Aus der

Universitätsklinik für Anästhesiologie und Intensivmedizin

Sektion für Experimentelle Anästhesiologie

Synergistic Interactions between Endogenous Neurosteroids and Zolpidem in Cultured Neocortical Tissue Slices

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

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2022

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Tag der Disputation:	03.05.2022

To my parents Ingrid and Willi.
Thank you for your amazing support.

Contents

List of Abbreviations

A	Amplitude
ACSF	Artificial cerebrospinal fluid
AP5	2-amino-5-phosphonopentanoic acid
API	Acute panic inventory
ATP	Adenosine triphosphate
BZ site	Benzodiazepine binding site
CCK-4	Cholecystokinin tetrapeptide
CI95	95% confidence interval
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
ct	Charge transfer
EGTA	Triethylene glycol diamine tetraacetic acid
frq	Frequency
GABA	γ-aminobutyric acid
$GABA_A$ receptor	γ-aminobutyric acid receptor type A
h0	Null hypothesis
HEK cells	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPSC	Inhibitory postsynaptic current
mIPSC	Miniature inhibitory postsynaptic current
mRNA	Messenger ribonucleic acid
MPTP	Mitochondrial permeability transition pore
P450scc	Cholesterol side-chain cleavage enzyme

PBR	Peripheral benzodiazepine receptor
PET	Positron emission tomography
Rec	Recording
sIPSC	Spontaneous inhibitory postsynaptic current
τ	Decay time
THDOC	Tetrahydrodeoxycorticosterone
TSPO	Translocator protein
TTX	Tetrodotoxin

List of Figures

List of Tables

1 Introduction

1.1 Interactions between Anaesthetics

Since their introduction, anaesthetics have been one of the most crucial innovations in clinical medicine, rendering possible an entire spectrum of novel therapeutic options. To date, they remain irreplaceable especially for surgical procedures, and modern medicine without them would be unimaginable. However, their exact mechanisms of action remain largely unknown. It is still unclear which receptors exactly are targeted by these substances and their possible interactions have so far been largely ignored. Little is known on how they influence each other and how this might affect the actions of different anaesthetics in research as well as in clinical use. Furthermore, recent studies have hinted that the effects of anaesthetics may be modulated by endogenous substances produced by the human body itself (Li et al. 2014, Drexler et al. 2016), yet in clinical anaesthesia, this has so far not been taken into account sufficiently.
Knowledge of these mechanisms may have a great impact on the patient-friendly and appropriate use of anaesthetics. Making use of possible synergistic effects between anaesthetic drugs could help in reducing the overall dose requirements of anaesthetics, thereby reducing unwanted side-effects of narcoses. This may lead to an improvement in the patient-friendly use of anaesthetics overall.
This study aims to examine this issue, specifically the effects and interactions of GABAergic drugs both on a synaptic and on a network level. It focuses on the interaction of zolpidem, a classical and well-known drug that mainly targets $GABA_A$ receptors containing an $\alpha 1$ subunit, and XBD173, which supposedly targets $GABA_A$ receptors indirectly via newly synthesized neurosteroids.

1.2 $GABA_A$ Receptors

1.2.1 Properties of $GABA_A$ Receptors

The γ-aminobutyric acid receptor type A ($GABA_A$ receptor) is the major inhibitory neurotransmitter receptor in the brain and plays a crucial role in the mediation of the effects of anaesthetic drugs. It is distributed over almost all brain regions (Young & Chu 1990). The $GABA_A$ receptor functions as a ligand-gated ion channel. In mature neurons, the binding of GABA at its distinct binding site leads to an influx of chloride ions and thus hyperpolarization of the cell membrane (in im-

mature neurons, the binding of GABA has been shown to cause depolarization, which is believed to be due to a modified chloride gradient (Spitzer 2010)).
$GABA_A$ receptor subunits consist of an N-terminal extracellular domain, four transmembrane domains, and a loop between the transmembrane regions 3 and 4 (Schofield et al. 1987). The receptor itself is composed of five subunits (Nayeem et al. 1994) out of a pool of 19 different subunits that have been distinguished so far (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) (Barnard et al. 1998, Olsen & Sieghart 2008). In theory, a large variety of different receptors could be formed if subunits could arrange randomly. In practice, only relatively few different assemblies have been identified so far (Olsen & Sieghart 2009). However, it is still unknown how many $GABA_A$ receptor subtypes exist. The majority of the receptors that have been identified to date are composed of α, β, and γ subunits. The most frequent composition consists of two α, two β, and one γ or one δ subunit, and it may contain two different α subunits (Rudolph & Knoflach 2011).

Due to its widespread distribution throughout the entire central nervous system, the $GABA_A$ receptor plays a role in virtually all brain functions (Young & Chu 1990, Olsen & Sieghart 2009). It is the target of numerous important drugs, such as benzodiazepines, steroids, and anaesthetics, which target distinct allosteric binding sites (Macdonald & Olsen 1994, Forman & Miller 2016) and serve both as clinical drugs and as important research tools.
Depending on their subunit composition, $GABA_A$ receptors display different pharmacological and electrophysiological properties (Rudolph & Möhler 2004). $GABA_A$ receptor subunit compositions differ in regional, cellular, and subcellular localization, thus fulfilling different roles in brain circuits and behavior (Olsen & Sieghart 2009).

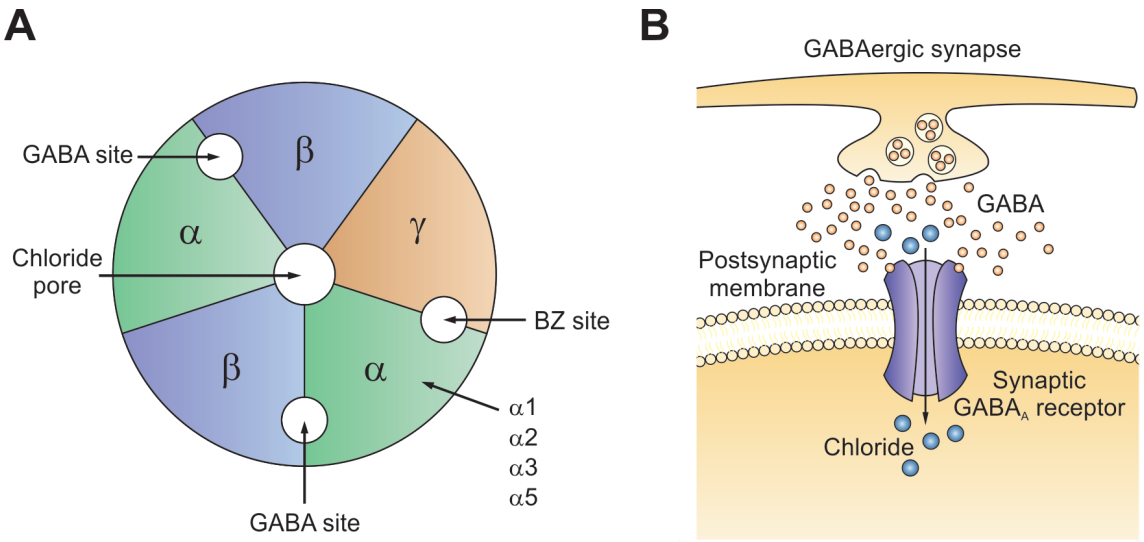


Figure 1: **Structure of the GABA$_A$ receptor** modified acc. to Rudolph & Knoflach (2011). GABA$_A$ receptors consist of five subunits out of a pool of 19 known subunits. They form a pore permeable to chloride ions. The majority of GABA$_A$ receptors are composed of two α subunits, two β subunits, and one γ subunit. These form two GABA binding sites which are located between the α and β subunits, as well as a binding site for benzodiazepines which is located between the α and the γ subunit. Only specific subtypes of α subunits form binding sites that benzodiazepines are able to bind to (α1, α2, α3, and α5), and the most frequent γ subunit forming the benzodiazepine binding site is the γ2 subunit, which is contained by about 90% of all GABA$_A$ receptors (**A**). GABA$_A$ receptors containing α4 and α6 subunits do not bind classical benzodiazepines. The binding of GABA to its designated binding site leads to an influx of chloride ions through the central pore, leading to a hyperpolarization of the postsynaptic membrane (**B**). Synaptic GABA$_A$ receptors are able to detect millimolar concentrations of GABA.

1.2.2 Synaptic and Extrasynaptic GABA$_A$ Receptors

GABA$_A$ receptors can be localized on both synaptic and extrasynaptic sites. Synaptic receptors mediate rapid phasic inhibition, whereas extrasynaptic receptors mediate tonic inhibition (Mody & Pearce 2004, Farrant & Nusser 2005). Synaptic transmission is mediated very rapidly by high concentrations of GABA (0.3-1.0 mM) that remain in the synaptic cleft only very briefly (about 1 ms) (Mody & Pearce 2004). This GABA transient activates synaptic GABA$_A$ receptors and thus leads to an inhibitory postsynaptic current (IPSC) that is shaped by the number and the distinct properties of the activated receptors, as well as by the extent and duration of the GABA transient. The majority of synaptic GABA$_A$ receptors contain a γ subunit, in particular γ2, which is essential for benzodiazepine sensitivity (Olsen & Sieghart 2008).
Extrasynaptic GABA$_A$ receptors can be activated by close to micromolar levels

of GABA that are constantly present in the extracellular space (Mody & Pearce 2004), as they display an unusually high affinity for GABA (Yeung et al. 2003). They mediate tonic inhibition. Extrasynaptic $GABA_A$ receptors often contain a δ subunit instead of a γ subunit. There are, however, experiments showing that tonic inhibition can also be enhanced by benzodiazepines, suggesting that there are also γ-containing receptors that take part in tonic conductance (Olsen & Sieghart 2009).

The charge transferred by tonically active $GABA_A$ receptors can be greater than three times the charge carried by phasic inhibition (Mody & Pearce 2004).

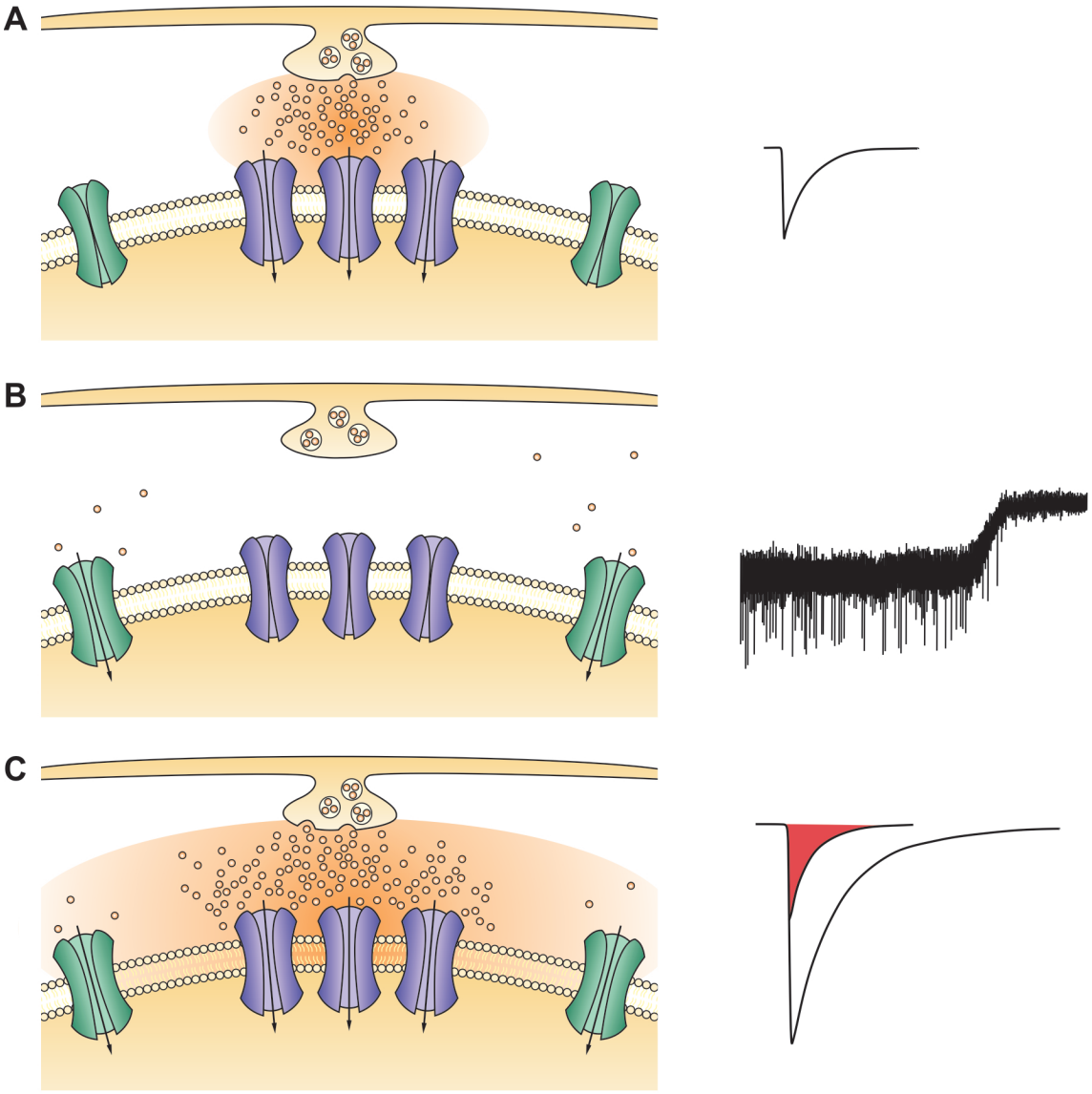


Figure 2: **Phasic, tonic, and spillover inhibition mediated by $GABA_A$ receptors** modified acc. to Weir et al. (2017). **A** shows the vesicular release of GABA which activates synaptic $GABA_A$ receptors, leading to an inhibitory postsynaptic current (IPSC) which is traced on the right, mediating a form of fast transient phasic inhibition. Spontaneous IPSCs may happen in the presence of action potentials as well as in their absence. Miniature IPSCs (mIPSCs) are a subgroup of IPSCs that are recorded in the presence of a sodium channel blocker (such as tetrodotoxin) and that are thus action potential-independent. **B** shows the activation of extrasynaptic $GABA_A$ receptors by ambient concentrations of GABA, leading to a constant tonic inhibition. On the right, this is displayed on a relatively slow time scale as a baseline shift induced by the $GABA_A$ receptor antagonist bicuculline (the downward spikes represent IPSCs which are suppressed as well due to bicuculline blocking synaptic $GABA_A$ receptors). **C** shows the activation of extrasynaptic $GABA_A$ receptors induced by a spillover of GABA from the synapse caused by high frequencies of presynaptic action potentials. This leads to a prolonged slow form of phasic inhibition, displayed on the right as an amplified IPSC compared to synaptic inhibition.

1.2.3 $GABA_A$ Receptors and Benzodiazepines

Benzodiazepines non-selectively target $GABA_A$ receptors containing $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ subunits, thus conveying multiple clinical effects such as sedation, hypnosis, anxiolysis, and muscle relaxation. They function as allosteric modulators of $GABA_A$ receptors, targeting the benzodiazepine binding site of the receptor. The benzodiazepine binding site is formed by an α subunit ($\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$) and a γ subunit (typically $\gamma2$), which means that the benzodiazepine pharmacology of different receptor subtypes is mainly determined by the α and γ subunits making up the binding site. Benzodiazepines show no effect on $\alpha4$- and $\alpha6$-containing receptors and a lower activity on $GABA_A$ receptors containing $\gamma1$ or $\gamma3$ subunits (Olsen & Sieghart 2008, Rudolph & Knoflach 2011). Benzodiazepines are valued not only for their clinical benefits, but also for their importance as research tools for analyzing the role of GABAergic inhibitory transmission in the central nervous system.

The modulation of $GABA_A$ receptors by benzodiazepines is self-limiting, and the conductance of the channel that can be achieved in the presence of GABA and benzodiazepines does not exceed the conductance that can be achieved in the presence of high concentrations of GABA alone. Also, benzodiazepines are unable to open the $GABA_A$ receptor in the absence of GABA (Rudolph & Knoflach 2011).

Regarding their clinical use, the fact that benzodiazepines target $GABA_A$ receptors in a non-selective manner may cause problems, since their multiple effects and side-effects are not entirely separable by dose. For example, benzodiazepines can be used as sedatives, in which case their sedative action is a wanted effect, or they can be used as anxiolytics, in which case sedation becomes an unwanted side-effect and might pose a problem. Further problems of benzodiazepines include their addictive properties and the development of tolerance, limiting their long-term use (Rudolph & Knoflach 2011).

1.2.4 Individual Effects of $GABA_A$ Receptors with Different Subunits

There is evidence that $GABA_A$ receptors with certain subunits modulate different effects. This phenomenon was examined in genetically modified mice, in which $GABA_A$ receptors with certain α subunits were rendered insensitive to diazepam, while the rest of the receptors were sensitive to GABA as usual. Certain effects of diazepam were absent in these mice, depending on which α subunit had been

modified:
In mice lacking functional α1 subunits, diazepam had no sedative action and no anterograde amnestic action. Anticonvulsant action was reduced in these animals, and anxiolytic-like action was present as usual (Rudolph et al. 1999, Rudolph & Knoflach 2011). The sedative, anterograde amnestic, and partly the anticonvulsant actions of diazepam were thus shown to be mediated by α1-containing $GABA_A$ receptors. Furthermore, the addictive properties of benzodiazepines have been linked to α1-containing $GABA_A$ receptors (Tan et al. 2010). Note that with a percentage of 60% of all $GABA_A$ receptors, the α1-containing $GABA_A$ receptor is the most wide-spread $GABA_A$ receptor subtype in the central nervous system (Rudolph & Knoflach 2011). This fact, as well as its role in the mediation of sedative effects, underlines its crucial importance for anaesthesia in general.
In mice lacking functional α2 subunits, diazepam displayed no anxiolytic-like action and its myorelaxant action was reduced. Its sedative action, however, was present as usual (Löw et al. 2000, Crestani et al. 2001). This indicates that the anxiolytic-like and, to a large extent, the myorelaxant actions are mediated by α2-containing $GABA_A$ receptors. Antidepression and antihyperalgesia have also been linked to α2-containing $GABA_A$ receptors (Rudolph & Knoflach 2011).
In mice lacking functional α3 subunits, diazepam displayed reduced myorelaxant action, while its sedative and anxiolytic-like actions remained present (Löw et al. 2000, Crestani et al. 2001). Furthermore, antihyperalgesia and sensorimotor gating have been linked to α3-containing $GABA_A$ receptors (Rudolph & Knoflach 2011). While their contribution to anxiolysis is still being discussed controversially, a recent study was able to link anxiolytic action to $GABA_A$ receptors with α2 subunits rather than α3 subunits, providing evidence for the theory that α3-containing $GABA_A$ receptors play no major role in the mediation of anxiolysis (Neumann et al. 2018).
In mice lacking functional α5 subunits, just as in mice lacking functional α3 subunits, the myorelaxant action of diazepam was reduced, while sedative and anxiolytic-like actions remained present (Crestani et al. 2002). It appears that the myorelaxant action is mediated in part by α2-, α3-, and α5-containing $GABA_A$ receptors. α5-containing $GABA_A$ receptors have been shown to play a role in the development of tolerance to the sedative action of benzodiazepines (van Rijnsoever et al. 2004), as well as in sensorimotor gating, cognitive impairment (Rudolph & Knoflach 2011, Sternfeld et al. 2004), and trace fear conditioning (Crestani et al. 2002).

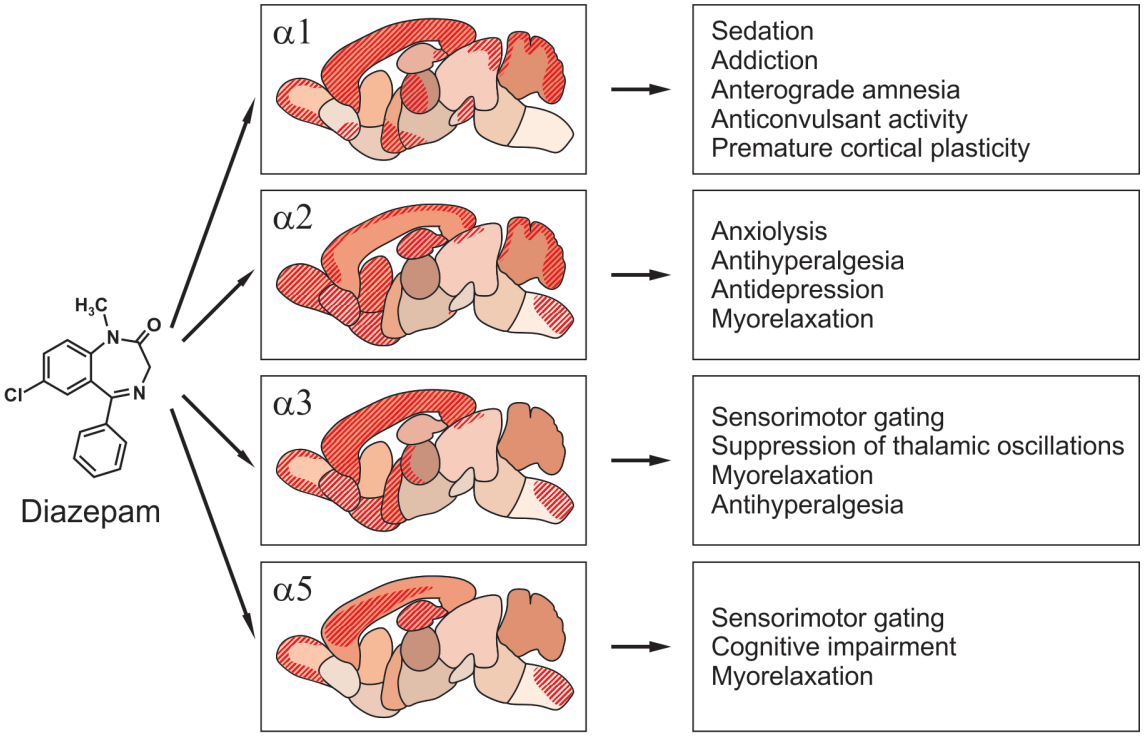


Figure 3: **Pharmacological effects and distribution of $GABA_A$ receptor α subunits in the mouse brain** modified acc. to Rudolph & Knoflach (2011). This figure provides an overview over the expression of different α subunits in the mouse brain, as well as the distinctive pharmacological effects that have been linked to $GABA_A$ receptors containing the respective subunit.

In conclusion, there is evidence that $GABA_A$ receptors containing an α1 subunit mediate primarily sedation and anterograde amnesia, receptors containing an α2 subunit mediate anxiolysis and myorelaxant effects, while receptors containing an α3 or α5 subunit are responsible, among other things, for myorelaxant effects. These findings may open up new possibilities for drug development. $GABA_A$ receptors with distinct subunits may function as novel targets for selective pharmaceutical drugs, reducing unwanted side-effects produced by non-selective $GABA_A$ receptor-modulating drugs like classical benzodiazepines.

1.2.5 Zolpidem as an α1-selective Drug

Zolpidem has a high affinity to $GABA_A$ receptors with an α1 subunit, while having a 20-fold lower affinity for α2-containing and α3-containing $GABA_A$ receptors, and close to no affinity to α5-containing $GABA_A$ receptors (Rudolph & Knoflach 2011). Although it is an imidazopyridine, zolpidem targets the same binding site as ben-

zodiazepines and displays a comparable profile of behavioral effects.
Zolpidem has a strong sedative effect and is thus used clinically for the treatment of insomnia. Considering the findings in mice lacking functional α1 subunits and the binding affinity of zolpidem to α1-containing $GABA_A$ receptors, it appears to be likely that zolpidem mediates its sedative effect via α1-containing $GABA_A$ receptors. Indeed, it could be demonstrated that zolpidem has no sedative effect on mice lacking functional α1 subunits (Crestani et al. 2000), thus confirming this theory.
For this work, zolpidem was used because of its nearly α1-selective properties, allowing for a comparison between its action profile and that of other GABAergic drugs.

1.3 Neurosteroids

1.3.1 Modulation of $GABA_A$ Receptors via Neuroactive Steroids

Certain steroid hormones possess the ability to influence the CNS rapidly, thus inducing effects like sedation and anaesthesia after being administered parenterally to test animals (Selye 1941). This effect is transmitted to a large extent, but not exclusively, via $GABA_A$ receptors. These neuroactive steroids function as positive allosteric modulators of the $GABA_A$ receptor, prolonging the open state of chloride channels and potentiating GABAergic neurotransmission. Neuroactive steroids thus produce anaesthestic, anxiolytic, analgesic, anticonvulsant, sedative, and hypnotic effects, and may even produce GABA-mimetic effects by activating $GABA_A$ receptors directly (Belelli & Lambert 2005). So far, two distinct types of neurosteroid binding sites have been identified, one of which has been shown to be responsible for the direct activation of $GABA_A$ receptors via neurosteroids, while the other one mediates the potentiation of the activating effects of GABA and neurosteroids (Hosie et al. 2006).

1.3.2 Neurosteroids as an Endogenous Regulating Mechanism

Originally, steroids were believed to be synthesized only in the endocrine glands (e.g. ovaries, testes, and adrenal glands), so they would have to cross the blood-brain barrier in order to affect neuronal activity. More recent evidence, however, shows that the brain itself is a steroid-synthesizing organ. Certain neu-

rons and glia are able to synthesize pregnane neurosteroids on their own, which serves as a paracrine mechanism to influence local neuronal activity (Mellon et al. 2001, Belelli & Lambert 2005).
Neurosteroid levels change under different physiological and pathophysiological conditions such as neuronal development, stress, pregnancy, ageing, psychoactive drugs, or psychological disturbances (Purdy et al. 1991, Belelli & Lambert 2005). Neurosteroids are thus able to act as endogenous ligands of the $GABA_A$ receptor, producing anxiolytic, analgesic, anticonvulsant, sedative, and hypnotic effects as a reaction to certain conditions of the body.
Note that the term “neurosteroids” refers to steroids produced by the body endogenously, whereas “neuroactive steroids” refers to both endogenous steroids and synthetically produced steroids.

Neurosteroids are synthesized from cholesterol in the mitochondria of steroid-synthesizing cells in the CNS. The first step in neurosteroidogenesis is the transport of cholesterol into the mitochondria, which is presumably mediated by the translocator protein 18kD (TSPO). This is an essential, rate-limiting step in steroid hormone biosynthesis. In the mitochondria, cholesterol is converted into pregnenolone by the cholesterol side-chain-cleaving cytochrome P450 enzyme (P450scc) located on the inner mitochondrial membrane. The neurosteroid pregnenolone then diffuses into the cytoplasm, where it is converted into progesterone by the microsomal 3β-hydroxysteroid dehydrogenase (3β-HSD)/Δ^5-Δ^4 isomerase. Progesterone is further metabolized into a variety of different neurosteroids by a cascade of several enzymes (Rupprecht et al. 2010). Due to their very lipophilic properties, the resulting neurosteroids are able to diffuse through the cell membrane easily, and can modify both synaptic and extrasynaptic $GABA_A$ receptors in a paracrine and in an autocrine manner.

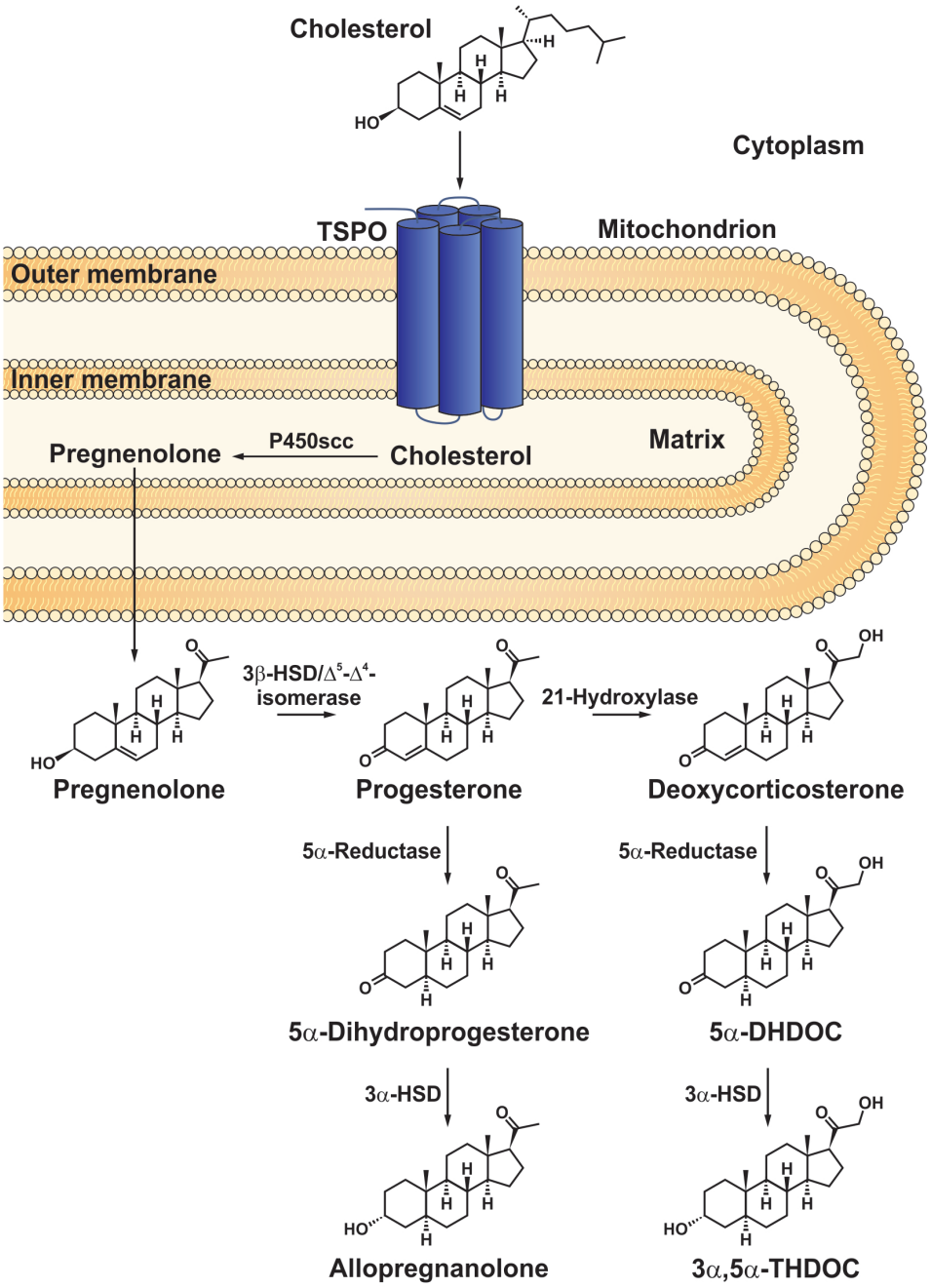


Figure 4: **Neurosteroidogenesis** modified acc. to Rupprecht et al. (2010). This figure provides an overview over the biosynthesis of neurosteroids taking place inside and outside the mitochondria of steroid-synthesizing cells. The rate-limiting step is the transport of cholesterol to the inner mitochondrial membrane via the translocator protein 18kD (TSPO). There, it is converted to pregnenolone via P450scc and then diffuses into the cytoplasm, where it is converted into various neurosteroid derivatives by a cascade of different enzymes.

1.3.3 Electrophysiological Effects of Neuroactive Steroids

Various neuroactive steroids are able to positively modulate $GABA_A$ receptor function in vitro. Whole-cell patch clamp experiments have shown that neuroactive steroids prolong the decay time of IPSCs (Lambert et al. 2003). Furthermore, some of them display an enhancing effect on tonic conductance (Stell et al. 2003, Farrant & Nusser 2005).
However, there appear to be significant differences in the modulation of phasic and tonic inhibition by neuroactive steroids. For example, 5α-tetrahydrodeoxycorticosterone (5α-THDOC) was shown to significantly potentiate tonic conductance in the dentate gyrus in mice at concentrations close to the physiological range (about 10 nM), while displaying no effect on phasic currents (Stell et al. 2003). It appears that the effects on phasic and tonic conductance are very diverse and there is emerging evidence that the interactions between neuroactive steroids and $GABA_A$ receptors differ throughout certain brain regions, certain neurons, and supposedly even throughout certain types of $GABA_A$ receptors on the same neuron, indicating that neuroactive steroids interact with $GABA_A$ receptors in a highly selective manner (Belelli & Lambert 2005).

1.3.4 Interactions between Neuroactive Steroids and $GABA_A$ Receptor-targeting Anaesthetics

There is evidence that neuroactive steroids are able to modify the effects of $GABA_A$ receptor-targeting drugs. The neuroactive steroid allopregnanolone was shown to potentiate the prolongation of spontaneous inhibitory postsynaptic current (sIPSC) decay times induced by the anaesthetic propofol in a synergistic manner, while both substances displayed additive effects on spontaneous action potential activity (Drexler et al. 2016). Pregnanolone was shown to significantly potentiate the effect of etomidate in HEK 293 cells expressing rat $\alpha1\beta2\gamma2$L $GABA_A$ receptors, as well as increase the sedative effect of etomidate in test animals (Li et al. 2014). Furthermore, it could be demonstrated that the neuroactive steroid alfaxalone does not only potentiate the increase in sIPSC decay time when co-applied with propofol or diazepam, but also amplifies sedation mediated by these two substances in test animals (Cao et al. 2018).
These findings are of great interest, since they suggest that when co-administered with neuroactive steroids, a much smaller dose of an anaesthetic is required to achieve a certain level of effect. This might offer a new approach to reduce the

dose requirements of anaesthetic drugs in clinical use, thereby also reducing unwanted side-effects and providing an overall improvement in the clinical use of anaesthetics.
This work aims to further elaborate on the interactions between neurosteroids and anaesthetics, specifically zolpidem which, unlike common general anaesthetics, preferredly targets α1-containing $GABA_A$ receptors.

1.4 Translocator Protein 18 kD (TSPO)

1.4.1 The Role of TSPO

The translocator protein 18kD (TSPO) is a protein located on the outer mitochondrial membrane (Anholt et al. 1986). It was previously known as the peripheral benzodiazepine receptor (PBR), as it was initially described as a receptor for benzodiazepines distinct from the central benzodiazepine receptor, the latter being part of the $GABA_A$ receptor complex (Papadopoulos et al. 2006).
TSPO is distributed almost ubiquitously in the body, but its expression seems to be especially high in steroid hormone-synthesizing cells (Selvaraj & Stocco 2015). Its protein sequence hardly varies from bacteria to humans, which indicates its crucial role in cellular and organismal physiology (Fan et al. 2012).

Early studies on TSPO were able to demonstrate its importance for the production of steroid hormones. It has been shown that TSPO mediates the transport of cholesterol into the mitochondria like a channel, thus playing an essential role in the regulation of steroidbiosynthesis (Krueger & Papadopoulos 1990, Papadopoulos et al. 1997). Many studies have since confirmed this theory, linking TSPO function directly to the synthesis of steroids (but see Selvaraj & Stocco 2015): Stimulation of TSPO by appropriate ligands has been shown to increase the level of neurosteroids in rats (McCauley et al. 1995, Serra et al. 1999), whereas the disruption of TSPO via homologous recombination leads to a decrease in steroid hormone production in rat Leydig tumor cells (Papadopoulos et al. 1997). TSPO knock out in mice has been found to be lethal during early embryonic development (Papadopoulos et al. 1997) and cells with a TSPO knock down of more than 70% were found to be unable to survive (Veenman et al. 2007).
TSPO has also been attributed a role in apoptosis. It has been suggested to be part of the mitochondrial permeability transition pore (MPTP), a pore that forms in the inner mitochondrial membrane prior to the apoptosis of the cell, thus increas-

ing the membrane's permeability and eventually leading to the death of the cell (Chelli et al. 2001, Casellas et al. 2002).

1.4.2 Regulation of TSPO and the Role of TSPO Ligands

Under physiological conditions, the expression of TSPO in the central nervous system remains low and is basically limited to microglia and astrocytes. In response to brain injury or inflammation however, the expression can increase dramatically (Chen & Guilarte 2008, Rupprecht et al. 2010).
This creates opportunities for new diagnostic means – e.g. detecting TSPO via drugs that bind to TSPO with a high affinity and that are labelled with PET tracers (Chauveau et al. 2008), which allows for the visualization of neuroinflammation in various neurodegenerative diseases – while also providing novel therapeutic approaches.
TSPO ligands are currently subject to research. In several disease models, TSPO-binding drugs have shown a positive effect on many pathologies of the central nervous system (Rupprecht et al. 2010). Most of these effects are mainly attributed to TSPO's role in steroid hormone synthesis, but the exact mechanisms remain unclear to date. It has to be noted that TSPO ligands have been demonstrated to produce various pharmacological effects, including anti-anxiety, anti-convulsant, anti-apoptotic, anti-inflammatory, and neuroprotective effects (Kita et al. 2004). The TSPO ligands PK11195 and Ro5-4864 have been found to affect MPTP opening (Casellas et al. 2002), thus being able to influence apoptosis.
For this work, the TSPO ligand XBD173 was used in order to examine its effect on GABAergic neurotransmission and its interaction with $GABA_A$ receptor-targeting anaesthetics.

1.5 XBD173

1.5.1 XBD173 as a TSPO Ligand

XBD173 (AC-5216, emapunil) is a selective ligand of TSPO. It is an 8-oxopurine derivative that binds to TSPO with nanomolar affinity, while displaying only negligible affinity to the majority of other neurotransmitter receptors, including $GABA_A$ receptors (Kita et al. 2004). Unlike most TSPO ligands, XBD173 is orally active,

thus facilitating its potential use as a medical drug.

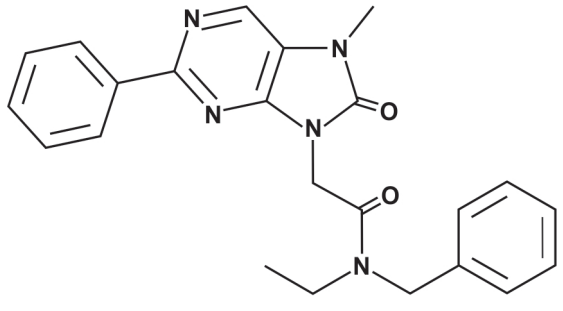


Figure 5: **Chemical structure of XBD173.**

XBD173 is assumed to enhance GABAergic neurotransmission indirectly via stimulation of the synthesis of GABAergic neurosteroids after binding to TSPO. This effect has been examined in vitro in mouse neocortical slices. Indeed, XBD173 was able to potentiate GABAergic neurotransmission in these experiments. The effect was prevented by finasteride, a 5α-reductase inhibitor which inhibits neurosteroidogenesis (Rupprecht et al. 2009). Furthermore, said study demonstrated a significant increase in allopregnanolone levels in rat brains after the administration of XBD173, but not after the administration of diazepam. These findings are in line with the assumed induction of neurosteroidogenesis mediated by XBD173.

1.5.2 Anxiolytic Properties of XBD173

Neuroactive steroids have been shown to produce anxiolytic-like effects in several animal models (Bitran et al. 1991, Rodgers & Johnson 1998, Gomez et al. 2002). Therefore, XBD173 was developed as a novel ligand of TSPO for the treatment of anxiety and stress disorders (Kita et al. 2004). Its pharmacological effects and anxiolytic profile have been demonstrated in various studies, especially in different models of anxiety. In all of these models – including the Vogel-type conflict test as a conditioned model of anxiety, as well as the light/dark box test, the social interaction test, and the elevated plus maze test as unconditioned models of anxiety – test animals that had been administered XBD173 showed clear anti-anxiety effects compared to test animals treated with a placebo. Those anti-anxiety effects were in part comparable to those displayed by test animals treated

with diazepam, a typical representative of classical benzodiazepines. In all experiments, the anti-anxiety effects of XBD173 could be prevented by PK11195, a TSPO ligand that functions as an antagonist at the applied concentrations (Kita et al. 2004, Rupprecht et al. 2009).
The anxiolytic potential of XBD173 was further examined in the CCK-4 challenge. During this test, rats were administered CCK-4, a potent panic-inducing agent which is used experimentally to induce panic-like anxiety, while their behavior was being observed. XBD173 was shown to effectively prevent panic-like behavior in the CCK-4 challenge in rats. The effect was comparable to that of alprazolam, a representative of benzodiazepines that was used as a control in this experiment (Rupprecht et al. 2009).

In order to further investigate the effects and side-effects of XBD173, as well as its potential therapeutic use, it was tested on healthy male volunteers.
The test subjects underwent the CCK-4 challenge as described above, and only subjects who showed a clear panic response were treated with either a placebo, 10, 30, or 90 mg XBD173, or 2 mg alprazolam per day for seven days, before undergoing a second CCK-4 challenge. The level of anxiety and the anxiolytic potential of the drugs used were assessed via the acute panic inventory (API). Results showed that the anxiolytic-like effects of 90 mg XBD173 were comparable to the effects of alprazolam, whereas side-effects in each XBD173 group were comparable to those in the placebo group. Sedation and development of withdrawal symptoms were hardly reported by test persons treated with any dose of XBD173, but were reported by a number of persons treated with alprazolam (Rupprecht et al. 2009).

1.5.3 Side-effect Profile of XBD173

Various experiments have been conducted in order to examine the side-effect profile of XBD173, especially regarding the question whether the side-effects of XBD173 resemble those of classical benzodiazepines. Among the most common side-effects of benzodiazepines are unwanted sedation, myorelaxation, anterograde amnesia, and development of tolerance and addiction. None of these effects could be observed after the administration of XBD173 in any of the experiments, including the traction test in mice (Kita et al. 2004), the passive avoidance test (Kita et al. 2004), and testing of the development of tolerance to XBD173 (Rupprecht et al. 2009).

Sedation after the administration of XBD173 could not be observed in any experiment, neither in rats, nor in humans (Rupprecht et al. 2009).
This suggests that XBD173 is able to mediate rapid anxiolytic and anti-panic effects while having a more beneficial side-effect profile than benzodiazepines. It was shown to produce anti-anxiety and anti-depressant-like effects in animal models, without causing the undesirable effects associated with conventional benzodiazepines.
To date, it is not exactly clear how XBD173 mediates these effects. A better understanding of its action profile may, however, open up new ways to reduce unwanted side-effects in the clinical treatment of anxiety and depression. This work focuses on examining how XBD173 interacts with zolpidem as a $GABA_A$ receptor-targeting drug.

1.6 Thesis

1.6.1 Neurosteroid-based Anaesthesia

To date, neurosteroids play only a minuscule role in clinical anaesthesia. Their interactions with anaesthetics and possibly beneficial effects on the outcome of anaesthesia have so far been largely ignored, despite there being promising evidence on the use of neurosteroids: They have been shown to act as potent intravenous anaesthetics causing significantly lesser side-effects than the most commonly used agent propofol (Monagle et al. 2015). Synergistic effects of neurosteroids and commonly used anaesthetic agents may allow for a significant reduction of dose requirements (Li et al. 2014, Drexler et al. 2016), thus reducing side-effects of general anaesthetics, such as dependence and addiction, development of tolerance, and prolonged sedation. There is compelling evidence that most of these unwanted effects are linked to drug interactions at α1-containing $GABA_A$ receptors (Tan et al. 2010, Rudolph & Knoflach 2011). Therefore, α1-receptor-sparing agents may possibly show an improved side-effect profile. Furthermore, neurosteroids provide neuroprotective properties (Schumacher et al. 2014), which may be utilized in order to improve the cognitive outcome of general anaesthesia
However, too little is known on how neurosteroids interact with other GABAergic drugs and how they might alter each other's effects. This work aims to elaborate on this issue, focusing on the effects and interactions of zolpidem, a GABAergic, highly α1-affine drug, and XBD173, a TSPO ligand which supposedly targets

$GABA_A$ receptors indirectly via neurosteroidogenesis. The main question that shall be investigated is how the action profiles of both substances differ and how XBD173 alters the effect of zolpidem on neuronal activity in neocortical slice cultures.

1.6.2 Molecular Action Profile of Neurosteroids

Studies on genetically engineered mice provide evidence that $GABA_A$ receptors containing an $\alpha 1$ subunit contribute to the sedative and hypnotic properties of anaesthetic agents (Rudolph et al. 1999, Rudolph & Knoflach 2011). This is in line with the observation that zolpidem, a drug that is used mainly for its sedative-hypnotic effects, primarily targets $\alpha 1$-containing $GABA_A$ receptors.
Despite XBD173 also targeting $GABA_A$ receptors, it displays very different clinical effects. On the behavioral level, XBD173 mediates a clear anxiolytic effect and appears to lack sedative properties (Rupprecht et al. 2009). Still, the exact mechanism of action of XBD173 remains unknown to date. While it supposedly targets $GABA_A$ receptors indirectly via neurosteroidogenesis, it lacks a number of effects that are typical for GABAergic drugs. This raises the question whether XBD173-induced neurosteroids possibly target certain $GABA_A$ receptor subtypes with a higher affinity than others. Since XBD173 shows no observable effect on GABA-evoked chloride currents in cells expressing exclusively $\alpha 1$-containing $GABA_A$ receptors (Rupprecht et al. 2009), and it lacks the sedative effect typically mediated by $\alpha 1$-containing $GABA_A$ receptors, it is well possible that the effects of XBD173 are mediated by $GABA_A$ receptor subtypes containing α subunits other than $\alpha 1$. This suggests that $\alpha 1$-containing $GABA_A$ receptors are not a major molecular target of neurosteroids.

The present study aims to challenge this hypothesis by conducting electrophysiological recordings on neocortical slice cultures. The interactions between zolpidem and XBD173 on a synaptic level were examined in whole-cell patch clamp experiments. Previous studies provided substantial evidence for synergistic interactions between neurosteroids and drugs acting as positive allosteric modulators of $GABA_A$ receptors, including etomidate, propofol, barbiturates, and benzodiazepines (Li et al. 2014, Drexler et al. 2016, Cao et al. 2018). However, these synergisms usually require two different agents to bind to different binding sites on the same $GABA_A$ receptor (Kent et al. 2019). Assuming that zolpidem and neurosteroids predominantly target different $GABA_A$ receptor subtypes, their

interaction on the synaptic level could well be additive rather than synergistic.
A second series of experiments was conducted in order to evaluate interactions between zolpidem and XBD173 on a network level via extracellular multi-unit recordings. In this test system, zolpidem has been shown to decrease high-frequency action potential firing by modulating α1-containing $GABA_A$ receptors (Neumann et al. 2019). Assuming that this receptor subtype is not a major target of neurosteroids, XBD173-induced neurosteroidogenesis should not be able to modulate high-frequency neuronal activity. The present work includes a series of experiments for testing this hypothesis.

2 Methods

2.1 Slice Cultures

2.1.1 Preparation of Cortical Slice Cultures

All experiments were performed in accordance with the EU guideline 2010/63/UE and German law and were approved by the local authority veterinary service and by our institution (Mitteilung vom 01.03.2017 “Pharmakologische Interaktionen zwischen Narkosemitteln und Neurosteroiden” nach §4 Abs. 3 Tierschutzgesetz).

Organotypic slice cultures from mouse neocortex were isolated and cultivated similar to the method described by Gähwiler (1981). For the experiments, neonatal wildtype C57/BL6J mice of both genders, aged four to five days, were used. The mice were first narcotized with the inhalational anaesthetic isoflurane (CP-Pharma, Burgdorf, Germany) and then decapitated. The brain was removed aseptically and immediately transferred into a petri dish with ice-cold Gey’s Balanced Salt Solution (Sigma-Aldrich, Taufkirchen, Germany) from a stock of 500 mL to which 4 mL 50% glucose and 5 mL 1M magnesium chloride had been added (both by AppliChem, Darmstadt, Germany). The infratentorial parts of the brain were removed and the brain was fixed on a teflon block using glue (UHU, Bühl, Germany), leaning against an agar block (Sigma-Aldrich, Taufkirchen, Germany) as support for the following slicing process. Using a vibratome (World Precision Instruments, Berlin, Germany), coronal slices of 300 µm thickness were cut. Thus, several slices were obtained from a single mouse brain. Cooling was maintained during the entire process. From the brain slices, small pieces of cortex were cut out with a spatula and were embedded in a 9 µL drop of chicken plasma (Sigma-Aldrich, Taufkirchen, Germany) on a glass coverslip (Kindler, Freiburg, Germany) coated with poly-D-lysine (Sigma-Aldrich, Taufkirchen, Germany). The plasma was then coagulated by adding a 9 µL drop of bovine thrombin (Sigma-Aldrich, Taufkirchen, Germany) and let dry, keeping the slice in its place. The slice cultures were transferred into tubes (Nunc, Wiesbaden, Germany) filled with 750 µL of a nutrition medium from a stock which consisted of 100 mL horse serum (Life Technologies, Karlsruhe, Germany), 200 mL Basal Medium Eagle (Sigma-Aldrich, Taufkirchen, Germany), 100 mL Hank’s Balanced Salt Solution (Sigma-Aldrich, Taufkirchen, Germany), 4 mL 50% glucose (AppliChem, Darmstadt, Germany), and 2 mL 200 mM glutamine solution (Sigma-Aldrich, Taufkirchen, Germany). Up to this step, the whole preparation process was done at the clean

bench in order to avoid contamination of the cultures.

2.1.2 Cultivation of Cortical Slice Cultures

After the preparation, the slice cultures were put into an incubator (Heraeus, Hanau, Germany), where they were incubated at 36 °C. For the first 90 minutes after the preparation and after each change of the nutrition medium, the incubation took place in a 5% carbon dioxide atmosphere for pH value stabilization. For this purpose, the lids of the culture tubes were not fully closed, allowing for a constant gas exchange between the atmosphere of the incubator and the culture tubes. The lids were closed firmly and cultures were incubated as usual after 90 minutes.
Cultures were incubated using the roller-tube technique, i.e. they were kept in a slightly tilted cylinder that slowly rotated with about ten rotations per hour. Thus, the slices had alternating contact with both medium and air, ensuring a sufficient supply with both nutrient and oxygen.
The nutrition medium was renewed 24 hours after preparation and from there on every three to four days. When the medium was changed for the first time, 10 µL of an anti-mitotic solution containing 1 mM cytarabine, 1 mM 5-fluoro-2'-deoxyuridine, and 1 mM uridine (all from Sigma-Aldrich, Taufkirchen, Germany) were added to limit the growth of glial tissue. After each change of the medium, cultures were aerated with 5% carbon dioxide as described above.
The cultures were cultivated for at least 10 days before being used for patch clamp recordings, and at least 14 days before being used for extracellular recordings.

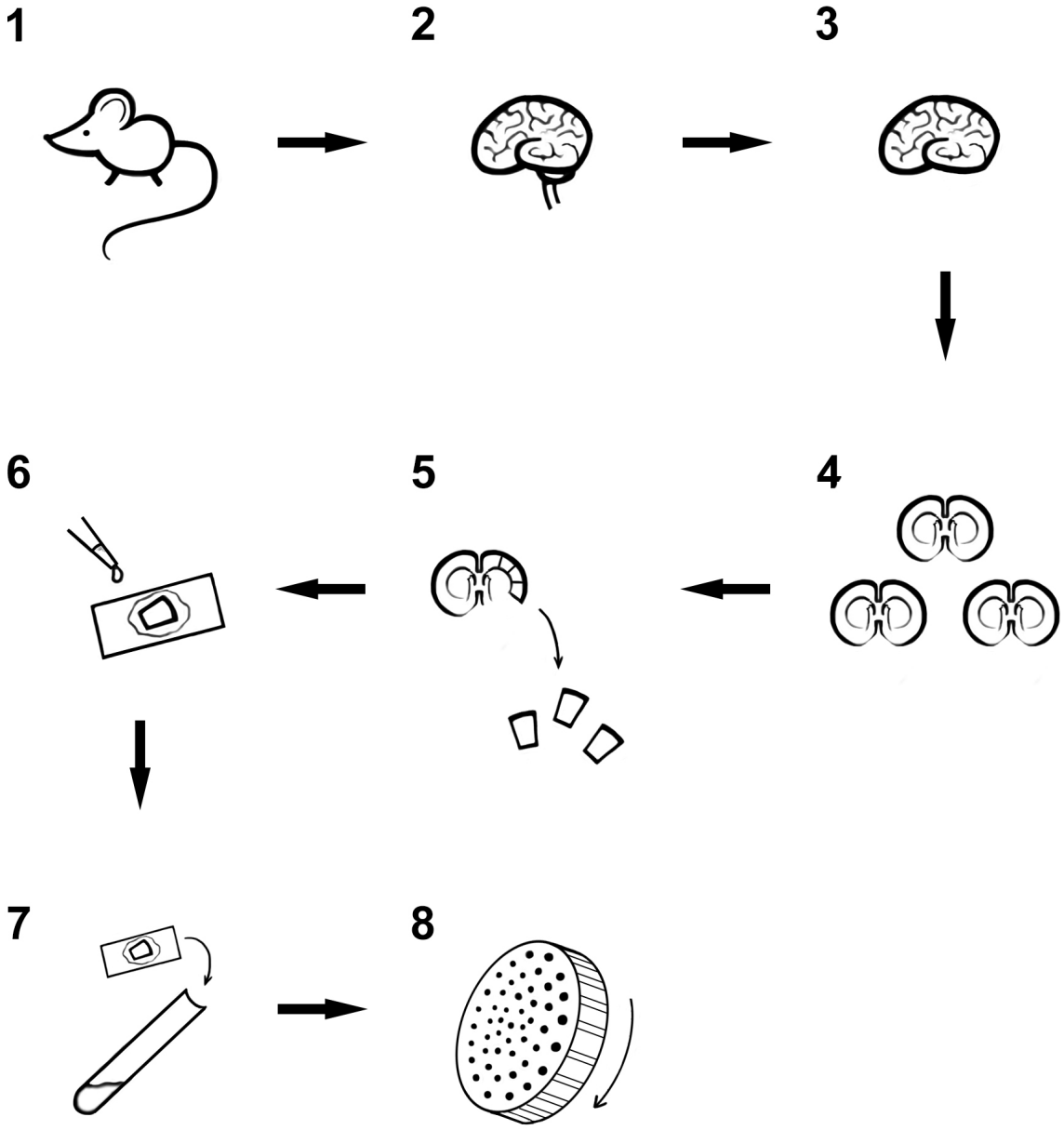


Figure 6: **Preparation of neocortical slice cultures.** The mouse brain is removed (2) and the infratentorial parts are cut off (3). The brain is then cut into slices of 300 µm thickness (4). Small pieces of neocortex are cut out (5) and embedded on a glass coverslip (6). The slice cultures are transferred into tubes filled with nutrition medium (7) and are then cultivated using the roller-tube technique (8).

The neocortical slice cultures that were obtained via the described method are referred to as organotypic slice cultures (Gähwiler et al. 1997). This means that the development of neuronal networks in vitro proceeds similar to their development in vivo, increasing the number of synaptic connections and the ability of neurons to activate each other over the time of cultivation, thus providing a suitable model for neuronal networks in the neocortex.

2.2 Patch Clamp Recordings

2.2.1 Experimental Set-up for Patch Clamp Recordings

Neocortical slice cultures aged between 10 and 29 days were used for patch clamp recordings. The slices were put into a recording chamber consisting of a metal frame with a glass bottom. During the recordings, the slices were constantly perfused with a solution containing 3 µM XBD173, respectively ethanol for sham applications, dissolved in artificial cerebrospinal fluid (ACSF), via IPC peristaltic pumps (Ismatec, Wertheim, Germany). The 3 µM XBD173 (respectively ethanol) solution was bubbled with 95% oxygen and 5% carbon dioxide for pH value stability. The recording chamber was kept at a constant temperature of 34 ℃ by a manually controlled heating wire.
Glass electrodes with a calibre at the tip of about 1 µm were made from thin-walled borosilicate capillaries (World Precision Instruments, Berlin, Germany) using a Model P-2000 laser puller (Sutter Instrument, Novato, USA) and were then filled with a solution containing 121 mM CsCl, 24 mM CsOH, 10 mM HEPES, 5 mM EGTA, 1 mM $MgCl_2$, and 4 mM ATP (Sigma-Aldrich, Taufkirchen, Germany and AppliChem, Darmstadt, Germany). The electrodes had resistances of 1.5-3 MΩ.
Test current responses were monitored during the entire process until a whole-cell configuration was obtained. The electrode was positioned above the slice and carefully lowered down to the surface of the slice under optical control on a television monitor using a 40x water-immersion objective (Zeiss, Oberkochen, Germany). Neurons with their somata on the surface of the slice were used for recordings. The electrode was slowly advanced towards a suitable cell body, maintaining a slightly positive pressure on the electrode via mouth-on technique. It was carefully pushed against the cell body just a little, until a small dimple as well as a slight increase in the resistance of the electrode were visible. The pressure was then decreased relatively quickly and lowered down to negative values,

slightly aspirating the cell membrane covering the opening of the electrode, thus establishing a firm connection between the cell membrane and the electrode. The pressure was immediately set to zero once resistances greater than 1 GΩ could be observed, indicating the achievement of a gigaseal. Once a gigaseal had been established, the holding voltage was set to -70 mV, before attempting to open the cell membrane connecting to the electrode by carefully applying quick negative pressures. The opening of the membrane was indicated by a characteristic pattern of the test current response.

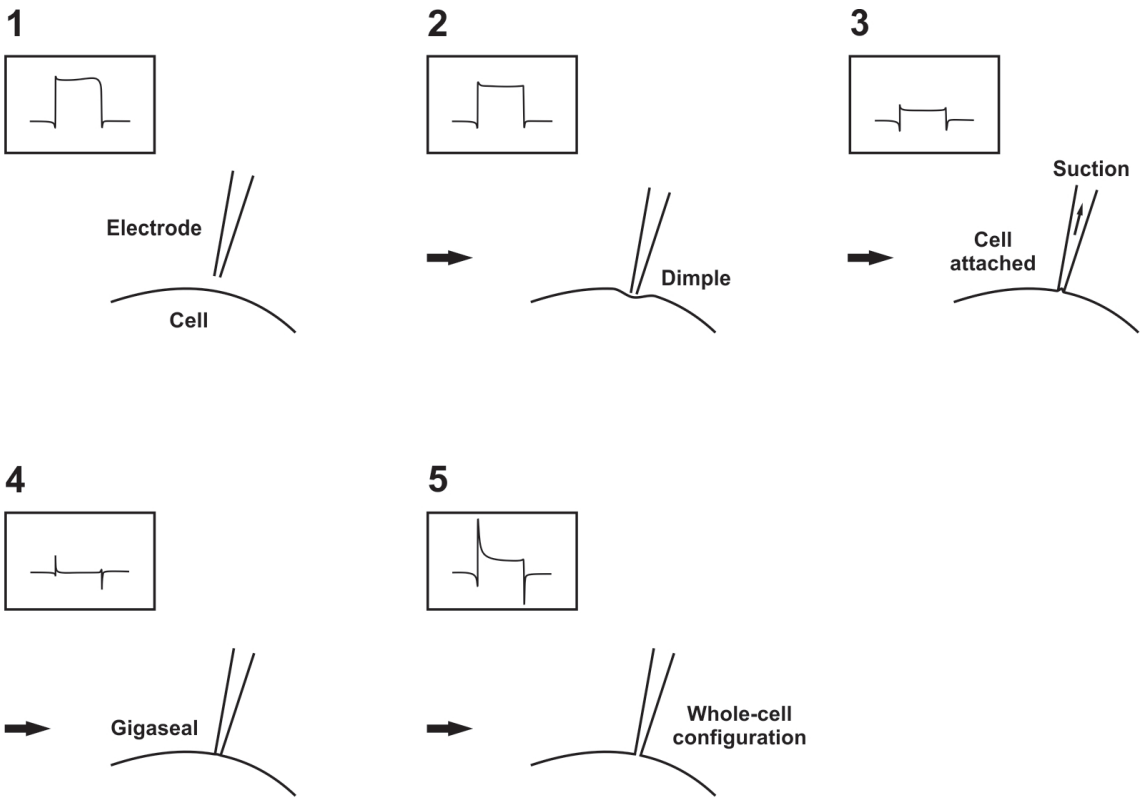


Figure 7: **Establishment of a whole-cell configuration.** The electrode is advanced towards the cell body maintaining a slightly positive pressure (1), until a small dimple becomes visible (2). The pressure is then lowered down to negative values, aspirating the cell membrane covering the opening of the electrode (3). The pressure is immediately set to zero once a gigaseal is achieved (4). The cell membrane is then opened by carefully applying quick negative pressures, until a whole-cell configuration is achieved (5). The current responses to a 1 mV test current pulse are displayed alongside.

Immediately after the whole-cell configuration was established, the NMDA re-

ceptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) and the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), as well as the sodium channel blocker tetrodotoxin (TTX) were applied, effectively inhibiting all neurotransmitter receptor activity except the activity of $GABA_A$ receptors. Spontaneously occurring currents of varying amplitude (inhibitory postsynaptic currents, IPSCs) could then be observed, resulting from the influx of chloride ions through $GABA_A$ receptors. Note that TTX blocks voltage gated sodium channels and thus the generation of action potentials, so the occurring IPSCs are action potential-independent and are therefore being called miniature IPSCs (mIPSCs).
Tonic currents were displayed by a slow shift of the baseline towards lower values. This shift was quantified by applying bicuculline, a selective $GABA_A$ receptor blocker, and calculating the change in baseline levels.

2.2.2 Preparation and Application of Test Substances for Patch Clamp Experiments

The ACSF consisted of 120 mM NaCl, 3.5 mM KCl, 1.13 mM NaH_2PO_4, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM $CaCl_2$, and 11 mM D-glucose (all by AppliChem, Darmstadt, Germany) dissolved in Ampuwa (Fresenius, Bad Homburg, Germany). XBD173 (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in ethanol 99.9% (Merck, Darmstadt, Germany), producing a 10 mM stock solution. This stock solution was then dissolved in ACSF, producing a 3 µM test solution that was used for the experiments. The test solution for sham experiments was obtained by dissolving an equivalent amount of ethanol 99.9% in ACSF, producing a 5.1 mM ethanol solution.
Zolpidem (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in ethanol 99.9%, producing a 20 mM stock solution. It was further dissolved in a 3 µM XBD173 solution, producing a test solution with concentrations of 400 nM zolpidem and 3 µM XBD173.
Bicuculline (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in a 3 µM XBD173 solution, producing a test solution with concentrations of 40 µM bicuculline and 3 µM XBD173. Likewise, for the sham group, both zolpidem and bicuculline were dissolved in a 5.1 mM ethanol solution.
AP5 and CNQX (both from Tocris, Wiesbaden, Germany) were dissolved in ACSF together, producing a solution with concentrations of 500 µM AP5 and 200 µM CNQX. TTX (Tocris, Wiesbaden, Germany) was dissolved in ACSF, producing a solution with a concentration of 20 µM TTX.

All test substances were prepared freshly every one to two days.

The XBD173 solution (respectively ethanol solution) was bubbled with 95% oxygen and 5% carbon dioxide for at least 30 minutes before and during its application. For patch clamp experiments, the XBD173 solution was applied via bath perfusion using IPC peristaltic pumps. Zolpidem and bicuculline were kept in gas-tight glass syringes of 50 mL and were applied via bath perfusion using a syringe pump (Harvard Apparatus, Holliston, USA) at a flow rate of 0.5 mL/min. The pump contained one syringe with 400 nM zolpidem and 3 µM XBD173, one with 40 µM bicuculline and 3 µM XBD173, and one with 3 µM XBD173 only, two of which were active at a time during the application, producing a total flow rate of 1 mL/min and effective concentrations of 200 nM zolpidem and 20 µM bicuculline in the recording chamber.
AP5, CNQX, and TTX were kept in gas-tight glass syringes of 10 mL and were applied via bath perfusion using a syringe pump at a flow rate of 0.05 mL/min, producing effective concentrations of 25 µM AP5, 10 µM CNQX, and 1 µM TTX in the recording chamber.
All pumps were connected to the recording chamber via Teflon tubing. The fluid from the chamber was drained using a peristaltic pump, allowing for a replacement of 95% of the medium in the chamber within two minutes.

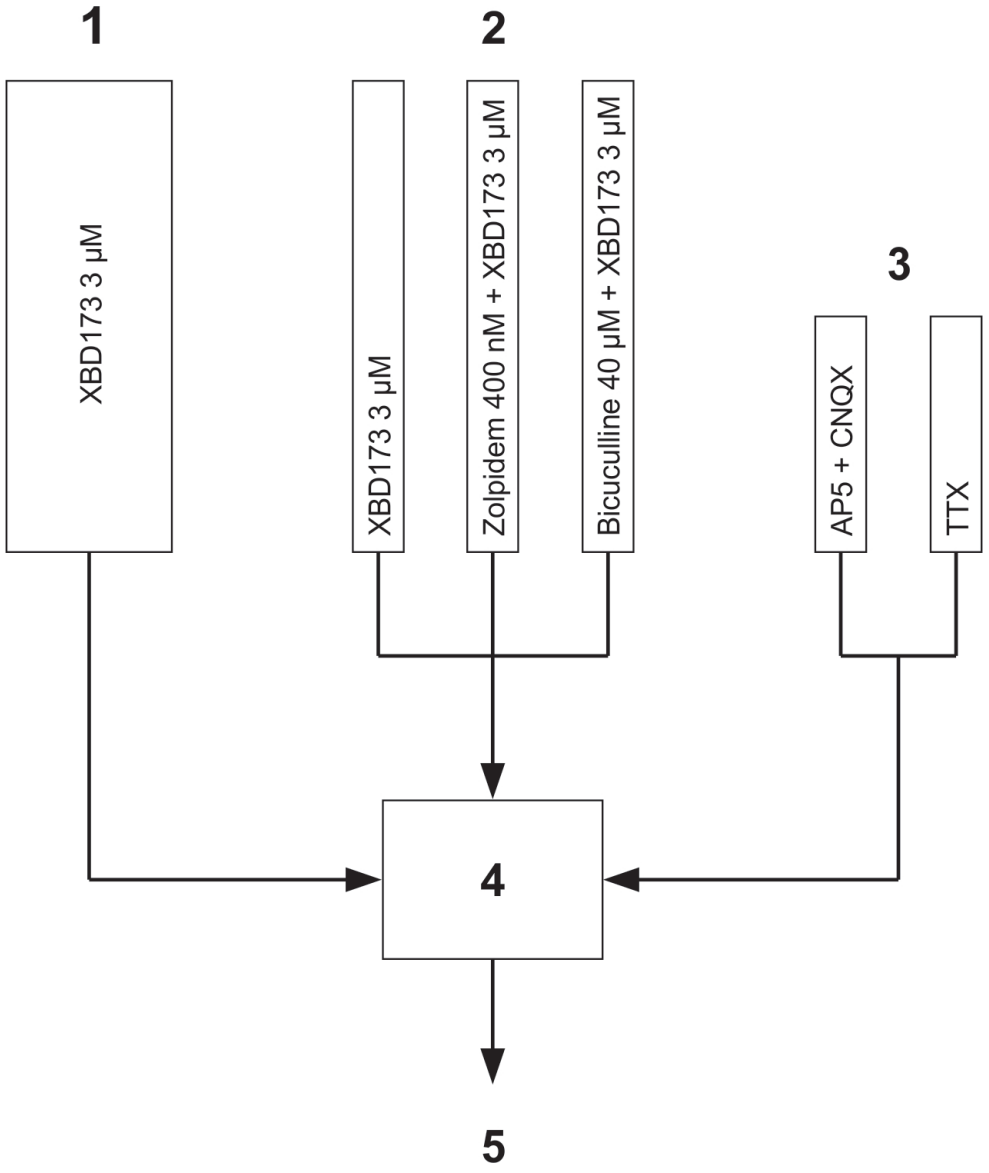


Figure 8: **Application of test substances for patch clamp recordings**.
1 = XBD173 3 µM solution, applied via a peristaltic pump
2 = XD173 3 µM solution, as well as zolpidem 400 nM plus XBD173 3 µM solution, and bicuculline 40 µM plus XBD173 3 µM solution, applied via syringe pumps at a flow rate of 0.5 mL/min. Two syringes were active at a time, producing concentrations of 200 nM zolpidem and 20 µM bicuculline in the recording chamber.
3 = AP5, CNQX, and TTX, applied via syringe pumps at a flow rate of 0.05 mL/min
4 = recording chamber
5 = draining via a peristaltic pump

2.2.3 Experimental Protocol for Patch Clamp Recordings

In order to assess the effect of XBD173 on GABAergic transmission and on the effect of zolpidem, one test group was treated with XBD173 (XBD173 group) and one with ethanol only, which served as the solvent for XBD173 (sham group).

In the XBD173 group, slices were perfused with 3 µM XBD173 for at least 30 minutes, before establishing a whole-cell configuration. This time span is relatively long due to the process of neurosteroidogenesis that is supposed to take place before the effects of XBD173 on neuronal activity may become apparent.
Immediately after establishing a whole-cell configuration, the application of AP5, CNQX, and TTX was started in order to block all neurotransmitter receptors except $GABA_A$ receptors. To ensure a stable effect of AP5, CNQX, and TTX, the recording was not started until at least seven minutes after the begin of their administration. The first recording was then taken over a period of three minutes, displaying mIPSC activity under the impact of XBD173 and the solvent. Thereafter, the additional application of 200 nM zolpidem was started, and five consecutive recordings of three minutes each were taken, monitoring the effect of zolpidem. In order to ensure a stable effect of zolpidem, the last of these five recordings was later used for data analysis.
Bicuculline was then added in order to block $GABA_A$ receptors as well, and another three consecutive three-minute recordings were taken, displaying the effect of bicuculline.

In the sham group, the experimental protocol was essentially the same, the only difference being that XBD173 was replaced by an amount of ethanol equivalent to the amount of the solvent in the XBD173 group. As described above, slices were perfused with a 5.1 mM ethanol solution for 30 minutes before establishing a whole-cell configuration. AP5, CNQX, and TTX were administered for seven minutes before taking the first recording. Zolpidem was added subsequently, and five recordings were taken consecutively. Thereafter, bicuculline was added, and three additional consecutive recordings were taken.

Figure 9: **Experimental protocol for patch clamp recordings.**

2.2.4 Data Analysis of Patch Clamp Recordings

Data were acquired on a personal computer using a MultiClamp 700B amplifier, Digidata 1440A interface, and pCLAMP 10 Software (all from Molecular Devices, San José, USA).
All data were analysed using MatLab routines (The MathWorks, Natick, USA) provided by our research group.

2.2.4.1 Detection and Fitting of mIPSCs

mIPSCs were detected by manually setting a threshold. All events exceeding this threshold were registered as mIPSCs.

For data analysis, the time courses of all registered mIPSCs were fitted for every single experiment by the bi-exponential function

$$y(t) = A_{fast} * \exp(-t/\tau_{fast}) + A_{slow} * \exp(-t/\tau_{slow})$$

A_{fast} = amplitude of the fast component
A_{slow} = amplitude of the slow component
τ_{fast} = decay time of the fast component
τ_{slow} = decay time of the slow component

mIPSCs that could not be fitted appropriately by the fitting routine were manually eliminated from further analysis. The fitted mIPSCs for every single experiment were then averaged.

The weighted decay time constant ($\tau_{weighted}$) was calculated by scaling the fast and slow decay time components by their respective amplitudes:

$$\tau_{weighted} = (A_{fast} * \tau_{fast} + A_{slow} * \tau_{slow}) / (A_{fast} + A_{slow})$$

2.2.4.2 Averaging of Tonic Currents

To assess tonic currents, a section of the recording immediately before the visible effect of bicuculline was taken, as well as a section immediately after the effect had reached its maximum expression. The baseline value for each section was averaged and the difference was calculated. This difference between the base line values before and after the impact of bicuculline displays the tonic currents.
The median difference and 95% confidence intervals were calculated from the differences of all single experiments for both the XBD173 group and the sham group separately.

2.3 Extracellular Recordings

2.3.1 Experimental Set-up for Extracellular Recordings

Neocortical slice cultures aged between 14 and 34 days were used for extracellular recordings. The method used detects action potential activity via extracellular multi-unit recordings.
Slices were put into a recording chamber consisting of a metal frame with a glass bottom. During the recordings, the slices were constantly perfused with ACSF via IPC peristaltic pumps. The ACSF was bubbled with 95% oxygen and 5% carbon dioxide for pH value stability. The recording chamber was constantly kept at a temperature of 34 ℃ by a feedback-controlled heating wire.
Glass electrodes with a calibre at the tip of about 1-2 µm were made from borosilicate capillaries by a DMZ universal puller (Zeitz, Martinsried, Germany) and were then filled with ACSF. The electrodes had resistances of 2-5 MΩ. They were positioned above the slice under an inverse microscope and were then advanced into the tissue until spikes could clearly be distinguished from the baseline noise. A reference electrode was placed in the surrounding ACSF and two measuring electrodes were placed in the tissue, allowing for two simultaneous recordings of different multi-units at the same time.

2.3.2 Preparation and Application of Test Substances for Extracellular Experiments

XBD173 was dissolved in ethanol 99.9%, producing a 10 mM stock solution. This stock solution was then dissolved in ACSF, producing a 3 µM test solution that was used for the experiments. The test solution for the sham experiments was obtained by dissolving an equivalent amount of ethanol 99.9% in ACSF, producing a 5.1 mM ethanol solution.
Zolpidem was dissolved in ethanol 99.9%, producing a 20mM stock solution. It was further dissolved in ACSF, producing a 200 nM test solution.
All test substances were prepared freshly every one to two days.

ACSF and all test substances were applied (via bath perfusion) and drained using IPC peristaltic pumps which were connected to the recording chamber via Teflon tubing. The flow rate was about 1 mL/min, allowing for a replacement of 95% of the medium in the chamber within two minutes. To ensure a steady state, recordings were not started until at least twelve minutes after the begin of the application of a new test substance. XBD173 recordings were not started until at least 30 minutes after the begin of the application of XBD173.
ACSF and all test substances were bubbled with 95% oxygen and 5% carbon dioxide 30 minutes before and during recording.

2.3.3 Experimental Protocol for Extracellular Recordings

Similarly to the patch clamp experiments, one test group was treated with XBD173 (XBD173 group) and one with ethanol (sham group).
In the XBD173 group, slices were perfused with ACSF for 30 minutes before neuronal activity was recorded for six minutes, serving as a control recording under drug-free conditions. Immediately thereafter, XBD173 was applied at a concentration of 3 µM for another 30 minutes. After this time span, a second six-minute recording was taken, assessing the effect of XBD173 (and its solvent ethanol) on neuronal activity. Subsequently, zolpidem was applied at a concentration of 200 nM for twelve minutes, while XBD173 was still being administered at the same concentration as before. A third six-minute recording was taken, assessing the effect of zolpidem in the presence of XBD173.

In the sham group, the experimental protocol was essentially the same, the only difference being that XBD173 was replaced by an equivalent amount of ethanol. As described above, slices were perfused with pure ACSF for 30 minutes before the first recording. Ethanol was then administered for another 30 minutes and the second recording was taken. Thereafter, zolpidem was applied at a concentration of 200 nM for twelve minutes before the third recording, assessing the effect of zolpidem in absence of XBD173.

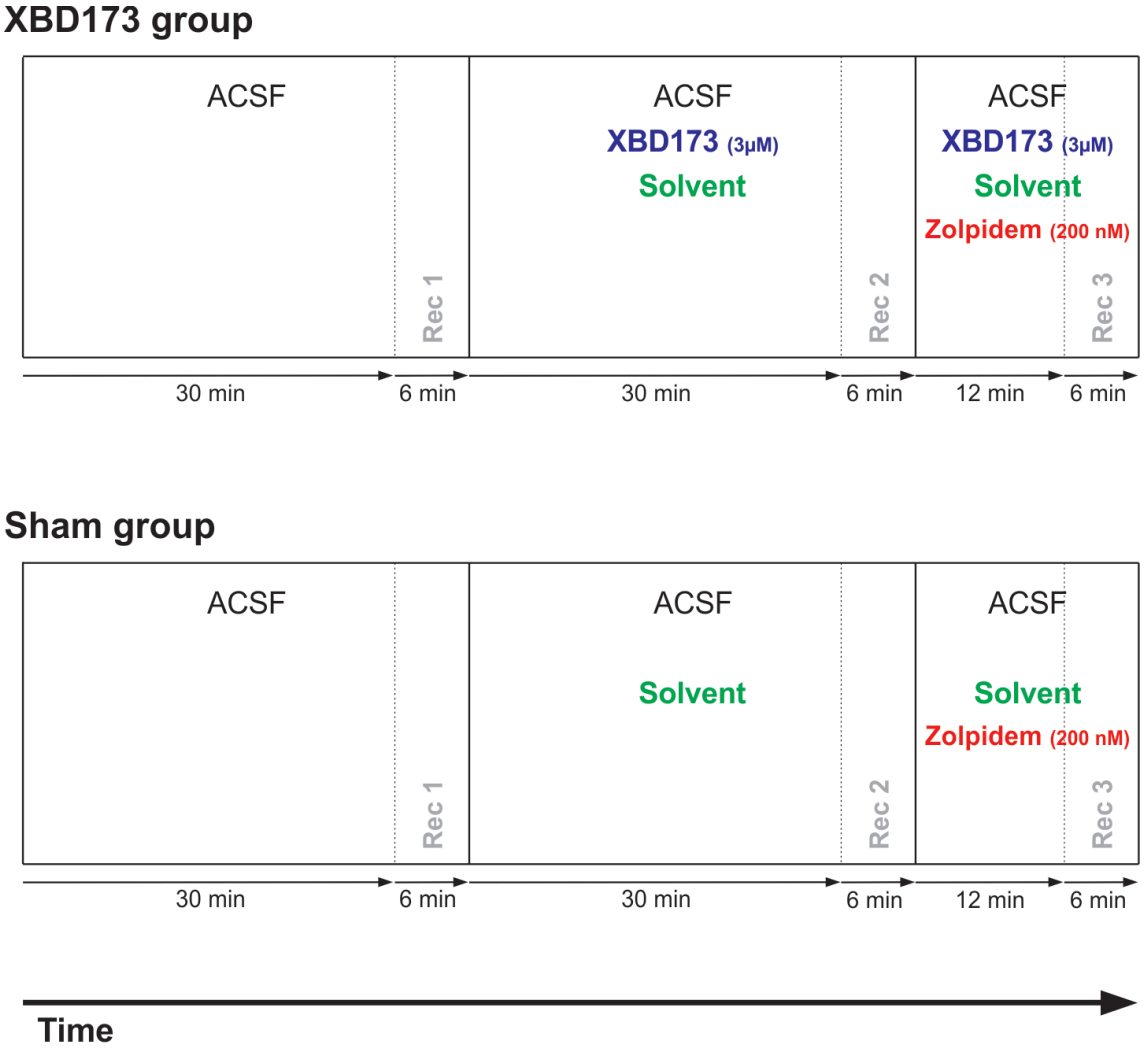


Figure 10: **Experimental protocol for extracellular multi-unit recordings.**

2.3.4 Data Analysis of Extracellular Recordings

Data were acquired on a personal computer using a MultiClamp 700B amplifier, Digidata 1440A interface, and Axoscope 10.7 software (Molecular Devices, San José, USA).
All data were analysed using MatLab routines provided by our research group.

2.3.4.1 Detection of Action Potentials

The raw signal was high-pass filtered below 300 Hz, eliminating slow field potential changes and allowing for the discrimination of single action potentials. These action potentials were distinguished from the baseline noise by manually setting a threshold. All spikes exceeding this threshold were registered as action potentials. The number of action potentials is considered a quantitative parameter for neuronal activity.

2.3.4.2 Burst Detection

Spontaneous neuronal activity in the neocortex displays a characteristic pattern. Phases of constant action potential activity – so called bursts – can be distinguished from phases of lower basic activity. These bursts are the object of interest when examining neuronal activity in the neocortex, as they feature various parameters that may act as indicators for changes in neuronal activity.
After registering single action potentials as described above, clusters of action potential activity were registered using MatLab routines.

2.4 Statistical Analysis of the Experimental Data

For patch clamp recordings, the time courses of all mIPSCs of a single recording were fitted by a bi-exponential function. A number of different parameters (see table 1) were calculated for each mIPSC and then averaged for all mIPSCs of one recording. Since the hypothesis that data samples derive from a normally distributed population had to be rejected for a number of experiments, as was determined using the Lilliefors test, median parameters and 95% confidence intervals were calculated and used for further statistical analysis. The results are

represented as boxplots, with the middle line representing the median, the upper and lower margin of the box representing the upper and lower quartile (75^{th} and 25^{th} percentile), and the upper and lower whisker representing the greatest and the smallest value not considered an outlier. p-values were calculated using a Mann-Whitney U test.
For the analysis of tonic currents, the baseline shift was calculated for each experiment. Outliers were eliminated from further analysis and the median baseline shift as well as 95% confidence intervals were calculated for both the sham group and the XBD173 group separately. p-values were calculated using a Mann-Whitney U test.

For extracellular recordings, clusters of action potential activity, so-called bursts, were registered. For each experiment, every single burst was split into bins of 5 ms. Median discharge rates were then calculated for each number of bin (i.e. calculating one median value from all the values of the first bin of each burst, one median value from the values of each second bin, and so on) and plotted against time, allowing for a visual comparison of the average burst pattern of two different conditions. Normalized activities were then calculated for each comparison between two conditions, and the normalized activities for each experiment were ranked by their magnitude, resulting in their cumulative frequency. The results are displayed as the normalized activity plotted against the cumulative frequency, as well as boxplots displaying the depression of discharge rates, with the middle line representing the median, the upper and lower margin of the box representing the upper and lower quartile, the lateral notches of the box representing the 95% confidence interval, and the upper and lower whisker representing the greatest and the smallest value not considered an outlier. p-values were derived via a Mann-Whitney U test.

3 Results

3.1 Patch Clamp Experiments

3.1.1 mIPSC Kinetics

In neocortical slice cultures, miniature inhibitory postsynaptic currents were recorded at 34 ℃ from neurons voltage-clamped at -70 mV. mIPSCs are the result of the action potential-independent presynaptic release of GABA modulating postsynaptic $GABA_A$ receptors, leading to an influx of chloride ions through and thus hyperpolarization of the postsynaptic membrane. Under voltage-clamp conditions and symmetrical concentrations of chloride ions on both sides of the cell membrane, this influx can be measured as inward currents from a holding potential of -70 mV.
The frequency of mIPSCs serves as an indicator for the probability of presynaptic GABA release, whereas their duration, indicated by the decay time, represents the duration of the open time of postsynaptic $GABA_A$ receptors.

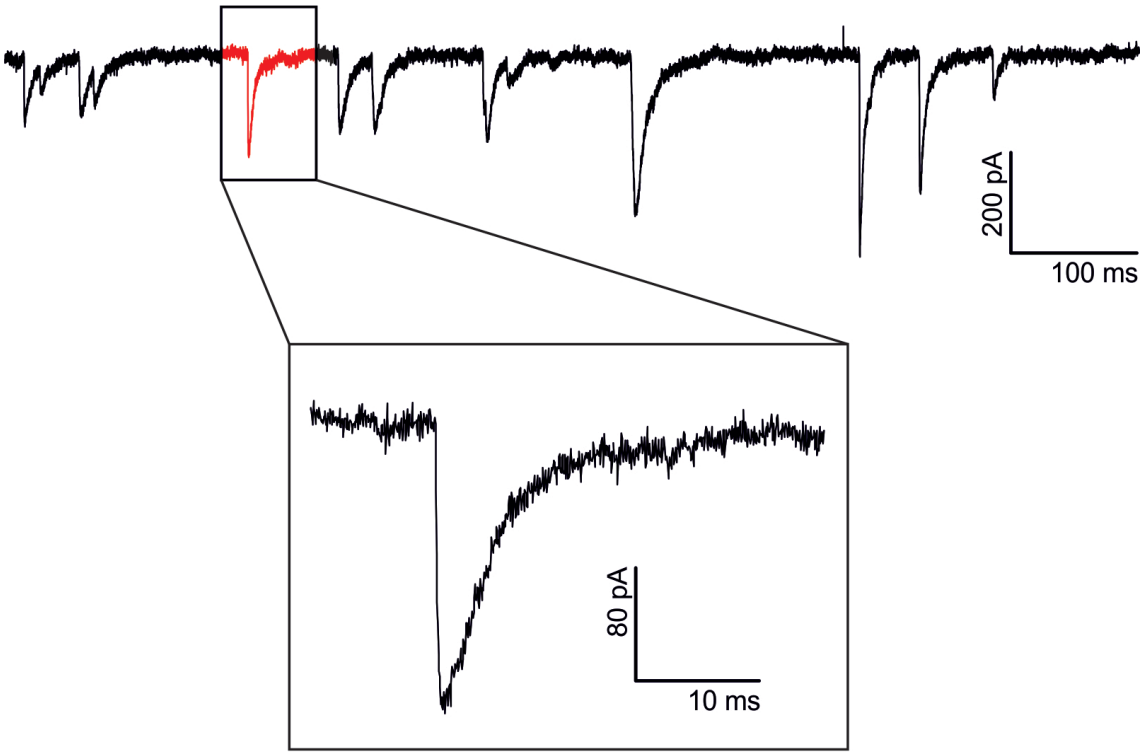


Figure 11: **An example of mIPSCs.**

Several hundred mIPSCs were sampled during a single recording. For data analysis, their time courses were fitted by a bi-exponential function as described above, distinguishing between a fast and a slow component of the current. Thus, a variety of different parameters such as the amplitude and the decay time were calculated for every single mIPSC and were then averaged. A complete list of the calculated parameters is displayed in table 1.

3.1.2 Effect of Zolpidem

A total of 26 slice cultures were examined in the patch clamp experiments, 12 of which were treated with 3 µM XBD173 during the recording, and 14 of which were treated with a sham application. All 26 slice cultures were subsequently treated with 200 nM zolpidem, as described in the experimental protocol. The effect of zolpidem alone was assessed by comparing averaged mIPSCs from all recordings under drug-free conditions in the sham group to averaged mIPSCs from all recordings under zolpidem conditions in the same group. These are paired samples since the recordings under drug-free conditions and under zolpidem conditions were carried out on the same cell in each experiment. Various parameters were examined, as listed in the table below.
Normal distribution did not apply to all parameters, as was determined using the Lilliefors test. Hence, medians and 95% confidence intervals were used for descriptive statistics.

	Drug-free			**Zolpidem**			**Change**
	Median	$CI95_{high}$	$CI95_{low}$	Median	$CI95_{high}$	$CI95_{low}$	%
A_{fast} (pA)	38.68	45.75	23.68	41.11	46.05	29.51	6.27
A_{slow} (pA)	8.33	15.10	4.56	13.26	20.76	9.21	59.14
A_{total} (pA)	52.09	69.58	30.98	63.23	76.76	43.37	21.38
$A_{fast/slow}$	4.80	6.86	3.49	3.21	4.28	2.09	-33.17
τ_{fast} (ms)	3.90	4.93	2.98	6.49	7.30	5.57	66.33
τ_{slow} (ms)	14.26	22.11	11.28	20.54	27.10	15.28	44.03
$\tau_{weighted}$ (ms)	6.86	9.96	5.32	12.02	15.12	10.60	75.21
$\tau_{fast/slow}$	0.14	0.20	0.12	0.19	0.21	0.15	34.95
width (ms)	3.46	4.63	2.47	5.93	7.39	5.51	71.17
ct_{fast} (pC)	0.13	0.24	0.09	0.25	0.34	0.19	84.95
ct_{slow} (pC)	0.29	0.43	0.12	0.47	0.67	0.32	61.98
ct_{total} (pC)	0.47	0.67	0.24	0.82	1.08	0.51	72.29
$ct_{fast/slow}$	0.50	0.73	0.38	0.49	0.58	0.30	-2.10
frq (Hz)	4.12	8.11	1.53	5.06	9.01	2.81	22.84

Table 1: **Medians and 95% confidence intervals for all recorded parameters under drug-free and zolpidem conditions.** The changes induced by zolpidem are displayed in percent of the drug-free value.
The following parameters were calculated:
A_{fast} = amplitude of the fast component of the current in pA
A_{slow} = amplitude of the slow component of the current in pA
A_{total} = amplitude of the entire current in pA
$A_{fast/slow}$ = ratio between the amplitudes of the fast and slow component
τ_{fast} = decay time of the fast component of the current in ms
τ_{slow} = decay time of the slow component of the current in ms
$\tau_{weighted}$ = weighted decay time constant in ms
$\tau_{fast/slow}$ = ratio between the decay times of the fast and slow component
width = width of the current in ms
ct_{fast} = charge transfer of the fast component of the current in pC
ct_{slow} = charge transfer of the slow component of the current in pC
ct_{total} = charge transfer of the entire current in pC
$ct_{fast/slow}$ = ratio between the charge transfer values of the fast and slow component
frq = frequency in Hz

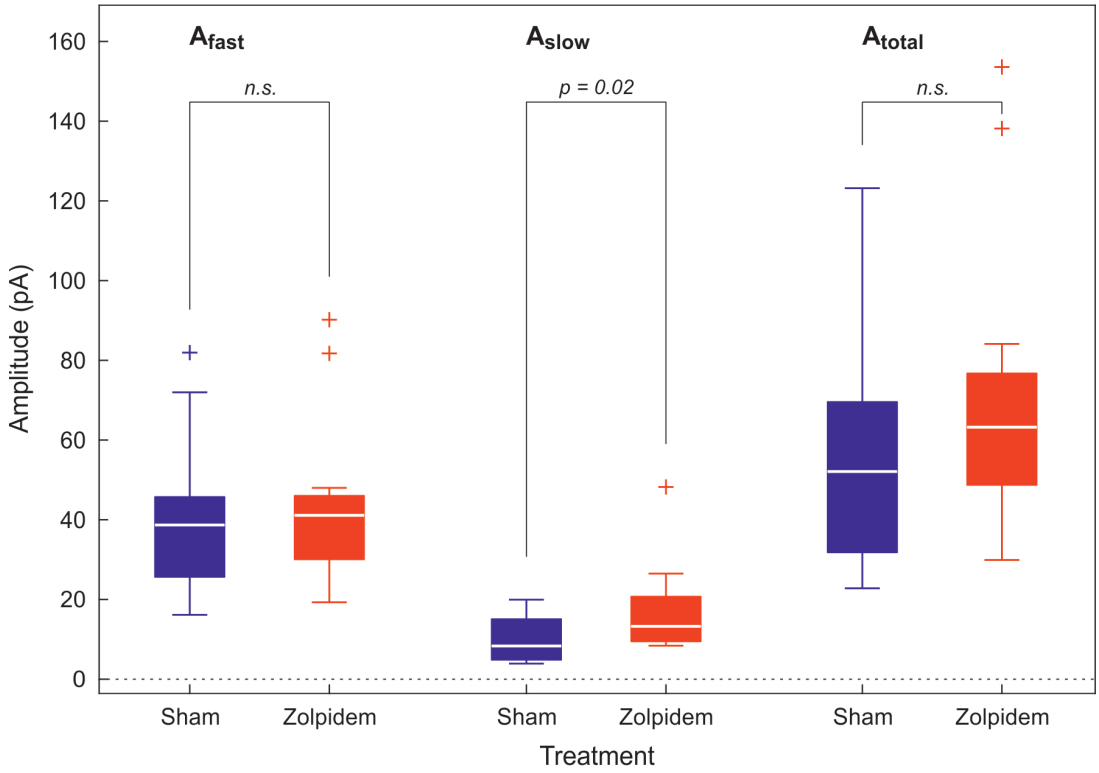
Zolpidem affected a number of parameters to a significant extent: Both the decay time of the fast and slow component as well as the weighted decay time constant were increased significantly (see table 2).
The charge transfer of both components was increased significantly, which is also represented by a significant increase in total charge transfer.
The amplitude of the slow component was enhanced significantly, thus shifting the amplitude ratio between the fast and the slow component more towards the slow component.
Zolpidem did not display a statistically significant effect on mIPSC frequency.

	n	A_{fast} (pA)	A_{slow} (pA)	A_{total} (pA)	$A_{fast/slow}$
Drug-free	14	38.68 (45.75; 23.68)	8.33 (15.10; 4.56)	52.09 (69.58; 30.98)	4.80 (6.86; 3.49)
Zolpidem	14	41.11 (46.05; 29.51)	13.26 (20.76; 9.21)	63.23 (76.76; 43.37)	3.21 (4.28; 2.09)
p-value		0.6	0.02	0.3	0.009

	n	τ_{fast} (ms)	τ_{slow} (ms)	$\tau_{weighted}$ (ms)	$\tau_{fast/slow}$
Drug-free	14	3.90 (4.93; 2.98)	14.26 (22.11; 11.28)	6.86 (9.96; 5.32)	0.14 (0.20; 0.12)
Zolpidem	14	6.49 (7.30; 5.57)	20.54 (27.10; 15.28)	12.02 (15.12; 10.60)	0.19 (0.21; 0.15)
p-value		0.0005	0.04	0.0009	0.01

	n	ct_{fast} (pC)	ct_{slow} (pC)	ct_{total} (pC)	$ct_{fast/slow}$
Drug-free	14	0.13 (0.24; 0.09)	0.29 (0.43; 0.12)	0.47 (0.67; 0.24)	0.50 (0.73; 0.38)
Zolpidem	14	0.25 (0.34; 0.19)	0.47 (0.67; 0.32)	0.82 (1.08; 0.51)	0.49 (0.58; 0.30)
p-value		0.008	0.01	0.005	0.3

Table 2: **Analysis of mIPSC amplitude, decay time, and charge transfer under drug-free and zolpidem conditions.** The table displays median values from recordings carried out on 14 slice cultures. p-values were derived via a Mann-Whitney U test.


A fast
A slow
A total
n.s.
p = 0.02
n.s.
Amplitude (pA)
160
140
120
100
80
60
40
20
0
Sham
Zolpidem
Sham
Zolpidem
Sham
Zolpidem
Treatment

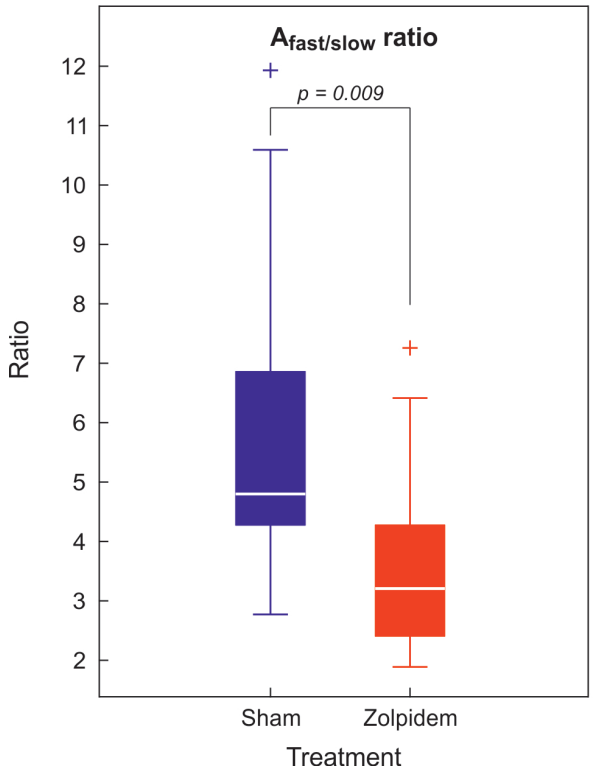
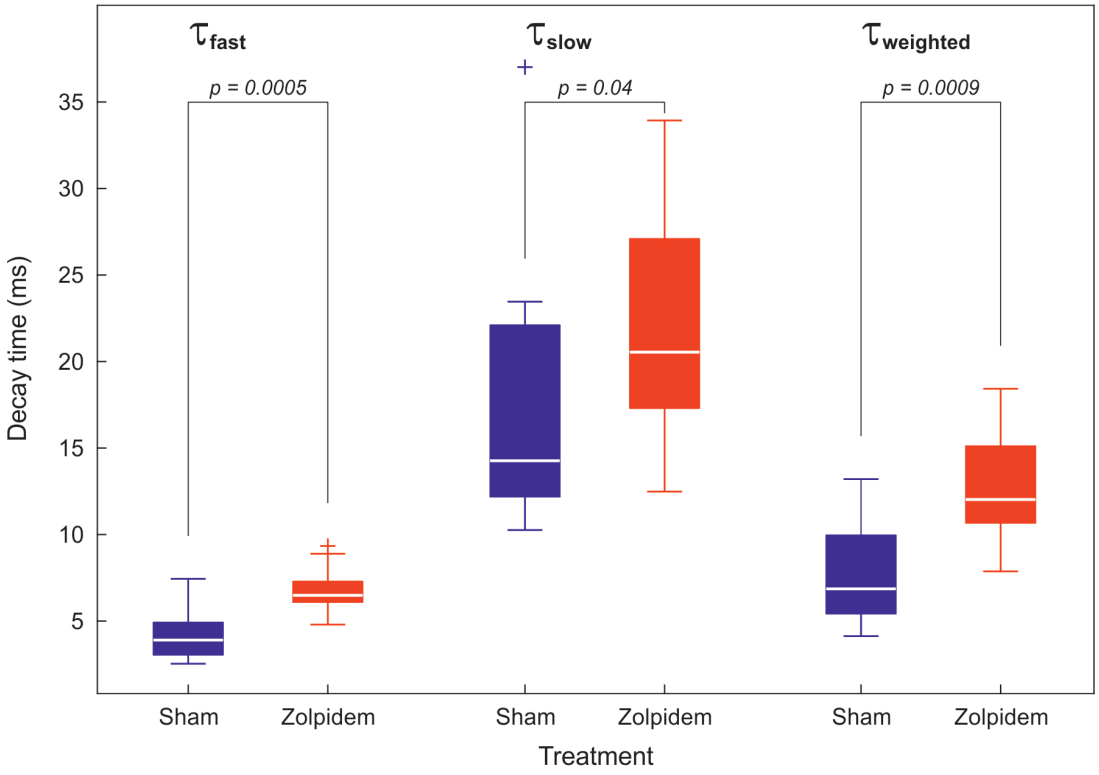

A fast/slow ratio
p = 0.009
Ratio
12
11
10
9
8
7
6
5
4
3
2
Sham
Zolpidem
Treatment

Figure 12: **Amplitudes of the fast and slow component as well as the total amplitudes and amplitude ratios between the fast and slow component of fitted mIPSCs under drug-free and zolpidem conditions.** The null hypothesis (h0), that in sham- and zolpidem-treated slices the amplitudes of the slow component are undistinguishable, could be rejected at the 5% significance level. Correspondingly, the same could be stated for the ratios between the fast and slow component at the 1% significance level. h0 could not be rejected for A_{fast} and A_{total}. This indicates a shift towards the slow component induced by zolpidem.


τfast
τslow
τweighted
p = 0.0005
p = 0.04
p = 0.0009
Decay time (ms)
35
30
25
20
15
10
5
Sham
Zolpidem
Sham
Zolpidem
Sham
Zolpidem
Treatment

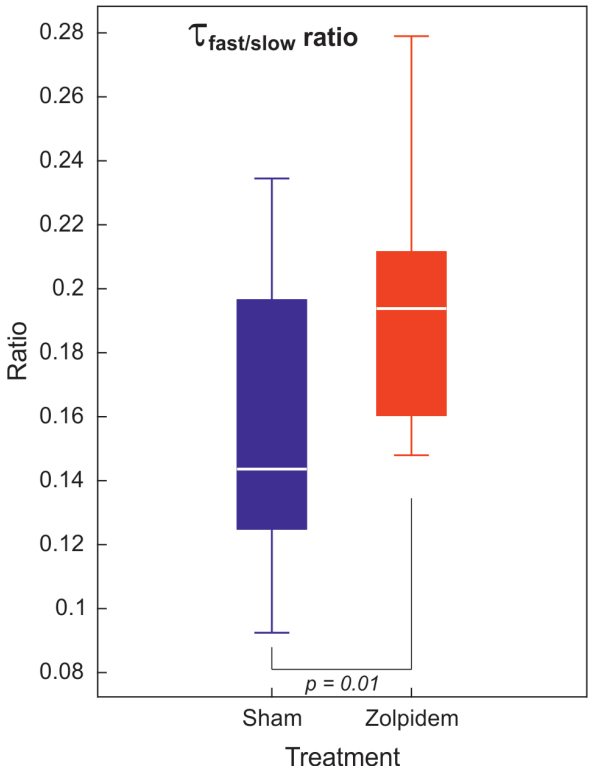
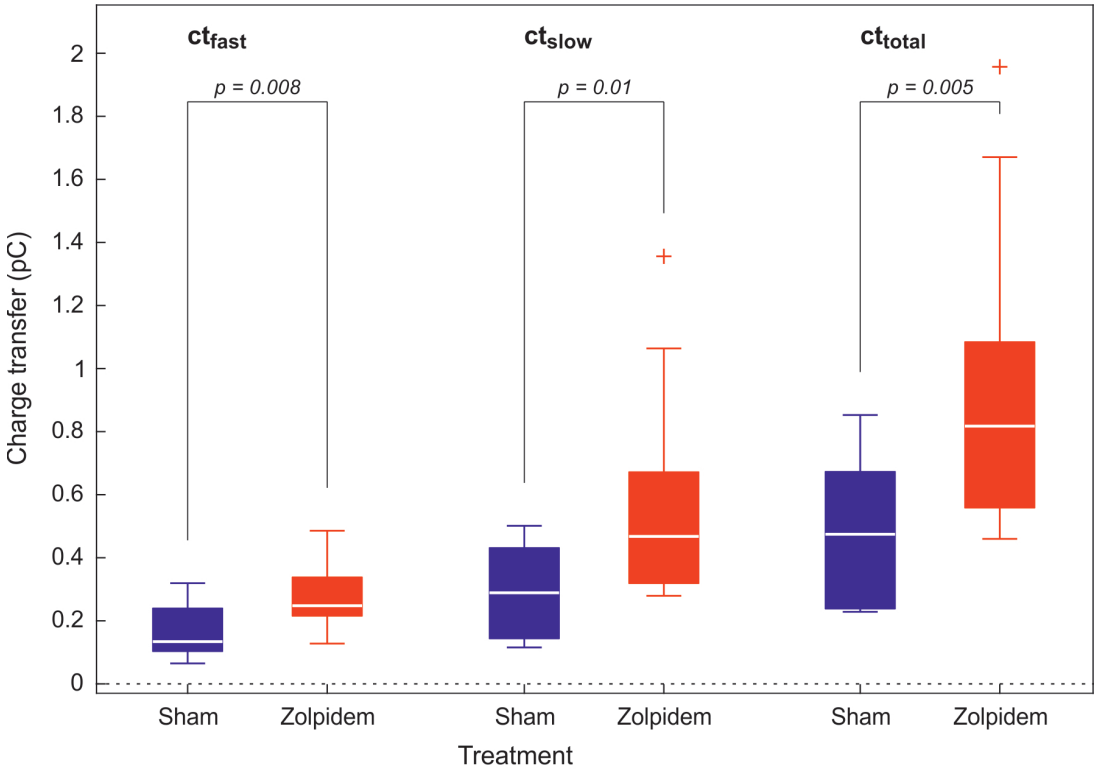

τfast/slow ratio
Ratio
0.28
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
p = 0.01
Sham
Zolpidem
Treatment

Figure 13: **Decay times of the fast and slow component as well as the weighted decay time constants and decay time ratios between the fast and slow component of fitted mIPSCs under drug-free and zolpidem conditions.** The weighted decay time constant ($\tau_{weighted}$) was calculated by scaling the fast and slow decay time components by their respective amplitudes. The null hypothesis, that in sham- and zolpidem-treated slices the weighted decay time constants are undistinguishable, could be rejected at the 1% significance level. Correspondingly, h0 could also be rejected for τ_{fast} and $\tau_{fast/slow}$ at the 1% significance level, as well as for τ_{slow} at the 5% significance level. This indicates a strong overall effect of zolpidem on the decay time.


ctfast
ctslow
cttotal
p = 0.008
p = 0.01
p = 0.005
Charge transfer (pC)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sham
Zolpidem
Sham
Zolpidem
Sham
Zolpidem
Treatment

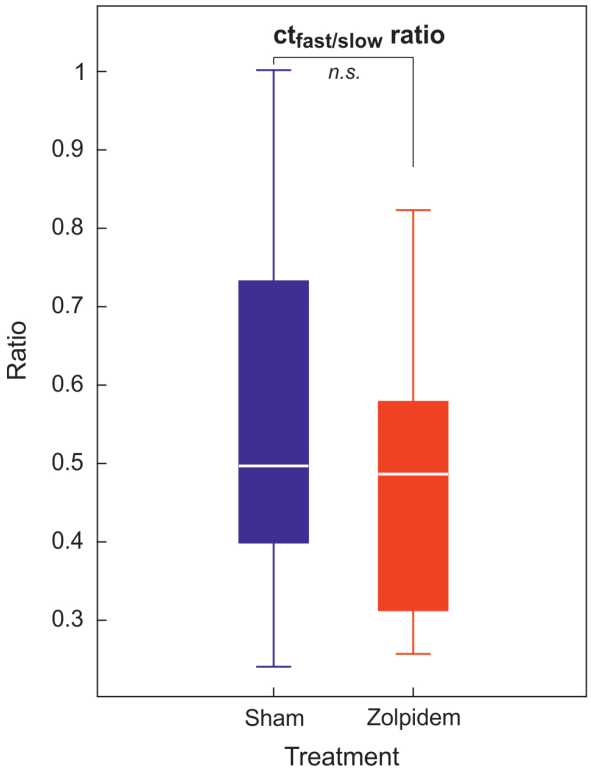

ctfast/slow ratio
n.s.
Ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
Sham
Zolpidem
Treatment

Figure 14: **Charge transfer values of the fast and slow component as well as the total charge transfer values and charge transfer ratios between the fast and slow component of fitted mIPSCs under drug-free and zolpidem conditions.** The null hypothesis, that in sham- and zolpidem-treated slices the total charge transfer of fitted mIPSCs is undistinguishable, could be rejected at the 1% significance level. Correspondingly, the same could be stated for ct_{fast} and ct_{slow}. This is in line with the zolpidem-induced changes to the decay time.

3.1.2.1 Summary

Zolpidem produced a clear effect on synaptic GABAergic transmission. At a concentration of 200 nM, it was able to strongly enhance mIPSC decay time and charge transfer, while also displaying a minor enhancing effect on mIPSC amplitudes.

3.1.3 Effect of XBD173

In order to assess the effect of XBD173, averaged mIPSCs from all recordings under drug-free conditions in the sham group were compared to averaged mIPSCs from all recordings under XBD173 conditions. These are unpaired samples, since data from two different groups were compared to each other. The calculated parameters were the same as above, and are listed in table 3.
Again, normal distribution did not apply to all parameters, so medians and 95% confidence intervals were used for descriptive statistics.

	Drug-free			**XBD173**			**Change**
	Median	$CI95_{high}$	$CI95_{low}$	Median	$CI95_{high}$	$CI95_{low}$	%
A_{fast} (pA)	38.68	45.75	23.68	31.51	39.79	19.09	-18.55
A_{slow} (pA)	8.33	15.10	4.56	12.13	15.52	8.51	45.52
A_{total} (pA)	52.09	69.58	30.98	49.66	59.07	31.01	-4.66
$A_{fast/slow}$	4.80	6.86	3.49	3.18	4.46	2.37	-33.28
τ_{fast} (ms)	3.90	4.93	2.98	5.36	5.62	3.22	37.35
τ_{slow} (ms)	14.26	22.11	11.28	22.07	25.13	15.45	54.71
$\tau_{weighted}$ (ms)	6.86	9.96	5.32	11.03	12.86	8.73	60.69
$\tau_{fast/slow}$	0.14	0.20	0.12	0.14	0.15	0.12	-2.50
width (ms)	3.46	4.63	2.47	5.27	5.96	3.34	52.11
ct_{fast} (pC)	0.13	0.24	0.09	0.14	0.15	0.11	2.07
ct_{slow} (pC)	0.29	0.43	0.12	0.35	0.42	0.25	20.64
ct_{total} (pC)	0.47	0.67	0.24	0.55	0.58	0.37	16.18
$ct_{fast/slow}$	0.50	0.73	0.38	0.34	0.46	0.26	-30.68
frq (Hz)	4.12	8.11	1.53	3.98	5.82	2.26	-3.44

Table 3: **Medians and 95% confidence intervals for all recorded parameters under drug-free and XBD173 conditions.** The changes induced by XBD173 are displayed in percent of the original value.

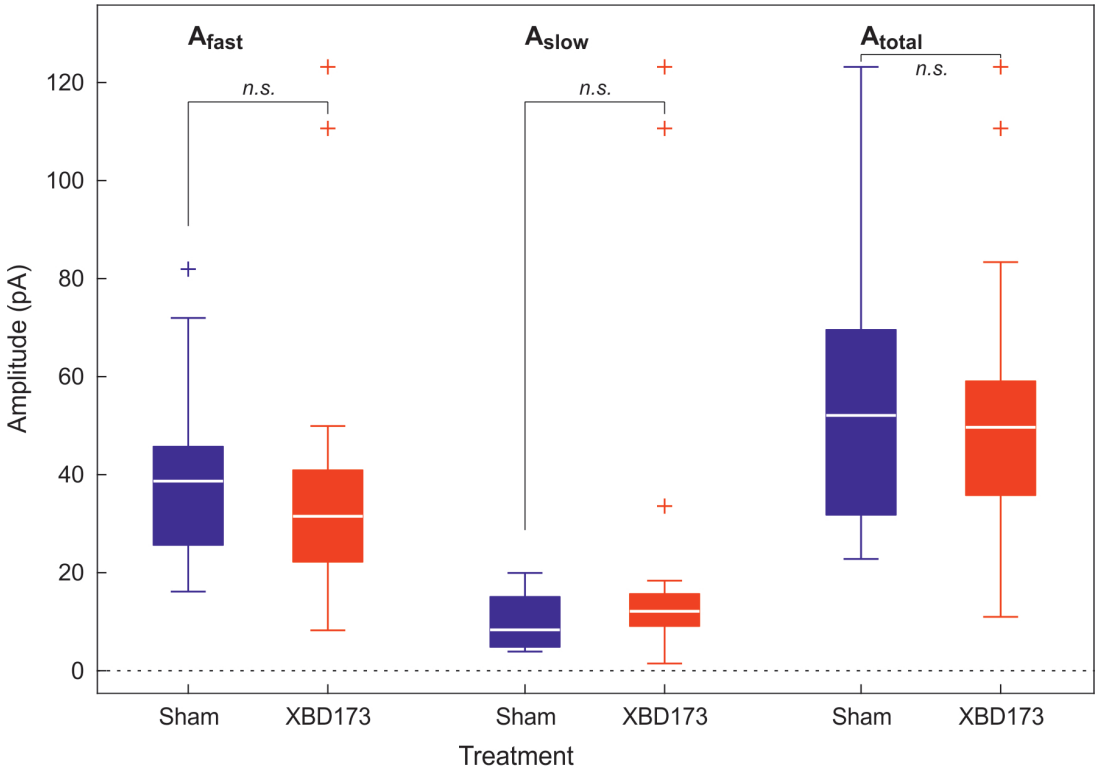
XBD173 significantly increased the weighted decay time constant.
It furthermore altered the ratio between the amplitudes of the fast and slow component as well as the ratio between the charge transfer values of the fast and slow component both significantly towards the slow component.
XBD173 did not display a statistically significant effect on mIPSC frequency.

	n	A_{fast} (pA)	A_{slow} (pA)	A_{total} (pA)	$A_{fast/slow}$
Drug-free	14	38.68	8.33	52.09	4.80
		(45.75; 23.68)	(15.10; 4.56)	(69.58; 30.98)	(6.86; 3.49)
XBD173	12	31.51	12.13	49.66	3.18
		(39.79; 19.09)	(15.52; 8.51)	(59.07; 31.01)	(4.46; 2.37)
p-value		0.3	0.4	0.5	0.03

	n	τ_{fast} (ms)	τ_{slow} (ms)	$\tau_{weighted}$ (ms)	$\tau_{fast/slow}$
Drug-free	14	3.90	14.26	6.86	0.14
		(4.93; 2.98)	(22.11; 11.28)	(9.96; 5.32)	(0.20; 0.12)
XBD173	12	5.36	22.07	11.03	0.14
		(5.62; 3.22)	(25.13; 15.45)	(12.86; 8.73)	(0.15; 0.12)
p-value		0.2	0.08	0.03	0.9

	n	ct_{fast} (pC)	ct_{slow} (pC)	ct_{total} (pC)	$ct_{fast/slow}$
Drug-free	14	0.13	0.29	0.47	0.50
		(0.24; 0.09)	(0.43; 0.12)	(0.67; 0.24)	(0.73; 0.38)
XBD173	12	0.14	0.35	0.55	0.34
		(0.15; 0.11)	(0.42; 0.25)	(0.58; 0.37)	(0.46; 0.26)
p-value		0.8	0.6	0.9	0.04

Table 4: **Analysis of mIPSC amplitude, decay time, and charge transfer under drug-free and XBD173 conditions.** The table displays median values from recordings carried out on 14 slice cultures for drug-free conditions and 12 slice cultures for XBD173 conditions. p-values were derived via a Mann-Whitney U test.


A fast
A slow
A total
n.s.
n.s.
n.s.
Amplitude (pA)
120
100
80
60
40
20
0
Sham
XBD173
Sham
XBD173
Sham
XBD173
Treatment

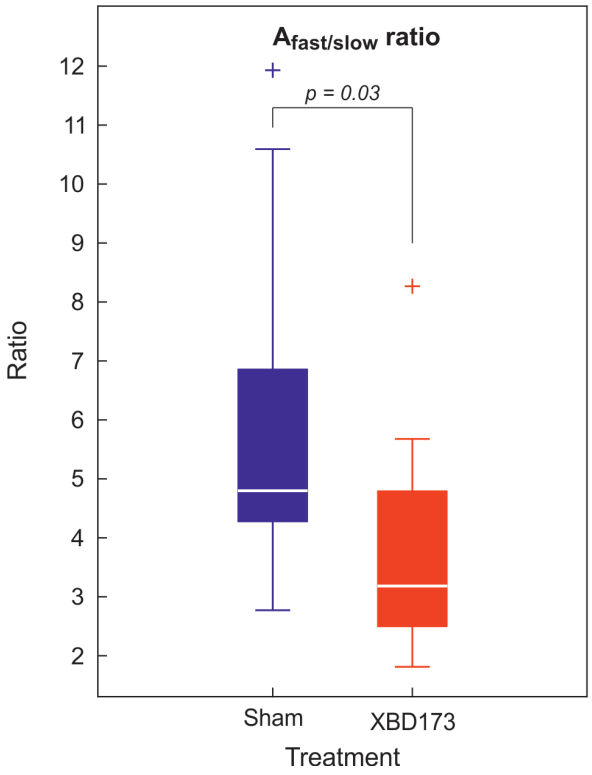
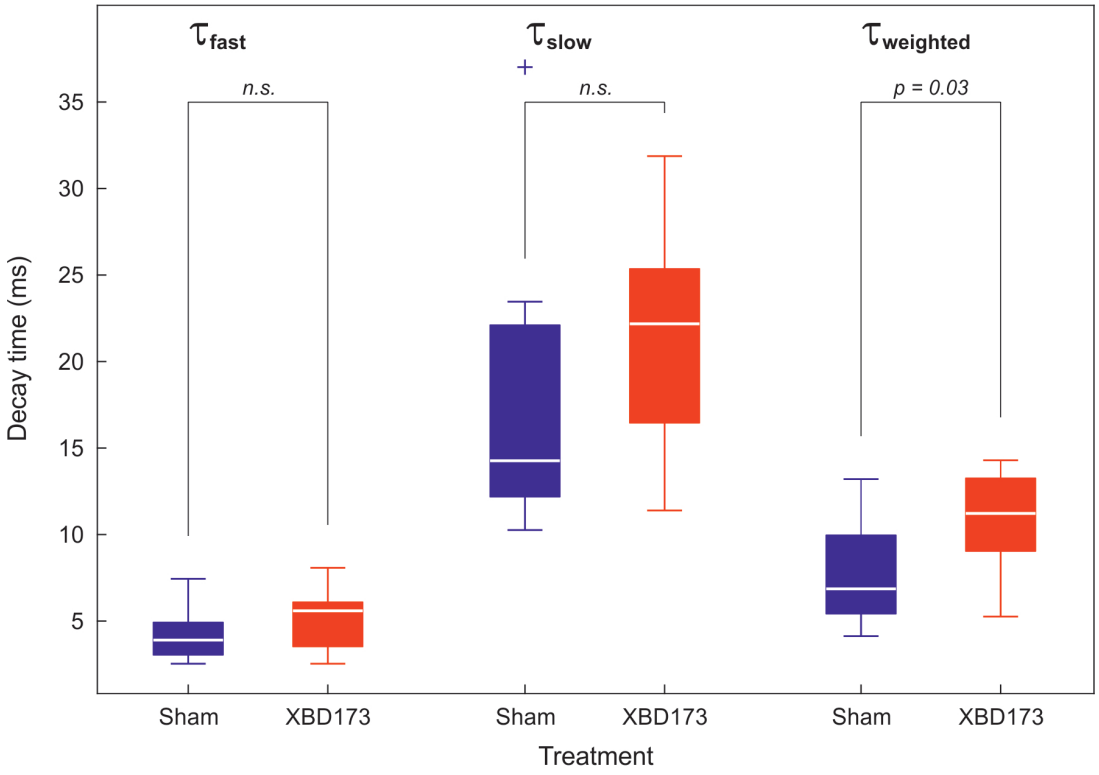

A fast/slow ratio
p = 0.03
Ratio
12
11
10
9
8
7
6
5
4
3
2
Sham
XBD173
Treatment

Figure 15: **Amplitudes of the fast and slow component as well as the total amplitudes and amplitude ratios between the fast and slow component of fitted mIPSCs under drug-free and XBD173 conditions.** The null hypothesis, that in sham- and XBD173-treated slices the amplitudes of the fast component and, respectively, the slow component are undistinguishable, could not be rejected at the 5% significance level. However, h0 could be rejected for $A_{fast/slow}$ at the 5% significance level, with $A_{fast/slow}$ being decreased by $\sim$ 33% by XBD173. This indicates a shift in amplitude towards the slow component induced by XBD173.


τfast
τslow
τweighted
n.s.
n.s.
p = 0.03
Decay time (ms)
35
30
25
20
15
10
5
Sham
XBD173
Sham
XBD173
Sham
XBD173
Treatment

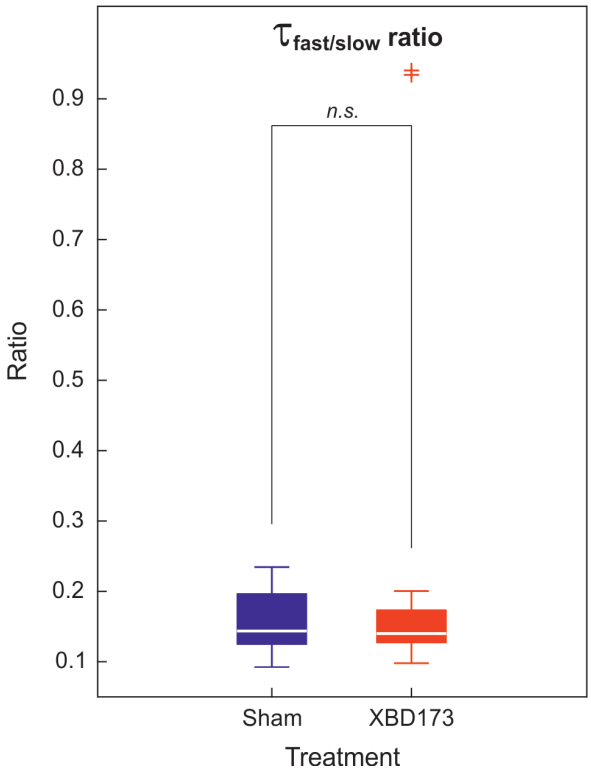
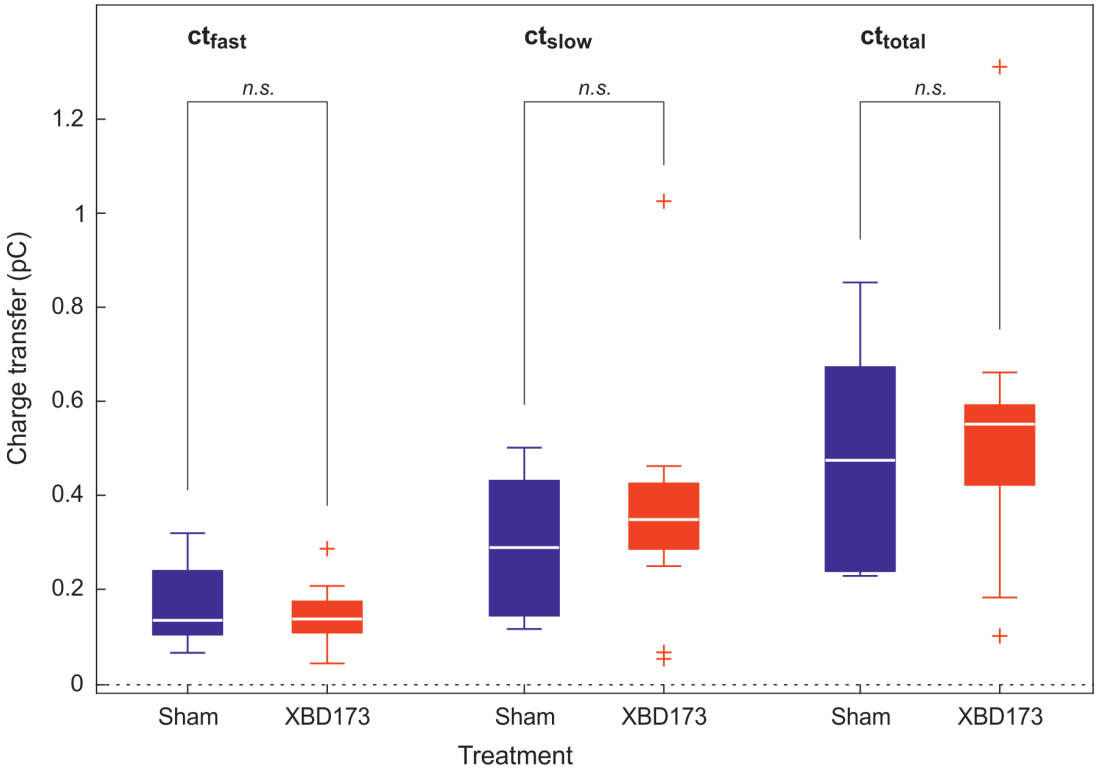

τfast/slow ratio
n.s.
Ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Sham
XBD173
Treatment

Figure 16: **Decay times of the fast and slow component as well as the weighted decay time constants and decay time ratios between the fast and slow component of fitted mIPSCs under drug-free and XBD173 conditions.** The null hypothesis, that in sham- and XBD173-treated slices the weighted decay time constants ($\tau_{weighted}$) are undistinguishable, could be rejected at the 5% significance level. h0 could not be rejected for τ_{fast} and τ_{slow}. However, XBD173 displayed a strong tendency to alter those parameters (displaying an increase of $\sim$ 37% for τ_{fast} and $\sim$ 55% for τ_{slow}). These findings suggest that the decay time changes for the fast and slow component alone are too subtle to reach statistical significance, but scaling them by their respective amplitudes reveals an overall significant effect of XBD173 on mIPSC kinetics.


ctfast
ctslow
cttotal
n.s.
n.s.
n.s.
Charge transfer (pC)
0
0.2
0.4
0.6
0.8
1
1.2
Sham
XBD173
Sham
XBD173
Sham
XBD173
Treatment

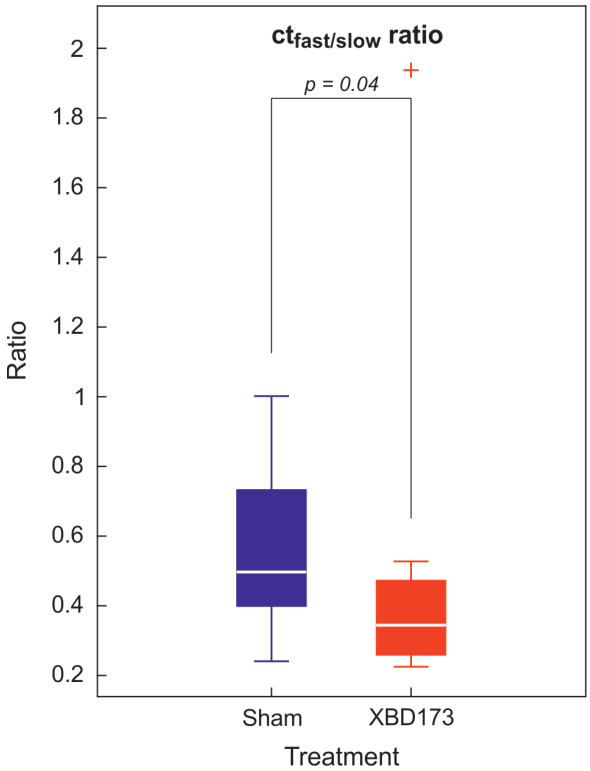

ctfast/slow ratio
p = 0.04
Ratio
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Sham
XBD173
Treatment

Figure 17: **Charge transfer values of the fast and slow component as well as the total charge transfer values and charge transfer ratios between the fast and slow component of fitted mIPSCs under drug-free and XBD173 conditions.** The null hypothesis, that in sham- and XBD173-treated slices the charge transfer values of the fast component and, respectively, the slow component are undistinguishable, could not be rejected at the 5% significance level. However, h0 could be rejected for $ct_{fast/slow}$ at the 5% significance level, with $ct_{fast/slow}$ being decreased by $\sim$ 31% by XBD173. This is in line with the XBD173-induced changes on mIPSC amplitudes and indicates a shift in charge transfer towards the slow component.

3.1.3.1 Summary

XBD173 was shown to modify mIPSC kinetics by increasing the weighted decay time constant and shifting the amplitude and charge transfer ratios both more towards the slow component. While it displayed a tendency to increase the overall decay time, this effect failed to reach statistical significance.

3.1.4 Zolpidem-induced Changes under Drug-free and XBD173 Conditions

Further data analysis addressed the question whether or not zolpidem-induced changes in mIPSC kinetics are modulated by enhancing neurosteroidogenesis. The effects of zolpidem in the absence of XBD173 and in the presence of XBD173 were assessed by quantifying the effect of zolpidem on each parameter under both drug-free and XBD173 conditions, i.e. the differences between drug-free conditions and zolpidem conditions were calculated for each single experiment, as well as the differences between XBD173 conditions and XBD173 plus zolpidem conditions respectively. These differences were then compared. This is a combination of paired and unpaired samples.

	Effect of Zolpidem without XBD173			**Effect of Zolpidem with XBD173**			**Change**
	Median	$CI95_{high}$	$CI95_{low}$	Median	$CI95_{high}$	$CI95_{low}$	%
A_{fast} (pA)	4.98	8.25	-6.37	0.83	4.60	-3.76	-83.26
A_{slow} (pA)	6.07	8.15	1.89	6.37	7.55	3.56	4.93
A_{total} (pA)	9.54	17.24	3.73	7.23	10.10	0.54	-24.18
$A_{fast/slow}$	-1.99	-0.97	-4.18	-1.14	-0.93	-1.46	-42.59
τ_{fast} (ms)	2.56	3.27	1.81	1.69	2.32	0.78	-33.77
τ_{slow} (ms)	5.51	6.65	2.16	6.74	7.78	2.72	22.30
$\tau_{weighted}$ (ms)	5.02	6.44	2.55	4.85	6.10	3.09	-3.48
$\tau_{fast/slow}$	0.04	0.06	0.02	0.00	0.02	-0.01	-90.63
width (ms)	2.67	3.32	1.79	2.42	2.87	1.73	-9.49
ct_{fast} (pC)	0.10	0.17	0.06	0.05	0.07	0.04	-49.97
ct_{slow} (pC)	0.24	0.29	0.11	0.24	0.32	0.19	-0.59
ct_{total} (pC)	0.41	0.46	0.23	0.33	0.41	0.22	-17.71
$ct_{fast/slow}$	0.39	0.73	0.26	0.17	0.22	0.10	-56.44
frq (Hz)	1.09	1.42	-0.12	0.75	2.07	-0.03	-31.38

Table 5: **Medians and 95% confidence intervals for all recorded differences between sham conditions and zolpidem conditions as well as between XBD173 conditions and XBD173 plus zolpidem conditions respectively.** The differences of the effects of zolpidem in absence and in presence of XBD173 are presented in percent of the value in absence of XBD173.

Zolpidem has different effects on the decay times in absence and in presence of XBD173. The effect of zolpidem on the decay time of the fast component was significantly reduced in presence of XBD173, which is also represented by a significantly smaller effect on the decay time ratio between the fast and slow component.

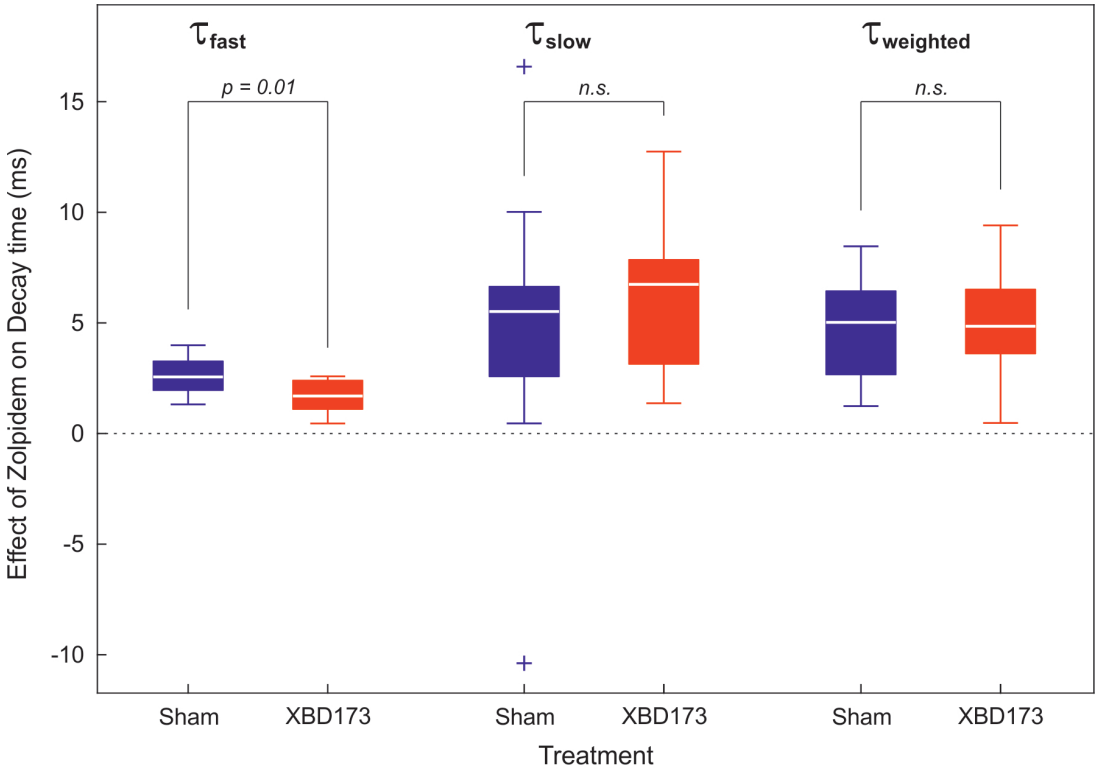
Likewise, the effect on the charge transfer during the fast component was significantly reduced in presence of XBD173, which is also represented by a significantly smaller effect on the charge transfer ratio between the fast and slow component.

The changes of the effects of zolpidem on mIPSC amplitude and frequency did not reach statistical significance.

	n	τ_{fast} (ms)	τ_{slow} (ms)	$\tau_{weighted}$ (ms)	$\tau_{fast/slow}$
Effect of Zolpidem without XBD173	14	2.56 (3.27; 1.81)	5.51 (6.65; 2.16)	5.02 (6.44; 2.55)	0.04 (0.06; 0.02)
Effect of Zolpidem with XBD173	12	1.69 (2.32; 0.78)	6.74 (7.78; 2.72)	4.85 (6.10; 3.09)	0.00 (0.02; -0.01)
p-value		0.01	0.5	1.0	0.004

	n	ct_{fast} (pC)	ct_{slow} (pC)	ct_{total} (pC)	$ct_{fast/slow}$
Effect of Zolpidem without XBD173	14	0.10 (0.17; 0.06)	0.24 (0.29; 0.11)	0.41 (0.46; 0.23)	0.39 (0.73; 0.26)
Effect of Zolpidem with XBD173	12	0.05 (0.07; 0.04)	0.24 (0.32; 0.19)	0.33 (0.41; 0.22)	0.17 (0.22; 0.10)
p-value		0.01	0.5	0.5	0.02

Table 6: **Differences between the effects of zolpidem on the decay time and charge transfer in absence and in presence of XBD173.** The table displays median values obtained by comparing the differences between drug-free conditions and zolpidem conditions to the differences between XBD173 conditions and XBD173 plus zolpidem conditions. p-values were derived via a Mann-Whitney U test.


τfast
τslow
τweighted
p = 0.01
n.s.
n.s.
15
10
5
0
-5
-10
Effect of Zolpidem on Decay time (ms)
Sham
XBD173
Sham
XBD173
Sham
XBD173
Treatment

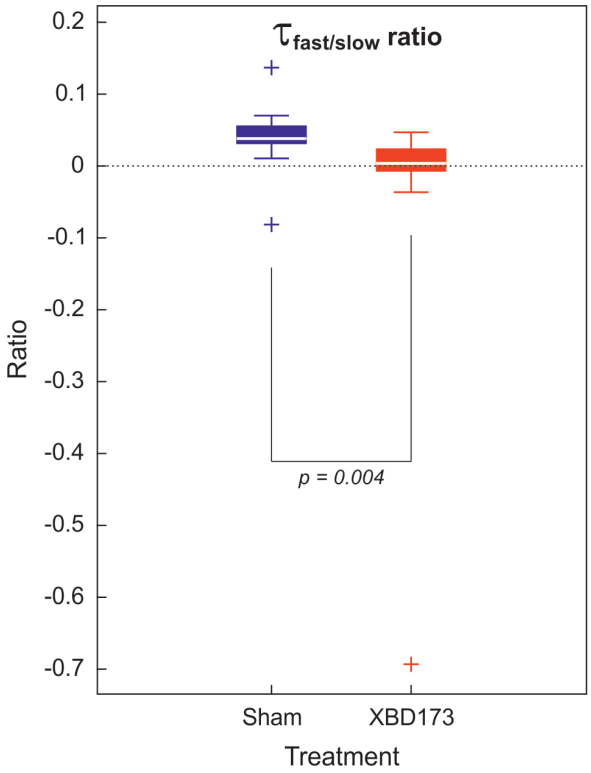
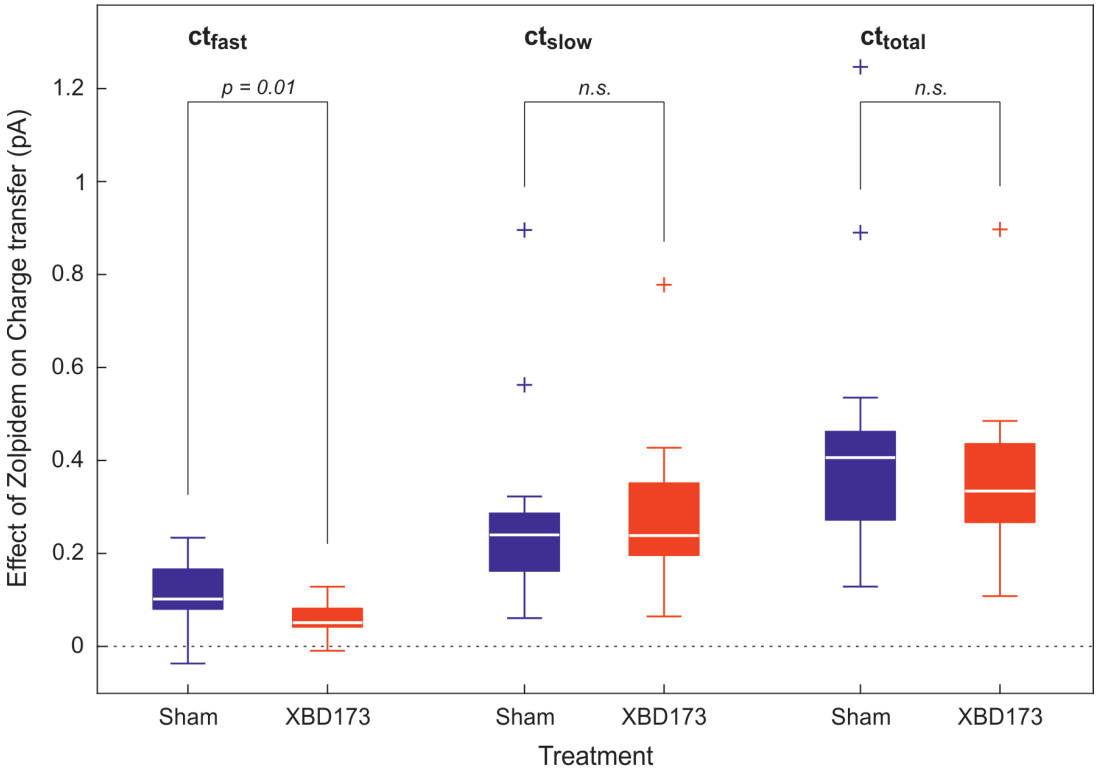

τfast/slow ratio
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
Ratio
p = 0.004
Sham
XBD173
Treatment

Figure 18: **Effects of zolpidem on the decay time of fitted mIPSCs in absence and in presence of XBD173.** In the presence of XBD173, the effect of zolpidem on the decay time of the fast component is reduced by $\sim$ 34%, which also leads to a smaller effect on the $\tau_{fast/slow}$ ratio. This finding is statistically significant. No statistically significant change of the effect on τ_{slow} and $\tau_{weighted}$ could be observed.


ctfast
ctslow
cttotal
p = 0.01
n.s.
n.s.
Effect of Zolpidem on Charge transfer (pA)
1.2
1
0.8
0.6
0.4
0.2
0
Sham
XBD173
Sham
XBD173
Sham
XBD173
Treatment

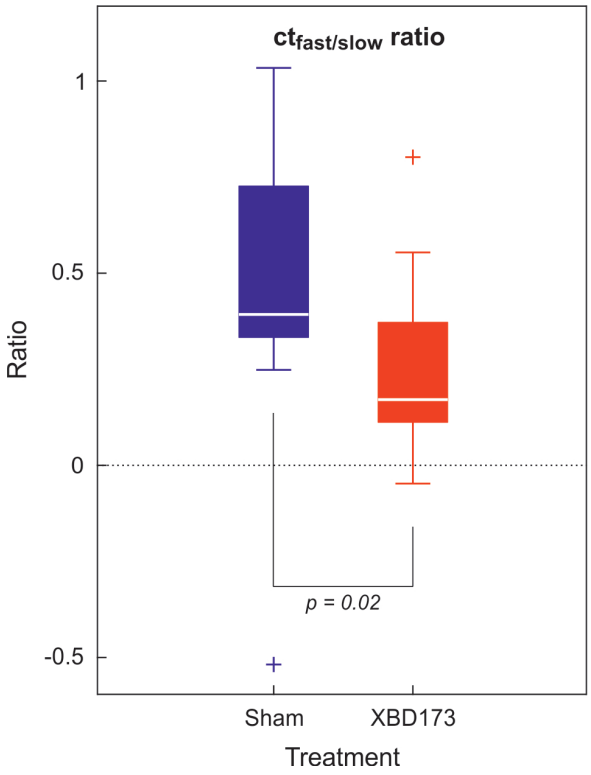

ctfast/slow ratio
Ratio
1
0.5
0
-0.5
p = 0.02
Sham
XBD173
Treatment

Figure 19: **Effects of zolpidem on the charge transfer of fitted mIPSCs in absence and in presence of XBD173.** In the presence of XBD173, the effect of zolpidem on the charge transfer during the fast component is reduced by $\sim$ 50%, which also leads to a smaller effect on the $ct_{fast/slow}$ ratio. This finding is statistically significant. No statistically significant change of the effect on ct_{slow} and ct_{total} could be observed. This is in line with the observed effects on the decay time.

3.1.4.1 Summary

In the presence of XBD173, the effects of zolpidem on the decay time and the charge transfer of the fast component were reduced significantly, indicating that XBD173 impedes the action of zolpidem on synaptic $GABA_A$ receptors.

3.1.5 Effect of Zolpidem on Tonic Currents under Sham and XBD173 Conditions

To assess the effect of zolpidem on tonic currents in organotypic slice cultures in the absence and in the presence of XBD173, bicuculline was applied and the baseline shift was recorded. This was done under sham plus zolpidem conditions as well as under XBD173 plus zolpidem conditions. The median baseline shift for each condition was then calculated.

Neither in the sham group nor in the XBD173 group could a significant baseline shift be observed after the application of bicuculline. This indicates that zolpidem has no notable effect on tonic currents. While there appears to be a tendency for the baseline shift to be enhanced in the presence of XBD173 (the median baseline shift was -7.01 pA (CI95: -4.90 pA / -21.28 pA) under sham plus zolpidem conditions, and -9.42 pA (CI95: -3.21 pA / -24.94 pA) under XBD713 plus zolpidem conditions), this effect failed to reach statistical significance.

3.1.6 Summary

The patch clamp experiments were able to show that both XBD173 and zolpidem significantly modify GABAergic synaptic transmission. This becomes apparent by changes in mIPSC kinetics that occur after the application of either substance. Zolpidem significantly increased the decay time and charge transfer, while also increasing the amplitude of the slow component.

XBD173 significantly increased the weighted decay time constant and shifted the amplitude ratio and the charge transfer ratio both more towards the slow component.
The effect of zolpidem on the decay time and the charge transfer during the fast component was significantly reduced in the presence of XBD173.

In summary, XBD173 and zolpidem alter mIPSC kinetics in different ways, suggesting different mechanisms of action. The effect of zolpidem is less pronounced in the presence of XBD173.

3.2 Extracellular Recordings

Patch clamp experiments as described above demonstrate the effects of XBD173 and zolpidem on GABAergic synaptic transmission only. In order to examine the effects of both drugs on a network level, extracellular recordings were done. These multi-unit recordings register neuronal population activity of synaptically interacting neurons.
A total of 183 neocortical slice cultures were used for extracellular recordings, 87 of which were treated with XBD173 and 96 of which were treated with a sham application.

3.2.1 Neuronal Activity under Control Conditions

In these experiments, spontaneous neuronal activity was recorded. The raw signal displays an alternation of active phases, so-called up states or bursts, that result from synchronous sodium-dependent, fast action potential activity of multiple neuronal cells, and silent phases (down states), during which no synchronous action potential activity is observed.
Local action potentials are rapid changes in the potential of a single cell. Beyond that, a slow change in potential can be observed during each burst. This so-called field potential is the result of the synchronous synaptic activity in the entire recorded cell unit.

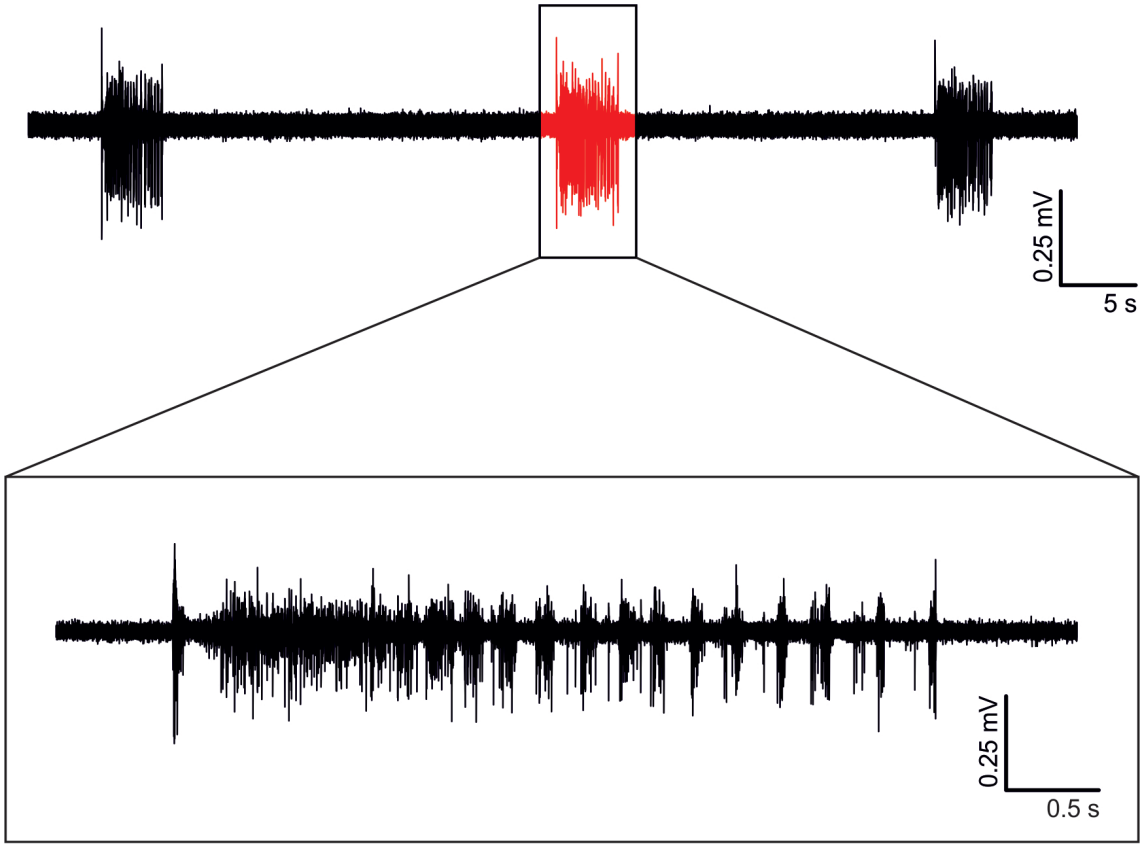


Figure 20: **A sample recording of spontaneous neuronal activity.** The raw signal was high-pass filtered below 300 Hz, allowing for the discrimination of single action potentials. Phases of high action potential activity (bursts) alternate with silent phases. Under control conditions, these bursts may vary greatly in length, action potential rate, and frequency, making for a heterogeneous pattern of activity. Action potential activity is particularly high at the beginning of the burst. This high activity then declines rapidly into a constant lower activity over a longer period of the burst.

3.2.2 Effect of XBD173

In order to assess the effect of XBD173 on cortical neuronal activity, control recordings from cortical slice cultures were done under drug-free conditions. Then, one group was treated with XBD173 for 30 minutes (XBD173 group), whereas the other group was treated with the solvent alone (sham group).

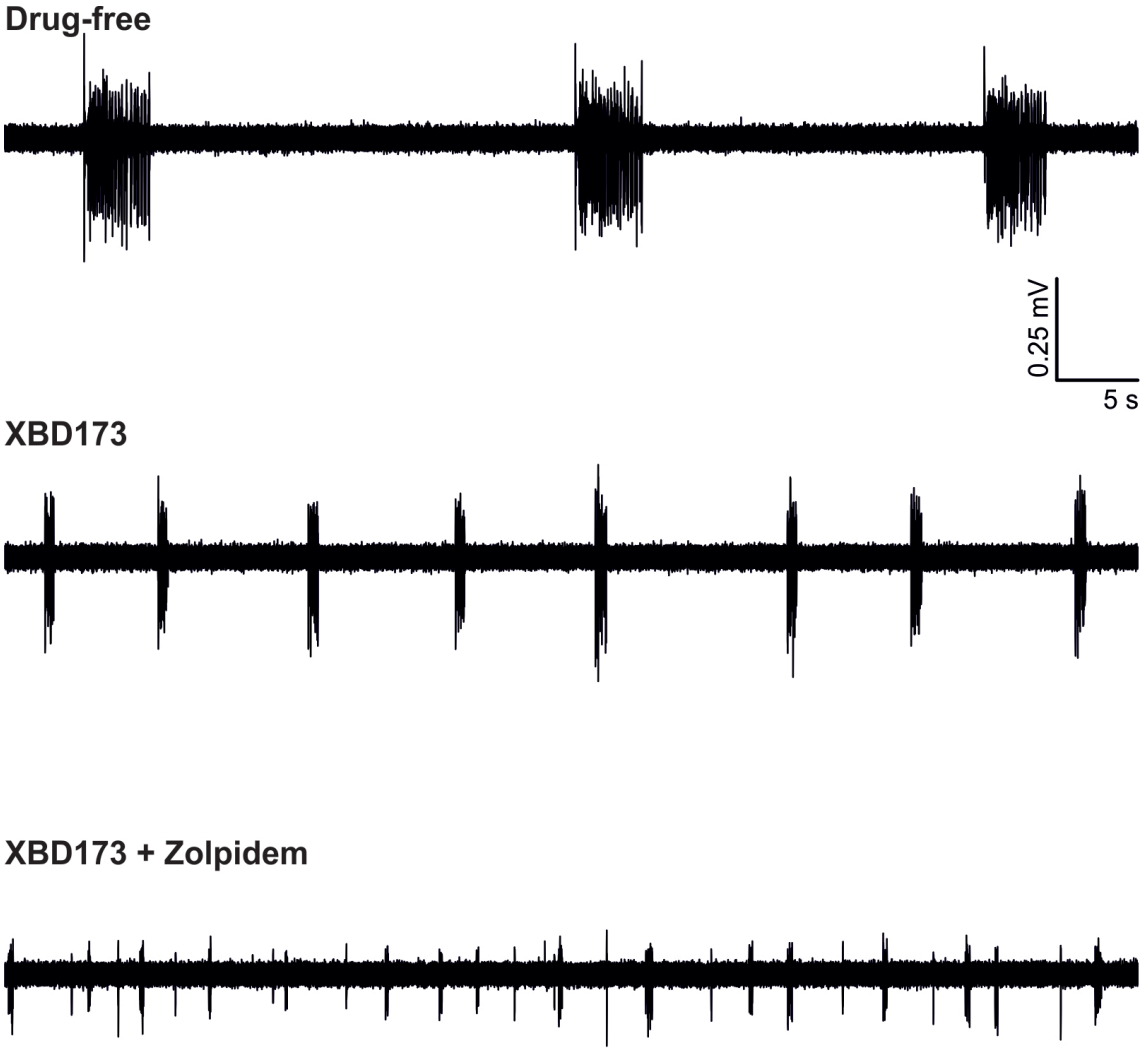


Figure 21: **A sample recording under drug-free conditions, as well as XBD173 conditions and XBD173 plus zolpidem conditions.** In general, a reduction of burst length and an increase in burst frequency could be observed after the application of both XBD173 and zolpidem.

To further analyze burst patterns, for each experiment, every single burst was split into bins of 5 ms. Median discharge rates were then calculated for each number of bin, resulting in the curve displayed below.

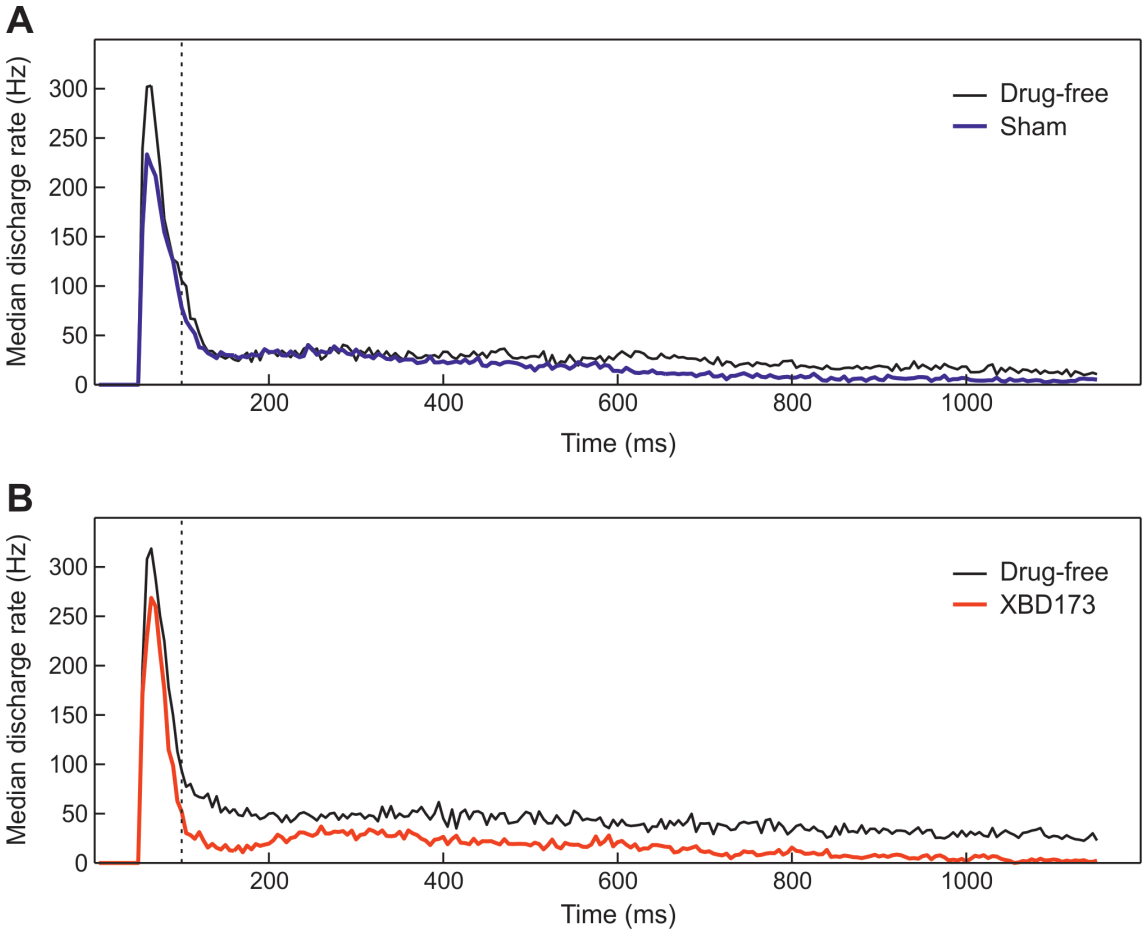


Figure 22: **Effect of XBD173 on the median discharge rates. A** displays the median discharge rates of all bursts under drug-free conditions (black curve) and under solvent only (sham) conditions (blue curve) over the course of 1200 ms. **B** displays the median discharge rates of all bursts under drug-free conditions (black curve) and under XBD173 conditions (red curve).

Data analysis showed that two phases of the burst could clearly be distinguished – an early phase characterized by very high firing rates that rapidly drop down to a lower level (referred to as “early phase” below), and a second phase characterized by constant, lower firing rates over a longer period of time (referred to as “late phase” below). For the analysis of the results, it proved sensible to examine the early and the late phase separately. Therefore, the border between the early and the late phase was drawn at 50 ms after the beginning of the burst.

To assess the effect of XBD173 on neuronal network activity, the median firing rates of all recordings under XBD173 conditions and of all recordings under sham conditions were compared to their respective drug-free control recordings. As visualized above, both the XBD173 curve (red) and the sham curve (blue) lie

below their respective control curves (black), indicating a decrease in activity in both the XBD173 group and the sham group.
This shall be examined more closely by inspecting the early phase and the late phase separately.

3.2.2.1 Effect of XBD173 during the Early Phase

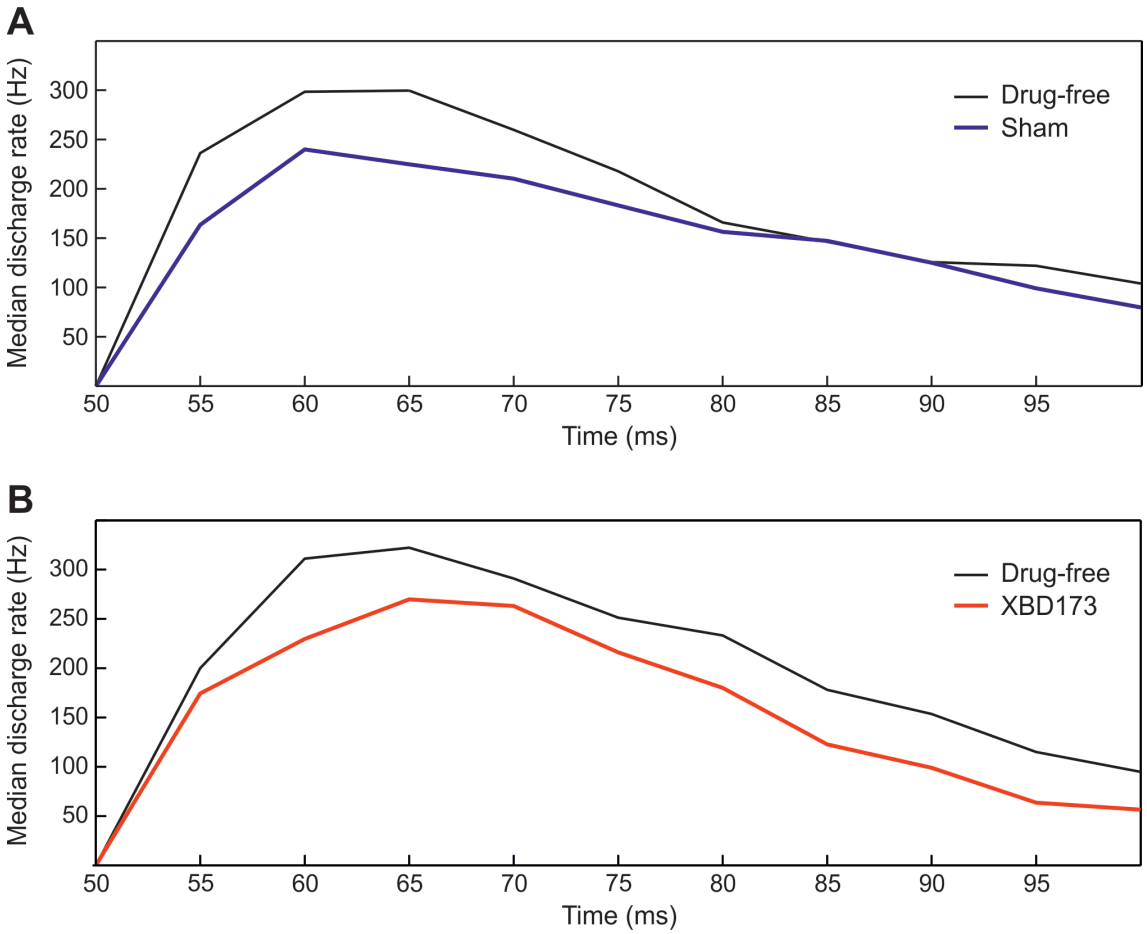


Figure 23: **Effect of XBD173 on the median discharge rates during the early phase.** This figure shows an excerpt from figure 22. **A** displays the median discharge rates of all bursts under drug-free conditions (black curve) and under sham conditions (blue curve) over the course of the first 50 ms of the burst. **B** displays the median discharge rates of all bursts under drug-free conditions (black curve) and under XBD173 conditions (red curve). A decrease in median discharge rates took place both in the XBD173 group and in the sham group.

In order to further assess the effect size in the XBD173 group and the sham group, normalized activities were calculated for both groups. The normalized activity was obtained by dividing the activity during the second recording (XBD173 condition, respectively sham condition) by the activity during the first recording (drug-free condition), in which the activity was defined as the average number of spikes of all bursts of the recording during a certain period of time (in this case during the first 50 ms of each burst). I.e. the closer to zero the value of the normalized activity gets, the greater the reduction in activity that has taken place

in between recordings.
Thus, a value for the normalized activity was obtained for each single experiment. Ranking those by their magnitude results in the cumulative frequency, indicating the relative amount of values that are smaller than a certain value.

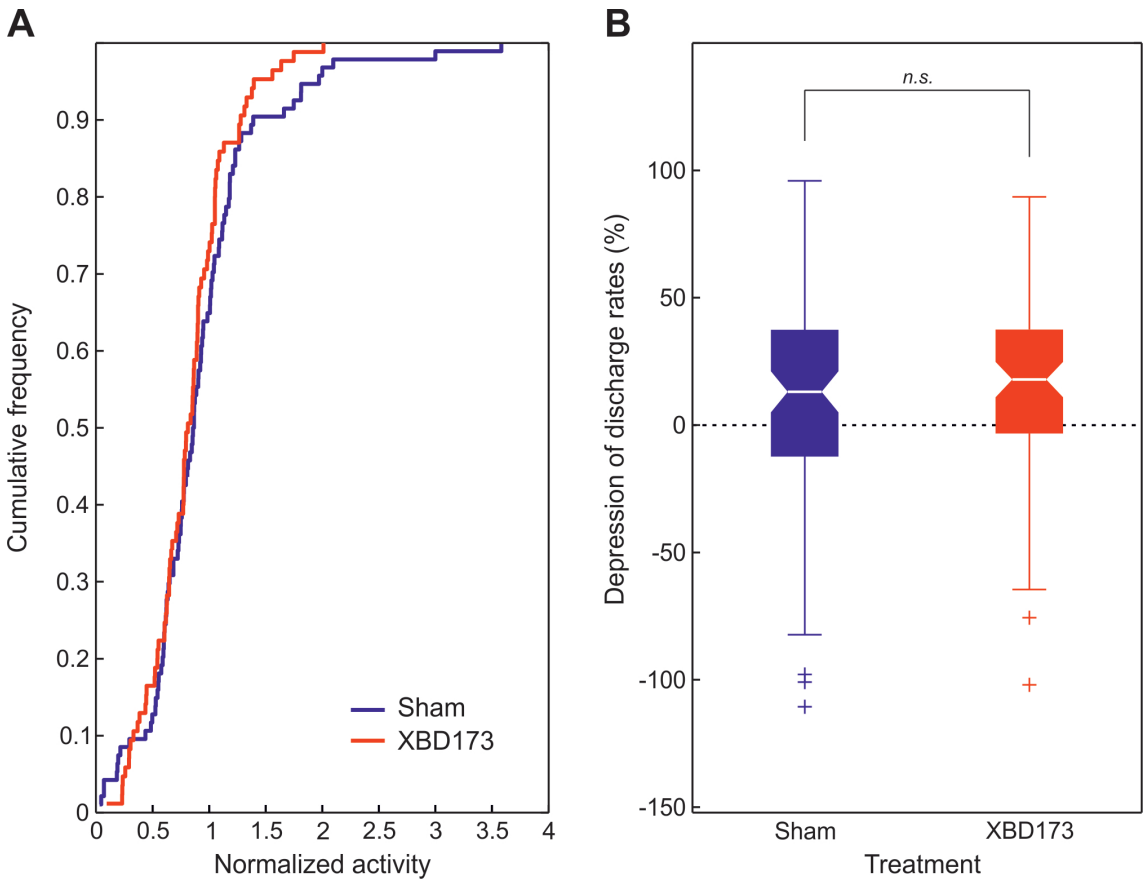


Figure 24: **Effect of XBD173 on the median discharge rates during the early phase.** In **A**, the cumulative frequency is plotted against the normalized activity. This allows for a direct visual comparison of the effects in both groups. The curves for XBD173 (red) and sham treatment (blue) are almost identical and overlap especially around the median value, indicating no significant difference between both groups. **B** displays the percentage depression of discharge rates after 30 minutes of sham treatment and 30 minutes of XBD173 treatment. The median depression of discharge rates is around 20% for both the XBD173 group and the sham group. p-values were derived via a Mann-Whitney U test, confirming that no statistically significant effect of XBD173 could be detected during the early phase of the burst. The null hypothesis, that in sham- and XBD173-treated slice cultures the depression of discharge rates during the early phase is undistinguishable, could not be rejected.

During the early phase, no significant difference between the XBD173 group and the sham group could be observed. Both displayed a decrease in activity of about 20%, indicating a rundown that existed in both groups similarly. Plotting the cumulative frequency against the normalized activity after application of XBD173 or solvent respectively reveals almost identical curves for both groups, with only a few outliers deviating in the boundary areas.
The Mann-Whitney U test delivered a p-value of 0.4, rendering a significant disparity between the two tested groups highly unlikely.

3.2.2.2 Effect of XBD173 during the Late Phase

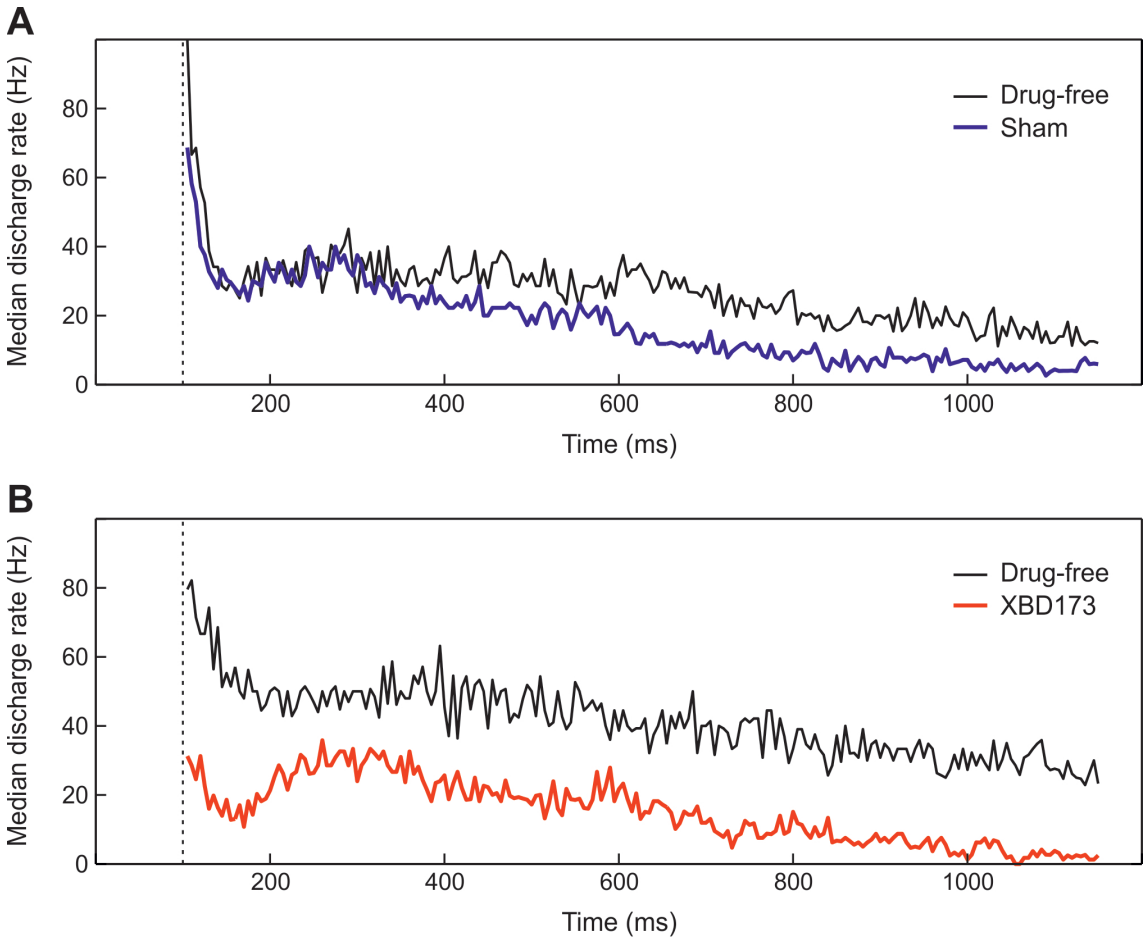


Figure 25: **Effect of XBD173 on the median discharge rates during the late phase.** This figure shows an excerpt from figure 22. **A** displays the median discharge rates of all bursts under drug-free conditions (black curve) and under sham conditions (blue curve) from 50 ms to 1200 ms of the burst. **B** displays the median discharge rates of all bursts under drug-free conditions (black curve) and under XBD173 conditions (red curve).

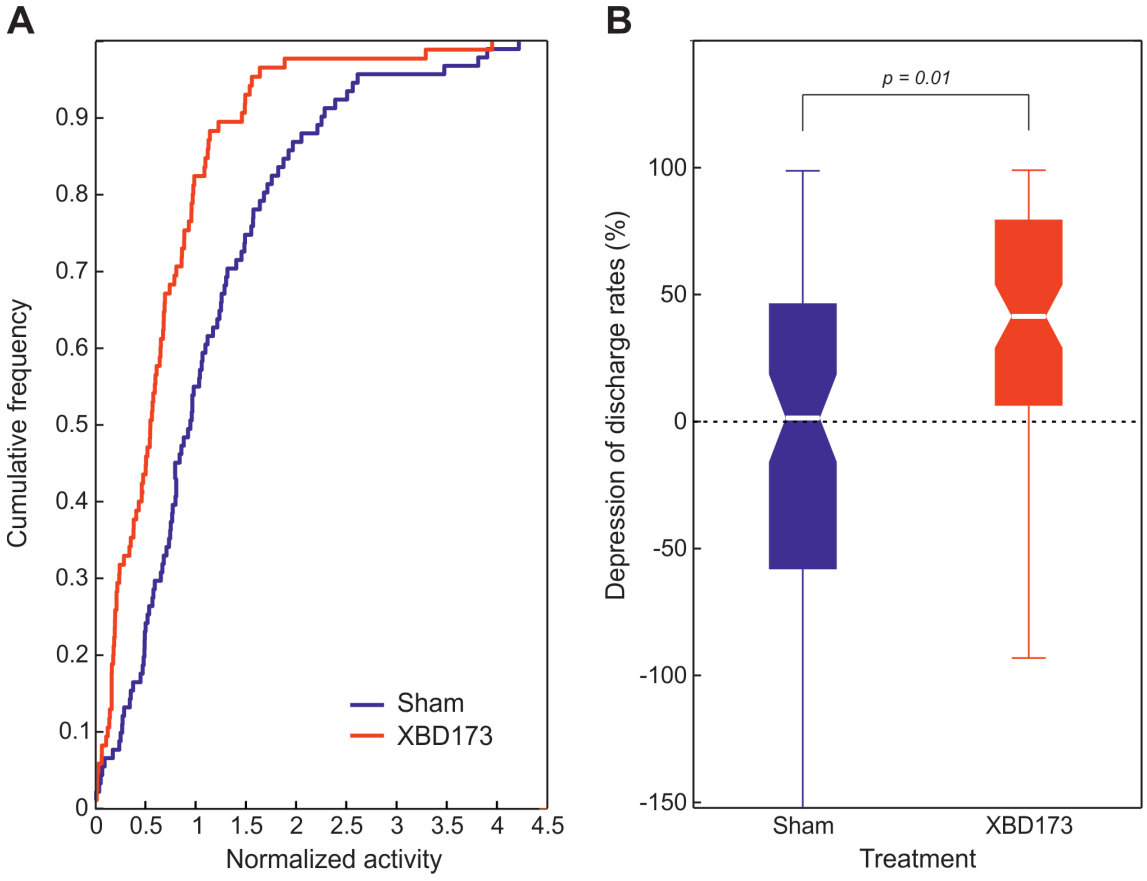


Figure 26: **Effect of XBD173 on the median discharge rates during the late phase.** In **A**, the cumulative frequency is plotted against the normalized activity as described above, but taking into calculation only the activity between 50 ms and 1200 ms of the burst. The curves for XBD173 (red) and sham treatment (blue) clearly diverge, indicating a significant difference between both groups. **B** displays the percentage depression of discharge rates after 30 minutes of sham treatment and 30 minutes of XBD173 treatment. The median depression of discharge rates is close to 0% for the sham group and around 40% for the XBD173 group. The null hypothesis, that in sham- and XBD173-treated slice cultures the depression of discharge rates during the late phase is undistinguishable, could be rejected at the 5% significance level.

During the late phase, a significant difference between the depression of discharge rates in the sham group and in the XBD173 group could be observed. The normalized activity in the XBD173 group takes smaller values, which is represented by a shift of the XBD173 curve to the left compared to the sham curve. This indicates that the inhibition that took place in the XBD173 group is significantly more pronounced than that in the sham group. While the median depression of discharge rates was close to 0% in the sham group, it was around 40% in the XBD173 group.

The Mann-Whitney U test delivered a p-value of 0.01, confirming the statistical

significance of the results.
In conclusion, XBD173 reduced neuronal activity during the late phase.

3.2.3 Effect of Zolpidem in Presence and in Absence of XBD173

In order to assess the effect of zolpidem in presence and in absence of XBD173, an additional recording was done after adding zolpidem both in the XBD173 group and the sham group. These recordings were then compared to the recordings under XBD173 only conditions or under sham conditions respectively. Median discharge rates were calculated in the same way as described above.

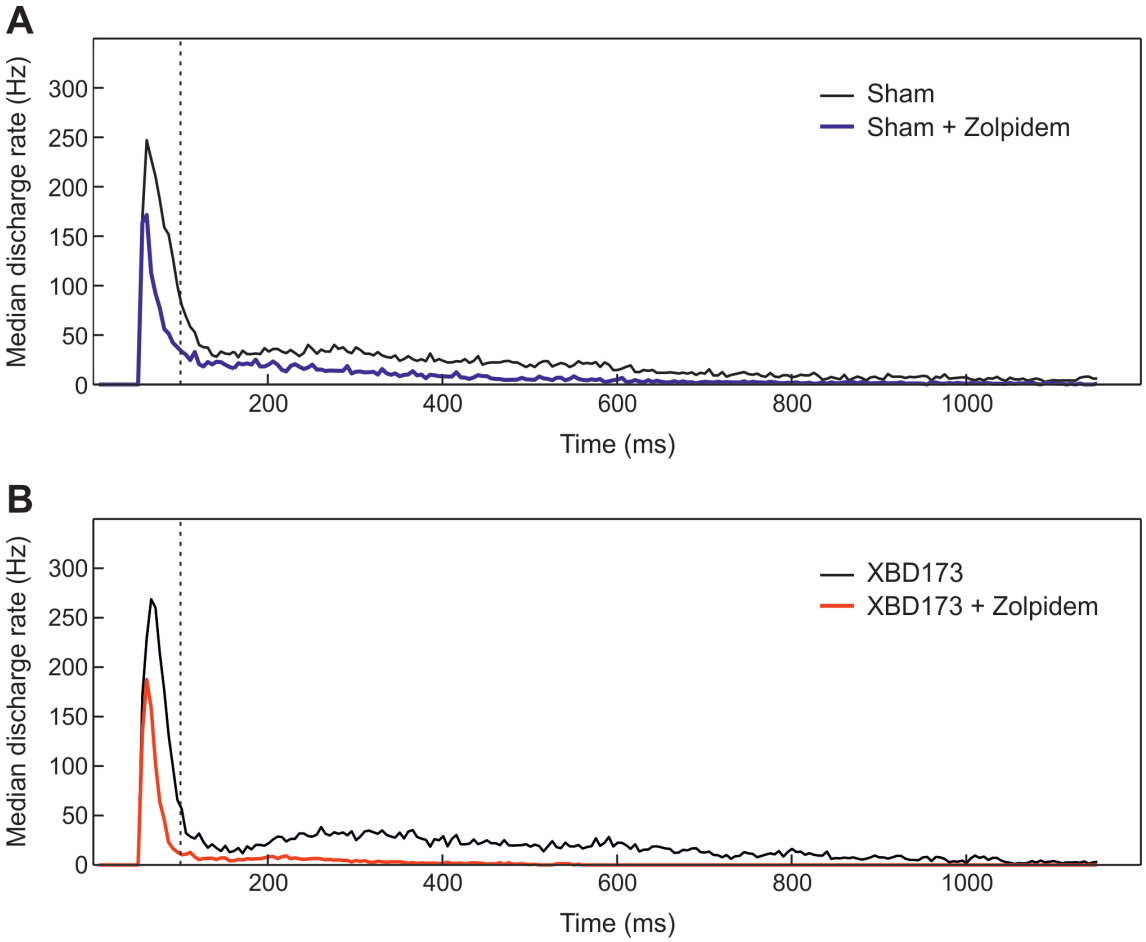


Figure 27: **Effect of zolpidem on the median discharge rates in presence and in absence of XBD173. A** displays the median discharge rates of all bursts under solvent only conditions (black curve) and under solvent plus zolpidem conditions (blue curve) over the course of 1200 ms. **B** displays the median discharge rates of all bursts under XBD173 conditions (black curve) and under XBD173 plus zolpidem conditions (red curve).
Note that for comparability, the third recording (solvent plus zolpidem, respectively XBD173 plus zolpidem; blue and red curve) is compared to the second recording (solvent only, respectively XBD173; these are identical to the recordings represented by the blue and red curve in figure 22 and are now represented by the black curves). This ensures that only the effect of zolpidem is being analyzed and the prior decrease in discharge rates induced by XBD173 is not taken into account.

As visualized above, both the XBD173 + zolpidem curve (red) and the sham + zolpidem curve (blue) lie under their respective control curves (black), indicating a clear decrease in discharge rates for both groups after the administration of zolpidem. This effect appears to be significantly more pronounced than the effect that could be observed after the administration of XBD173 or solvent alone and shall be examined more closely by inspecting the early phase and the late phase separately.

3.2.3.1 Interactions between XBD173 and Zolpidem during the Early Phase

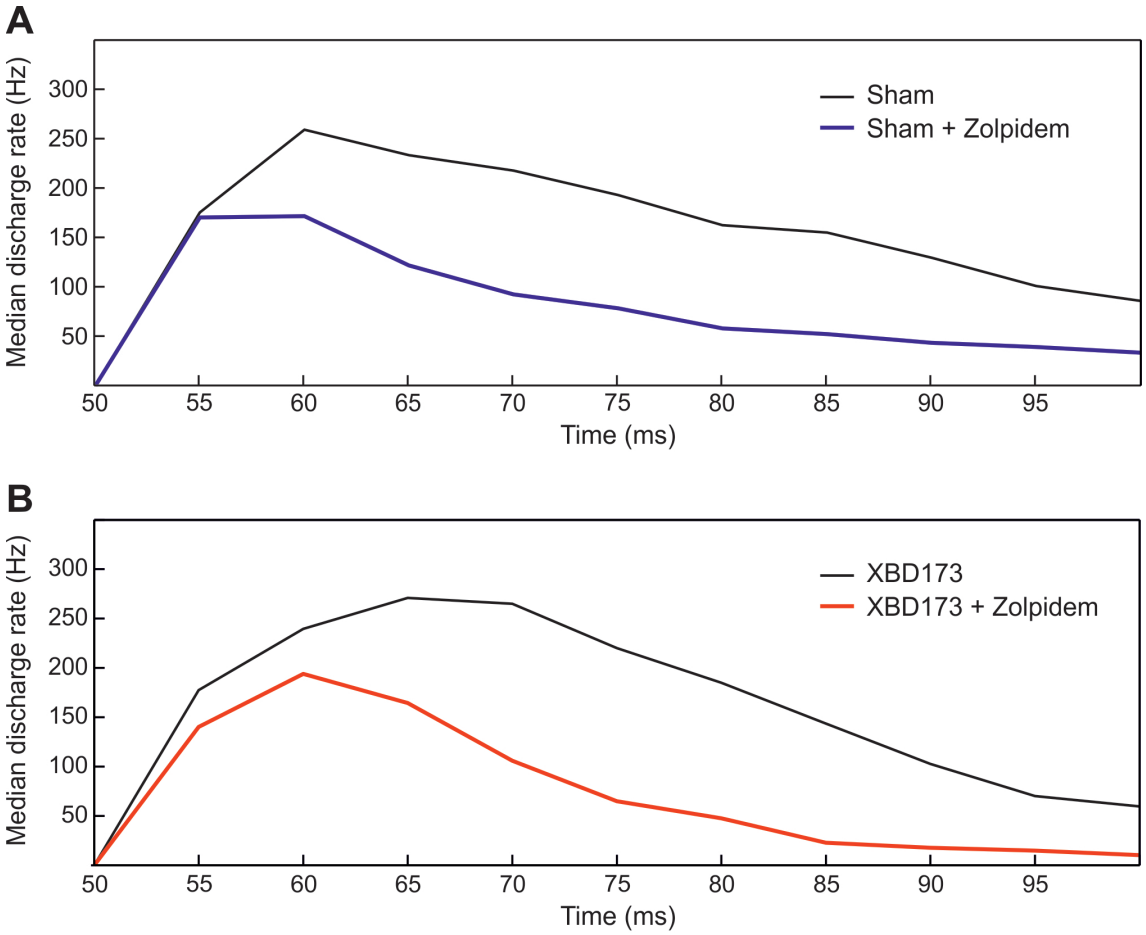


Figure 28: **Effect of zolpidem on the median discharge rates during the early phase in presence and in absence of XBD173.** This figure shows an excerpt from figure 27. **A** displays the median discharge rates of all bursts under solvent only conditions (black curve) and under solvent plus zolpidem conditions (blue curve) over the course of the first 50 ms of the burst. **B** displays the median discharge rates of all bursts under XBD173 conditions (black curve) and under XBD173 plus zolpidem conditions (red curve). A decrease in median discharge rates took place both in the XBD173 group and in the sham group.

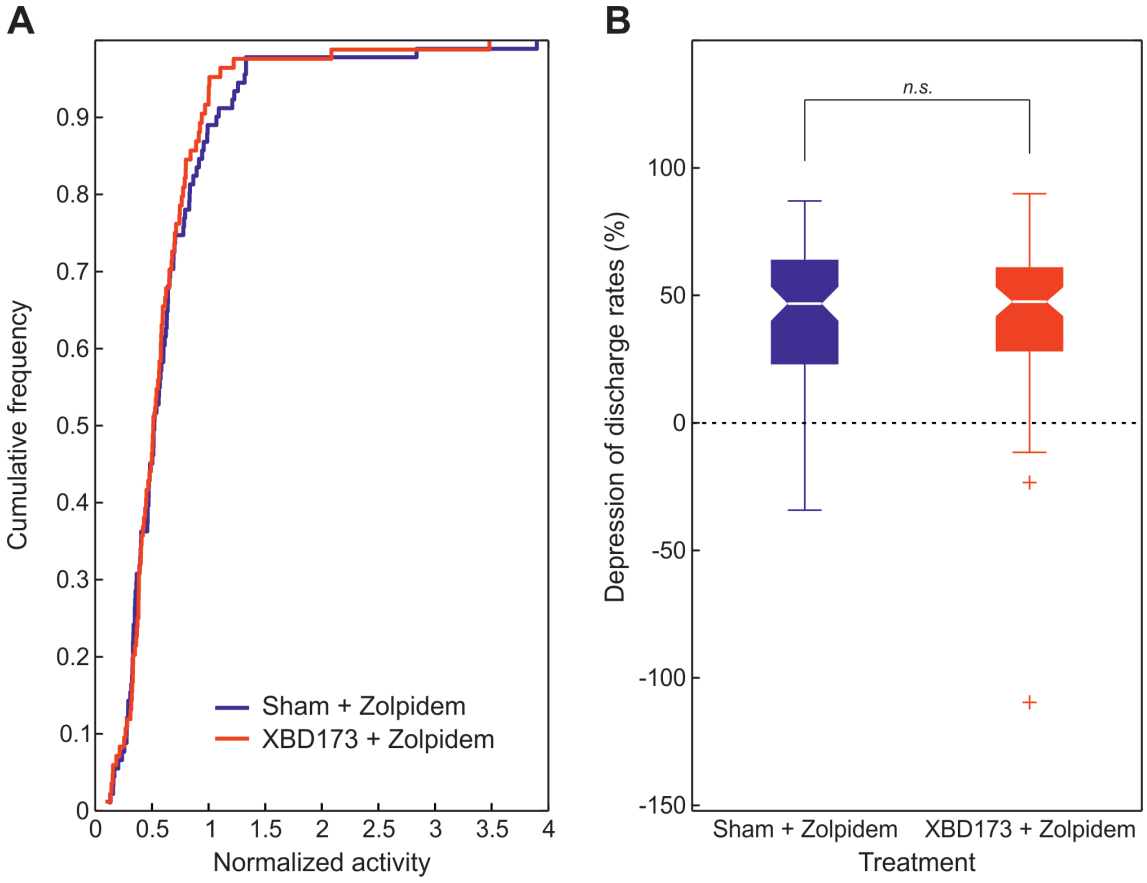


Figure 29: **Effect of zolpidem on the median discharge rates during the early phase in presence and in absence of XBD173.** In **A**, the cumulative frequency is plotted against the normalized activity, taking into calculation only the activity during the first 50 ms of the burst. The curves for XBD173 plus zolpidem (red) and solvent plus zolpidem treatment (blue) show no significant difference. **B** displays the percentage depression of discharge rates after treatment with solvent plus zolpidem and treatment with XBD173 plus zolpidem. The median depression of discharge rates is around 50% for both the XBD173 group and the sham group. p-values were derived via a Mann-Whitney U test, confirming that no statistically significant difference between the effect of zolpidem in presence and in absence of XBD173 could be detected during the early phase of the burst. The null hypothesis, that in sham- and XBD173-treated slice cultures the depression of discharge rates induced by zolpidem during the early phase is undistinguishable, could not be rejected.

In the early phase, no significant difference between the XBD173 group and the sham group could be observed. Both display a decrease in activity of about 50%, which is due to the effect of zolpidem and due to a general rundown effect. Plotting the cumulative frequency against the normalized activity after application of zolpidem both in the sham group and the XBD173 group reveals almost identical curves for both groups, with only a few outliers deviating in the boundary areas.

The Mann-Whitney U test delivered a p-value of 0.8, rendering a significant disparity between the two tested groups highly unlikely.
In conclusion, XBD173 did not significantly affect the actions of zolpidem during the early phase.

3.2.3.2 Interactions between XBD173 and Zolpidem during the Late Phase

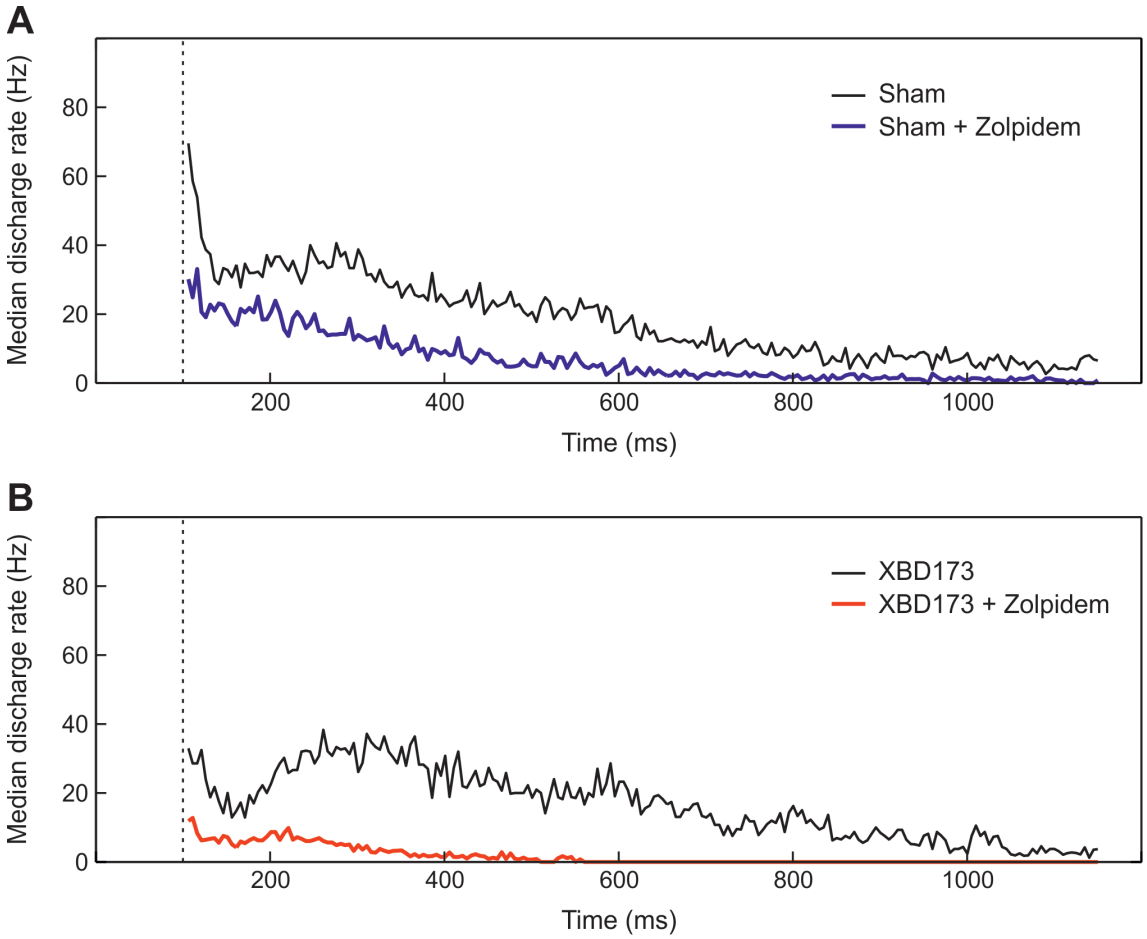


Figure 30: **Effect of zolpidem on the median discharge rates during the late phase in presence and in absence of XBD173.** This figure shows an excerpt from figure 27. **A** displays the median discharge rates of all bursts under solvent only conditions (black curve) and under solvent plus zolpidem conditions (blue curve) from 50 ms to 1200 ms of the burst. **B** displays the median discharge rates of all bursts under XBD173 conditions (black curve) and under XBD173 plus zolpidem conditions (red curve). A decrease in median discharge rates took place both in the XBD173 group and in the sham group.

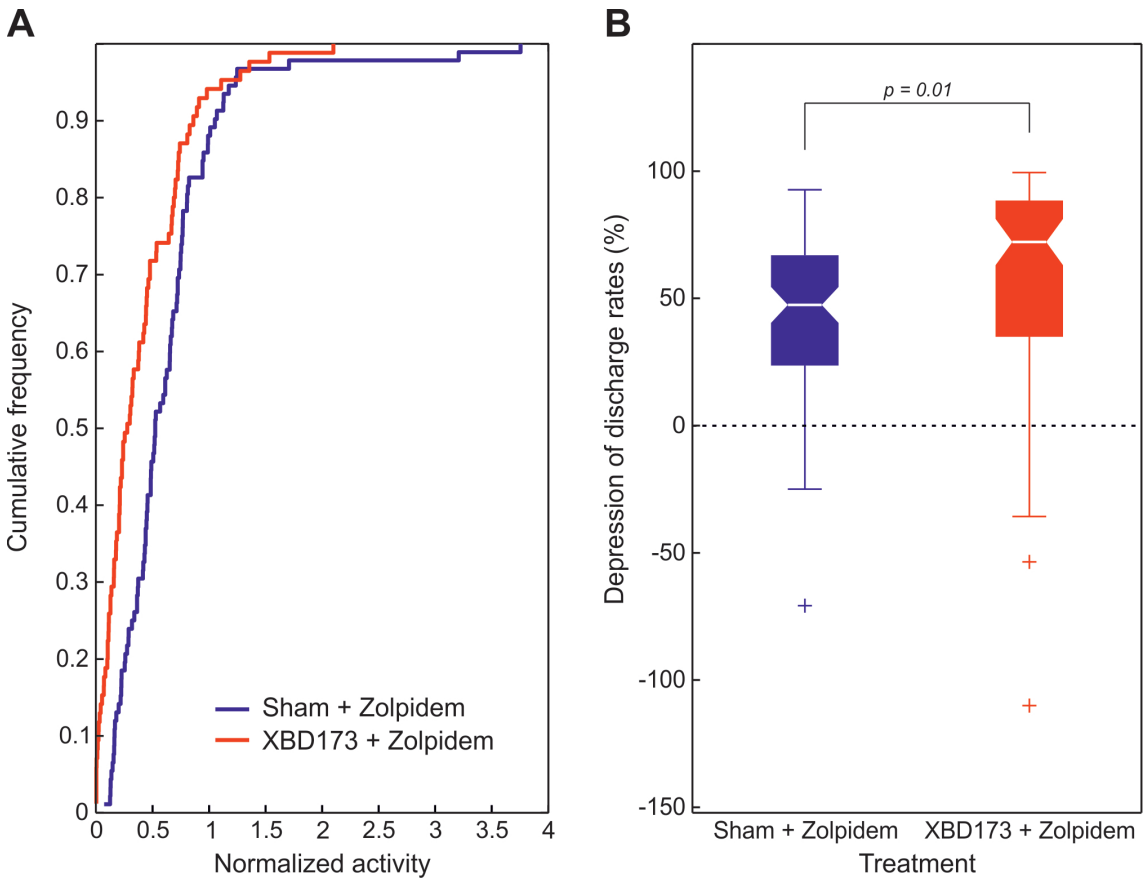


Figure 31: **Effect of zolpidem on the median discharge rates during the late phase in presence and in absence of XBD173.** In **A**, the cumulative frequency is plotted against the normalized activity as described above, but taking into calculation only the activity between 50 ms and 1200 ms of the burst. The curves for XBD173 plus zolpidem (red) and solvent plus zolpidem treatment (blue) clearly diverge, indicating a significant difference between both groups. **B** displays the percentage depression of discharge rates after treatment with solvent plus zolpidem and treatment with XBD173 plus zolpidem. The median depression of discharge rates is around 50% for the sham group and around 75% for the XBD173 group. The null hypothesis, that in sham- and XBD173-treated slice cultures the depression of discharge rates induced by zolpidem during the late phase is undistinguishable, could be rejected at the 5% significance level.

During the late phase, a significant difference between the depression of discharge rates induced by zolpidem in the sham group and in the XBD173 group could be observed. The normalized activity in the XBD173 group takes smaller values, which is represented by a shift of the XBD173 curve to the left compared to the sham curve. This indicates that the effect of zolpidem is significantly enhanced in the presence of XBD173 compared to the effect of zolpidem in the absence of XBD173. While the median depression of discharge rates was around 50% in the sham group, which matches the effect of zolpidem during the early phase, it was around 75% in the XBD173 group.

The Mann-Whitney U test delivered a p-value of 0.01, confirming the statistical significance of the results.
In conclusion, XBD173 increased the effect of zolpidem during the late phase.

3.2.4 Summary

The extracellular experiments were able to show that both XBD173 and zolpidem significantly alter neuronal discharge patterns in organotypic neocortical slice cultures.
Treatment with XBD173 significantly reduced action potential activity in those slice cultures. This inhibitory action was evident during episodes of ongoing activity, so-called bursts. XBD173 was shown to be effective during late phases, but not during early phases (first 50 ms) of sustained action potential firing.
Zolpidem decreased action potential firing during early and late phases of sustained neuronal activity. The effect of zolpidem was modulated by XBD173 only during late phases, but not during early phases of sustained action potential firing.
In conclusion, XBD173 and zolpidem alter neuronal discharge patterns in distinct ways, suggesting different mechanisms of action. The data obtained from the experiments suggests a synergistic effect of XBD173 and zolpidem during the late phases of sustained action potential firing.

4 Discussion

4.1 Background for this Study

This study's aim was to examine the mechanisms of action of neurosteroids, as well as their interactions with other GABAergic drugs. In order to do so, it focused on two distinct substances – XBD173, a TSPO ligand which supposedly enhances the endogenous production of neurosteroids, and zolpidem, a sedative drug that primarily targets α1 subunit-containing $GABA_A$ receptors. Despite targeting $GABA_A$ receptors either directly or indirectly, both substances display very different behavioral effects. While there is evidence that α1-containing $GABA_A$ receptors play the main role in the modulation of sedative effects and zolpidem has been shown to target primarily said α1-containing $GABA_A$ receptors, thus promoting a strong sedative effect, XBD173 lacks sedative properties as well as the majority of benzodiazepine-typical side-effects that are supposedly mediated via α1-containing $GABA_A$ receptors. This suggests that, while neurosteroids promoted by XBD173 are effective $GABA_A$ receptor modulators, α1-containing $GABA_A$ receptors are not their primary target. Therefore, XBD173 and zolpidem are expected to display different action profiles, targeting different receptor subtypes, with the potential of possibly modulating each others actions.
In order to either confirm or reject this hypothesis, electrophysiological recordings of organotypic slice cultures from the neocortex of neonatal mice were done. The effects and interactions of zolpidem and XBD173 on synaptic GABAergic transmission were assessed in whole-cell patch clamp experiments. Furthermore, extracellular multi-unit recordings were done in order to assess the effect of zolpidem and XBD173 on neuronal network activity.

4.2 Summary of Results

This work showed that both XBD173 and zolpidem significantly reduce the activity of cortical neurons, and provided evidence for interactions between XBD173 and zolpidem on a synaptic as well as on a network level.
The patch clamp experiments demonstrated that both zolpidem and XBD173 have significant effects on GABAergic synaptic transmission. While zolpidem alone produced a strong increase in decay time and charge transfer of mIPSCs, this effect was reduced significantly in the presence of XBD173.
The extracellular multi-unit recordings showed that both zolpidem and XBD173

have a significant effect on neuronal network activity in organotypic slice cultures, each modifying burst patterns in a distinct way. Zolpidem was able to reduce neuronal activity during the entire duration of the burst, while XBD173 was shown to reduce neuronal activity only during the late phase of the burst but not during the early phase. XBD173 was shown to enhance the effect of zolpidem during said late phase, which coincides with a synergistic effect of XBD173 and zolpidem.

4.3 Interpretation of Patch Clamp Results

4.3.1 Effect of Zolpidem on mIPSC Kinetics

Zolpidem was shown to significantly increase the overall decay time and charge transfer of mIPSCs. This indicates a prolonged state during which postsynaptic $GABA_A$ receptors are open, translating into an overall increase in GABAergic synaptic inhibition. Since zolpidem did not enhance the frequency of mIPSCs, it appears to have no effect on the probability of $GABA_A$ receptors opening, but rather only on the length of their open status. This finding is in line with other studies demonstrating that zolpidem increases IPSC decay time, while displaying no significant effect on the total amplitude and frequency (Jia et al. 2009, Chen et al. 2004).
A slight increase in amplitude after the administration of zolpidem could be observed only during the slow component of fitted mIPSCs. This could be a direct consequence of the prolonged decay time and thus slower decline of the current, or it could point towards general changes in the kinetics of synaptic GABAergic transmission induced by zolpidem.

4.3.2 Effect of XBD173 on mIPSC Kinetics

XBD173 was shown to modify mIPSC kinetics significantly. While not displaying an effect on the decay times of the fast and slow component as strong as that of zolpidem, it did increase the weighted decay time constant significantly.
To date, there are hardly any studies examining the effect of XBD173 on GABAergic transmission directly. Rupprecht et al. (2009) were able to demonstrate a significant increase in IPSC amplitude, decay time, and charge transfer in mouse neocortical slices at a concentration of 5 µM XBD173. This effect, however,

could not be reproduced to the same extent by the study at hand. While XBD173 was able to increase the weighted decay time constant and did show a strong tendency to alter the overall decay time as well (displaying a change of about 33% for the fast component and about 55% for the slow component), the latter effect did not reach statistical significance. While this might be due to a slightly smaller concentration of XBD173 used for these experiments, or due to a relatively small number of experiments – it is entirely possible that these parameters might reach statistical significance for a higher number of experiments, so an effect of XBD173 on the length of the open state of postsynaptic $GABA_A$ receptors can therefore not be ruled out just yet – so far it could solely be demonstrated that XBD173 alters mIPSC kinetics in a different way than zolpidem does and that its effect on the decay time is less pronounced. While having some significant effects on mIPSC kinetics, when compared to zolpidem, XBD173 does not display such a clear increase in GABAergic inhibition.

4.3.3 Interactions between Zolpidem and XBD173 in Patch Clamp Experiments

When applying zolpidem to neocortical slice cultures treated with XBD173, it could be observed that the effect of zolpidem on GABAergic synaptic transmission was reduced. Especially the effects on the decay time as well as the charge transfer of the fast component were significantly less pronounced in the presence of XBD173, thus shifting the ratio of both parameters more towards the slow component. It appears that, via some mechanism unknown so far, XBD173 impairs the effect of zolpidem on synaptic $GABA_A$ receptors. A possible explanation for this might be that XBD173 or neurosteroids are able to bind to α1-containing $GABA_A$ receptors as well, but making them less susceptible to the binding of zolpidem or GABA itself. While this does not support the thesis that XBD173 and zolpidem target $GABA_A$ receptors with different α subunits, it still offers a viable explanation for the distinct clinical effects of both substances.
It has to be noted, however, that synergisms between neurosteroids and anaesthetics on a synaptic level have been demonstrated in various studies: Drexler et al. (2016) were able to show that the neurosteroid allopregnanolone significantly enhances the decay time of IPSCs and that, when administered with propofol, both substances display a synergistic effect on IPSC decay time. Furthermore, it could be demonstrated that the neuroactive steroid alfaxalone significantly increases the effect of both propofol and diazepam on IPSC decay

time in rat hippocampal neurons (Cao et al. 2018). The discrepancy between those studies and the results of this work might be due to different mechanisms of action of the substances used or due to a concentration-dependent effect of neurosteroids. A third possible explanation lies within the heterogeneity of endogenously produced neurosteroids. While all studies so far examined the effects of a single substance – most commonly allopregnanolone or alfaxalone – there exists a much greater variety of different neurosteroids that are produced endogenously and thus may be promoted by XBD173. It could be demonstrated that allopregnanolone is one of the substances that are induced by XBD173 (Rupprecht et al. 2009), but most likely it is only one single substance in a much greater pool of different substances that may exert their effects on $GABA_A$ receptors. Many neurosteroids have been shown to have inhibitory effects (Belelli & Lambert 2005), but there are also neurosteroids, such as pregnenolone sulfate, that function as antagonists on $GABA_A$ receptors (Majewska et al. 1988, Seljeset et al. 2015), supposedly promoting an excitatory effect over an inhibitory one.
To date, it is unclear which substances exactly are promoted by XBD173 and it is well possible that many of them display very different mechanisms of action, or even contrary effects. For all we know, the observed effects of XBD173 might just be the result of both inhibitory and excitatory actions mediated by different neurosteroids. This may explain why the results of this study differ so significantly from the results of other studies researching the effects of single neuroactive steroids on synaptic GABAergic transmission.

4.3.4 Effect of Zolpidem on Tonic Currents

The effect of zolpidem on tonic currents was assessed by recording the baseline shift after the application of bicuculline. This was done both under XBD173 and under sham conditions. The magnitude of the baseline shift served as a marker for extrasynaptic transmission.
As it has been reported before (Jia et al. 2009), zolpidem did not significantly modify tonic conductance. XBD173 displayed a slight tendency to increase the baseline shift induced by bicuculline, but also failed to produce a significant effect. It has to be noted, however, that the isolated effect of XBD173 on tonic inhibition was not examined in these experiments, since the baseline shift was recorded after the application of zolpidem in both experimental groups. Therefore, it is unclear what the effect of XBD173 may look like in the absence of other anaesthetic substances. This might be worth examining in further experiments,

since so far, there have been multiple studies demonstrating that neurosteroids appear to be able to enhance tonic conductance in some neurons (Belelli & Lambert 2005, Drexler et al. 2016).

One study provided some particularly interesting findings: Stell et al. (2003) were able to demonstrate that small concentrations of the endogenous neurosteroid tetrahydrodeoxycorticosterone (THDOC) – around 10 nM, which is close to the physiological concentration range of THDOC – were able to significantly increase tonic conductance in dentate gyrus and cerebellar granule cells. This effect appears to be concentration-dependent, since it was significantly more pronounced at a concentration of 100 nM THDOC. What is interesting to note is that the low concentration of 10 nM THDOC produced no significant effect on sIPSC 10-90% rise time, peak amplitude, or decay time. At a concentration of 100 nM however, while still not affecting rise time and peak amplitude significantly, THDOC produced variable effects on sIPSC decay time, increasing the decay time of the slow component in some cells. At concentrations as great as 1 µM, THDOC consistently increased the decay time and charge transfer of sIPSCs. These concentrations also led to an 8-fold increase in tonic conductance in dentate gyrus granule cells.
This study, while demonstrating that the endogenous neurosteroid THDOC is able to modify tonic conductance even at small concentrations in certain cells, also provides evidence that the effect of neurosteroids on synaptic GABAergic transmission, i.e. on IPSC kinetics, is dependent on neurosteroid concentration levels. While very high concentrations of THDOC consistently increased IPSC decay time, smaller concentrations produced more subtle changes to IPSC kinetics. They appeared to shift the decay time more towards the slow component, which is similar to what could be observed in the patch clamp recordings done for the work at hand. This makes it likely that the effects of XBD173 on tonic conductance as well as on synaptic GABAergic transmission may also change with varying concentrations.

4.4 Interpretation of Extracellular Results

4.4.1 Interpretation of Burst Patterns

In the extracellular experiments, spontaneous neuronal network activity was recorded. In the neocortex, this neuronal activity displays a distinct pattern,

taking the form of so-called bursts – phases of high action potential activity alternating with silent phases during which no action potential activity can be observed. While action potentials are rapid changes in the potential of a single cell, the entirety of the burst results from the synchronous action potential activity of multiple neurons.
Bursts display characteristic kinetics which are a lot more complicated than those of isolated IPSCs. Spontaneous neuronal activity is the result of the interaction between excitation and inhibition and reacts sensitively to changes to either one. Drugs like zolpidem or benzodiazepines in general are able to shift this balance more towards inhibition by positively modifying $GABA_A$ receptors. This is represented by characteristic changes to the burst pattern, generally seen as a reduction of action potential activity. There is, however, a lot more to burst kinetics than just GABAergic modulation. Since the entirety of the neuronal network has to be taken into account in extracellular experiments, there is a wide spectrum of receptors – both inhibitory and excitatory – that partake in the process and that provide viable targets for modifying neuronal activity.

In the experiments, two phases of the burst with very different characteristics could clearly be observed. The early phase covering roughly the first 50 ms of the burst was characterized by a rapid increase in action potential activity to a very high level which then declined just as rapidly, while the late phase covered a much longer time span and was characterized by sustained action potential activity on a low level. This suggests that different types or subtypes of receptors are active and involved at different points in time during a burst.

4.4.2 Action Profiles of Zolpidem and XBD173

XBD173 at a concentration of 3 µM was shown to significantly modify action potential activity in neocortical slice cultures. It reduced action potential activity during the late phase of the burst by about 40%, but it displayed no effect on the early phase. This indicates that XBD173, or rather neurosteroids, do not target receptors that are active during the early phase of the burst (or they target both inhibitory and excitatory receptors in a perfectly balanced way, which is highly unlikely). This novel finding has not been described until now. Since XBD173 does have an effect during the late phase of the burst, there have to be some receptor types or subtypes that are active only during that part of the burst and that are targeted by either XBD173 or neurosteroids. This might be a certain

subtype of $GABA_A$ receptor, but it is similarly possible that this effect is mediated via a completely different type of receptor.

Zolpidem at a concentration of 200 nM was shown to mediate an overall inhibitory effect on neuronal activity, reducing action potential activity both during the early and during the late phase of the burst – comparable concentrations of zolpidem have been demonstrated to significantly reduce action potential activity in previous studies, while there is evidence that zolpidem loses its α1-selectivity at higher concentrations (Neumann et al. 2019), hence this concentration was used for the study at hand.
Comparing this pattern to the effect mediated by XBD173 clearly suggests that zolpidem very likely targets different receptors than XBD173 (or rather neurosteroids) at least to some extent, since XBD173 displayed no significant effect whatsoever on the early phase. Since zolpidem was shown to have a very high affinity to α1-containing $GABA_A$ receptors and this experiment showed that zolpidem causes a strong depression of discharge rates during the early phase, it is very likely that α1-containing $GABA_A$ receptors are active at least but not necessarily exclusively during this phase.

In fact, this is in line with the study by Neumann et al. (2019) demonstrating that zolpidem reduces firing rates mainly during the early phases of high action potential activity. This study was able to confirm these findings using $GABA_A$R-α1(H101R) mutant mice, in which α1-containing $GABA_A$ receptors were rendered insensitive to benzodiazepines and zolpidem. While zolpidem depressed peak firing rates in wildtype mice, it did not do so in $GABA_A$R-α1(H101R) mice, indicating that these phases of high action potential activity are indeed modified by α1-containing $GABA_A$ receptors. Furthermore, the study examined the action profile of SH-053-2'F-S-CH3, a benzodiazepine that primarily targets α2-, α3-, and α5-containing $GABA_A$ receptors. SH-053-2'F-S-CH3 had no effect on the early peak firing rates, but displayed a depression of discharge rates during the phases of moderate action potential activity. Overall, these findings strongly suggest that phases of high and moderate action potential activity are mediated by different $GABA_A$ receptor subtypes. While early peak firing rates are controlled mainly by α1-containing $GABA_A$ receptors, late phases of moderate activity are controlled by α2-, α3-, and α5-containing $GABA_A$ receptors.
Just like SH-053-2'F-S-CH3, XBD173 showed no effect on the early phase of high action potential activity. This strongly suggests that it does not positively

affect α1-containing $GABA_A$ receptors, and it supports the theory that XBD173 and zolpidem have different mechanisms of action. According to these findings, it is well possible that XBD173 ultimately targets $GABA_A$ receptors with α2, α3, and/or α5 subunits, but not with α1 subunits.

However, it also has to be noted that several neurosteroids that positively modulate the function of the $GABA_A$ receptor have been found, when administered directly, to induce α1-mediated effects such as muscle relaxation, sedation, and anaesthesia as well. At higher concentrations they even appear to have GABA-mimetic effects, activating $GABA_A$ receptors directly (Belelli & Lambert 2005). XBD173, unlike exogenously administered neurosteroids, did not produce these effects (Rupprecht et al. 2009). A possible explanation for this difference is that the change in endogenous neurosteroid levels induced by TSPO ligands such as XBD173 is small compared to that after their exogenous administration – which may also be due to the experimental design, as is discussed below – and it is well possible that the actions of neurosteroids depend on their concentration, suggesting that at lower concentrations, they may have a higher affinity to α2-, α3-, α4-, α5-, or α6-containing receptors than they have to α1-containing receptors.

4.4.3 Synergistic Effects between Zolpidem and XBD173

During the early phase of high action potential activity, the effect of zolpidem was in no way modified by the presence or absence of XBD173. This is in line with the observation that XBD173 exerts no effect during this phase. This observation is particularly interesting considering that XBD173 was shown to impede the effect of zolpidem in the patch clamp experiments. In the extracellular recordings, however, zolpidem's action during the early phase remained unaffected, while during the late phase, the inhibitory effect of zolpidem was even enhanced by roughly 50% in the presence of XBD173, indicating a synergistic effect between both substances during this phase. Considering the results of the patch clamp experiments, this synergistic effect observed in the extracellular experiments cannot be transmitted by synaptic $GABA_A$ receptors alone.
The most likely explanation for this is that either substance may target receptors other than $GABA_A$ receptors. Unlike in the patch clamp experiments, the range of contributing receptors in the extracellular experiments was not narrowed down to $GABA_A$ receptors alone by applying substances that block all other

neurotransmitter receptor activity, but rather featured the entirety of receptors in the neocortex. This network is a much more complex system than mere GABAergic synapses, with many different receptors exerting both their inhibitory and excitatory influences, such as glutamate receptors, glycine receptors, and potassium channels. While both XBD173 and zolpidem have been shown to affect $GABA_A$ receptors in previous studies (Jia et al. 2009, Rupprecht et al. 2009), which could also be confirmed by the work at hand, it is very well possible that especially neurosteroids, whose mechanisms of action are quite unexplored to date, target receptors other than $GABA_A$ receptors as well, thus adding to the very complex interactions between different neuronal receptors and neurotransmitters. A possible explanation for the synergistic effects of zolpidem and XBD173 might also be that XBD173 itself or neurosteroids act as antagonists on excitatory receptors, shutting these receptors down during the late phase of the burst and allowing the inhibitory effect of zolpidem to predominate, thus leading to the observed synergism.
The involvement of all kinds of receptors in neuronal discharge patterns also makes it difficult to fathom how the changes to mIPSC kinetics induced by either substance translate into changes in the burst patterns. While the enhanced inhibition induced by zolpidem that was demonstrated in the patch clamp experiments is well in line with the observed overall suppression of discharge rates in the extracellular recordings, the changes to mIPCS kinetics induced by XBD173 were a lot more subtle and more complex and in a network context might well be overwritten by the impact of other receptors.

Nonetheless, $GABA_A$ receptors are still likely to play a crucial role in this process. While the observed interactions between zolpidem and XBD173 cannot be explained by the involvement of synaptic $GABA_A$ receptors alone, it could be demonstrated that $GABA_A$ receptors are in fact one endpoint of the effect of XBD173, so at least part of its effect is very likely mediated via $GABA_A$ receptors. There is strong evidence that these are, at least in part, other subtypes of $GABA_A$ receptors than those targeted by zolpidem – confirming one of the main theses of this work – but it remains unclear which receptor subtypes these are exactly.
While synaptic $GABA_A$ receptors have been shown to be targets of XBD173 or its substrates, it is still entirely possible that extrasynaptic $GABA_A$ receptors play a role in the process as well. It could be shown that zolpidem produces no significant effect on tonic inhibition, but so far it is unclear whether XBD173 might have an impact here, since its effect on tonic conductance was not tested isolatedly,

but only in the presence of zolpidem. The baseline shift after the application of bicuculline was slightly enhanced in the presence of XBD173, but this effect failed to reach statistical significance. This may have been due to the experimental design – since the baseline shift was recorded only in the presence of zolpidem, which might have impeded the effect of XBD173 – and relatively small number of experiments. It may be worthwhile examining the effect of XBD173 on tonic conductance without the influence of other substances. Up to now, an enhancing effect of XBD173 on tonic conductance could neither be confirmed nor ruled out entirely. There are several studies showing that neurosteroids are able to produce an effect on tonic conductance (Belelli & Lambert 2005, Drexler et al. 2016). It is well possible that neurosteroids promoted by XBD173 are able to target extrasynaptic $GABA_A$ receptors, thus enhancing tonic currents that contribute to the observed synergistic effect, but this remains to be seen.

Lastly, a possible explanation could be a synergism on the cellular level, i.e. the change in $GABA_A$ receptor activity might influence secondary proteins which in turn have an impact on the formation of action potentials. This involves a wide field of possible complex interactions which are difficult to assess experimentally.

4.5 Effects of XBD173 and the Role of TSPO

The experiments were able to demonstrate a clear effect of XBD173 on synaptic GABAergic transmission as well as on neuronal network activity. Supposedly, this is due to XBD173 affecting $GABA_A$ receptors indirectly, i.e. by targeting TSPO, thus promoting the synthesis of neurosteroids which then affect the $GABA_A$ receptor. It is, however, well possible that XBD173 targets other receptors directly as well. The hypothesis that XBD173 targets $GABA_A$ receptors directly was rejected by Kita et al. (2004), yet to date, there is still very little research on the exact mechanisms of action of XBD173.
Originally, XBD173 was designed to specifically target TSPO, thus promoting neurosteroidogenesis, and it has been shown to have a negligible affinity to receptors other than TSPO (Kita et al. 2004). Another study demonstrated an increase in allopregnanolone levels after the administration of XBD173 in test animals (Rupprecht et al. 2009). This makes the hypothesis that XBD173 targets $GABA_A$ receptors indirectly via neurosteroidogenesis a likely explanation for its effects on synaptic transmission.
The function of TSPO and its role in neurosteroidogenesis are, however, subject

to intense discussion. Early studies on TSPO were able to show that it plays a critical role in the production of steroid hormones. It was demonstrated that TSPO mediates the transport of cholesterol into the mitochondria, thus regulating the essential, rate-limiting step of steroid biosynthesis (Krueger & Papadopoulos 1990, Papadopoulos et al. 1997). This theory has since been supported by many studies: Certain TSPO ligands have been found to increase the level of neurosteroids in rats (McCauley et al. 1995, Serra et al. 1999), whereas steroid hormone production was decreased in rat Leydig tumor cells with TSPO disrupted by homologous recombination (Papadopoulos et al. 1997). Complete TSPO knock out in mice has been found to be lethal during embryonic development (Papadopoulos et al. 1997). Also, cells with a TSPO knock down greater than 70% were found to be unable to survive (Veenman et al. 2007).

More recent studies, however, brought up results that contradict the results of those older studies, questioning TSPO's role in steroidbiosynthesis. Various studies were able to show that mice with global TSPO deletion (TSPO-/-) did not display any evident abnormalities. TSPO deletion affected neither the ability to produce steroid hormones, nor the viability or fertility of the test animals, and TSPO-/- mice were found to be able to generate both gonadal and adrenal steroid hormones at normal levels (Tu et al. 2014, Banati et al. 2014). Expression studies found that cell lines with no expression of TSPO mRNA or protein were able to produce normal levels of steroid hormones (Tu et al. 2014), and after administration of the TSPO ligand PK11195, both cell lines with and without TSPO showed the same level of increase in steroid hormone production, suggesting that the effect of PK11195 is not mediated via TSPO (Tu et al. 2015).
In conclusion, the majority of the more recent studies suggest that TSPO is not involved in the transport of cholesterol into the mitochondria, nor in the regulation of steroidbiosynthesis. The actual function of TSPO is currently being discussed and remains to be identified.

This work was able to demonstrate that the $GABA_A$ receptor is one endpoint of XBD173's effect on neuronal transmission, but so far it has not been able to provide proof for its mechanism of action. In this context, it has to be considered that the effect following the administration of XBD173 may not be the result of neurosteroidogenesis, but rather of XBD173 targeting the receptor itself.
Further experiments on this issue might include shorter wash-in times and measuring the levels of certain neurosteroids in order to work out which ef-

fects of XBD173 are actually mediated by neurosteroids and which might be mediated by XBD173 itself. The same issue could be tackled by conducting experiments with antagonists of enzymes involved in neurosteroidogenesis – such as finasteride, which inhibits the function of the 5α-reductase – to verify the role of neurosteroidogenesis in the process. Similar experiments have been done by Rupprecht et al. (2009), indicating a reduced effect of XBD173 in the presence of finasteride, rendering an involvement of TSPO more likely. And lastly, it remains unclear how much of the effects – or lack thereof – are simply concentration-based. Varying the concentration of XBD173 in similar experiments might shed some light on this issue.

4.6 Discussion on the Experimental Design

Just like any experiment, electrophysiological recordings can hardly be done under ideal conditions, so there were a few drawbacks that had to be put up with. Firstly, it would have been preferable for the experimental protocol of the patch clamp recordings to include an initial control recording under ACSF-only conditions in order to increase the comparability of the results. However, this was technically impossible within the given experimental setting because of the difficulty to hold clamped cells long enough in order to make that additional recording. Once the whole-cell configuration has been established, the cell deteriorates quite quickly due to the lack of structural integrity of the cell membrane, allowing for recording times of around 15 to 30 minutes. This makes it nearly impossible to record long enough to include an additional 30 minutes of incubation time while maintaining whole-cell configuration. Therefore, the comparison between the effects in the XBD173 group and the sham group were done by the means of unpaired samples, but unfortunately it was not possible to compare a recording under XBD173 conditions to a drug-free control recording of the same cell.

Another aspect of interest is the concentration of the substrates in the testing chamber. During the experiments, the liquid volume in the testing chamber was constantly being renewed by a steady influx and efflux of the test substances zolpidem and XBD173. Both substances were constantly being applied at the same rate, leading to a steady state with stable concentrations. However, the neurosteroids which are presumed to mediate part of the effect were supposedly synthesized by the tissue itself rather than applied externally. While they were

constantly being drained together with the rest of the liquid volume in the testing chamber, it is not clear at which rate they were being synthesized. Since it is unclear to which extent the efflux of the liquid volume may possibly have affected the concentration of neurosteroids in the testing chamber, the concentration of neurosteroids may have actually been lower than it would have been without a constant wash out. This may have lead to a smaller observable effect.
It also remains unclear how the observed effects might scale with different concentration levels of the substrates. Do higher concentration levels of neurosteroids produce a greater effect, or do neurosteroids at greater concentrations even extend their impact on other receptor types or subtypes, thus displaying different patterns of effect? To further examine this issue, it might prove helpful to run a similar experimental protocol with higher concentrations of XBD173 or with a direct administration of neurosteroids, and to compare the patterns of effect on neuronal activity to the changes observed in the experiments done for the work at hand. This might not only clarify the role of neurosteroid concentration levels but also support the thesis of neurosteroids playing a crucial role in XBD173's mechanism of action – if the thesis of neurosteroid involvement is correct, then the direct administration of different neurosteroids at comparable concentrations could possibly lead to a similar pattern of effect on neuronal activity. Longer application times of XBD173 (e.g. 90 minutes instead of 30 minutes) might also be able to generate a similar effect.

Thirdly, the role of ethanol has to be considered. In the experiments, ethanol served as the solvent for XBD173. It has been shown that ethanol is capable of reducing neuronal activity by itself, therefore it supposedly is able to alter the effects of the test substances. However, as an attempt to mitigate the influence of ethanol on the experimental outcome, concentrations of ethanol equivalent to the concentrations of the solvent in the XBD173 test solution were applied to all control groups, effectively negating the impact of ethanol in the analysis of the results. It has to be noted that a general rundown effect could be observed in all experimental groups similarly. This is most likely due to the effect of ethanol and due to a general decrease in cell activity under the experimental conditions.

4.7 Conclusion and Outlook

To date, the exact mechanisms of action of many anaesthetics remain largely unknown, despite anaesthetics being some of the most frequently used drugs in modern clinical medicine. While their exact molecular targets as well as their interactions with each other often are still unclear, a deeper understanding of these mechanisms may greatly impact the patient-friendly use of clinical anaesthetics. Identifying specific receptors that mediate specifically either wanted effects or unwanted side-effect may provide an approach to reduce side-effects by bypassing certain drug targets, and synergistic effects between certain drugs could be used specifically in order to reduce dose requirements of general anaesthetics, thereby reducing unwanted side-effects of narcoses as well. A greater knowledge on this issue may serve as a starting point for further clinical studies.

This work pursued the study on XBD173 by Rupprecht et al. (2009) and compared the effects of XBD173 to those of zolpidem, a drug specifically targeting α1 subunit-containing $GABA_A$ receptors. It aimed to shed some light on the role of neurosteroids as endogenously produced substances in the cortex and on their interactions with anaesthetics. The study at hand was able to demonstrate the action profiles of zolpidem and XBD173 on a synaptic level as well as on a network level. It showed that both substances mediate neuronal activity in different ways, indicating different mechanisms of action. Hereby, one of the main theses of this work could be confirmed.

While the patch clamp experiments demonstrated that both substances are able to modify synaptic $GABA_A$ receptors, zolpidem produced a much clearer effect on GABAergic inhibition by significantly increasing mIPSC decay time and charge transfer. XBD173, on the other hand, appeared to alter mIPSC kinetics in a much more complex way, shifting many parameters towards the slow component of the current and increasing the weighted decay time constant. Rather than enhancing the effect of zolpidem on synaptic GABAergic transmission, its effect appeared to be impeded by XBD173. This is particularly interesting considering the results of the extracellular recordings – in these experiments, XBD173 did not only decrease action potential activity during the late phase of the burst, but when applied together with zolpidem, both substances displayed a synergistic effect during said phase.

First of all, these results offer a valid explanation for the different clinical effects of both substances – zolpidem having distinct sedative properties, while XBD173 provides an anxiolytic effect above all else. It could be demonstrated that

XBD173 has no effect on the early phase of the burst which has been shown to be modulated to a great extent by α1-containing $GABA_A$ receptors (Neumann et al. 2019). This is in line with the observation that XBD173 basically lacks clinical effects that are mediated via α1-containing $GABA_A$ receptors. Secondly, it has to be put on record that the synergistic effect of XBD173 and zolpidem during the late phase of the burst, as suggested by the results of this work, cannot be explained by the involvement of synaptic $GABA_A$ receptors alone. There has to be some other type or subtype of receptor involved. Up to now, it remains unclear which receptors these are exactly. While the assumption that different subtypes of $GABA_A$ receptors are targeted by zolpidem and XBD173 offers a viable explanation for the changes observed during the multi-unit recordings, this hypothesis fails to explain the combined results of both the extracellular and patch clamp results. Either extrasynaptic $GABA_A$ receptors play a role in this – which so far could neither by confirmed nor denied – or it has to be explained by the involvement of receptors other than $GABA_A$ receptors.

Despite being unable to attribute the effects of XBD173 to a single certain type or subtype of receptor, these findings do not limit the potential use of XBD173 as a treatment for anxiety without benzodiazepine-like side-effects as it was proposed by Rupprecht et al. (2009), nor do they negate the potential of using synergistic effects between different anaesthetic drugs in order to reduce dose requirements of anaesthetics. Quite the opposite: a synergistic effect between XBD173 and zolpidem as a GABAergic drug could be demonstrated in this work, which should spark interest in further experimental and clinical studies researching how synergisms like this may transition into clinical effects and how neurosteroid-based anaesthesia – either via direct administration of neurosteroids or via promotion of endogenous neurosteroidogenesis – may provide novel options to improve clinical anaesthesia in general. However, it might be necessary to shift the spotlight away from a narrow view on $GABA_A$ receptors only, more towards researching the effects of other receptors as well.

5 Abstract

Despite their long-standing and widespread use in modern medicine, the mechanisms of action of many anaesthetics still remain unknown. To date, it is mostly unclear which receptors exactly are targeted by these substances and how they interact with each other as well as with endogenous substances produced by the human body itself.
This study aims to examine this issue, specifically the effects and interactions of GABAergic drugs both on a synaptic as well as on a network level. It focuses on the action profiles and the interactions of zolpidem, a sedative drug that mainly targets α1 subunit-containing $GABA_A$ receptors, and XBD173, a TSPO ligand which supposedly targets $GABA_A$ receptors indirectly via neurosteroidogenesis.
For this study, electrophysiological recordings of spontaneous neuronal activity in organotypic slice cultures from the neocortex of neonatal mice were done. Neuronal activity on a synaptic level was assessed via patch clamp experiments, while network activity was assessed using extracellular multi-unit recordings.
The patch clamp experiments demonstrated that both XBD173 and zolpidem are able to affect synaptic GABAergic transmission in organotypic neocortical slice cultures, although they modify mIPSC kinetics in different ways. While zolpidem alone produced a strong increase in mIPSC decay time, XBD173 altered mIPSC kinetics in a much more complex way and, when co-applied with zolpidem, significantly reduced its effect.
In the extracellular experiments, XBD173 and zolpidem both significantly reduced action potential activity, while again displaying different patterns of effect. Zolpidem strongly decreased high frequency action potential activity, while XBD173 only affected phases of moderate sustained action potential activity. During those phases, a synergistic effect could be observed after the combined application of zolpidem and XBD173.
In conclusion, this study was able to confirm that zolpidem and XBD173 have different profiles of action in organotypic neocortical slice cultures, offering a plausible explanation for their distinct clinical effects. It also demonstrated a synergistic effect between both substances on a neuronal network level. Considering the combined results of the patch clamp and extracellular experiments, however, this effect cannot be mediated by synaptic $GABA_A$ receptors alone, indicating that there have to be other receptors involved in the process.
These findings may offer novel options for clinical anaesthesia – making use of synergistic effects, which may come in the form of synergistic interactions

between classical anaesthetics and neurosteroids, could quite possibly offer a new way to reduce anaesthetic dose requirements and thus their unwanted side-effects, thereby improving the patient-friendly use of anaesthetics overall.

6 Zusammenfassung

Trotz ihres bewährten und weitverbreiteten Einsatzes in der modernen Medizin sind die Wirkmechanismen vieler Anästhetika noch immer nicht bis ins Detail bekannt. Bis heute ist nicht abschließend geklärt, an welchen Rezeptoren sie genau wirken und wie sie miteinander oder mit körpereigenen Substanzen interagieren.

Diese Studie möchte dies genauer untersuchen, insbesondere die Wirkweisen und Interaktionen GABAerger Wirkstoffe, sowohl auf der neuronalen Netzwerk- als auch auf der synaptischen Ebene. Der Fokus liegt dabei auf den Wirkprofilen und Wechselwirkungen zwischen Zolpidem, einem sedierend wirkenden Medikament, das vor allem an $GABA_A$-Rezeptoren mit einer α1-Untereinheit wirkt, und XBD173, einem TSPO-Liganden, der auf $GABA_A$-Rezeptoren mutmaßlich indirekt via Neurosteroidbiosynthese wirkt.

Für diese Studie wurden elektrophysiologische Messungen spontaner neuronaler Aktivität in organotypischen Schnittkulturen aus dem Neokortex neonataler Mäuse durchgeführt. Die neuronale Aktivität auf der synaptischen Ebene wurde anhand von Patch-Clamp-Messungen untersucht; zur Erfassung der Netzwerkaktivität wurden extrazelluläre Multi-Unit-Aufzeichnungen durchgeführt.

Die Patch-Clamp-Versuche konnten zeigen, dass sowohl XBD173 als auch Zolpidem die synaptische GABAerge Transmission in organotypischen Schnittkulturen des Neokortex beeinflussen, obgleich sie die Kinetik inhibitorischer postsynaptischer Ströme auf unterschiedliche Weise modifizieren. Während Zolpidem alleine eine deutliche Verlängerung der Abklingzeit der mIPSCs bewirkte, führte XBD173 zu komplexeren Änderungen der mIPSC-Kinetik. Bei der gemeinsamen Applikation beider Substanzen war der Effekt von Zolpidem signifikant vermindert.

In den Extrazellulär-Versuchen konnte demonstriert werden, dass XBD173 und Zolpidem beide die Aktionspotenzial-Aktivität signifikant vermindern, wobei sie wiederum unterschiedliche Wirkungsmuster zeigten. Zolpidem zeigte einen starken Effekt auf hochfrequente Aktionspotenzial-Aktivität, während XBD173 nur Phasen konstanter niedriger Aktivität beeinflusste. Während ebendieser Phasen konnte zudem eine synergistische Wirkung von Zolpidem und XBD173 beobachtet werden.

Zusammenfassend konnte diese Studie bestätigen, dass XBD173 und Zolpidem in organotypischen Schnittkulturen des Neokortex unterschiedliche Wirkprofile zeigen, was eine plausible Erklärung für ihre unterschiedlichen klinischen Ef-

fekte liefert. Ebenso konnte ein synergistischer Effekt beider Substanzen auf der Netzwerk-Ebene aufgezeigt werden. Wenn man jedoch sowohl die Ergebnisse der Patch-Clamp-Versuche als auch der Extrazellulär-Messungen bedenkt, so kann dieser Effekt nicht allein durch die Wirkung an synaptischen $GABA_A$-Rezeptoren bedingt sein – vielmehr müssen hier auch andere Rezeptoren involviert sein.
Diese Erkenntnisse könnten womöglich neue Möglichkeiten in der klinischen Anästhesie eröffnen: Durch das Ausnutzen synergistischer Effekte, zum Beispiel in Form synergistischer Wirkungen zwischen herkömmlichen Anästhetika und Neurosteroiden, ließe sich womöglich die notwendige Dosis der verwendeten Narkosemedikamente verringern und somit ungewollte Nebenwirkungen reduzieren, was zu einer insgesamt verbesserten, patientenfreundlicheren Anwendung von Anästhetika führen könnte.

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8 Declaration of Own Contribution

This study was done at the Experimental Anaesthesiology Section, Department of Anaesthesiology and Intensive Care Medicine, Eberhard Karls University Tübingen, under the supervision of Prof. Dr. Bernd Antkowiak.
The concept of the study was developed by Prof. Dr. Antkowiak and myself.
The preparation and cultivation of neocortical slice cultures was done with the collaboration of Prof. Dr. Antkowiak, Dipl.-Biol. Claudia Holt, Dipl.-Biol. Ina Pappe, and myself. All experiments and data analysis were carried out by myself after initial training by the aforementioned. The statistical analysis of the results was done by myself with the help of Prof. Dr. Antkowiak, using self-written MatLab scripts and scripts provided by our research group. All figures were created by myself with the help of Dipl.-Biol. Claudia Holt. Figures displaying contents from other research papers are labelled accordingly.
I hereby assure that I wrote this manuscript myself and that I did not use any but the sources I quoted.

Tübingen, July 5th, 2021

9 Acknowledgements

Throughout the process of creating this dissertation, I received a great deal of support and assistance, which shall not go unmentioned. I would like to thank everyone who contributed their professional and personal support to the successful completion of this work.

First of all, I would like to thank my supervisor, Professor Dr. Bernd Antkowiak, for giving me the opportunity to work on this thesis, and even more so for the excellent mentoring, for all the advice and feedback throughout the entire process of creating this work.
I also wish to thank Claudia Holt and Ina Pappe, who guided me through the experimental part of this work and who always had an open ear for my problems and concerns. Your help was invaluable!
Furthermore, I would like to thank my parents Ingrid and Willi, as well as my brother Martin, for their constant support and encouragement. Special thanks goes to my dad for his coaching and all his feedback on my work.
Finally, I would like to thank my partner Julian for supporting me and always being there for me.