-gated ion channels, the majority of them containing two α, two β and one γ subunit (Barnard et al., 1998). Mutagenesis studies have identified amino acid residues in GABA<sub>A</sub> receptor β subunits that are crucial for the actions of the general anesthetics etomidate and propofol in vitro (Belelli et al., 1997; Krasowski et al., 1998; Mihic et al., 1997; Siegwart et al., 2002; Siegwart et al., 2003).

It has been shown that β3(N265M) mice are insensitive to the immobilizing and respiratory depressant action of etomidate and propofol and have a reduced sensitivity to the hypnotic action of these drugs, suggesting that β3-containing GABA<sub>A</sub> receptors mediate these actions, while etomidate retains its sedative (motor depressant) action at subanesthetic doses (Jurd et al., 2003; Zeller et al., 2005). In line with these findings, β2(N265S) mice are still sensitive to the immobilizing and hypnotic actions of etomidate, but lack the sedative response to low doses of etomidate (Reynolds et al., 2003). Furthermore, the hypothermic response to etomidate is strongly decreased in β2(N265S) mice (Cirone et al., 2004) and only moderately decreased in β3(N265M) mice (Zeller et al., 2005), indicating that the hypothermic response to etomidate is mediated in large part by β2-containing GABA<sub>A</sub> receptors and to a more limited degree by β3-containing GABA<sub>A</sub> receptors.

In contrast to etomidate and propofol, which exert most if not all of their clinically relevant actions via β2- and β3-containing GABA<sub>A</sub> receptors, the barbiturate pentobarbital has a wider range of targets, modulating the activity not only of GABA<sub>A</sub> receptors (Thompson et al., 1996), but also of nicotinic acetylcholine receptors, AMPA receptors, kainate receptors and glycine receptors (Krasowski and Harrison, 1999).
Results

In this study, we investigated in wild type and β3(N265M) mice the following actions of pentobarbital: loss of righting reflex (LORR) as a measure of the hypnotic activity, loss of the hindlimb withdrawal reflex (LHWR) as a measure of the immobilizing activity, respiratory depression, heart rate, core body temperature and the electrocardiogram (ECG). We show that some clinically important but not all of the actions of pentobarbital are mediated by β3-containing GABA_A receptors and that there are striking differences compared to the β3 subtype dependence of etomidate and propofol actions.

Material and Methods

Animals

Generation, characterization and breeding of β3(N265M) mice has been described previously (Jurd et al., 2003). Mice used for telemetry were 3 months old at the time of surgery, 4 months old at the beginning of the experiments and 10 months at the end of the telemetry experiments. Mice used for blood gas analysis and reflex tests were 5 to 8 weeks old. Mice were of a mixed background (129/Sv (12.5%)×129/SvJ (87.5%) mice). Mice were all female.

Behavioral Analysis of Intravenous Anesthetics

Female mice were treated with increasing doses of pentobarbital (Nembutal, 50, 62.5, 75 mg/kg, Abbott AG, Baar, Switzerland/Abbott Laboratories, Chicago, USA) administered intravenously (i.v.) into the tail vein in a volume of 4 μl/kg body weight. The duration of the LORR and LHWR was recorded as described previously (Arras et al., 2001). Briefly, the LORR was assessed by measuring the time a mouse remains on its back on a flat surface. The LHWR, which is always shorter than the LORR and starts after onset of LORR and stops before LORR is regained, was determined by pinching a mouse with a pair of tweezers into the interdigital skin of the hindlimb. The reflex was rated as being present when a mouse retracts its hindlimb upon pinching. Each mouse was tested only once.

Blood Gas Measurements

Arterial blood samples were taken from the carotid artery 130 seconds (range 110 to 140 seconds) after injection of 75 mg/kg pentobarbital i.v. or 30 mg/kg alphaxalone i.v. following the procedure described by Arras et. al. (Arras et al., 2001). Briefly, the ventral aspect of the neck was incised, the right common carotid artery was dissected, and a small hole was cut in the artery, using a fine-bladed pair of scissors. Arterial blood was collected in a heparinised syringe. Oxygen partial pressure (paO_2, mmHg), carbon dioxide partial pressure (paCO_2, mmHg), acid-base balance (pH value), and standard bicarbonate concentrations (HCO_3^-, mmol/L) were determined immediately by use of a blood gas analyser (AVL Compact 3, AVL List, Graz, Austria). For ethical reasons no arterial blood samples were taken from non-anesthetized mice. Instead, data previously obtained from wild type mice (strain HanIbm:NMRI), male, obtained from RCC (Research and Consulting Company, Biotechnology and Animal Breeding Division, Füllinsdorf, Switzerland)) and reported in Arras et al. (2001) were used for comparison.

Surgery

16 female mice (8 β3(N265M) mice and 8 wild-type controls) were implanted under isoflurane anaesthesia (3-5% in oxygen) with intra-peritoneal radiotelemetry transmitters for measuring core body temperature and ECG (model No. ETA-F20, Data Sciences International (DSI), St. Paul, MN). The transmitter body was implanted under sterile conditions in the abdominal cavity and the sensing leads were positioned as described previously (Späni et al., 2003). Mice received postoperative antibiotics (20 mg/kg sulfadoxin, 5 mg/kg trimethoprim, Borgal 7.5 %, Hoechst Roussel vet, Provet AG, Lyssach, Switzerland) and postoperative pain treatment for 5 days (2.5 mg/kg flunixin s.c., Finadyne, BERNNA Veterinärprodukte AG, Berne, Switzerland). Mice were allowed to recover for 4 weeks before the first
Targets mediating the anesthetic actions of pentobarbital (Paper 2)

experiment. To ascertain full recovery after surgery we measured core body temperature and heart rate over 72 h before starting the experiments.

Experimental Conditions
Mice implanted with telemetry transmitters were singly housed in standard laboratory conditions with a 12 h light/dark schedule (lights on 8:00 am, lights off 8:00 pm) and free access to food and water. Experiments were performed between 9 am and 12 am. Mice used for reflex tests and blood gas analysis were group housed and experiments were performed between 8 am and 5 pm.

Effect of Anesthetics on Core Body Temperature (CBT), Heart Rate (HR) and ECG Parameters (PQ, QT)
For drug and vehicle administration experiments, a baseline was recorded between 0 and 2 hours after lights on and drugs were administered immediately afterwards. Drug effects were compared to vehicle effects, which did not differ significantly from baseline. Before injection of pentobarbital 60 mg/kg i.p. (Nembutal, Abbott AG, Baar, Switzerland / Abbott Laboratories, Chicago, USA), mice were already treated with other anesthetics (see also (Zeller et al., 2005)). Vehicle solutions were as follows: Saffan® 14 % Cremophor EL, pentobarbital 10 % EtOH, 40 % propylene glycol. The time interval between single injections was 7 days. Half of the mice in each group were injected first with vehicle and then with the corresponding anesthetic, the other half vice versa. Mice were used for several experiments because of the transmitter are very expensive and their implantation is time-consuming. After turning on the transmitters with a magnet, a one hour baseline was measured with data sampling for 30 s every 3 minutes. Five minutes before injection the sampling schedule was switched to continuous ECG recording and body temperature and heart rate were sampled every 30 s. Two hours after the return of righting reflex the continuous sampling was switched to a data sampling for 30 s every 3 minutes and then continued for another 15 hours. Data were acquired with the Dataquest ART 3.0 acquisition system (DataSciences International, St. Paul, MN, USA). All signals (CBT, HR and ECG parameters) were recorded simultaneously in the same experiment. CBT and HR were calculated by the acquisition software (Dataquest A.R.T. 3.01, DataSciences International). The ECG signal was further processed to derive time domain parameters (PQ, QT) with the Physiostat™ ECG Analysis 4.00 (DataSciences International) software.

Statistical Analysis
Results are expressed as mean±SEM. For analysis of reflex and blood gas data the unpaired Student's t-test was used. For analysis of telemetry data statistical differences were assessed by using the paired Student's t-test for testing whether the effect of anesthetic is significant compared to the vehicle and the unpaired Student's t-test for determining potential differences between wild type and mutant mice. The minimum CBT or HR after injection of anesthetic and the mean of vehicle values over a time period of two hours after injection were determined and compared to the mean of one hour baseline before injection.

Results

β3(N265M) mice are resistant to pentobarbital-induced hypnosis and immobility
We measured two different endpoints to assess the anesthetic action of pentobarbital in wild type and β3(N265M) mice. The loss of righting reflex (LORR) was taken as a measure of hypnosis (loss of consciousness) and the loss of hindlimb withdrawal reflex (LHWR) was taken as a measure of immobility (surgical tolerance, loss of response to a noxious stimulus). Both reflexes are used widely in animal research to assess the effectiveness of anesthetics. The dose range of pentobarbital that could be used was very small (50 to 75 mg/kg pentobarbital i.v.). At lower doses, neither
genotype showed a reliable loss of reflexes and at higher doses all animals died (data not shown). Pentobarbital at doses of 50, 62.5 and 75 mg/kg i.v. induced a LORR in both wild type and β3(N265M) mice, and the duration of LORR was significantly reduced in β3(N265M) mice compared to wild type (62%, 57% and 64% of the duration of LORR in wild type mice) (Fig. 1). The LHWR was very short in wild type mice treated with 50 mg/kg pentobarbital, but robust at the higher doses. LHWR was completely abolished in almost all β3(N265M) mice at all doses tested. At 62.5 mg/kg and 75 mg/kg all wild type mice lost the hind-limb withdrawal reflex, whereas at 62.5 mg/kg none out of 11 of the β3(N265M) mice and at 75 mg/kg one out of 9 β3(N265M) mice lost the hindlimb withdrawal reflex (Fig. 1). At 75 mg/kg, 2 out of 12 wild type and 2 out of 11 β3(N265M) mice died. At 62.5 mg/kg one out of 12 wild type mice died and at 50 mg/kg one out of 17 β3(N265M) mice died. In contrast to what was observed for etomidate and propofol where at the highest dose 50% of wild type mice but none of the β3(N265M) mice died (Jurd et al., 2003), after injection of pentobarbital, no genotype difference in lethality was observed.

In summary, β3(N265M) mice are completely resistant to pentobarbital-induced loss of hind-limb withdrawal reflex, and are partially resistant to pentobarbital-induced loss of righting reflex, compared to wild type mice. These results are very similar to those obtained previously for etomidate and propofol in these mice (Jurd et al., 2003) and indicate that the immobilizing action and in part the hypnotic action of pentobarbital are mediated by β3-containing GABA<sub>A</sub> receptors.

Figure 1. Behavioural responses to pentobarbital in β3(N265M) and wild type mice. A. Reduction in the duration of the loss of righting reflex (LORR) induced by pentobarbital in β3(N265M) mice compared to wild type mice. B. Pentobarbital failed to induce loss of hindlimb withdrawal reflex in β3(N265M) mice in contrast to wild type mice. n=7–17. * p<0.05, ** p<0.01, *** p<0.001.
\(\beta 3(N265M)\) mice are susceptible to pentobarbital-induced respiratory depression

To assess respiratory depression induced by the general anesthetic pentobarbital, arterial blood gases and pH values were determined after intravenous injection in \(\beta 3(N265M)\) and wild type mice (Fig. 2). After i.v. injection of 75 mg/kg pentobarbital, both genotypes showed a marked respiratory depression. The oxygen partial pressure (paO\(_2\)) was 53±7 mmHg in wild type mice and 64±5 mmHg in \(\beta 3(N265M)\) mice. The normal range for paO\(_2\) in awake mice is 101±3 mmHg (Arras et al., 2001). The carbon dioxide partial pressure (paCO\(_2\)) was 54±4 mmHg in wild type mice and 45±2 mmHg in \(\beta 3(N265M)\) mice. The normal range for paCO\(_2\) in wild-type mice is 25±1 mmHg (Arras et al., 2001). The pH was 7.15±0.02 in wild type mice and 7.14±0.02 in \(\beta 3(N265M)\) mice (normal value in mice: pH=7.44±0.01, (Arras et al., 2001)). The unpaired Student’s t-test reveals a significant decrease of the oxygen partial pressure, an increase in carbon dioxide partial pressure and a decrease in pH after pentobarbital in both \(\beta 3(N265M)\) and wild type mice compared to blood gas parameters in awake mice (p<0.001 for both genotypes and all parameters measured)(Arras et al., 2001), but no genotype difference (pentobarbital: paO\(_2\) p=0.435, paCO\(_2\) p=0.144, pH p=0.475). The results are similar to those previously reported for the neurosteroidal anesthetic alphaxalone/alphadolone (Zeller et al., 2005), whose actions are not affected by the \(\beta 3(N265M)\) point mutation (Belelli et al., 1999; Siegwart et al., 2002). These results indicate that the respiratory depressant action of pentobarbital is not dependent on \(\beta 3\)-containing GABA\(_A\) receptors, in contrast to the respiratory depressant effects of etomidate and propofol.
Results

Figure 2. Assessment of pentobarbital-induced respiratory depression by blood gas analysis.
A,B. In β3(N265M) mice injected with pentobarbital, paO₂ was decreased and paCO₂ was increased similar to wild type mice, indicating the independence of the respiratory depressant effect of pentobarbital on β3-containing GABA<sub>A</sub> receptors. As a comparison, the values for the neurosteroidal anesthetic alphaxalone which have already been published before (Zeller et al., 2005) are displayed as well. Alphaxalone, whose action is not affected by the β3(N265M) mutation in vitro, elicits changes in blood gases without a difference between genotypes. C. After pentobarbital and alphaxalone, pH was decreased in both β3(N265M) mice and wild type mice. n=10-17. For all three parameters, there was no genotype difference (pentobarbital: paO₂ p=0.435, paCO₂ p=0.144, pH p=0.475, alphaxalone: paO₂ p=0.515, paCO₂ p=0.183, pH p=0.757).

The heart rate depressant effect of pentobarbital is present but reduced in β3(N265M) mice

To determine the cardiac depressant effect of pentobarbital in β3(N265M) mice, heart rate (HR) was measured using a radiotelemetry system in unrestrained animals (Zeller et al., 2005). The baseline heart rate is similar for both genotypes without any handling stress (561±19 bpm for wild type mice, 554±24 bpm for β3(N265M) mice, data not shown). After injection of 60 mg/kg pentobarbital i.p., HR decreases in wild type mice from 620±53 beats per minute (bpm) to 220±17 bpm (-65%, p<0.01) and in β3(N265M) mice from 636±15 bpm to 363±21 bpm (-43%, p<0.01) (Fig. 3). HR after vehicle injection is slightly increased in both genotypes (Fig. 3a) compared to the baseline, probably due to handling stress. The HR decrease induced by pentobarbital is significantly less pronounced in β3(N265M) mice compared to wild type mice (Fig. 3b, p<0.01, maximum HR decrease after injection compared to vehicle). Our results suggest that there is a minor contribution of β3-containing GABA<sub>A</sub> receptors to the heart rate depressant action of pentobarbital.
Figure 3. Pentobarbital-induced heart rate depression. A. After injection of pentobarbital heart rate (HR) decreases in both wild type and β3(N265M) mice. B. Maximum HR change after injection of anesthetic or vehicle compared to 1 hour baseline before injection. A value of zero would designate no deviation from baseline, whereas a positive value designates an increase of heart rate compared to the baseline and a negative value designates a decrease of heart rate compared to the baseline. For comparison, values for alphaxalone, a neurosteroid whose action at the GABA\textsubscript{A} receptor is not influenced by the β3(N265M) point mutation, are displayed as well (Zeller et al., 2005). Pentobarbital: n=5 for wt, n=7 for β3(N265M); alphaxalone i.v.: wt n=6, β3(N265M) n=6. * p<0.05, ** p<0.01, *** p<0.001 when the effect of the vehicle or anesthetic is compared to the baseline; ## p < 0.01 for genotype difference.

The hypothermic effect of pentobarbital is present but reduced in β3(N265M) mice

Most general anesthetics induce hypothermia. We therefore measured the changes in core body temperature (CBT) after injection of pentobarbital. After injection of 60 mg/kg pentobarbital i.p., the CBT decreased significantly in both genotypes, from 36.5±0.3°C and 36.8±0.2°C to 28.9±0.3°C (-21%, p<0.01) and 30.9±1.1°C (-16%, p<0.01) in wild type and β3(N265M) mice, respectively (Fig. 4). The decrease of CBT is pronounced in both genotypes after pentobarbital application, but significantly less in β3(N265M) mice compared to wild type mice (p<0.05). Thus, while the decrease in CBT appears to be largely mediated by other targets, presumably β2-containing GABA\textsubscript{A} receptors, there is clearly a minor component of hypothermia mediated by β3-containing GABA\textsubscript{A} receptors.
Results

Figure 4. Pentobarbital-induced hypothermia. A. After injection of pentobarbital, core body temperature (CBT) decreases in both wild type and β3(N265M) mice. B. Maximum CBT change after injection of anesthetic or vehicle compared to 1 hour baseline before injection. For comparison, values for alphaxalone are displayed as well. Pentobarbital: n=5 for wt, n=7 for β3(N265M); alphaxalone i.v.: wt n=6, β3(N265M) n=6. * p<0.05, ** p<0.01, *** p<0.001 when the effect of the vehicle or anesthetic is compared to the baseline; # p < 0.05 for genotype difference.

Effects of pentobarbital on ECG parameters

General anesthetics are known to change the duration of various intervals of the ECG in humans. To our knowledge, with the exception of ketamine (Mitchell et al., 1998), this has not been demonstrated in mice. We investigated the actions of pentobarbital on the ECG in wild type and in β3(N265M) mice. Pentobarbital prolonged the PQ, QRS and QT intervals from 31.8±1.3 ms, 12.2±0.5 ms, 23.5±0.6 ms to 45.5±1.2 ms (p<0.01 versus vehicle), 16±2 ms (p=0.113 versus vehicle), 27.6±1.3 ms (p<0.05 versus vehicle) in wild type mice and from 32.2±0.7 ms, 11.2±0.5 ms, 19.7±0.9 ms to 35.1±1.4 ms (p=0.217 versus vehicle), 13.8±5 ms (p<0.05 versus vehicle), 25.6±1 ms (p<0.01 versus vehicle) in β3(N2565M) mice. In wild type mice, PQ and QT interval were increased significantly compared to vehicle, the QRS interval was not significantly increased. In β3(N2565M) mice, the QRS and QT intervals were increased, while the PQ interval was not significantly different from baseline, presumably due to a high variability (Table 1, Figure 5). There is no significant genotype difference for the ECG intervals. Heart rate variability (HRV) is measured as the standard deviation of the inter-beat-interval (RR interval). 60 mg/kg pentobarbital i.p. increases HRV 8-fold in wild type and 4-fold in β3(N265M) mice (p<0.01 in wt, p<0.05 in β3(N265M) versus vehicle, p<0.05 between genotypes after
drug). For comparison purposes, we also studied an alphaxalone/alphadolone mixture, subsequently referred to as alphaxalone, whose action is not influenced by the β3(N265M) point mutation. Alphaxalone induces similar changes of all analyzed ECG parameters in wild type and β3(N265M) mice. HRV increases in wild type mice 5-fold and in β3(N265M) mice 3.5-fold (p<0.05 versus vehicle in both genotypes, p=0.317 between genotypes). QT, QRS and PQ are prolonged from 23.5±0.6 ms, 12.2±0.5 ms and 31.8±1.3 ms to 26.6±0.8 ms, 14±0.8 ms and 44.6±1 ms (p=0.186, p=0.181, p<0.001 for QT, QRS, PQ, respectively, versus vehicle) in wild type mice and from 19.7±0.9 ms, 11.2±0.5 ms and 32.2±0.7 ms to 24.9±1 ms, 13±1.2 ms and 43.9±0.8 ms (p=0.113, p=0.811, p<0.05 for QT, QRS, PQ, respectively, versus vehicle in β3(N265M) mice (p=0.644, p=0.964, p=0.139 between genotypes).

<table>
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<tr>
<th>(msec)</th>
<th>Baseline</th>
<th>Pentobarbital Vehicle</th>
<th>Pentobarbital 60 mg/kg i.p.</th>
<th>Alphaxalone Vehicle</th>
<th>Alphaxalone 15 mg/kg i.v.</th>
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<tr>
<td></td>
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<td>wt</td>
<td>β3(N265M)</td>
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<tr>
<td>RR</td>
<td>107±3.6</td>
<td>108±4.7</td>
<td>130±29</td>
<td>117±3.5</td>
<td>265±13.6**</td>
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<td>5.4±0.8</td>
<td>6.4±2.8</td>
<td>3.2±0.7</td>
<td>40.4±5.8**</td>
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<tr>
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<td>24.1±0.9</td>
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<tr>
<td>QRS</td>
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<td>11.8±0.6</td>
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<td>16±2</td>
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<td>PQ</td>
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<td>33.8±2.1</td>
<td>36.4±2.7</td>
<td>45.5±1.2*</td>
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Table 1. Effects of pentobarbital on baseline ECG parameters. All values are mean±SEM. RR inter-beat-interval. Group sizes: pentobarbital: wt n=5, β3(N265M)n=7; alphaxalone i.v.: wt n=6, β3(N265M) n=6. * p<0.05, ** p<0.01, *** p<0.001 compared to baseline, # p<0.05 wild type compared to β3(N265M) mice. If not indicated, the deviation from baseline or the genotype difference is statistically not significant.

Thus, HRV is slightly less increased in β3(N265M) mice compared to wild type after injection of pentobarbital, whereas the HRV increase is similar in both genotypes after alphaxalone, suggesting that the β3(N265M) mice respond normally to anesthetic-induced ECG changes and that the pentobarbital-induced increase in HRV is mediated by β3-containing GABA<sub>A</sub> receptors. Both anesthetics induced prolongation of QT, QRS and in particular PQ intervals, but these changes, presumably due to high variability, only partly reached statistical significance. Most importantly, there was no genotype difference both for pentobarbital and alphaxalone, indicating that β3-containing GABA<sub>A</sub> receptors do not play a role in pentobarbital-induced ECG interval prolongation.
Figure 5. Pentobarbital-induced changes of ECG intervals. **A,C.** With injection of 60 mg/kg pentobarbital i.p., PQ and QT intervals are prolonged. The prolongation is slightly less pronounced in β3(N265M) mice compared to wild type. **B,D.** Maximum change of PQ and QT after injection of pentobarbital compared to 1 hour baseline before application. For comparison, values for alphaxalone are displayed as well. Pentobarbital: n=5 for wt, n=7 for β3(N265M); alphaxalone i.v.: wt n=6, β3(N265M) n=6. * p<0.05.
Discussion

We studied the effects of general anesthetics in mice harbouring an asparagine to methionine point mutation in position 265 of the β3 subunit of the GABA_A receptor. This point mutation renders recombinant β3-containing GABA_A receptors insensitive to the actions of the general anesthetics etomidate and propofol, but not to the neurosteroidal anesthetic alphaxalone (Pistis et al., 1999; Siegwart et al., 2002). The β3(N265M) mutation completely abolishes the direct (i.e. GABA-independent) action of pentobarbital, and shifts the concentration-response curve for the modulatory action of pentobarbital to the right (Pistis et al., 1999). In β3(N265M) mice the suppression of noxious-evoked movements in response to the anesthetics propofol and etomidate was completely abolished and the hypnotic response was also decreased significantly (Jurd et al., 2003). In addition, the respiratory depressant action of etomidate and propofol was strongly reduced. The β3(N265M) mice also show a slightly reduced hypothermia in response to etomidate, but not propofol (Zeller et al., 2005).

We investigated the actions of pentobarbital in β3(N265M) mice by assessing different anesthetic endpoints like immobility (suppression of noxious-evoked movements), hypnosis, respiratory depression, hypothermia, heart rate depression and influence on ECG. The β3(N265M) mice show a strongly reduced duration of the loss of righting reflex in response to pentobarbital, and a complete absence of the loss of the hindlimb withdrawal reflex. This reduction or loss of response to pentobarbital is similar to the altered response of the β3(N265M) mice to etomidate and propofol. The suppression of noxious-evoked withdrawal reflexes (immobility) is thought to be mediated by spinal cord circuits (Antognini and Schwartz, 1993; Antognini et al., 2000; Rampil, 1994; Rampil et al., 1993). β3-containing GABA_A receptors are indeed the predominant GABA_A receptor subtype expressed in dorsal root ganglia, the superficial dorsal horn of the spinal cord and motor neurons (Ma et al., 1993; Persohn et al., 1991). Our data now indicate that the action of pentobarbital on spinal cord-mediated reflexes occurs via β3-containing GABA_A receptors. Generation of respiratory rhythms occurs in a network of neurons originating from the pre-Bötzinger complex (Richter et al., 2003). Synaptic interactions involving AMPA,
NMDA, \( \text{GABA}_A \), \( \text{GABA}_B \) and glycine receptors are thought to play a major role in regulating this network. We have shown previously that etomidate and propofol-induced respiratory depression is mediated by \( \beta_3 \)-containing \( \text{GABA}_A \) receptors. It is currently unknown which neurons specifically mediate this effect. We investigated in this study whether pentobarbital-induced respiratory depression is also mediated by \( \beta_3 \)-containing \( \text{GABA}_A \) receptors. After injection of pentobarbital, \( \beta_3 \)(N265M) mice show a very pronounced respiratory depression similar to wild type mice. This indicates that pentobarbital-induced respiratory depression is not mediated by \( \beta_3 \)-containing \( \text{GABA}_A \) receptors or if it is to some degree, that pentobarbital can also induce respiratory depression via other targets. This anesthetic endpoint is therefore mediated by different receptors or circuits in etomidate- and propofol-induced anesthesia compared to pentobarbital-induced anesthesia.

Respiratory depression can be achieved by either inhibition of the overall glutamatergic drive or an enhanced overall GABAergic inhibitory drive to the neurons of the pre-Bötzinger complex or a combination of decreased excitation and enhanced inhibition (Stucke et al., 2005a; Stucke et al., 2005b). Sevoflurane, for example, has both effects (Stucke et al., 2005a). Etomidate and propofol apparently bind quite exclusively to \( \text{GABA}_A \) receptors and might therefore induce respiratory depression mostly by increasing GABAergic inhibition. Pentobarbital modulates the activity of more additional targets, e.g. it negatively modulates the activity of neuronal nACh, AMPA and Kainate receptors (Krasowski and Harrison, 1999; Petrenko et al., 2004) and might therefore have effects on both the excitatory and the inhibitory drive of the neurons of the pre-Bötzinger complex. The increase of inhibitory drive might be abolished in \( \beta_3 \)(N265M) mice, but the remaining excitatory drive may be sufficient to induce respiratory depression. Pentobarbital might therefore induce respiratory depression exclusively by decreasing the excitatory drive of the neurons of the pre-Bötzinger complex and for that reason \( \beta_3 \)(N265M) mice are still susceptible to pentobarbital-induced respiratory depression. It is tempting to speculate that this essential difference underlies the significantly smaller therapeutic range of barbiturates compared to etomidate and propofol. The propensity of barbiturates to cause potentially lethal respiratory depression is also exploited in assisted suicide and as an euthanizing agent in veterinary medicine.
Hypothermia is a common side effect of anesthesia. It has been shown that etomidate-induced hypothermia is largely mediated by both $\beta_2$- and $\beta_3$-containing GABA$_A$ receptors, with the $\beta_2$-containing GABA$_A$ receptors playing a dominant role (Cirone et al., 2004). We now measured the effect of pentobarbital on core body temperature. We show that pentobarbital-induced hypothermia is mediated to a limited degree by $\beta_3$-containing GABA$_A$ receptors. Other targets mediating the majority of pentobarbital-induced hypothermia might be $\beta_2$-containing GABA$_A$ receptors, but also other receptors.

General anesthetics are known to reduce heart rate in both mice and humans (Mitchell et al., 1998; Zeller et al., 2005). The heart rate depression is much less pronounced in humans where e.g. thiopental and low doses of propofol slightly increase heart rate, whereas higher doses of propofol and etomidate depress the heart rate (Kienbaum and Peters, 2001). However, in mice, heart rate depression is usually stronger, either due to different regulation of the cardiac system in mice and humans or due to the higher dosages usually used in experimental research (Appleton et al., 2004; Mitchell et al., 1998). We have shown previously that etomidate, propofol and alphaxalone depress the heart rate strongly in both $\beta_3$(N265M) and wild type mice. Heart rate depression is slightly reduced in $\beta_3$(N265M) mice after etomidate. In this report, we show that pentobarbital depresses heart rate less in $\beta_3$(N265M) mice compared to wild type mice. This shows that pentobarbital-induced heart rate depression is partly mediated by $\beta_3$-containing GABA$_A$ receptors, but mainly by other targets.

General anesthetic agents also alter electrocardiography (ECG) intervals, and they decrease heart rate variability in humans (Ledowski et al., 2005). Here, we report that HRV is increased by pentobarbital in mice. HRV is considered to be an indicator of cardiac vagal control, and drugs increasing HRV have been shown to reduce mortality and sudden death in patients with several chronic cardiac conditions in clinical trials (Routledge et al., 2002). This might indicate that general anesthetics induces a sympathetic blockade in mice which results in prolongation of time domain intervals such as QT, QRS and PQ and in an increase in HRV (Gehrmann et al., 2000). The increase in HRV after pentobarbital is slightly but significantly reduced in
β3(N265M) compared to wild type mice. Prolongation of QT, QRS and PQ intervals is similar and not statistically different in β3(N265M) and wild type mice. Although β-adrenergic receptors are thought to regulate HRV (Ecker et al., 2006), HRV might also be influenced by central nervous system mechanisms. Our results suggest that β3-containing GABA<sub>A</sub> receptors might play a role in this latter regulation.

In summary, in this study, we provide evidence that some anesthesia-related endpoints of pentobarbital, in particular LHWR and in part LORR, are mediated by β3-containing GABA<sub>A</sub> receptors. Particularly striking is that the respiratory depressing action of pentobarbital is independent of this receptor subtype, whereas the respiratory depressing actions of etomidate and propofol are mediated by this receptor subtype, consistent with a wider spectrum of relevant targets for pentobarbital. Our results show that it is possible to separate the immobilizing and the respiratory depressing action of general anesthetics.

**Acknowledgements**

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References


6.3. Mapping the contribution of β3-containing GABAA receptors to volatile and intravenous general anesthetic endpoints (Paper 3)

Anja Zeller¹, Margarete Arras ², Rachel Jurd¹, Uwe Rudolph¹,³

¹Institute of Pharmacology and Toxicology, University of Zürich, Winterthurerstr. 190, CH-8057 Switzerland, ²Institute of Laboratory Animal Science, University of Zürich, Winterthurerstr. 190, CH-8057 Switzerland, ³Laboratory of Genetic Neuropharmacology, McLean Hospital, Harvard Medical School, Belmont, MA 02478

Abstract

Background: Agents belonging to diverse chemical classes are used clinically as general anesthetics. The molecular targets mediating their actions are however still only poorly defined. Both chemical diversity and substantial differences in the clinical actions of general anesthetics suggest that general anesthetic agents may have distinct pharmacological targets. It was demonstrated previously that the immobilizing action of etomidate and propofol is completely mediated, and the immobilizing action of isoflurane partly mediated, by β3-containing GABAA receptors. This was determined by using the β3(N265M) mice, which carry a point mutation known to decrease the actions of general anesthetics at recombinant GABAA receptors. In this communication, we analyzed the contribution of β3-containing GABAA receptors to the pharmacological actions of isoflurane, etomidate and propofol by means of β3(N265M) mice. Results: Isoflurane decreased heart rate and core body temperature to a smaller degree in β3(N265M) mice than in wild type mice, indicating a minor but significant role of β3-containing GABAA receptors in these actions. Prolonged time intervals in the ECG and increased heart rate variability were indistinguishable between genotypes, suggesting no involvement of β3-containing GABAA receptors. The anterograde amnesic action of propofol was indistinguishable in β3(N265M) and wild type mice, suggesting that it is mediated independently of β3-containing GABAA receptors. The increase of heart rate variability and prolongation of ECG intervals by etomidate and propofol were also less pronounced in β3(N265M) mice than in wild type mice, pointing to a limited involvement of β3-containing GABAA receptors in these actions. The lack of etomidate- and propofol-induced immobilization in β3(N265M) mice was also observed in congenic 129/SvJ and
Results

C57BL/6J backgrounds, indicating that this phenotype is stable across different backgrounds. **Conclusion:** Our results provide evidence for a defined role of β3-containing GABA_A receptors in mediating some, but not all, of the actions of general anesthetics, and confirming the multisite model of general anesthetic action. This pharmacological separation of anesthetic endpoints also suggests that subtype-selective substances with an improved side-effect profile may be developed.

Keywords: anesthesia, animal model, GABA_A receptor, hypothermia, heart rate depression, heart rate variability, ECG intervals, isoflurane, etomidate, propofol

**Background**

The introduction of general anesthetics into medical practice 160 years ago has revolutionized surgical practice, however, the mechanisms of action of this class of drugs are still only poorly understood. Although general anesthetics have been shown to modulate the activity of a number of proteins, e.g. ligand-gated ion channels (Krasowski and Harrison, 1999) and two-pore domain potassium channels (Franks and Honore, 2004) *in vitro*, the identification of targets mediating specific actions of general anesthetics *in vivo* has only just begun.

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). Mutagenesis studies have identified amino acid residues in GABA_A receptor β subunits (e.g. N265 in the β3 subunit) to be crucial for the actions of the general anesthetics propofol and etomidate *in vitro* (Belelli et al., 1997; Krasowski et al., 1998b; Mihic et al., 1997; Siegwart et al., 2002; Siegwart et al., 2003). It has been shown that β3(N265M) mice are insensitive to the immobilizing and respiratory depressant action of etomidate and propofol and have a reduced sensitivity for the hypnotic action of these drugs (Jurd et al., 2003; Zeller et al., 2005), suggesting that β3-containing GABA_A receptors mediate these actions, while etomidate retains its sedative (motor depressant) action at subanesthetic doses. In line with these findings, β2(N265S) mice are still sensitive to the immobilizing and hypnotic actions of etomidate, but lack the sedative response to low doses of etomidate (Reynolds et al., 2003).
β3-containing GABA<sub>A</sub> receptors mediate endpoints of volatile and intravenous anesthetics (Paper 3)

Inhalation anesthetics like isoflurane show a wider range of targets in vitro, including the GABA<sub>A</sub> receptor, glycine receptor, 5-HT3 receptor, kainate receptor, nicotinic acetylcholine receptor, AMPA receptor, and NMDA receptor (Krasowski and Harrison, 1999). It was recently shown that the inhalational anesthetics isoflurane, enflurane and halothane exert their immobilizing action only partly via β3-containing GABA<sub>A</sub> receptors (Jurd et al., 2003; Lambert et al., 2005; Liao et al., 2005; Quinlan et al., 1998), suggesting that other targets of these volatile anesthetics mediate most of their immobilizing action (Franks and Honore, 2004). The extinction of the conditioned fear response by isoflurane, which is related to the amnestic action of isoflurane, on the other hand, has been suggested to be mediated by cortical α1-containing GABA<sub>A</sub> receptors (Sonner et al., 2005), which are frequently associated with β2 subunits (Benke et al., 1994).

In this study, we assessed the effects of isoflurane on heart rate, core body temperature and the ECG, the anterograde amnesic action of propofol, and the effects of propofol and etomidate on the ECG. We further assessed the immobilizing and hypnotic action of etomidate and propofol in mice carrying the β3(N265M) mutation on congenic C57BL/6J and 129X1/SvJ backgrounds to confirm the phenotype of the β3(N265M) mutation on two additional genetic backgrounds.

**Material and Methods**

**Animals**
Generation, characterization and breeding of β3(N265M) mice has been described previously (Jurd et al., 2003). Mice used for telemetry were 3 months old at the time of surgery, 4 months old at the beginning of the experiments and 10 months at the end of the telemetry experiments. Mice used in the passive avoidance paradigm were 6 to 8 weeks old, and mice used for reflex tests were 4 to 7 months old. Telemetry experiments were performed on 129/Sv x 129X1/SvJ (12.5%/87.5%) mice, anterograde amnesia was performed on a congenic 129X1/SvJ background (10 backcrosses with 129X1/SvJ), and loss of righting reflex and loss of hindlimb withdrawal reflex were performed on a congenic 129X1/SvJ background (10 backcrosses with 129X1/SvJ) and a congenic C57BL/6J background (9 generations of backcrosses with C57BL/6J mice). All animal experiments have been approved by the cantonal veterinary office in Zurich.

**Surgery**
16 female mice (8 β3(N265M) mice and 8 wild type controls) were implanted under isoflurane anaesthesia (3-5% in oxygen) with intraperitoneal radiotelemetry transmitters for measuring core body temperature, ECG and activity (model No. ETA-F20, Data Sciences International (DSI), St. Paul, MN). The transmitter body was implanted under sterile conditions in the abdominal cavity and the sensing leads were positioned as described previously (Späni et al., 2003). Mice received postoperative antibiotics (20 mg/kg sulfadoxin, 5 mg/kg trimethoprim, Borgal 7.5 %, Hoechst Roussel vet, Provet AG, Lyssach, Switzerland) and postoperative pain treatment for 5 days (2.5 mg/kg flunixin s.c., Finadyne, BERNA Veterinärprodukte AG, Berne, Switzerland). Mice were allowed to recover for 4 weeks before the first experiment. To ascertain full recovery after surgery we measured core body temperature and heart rate over 72 h.
Experimental Conditions
Mice implanted with telemetry transmitters were singly housed in standard laboratory conditions with a 12 h light/dark schedule (lights on 8:00 am, lights off 8:00 pm) and free access to food and water. Experiments were performed between 9 am and 12 am. Mice used for passive avoidance and reflex tests were group housed. The passive avoidance experiment was performed between 9 am and 12 am, the reflex tests between 8 am and 5 pm.

Effect of Anesthetics on Core Body Temperature (CBT), Heart Rate (HR) and ECG Parameters (PQ, QRS, QT, heart rate variability(HRV))
For drug and vehicle administration experiments, a baseline was recorded between 0 and 2 hours after lights on and drugs were administered immediately afterwards. Drug effects were compared to vehicle effects, which did not differ significantly from baseline, except for isoflurane, where the drug effect was compared to baseline. Mice were treated (in this order) with 30 mg/kg propofol i.v. (Sigma-Aldrich Chemicals, Buchs, Switzerland), 10 mg/ kg etomidate i.v. (Janssen-Cilag, Neuss, Germany), 15 mg/kg alphaxalone i.v. (Saffan®, alphaxalone/alphadolone 15/5 mg/kg), isoflurane 1.2% in air (Arovet, Zollikon, Switzerland), pentobarbital 60 mg/kg i.p. (Nembutal, Abbott AG, Baar, Switzerland / Abbott Laboratories, Chicago, USA), 180 mg/kg propofol i.p., 20 mg/kg etomidate i.p. (see also (Zeller et al., 2005)). Vehicle solutions were as follows: propofol, 14 % Cremophor EL, etomidate 35 % propylene glycol, Saffan® 0.9 % saline. The doses used for the i.v. route have been previously examined for their effects on loss of reflexes (Jurd et al., 2003). Intravenous injections were performed in the tail vein after warming the tail in 39°C warm water to achieve vasodilatation. The doses for the i.p. route were determined in pre-tests. The conditions for isoflurane application were chosen to ensure that animals would not die of hypothermia, since animals were not warmed up with an external heating source. For assessment of the effect of isoflurane, a sealed Plexiglas chamber was used as described previously (Lambert et al., 2005). Only one mouse at one time was placed into the chamber to avoid overlapping of the radio transmitter signals. After turning on the transmitters with a magnet, a one hour baseline was measured with data sampling for 30 s every 3 minutes. Five minutes before injection the sampling schedule was switched to continuous ECG recording and body temperature and heart rate were sampled every 30 s. Two hours after the return of the righting reflex the continuous sampling was switched to a data sampling for 30 s every 3 minutes and then continued for another 15 hours. Data were acquired with the Dataquest ART 3.0 acquisition system (DataSciences International, St. Paul, MN, USA). All signals (CBT, HR and ECG parameters) were recorded simultaneously in the same experiment. CBT and HR were calculated by the acquisition software (Dataquest A.R.T. 3.01, Data Sciences International). The ECG signal was further processed to derive time domain parameters (PQ, QRS, QT) with the Physiostat™ ECG Analysis 4.00 (DataSciences International) software.

Amnestic effect of propofol in a passive avoidance task
The amnestic effect of propofol was tested in a single-trial passive avoidance task. Propofol was injected 20 min before training at 0, 25, 50, 75 and 100 mg/kg i.p. After injection, mice were put back in their home cage and after 20 min, when the drug effect was fully developed, they were placed in a lit chamber (860 lux light intensity). Mice were allowed to explore the chamber for 30 sec, then a door was opened to a dark chamber and latency of the mouse to enter the dark chamber was measured (imprint). The door was closed and 10 sec later two foot shocks with 0.5 mA strength at an interval of 3 sec were applied. Retention was tested 24 h after training as latency to re-enter the dark chamber (retrieval). The experimenter was blinded to genotype and substance.

Statistical Analysis
Results are expressed as mean±SEM. For analysis of telemetry data statistical differences were assessed by using the paired Student’s t-test for testing whether the effect of anesthetic is significant compared to the vehicle, and the unpaired Student’s t-test for determining potential genotype differences between wild type and mutant mice. The minimum CBT or HR after injection of anesthetic and the mean of vehicle values over a time period of two hours after injection were determined and compared to the mean of one hour baseline before injection. For analysis of passive avoidance data, repeated measures ANOVA followed by a post hoc Bonferroni test was used. For analysis of reflex data, the unpaired Student’s t-test was used.
β3-containing GABA_A receptors mediate endpoints of volatile and intravenous anesthetics (Paper 3)

Results

The heart rate depressant effect of isoflurane is present but reduced in β3(N265M) mice

It has previously been shown that the immobilizing action of isoflurane is mediated only in part by β3-containing GABA_A receptors (Lambert et al., 2005; Liao et al., 2005). We now investigated whether the heart rate depressant action of isoflurane is dependent on β3-containing GABA_A receptors. We chose a concentration of 1.2% isoflurane, which represents approximately 0.7 "MAC" in wild type mice and 0.6 "MAC" in β3(N265M) mice with respect to the loss of the hindlimb withdrawal reflex (Lambert et al., 2005). After application of 1.2% isoflurane for 40 min, the heart rate of wild type mice decreased from a baseline value of 613±18 bpm to 408±27 bpm (-34%, p<0.001), whereas in β3(N265M) mice it decreased from a baseline value of 585±13 to 466±9 bpm (-21%) (p<0.01) (Fig. 1). The heart rate after isoflurane is significantly smaller in β3(N265M) mice compared to wild type mice under these experimental conditions (p<0.001), however, the difference is rather small and thus the heart rate depressant action of isoflurane is largely mediated by targets other than β3-containing GABA_A receptors. Alphaxalone, whose action is not affected by the point mutation (Siegwart et al., 2002), and which was used as a negative control, displayed no genotype difference with respect to heart rate depression (Zeller et al., 2005), indicating that the heart rate depressant action as such is not affected by the point mutation.

Figure 1. Isoflurane-induced heart rate depression. A. After application of isoflurane heart rate (HR) decreases in both wild-type and β3(N265M) mice. B. Maximum HR change after application of anesthetic compared to 1 hour baseline before application. For comparison, values for alphaxalone, a neurosteroid whose action at the GABA_A receptor is not influenced by the β3(N265M) point mutation, are displayed as well (Zeller et al., 2005). Isoflurane: n=7, alphaxalone i.v.: wt n=6, β3(N265M) n=6. * p<0.05, ** p<0.01, *** p<0.001.
The hypothermic effect of isoflurane is present but reduced in $\beta_3$(N265M) mice

Isoflurane decreases the core body temperature (CBT). When mice are placed in a chamber with 1.2% isoflurane (for 40 minutes), the CBT starts to decrease within 5 minutes. The CBT decreased from 36.4±0.4 °C and 36.1±0.1 °C to 30.9±0.4 °C (p<0.001) and 31.6±0.2 °C (p<0.001) (-16% and -12%) in wild type and $\beta_3$(N265M) mice, respectively (p<0.05 between genotypes) (Fig. 2). Immediately after the mouse is taken out of the isoflurane chamber, the CBT increases again. The decrease of CBT in the presence of isoflurane is pronounced in both genotypes although slightly but significantly smaller in $\beta_3$(N265M) mice, indicating a role for the $\beta_3$-containing GABA$_A$ receptors in this drug action. The hypothermic response to alphaxalone was not different between $\beta_3$(N265M) and wild type mice, indicating that $\beta_3$(N265M) mice respond properly to a hypothermic challenge.

Figure 2. Isoflurane-induced hypothermia. A. After application of isoflurane core body temperature (CBT) decreases in both wild-type and $\beta_3$(N265M) mice. B. Maximum CBT change after application of anesthetic compared to 1 hour baseline before application. For comparison, values for alphaxalone are displayed as well (Zeller et al., 2005). Isoflurane: n =7, alphaxalone i.v.: wt n=6, $\beta_3$(N265M) n=6. * p<0.05, ** p<0.01.
**Effects of isoflurane on ECG parameters**

General anesthetics are known to alter ECG parameters in humans. In mice, ECG changes have only been reported for ketamine (Mitchell et al., 1998). We determined the effects of isoflurane on the ECG in wild type and β3(N265M) mice (Fig. 3, Table 1). In wild type mice, isoflurane increased heart rate variability, the PQ, QT, and QRS intervals. There was no genotype difference in these parameters suggesting no role of β3-containing GABA_A receptors in the effects of isoflurane on these ECG parameters. There were no genotype difference after alphaxalone, indicating an unaltered responsiveness of β3(N265M) mice to changes in the ECG.

**Figure 3. Isoflurane-induced changes of ECG intervals. A,C.** With application of isoflurane, QT and PQ intervals are prolonged. The prolongation is slightly less pronounced in β3(N265M) mice compared to wild type. **B,D.** Maximum change of PQ and QT after application of isoflurane compared to 1 hour baseline before application. For comparison, values for alphaxalone are displayed as well. Isoflurane: n=7; alphaxalone i.v.: wt n=6, β3(N265M) n=6.
**Results**

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**Table 1. Effects of isoflurane-induced anesthesia on baseline ECG parameters.** All values are mean ± SEM. RR inter-beat-interval. Group sizes: isoflurane: wt n = 7, β3(N265M) n = 7; alphaxalone i.v.: wt n = 6, β3(N265M) n = 6. * p<0.05, ** p<0.01, compared to baseline.

β3(N265M) mice on different genetic backgrounds show similar response to propofol and etomidate

In view of extensive literature on the influence of genetic background in genetically modified mice, we checked whether the response of β3(N265M) mice to general anesthetics is influenced by this. All experiments done in these mice published so far (Jurd et al., 2003; Zeller et al., 2005) and all experiments presented so far in this communication were done with mice harbouring the β3(N265M) point mutation on a mixed background of 129X1/Sv x 129/SvJ. To test whether the genetic background influences the response to etomidate- and propofol-induced loss of righting reflex (LORR) and loss of hindlimb withdrawal reflex (LHWR), these mice were backcrossed 9 and 10 times, respectively, either to C57/BL/6J or 129X1/SvJ wild type mice, to obtain the mutation on a congenic background. These mice were tested with etomidate (10 mg/kg i.v.) and propofol (30 mg/kg i.v.) (Fig. 4). After injection of etomidate LORR was 30±6 min and 18±4 min, in the C57BL/6J wild type and 129X1/SvJ wild type mice, respectively. The duration of LHWR was 7.3±0.9 min and 9.1±1.3 min in C57BL/6J and 129X1/SvJ wild type mice, respectively. Similar to what was previously observed in the mixed background 129/Sv x 129/SvJ β3(N265M) mice (Jurd et al., 2003), also in β3(N265M) mice on the C57BL/6J and 129X1/SvJ backgrounds, 10 mg/kg etomidate lead to a significantly decreased LORR (14±2 min, 6±1 min, respectively, p<0.05, versus wild type) and to an abolished LHWR (0 min on both backgrounds, p<0.001 versus the corresponding wild type). Thus, we have
observed the same phenotype in three different backgrounds, suggesting that it is robust across different genetic backgrounds.

Figure 4. Behavioural responses to propofol and etomidate in β3(N265M) and wild type mice on pure C57/Bl6J or 129/SvJ background. A,C. Reduction in the duration of the loss of righting reflex (LORR) induced by propofol and etomidate in A) C57/Bl6J β3(N265M) and C) 129X1/SvJ β3(N265M) mice is not influenced by the genetic background. B,D. Failure to induce loss of hindlimb withdrawal reflex induced by propofol and etomidate in B) C57/Bl6J β3(N265M) and D) 129X1/SvJ β3(N265M) mice is not influenced by the genetic background. n=10. * p<0.05, *** p<0.001.

Propofol induces anterograde amnesia in β3(N265M) mice

General anesthetics are known to cause anterograde amnesia. We tested whether propofol-induced anterograde amnesia is mediated by β3-containing GABA<sub>A</sub> receptors. Increasing doses of propofol (25, 50, 75 and 100 mg/kg i.p.) were employed (Fig. 5). The increasing imprint latency on the training day at the two highest doses of 75 and 100 mg/kg propofol might indicate that in both genotypes mice are slightly sedated at these doses. Most importantly, propofol decreased retrieval, i.e. the latency to re-enter the dark compartment, to a similar degree in wild
type and β3(N265M) mice. ANOVA indicated a difference between the drug doses [F(1, 112)=16.723, p<0.001 overall, F(4,112)=4.997, p<0.001 for drug effect], but no genotype effect [F(1,112)=3.413, p=0.067 for genotype effect].

These results indicate that propofol produces anterograde amnesia in both wild type and β3(N265M) mice and thus by targets independent of the β3-containing GABA<sub>A</sub> receptors.

**Figure 5.** Propofol induces anterograde amnesia in β3(N265M) mice. Dose-dependent effect of propofol on imprint and retrieval in both β3(N265M) and wild type mice. n=15.

**Effects of etomidate and propofol on ECG parameters**

When applying etomidate and propofol via the i.v. route and the i.p. route, we observed a strong decrease in heart rate (see also (Zeller et al., 2005)) and a prolongation of all time domain parameters measured (RR interval, PQ interval, QRS interval, and QT interval (Table 2, 3)). The heart rate variability (HRV) increases 2.5 to 8-fold (etomidate 20 mg/kg i.p. and etomidate 10 mg/kg i.v.) in wild type mice and 3 to 5-fold in β3(N265M) mice (propofol 30 mg/kg i.v. and etomidate 10 mg/kg i.v.). The HRV in β3(N265M) mice after injection of etomidate 10 mg/kg i.v. is significantly smaller than HRV in wild type mice after etomidate 10 mg/kg i.v. (p<0.05). No genotype differences were noted for all other drug applications. QT, QRS and PQ
β3-containing GABA<sub>A</sub> receptors mediate endpoints of volatile and intravenous anesthetics (Paper 3)

Intervals are prolonged in wild type mice and β3(N265M) mice after injection of etomidate and propofol i.v. and i.p.. There is no genotype effect after injection of any anesthetic for the QRS interval, for the QT interval there is a genotype difference only after propofol 180 mg/kg i.p. (p<0.05) and for PQ there is a genotype difference only after etomidate 10 mg/kg i.v. (p<0.05). In wild type mice, potential changes compared to vehicle are not significant for any interval after propofol 30 mg/kg i.v., only for PQ after propofol 180 mg/kg i.p. (p<0.05), after etomidate 10 mg/kg i.v. the increases of QRS and PQ are significant (p<0.05, p<0.001, respectively) and after etomidate 20 mg/kg i.p. the increases of QT, QRS, and PQ are significant. In β3(N265M) mice, the increase compared to vehicle is significant after propofol 30 mg/kg i.v. for PQ, after etomidate 10 mg/kg i.v. for QT and PQ, and after etomidate 20 mg/kg i.p. for none of the ECG intervals. Genotype differences were found for none of the intervals after propofol 30 mg/kg i.v., for QT after propofol 180 mg/kg i.p., for PQ after etomidate 10 mg/kg i.v. and for none of the intervals after etomidate 20 mg/kg i.p.. In summary, as mentioned previously for isoflurane, etomidate and propofol lead to changes in the ECG, which are largely independent of β3-containing GABA<sub>A</sub> receptors.
Table 2. Effects of propofol- and etomidate- induced anesthesia on baseline ECG parameters. All values are mean ± SEM. Group sizes: propofol i.v.: wt n = 7, β3(N265M) n= 5, etomidate i.v.: wt n = 7, β3(N265M) n = 7, alphaxalone i.v.: wt n= 6, β3(N265M) n = 6, propofol i.p.: wt n = 4, β3(N265M) n = 6, etomidate i.p.: wt n = 5, β3(N265M) n= 5. * p<0.05, ** p<0.01, *** p<0.001 compared to baseline, # p<0.05 wild type compared to β3(N265M) mice. Statistical comparison of drug values with vehicle values and of drug values with baseline values yields similar results.
### Table 3. Effects of vehicles on ECG parameters.

Vehicle values are the mean of 2 hours after injection of the vehicle. All values are mean ± SEM. For group sizes, see legend to Table 2.
Discussion

In this report, we investigated the contribution of β3-containing GABA<sub>A</sub> receptors to various physiological and behavioural endpoints of the inhalational general anesthetic isoflurane and the intravenous general anesthetics propofol and etomidate. We show that the hypothermic and cardiac depressant actions of isoflurane are to a small but significant degree mediated by β3-containing GABA<sub>A</sub> receptors, and that the anterograde amnestic action of propofol is not mediated by β3-containing GABA<sub>A</sub> receptors. We also found that the resistance of the β3(N265M) to the immobilizing action of etomidate and propofol and their partial resistance to the hypnotic action of etomidate and propofol are present on a total of three different genetic backgrounds.

We studied the effects of general anesthetics in mice harbouring an asparagine to methionine point mutation in position 265 of the β3 subunit of the GABA<sub>A</sub> receptor. This point mutation renders β3-containing GABA<sub>A</sub> receptors insensitive to the general anesthetics propofol and etomidate in a recombinant system, but not to alphaxalone (Siegwart et al., 2002). The action of the volatile anesthetic enflurane is also strongly reduced by the N265M point mutation in a recombinant system (Jurd et al., 2003; Siegwart et al., 2003). In addition, an in vivo increase of the EC<sub>50</sub> values for enflurane, halothane and isoflurane by 16%, 21%, and 24% has been reported for their immobilizing action in β3(N265M) mice (Jurd et al., 2003; Lambert et al., 2005). In β3(N265M) mice the suppression of noxious-evoked movements by etomidate and propofol, as measured by the loss of the hindlimb withdrawal reflex was completely abolished and the obtundung or hypnotic response, as determined by the loss of the righting reflex, was also decreased significantly (Jurd et al., 2003). In addition, the respiratory depressant action of propofol and etomidate was strongly reduced in the β3(N265M) mice (Zeller et al., 2005). The β3(N265M) mice also display a slightly reduced hypothermia in response to etomidate, but not to propofol (Zeller et al., 2005).

It was previously shown that isoflurane induces LORR and LHWR largely via targets other than β3-containing GABA<sub>A</sub> receptors, although these receptors play an
β3-containing GABA<sub>A</sub> receptors mediate endpoints of volatile and intravenous anesthetics (Paper 3)

appreciable role in mediating LHWR (Lambert et al., 2005; Liao et al., 2005). In this study, we report that core body temperature, heart rate and ECG changes in response to isoflurane are also partly mediated by β3-containing GABA<sub>A</sub> receptors, although other targets mediate a larger part of these responses. The decrease in the heart rate may be secondary to the decrease in body temperature and thus represent a dependent and not an independent parameter. It is noteworthy that isoflurane typically increases heart rate in humans. This might be due to fact that during anesthesia in man the temperature is typically controlled to some degree, or there might be a species difference.

General anesthetics are known to reduce heart rate in mice and man (Zeller et al., 2005). The heart rate depression is much less pronounced in man where e.g. thiopental and low doses of propofol even slightly increase heart rate, whereas higher doses of propofol and etomidate depress heart rate (Zeller et al., 2005). Heart rate depression observed in mice is usually stronger, either due to potential species differences in the regulation of the cardiac system or due to the higher dosages usually used in mice (Mitchell et al., 1998), it could also be secondary due to hypothermia, which is better controlled in man. We have shown previously that etomidate, propofol and alphaxalone depress heart rate strongly in both β3(N265M) and wild type mice (Zeller et al., 2005). Heart rate depression is slightly reduced in β3(N265M) mice after application of etomidate (Zeller et al., 2005), and we now show that the heart rate depressant action of isoflurane is also slightly but significantly reduced in β3(N265M) mice. Likewise, the hypothermic action of isoflurane is slightly reduced in β3(N265M) mice, indicating that β3-containing GABA<sub>A</sub> receptors play a minor role in the heart rate depressing action of isoflurane, similar to what was previously observed for etomidate (Zeller et al., 2005).

General anesthetic agents also alter electrocardiographic (ECG) intervals and decrease heart rate variability in humans as was shown for induction with the barbiturate thiopentone and subsequent inhalation of isoflurane-nitrous oxide (Ledowski et al., 2005). Here, we report that heart rate variability (HRV) is increased by general anesthetics in mice. HRV is considered to be an indicator of cardiac vagal control, and drugs increasing HRV have been shown to reduce mortality and sudden death in severe heart failure in clinical trials (Routledge et al., 2002). Our finding
might indicate that general anesthetics reduces sympathetic tone in mice (Gehrmann et al., 2000) which results in prolongation of time domain intervals such as QT, QRS and PQ and in an increase in HRV. We showed an increase of HRV after all anesthetics tested, but most pronounced in wild type mice after application of etomidate i.v.. The increase in HRV after etomidate is slightly but significantly reduced in β3(N265M) compared to wild type mice. Prolongation of QT, QRS and PQ intervals is similar in β3(N265M) and wild type mice. To our knowledge, no central mechanisms of HRV regulation have been investigated in mice. In the periphery, β-adrenergic receptors expressed in cardiac tissue are thought to regulate HRV (Ecker et al., 2006). β1 and β2-ARs play differential roles in the modulation of HRV, each receptor subtype regulating different frequency components of HRV (Ecker et al., 2006). β3-containing GABA_A receptors might play a role in the central regulation of HRV.

Based on differential sensitivities to propofol of inbred long sleep (ILS) and inbred short sleep (ISS) mice, it has been postulated that a gene responsible for the LORR induced by propofol, termed Lorp1, would be located in a 99% confidence interval from 71.4-89.7 Mb on mouse chromosome 7 (Simpson et al., 1998), and in addition, an etomidate-sensitivity QTL has also been identified in this chromosome region (Christensen et al., 1996; Downing et al., 2003). So far, the identity of the Lorp1 gene is unknown. Interestingly, the Gabrb3 gene encoding the β3 subunit of the GABA_A receptor is also located on mouse chromosome 7, between 57.4 and 57.7 Mb. To ascertain that the phenotype previously described in 129/Sv x 129/SvJ mice, i.e. partial loss of LORR and complete loss of LHWR in response to etomidate and propofol in β3(N265M) mice (Jurd et al., 2003) is really due to the point mutation in the GABA_A receptor β3 subunit, the mutant mice were bred for 10 and 9 generations, respectively, onto the 129X1/SvJ and C57BL/6J backgrounds, to yield congenic mice. In all backgrounds examined, we observed the same phenotype, demonstrating that this phenotype is very robust across different backgrounds, and thus that the observed phenotype is really associated with the N265M point mutation in the Gabrb3 gene. Thus, our analysis shows that Gabrb3 and Lorp1 are separate genes.
We further tested β3(N265M) mice on the congenic 129X1/SvJ background, in the passive avoidance paradigm, to examine whether the anterograde amnesic action of propofol would be mediated by β3-containing GABA<sub>A</sub> receptors. Our results suggest that this anesthetic endpoint is independent of β3-containing GABA<sub>A</sub> receptors. This result is consistent with previous findings that both the anterograde amnesic action of diazepam (Rudolph et al., 1999) (studied using the same paradigm) as well as the anterograde amnesic action of isoflurane (determined as an extinction of conditioned fear response) (Sonner et al., 2005) are mediated by α1-containing GABA<sub>A</sub> receptors. Since α1β2γ2 is the most abundant GABA<sub>A</sub> receptor subtype (Fritschy et al., 1992), it is tempting to speculate, and consistent with all data currently available, that this receptor subtype mediates the anterograde amnesic actions not only of sedative-hypnotic agents like diazepam (Rudolph et al., 1999), but also of general anesthetic agents. However, in α5<sup>−/−</sup> mice, long term potentiation (LTP) in CA1 is reduced by etomidate in wild type but not in α5<sup>−/−</sup> mice (Cheng et al., 2006). Furthermore, learning in the Morris water maze and in fear conditioning is impaired by etomidate in wild type mice, but less pronounced in α5<sup>−/−</sup> mice (Cheng et al., 2006). These data indicate a role for α5-containing GABA<sub>A</sub> receptors in drug-induced amnesia, in addition to its involvement in certain hippocampus-dependent forms of associative learning like trace fear conditioning (Cheng et al., 2006; Crestani et al., 2002).

Our current knowledge on the role of β3-containing GABA<sub>A</sub> receptors in the action of the general anesthetics etomidate, propofol and isoflurane is summarized in Table 4. The genetic dissection of the pharmacological spectrum of general anesthetics is of interest for the design of novel general anesthetic compounds, in which the various desired and undesired effects can be separated.

**Conclusion**

We show that β3-containing GABA<sub>A</sub> receptors mediate a small, but significant, part of isoflurane-induced heart rate depression and hypothermia, which is in line with the small but significant contribution of β3-containing GABA<sub>A</sub> receptors to isoflurane-induced immobility and hypnosis reported previously. We also found that isoflurane-induced ECG changes are not mediated by β3-containing GABA<sub>A</sub> receptors. These
data indicate that isoflurane exerts its effects via many targets, β3-containing GABA<sub>Α</sub> receptors being one of them. Furthermore, we found a dissociation between the immobilizing and anterograde amnestic action of propofol. Whereas as previously shown the immobilizing action of etomidate is mediated by β3-containing GABA<sub>Α</sub> receptors, the anterograde amnestic action is independent of this receptor subtype. Prolongation of ECG intervals induced by etomidate and propofol was in part mediated by β3-containing GABA<sub>Α</sub> receptors. By demonstrating that etomidate- and propofol-induced immobilization is mediated exclusively and hypnosis is mediated partly by β3-containing GABA<sub>Α</sub> receptors in two congenic backgrounds, we show that this phenotype is robust across three backgrounds and that the Gabrb3 locus is different from the Lorp1 locus.

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Table 4. Proposed roles of β3-containing GABA<sub>Α</sub> receptors in the actions of the general anesthetics etomidate, propofol and isoflurane. +++ designates complete mediation of this action by β3-containing GABA<sub>Α</sub> receptors. ++ designates partial mediation (approximately 50%), + designates a small but significant contribution of β3-containing GABA<sub>Α</sub> receptors. --- designates no mediation at all or a very minor contribution through β3-containing GABA<sub>Α</sub> receptors. n.d. not determined. Data from <sup>1</sup>Jurd et al., 2003; <sup>2</sup>Reynolds et al., 2003b; <sup>3</sup>Cirone et al., 2004; <sup>4</sup>Lambert et al., 2005; <sup>5</sup>Zeller et al., 2005).
List of abbreviations

GABA  \( \gamma \)-aminobutyric acid
GABA\(_A\) receptor  GABA type A receptor
HR  heart rate
CBT  core body temperature
ECG  electrocardiogram

Authors' contributions

AZ designed and carried out telemetry experiments, reflex tests, passive avoidance test, analysed all data and drafted the manuscript.
MA consulted on telemetry experiments and statistical analysis.
RJ generated the \( \beta_3(N265M) \) mouse model and participated in the preparation of the manuscript.
UR conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

β3-containing GABAA receptors mediate endpoints of volatile and intravenous anesthetics (Paper 3)


6.4. Identification of GABA_A receptor α subunits mediating the hypnotic and immobilizing actions of diazepam (Paper 4)

Anja Zeller¹, Ruth Keist¹, Uwe Rudolph¹,²
¹ Institute of Pharmacology and Toxicology, University of Zürich, Winterthurerstr. 190, CH-8057 Switzerland, ² Laboratory of Genetic Neuropharmacology, McLean Hospital, Harvard Medical School, Belmont, MA 02478, USA

Abstract

GABA_A receptors have been shown to mediate the hypnotic and immobilizing action of the intravenous anesthetics etomidate and propofol. More specifically, β3-containing GABA_A receptors mediate their immobilizing action, while β2- and β3-containing GABA_A receptors mediate their hypnotic action. However, the precise subunit composition of the GABA_A receptors mediating these actions is unknown. Therefore, we determined diazepam-induced hypnosis and immobility in α1(H101R), α2(H101R), α3(H126R), and α5(H105R) mice carrying point mutations rendering the respective GABA_A receptors insensitive for diazepam. While duration of the loss of righting reflex, indicative for hypnosis, was reduced in α5(H105R) mice, the loss of the hindlimb withdrawal reflex, indicative of immobilization, was reduced in α3(H126R), and α5(H105R) mice. Thus, α5-containing receptors partly mediate hypnosis, while α3- and α5-containing GABA_A receptors mediate immobility. Based on our knowledge of β subunit selectivity of general anesthetic action and the fact that diazepam-sensitive GABA_A receptors typically contain the γ2 subunit, we predict that α5β2/3γ2 receptors are mediating hypnosis while α3β32 and α5β3γ2 GABA_A receptors mediate immobilization.

Keywords: animal model, GABA_A receptor, immobility, hypnosis, diazepam
**Introduction**

GABA\(_A\) (\(\gamma\)-aminobutyric acid) receptors are molecular substrates for the regulation of vigilance, anxiety, muscle tension, epileptogenic activity and memory functions, all of which can be influenced by drugs binding to the benzodiazepine-binding site. It has been shown by means of introducing point mutations into GABA\(_A\) receptor \(\alpha\) subunits which abolish benzodiazepine binding that specific \(\alpha\) subunits of the GABA\(_A\) receptor mediate well defined actions of benzodiazepines. \(\alpha1\)-containing GABA\(_A\) receptors mediate the sedative and anterograde amnesic action of diazepam (Rudolph et al., 1999a), \(\alpha2\)-containing GABA\(_A\) receptors the anxiolytic-like action and in part the muscle relaxant action of diazepam (Low et al., 2000), \(\alpha3\)-containing GABA\(_A\) receptors in part the muscle relaxant action of diazepam at higher doses (Collins et al., 2002; Crestani et al., 2001) and \(\alpha5\)-containing GABA\(_A\) receptors are involved in a certain forms of learning and memory (Crestani et al., 2002). \(\alpha5\) knockout mice display a better performance in the Morris water maze test, a spatial learning task dependent on hippocampal function (Collinson et al., 2002).

All the actions of diazepam described above are achieved at relatively low doses. The anxiolytic effect is observed at 0.5–3 mg/kg p.o. (Löw et al., 2000), the anterograde amnesic action at 6 mg/kg p.o. (Rudolph et al., 1999b) and the muscle relaxant and sedative action of diazepam are typically seen at 3 to 30 mg/kg p.o. (Rudolph et al., 1999). In clinical anesthesia, benzodiazepines are used to sedate patients preoperatively and for induction and maintenance of anesthesia. With sufficient doses of general anesthetic drugs, patients loose consciousness (hypnosis) and then finally do not respond to noxious stimuli (immobility, surgical tolerance) (Rudolph and Antkowiak, 2004). Very high doses of diazepam have been shown to induce hypnosis and immobility in mice and rats (Kissin, 1997; Kralic et al., 2002b).

The goal of this study was to investigate the contribution of individual \(\alpha\) subunits of the GABA\(_A\) receptor to hypnosis and immobility.
### Material and Methods

#### Animals

Generation, characterization and breeding of $\alpha_{1}(H101R)$, $\alpha_{2}(H101R)$, $\alpha_{3}(H126R)$, $\alpha_{5}(H105R)$ mice has been described previously (Crestani et al., 2002; Crestani et al., 2001; Low et al., 2000; Rudolph et al., 1999). $\alpha_{1}(H101R)/\alpha_{2}(H101R)/\alpha_{3}(H126R) (=\alpha_{123})$ mice and $\alpha_{1}(H101R)/\alpha_{2}(H101R)/\alpha_{5}(H105R) (=\alpha_{125})$ triple mutant knockin mice were derived from interbreeding the single knockin strains. Mice were congenic on the 129/SvJ background. Mice used for reflex tests were 4 to 7 months old. Mice were used only once.

#### Behavioral analyses of intravenous anesthetics

The duration of the loss of righting reflex (LORR) and loss of hind-limb withdrawal reflex (LHWR) was recorded as described previously (Arras et al., 2001). Briefly, the LORR was assessed by measuring the time a mouse remains on its back on a plane surface. The LHWR, which is always shorter than the LORR and starts after onset of LORR and stops before LORR is regained, was determined by pinching a mouse with a pair of tweezers into the interdigital skin of the hindlimb. The reflex was rated as being present when a mouse retracts its hindlimb upon pinching. Pre-tests were done to determine doses and injection routes of diazepam with which mice lose either one or both reflexes. To study LORR, 40 mg/kg diazepam were injected i.v. To study LHWR, 120 mg/kg diazepam i.p. were administered in a volume of 8 $\mu$l/ g body weight, and 30 min later, when the effect of the first dose was fully developed, 40 mg/kg diazepam i.v. were administered in a volume of 4 $\mu$l/ g body weight into the tail vein. Only mice that recover from this anesthesia overnight were included in the analysis. The genotype difference of the diazepam effect was assessed by one way ANOVA followed by a Bonferroni post hoc test. Results are given as mean±SEM.

### Results

#### LORR time is reduced in $\alpha_{5}(H105R)$ mice compared to wild type

At 40 mg/kg i.v. all genotypes lose the righting reflex, but none loses the hindlimb withdrawal reflex. Only this dose and this route of injection resulted in a reliable LORR in every single mouse on one hand and in a reasonable time frame of LORR. Wild type mice lose the righting reflex for 41.4±1.7 min (34 to 54 min), $\alpha_{1}(H101R)$ mice for 35.3±5.4 min (12 to 80 min), $\alpha_{2}(H101R)$ mice for 32±3.9 min (17 to 60 min), $\alpha_{3}(H126R)$ mice for 38.7±5.8 min (10 to 86 min), $\alpha_{5}(H105R)$ mice for 13.6±4 min (0 to 43 min), only one mouse out of 9 $\alpha_{5}(H105R)$ mice did not lose the righting reflex.

One-way ANOVA revealed a significant genotype interaction [F(4,63)=5.294, P<0.001]. Bonferroni post hoc test revealed a significant reduction of LORR in $\alpha_{5}(H105R)$ mice compared to wild type (p<0.001).

#### LHWR is absent in $\alpha_{3}(H126R)$, $\alpha_{5}(H105R)$, $\alpha_{123}$ and $\alpha_{125}$ triple knock-in mice

To achieve LHWR for approximately 15 min in wild type mice, two subsequent injections of diazepam were necessary. The first injection consisted of a very high
dose of diazepam (120 mg/kg) i.p., followed 30 min later, when the diazepam effect had fully developed, by a rather high dose of diazepam (40 mg/kg) i.v.. The i.p.
 injection alone induced a long-lasting LORR of at least 4 hours, often up to 6 hours. Nevertheless, despite this high dose of diazepam, mice did not lose the hindlimb withdrawal reflex. Only upon i.v. injection of the second dose of diazepam, LHWR was achieved almost immediately after injection. This complex injection schedule was necessary because of the low solubility of diazepam at the high concentrations necessary for loss of these reflexes. Wild type mice lose the hindlimb withdrawal reflex for 14.8±4.6 min (2 to 30 min), α1(H101R) mice for 8.0±3.0 min (3 to 16 min), α2(H101R) mice for 10.8±1.6 min (8 to 18 min), α3(H126R) mice for 1.2±0.7 min (0 to 4 min), 3 out of 5 α3(H126R) mice did not lose the hindlimb withdrawal reflex, α5(H105R) mice for 1.2±0.7 min (0 to 4 min), 4 out of 6 α5(H105R) mice did not lose the hindlimb withdrawal reflex, none out of 7 α123 knockin mice and none out of 6 α125 knockin mice lost the hindlimb withdrawal reflex. One-way ANOVA revealed a significant genotype interaction \[F(6,39)=10.276, P<0.001\]. Bonferroni post hoc test revealed a significant reduction of LHWR in α3(H126R) and α5(H105R) mice compared to wild type (p<0.001) and also in α123 and α125 knockin mice (p<0.001).

Figure 1. Behavioural responses to diazepam in α1(H101R), α2(H101R), α3(H126R), α5(H105R), α123 and α125 triple knock-in and wild type mice. A. Reduction in the duration of the loss of righting reflex (LORR) induced by diazepam in α5(H105R) mice compared to wild type mice. B. Reduction in the duration of loss of hindlimb withdrawal reflex (LHWR) in α3(H126R), α5(H105R), α123, α125 mice compared to wild type mice. ** p<0.01, n=9–13 for LORR, n=5–7 for LHWR.
Results

Discussion

In this report, we investigated the contribution of different $\alpha$ subunits of the GABA$_A$ receptor to the hypnotic and immobilizing actions of diazepam. Building on a previous observation by others that rodents can be immobilized with high doses of diazepam which are relatively close to the lethal dose (Kissin, 1997), we tested the effect of diazepam on two major anesthetic endpoints, hypnosis and immobility, in mice carrying histidine to arginine point mutations at a homologous residue in the $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$ subunits, respectively. Since we have previously shown that the histidine to arginine point mutations dramatically decreases the sensitivity to diazepam by at least 200 fold (Crestani et al., 2002; Low et al., 2000; Rudolph et al., 1999), we were confident that the GABA$_A$ receptors carrying the point mutations would also be insensitive to the high concentrations of diazepam used to achieve LORR and LHWR. We show that in $\alpha_5$(H105R) mice the hypnotic action of diazepam is reduced. In $\alpha_3$(H126R) and $\alpha_5$(H105R) mice the immobilizing action of diazepam is completely abolished.

Using the horizontal wire test and the inverted screen test, our group previously reported that at doses up to 10 mg/kg of diazepam the myorelaxant action of diazepam is largely mediated by $\alpha_2$-containing GABA$_A$ receptors, whereas at higher doses $\alpha_3$- and $\alpha_5$-containing GABA$_A$ receptors also play a role (Crestani et al., 2002; Crestani et al., 2001). It is not known which particular circuits are crucial for diazepam-induced muscle relaxation. The partial resistance of $\alpha_5$(H105R) mice to both diazepam-induced LORR and diazepam-induced myorelaxation in the horizontal wire test indicates that $\alpha_5$-containing GABA$_A$ receptors are involved in mediating both tasks, which might be partly overlapping in nature. However, whereas in the horizontal wire and inverted screen tests we observed a small contribution of $\alpha_3$-containing GABA$_A$ receptors to the response, this was not the case for LORR, indicating that $\alpha_3$-containing GABA$_A$ receptors mediate a component of the myorelaxant action of diazepam which may be less relevant for LORR.

Based on the examination of diazepam-induced LHWR in point-mutated mice, we conclude that both $\alpha_3$- and $\alpha_5$-containing GABA$_A$ receptors mediate immobility. This
receptor subtype profile for immobility (LHWR) (α3, α5) is thus different from the profile for hypnosis (LORR) (α5) and the profile for myorelaxation (horizontal wire test)(α2, α3, α5). We find it most surprising that the receptor subtype that plays a major role in mediating the myorelaxant action of diazepam has no apparent role in the immobilizing action. This might be explained in the way that at the high doses of diazepam used in these experiments receptor subtypes other than α2 can mediate the “myorelaxation”, i.e. the efferent portion of the LHWR. The presence of both the α3- and α5-containing GABA_A receptors may be required in the afferent portion of the LHWR. The latter conclusion is based on the observations that diazepam action on α1- , α2-, and α5-containing GABA_A receptors in α3(H126R) mice and on α1- , α2-, and α3-containing GABA_A receptors in α5(H105R) mice is not sufficient to achieve immobilization, while based on our previous results diazepam action on these subunit combinations should result in myorelaxation. In triple knock-in mice we checked whether diazepam action selectively on α5-containing GABA_A receptors or α3-containing GABA_A receptors is sufficient to achieve immobilization. The observation that there is no LHWR in α123 and α125 triple mutant mice indicates that diazepam action selectively on α3- or α5-containing GABA_A receptors is insufficient to achieve immobilization. All of our results reported here indicate that immobilization can only be achieved by the concomitant positive allosteric modulation of both the α3-containing GABA_A receptors and α5-containing GABA_A receptors. Individually, α3-containing GABA_A receptors and α5-containing GABA_A receptors are necessary and sufficient for immobilization. Combined, we propose that they are both necessary and sufficient for immobilization (Table 1).

Using point-mutated knock-in mice with etomidate-insensitive β2 or β3 subunits, respectively, it was shown that sedation induced by subanesthetic doses of etomidate is mediated by GABA_A receptors containing the β2 subunit (Reynolds et al., 2003) but not by GABA_A receptors containing the β3 subunit (Zeller et al., 2005). Since the γ2 subunit is a necessary part of the benzodiazepine binding site, it can be suggested that receptors with the subunit composition α1β2γ2 mediate sedation. The α1β2γ2 GABA_A receptor constitutes approximately 60% of all diazepam-sensitive GABA_A receptors (Benke et al., 2004). This GABA_A receptor subtype and the neuronal circuits where the α1β2γ2 subunit combination is expressed are expected to
mediate the sedative action of drugs binding to the GABA<sub>A</sub> receptor. The hypnotic action of etomidate and propofol appears to be mediated by both GABA<sub>A</sub> receptors containing the β2 subunit and GABA<sub>A</sub> receptors containing the β3 subunit (Jurd et al., 2003, Reynolds et al., 2003), whereas the immobilizing action of etomidate and propofol has been shown to be mediated by β3-containing but not by β2-containing GABA<sub>A</sub> receptors (Jurd et al., 2003; Reynolds et al., 2003). While the neuronal populations mediating the loss of the righting reflex are thought to be mediated by subcortical and midbrain regions (Rudolph and Antkowiak, 2004), there is good evidence that immobility is mediated at least to a large part by circuits in the spinal cord (Antognini and Schwartz, 1993; Antognini et al., 2000; Rampil, 1994; Rampil et al., 1993). The finding that the β3 subunit is a predominant subunit in the spinal cord (Bohlhalter et al., 1996) is consistent with β3-containing GABA<sub>A</sub> receptors in the spinal cord playing a significant role in anesthesia-induced immobility. Both β2- and β3-containing GABA<sub>A</sub> receptors mediate part of the hypnotic action of etomidate and propofol (Jurd et al., 2003; Reynolds et al., 2003).

The α3 subunit of the GABA<sub>A</sub> receptor is abundantly expressed in the spinal cord. The α2 subunit is expressed in motoneurons, and α1 and α5 are expressed mostly in lamina III of the dorsal horn and in the ventral horn. The dorsal horn receives afferent mechanosensory input, whereas the ventral horn mediates efferent motor output. In addition to the abundantly expressed subunit combination α3β2/3γ2, the subunit combinations α1β2/3γ2, α5β2/3γ2, α1/5β2/3γ2 are expressed more differentially in different laminae (Bohlhalter et al., 1996). The β3 subunit is mostly expressed in the superficial dorsal horn (Todd et al., 1996). The β3 subunit has previously been shown to be essential for etomidate and propofol-induced immobility (Jurd et al., 2003). The α3 and α5 subunits and the β3 subunit of the GABA<sub>A</sub> receptor are highly expressed in the ventral horn of the spinal cord. The receptor complex α3β3γ2 and α5β3γ2 might indeed be involved in relaying mechanosensory (proprioceptive) input to spinal cord motoneurons which in turn evoke the spinal cord-mediated reflexes. Our results show that GABA<sub>A</sub> receptor heterogeneity in the spinal cord is of functional relevance (Table 1).
Further experiments are necessary to elucidate the exact function of the individual GABA<sub>A</sub> receptor α subunits in the spinal cord circuits, e.g. determination of the electrophysiological response of spinal cord neurons to application of low and high doses of diazepam. In addition, it would be interesting to study the subcellular distribution of individual GABA<sub>A</sub> receptor α subunits, i.e. synaptic versus extrasynaptic, in defined motoneurons and interneurons. In the spinal cord, one could further elucidate the connectivity and function of spinal cord circuits and their modulation by GABA<sub>A</sub> receptor modulators. Presumably, the function of individual GABA<sub>A</sub> receptor α subunits in spinal cord circuits is different than their function in the brain.

![Table 1. Subunit of the GABA<sub>A</sub> receptor mediating the specific action of a specific CNS depressant.](image)

Summary of data obtained with β3(N265M) (1Jurd et al., 2003; 2Zeller et al., 2005), β2(N265S) (3Cirone et al., 2004; 4Reynolds et al., 2003), α1(H101R) (5Rudolph et al., 1999), α2(H101R), α3(H126R) (6Low et al., 2000), α5(H105R) (7Crestani et al., 2002) knock-in and α5 knock-out (8Cheng et al., 2006) mice. Data obtained during this study are highlighted in bold. 9unpublished data from A. Zeller.
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6.5. Forebrain pyramidal neurons mediate diazepam-induced sedation (Paper 5)

Anja Zeller¹, Jean-Marc Fritschy¹, Florence Crestani¹, Gregg E. Homanics², Takuji Iwasato³,⁴, Shigeyoshi Itohara⁴, Uwe Rudolph¹,⁵

¹Institute of Pharmacology and Toxicology, University of Zürich, Winterthurerstr. 190, CH-8057 Switzerland, ²Departments of Anesthesiology and Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, ³PRESTO, Japan Science and Technology Agency, Saitama, Japan, ⁴Laboratory for Behavioral Genetics, Brain Science Institute (BSI), Riken, Saitama, Japan, ⁵Laboratory of Genetic Neuropharmacology, McLean Hospital and Department of Psychiatry, Harvard Medical School, Belmont, MA 02478, USA

Abstract

The sedative action of diazepam is mediated by α1-containing GABA_A receptors, however, the neuronal circuits mediating this action are unknown. While diazepam does not sedate α1(H101R) mice with diazepam-insensitive α1-containing GABA_A receptors, global α1-deficient mice are more sensitive to the sedative, i.e. motor depressant effect of diazepam, most likely due to compensatory mechanisms, e.g. upregulation of the α2 and α3 subunit of the GABA_A receptor. We generated mice lacking the α1 subunit specifically in forebrain pyramidal neurons (forebrain-specific α1⁻/⁻ mice) and mice expressing only one diazepam-insensitive α1(H101R) allele in forebrain pyramidal neurons but both a functional wild type allele and the α1(H101R) allele in all other cells of the CNS (compound heterozygous forebrain-specific knockout /global heterozygous knock-in mice). Forebrain-specific α1⁻/⁻ mice were more sensitive to the motor impairing/sedative action of diazepam than wild type mice in a circular arena. We found a compensatory upregulation of the GABA_A receptor α2 and α3 subunits selectively in cerebral cortex and hippocampus, similar to the changes reported previously for the α1 global knockout in these brain regions. No difference in the motor impairing/sedative action of diazepam was observed between forebrain-specific α1⁻/⁻ mice, which also displayed upregulation of the α3 subunit, and global α1⁻/⁻ mice. Thus, a tissue-specific homozygous or heterozygous knockout of the α1 subunit restricted to the glutamatergic forebrain pyramidal neurons mimics an essential aspect of the phenotype of the global α1 knockout. Thus, our results
suggest that $\alpha_1$-containing GABA$_A$ receptors in forebrain pyramidal neurons mediate the sedative action of diazepam.

Keywords: sedation, diazepam, GABA$_A$ receptor, cortex

Introduction

GABA$_A$ receptors mediate the majority of GABAergic inhibition in the adult mammalian central nervous system. GABA$_A$ receptors are pentameric ligand-gated ion channels, the majority of them containing two $\alpha$, two $\beta$ and one $\gamma$ subunit (Backus et al., 1993; Barnard et al., 1998; Chang et al., 1996). These receptors are the targets of many clinically important drugs including benzodiazepines (Rudolph and Mohler, 2004), barbiturates, neurosteroids (Belelli and Lambert, 2005) and general anesthetics (Rudolph and Antkowiak, 2004). Benzodiazepine binding to GABA$_A$ receptors influences a wide range of behavioural patterns like sedation/vigilance, anxiety, muscle tension, epileptogenic activity and memory function. The $\alpha_1$-containing GABA$_A$ receptors mediate the sedative (McKernan et al., 2000; Rudolph et al., 1999), amnestic, and partly the anticonvulsant action of diazepam (Rudolph et al., 1999). This was shown by introducing a histidine-to-arginine point mutation at position 101 of the murine GABA$_A$ receptor $\alpha_1$ subunit gene. The receptor containing the point-mutated subunit is insensitive to allosteric modulation by benzodiazepine-site ligands both in vitro and in vivo, while regulation by the physiological neurotransmitter GABA is preserved (Rudolph et al., 1999b). $\alpha_1$(H101R) mice are resistant to the motor depressant effect of diazepam, showing that the sedative action of diazepam is mediated by this receptor subtype (McKernan et al., 2000; Rudolph et al., 1999). However, the global $\alpha_1$-deficient mice are more sensitive to the sedative (motor depressant) and hypnotic (loss of righting reflex) action of diazepam. One drawback of behavioural analysis in the $\alpha_1$ knock-out compared to the $\alpha_1$ knock-in mouse is that the $\alpha_1$ knock-out shows upregulation of other GABA$_A$ receptor $\alpha$ subunits, most pronounced of the $\alpha_2$, $\alpha_3$ and $\alpha_4$ subunit (Kralic et al., 2006). It is therefore not certain whether phenotypes observed in the $\alpha_1$ knock-out mice are really due to the absence of the $\alpha_1$ subunit or rather due to the
overexpression of other GABA\(_\alpha\) receptor \(\alpha\) subunits, other compensatory mechanisms or developmental deficits. However, L838-417, which acts as a partial agonist at \(\alpha2\)-, \(\alpha3\)- and \(\alpha5\)-containing GABA\(_\alpha\) receptors and as a antagonist at \(\alpha1\)-containing GABA\(_\alpha\) receptors has no sedative effect in mice (McKernan et al., 2000b). Functional imaging experiments in humans show that low, sedative concentrations of anesthetics first reduce activity and blood flow in cortical regions, and only higher concentrations inducing strong sedation or hypnosis also influence subcortical structures like thalamus (Heinke and Koelsch, 2005; Rudolph and Antkowiak, 2004). These results would be consistent with a role for cortical neurons in the sedative action of CNS depressants. While the GABA\(_\alpha\) receptor \(\alpha1\) subunit is strongly expressed in the cortex (Fritschy and Mohler, 1995), there is no evidence linking \(\alpha1\)-containing GABA\(_A\) receptors in the cortex to sedation. Recently, Sonner and co-workers, reported that the amnestic effect of the volatile general anesthetic isoflurane, assessed as loss of fear conditioning, is dependent on \(\alpha1\) GABA\(_\alpha\) receptors in the forebrain (Sonner et al., 2005), indicating that amnesia is mediated by a defined subset \(\alpha1\)-containing GABA\(_\alpha\) receptors.

In this study, we investigated the hypothesis that forebrain \(\alpha1\) GABA\(_\alpha\) receptors mediate the sedative, i.e. motor depressant effect of diazepam, We used genetically engineered mice that either conditionally lack the \(\alpha1\) subunit in the pyramidal cells of the hippocampus and cortex, or harbour only one point-mutant and diazepam-insensitive \(\alpha1\)(H101R) allele in this cell population. We found that defined genetic ablations of the \(\alpha1\) subunit in forebrain pyramidal neurons mimic essential aspects of the phenotype of the global \(\alpha1\)/- mice, including upregulation of other \(\alpha\) subunits and increased sedation, demonstrating that forebrain pyramidal cells mediate drug-induced changes in motor activity. This suggests that \(\alpha1\)-containing GABA\(_\alpha\) receptors in forebrain pyramidal neurons mediate the sedative action of diazepam.
Material and Methods

Animals

To generate mice lacking the α1 subunit of the GABA_A receptor in pyramidal cells of cortex and hippocampus, we crossed mice carrying the α1 floxed allele (exon 8 of the Gabra1 gene is flanked by loxp sites) (H^{floxed}H^{floxed}), (Vicini et al., 2001), obtained from the Jackson Laboratory, Bar Harbor, ME, (B6.129(FVB)-Gabra1<sup>Wm1Geh</sup>/J, at least six backcrosses onto C57BL/6J) and Emx1-cre Tg3 PAC transgenic mice (Emx1-cre<sup>9</sup>), (Iwasato et al., 2004), B6-Tg(Emx-cre)). The H indicates a wild type histidine codon at position 101 of the amino acid sequence of the GABA_A receptor α1 subunit. The Emx1-cre transgene is specifically expressed in the pyramidal cells of cortex and hippocampus. Expression has also been noted in the septum, amygdala, piriform cortex and olfactory bulb (Iwasato et al., 2004). To obtain the experimental animals used in this study, mice homozygous for the α1 floxed allele and hemizygous for the Emx1-cre transgene (forebrain-specific α1<sup>−/−</sup>, H^{floxed}/Emx1-cre<sup>−/+</sup>) were bred with mice homozygous for the α1 floxed allele but not carrying the Emx1-cre transgene (wild type, H^{floxed}/Emx1-cre<sup>+/+</sup>) for breeding scheme see Table 1 and Fig. 1). The Emx1-cre transgene has been reported to be expressed in some animals in the germline, which can be detected in the liver of the offspring, since there is no somatic expression in this organ (Iwasato et al., 2004). We genotyped liver biopsies from all mice used in experiments when individual parental animals carried both the Emx1-cre transgene and the α1 floxed allele. Recombination in the liver biopsy sample indicates that cre-mediated recombination already occurred in the germline of the parent harboring both the α1 floxed allele and the Emx1-cre transgene. Mice with germline recombination (36 % of all mice analyzed when one of the parents had one or two floxed alleles) were excluded from analysis. Mice homozygous for the α1 floxed allele and hemizygous for the Emx1-cre transgene (H^{floxed}/Emx1-cre<sup>−/+</sup>) are predicted to lack the α1 subunit specifically in the glutamatergic pyramidal cells of the telencephalon (hippocampus and cortex, forebrain-specific α1<sup>−/−</sup>). Experimental controls were mice homozygous for the floxed allele but not expressing the Emx1-cre transgene (H^{floxed}/Emx1-cre<sup>−/−</sup>, wild type), which are expected to be phenotypically indistinguishable from wild type mice. The α1 floxed mice were on the C57BL/6J background, the Emx1-cre mice were kept in Zurich on the C57BL/6JOlaHsd Background. Since two generations were required to generate the H^{floxed}/Emx1-cre<sup>−/+</sup> parents used, the mice used are approximately 75% C57BL/6J and 25% C57BL/6JOlaHsd. The following PCR reactions were used for genotyping: for the α1 floxed allele as described in (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=584); for the cre transgene 5’-TGA CAG CAA TGC TGT TTC ACT GG-3’ and 5’-GCA TGA TCT CCG GTA TTG AAA CTC C-3’, product size 570 bp; to assess 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 has occurred and exon 8 was excised.

To obtain mice which in forebrain pyramidal cells express exclusively one diazepam-insensitive α1(H101R) allele but both a floxed α1 allele as a functional wild type allele and the α1(H101R) allele in all other cells of the CNS (H^{floxed}/Emx1-cre<sup>−/+</sup>, forebrain-specific α1<sup>−/−</sup>, wild type), we first bred Emx1-cre<sup>−/+</sup> mice (background C57BL/6JOlaHsd) with homozygous α1(H101R) mice (α1<sup>R/R</sup> mice, background C57BL/6J) (Rudolph et al., 1999b). Mice derived from this breeding which had one α1(H101R) point mutated allele and one α1(H) wild type allele and were hemizygous for the Emx1-cre transgene (H^{floxed}/Emx1-cre<sup>−/+</sup>, forebrain-specific α1<sup>−/−</sup>) were bred to mice homozygous for the α1 floxed allele (H^{floxed}/H^{floxed}). Offspring was H^{floxed}/Emx1-cre<sup>−/+</sup> (α1<sup>H101R</sup>), H^{floxed}/Emx1-cre<sup>−/+</sup> (forebrain-specific α1<sup>−/−</sup>), H^{floxed}/H^{floxed} (pseudo wild type), and H^{floxed}/H^{floxed} (forebrain-specific α1<sup>−/−</sup>) (Table 1, Fig. 1.). In these breedings, liver biopsies were analyzed to exclude germline transmission. In addition to the already described genotyping PCRs, the following PCR primers were used to detect the α1(H101R) point mutated allele: 5’-CAATGGTAGAGTCGGGAGATAAGATAT-3’ and 5’-AACACAACACACACACACACACCAAGACCAGACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
Results

Figure 1. Breeding schemes. A. Breeding scheme to obtain $H^{\text{floxed}}$/$H^{\text{floxed}}$/Emx1-cre<sup>tg</sup>-/+ mice. B. Breeding scheme to obtain $H^{\text{floxed}}$/H<sup>α1</sup>R/Emx1-cre<sup>tg</sup>-/+ mice. $H^{\text{floxed}}$=α<sub>1</sub> floxed allele, $H^{\alpha1}$=α<sub>1</sub> wild type allele with a codon for histidine at amino acid position 101, $R^{\alpha1}$=α<sub>1</sub> point-mutated allele with a codon for arginine at amino acid position 101, Emx1-cre<sup>tg</sup>-/+ = absence or presence of cre transgene.

### Table 1. Description of the genotypes of mice used in the present study

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<th>Floxed Allele of α&lt;sub&gt;1&lt;/sub&gt;</th>
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Table 1. Description of the genotypes of mice used in the present study. The left half (Genotype) lists the genotypes. $H^{\text{floxed}}$=α<sub>1</sub> floxed allele, $H^{\alpha1}$=α<sub>1</sub> wild type allele with a codon for histidine at amino acid position 101, $R^{\alpha1}$=α<sub>1</sub> point-mutated allele with a codon for arginine at amino acid position 101, Emx1-cre<sup>tg</sup>-/+ = absence or presence of cre transgene. The right half (Phenotype) describes the functional genotype in forebrain principal neurons which express the Emx-transgene and the functional genotype in all non-cre expressing cells in the brain including inhibitory interneurons in the forebrain. "-/-" indicates excision of the floxed exon in the α<sub>1</sub> gene resulting in a knockout (null) allele. For the description of the functional genotypes a floxed allele is described as an H allele, since it carries an exon for H at position 101 of the α<sub>1</sub> gene. It is assumed that the loxP sites present in introns do not have an appreciable effect on gene expression.
Antibodies
All antibodies used were raised in-house and are described in detail elsewhere (Kralic et al., 2006).

Immunohistochemistry
The regional distribution and relative immunoreactivity level of GABA_A receptor α subunits were investigated in wild type-like (H_floxH/Emx1-cre<sup>β</sup>) and mutant mice (H_floxH/Emx1-cre<sup>β</sup>+, H<sup>flox</sup>R/Emx1-cre<sup>β</sup>+, H<sup>flox</sup>H/flox/Emx1-cre<sup>β</sup>). Adult mice were deeply anesthetised with pentobarbital (50 mg/kg, i.p.) and perfused through the aorta with 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4). Brains were postfixed for 3 hours, cryoprotected in sucrose, frozen and cut parasagitally at 40 μm with a sliding microtome. Sections were collected in PBS and stored in an antifreeze solution prior to staining. Free-floating sections were incubated overnight at 4°C with primary antibodies for GABA_A receptor α subunits diluted in Tris buffer containing 2% normal goat serum and 0.2% Triton X-100. Sections were washed and incubated for 30 minutes at room temperature in biotinylated secondary antibodies (1:300, Jackson Immuno research, West Grove, PA) in the same buffer as the primary antibodies. After washing, sections were incubated in the ABC complex (1:100 in Tris buffer, Vectastatin Elite Kit, Vector Laboratories, Burlingham, CA) and after another wash finally reacted with diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in Tris buffer (pH 7.7) containing 0.015% hydrogen peroxide. The colour reaction was stopped after 5-20 minutes with ice-cold PBS. 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). 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) in digital images from sections of wild type and mutant processed simultaneously under identical conditions. The relative staining intensity in regions of interest was measured in the cerebellum for the α3 subunit and in the inferior colliculus for the α2 subunit and subtracted. Despite parallel processing, overall staining intensities differed between genotypes. Therefore, all ROD values were normalized to the ROD intensity of the striatum where no recombination occurs. Results, expressed as mean±SEM, were analysed using nonparametric Kruskal-Wallis test and Mann-Whitney tests for post hoc mean comparison.

Behavioural procedures
Mice were raised in group-housed cages in a 12h light-dark cycle with lights on at 8 a.m.. For the motor activity test the 12h light-dark cycle was reversed (lights off at 8 a.m.) and genders were separated at least two weeks before the beginning of the experiment. Testing was performed between 9 a.m. and 12 a.m. Motor activity was measured in individual circular alleys (Imetronic, Pessac, France). The dose of diazepam was chosen based on previous experiments (Rudolph et al., 1999b) as a dose at which wild type mice clearly show sedation, but α1(H101R) mice do not. Naïve mice were injected with vehicle (0.3% Tween in saline) or 10 mg/kg diazepam p.o and 30 min later placed in the individual circular alleys and motor activity, measured as the number of photocell interruptions, was measured during a one hour period. The terms sedation and sedative, as defined by Katzung (Katzung, 1995), indicate the drug-induced decrease in the animal’s spontaneous activity. Measurement of motor activity in rodents represents a standard behavioral assay for testing the sedative potential of drugs (Vogel, 2002). Male and female mice were measured separately. Genders were combined after two-way ANOVA showed no significant difference between genders for any treatment and genotype. The sedative effect of diazepam within each genotype was assessed by two-way repeated measures ANOVA followed by a Bonferroni post hoc test. The genotype difference of the diazepam effect was assessed by two-way repeated measures ANOVA followed by a Bonferroni post hoc test. Results are expressed as mean±SEM.
Results

Conditional knockout of the GABA$_\text{A}$ receptor $\alpha$1 subunit in pyramidal cells in hippocampus and cortex (forebrain-specific $\alpha$1$^{-/-}$).

We analyzed the expression of GABA$_\text{A}$ receptor subunits $\alpha$1, $\alpha$2, $\alpha$3 in forebrain-specific $\alpha$1$^{-/-}$/EMX1-cre mice.

In the cortex, the $\alpha$1 subunit immunoreactivity (IR) is prominent and nearly evenly distributed across all areas in wild type mice (Fig. 2ac). As reported previously (Fritschy and Mohler, 1995), in the parietal cortex, the $\alpha$1 subunit staining is most prominent in layers I and III-IV (Fig. 2a). In the hippocampus in wild type mice, the $\alpha$1-IR is intense and diffuse in all layers except the pyramidal cell layer and the granule cell layer. No structures or single neurons can be distinguished except in CA3 where a few interneurons and their dendrites are visible in the stratum lucidum (Fig. 3a). In contrast, in forebrain-specific $\alpha$1$^{-/-}$ mice the $\alpha$1-IR is clearly absent from all excitatory neurons of the cerebral cortex (Fig. 3d). Interneurons retain expression of the $\alpha$1 subunit. Overall, the expression of the $\alpha$1 subunit in the hippocampus and cortex of forebrain-specific $\alpha$1$^{-/-}$ mice is reduced (Fig. 2a,b). In the forebrain-specific $\alpha$1$^{-/-}$ mice, only in the hippocampal interneurons a strong IR for the $\alpha$1 subunit remains visible (Fig. 3b).

In cerebral cortex and hippocampus of forebrain-specific $\alpha$1$^{-/-}$ mice, expression of the $\alpha$1 subunit is absent in pyramidal cells. In the interneurons from both cerebral cortex and hippocampus $\alpha$1 expression was fully retained. All other brain regions analyzed displayed no obvious change of $\alpha$1 expression, confirming the specificity of the cre recombinase driven by the Emx1 promoter (data not shown).

Upregulation of GABA$_\text{A}$ receptor $\alpha$2 and $\alpha$3 subunit staining in forebrain-specific $\alpha$1$^{-/-}$ mice

In the neocortex, the $\alpha$2 subunit immunoreactivity (IR) is confined to the outer layers and virtually absent in layers V and VI in wild type mice (Fig. 2c). Wild type mice displayed a pronounced IR for the $\alpha$2 subunit in the hippocampus (Fig. 2c). The $\alpha$2-IR is more prominent in the dentate gyrus than in the Ammon’s horn (Fig. 2c).

In wild type mice, the $\alpha$3-IR was most abundant in cortical layers V and VI and particularly intense in the frontal cortex, but almost absent in layer IV (Fig. 3e). $\alpha$3-IR in the hippocampus was only observed in CA1 (Fig. 3e). In forebrain-specific $\alpha$1$^{-/-}$
mice, α2-IR is increased in the frontal cortex and in the outer layers of the parietal cortex (Fig. 2d, Table 2, +53% and +148%, p<0.05). There is a slight increase in α2-IR in the hippocampus (Fig. 2d, Table 2, 6% to 61%). In forebrain-specific α1−/− mice expression of α3 was increased in the frontal and parietal cortex (Fig. 3f, Table 3, +95% to 170%, p<0.05) and in layer IV of the parietal cortex. In the hippocampus, the expression of α3 is increased in all layers of CA1 (Table 3, +54% to 84%) and in the granule cell layer of the dentate gyrus, but not in CA3.

These findings indicate that deletion of the α1 gene in cortical pyramidal cells is sufficient to cause an upregulation of the α2 and α3 subunit, a phenomenon which was also seen in global α1−/− mice.

Figure 2. Expression of GABA<sub>A</sub> receptor α1, α2, and α3 subunits in hippocampus and cortex in forebrain-specific α1−/− mice. A. Expression pattern of α1 in wild type mice. B. Reduction of α1 expression in hippocampus and cortex in forebrain-specific α1−/− mice. C. expression of α2 in wild type mice. D. Increase of expression of α2 in forebrain-specific α1−/− mice. E. Expression of α3 in wild type mice. F. Increased expression of α3 in forebrain-specific α1−/− mice. The increase in expression of α2 appears to be less pronounced than that of α3.
### Results

Results are expressed as mean ± SEM. Background was measured in the cerebellum and subtracted. For abbreviations see legend to Table 2.

#### Table 2. Quantification of the expression of the GABA<sub>A</sub> receptor α2 subunit in forebrain-specific α<sub>1<sup>R</sup></sub> mice. Densitometry was performed in sections processed for immunoperoxidase staining (adult mice, n=4 per genotype). For each region, three sections were analysed per animal, background was measured in the inferior colliculus and subtracted. DG, dentate gyrus; OD, optical density values (arbitrary scale); s. im, stratum lacunosom-moleculare; s. lucidum, stratum lucidum; s. mol, stratum molecular; s. or, stratum oriens; s. rad, stratum radiatum. Results are expressed as mean±SEM; NS, not significant (P<0.05).

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<th>Region</th>
<th>OD WT H&lt;sup&gt;em&lt;/sup&gt;H&lt;sup&gt;Emx1-Cre&lt;/sup&gt;&lt;sup&gt;loxp&lt;/sup&gt;</th>
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<td>96 ± 5</td>
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<td>78 ± 10</td>
<td>NS</td>
<td>84 ± 9</td>
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<td>DG, s.mol</td>
<td>1.6 ± 0.1</td>
<td>114 ± 3</td>
<td>NS</td>
<td>97 ± 8</td>
<td>NS</td>
<td>93 ± 5</td>
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<tr>
<td>DG, hilus</td>
<td>0.4 ± 0.0</td>
<td>128 ± 13</td>
<td>NS</td>
<td>68 ± 11</td>
<td>NS</td>
<td>62 ± 10</td>
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<tr>
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<td>NS</td>
<td>100 ± 6</td>
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<tr>
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<tr>
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<td>178 ± 17</td>
<td>&lt;0.05</td>
<td>71 ± 19</td>
<td>NS</td>
<td>109 ± 13</td>
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#### Table 3. Quantification of the expression of the GABA<sub>A</sub> receptor α3 subunit in forebrain-specific α<sub>1<sup>R</sup></sub> mice. Densitometry was performed in sections processed for immunoperoxidase staining (adult mice, n=4 per genotype). For each region, three sections were analysed per animal, background was measured in the cerebellum and subtracted. For abbreviations see legend to Table 2. Results are expressed as mean±SEM; NS, not significant (P>0.05).

<table>
<thead>
<tr>
<th>Region</th>
<th>OD WT H&lt;sup&gt;em&lt;/sup&gt;H&lt;sup&gt;Emx1-Cre&lt;/sup&gt;&lt;sup&gt;loxp&lt;/sup&gt;</th>
<th>OD % Forebrain-specific α&lt;sub&gt;1&lt;sup&gt;R&lt;/sup&gt;&lt;/sub&gt;</th>
<th>P</th>
<th>OD % Forebrain-specific α&lt;sub&gt;1&lt;sup&gt;H&lt;/sup&gt;&lt;/sub&gt;</th>
<th>P</th>
<th>OD % Forebrain-specific α&lt;sub&gt;1&lt;sup&gt;R&lt;/sup&gt;&lt;/sub&gt;</th>
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<td>NS</td>
<td>95 ± 11</td>
<td>NS</td>
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<td>CA1, s.pyr</td>
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<td>154 ± 10</td>
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<td>87 ± 10</td>
<td>NS</td>
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<td>NS</td>
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<td>Parietal Cortex, I-III</td>
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<td>107 ± 10</td>
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<td>Parietal/Visual Cortex, IV</td>
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<td>Parietal/Visual Cortex, V-VI</td>
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<td>199 ± 15</td>
<td>&lt;0.05</td>
<td>86 ± 8</td>
<td>NS</td>
<td>124 ± 11</td>
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Forebrain pyramidal neurons mediate diazepam-induced sedation (Paper 5)

Figure 3. Expression of α1 in forebrain-specific α1−/− mice. A, C. Wild type-like expression pattern of α1 in wild type mice. B, D. Loss of α1 expression in excitatory neurons in forebrain-specific α1−/− mice in hippocampus and cortex. Expression of α1 in interneurons is intact (arrowhead).

Sensitivity of forebrain-specific α1−/− mice to the motor depressant actions of diazepam

Diazepam (10 mg/kg p.o.) induces an overall decrease in motor activity in forebrain-specific α1−/− mice and wild type mice (Figure 4). Two-way repeated measures ANOVA revealed a significant genotype-treatment interaction [F(1,36)=13.09, P<0.01]. The drug effect was stronger in the forebrain-specific α1−/− mice [F(2, 54)=15.14, P<0.001]. No genotype difference was observed with vehicle treatment. These results suggest that neuronal circuits affected by the cell-specific knock-out of the α1 subunit contribute to diazepam-mediated sedation.
Figure 4. Diazepam-induced sedation in forebrain-specific $\alpha_1^{-/-}$ mice. Diazepam was administered p.o. 30 min before mice were placed into the circular alleys and then motor activity was measured for 1 h. Diazepam induced a decrease in motor activity in both wild type mice and in forebrain-specific $\alpha_1^{-/-}$ mice, however, the decrease of motor activity was higher in the forebrain-specific $\alpha_1^{-/-}$ mice. (n=19. *p<0.05, ***p<0.001)

Presence of one $\alpha_1$ allele leads to a decreased expression of $\alpha_1$ in forebrain excitatory neurons

Wild type (H$^{\text{floxed}}$/Emx1-cre$^{\text{tg}}$, $\alpha_1^{H/H}$) and heterozygous knock-in $\alpha_1^{H/R}$ mice show a wild type-like expression pattern of the GABA$\_A$ receptor $\alpha_1$ subunit (data not shown). However, in both forebrain-specific $\alpha_1^{-/-H}$ and forebrain-specific $\alpha_1^{-/-R}$ mice the single remaining wild type or point-mutant $\alpha_1$ allele appears to be insufficient to provide expression of the $\alpha_1$ subunit in the excitatory cells of the cortex and hippocampus at the level observed in wild type mice (Fig. 5a,b). At low magnification, no apparent differences in cortical staining pattern are visible between wild type (Fig. 3c) and forebrain-specific $\alpha_1^{-/-H}$ and $\alpha_1^{-/-R}$ mice (Fig. 5c,d). However, at higher magnification, differences between wild type mice (inset Fig 3c) and forebrain-specific $\alpha_1^{-/-H}$ mice and $\alpha_1^{-/-R}$ mice become visible (inset Fig. 5c,d). Individual interneurons and their dendrites (arrowheads) can be distinguished in forebrain-specific $\alpha_1^{-/-H}$ and $\alpha_1^{-/-R}$ mice, but not in wild type mice, whereas diffuse staining of the neuropil - representing $\alpha_1$ in pyramidal cells - is reduced.
Forebrain pyramidal neurons mediate diazepam-induced sedation (Paper 5)

Figure 5. Reduction of expression of $\alpha_1$ in global heterozygous knock-in/forebrain-specific heterozygous knock-out mice. A, B. Slight reduction of $\alpha_1$-IR in excitatory neurons in forebrain-specific $\alpha_1^{-H}$ mice compared to wild type mice in Fig 1A. Individual interneurons and their dendrites become visible (arrowheads). B, D, Pronounced reduction of $\alpha_1$-IR in excitatory neurons in forebrain-specific $\alpha_1^{-R}$ mice. In all hippocampal regions and in the cortex, individual interneurons and their dendrites become visible (arrowheads).

Compensatory upregulation of GABA$_A$ receptor $\alpha_3$ subunit in forebrain-specific $\alpha_1^{-H}$ and $\alpha_1^{-R}$ mice

Wild type and heterozygous knock-in $\alpha_1^{H/R}$ mice show a wild type-like expression pattern of both the GABA$_A$ receptor $\alpha_2$ and $\alpha_3$ subunit. In forebrain-specific $\alpha_1^{-H}$ and $\alpha_1^{-R}$ mice, no increase of the $\alpha_2$-IR is visible in the cortex and only a slight increase in the hippocampus (Fig. 6a,b vs. Fig. 3c, Table 2). In forebrain-specific $\alpha_1^{-H}$ mice, no increase of the $\alpha_3$-IR is seen in the cortex (Fig 6c vs. Fig.2e, Table 3). In forebrain-specific $\alpha_1^{-R}$ mice the $\alpha_3$-IR was increased in the parietal cortex in all layers, most pronounced in layer IV where almost no $\alpha_3$ was detected in wild type mice (Fig. 6d vs. Fig. 2e, Table 3, +24 to 40%, p<0.05). There is no increase of $\alpha_3$-IR
in the hippocampus in forebrain-specific $\alpha_1^{-H}$ and $\alpha_1^{-R}$ mice (Fig. 6c,d vs. Fig 3e, Table 3).

The upregulation of $\alpha_3$ is less pronounced in forebrain-specific $\alpha_1^{-H}$ and $\alpha_1^{-R}$ mice than in forebrain-specific $\alpha_1^{-}$ mice where $\alpha_1$ is completely lost in cortical excitatory neurons.

Figure 6. Expression of GABA$_A$ receptor $\alpha_2$ and $\alpha_3$ subunits in hippocampus and cortex in forebrain-specific $\alpha_1^{-H}$ and forebrain-specific $\alpha_1^{-R}$ mice. A,B. Slight increase of $\alpha_2$-IR in forebrain-specific $\alpha_1^{-H}$ and forebrain-specific $\alpha_1^{-R}$ mice in hippocampus and cortex compared to wild type (Fig. 4c). C,D. Increase of $\alpha_3$-IR in forebrain-specific $\alpha_1^{-H}$ and forebrain-specific $\alpha_1^{-R}$ mice in hippocampus and cortex compared to wild type (Fig. 4e).

Forebrain-specific $\alpha_1^{-H}$ and $\alpha_1^{-R}$ mice are sedated by diazepam

Diazepam (10 mg/kg p.o.) induces an overall decrease in motor activity in forebrain-specific $\alpha_1^{-H}$ and $\alpha_1^{-R}$ and wild type mice (Fig. 7). Two-way repeated measures ANOVA revealed a significant genotype-treatment interaction [$F(3,80)=4.68, P<0.01$]. The drug effect was stronger in the forebrain-specific $\alpha_1^{-H}$ mice compared to forebrain-specific $\alpha_1^{-R}$ mice [$F(3, 40)=5.1, P<0.01$]. No genotype difference was observed with vehicle treatment.

The presence of the H101R point mutated allele decreased the sensitivity of the mice for diazepam-induced reduction of locomotor activity similarly in $\alpha_1^{H/R}$ and forebrain-specific $\alpha_1^{-R}$ mice. Both $\alpha_1^{H/R}$ and forebrain-specific $\alpha_1^{-H}$ mice are more susceptible to diazepam-induced reduction of locomotor activity than $\alpha_1^{H/R}$ and forebrain-specific $\alpha_1^{-R}$ mice revealing an effect of the heterozygous point-mutated $\alpha_1(H101R)$ allele.
These results suggest that the $\alpha_3$-containing $\text{GABA}_A$ receptors, which are upregulated, mediate diazepam-induced sedation in these mice. Therefore, modulation of the activity of neuronal circuits by diazepam in the cerebral cortex and/or the hippocampus are a major determinant of diazepam-induced sedation in mice.

**Figure 7.** Diazepam-induced sedation in forebrain-specific $\alpha_1^{-/-}$ and forebrain-specific $\alpha_1^{-/-R}$ mice. Diazepam was administered p.o. 30 min before mice were placed into the circular alleys and then motor activity was measured for 1 h. Motor activity after administration of vehicle is indistinguishable in all four genotypes (wild type, forebrain-specific $\alpha_1^{-/-}$, $\alpha_1^{H/R}$, forebrain-specific $\alpha_1^{-/-R}$). Diazepam induced a decrease in motor activity in all four genotypes. The effect of diazepam was smaller in forebrain-specific $\alpha_1^{-/-R}$ and in $\alpha_1^{H/R}$ than in forebrain-specific $\alpha_1^{-/-}$ and wild type mice. $n=21$, ** $p<0.01$, *** $p<0.001$
Discussion

In this report, we investigated the potential involvement of cortical pyramidal neurons in diazepam-induced motor impairment. First, we showed that mice lacking \( \alpha_1 \)-containing GABA\(_A\) receptors specifically in cortical pyramidal cells display an upregulation of the GABA\(_A\) receptor \( \alpha_3 \) subunit and are more sensitive to the motor impairing effect of diazepam than their corresponding wild type mice. Second, we show that forebrain-specific \( \alpha_1^{\text{R}} \) and \( \alpha_1^{\text{H}} \) mice are not less sensitive to diazepam compared to global \( \alpha_1^{\text{H/R}} \) and \( \alpha_1^{\text{H/H}} \) mice, respectively. Forebrain-specific \( \alpha_1^{\text{R}} \) and \( \alpha_1^{\text{H}} \) mice also display upregulation of the GABA\(_A\) receptor \( \alpha_3 \) subunit. Our results suggest that the compensatory upregulation of the \( \alpha_3 \) subunit in the cerebral cortex of mice lacking \( \alpha_1 \) in forebrain pyramidal neurons is sufficient to increase the diazepam-induced sedation, and that even in forebrain-specific \( \alpha_1^{\text{R}} \) and \( \alpha_1^{\text{H}} \) mice, which still express an \( \alpha_1 \) allele in the forebrain, there is compensatory upregulation of the \( \alpha_3 \) subunit which provides some sensitivity for diazepam-induced sedation. The forebrain-specific \( \alpha_1^{-/-} \) mice mimic the intriguing phenotype of global \( \alpha_1^{-/-} \) mice: upregulation of the \( \alpha_2 \) and \( \alpha_3 \) subunits (Kralic et al., 2002a; Kralic et al., 2006) and increased diazepam-induced sedation (Kralic et al., 2002b; McKernan et al., 2000), indicating that the knockout of the \( \alpha_1 \) subunit in forebrain pyramidal neurons with the subsequent compensatory changes is a sufficient condition for this phenotype. This suggests a causal relationship between the cortical pyramidal cells and diazepam-induced sedation. Since \( \alpha_1^{(H101R)} \) mice are insensitive to the sedative action of diazepam (Rudolph et al., 1999; McKernan et al., 2000), we assume that the \( \alpha_1 \)-containing GABA\(_A\) receptors in cortical pyramidal cells are mediating diazepam-induced sedation.

The GABA\(_A\) receptor \( \alpha \) subunits are differentially distributed at the cellular and at the subcellular level. More specifically, cortical pyramidal cells express multiple \( \alpha \) subunits. Whereas the somatic synapses from parvalbumin-positive interneurons contain the \( \alpha_1 \) subunit, the somatic synapses from CCK/VIP-positive interneurons and the axo-axonic synapses from chandelier cells contain the \( \alpha_2 \) subunit. The \( \alpha_5 \) subunit is found at the bases of the dendritic spines (Freund, 2003; Mohler et al., 2005). The fact that in wild type animals sedation is apparently exclusively mediated...
by α1-containing GABA_A receptors indicates that the neuronal pathway mediating diazepam-induced sedation includes the parvalbumin-positive neurons. Interneurons also express the α1 subunit, and since the EMX1-cre transgene is expressed in pyramidal cells but not in interneurons, in forebrain-specific α1⁻/⁻ mice diazepam likely depresses the activity of parvalbumin-positive interneurons. Thus, the inhibitory influence of the interneurons on the pyramidal neurons might be diminished, potentially counteracting sedation. Since these mice are sedated more strongly than wild type mice, this mechanism appears to be unlikely. We rather believe that the upregulated α3-containing GABA_A receptors may replace the missing α1-containing GABA_A receptors on the pyramidal neurons and mediate diazepam-induced sedation.

Mice harboring a point mutation in the α1 subunit of the GABA_A receptor are insensitive to the sedative action of diazepam (Rudolph et al., 1999), whereas α1-deficient mice are more sensitive to the sedative action of diazepam (Kralic et al., 2002b; McKernan et al., 2000).

The ablation of the α1 subunit in the α1 knock-out results in massive compensatory upregulation by up to 300% compared to wild type (Kralic et al., 2006) of other α subunits of the GABA_A receptor. Although upregulation of other α subunits occurs, e.g., of the α2 and α3 subunit in the cerebellum (Kralic et al., 2006), these compensatory upregulations do not manage to replace for the ablated α1 subunit as shown by a decrease of GABAergic currents in cerebellar slices (Vicini et al., 2001) or cerebellar Purkinje cells (Sur et al., 2001). The ablation of the α1 subunit selectively in pyramidal cells of the hippocampus and cortex also induces compensatory upregulations, in particular of the α3 subunit. However, these upregulations are restricted to those brain regions where the α1 subunit is knocked out. The forebrain-specific α1⁻/⁻ not only shows compensatory upregulation similar to that seen in the global α1⁻/⁻ but also shows a similarly increased sensitivity to diazepam-induced motor impairment. It is conceivable that the different kinetic properties of the α3-containing GABA_A receptors apparently replacing the α1-containing GABA_A receptors in pyramidal cells, are at least in part responsible for this increased sensitivity. With regard to the this increased diazepam-sensitivity, the upregulation of the α3 subunit seems to overcompensate and render the cortical
circuit more sensitive to the depressive action of diazepam. The prolongation of current deactivation that is seen in interneurons and pyramidal cells of the global $\alpha_{1-}^{-}\text{-}mice$ indicates that the upregulation of the $\alpha_2$ and $\alpha_3$ subunits has functional consequences in vitro (Goldstein et al., 2002; Vicini et al., 2001; Vicini and Ortinski, 2004) and thus might have an impact on behavior. $\alpha$ subunit composition determines activation and deactivation kinetics of $\text{GABA}_A$ receptors, and $\text{GABA}_A$ receptors containing the $\alpha_2$ or $\alpha_3$ subunit have slower deactivation kinetics compared to $\alpha_1$-containing $\text{GABA}_A$ receptors (Gingrich et al., 1995; Lavoie et al., 1997) In the forebrain-specific $\alpha_{1-}^{-}\text{-}mice$, the upregulation of the $\alpha_3$ subunit might almost fully compensate for the loss of the $\alpha_1$ subunit when no drug challenges the system and might lead to a normal spontaneous behavior. As however the $\alpha_3$ subunit is more sensitive to diazepam, the upregulation of this subunit in the forebrain renders the forebrain-specific $\alpha_{1-}^{-}\text{-}mice$ more sensitive to the sedative effect of diazepam. Thus, a selective knock-out of the $\alpha_1$ subunit in forebrain pyramidal cells shows the same response to diazepam-induced motor impairment as the whole brain knock-out of the $\alpha_1$ subunit. This indicates that the brain region mediating specifically the motor impairing/sedative effect of diazepam might be the cortex and/or hippocampus.

fMRI experiments in humans show that low, sedative doses of the 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. Similarly, it was shown in vivo experiments in rats that sedative doses of the volatile anesthetics isoflurane and enflurane depress the cortical firing rate by 65% (Hentschke et al., 2005). This correlation between behavioural 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. In the present study, we chose an experimental approach designed to examine a causal relationship between cortical pyramidal cells and diazepam-induced sedation and show that sedation induced by benzodiazepines is mediated by cortical circuits.
To exploit the diazepam-insensitivity of the global $\alpha_1$ (H101R) mice and the lack of any decrease of expression of the $\alpha_1$ subunit or compensatory changes in expression of other subunits in $\alpha_1$ (H101R) mice, we generated a mouse model expressing selectively in forebrain pyramidal cells only one $\alpha_1$ (H101R) allele and in all other cells of the brain both a point-mutated and a wild type allele ($\alpha_1^{H/R}$). We expected this mouse to be completely resistant to the motor impairing effect of diazepam. However, the forebrain-specific point-mutant mouse was similarly sensitive to the motor impairing effects of diazepam as the whole brain heterozygous point mutant ($\alpha_1^{H/R}$). This might be due to the slight decrease of $\alpha_1$ subunit expression in the forebrain-specific point-mutant mouse (which potentially decreases the sensitivity to diazepam) and the concomitant slight compensatory upregulation of the $\alpha_3$ subunit (which potentially increases the sensitivity to diazepam). Similarly, the forebrain-specific heterozygous knock-out of the $\alpha_1$ subunit in forebrain pyramidal cells shows a similar sensitivity to the motor impairing effects of diazepam as the whole brain wild type ($\alpha_1^{H/H}$), also due to slight decrease of $\alpha_1$ subunit expression and slight increase of $\alpha_3$ subunit expression. We speculate that these two effects – the decrease of $\alpha_1$ subunit expression and the subsequent increase of $\alpha_3$ subunit expression counteract each other and therefore the forebrain-specific partial ablation of the $\alpha_1$ subunit does not result in a change in response to the sedative effect of diazepam.

The major input and output of the cerebral cortex is mediated by pyramidal cells. Pyramidal cells of global $\alpha_1^{-/-}$ mice have decreased frequency and amplitude, but increased decay time (Goldstein et al., 2002), indicating profound changes in the kinetics of GABA$_A$ receptors, probably due to compensatory upregulation of $\alpha_2$- and $\alpha_3$-containing GABA$_A$ receptors. The increase of the $\alpha_2$ and $\alpha_3$ subunit observed in forebrain-specific $\alpha_1^{-/-}$ mice might occur in pyramidal cells and thus increase the response of these cells to diazepam, leading to an increased inhibition of pyramidal cells in forebrain-specific $\alpha_1^{-/-}$ mice compared to wild-type mice. This might lead to a decrease in overall cortical activity, as observed as an effect of volatile anesthetics (Hentschke et al., 2005), or to a decreased cortical output.
The heterozygous point mutant mouse ($\alpha^{1H/R}$) is, compared to wild type mice, partly resistant to the motor impairing effect of diazepam and thus has an intermediary phenotype in relation to wild type and $\alpha^{1R/R}$ mice, which are insensitive to diazepam-induced sedation. This shows that a global partial reduction of diazepam-sensitive $\alpha$1-containing receptors (which in this case are replaced by diazepam-insensitive point-mutated receptors) leads to a decreased sensitivity to diazepam-induced sedation. The motor activity after diazepam is less decreased in $\alpha^{1H/R}$ than in wild type ($\alpha^{1H/H}$) mice, but still more decreased as compared to the homozygous knock-in ($\alpha^{1R/R}$) mice which are fully resistant to the motor impairing effect of diazepam. The fact that the degree of sedation is comparable in $\alpha^{1H/R}$ mice and forebrain-specific $\alpha^{1^{-R}}$ mice indicates that the loss of diazepam-sensitive $\alpha$1-containing receptors (which in this case is a net loss of $\alpha$1-containing receptors) in the forebrain is functionally compensated by the upregulation of the $\alpha$3 subunit.

In summary, we show that diazepam-induced motor impairment as a measure for sedation is altered in forebrain-specific $\alpha$1 knock-out mice indicating the involvement of pyramidal cells of the hippocampus and/or cortex in mediating this effect of diazepam.
References


Results


7. Overall Discussion

At the time the dissertation project was initiated, the first demonstration of a target mediating *in vivo* actions of general anesthetics had been made. It was found that etomidate and propofol, two commonly used general anesthetics, exert their immobilizing and in part their hypnotic action via $\beta_3$-containing GABA$_A$ receptors (Jurd et al., 2003). Starting from this observation, the two major broad goals of this dissertation were 1) to discover which actions of CNS depressing drugs are mediated by defined molecular targets and 2) to identify the brain region involved in mediating one defined endpoint of the CNS depressant drug diazepam. More specifically, it was the aim of this dissertation to identify which other functions of etomidate and propofol are mediated by $\beta_3$-containing GABA$_A$ receptors, since a pharmacological dissociation of desired and undesired actions could lead the way to the development of novel general anesthetics with an improved clinical profile (Paper 1). We also wanted to evaluate the role of this target for the actions of other general anesthetics, the barbiturate pentobarbital and the volatile anesthetic isoflurane (Papers 2 and 3). Furthermore, we wanted to identify the $\alpha$ subunit(s) involved in the immobilizing and hypnotic actions of general anesthetics (Paper 4). Finally, we wanted to localize the brain region in the CNS relevant for the sedative action, i.e. the decrease of motor activity, of CNS depressant drugs (Paper 5). While it was known that the sedative action is mediated by receptors containing the GABA$_A$ receptor $\alpha_1$ subunit, it had not been shown in which brain region this occurs.

The major findings were that $\beta_3$(*N265M*) mice are resistant to respiratory depression induced by etomidate and propofol, but not to anterograde amnesia, heart rate depression, hypothermia and ECG changes induced by etomidate and/or propofol (Paper 1). $\beta_3$(*N265M*) mice have also been found to be fully resistant to the immobilizing action of pentobarbital and partly resistant to its hypnotic action. The respiratory depressant action of pentobarbital was however still present in $\beta_3$(*N265M*) mice. Hypothermia and heart rate depression induced by pentobarbital were slightly reduced in $\beta_3$(*N265M*) mice (Paper 2).
Hypothermia and heart rate depression induced by the volatile anesthetic isoflurane are mediated to a small but significant degree by β3-containing GABA<sub>A</sub> receptors (Paper 3).

By studying α<sub>1</sub>(H101R), α<sub>2</sub>(H101R), α<sub>3</sub>(H126R), and α<sub>5</sub>(H105R) mice carrying point mutations rendering the respective GABA<sub>A</sub> receptors insensitive for diazepam, we showed that diazepam-induced immobilization requires both α<sub>3</sub>- and α<sub>5</sub>-containing GABA<sub>A</sub> receptors and hypnosis depends at least in part on α<sub>5</sub>-containing GABA<sub>A</sub> receptors (Paper 4).

By generating and analyzing a conditional knock-out of the α<sub>1</sub> subunit of the GABA<sub>A</sub> receptor in the pyramidal cells of cortex and hippocampus, we found that diazepam-induced sedation is mediated by cortical circuits (Paper 5).

### 7.1. Identification of molecular targets mediating actions of general anesthetics

While there is a plethora of potential targets for general anesthetics based on studies with recombinant receptors, the *in vitro* identification of a protein as a “target” for a general anesthetic does not teach us anything about which *in vivo* actions of the drug are mediated by which target. Moreover, even if one or several “targets” have been identified for a general anesthetic *in vitro*, it may happen that the true target mediating a certain anesthetic endpoint *in vivo* has not been discovered. The most stringent demonstration that a particular target mediates an anesthetic endpoint is to generate either knock-out animals lacking the target or knock-in animals carrying a point mutation rendering this target molecule insensitive to the action of the general anesthetic. If a specific action of the general anesthetic in question is no longer present in the mutant knock-out or knock-in mice, this indicates that the target indeed mediates this action. If a specific action of the general anesthetic in question is still present in the mutant knock-out or knock-in mice, this suggests that other targets are mediating this action. For theoretical reasons, the latter outcome does not positively identify which the target mediating the action still present in the mutant mice is.

#### 7.1.1. Immobility and hypnosis

When this work was initiated, it was known from studies in β3(N265M) mice, that the immobilizing action of etomidate and propofol is mediated essentially completely by
β3-containing GABA_A receptors, and that the hypnotic action of these drugs is mediated in part by β3-containing GABA_A receptors (Jurd et al., 2003). While our work was in progress, (Reynolds et al., 2003b), studying β2(N265S) mice, found that the hypnotic action of etomidate is mediated in part by β2-containing GABA_A receptors and that these mice are still susceptible to the immobilizing action of etomidate. Thus, complementary information was obtained studying the β3(N265M) and the β2(N265S) mouse mutants.

We found that the immobilizing action of pentobarbital is also mediated essentially completely by β3-containing GABA_A receptors, and the hypnotic action of pentobarbital in part by β3-containing GABA_A receptors (Paper 2). The immobilizing action of the volatile anesthetics enflurane, halothane and isoflurane has been found to be mediated to a limited degree by β3-containing GABA_A receptors in both β3 knock-out and β3 knock-in mouse models (Jurd et al., 2003; Lambert et al., 2005; Liao et al., 2005; Quinlan et al., 1998). While the difference in EC_{50} of 16%, 21%, and 24% for enflurane, halothane and isoflurane might appear to be small, there is a concentration range in which most of the wild type animals are immobilized, while most of the β3(N265M) animals are not immobilized, indicating that β3-containing GABA_A receptors play a significant role at clinically relevant concentrations of these volatile anesthetics. However, with increasing concentrations of volatile anesthetics, all β3(N265M) animals can be immobilized, presumably via other targets. The hypnotic action of the volatile anesthetics does not appear to be mediated by β3-containing GABA_A receptors, with the notable exception of isoflurane, where the EC_{50} for hypnosis is 12% higher in β3(N265M) compared to wild type mice, but no difference was found for halothane and enflurane.

The studies mentioned above and our own work have used a mutation in the β3 subunit to define a receptor population involved in the immobilizing and hypnotic actions of general anesthetics. However, at this point, we do not know which other subunits are contained in the GABA_A receptor which mediates these actions. Based on an report by Kissin (1997) which described that rats can be immobilized with high concentrations of diazepam relatively close to the lethal concentrations of diazepam we decided to study the hypnotic and immobilizing actions of diazepam in mice carrying point mutations in the diazepam-sensitive α subunits, i.e. α1(H101R), α2(H101R), α3(H126R), and α5(H105R). Lack of the hypnotic or immobilizing action
Overall Discussion

of diazepam in one of these mutants would indicate that the respective action of
diazepam is mediated by this particular $\alpha$ subunit. Moreover, since the $\gamma_2$ subunit is
required for the formation of the benzodiazepine binding site, using both mutations in
$\beta$ and $\alpha$ subunits we are able to describe the subunit composition of the GABA$_A$
receptor subtype mediating the hypnotic and immobilizing actions of CNS depressing
drugs. We found that $\alpha_3$(H126R), and $\alpha_5$(H105R) mice are completely resistant to
the immobilizing action of diazepam. In triple mutant mice carrying histidine to
arginine point mutations in the $\alpha 123$ subunit, diazepam can only act on $\alpha_5$-containing
GABA$_A$ receptors, and in $\alpha 125$ triple mutant mice diazepam can only act on $\alpha_3$-
containing GABA$_A$ receptors. In both triple mutant mouse lines, diazepam is unable
to induce immobilization. Thus, $\alpha_3$- and $\alpha_5$-containing GABA$_A$ receptors are required
but not sufficient for immobilization. $\alpha_5$(H105R) mice are partly resistant to the
hypnotic action of diazepam, thus providing further evidence that the GABA$_A$ receptor
subtypes mediating immobilization and hypnosis are partially overlapping and
partially distinct (Paper 4).

7.1.2. Respiratory depression

Already in the initial studies on the immobilizing and hypnotic actions of etomidate
and propofol, Jurd et al. described that at high doses of these drugs 50% of wild type
animals die but none of the $\beta_3$(N265M) mice. They speculated that wild type mice
might die due to respiratory depression or cardiac arrest. In this dissertation, we
examined whether the respiratory depressant action of etomidate and propofol would
be mediated by $\beta_3$-containing GABA$_A$ receptors (Paper 1). By performing blood gas
analysis, we found that the $\beta_3$(N265M) mice are largely resistant to the respiratory
depressant actions of etomidate and propofol, in contrast to the wild type mice.
These results show that the respiratory depressant action of etomidate and propofol
is mediated by $\beta_3$-containing GABA$_A$ receptors. This finding explains the high lethality
of wild type but not of $\beta_3$(N265M) mice at high doses of etomidate and propofol
reported by (Jurd et al., 2003).

When we examined the respiratory depressant action of pentobarbital (Paper 2), we
were surprised to see that it is largely unchanged in the $\beta_3$(N265M) mice compared
to wild type mice. Interestingly, the fact that pentobarbital-induced immobility and in
part hypnosis are mediated by $\beta_3$-containing GABA$_A$ receptors demonstrates that
Identification of molecular targets mediating actions of general anesthetics

pentobarbital exerts some of its action via these receptors. This indicates that pentobarbital-induced respiratory depression is not mediated by β3-containing GABA\textsubscript{A} receptors or if it is to some degree, that pentobarbital can also induce respiratory depression via other targets. This anesthetic endpoint is therefore mediated by different receptors or circuits in etomidate- and propofol-induced anesthesia compared to pentobarbital-induced anesthesia.

7.1.3. Anterograde amnesia

When this dissertation was initiated, there was no information available as to which target would mediate the anterograde amnesic action of general anesthetics. It was however known that the anterograde amnesic action of diazepam, as determined in the passive avoidance test, is mediated by α1-containing GABA\textsubscript{A} receptors (Rudolph et al., 1999).

We examined the anterograde amnesic action of propofol in the β3(N265M) mice in the passive avoidance test (Paper 3) and found that the anterograde amnesic action was still present in the β3(N265M) mice, indicating that this action is mediated by other targets. The target of propofol-induced anterograde amnesia remains unknown to date. Hippocampal α5-containing GABA\textsubscript{A} receptors have been found to be involved in mediating etomidate-induced learning impairment (Cheng et al., 2006).

Studying mice with a forebrain-specific knockout of the α1 subunit of the GABA\textsubscript{A} receptor, Sonner et al. (2005) found that these mice were significantly less sensitive to the amnestic effects of isoflurane in fear conditioning to context. The two published studies and our work are compatible with but do not prove the notion that α1β2γ2 GABA\textsubscript{A} receptors mediate the anterograde amnestic actions of CNS depressing drugs.

7.1.4. Hypothermia

While our work was in progress, Cirone et al. (2004), studying β2(N265S) mice, reported that the hypothermic action of etomidate is largely but not completely mediated by β2-containing GABA\textsubscript{A} receptors. We tested the hypothermic effect of etomidate, propofol, pentobarbital and isoflurane in β3(N265M) and wild type mice. After application of isoflurane, the hypothermia was significantly less pronounced in β3(N265M) compared to wild type mice. After all other substances, no genotype
difference was detected. This shows that all intravenous anesthetics tested induce hypothermia via targets other than β3-containing GABA_A receptors. However, volatile anesthetics might exert a small but significant part of their hypothermic effect via β3-containing GABA_A receptors.

7.1.5. Sedation

At subanesthetic concentrations, general anesthetics like etomidate decrease the motor activity. To test whether the β3-containing GABA_A receptor is involved in this action, we assessed the reduction of motor activity by low doses of etomidate in β3(N265M) mice (Paper 1). Etomidate decreased motor activity of β3(N265M) mice and wild type mice to a similar degree, indicating that this action of etomidate is not mediated by β3-containing GABA_A receptors. Thus, we have a very remarkable pharmacological dissociation between the sedative (not β3-dependent) and the immobilizing action (β3-dependent) of etomidate. The fact that sedation is not dependent on the β3 subunit is not really surprising since the sedative action of diazepam is mediated by α1-containing GABA_A receptors (Rudolph et al., 1999; McKernan et al., 2000) and the α1 subunit is most frequently associated with the β2 subunit but not the β3 subunit in α1β2γ2 receptor complexes (Benke et al., 1994; Whiting, 2003).

7.1.6. Heart rate depression

We tested the heart rate depressant effect of etomidate, propofol, pentobarbital and isoflurane in β3(N265M) and wild type mice. After application of isoflurane, the heart rate depressant effect was significantly less pronounced in β3(N265M) compared to wild type mice. After all other substances, no genotype difference was detected. This indicates that β3-containing GABA_A receptors play a minor role in the heart rate depressant action of isoflurane and likely no role in the heart rate depressant actions of the other substances studied.

7.1.7. ECG changes

Heart rate variability (HRV) is considered to be an indicator of cardiac vagal control, and drugs increasing HRV have been shown to reduce mortality and sudden death in
severe heart failure in clinical trials (Routledge et al., 2002). Anesthesia reduces sympathetic tone in mice (Gehrmann et al., 2000) which results in prolongation of time domain intervals such as QT, QRS and PQ and in an increase in HRV. Application of etomidate, propofol, pentobarbital, alphaxalone and isoflurane increased heart rate variability and prolonged ECG intervals, such as QT, QRS and PQ in both β3(N265M) and wild type mice. Control of heart rate and adaptive changes in heart rate variability and ECG intervals are therefore not mediated by β3-containing GABA<sub>A</sub> receptors. The targets mediating heart rate depression and ECG changes induced by the above mentioned general anesthetics might be completely different, e.g. these actions might be mediated peripherally by α<sub>2B</sub>-Adrenoceptors (Paris et al., 2003).

7.1.8. Summary of molecular targets mediating actions of general anesthetics

Based on our studies on β3(N265M) mice, it appears that etomidate and propofol on one hand and barbiturates on the other hand have partly overlapping targets, but also partly different targets (Fig. 1). Immobilization induced by all three anesthetics is fully mediated by β3-containing GABA<sub>A</sub> receptors and hypnosis is mediated by both β2- and β3-containing GABA<sub>A</sub> receptors. Pentobarbital might exert part of its hypnotic effect via β2-containing GABA<sub>A</sub> receptors, if it indeed shows the same pattern of targets like etomidate and propofol in terms of these two endpoints of general anesthetics. However, only respiratory depression induced by etomidate and propofol, but not by pentobarbital, is mediated by β3-containing GABA<sub>A</sub> receptors. This indicates that the desired immobilization and hypnosis and the unwanted respiratory depression as effects of general anesthetics can indeed be separated. A substance that would be only partly active at the GABA<sub>A</sub> receptor and which would not bind to the, up to now unknown, receptor that mediates pentobarbital-induced respiratory depression might have more desired effects and less unwanted side-effects of general anesthetics. In addition, this shows that indeed every single anesthetic has its own pattern of targets through which it mediates its in vivo actions and one cannot deduce the mechanism of action from one anesthetic to another.
The volatile anesthetic isoflurane also acts partly via $\beta_3$-containing GABA$_A$ receptors. Isoflurane-induced immobility, hypnosis, heart rate depression and hypothermia are all mediated to a small but significant degree by $\beta_3$-containing GABA$_A$ receptors. Whereas most intravenous anesthetics probably exert most of their clinically relevant actions through a limited number of targets, inhalation anesthetics may bind to an almost endless number of targets in the brain (Eckenhoff, 2001). This is however controversial (Krasowski and Harrison, 1999; Sonner et al., 2003; Sonner et al., 2005; Yamakura et al., 2001) and also volatile anesthetics might exert their clinical actions through a limited number of targets. It might therefore more difficult to dissect desired and undesired effect of inhalation anesthetics at the level of target receptors.
7.2. Identification of neuronal circuits mediating different effects of CNS depressant drugs

There is no universally accepted definition of general anesthesia. An overview of the pharmacological actions usually considered relevant and desired for anesthesia is given below (Table 1). Comparing behavioural states and anesthetic endpoints in mice and humans is not necessarily straightforward, therefore the definitions of anesthetic endpoints given below is still a matter of debate.

<table>
<thead>
<tr>
<th>Endpoints of Anesthesia</th>
<th>Mice</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedation</td>
<td>Decrease in motor activity</td>
<td>Failure to respond to verbal commands</td>
</tr>
<tr>
<td>(Anterograde) amnesia</td>
<td>Passive avoidance</td>
<td>Remembrance of intraoperative events, e.g. complications</td>
</tr>
<tr>
<td>Hypnosis</td>
<td>LORR</td>
<td>LOC</td>
</tr>
<tr>
<td>Immobility</td>
<td>LHWR</td>
<td>No response to noxious stimulus, e.g. surgical incision</td>
</tr>
<tr>
<td>Muscle relaxation</td>
<td>horizontal wire test</td>
<td>Muscle rigidity</td>
</tr>
<tr>
<td>Autonomic response to noxious stimuli</td>
<td>Increases in breathing, blood pressure, heart rate after noxious stimulus</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Stages of anesthesia as measured in mice and humans. (Eger and Sonner, 2006; Harris et al., 2006; Rudolph and Antkowiak, 2004). LORR, loss of righting reflex; LHWR, loss of hindlimb withdrawal reflex; LOC, loud voice plus mild prodding.

7.2.1. Immobility

Experiments carried out more than 10 years ago in goats and rats indicate that the spinal cord mediates the loss of response to noxious stimuli. Goats lack several arteries and veins connecting the cerebral and peripheral blood system. It is therefore possible to apply inhalation anesthetics such as isoflurane to either the brain or the spinal cord selectively. (Antognini and Schwartz, 1993) could show in this system that delivery of isoflurane specifically to the brain as opposed to the spinal cord increases the concentration necessary to suppress noxious stimuli-evoked movement almost threefold. In the same experimental system, they showed that propofol administered to the spinal cord depressed dorsal horn neuronal responses to noxious stimulation whereas it had no depressant effect when administered to the
cranial circulation (Antognini et al., 2000). Similarly, Rampil and co-workers showed that precollicular decerebration and even spinal cord transection did not change the concentration of isoflurane necessary to suppress motor response to tail or fore- and hindlimb clamp in rats (Rampil, 1994; Rampil et al., 1993). Thus, both groups showed in different systems that the spinal cord is the essential site for anesthetic-induced immobility.

As it is known that immobility is largely mediated by spinal cord circuits and that etomidate- and propofol-induced immobility are mediated by β3-containing GABA<sub>A</sub> receptors, our finding that the α3- and α5-containing GABA<sub>A</sub> receptors mediate the immobilizing action of diazepam suggests that the α3β3γ2 and the α5β3γ2 GABA<sub>A</sub> receptor complexes in the spinal cord mediate immobilization induced by CNS depressant drugs. All these subunits are highly expressed and often co-localized in the spinal cord (Bohlhalter et al., 1996; Todd et al., 1996).

### 7.2.2. Hypnosis

Hypnosis is most difficult to compare between mice and humans because very different behavioural responses are measured (see Table 1 above). In addition, the terms hypnosis and sedation are sometimes used as synonyms (Nelson et al., 2002), although these responses are observed in different doses range with sedation occurring at lower doses and hypnosis occurring at higher doses (Zeller et al., 2005). At sedative concentrations, propofol reduces neuronal activity in cortical networks in humans and rodents (Heinke et al., 2004; Heinke and Koelsch, 2005; Hentschke et al., 2005) as well as in mice (Antkowiak, 1999). At higher, hypnotic concentrations, subcortical structures, including the thalamus, midbrain reticular formation and hypothalamus, are also affected in rodents (Shirasaka et al., 2004; Ying et al., 2006). Both the brain regions and the target molecules mediating hypnosis are difficult to define experimentally. β2- and β3-containing GABA<sub>A</sub> receptors and α5-containing GABA<sub>A</sub> receptors mediate hypnosis induced by etomidate and propofol in part (see above section 7.1.1). We can therefore speculate that several different GABA<sub>A</sub> receptor subtypes containing both the β2 or the β3 subunit and the α5 subunit mediate hypnosis. These α5β2/3γ2 receptor complexes are probably located in a subcortical circuit including several nuclei in the Pons, midbrain and hypothalamus (Nelson et al., 2002). The tuberomammillary nucleus (TMN), a posterior
hypothalamic cell group, is important in promoting arousal. Injection of the GABA$_A$ receptor agonist muscimol into the TMN induced hypnosis (Nelson et al., 2002). The $\alpha_5$, $\beta_2/3$ and $\gamma_2$ subunit are all expressed in the TMN (Fritschy and Mohler, 1995). This hypothalamic nucleus therefore might be an important site for mediating anesthetic-induced hypnosis. Although not identical, sleep and general anesthesia share common features, including a similar EEG pattern and depression of sensory input and motor output. The hypothalamus might be an important site in the regulation of both sleep and anesthesia.

### 7.2.3. Sedation

One common effect of CNS depressants is sedation. It is likely that the $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptor complex mediates sedation induced by benzodiazepines and general anesthetics. Sedation induced by diazepam is mediated by $\alpha_1$-containing GABA$_A$ receptors (Rudolph et al., 1999). Sedation induced by etomidate is mediated by $\beta_2$-containing GABA$_A$ receptors (Reynolds et al., 2003b). Furthermore, we showed that the sedative action of etomidate is unaffected in $\beta_3$(N265M) mice (Paper 1). Both $\alpha_1$ and $\beta_2$ subunits are highly expressed in the cortex (Fritschy and Mohler, 1995; Miralles et al., 1999) and the cortex is also the site where small, sedative doses of general anesthetics first attenuate neuronal activity (Rudolph and Antkowiak, 2004). A conditional knock-out of the $\alpha_1$ subunit of the GABA$_A$ receptor in the pyramidal cells of the cortex and hippocampus indicates that diazepam-induced sedation is indeed mediated by cortical circuits (Paper 5).

### 7.2.4. Amnesia

Amnesia is considered to be induced already at drug doses causing only mild sedation. The brain region mediating amnesia remains controversial. Several mechanisms have been proposed in humans, including relatively unspecific depression of neuronal activity predominantly in the cortex or by specific depressant effects on the hippocampus, the insula, amygdala, and the prefrontal cortex (Heinke and Koelsch, 2005).

In mice, anesthetic-induced amnesia has been shown to be mediated both by $\alpha_1$-containing GABA$_A$ receptors in the cortex (isoflurane, (Sonner et al., 2005) or by $\alpha_5$-containing GABA$_A$ receptors in the hippocampus (etomidate (Cheng et al., 2006).
we could show that the anterograde amnestic effect of propofol is not mediated by β3-containing GABA<sub>A</sub> receptors (Paper 3), it remains speculative with which β subunits the α subunits mediating amnesia are associated. The α1 subunit in the cortex is most frequently associated with the β2 and γ2 subunit, suggesting α1β2γ2 as the receptor subtype mediating the amnestic action of general anesthetics. The α5 subunit in the hippocampus is associated with the β1 or β3 subunits, but not with the β2 subunit (Benke et al., 1994). Since β3-containing GABA<sub>A</sub> receptors apparently do not play a significant role for anesthetic-induced amnesia, it might be speculated that α5β1γ2 receptors might mediate this effect.

### 7.3. Identification of GABA<sub>A</sub> receptor complexes mediating different actions of CNS depressant drugs

<table>
<thead>
<tr>
<th>Anesthetic endpoint</th>
<th>Drug</th>
<th>Proposed GABA&lt;sub&gt;A&lt;/sub&gt;R complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etomidate /propofol</td>
<td>Pentobarbital</td>
<td>Proposed GABA&lt;sub&gt;A&lt;/sub&gt;R complex</td>
</tr>
<tr>
<td>Sedation</td>
<td>β2&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>(Anterograde) amnesia</td>
<td>α5&lt;sup&gt;7&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Hypnosis</td>
<td>β2/3&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Immobility</td>
<td>β3&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Respiratory depression</td>
<td>β3&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Not β3</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>β2&lt;sup&gt;3&lt;/sup&gt;(β3)</td>
<td>Not β3</td>
</tr>
<tr>
<td>Muscle relaxation</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 2. Subunit of the GABA<sub>A</sub> receptor mediating the specific action of a specific CNS depressant. Summary of data obtained with β3(N265M) (1Jurd et al., 2003), β2(N265S) (2Reynolds et al., 2003b; 3Cirone et al., 2004), α1(H101R) (4Rudolph et al., 1999), α2(H101R), α3(H126R) (5Low et al., 2000), α5(H105R) (6Crestani et al., 2002) knockin and α5 knockout (7Cheng et al., 2006) mice. Data in bold were obtained during this thesis.

By means of the β3(N265M) mice, we could throw light on which GABA<sub>A</sub> receptor subtype mediates which in vivo actions of several classes of general anesthetics.
Identification of GABA\textsubscript{A} receptor complexes mediating different actions of CNS depressant drugs

Approximately 90\% of all GABA\textsubscript{A} receptors in the brain are thought to contain an $\alpha\beta\gamma$ subunit combination. We therefore wanted to find out which $\alpha$ subtype associated with which $\beta$ subtype mediates specific actions of general anesthetics. The benzodiazepine diazepam, used clinically as a sedative, anxiolytic and anti-convulsant, binds to $\alpha1,2,3,5\beta\gamma2$ GABA\textsubscript{A} receptors. Studies with knockin mice where the benzodiazepine-binding site is abolished by introducing a specific point mutation in the $\alpha$ subunit could elucidate which $\alpha$ subunit mediates which diazepam-effect (Table 1).

By means of $\alpha1,2,3,5$ histidine to arginine knock-in mice which are insensitive to specific actions of diazepam \textit{in vivo}, we could show that diazepam-induced immobilization is fully mediated by $\alpha3$- and $\alpha5$-containing GABA\textsubscript{A} receptors and hypnosis is partly mediated by $\alpha5$-containing GABA\textsubscript{A} receptors.

Thus, taking together information from our studies and other sources, it is now possible to link a specific receptor complex expressed in a specific brain region to a certain effect of a general anesthetic. As it is known that immobility is mediated by spinal cord circuits and it is known that etomidate- and propofol-induced immobility are mediated by $\beta3$-containing GABA\textsubscript{A} receptors, we can now speculate that it is the $\alpha3\beta3\gamma2$ and the $\alpha5\beta3\gamma2$ GABA\textsubscript{A} receptor complex in the spinal cord that mediates immobilization induced by CNS depressant drugs.

Both the brain regions and the targets mediating hypnosis are more difficult to define experimentally. Both $\beta2$-, $\beta3$- and $\alpha5$-containing GABA\textsubscript{A} receptors mediate hypnosis induced by CNS depressant agent in part. We can therefore speculate that several different GABA\textsubscript{A} receptor subtypes containing both the $\beta2$ and $\beta3$ subunit and the $\alpha5$ subunit mediate hypnosis.
Overall Discussion

This thesis contributes novel insights into the sites of action of CNS depressants in terms of both receptor targets and brain regions. We could show that different actions of CNS depressant drugs are mediated by specific subtypes of the GABA_A receptor. Furthermore, our work provides evidence that the sedative action of CNS depressant drugs is mediated by defined cortical circuits.
8. References


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9. Appendices

9.1. Acknowledgements

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I would like to thank Dr. Margarete Arras for teaching me with exceptional patience i.v. injections, reflex tests, surgery and implantation of telemetry transmitters, how to use the telemetry system and software and finally how to analyse data statistically. I enjoyed very much working with her.

I would like to thank Prof. Jean-Marc Fritschy, first of all for accepting me and Carolin Straub willingly in his group after Uwe Rudolph moved to the Harvard Medical School in Boston. He invested many hours in giving me practical support in all my projects implying immunohistochemistry.

I would like to thank Ruth Keist for her teaching me molecular biology and giving me hours of support, especially during the first year of my thesis. Whenever I had any questions, she always found time to answer them thoroughly and she also helped my doing unpleasant lab work. I candidly admire her extensive knowledge about any practical problems that can ever turn up in a molecular biology lab.

I would like to thank Corinne Sidler who introduced my into immunohistochemistry and also supported me in my lab work whenever it was too much for me alone too do. I would like to thank Thomas Grampp, Ela Balic and Dietmar Benke for helping me with the biochemical aspects of my project.

I would like to thank my friend Jörk Pischke for always having an open ear for my endless daily problems and for encouraging me that I could cope with any problem or hurdle.
## 9.2. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
<td>GABA type A receptor</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>dinitrogen oxide, nitrous oxide, laughing gas</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>LORR</td>
<td>loss of righting reflex</td>
</tr>
<tr>
<td>LHWR</td>
<td>loss of hindlimb withdrawal reflex</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>CBT</td>
<td>core body temperature</td>
</tr>
<tr>
<td>ECG</td>
<td>electrocardiogramm</td>
</tr>
<tr>
<td>HRV</td>
<td>heart rate variability</td>
</tr>
<tr>
<td>QT, QRS, PQ</td>
<td>ECG intervals</td>
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</tbody>
</table>
Appendices

9.3. Curriculum vitae

Anja Zeller, M.Sc.

Personal data
born 01.03.1978 in Rudolstadt /Germany
Nationality German, Ausländerausweis C

Work Address:
Institute of Pharmacology and Toxicology
University of Zurich
Winterthurerstr. 190
8057 Zurich
+41 43 288 91 47
8057 Zurich
+41 44 635 59 95
e-mail: anja.zeller@pharma.unizh.ch

Private Address:
Langfurren 23
8057 Zurich
+41 43 288 91 47

Education
02/2003 - 12/2006 Ph.D. student at the University of Zurich, Institute of Pharmacology and Toxicology, group of Prof. Uwe Rudolph, Ph.D. Thesis: “GABA_A receptor subtypes as neuronal substrates for selective actions of benzodiazepines and general anesthetics”

2003/04 PhD program, Neuroscience Centre Zurich, University of Zurich and ETH Zurich: Introductory Course in Neuroscience, grade A (5.5)

9/1998– 10/2002 University of Berne: Master of Science in Biochemistry, final exam grade A (5.5)
1 year diploma work in the research group of Prof. Bernhard Erni (topic: Molecular investigation of the Dihydroxyacetone-Kinase Operon of Lactococcus lactis)
2 years basic study in chemistry
1 year basic study in biochemistry
(practical courses in biochemistry, organic chemistry, molecular biology)

1994-1998 Mathematisch- Naturwissenschaftliches Gymnasium (high school for mathematics and physical science)
Matura (school leaving examination): grade B (5)
1998 Matura Prize for the best final exam essay (Paul Haupt-Preis)
Prize for outstanding results in the subjects German, French, History and English (Burgener-Preis)

Practical experience
2006 practical training for human biology students in microscopy and immunohistochemistry

2003-2006 Laboratory work as a Ph.D. student, Institute of Pharmacology and Toxicology, University of Zurich
- DNA: cloning, PCR, sequencing
- RNA: preparation from brain tissue, RT-PCR
Curriculum vitae

- protein: western-blot, ELISA, immunoaffinity purification of antibodies
- immunohistochemistry: preparation of tissue, DAB and immunofluorescence staining
- behaviour: motor activity, passive avoidance
- assessment of drug effects: reflex testing (LORR, LHWR) after iv and ip injection in mice, blood gas measurement

1999/2000
2 x 6 weeks as laboratory assistant at Berna Biotech AG, Rehagstr. 79, 3018 Bern

1996
One week practical lab work at Roche: “Improving enantiomeric yield by means of different solvents”

Volunteer activities
1998-2002 student representation in the students society “Fachverein für Chemie und Biochemie” of the University of Berne

Publications


Manuscripts in preparation

Mapping the contribution of $\beta_3$-containing GABA$\text{A}$ receptors to volatile and intravenous general anesthetic endpoints. Zeller A., Arras M., Jurd R., Rudolph U., in revision

Diazepam-induced sedation is not dependent on $\alpha_1$-containing GABA$\text{A}$ receptors in cortical pyramidal cells. Zeller et al., in collaboration with Florence Crestani and Jean-Marc Fritschy, Institute of Pharmacology and Toxicology, University of Zurich

Identification of GABA$\text{A}$ receptor subtypes defined by their $\alpha$ subunits mediating the immobilizing action of diazepam. Collaboration with Christian Grasshoff and Bernd Antkowiak, Section of Experimental Anesthesiology, University of Tübingen

Poster presentations
Molecular targets of general anesthetics: Genetic dissection of the pharmacological spectrum of intravenous and inhalational anesthetics using GABA$\text{A}$ receptor beta3(N265M) knock-in mice. 5th FENS Meeting, 8.-12.07.2006, Vienna, Austria

Distinct molecular targets for the central respiratory and cardiac actions of the general anesthetics etomidate and propofol. SfN 35th Annual Meeting, 12.-16.11.2005, Washington, USA
β3-containing GABA<sub>A</sub> receptors mediate the respiratory depressant effect of propofol and etomidate

Mechanisms of general anaesthetic action: contribution of β3-type GABA<sub>A</sub> receptors analysed in β3(N265M) knock-in mice
Joint Meeting of the Union of the Swiss Societies for Experimental Research (USGEB), the Swiss Society of Neuroscience (SSN), the Swiss Society of Biological Psychiatry (SSBP), 17-19.2.2005, Zürich, Switzerland

GABA<sub>A</sub> receptor subtype-specificity of propofol and etomidate actions determined in β3(N265M) knock-in mice by telemetry and blood gas analysis
ZNZ Symposium 2004, 15.10.2004, Zürich, Switzerland

**Talks**

Genetic analysis of GABA<sub>A</sub> receptors as neuronal targets for general anesthetic actions
18.11.2005, McLean Hospital, Harvard Medical School

β3-containing GABA<sub>A</sub> receptors mediate the respiratory depressant effect of propofol and etomidate
46. Frühjahrstagung der Deutschen Gesellschaft für klinische Pharmakologie und Toxikologie (DGPT), 15.-17.3.2005, Mainz, Germany

**Grants**

Travel Grant from the Neuroscience Centre Zurich to attend the 5th FENS meeting, 8.-12.07.2006, Vienna, Austria

Travel Grant from Hartmann Müller-Stiftung to attend the 35th SfN meeting 2005 in Washington, DC, USA

Travel Grant from the Neuroscience Centre Zurich to attend the meeting Mechanisms of Anesthesia MAC2005, 25.-27.02.2005

Travel Grant “Young Investigator Travel Award in MAC2005” to attend the MAC2005, 25-27.02.2005

**Languages**

German native language
English fluently written and spoken (daily working language)
French good (Matura)

**PC knowledge**

Macintosh and PC, Windows, Microsoft Office, EndNote, SPSS, Adobe Illustrator and Photoshop, Clone Manager,
Telemetry for recording and analysis of telemetry data: DSI Dataquest ART Acquisition, Analysis, ECG Analysis