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Modulation of $GABA_A$ receptor subunits by neurosteroids and anaesthetics: Identification of molecular binding sites critical to neuroprotection in the mouse hippocampus

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Abstract

Most of the currently clinical used anaesthetics in perioperative anaesthesia potentiate the function of γ-aminobutyric type A receptors ($GABA_A$Rs), resulting in amnesia, sedation, hypnosis, and anxiolysis. Nonetheless, anaesthetics like benzodiazepines (BZDs) have been associated with adverse side effects such as tolerance development, addiction, anterograde amnesia, and cognitive impairment. Some studies proposed the use of neurosteroids as anxiolytic agents in perioperative anaesthesia since they present a similar anxiolytic profile, but in absence of detrimental effects on memory. Both neurosteroids and BZDs bind to the $GABA_A$Rs, however the binding sites and affinities to the different receptor subtypes are still not fully understood. In the present study, acute brain slices from wild-type and several transgenic mouse lines of both sexes presenting knock-in and knock-out mutations were used for monitoring of long-term potentiation (LTP), paired-pulse inhibition (PPI), hypoxia/hypoglycaemia (H/H) and whole-cell patch-clamp recordings. We described the $GABA_A$R subunits responsible for the effect of different BZDs such as midazolam and diazepam, and for the neurosteroids allopregnanolone and THDOC, which are potentially released after administration of XBD173, ligand of the translocator protein 18kDA (TSPO) that induces neurosteroidogenesis. The BZD midazolam at only 10nM inhibited LTP mainly by enhancing α_1-$GABA_A$Rs and this effect was prevented with the application of flumazenil, a BZD-binding site antagonist. Diazepam and zolpidem at 1μM inhibited LTP via α_1-$GABA_A$Rs, confirming the key role of this subunit at regulating LTP. At nanomolar concentrations, neither XBD173 nor the application of potential neurosteroids synthesised after XBD173 administration, such as allopregnanolone and THDOC, showed detrimental effects on LTP. Interestingly, preapplication of XBD173 prevented the LTP blockage after midazolam administration, suggesting a potential beneficial interaction between naturally occurring neurosteroids and BZDs. Midazolam administration increased spontaneous inhibitory postsynaptic currents (sIPSCs) at concentrations of 100nM and 1μM. Both THDOC and XBD173 augmented the decay time and amplitude of sIPSCs, while allopregnanolone only enhanced the decay time of sIPSCs. After H/H-induced excitotoxicity, midazolam at 10nM mediated neuroprotective actions via potentiating α_5-$GABA_A$Rs subtype. Moreover, XBD173 and THDOC exerted neuroprotection after the *in vitro* excitotoxicity model by modulation of δ-$GABA_A$R subunits. When monitoring GABAergic-interneuron inhibition, midazolam at 100nM increased this inhibition by enhancing α_1- and α_2-$GABA_A$Rs expressed in the targeted circuit, which modulated interneurons from the *Stratum lacunosum moleculare* (SLM) to

the somata of CA1 pyramidal neurons. Diazepam at 1µM slightly modulated interneuron inhibition, but no effect was seen after zolpidem. A change in interneuron-mediated inhibition after release or direct application of neurosteroids was not detected. Altogether, these findings indicate that midazolam at low concentrations inhibits LTP via enhancing α_1-$GABA_A$Rs and thus might interfere with hippocampal-related learning and memory processes. In contrast, the induction of biosynthesised neurosteroids via TSPO activation mediates neuroprotective mechanisms during excitotoxicity without the BZD-related undesired effects on memory-related processes, since LTP was not inhibited. Although further investigations on the specific $GABA_A$R subunits mediating the effects of these compounds are needed, our results suggest TSPO ligands and neurosteroids based anxiolysis as a promising alternative in perioperative anaesthesia.

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A	arginine
AAC	axo-axonic cells
aCSF	artificial cerebrospinal fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BZD	benzodiazepine
CA	*Cornu Ammonis*
CCKBC	cholecystokinin-expressing basket cells
CGP55845	(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid hydrochloride
CNS	central nervous system
D-AP5	(2R)-2-amino-5-phosphonopentanoic acid
DG	dentate gyrus
DMSO	dimethyl sulfoxide
EC_{20}	20% effect concentration
FDA	food and drug administration
fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
$GABA_BR$	γ-aminobutyric acid type B receptor
$GABA_CR$	γ-aminobutyric acid type C receptor
H	histidine
HFS	high-frequency stimulation
H/H	hypoxia/hypoglycaemia
IPSC	inhibitory postsynaptic current
IQR	interquartile range
IS	intracellular solution
IUPAHR	International Union of Basic and Clinical Pharmacology
KI	knock-in
KO	knock-out
LTP	long-term potentiation
MRK-016	3-tert-Butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1H-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-d]-[1,2,4]triazine
mRNA	messenger ribonucleic acid
NAM	negative allosteric modulator

NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NGFC	neurogliaform cells
NMDAR	N-methyl-D-aspartate receptor
OGD	oxygen and glucose deprivation
PAM	positive allosteric modulator
PBR	peripheral benzodiazepine receptor
PPI	paired-pulse inhibition
PS	population spike
PVBC	parvalbumin-expressing basket cells
SCCP	Schaffer collateral-commissural pathway
sIPSC	spontaneous inhibitory postsynaptic current
SLM	*Stratum lacunosum moleculare*
SOMC	somatostatin-positive cells
TBI	traumatic brain injury
THDOC	allotetrahydrodeoxycorticosterone
TSPO	translocator protein 18kDa
VDAC	voltage-dependent anion channel
WT	wild type
XBD173	also named Emapunil, systematic name: N-benzyl-N-ethyl-2(7-methyl-8-oxo-2-phenylpurin-9-yl)acetamide

1. Introduction

1.1. Anaesthesia

The first application of anaesthesia can be traced back to ancient times with the Babylonians, Greeks, Chinese and Inca civilisations. In Europe, the first record of its use manifests in the 1200s with the Italian physician and bishop Theodoric of Lucca. To alleviate the surgical pain, he used sponges soaked with opium and mandragora, and hashish and Indian hemp were commonly known as painkillers (Harrah, 2022).

During the intellectual movement of the Enlightenment, the carbon dioxide, oxygen, and nitrous oxide gases were identified, and they became of interest to the scientific community. It was observed that inhalation of nitrous oxide (also called “laughing gas”) caused euphoria and analgesia. Experimentation with this gas and ether let the scientists H. Davy and M. Faraday conclude in 1818 that it is difficult to quantify the dose of these anaesthetics to control the desired effect (The History of Anaesthesia, 2022).

But it was not until 1846, when O. W. Holmes coined the term “anaesthesia”, from the Greek alphabet and defined it as the “loss of sensation”. It was used to describe a person who underwent surgery in lack of suffering after receiving an inhaling vapour (Garcia et al., 2010). The same year, W. Morton witnessed a breakthrough in medical science that demonstrated that a patient, previously anaesthetised with ether, did not suffer any pain during a tumour removal from his neck. And one year later, the use of chloroform in medical practice was introduced by J. Simpson (Rudolph & Antkowiak, 2004).

Even though objections about general anaesthesia were present, it was used daily in the clinical practice. However, scientists still did not know the exact pharmacology of the used drugs and there were still reports of people dying as a result of administered anaesthesia. Furthermore, physicians were unfamiliar with the adverse effects. These events led to a scientific and practical growth of anaesthesia (The History of Anaesthesia, 2022). Since that pioneering time, anaesthetic practice has changed significantly and new discoveries such as the effects of different concentrations of inhaled anaesthetics have been made. It is now known that low concentrations of, for example, xenon and ketamine, can induce analgesia, amnesia, and hypnoses and that high concentrations may cause muscle relaxation, reduced motor responses to harmful stimuli and deep sedation. On the contrary, some anaesthetics (e.g., sevoflurane) present neuroprotective effects against ischaemia (Campagna et al., 2003; Park et al., 2011).

Nowadays, general anaesthetics are commonly used in clinical medicine because they contribute to immobility, pain relief and can induce unconsciousness in the operation room. They also have been used in neuroscience research to understand the molecular basis of action of general anaesthetics by different methods (Rudolph & Antkowiak, 2004).

1.2. Perioperative management

Perioperative management is a multidisciplinary medical field that has been motivated by an increase in complex surgeries, as well as improved anaesthetics and surgical techniques. Patients experiencing perioperative medicine are usually treated by anaesthetists (Schonborn & Anderson, 2019) because it involves pre-, intra-, and postoperative care to assist patients undergoing diagnostic and surgical procedures (Antkowiak & Rammes, 2019). It is however intriguing that after all the medical advances, the pharmacotherapy used to induce loss of consciousness in patients is imprecise and not fully understood (Garcia et al., 2010), and it frequently results in development of chronic pain and cognitive dysfunction after surgery. Current perioperative management make use of anaesthetics producing clinically desired impact by enhancing γ-aminobutyric acid (GABA) type A receptors ($GABA_ARs$) (Antkowiak & Rammes, 2019). For instance, propofol, etomidate and benzodiazepines (BZDs) like midazolam and diazepam, cause unconsciousness mainly by activating $GABA_ARs$ (Rudolph & Knoflach, 2011; Weir et al., 2017). They induce desired effects as anxiolysis, amnesia, sedation, hypnosis, muscle relaxation, and immobility in a concentration-dependent manner, and it is also subjected to the $GABA_AR$ subunit specificity (Wieland et al., 1992; Grasshoff et al., 2006). Nonetheless, anaesthetics also produce cardiovascular depression, postoperative nausea, delirium, and cognitive dysfunction upon surgery (Feng et al., 2017; Malapero et al., 2017; Safavynia & Goldstein, 2019). Perioperative anxiolysis is of importance since anxiety increases the patient's anaesthetic dose, and this allows anaesthetists to predict the possible postoperative pain (Ip et al., 2009). However, the use of BZDs specially in the ICU, is linked to several adverse effects, such as tolerance development during long-term sedation, delirium, anxiety, depression, cognitive dysfunction, symptoms of post-traumatic stress disorder, and longer hospitalisation (Curran, 1986; Van Rijnsoever et al., 2004; Vinkers & Olivier, 2012; Kok et al., 2018). Anxiety-related symptoms can be minor and temporary, yet many patients have severe and recurring symptoms that can impair overall life quality and therefore require long-term treatment (Bandelow & Michaelis, 2015). Symptoms are very treatable, but only 37% of those suffering will receive treatment (Anxiety and Depression Association of America [ADAA], 2021). This could be because patients are often misdiagnosed or that

the medication is simply not advanced enough (Reddy & Estes, 2016; Balon & Starcevic, 2020). Hence, current anaesthesiology research is focused on developing novel compounds in absence of these listed undesired side effects, offering new and improved perspectives for anaesthesia care and pain treatment (Knabl et al., 2008; Ralvenius et al., 2015).

1.3. $GABA_A$ receptor

The $GABA_AR$ plays a main role in anaesthesia, sedation and anxiolysis actions. Different agents modulate this receptor and enhance its action (Brohan & Goudra, 2017).

Since the GABA molecule was discovered, it has been known as the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Bloom & Iversen, 1971; Brohan & Goudra, 2017) and it is estimated to be used in one third of all synapses from the hippocampus, thalamus, and neocortex (MacIver, 2014). This neurotransmitter acts through the $GABA_AR$ or GABA type B receptor ($GABA_BR$), exerting its effects mainly via $GABA_AR$ (Pelkey et al., 2017), which is the one we have focused on in this research.

The ionotropic $GABA_ARs$ are part of the cys-loop ligand-gated ion channel superfamily. They are composed of five different subunits forming a central ion channel permeable to chloride ions (Rudolph & Knoflach, 2011), where all subunits consist of a long N-terminal extracellular domain, four transmembrane domains, and a large intracellular loop (Fig. 1) (Sieghart & Sperk, 2002). As of yet, 19 different subunits have been detailed ($\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, θ, π and $\rho_{1–3}$) (Olsen & Sieghart, 2008). It is important to point out that a homopentameric of ρ receptors have been described as type C ($GABA_CRs$) (Bormann, 2000), but the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (IUPHAR) discourages the use of this term, and they are generally categorised as an isoform of $GABA_ARs$ (Olsen & Sieghart, 2008).

Under normal conditions in mature neurons, GABA binds to the receptor and the activation of $GABA_ARs$ leads to an increase in the inward chloride current, resulting in a hyperpolarisation of the cell membrane potential. Consequently, a reduction of the postsynaptic neuronal action potential takes place, thus increasing neuronal inhibition (Uusi-Oukari & Korpi, 2010).

Theoretically, multiple subunit combinations are possible, but just a dozen configurations have physiological relevance and are expressed to a significant extent in the CNS (Olsen & Sieghart, 2008), displaying the different subunit distribution among brain regions and neuronal subtypes. The receptor composition seems to follow certain rules and the most likely stoichiometry is two α-, two β- and either one γ- or one δ-subunit (Fig. 1) (Barnard

et al., 1998; Farrant & Nusser, 2005), being the most abundantly expressed receptor subtype formed from $\alpha_1\beta_2\gamma_2$ subunits (Barnard et al., 1998; Pirker et al., 2000).

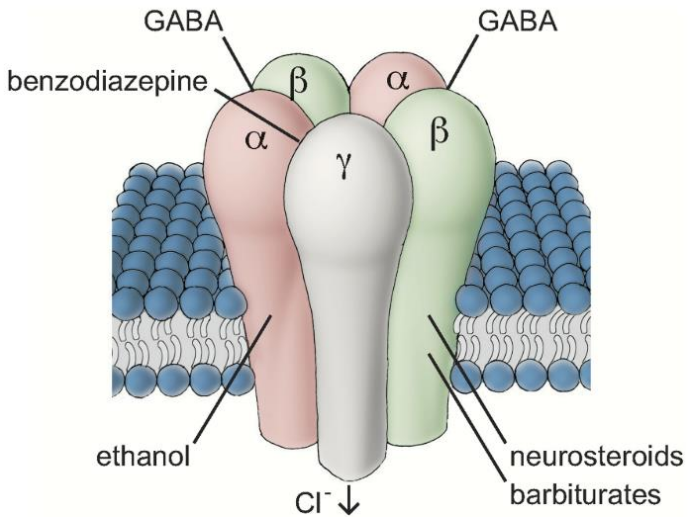

Fig. 1: Schematic $GABA_AR$ illustration and its binding sites; according to (Uusi-Oukari & Korpi, 2010).

Pentameric receptor composed of two α-, two β- and one γ-subunit. They also contain binding sites for several clinically relevant drugs. The binding of GABA is done at the interface between α- and β-subunits, opening the chloride channel. The BZD binding site is at the interface between α- and γ_2 subunits. Ethanol, barbiturates and neurosteroids have different binding sites in the intracellular domain. Text adapted from (Uusi-Oukari & Korpi, 2010).

As the $GABA_AR$ is widespread in the CNS, it is crucial for the physiological function of the brain, and it is the target of numerous drugs, including anaesthetics (Olsen & Sieghart, 2008). Moreover, it is evident that depending on the subunit composition, different drugs and endogenous ligands will be able to bind to the $GABA_ARs$ with more or less affinity. Certain positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) for the $GABA_AR$ exist. For example, a relevant type of PAMs are BZDs (Whiting et al., 1995; Sigel & Buhr, 1997) that increase neuronal inhibition, and a typical NAM would be MRK-016, which is a specific agent for α_5-subunits binding and it is known to be a cognitive enhancer (Atack et al., 2009).

1.3.1. Subunit abundance

As mentioned before, the prevalent and naturally occurring combination of $GABA_ARs$ is with $\alpha_1\beta_2\gamma_2$ subunits (Barnard et al., 1998; Pirker et al., 2000). For the α subunit, α_1 are the most abundant type (Sieghart & Sperk, 2002) and they are highly expressed in cortex, thalamus, pallidum, and hippocampus (Wisden et al., 1992). It was shown that when α_1 subunits are knock-out (KO), the total $GABA_AR$ content in the mouse brain is reduced by 50% (Sur et al., 2001). α_2 are expressed in hippocampus, cortex, striatum, and nucleus accumbens and α_3 were found in cortex and thalamus, whereas α_5 are highly

expressed in hippocampus and in deep cortical layers (Rudolph & Knoflach, 2011). α_4 subunits are limitedly expressed in the forebrain and α_6 subunits in the cerebellum (Wisden et al., 1992).

Among β subtypes, β_2 are the most common subunits, resulting in a 50% $GABA_AR$ reduction when these subunits are KO (Sur et al., 2001). β_1 are the least widespread subunits and β_3 are expressed, though in a discreet manner (Olsen & Sieghart, 2008). For γ, γ_2 subunits are the most abundant type in the rat brain in most regions, detected by mRNA *in situ* hybridisation (Wisden et al., 1992) and it is estimated to be about 75-80% of the total γ subunits (Olsen & Sieghart, 2008). Nevertheless, δ were also found replacing γ in the $GABA_AR$ and are highly expressed in the cerebellum, hippocampus, and thalamus (Laurie et al., 1992).

For synaptic localisation of $GABA_ARs$, γ_2 subunits are usually associated with α_1, α_2, or α_3 subunits. In the extrasynaptic site, γ_2 subunits can be found mostly with α_4, α_5 or α_6 subunits, but the combination with α_1, α_2, or α_3 subunits is also possible. In contrast to γ_2, δ subunits are known to be principally found in the extrasynaptic location (Farrant & Nusser, 2005).

1.3.2. GABAergic inhibition

Two different types of $GABA_AR$-mediated inhibition are responsible for controlling the excitability in the brain, named phasic (synaptic) and tonic (extrasynaptic) inhibition. Phasic inhibition is mainly mediated by $GABA_ARs$ composed of α_{1-3}, β_{2-3}, γ_2 subunits, but α_5 subunits can also be present in the synaptic site (Glykys & Mody, 2006; Serwanski et al., 2006). It can be measured with inhibitory postsynaptic currents (IPSCs), which emerge from a rapid and precise GABA release. In the presynaptic site, a local calcium influx arises from the action potential arrival, triggering the release of synaptic vesicles. Each vesicle releases thousands of GABA molecules in the synaptic cleft, resulting in a peak concentration of GABA which induces ion channel opening in the postsynaptic $GABA_ARs$ (Fig. 2). A characteristic of the phasic inhibition is the short duration that postsynaptic receptors are exposed to the GABA transient release (Mody & Pearce, 2004; Farrant & Nusser, 2005). In the hippocampus, IPSCs can be further subdivided into $GABA_{A,fast}$ and $GABA_{A,slow}$ IPSCs depending on the kinetics. While $GABA_{A,fast}$ IPSCs (3-20ms) are mediated by somatic synapses, $GABA_{A,slow}$ IPSCs (>30ms) are occurring in distal dendritic sites (Pearce, 1993; Capogna & Pearce, 2011).

Contrarily, tonic inhibition is triggered by extrasynaptic $GABA_AR$s, which mostly contain α_4, α_5, α_6 or δ subunits (Fig. 2). These high affinity binding subunits allow the activation of the receptor when low concentrations of ambient GABA are present (Glykys & Mody, 2006; Belelli et al., 2009).

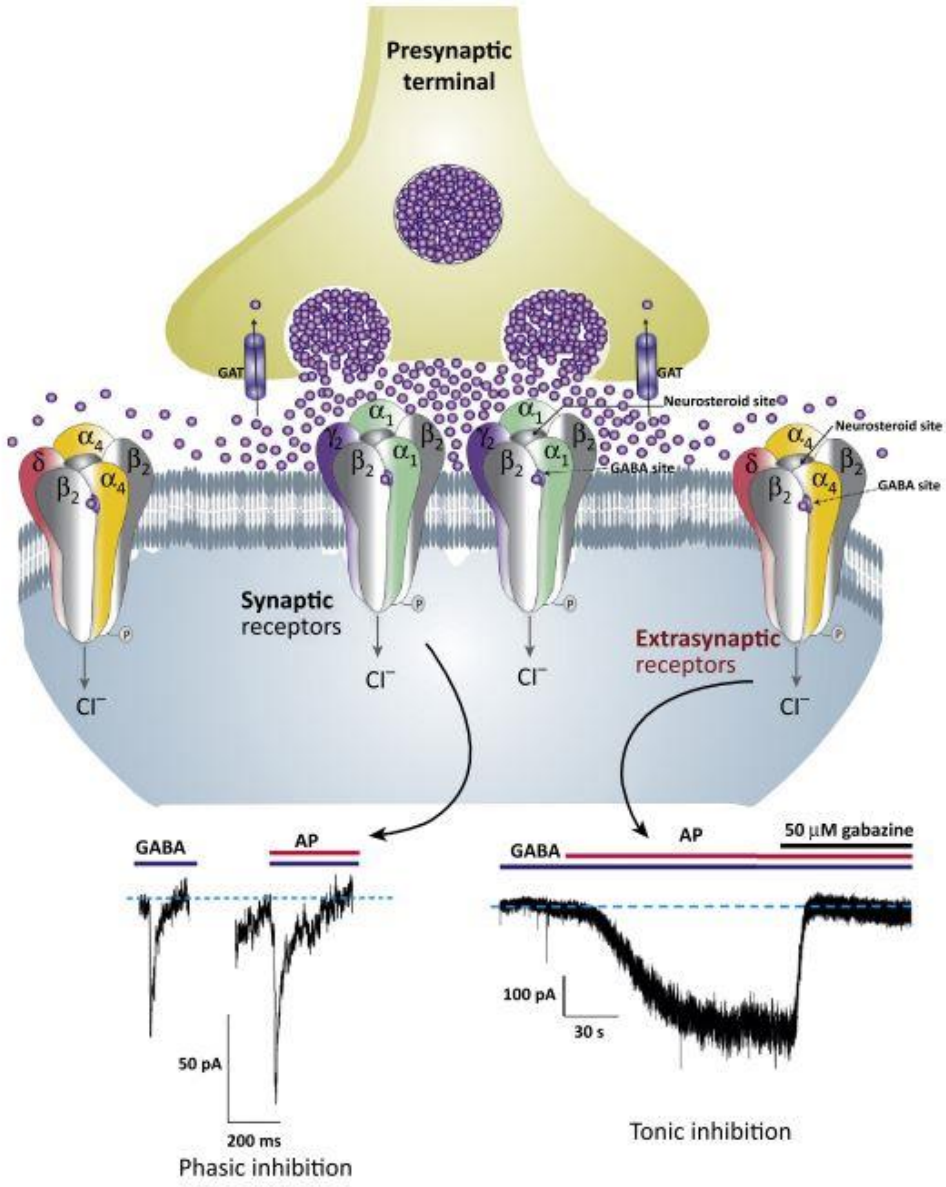

Fig. 2: Synaptic and extrasynaptic $GABA_AR$s: neurosteroid mechanism of action; according to (Reddy & Estes, 2016).

The neurosteroid binding site in the $GABA_AR$ differs from the site for BZDs, GABA and barbiturates. Synaptic $GABA_AR$s composed of $2\alpha2\beta_1\gamma$ subunits mediate phasic inhibition when high GABA levels are released, while extrasynaptic $GABA_AR$s composed of $2\alpha2\beta_1\delta$ subunits mediate tonic inhibition when low concentration of ambient GABA is present. Neurosteroids can enhance both receptor types, thus facilitating maximal neuronal inhibition. There are GABA transporters (GAT) that remove GABA. Traces represent phasic currents as IPSCs in presence of GABA (left) and allopregnanolone (right; AP) and tonic currents, also in presence of GABA (left) and AP (right). This is recorded from a dentate gyrus granule cell. The $GABA_AR$ antagonist gabazine is used to confirm GABAergic currents in the tonic inhibition example. Text adapted from (Reddy & Estes, 2016).

In pubertal mice, increased GABAergic inhibition through both phasic and tonic mechanisms resulted in a detrimental synaptic plasticity (Shen et al., 2020). This might indicate that these two components are related to deficits in the learning process during development. Tonic inhibition is particularly interesting for clinical and pharmacological significance because defective tonic conductance is involved in cognition and memory impairment, anxiety, and depression, among others (Smith et al., 2007; Martin et al., 2010; Damgaard et al., 2011; Holm et al., 2011). Evidence suggests that extrasynaptic $GABA_AR$s are principal targets of anaesthetics and antiseizure compounds, and proposing neurosteroid modulation as a therapeutic tool (Brickley & Mody, 2012).

1.3.3. Knock-in and knock-out mutations

Genetic modulation of individual $GABA_AR$ subunits has shown the biophysical and pharmacological properties depending on subunit composition. Consequently, allowing us to discover the targeted modulation of these subunits by different compounds (Wieland et al., 1992).

Individual $GABA_AR$ α subunits were genetically modified in mice and by applying diazepam, the role of these individual subunits was elucidated. Histidine (H) to arginine (R) point mutations at a conserved residue in the N-terminal extracellular region of α_1, α_2, α_3 or α_5 subunits rendered diazepam insensitive for their binding at the BZD binding site. However, this did not affect the physiological function of the receptor because the neurotransmitter GABA was still able to bind to the receptor (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2002; Kelly et al., 2002).

In α_1 and α_2 subunits, the mutation was in position 101, symbolised as α_1(H101R) and α_2(H101R), respectively. In α_3, the mutated position is 126 [α_3(H126R)] and in 105 for α_5 subunits [α_5(H105R)]. These point mutations resulted in different knock-in (KI) mouse lines, where the targeted α subunits are present in the receptor but insensitive to BZD binding (Rudolph et al., 1999; Crestani et al., 2002).

Furthermore, another type of transgenic mice appeared, called KO. In this case, the gene codifying for a certain $GABA_AR$ subunit is perturbed (either deleted from the gene sequence or preventing the transcription by a stop codon) and therefore unable to be expressed. In the KO lines, the certain mutated subunit is physically lacking in the $GABA_AR$ (Mihalek et al., 1999).

1.4. Benzodiazepines (BZDs)

In the 1950s, physicians and scientists first observed the wide range of therapeutic actions that BZDs offer, including sedation, anxiolysis, muscle relaxation, and seizure suppression. This discovery opened the door for further research to replace and improve treatments with BZDs in an effort to create the desired sedative-hypnotic (sleep-inducing) effect (Rudolph & Knoflach, 2011).

The $GABA_AR$ binding site for BZDs consists of an α- (1, 2, 3 or 5) and a γ-subunit (generally γ_2). BZDs classically used in the clinic do not bind to receptors presenting α_4 or α_6 subunits. Therefore, the α subtype accompanying the γ_2 subunit will determine the sensitivity of the receptor to the certain BZD (Rudolph & Knoflach, 2011). It is noteworthy that some receptors may be composed of two different α subunits in the same pentamer (Minier & Sigel, 2004). By using genetic techniques and pharmacological approaches, it has been demonstrated that the sedative and addictive effects of BZDs are mediated by α_1-containing $GABA_ARs$ (Rudolph et al., 1999), whereas $\alpha_{2/3}$-containing receptors are responsible for anxiolytic and muscle-relaxant actions (Löw et al., 2000). $GABA_ARs$ containing α_5-subunits mediate at least some memory-impairing effects of BZDs (Crestani et al., 2002; Rudolph & Antkowiak, 2004), entailing the depression of learning and memory processes that are hippocampal-related (Lister, 1985; Evans & Viola-McCabe, 1996; Tokuda et al., 2010).

Once the BZD binds to the receptor, the drug displays an allosteric effect by facilitating the GABA binding to the receptor and therefore augmenting its opening frequency and neuronal inhibition (Haefely, 1984).

1.4.1. Midazolam

Midazolam is a water-soluble BZD that exerts a more potent and faster onset when compared to diazepam (Cole et al., 1983) and with an elimination half-life of normally 1.5-3.5h (Dundee et al., 1984), whereas for diazepam is 20h (Kaplan et al., 1973). All these characteristics make midazolam a good candidate for exerting either conscious sedation for short procedures or pre-operative sedation, as well as for general anaesthesia (Suri, 2000). Midazolam is principally used in perioperative anaesthesia and its administration results in anxiolysis, amnesia, sedation, and hypnosis (Tokuda et al., 2010).

Midazolam enhances $GABA_AR$-mediated inhibition by increasing the number of GABA molecules binding to the receptor at low concentrations, but it can also directly activate

the receptor when applied at high concentrations (Eom et al., 2011). It has been shown that midazolam acts principally via $GABA_A$Rs, harbouring either α_1, α_2, α_3 or α_5 subunits. Nonetheless, it is highly potent at receptors containing α_1, which are the subunits responsible for mediating sedative effects (Rudolph et al., 1999). The potentiation exerted by midazolam in $GABA_A$Rs containing $\alpha_1\beta_2\gamma_2$ subunits is doubled when compared to the efficacy for $\alpha_2\beta_2\gamma_2$-$GABA_A$Rs (Ralvenius et al., 2015).

Midazolam strongly generates anterograde amnesia (i.e., a loss of memory of situations happening forward in time), a common side effect after BZDs administration (Hennessy et al., 1991; Mejo, 1992), and post-operative cognitive impairment is also evident after midazolam application (Thomas-Antérion et al., 1999). In accordance with this, it has been shown that midazolam blocks hippocampal long-term potentiation (LTP), which is a cellular correlate for memory and learning processes (Satoh et al., 1986; Evans & Viola-McCabe, 1996), and therefore inhibiting hippocampal synaptic plasticity. $GABA_A$Rs containing α_5-subunits are associated with synaptic plasticity modulation, and since they are mainly situated extrasynaptically in the hippocampus (Pirker et al., 2000; Sur et al., 2001), they are responsible for the tonic inhibitory conductance generated in CA1 pyramidal neurons (Cheng et al., 2006; Martin et al., 2009; 2010; Rodgers et al., 2015). However, these experiments were performed using etomidate and the effect of midazolam on LTP and the specific $GABA_A$R subunits mediating the amnesic properties in the hippocampus are still unidentified.

1.4.2. Diazepam

The classical BZD diazepam (e.g., Valium) is widely used in the clinic because of its anxiolytic, sedative, hypnotic, anticonvulsant, and muscle relaxant properties (Benson et al., 1998). Diazepam presents high affinity for $GABA_A$Rs containing α_1, α_2, α_3 or α_5 subunits, but α_4 and α_6 subunits are insensitive for diazepam (Hadingham et al., 1996). Evidence supports that diazepam binds to α_1- and α_2-$GABA_A$Rs, exerting sedative or anxiolytic effects, respectively (Rudolph et al., 1999; Löw et al., 2000). However, the principal disadvantages are the extended and undesired duration of the sedative and anxiolytic effects because of its long elimination half-life.

It has been shown that diazepam has a detrimental effect on memory function, causing anterograde amnesia (Suri, 2000) and blocking hippocampal LTP induction (del Cerro et al., 1992).

1.5. BZD-binding site compounds

1.5.1. Zolpidem

Zolpidem (e.g., Ambien) is a non-BZD molecule with a different pharmacological profile. However, it binds to the BZD binding site of the $GABA_AR$ — its effects are antagonisable by flumazenil (Lheureux et al., 1990; Patat et al., 1994) —, and it shows a hypnotic effect of rapid onset and short duration. It also produces anxiolytic and anticonvulsant effects, motor incoordination, and learning and memory impairment (Sanger & Depoortere, 1998). It is widely used in clinics for insomnia treatment.

It has been shown that at low nanomolar concentrations, zolpidem is highly selective for $GABA_ARs$ presenting $\alpha_1\beta\gamma_2$ subunits (Weiner et al., 1997; Möhler et al., 2002), at higher nanomolar concentrations it presents an intermediate affinity for receptors containing α_2- and α_3-subunits, but very low/no affinity for α_5 subunits (Crestani et al., 2002; Belelli, 2005). Furthermore, it is insensitive for α_4 and α_6 subunits (Uusi-Oukari & Korpi, 2010) and only functional when γ_2 subunits are present (Carver & Reddy, 2013).

Even though zolpidem has no affinity for the $GABA_ARs$ containing α_5-subunits, it can modulate synaptic plasticity by inhibiting LTP when applied at micromolar concentrations (Higashima et al., 1998).

1.5.2. Flumazenil

Flumazenil is a BZD antagonist, blocking the modulation of compounds from binding to the BZD binding site of the $GABA_AR$, both PAM and NAM (Goetz et al., 2007). It presents a rapid onset of action and then antagonises the sedative actions of different BZDs as midazolam and diazepam. Contrary, flumazenil cannot reverse other sedative and hypnotic effects caused by barbiturates, inhalational anaesthetics, opioids, ethanol, or propofol. Flumazenil's antagonism for the BZD binding site is highly selective for $GABA_ARs$ containing α_1, α_2, α_3 and α_5 subunits (Ki ~1nM), and less affine for α_4 and α_6 subtypes (Ki ~150nM) (Sieghart, 1995; Möhler et al., 2002; Pym et al., 2005). Because of all these characteristics, it is usually clinically applied in cases of BZD intoxication (Goetz et al., 2007).

1.5.3. MRK-016

MRK-016 is a pyrazolotriazine presenting an affinity of 0.8-1.5nM for the BZD binding site of native rat brain, with similar affinity for $GABA_ARs$ containing α_1, α_2, α_3 or α_5 subunits, but has a greater efficacy at inhibiting receptors containing α_5 compared to α_1,

α_2 and α_3 subtypes, exerting a NAM effect selective for α_5-containing $GABA_ARs$ (Atack et al., 2009).

MRK-016 acts as a cognition enhancer without displaying convulsant or anxiogenic effects in animals, and it was administered in clinical trials to young males, resulting in drug tolerance in absence of epileptic, hallucinogen, and anxiolytic effects (Jones et al., 2006). Nevertheless, it was poorly tolerated in elderly humans and lacking any cognitive improvement; thus, clinical investigations were ceased (Atack et al., 2009). It is possible that the poor tolerance and lack of efficacy in older subjects was caused by an age-related effect or a scarce brain penetration of the molecules (Petrache et al., 2020).

In the last years, investigators aimed to discover NAMs that selectively reduce α_5-$GABA_AR$ function like MRK-016 to clinically used them as cognitive enhancers or "smart drugs" (Jacob, 2019). However, this research is ongoing.

1.6. Neurosteroids

Baulieu proposed the term "neurosteroid" to describe steroid molecules that are synthesised and accumulated in the brain and are independent of peripheral glands (Baulieu & Robel, 1990), whilst Paul introduced the term "neuroactive steroid" referring to either endogenous or synthetic steroid that can rapidly alter neuronal excitability (Paul & Purdy, 1992). Currently, both endogenous and synthetically originated steroids are here included when referred to neurosteroids.

Neurosteroids act via either "classical" or "non-classical" receptors in both central and peripheral nervous systems. The classical approach is genomic and neurosteroids bind to intracellular receptors that regulate gene transcription, usually performed by steroid hormones, and this takes several days. On the contrary, the non-classical path is non-genomic, quicker (seconds-minutes) and involves neurotransmitter-dependent receptors (Slater et al., 1994; Rupprecht, 2003; Colciago et al., 2020). Neurosteroids presenting acute actions alter neuronal excitability primarily by interaction with $GABA_ARs$ (Majewska et al., 1986; Rupprecht & Holsboer, 1999). They enhance these receptors at nanomolar concentrations by acting as PAMs (Paul & Purdy, 1992; Lambert et al., 1995), although at high concentrations in the micromolar range, they present agonistic activity even in the absence of GABA (Puia et al., 1990).

The precursor of endogenous neurosteroids is pregnenolone, and it is synthesised from cholesterol in the mitochondria (Prasad et al., 1994; Lacapère & Papadopoulos, 2003). Once in the cytosol, pregnenolone is further processed into different types of neurosteroids (Fig. 3).

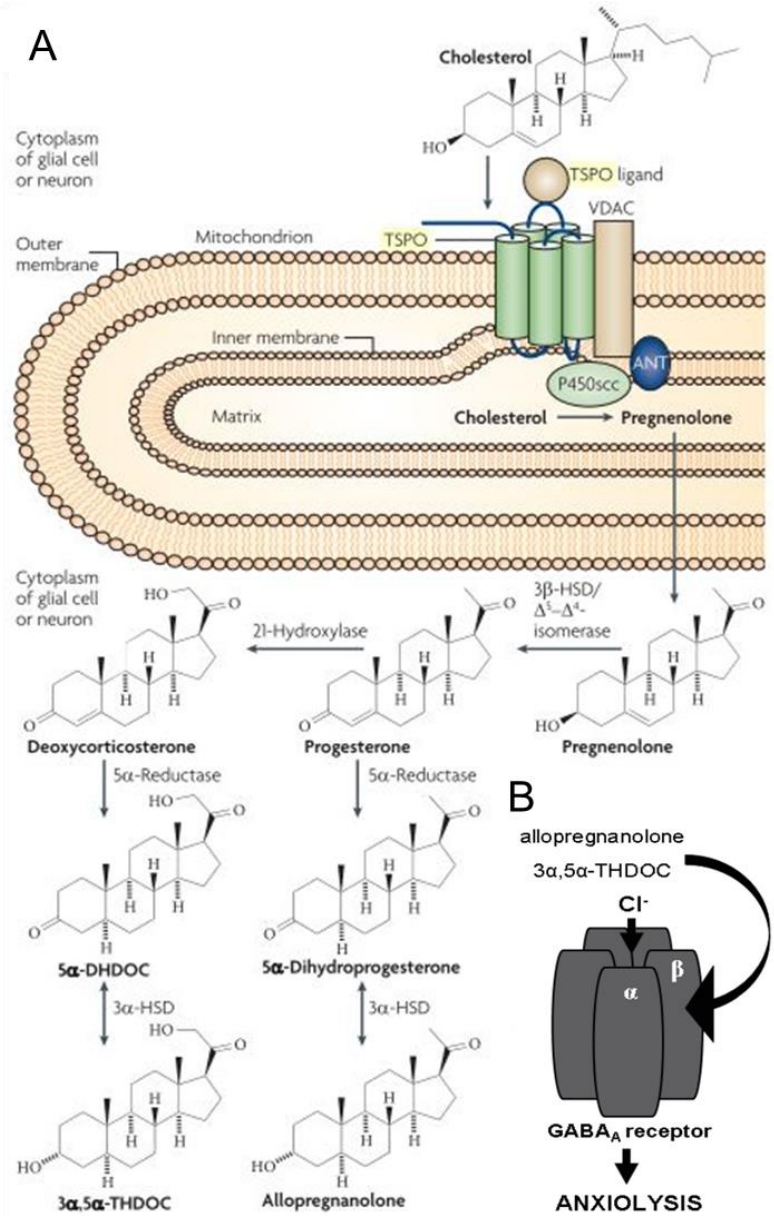

Fig. 3: Neurosteroidogenesis and effect of neurosteroids on $GABA_A$Rs.

(A) Neurosteroidogenesis induced via translocator protein (18kDa, TSPO) ligand; adapted from (Rupprecht et al., 2010). The cholesterol side-chain-cleaving cytochrome P450 enzyme, situated at the inner mitochondrial membrane, converts cholesterol to pregnenolone (precursor of neurosteroids). Pregnenolone diffuses to the cytoplasm, and it is transformed into progesterone via microsomal 3β-hydroxysteroid dehydrogenase (3β-HSD)/Δ^5-Δ^4 isomerase. Then, it is metabolised to deoxycorticosterone via 21-hydroxylase. Both progesterone and deoxycorticosterone are reduced to 5α-dihydroprogesterone and 5α-dihydro-corticosterone (5α-DHDOC), respectively, by 5α-reductase. A further reduction takes places via 3α-hydroxysteroid dehydrogenase (3α-HSD), resulting in the neurosteroids allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC). (**B)** The neurosteroids allopregnanolone and THDOC bind to the $GABA_A$Rs acting as PAMs and consequently inducing anxiolysis; adapted from (Nothdurfter et al., 2012). Text adapted from (Rupprecht et al., 2010; Nothdurfter et al., 2012).

Neurosteroidogenesis is neuron and region specific. This distribution depends on the expression of the different enzymes responsible for neurosteroid formation, as well as the relative TSPO abundance. Consequently, GABAergic neuronal inhibition is specifically brain-region enhanced (Rupprecht et al., 2010). Some resulting neurosteroids are allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC), both 3-α hydroxy ring A-reduced pregnane steroids, and they are among the most potent neurosteroids that can modulate the $GABA_A$Rs (Majewska et al., 1986; MacKenzie & Maguire, 2013; Zorumski et al., 2013). The exact receptor binding site is poorly defined, but there are two proposed binding sites, one in the α-subunit transmembrane domain for allosteric modulation and another in the α-β subunit interface for direct activation (Hosie et al., 2007; 2009). Particularly, photoaffinity labelling studies showed a specific binding site in α_1 and β_3 subunits, but deeper examinations remain to be done (Chen et al., 2019; Sugasawa et al., 2020).

In contrast to BZDs, neurosteroids can modulate all $GABA_A$Rs isoforms, as well as the ones including α_4 and α_6 subunits or in the absence of γ_2. However, neurosteroids present the highest sensitivity for δ-containing receptors (Brown et al., 2002; Carver & Reddy, 2016) at relevant and low physiological concentrations (10-100nM). Absence of functional δ subunits in mice revealed lower sensitivity to sedative and anxiolytic effects of neurosteroids.

In both rodents and humans, allopregnanolone administration resulted in induction of sedative, anaesthetic, analgesic, anxiolytic, and anticonvulsant actions (Belelli et al., 2009). Additionally, in both models it can be seen that the levels of allopregnanolone in the brain and plasma fluctuate during different physiological and pathological situations, such as during menstrual cycle or when experiencing stress (Bixo et al., 1997; Droogleever Fortuyn et al., 2004).

Essentially, evidence shows that neurosteroids present a high sensitivity at extrasynaptic δ-$GABA_A$Rs, allowing the treatment development targeting tonic inhibition in many mental disorders (Mihalek et al., 1999; Wohlfarth et al., 2002; Reddy & Estes, 2016). Synthesis of neurosteroids descends during stressful conditions, with age, and in chronic inflammatory and neurodegenerative diseases (Charalampopoulos et al., 2008; Borowicz et al., 2011). For instance, prolonged neuroinflammation was found to be related to delayed recovery after stroke patients (Liu et al., 2012; Liguz-Lecznar & Kossut, 2013), and impaired memory and plasticity (Greifzu et al., 2011; Doyle et al., 2015). He et al. (2004) demonstrated that after a traumatic brain injury (TBI) in rats, allopregnanolone diminishes neuronal loss and increases cognitive recovery. Moreover,

the neuroprotective and antineurodegenerative effects of allopregnanolone are involved in neurogenesis and synapse stabilisation (Diaz Brinton, 1994; Diaz Brinton & Ming Wang, 2006). Everything considered, these data suggest that neurosteroids can exert neuronal protection, as well as induction of neuroregeneration (Papadopoulos & Lecanu, 2009; see review from Guennoun, 2020).

Neurosteroids acting through $GABA_AR$s exert anaesthetic and hypnotic properties, opening a window of opportunity to either be used in perioperative anaesthesia or to create a novel anaesthetic with a neurosteroid-based action profile. Moreover, they could also be of advantage as therapeutic treatments for anxiety and stress disorders, depression, epilepsy, and neurodegenerative disorders (against excitotoxic or ischaemic events) (Majewska et al., 1986; Rupprecht, 2003; Carver & Reddy, 2013; Zorumski et al., 2013; Gunn et al., 2015). It is worth noting that intravenous allopregnanolone (i.e., brexanolone) administration was FDA-approved on March 19th, 2019, being the first specific treatment for postpartum depression (Pinna, 2020).

1.7. Translocator protein 18kDa

The translocator protein 18kDa (TSPO) was first identified in 1977 as a BZD binding site in peripheral organs and hence named accordingly as “peripheral benzodiazepine receptor (PBR)” (Braestrup & Squires, 1977). After extensive research, this receptor was renamed to translocator protein 18kDa (TSPO). The rename is accurate because not only BZDs bind to the receptor, but cholesterol is also a relevant TSPO ligand. The word “peripheral” for its location can be misleading because it is also expressed in the CNS and mainly but not exclusively in the mitochondria. It is always referred to as the 18kDa protein, which is the smallest functional unit for the known PBR (Papadopoulos et al., 2006).

1.7.1. Characteristics

The TSPO 18kDa is a mitochondrial transmembrane protein formed by 169 amino acids and organised as a five transmembrane helix structure (Joseph-Liauzun et al., 1998; Gavish et al., 1999). It is usually located in the outer mitochondrial membrane, allowing the translocation of cholesterol to the inside of the mitochondria, which is the rate-limiting step of the synthesis of neurosteroids (Anholt et al., 1986; Krueger & Papadopoulos, 1990; Rupprecht et al., 2010). Neurosteroidogenesis is regulated by TSPO, and its ligands originate an endogenous neurosteroid biosynthesis cascade in numerous areas of the brain (Rupprecht et al., 2009). Other proteins such as voltage-dependent anion

channel (VDAC) and the adenine nucleotide transporter are associated with TSPO in the mitochondrial membrane (McEnery et al., 1992) (Fig. 3).

Several organs express TSPO, however the tissues containing steroid-synthesising cells present higher TSPO expression levels (Lacapère & Papadopoulos, 2003; Papadopoulos et al., 2006). In the CNS, it is expressed in reactive astrocytes, glia and microglia (Maeda et al., 2007), facilitating a biomarker for inflammation and neurodegeneration (Rupprecht et al., 2010). TSPO expression levels are reduced in anxiety patients and the use of its ligands to induce neurosteroidogenesis may constitute a novel pharmacological approach to treat psychiatric disorders (Taliani et al., 2009; Rupprecht et al., 2010; Longone et al., 2011).

1.7.2. TSPO ligands: XBD173

Cholesterol and porphyrins are high-affinity TSPO endogenous ligands, as well as endozepines, which are neuropeptides capable of replacing BZDs from their binding site in the $GABA_AR$ (Costa & Guidotti, 1985). Cholesterol is the only ligand that binds to the C-terminus domain of TSPO (Li et al., 2001), while the rest of ligands binds to the N-terminus domain (Farges et al., 1994; Anzini et al., 2001).

Synthetic TSPO ligands have been developed primarily as neuroimaging markers and diagnostic instruments to study brain inflammation (Chauveau et al., 2008; Kim & Pae, 2016), besides revealing these ligands as potential therapeutic agents. Certain ligands, including XBD173, stimulated neurosteroidogenesis and exerted a consequent anxiolytic effect in rodents (Serra et al., 1999; Verleye et al., 2005; Rupprecht et al., 2009).

XBD173 is an 8-oxopurine derivative and a high affinity TSPO ligand in both rats and humans, nonetheless with an imperceptible affinity for $GABA_ARs$ (Kita et al., 2004). TSPO modulation via XBD173 activates neurosteroidogenesis; hence, the new formed neurosteroids, potentially allopregnanolone and THDOC, enhance $GABA_ARs$ action and subsequently causing anxiolytic and antipanic effects in rodents and humans (Kita et al., 2009; Rupprecht et al., 2009).

In essence, the research groups concerning Kita et al. (2009) and Rupprecht et al. (2009) presented the properties of XBD173, detailing the rapid onset of anxiolytic properties similar to BZDs, yet without the side effects. There is no evidence showing that XBD173 presents sedation, tolerance development, addiction, anterograde amnesia or withdrawal effects. While, unfortunately, all of the above are present when BZDs are administered in patients (Hennessy et al., 1991; Suri, 2000). Thus, XBD173 may provide

a new therapeutic opportunity for inducing anxiolysis in perioperative anaesthesia and for the treatment of psychiatric related disorders (Gunn et al., 2015; Paul et al., 2020).

1.8. Hippocampus

The term hippocampus (derived from the word “seahorse” in Greek) was first coined by Arantius in 1587, given its shape. In 1893, Santiago Ramón y Cajal was the first scientist who described the stratification of the hippocampus with his classical drawings, distinguishing cells with long and short axons. This discovery made evident that hippocampal neurons may target several cells and areas (Andersen et al., 2007).

1.8.1. Characteristics

Hippocampus is a structure located between the medial temporal lobe and the ventricle’s temporal horn of the brain, it belongs to the limbic system, and it has a characteristic C-shaped structure with densely packed neurons (Amaral & Witter, 1989; Rajmohan and Mohandas, 2007; Anand & Dhikav, 2012).

Two well-defined regions can be distinguished within the hippocampus, the *Cornu Ammonis* (CA), divided into three major fields (CA1-CA3) and the dentate gyrus (DG). The term of hippocampal formation is used for some neuroanatomists when also including other areas as the subiculum and the entorhinal cortex (Anand & Dhikav, 2012; Schultz & Engelhardt, 2014). The entorhinal cortex, via the perforant pathway, is the main source of input in the hippocampus. This information is processed and forwarded back to the cortex through the subiculum (Amaral & Witter, 1989; Bartesaghi & Gessi, 2003). In this hippocampal formation, an intrinsic trisynaptic circuit had been described. This circuit starts at the perforant pathway, where projections from the entorhinal cortex are directed to the DG. In there, the mossy fibres innervate the CA3 pyramidal neurons and successively, CA3 exhibits axonal projections to CA1 pyramidal cells via the Schaffer collaterals. To complete this trisynaptic circuit, CA1 axons project to the subiculum and back to the entorhinal cortex (Fig. 4) (Doller & Weight, 1982; Amaral & Witter, 1989; Yeckel & Berger, 1990).

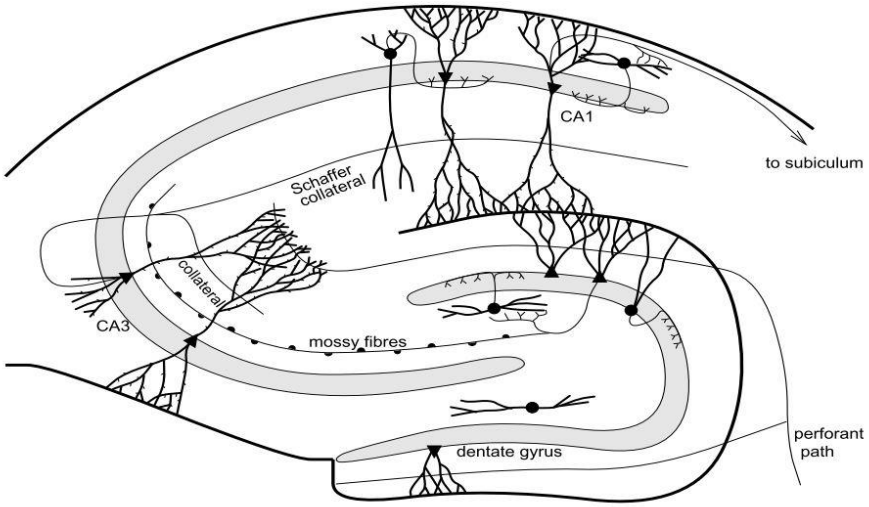

Fig. 4: Schematic hippocampal formation diagram, according to (Schultz et al., 1999).

Via the perforant path inputs, the information arrives to the hippocampus from entorhinal cells. This is then projected into the DG, CA3 and CA1, respectively. Apart from the perforant path inputs, CA3 also receives mossy fibres synapses from the DG cells. CA3 pyramidal cells axons have projections within CA3 and towards CA1 via the Schaffer collateral path. The information exits the hippocampus back to the entorhinal cortex via CA1 to subiculum projections. Text adapted from (Schultz et al., 1999).

The balance between excitatory and inhibitory inputs is fundamental for brain function. $GABA_A$Rs are well distributed in the hippocampus, and they work together with GABAergic interneurons in processing the information and keeping the hippocampal homeostasis (Jones, 1993; Pettit & Augustine, 2000). Interneurons can release GABA from the axon terminals, therefore becoming key modulators of neuronal excitability. Both pyramidal neurons and interneurons exert GABAergic inhibition, hence displaying a complex network of microcircuits where modulators of these cells play an important role in regulating this circuit (Bezaire & Soltesz, 2013; Booker & Vida, 2018). In the hippocampal GABAergic circuit, inhibitory interneurons are only 10-15% of the total population of neurons, yet essential in the regulation of GABAergic inhibition (Pelkey et al., 2017).

1.8.2. Function

The hippocampus is known to be the memory centre of the brain, playing a fundamental role in the formation of new memories. Scoville and colleagues were the first to observe a loss of memory (i.e., anterograde amnesia) after a bilateral hippocampal resection in a psychotic patient and in a patient with untreatable seizures (Scoville & Milner, 1957). Consequent confirmation of the association between hippocampus and memory was described in different animal models (Moss et al., 1981; Eichenbaum et al., 1992; Squire, 1992). However, the hippocampus only involves declarative/explicit memory, which is

the one that allows recollection of events and facts in a conscious way (Squire, 1992). Due to its memory involvement, it is evident that drugs with amnesic properties could modulate hippocampal data processing.

Specific $GABA_AR$ subtypes are critical for information processing in the brain, and there is evidence attributing α_5-$GABA_ARs$ responsible for mediating CA1 tonic inhibition (Caraiscos et al., 2004). α_5-$GABA_ARs$ are highly expressed in the hippocampus, representing 25% of the total hippocampal $GABA_ARs$ (Olsen & Sieghart, 2009), specifically abundant in extrasynaptic receptors of pyramidal cells and in interneurons from the CA1 area (Hörtnagl et al., 2013; Jacob, 2019). This restricted α_5-$GABA_ARs$ hippocampal expression allows us to associate this specific receptor subtype with learning and memory mechanisms (Caraiscos et al., 2004). It is therefore possible to study LTP and memory impairment when $GABA_ARs$ containing α_5 subunits are enhanced by anaesthetic agents (Crestani et al., 2002; Martin et al., 2009).

1.9. Aim of this dissertation

A common molecular mechanism of action shared among many general anaesthetics, BZDs and various endogenous neurosteroids is that they all positively allosterically modulate $GABA_ARs$. However, the affinity and efficacy of mentioned compounds are defined by the subunit composition of these receptors.

The exact neuronal processes leading to sedation during anaesthesia are still unclear, but extensive evidence supports that after being exposed to anaesthetic agents, consequent cognitive decline with neuronal degeneration of hippocampal neurons is present. It is also known that in the CNS during physiological processes such as the menstrual cycle, inflammation or stress, modification of neurosteroidogenesis is possible by TSPO activation. Therefore, to improve general anaesthesia and its posterior adverse side effects, and to elucidate suitable alternatives for BZDs currently used in perioperative anaesthesia, precise knowledge of these processes is required.

The aim of this study is to investigate the specific $GABA_AR$ subtypes modulated by several PAMs of these receptors, such as BZDs and biosynthesised neurosteroids, by means of electrophysiology in acute brain mouse slices. Combining extracellular and patch-clamp intracellular measurements in native and genetically modified $GABA_ARs$, we were able to describe the action of anaesthetic agents and neurosteroids on the synaptic, cellular and network level.

In order to address the gaps of knowledge, my project has the following objectives:

- To describe the $GABA_AR$ subunits responsible for LTP regulation in the CA1 hippocampal region after administration of BZDs, XBD173 and neurosteroids.

- To compare the kinetics of midazolam and TSPO-induced neurosteroidogenesis at modulating inhibitory synaptic transmission on the single-cell level.

- To identify the $GABA_AR$ subunits responsible for hippocampal neuroprotection after a severe ischaemic model in the presence of neurosteroids or midazolam.

- To define the $GABA_AR$ subunits responsible for the effects of midazolam and neurosteroids on inhibitory interneuron function by focusing on small network inhibition via monitoring a specific interneuron circuit at the CA1 region.

2. Materials and methods

2.1. Laboratory animals

All procedures were approved by the animal care committee (Technische Universität München, Germany) and were conducted in accordance with the German law on animal experimentation. Mice were housed in cages with *ad libitum* intake of food and water in an environmentally controlled room (23±0.5°C) respecting the 12h light/dark cycle.

Concerning extracellular recordings, 6 to 10 weeks-old female and male C57Bl6/N mice were used as wild-type (WT) model. The intracellular patch-clamp recordings were performed in younger WT mice from 3 to 5 weeks old, and they were all obtained from Charles River, Italy. As an exception, older female and male mice (17-19 weeks old) were used for $\alpha_{1/3/5}$KI and $\alpha_{2/3/5}$KI lines due to restrictions in mice production. From the several KI mice lines (female and male mice of 6-10 weeks old) used for the different experiments, α_1KI, α_5KI and $\alpha_{1/2/3}$KI were shipped from Calco, Italy and $\alpha_{1/3/5}$KI and $\alpha_{2/3/5}$KI lines were obtained from U. Zeilhofer's group in the University of Zurich, Switzerland (Table 1). In the mentioned transgenic lines, H residue was replaced by A in the genomic sequences coding for the specific α subunits of the $GABA_AR$ (Crestani et al., 2002), resulting in a desensitisation of those particular subunits in the BZD binding site, turning them resistant to modulation by allosteric modulators acting at the BZD binding site (Ralvenius et al., 2015). Although, the physiological function of the $GABA_ARs$ is not altered because the natural GABA ligand can still bind to them.

Moreover, female and male mice from the GABAδKO mouse line were used, which were bred in our own group and in this case, the animals presented a KO mutation. This implies that the gene for the δ subunits of the $GABA_ARs$ are missing, and therefore these subunits are physically lacking in the receptor (Mihalek et al., 1999). The purpose of using this line is because it exhibits a reduction in the sensitivity to the hypnotic and anxiolytic effects of neurosteroids (Boehm et al., 2006).

Table 1: Description of the mouse lines used in this project.

Mouse line	Line designation	Origin
WT	C57Bl6/N	Charles River (Italy)
α_1KI	129X1.129P2-Gabra1<tm1.1Uru/ Uru>10Gabra1SvRR	Calco (Italy)
α_5KI	Tbx18.CreERT2	Calco (Italy)

$\alpha_{1/2/3}$KI	129X1.129P2/129P2/129T2Gabra1<tm1.1Uru>Gabra2<tm1.1Uru>Gabra3<tm1.1Uru>GABAa123SvJ	Calco (Italy)
$\alpha_{1/3/5}$KI	129X1.129P2/129T2/129X1-Gabra1<tm1.1Uru>Gabra3 <tm1.1Uru>Gabra5<t1.1Uru>	Zurich (Switzerland)
$\alpha_{2/3/5}$KI	129X1.129P2/129T2/129X1>Gabra2<tm1.1Uru>Gabra3<tm1.1Uru>Gabra5 <tm1.1Uru>	Zurich (Switzerland)
GABAδKO	B6.129-Gabrdtm1Geh/J	Munich (Germany)

The electrophysiological experiments were carried out on the brains of all mice in the premises of the Clinic for Anaesthesiology and Intensive Care Medicine at the Klinikum rechts der Isar of the Technische Universität München.

2.2. Preparation of brain slices

The use of acute brain slices is a known and widespread methodology in the particular field of *in vitro* electrophysiology in neuroscience. However, laboratories are modifying this technique according to their own needs and therefore different protocols are available (Papouin & Haydon, 2018). Acute hippocampal brain slices allow us to perform an electrophysiological study of the hippocampus, whilst maintaining the most important elements of synaptic and circuit organisation also perceived *in vivo* (Lein et al., 2011).

Mice were deeply anaesthetised with vaporised isoflurane before decapitation with a guillotine. The brain was rapidly removed from the head and immediately place in ice-cold Ringer solution with the following composition (concentration in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.2 $CaCl_2$, 6 $MgCl_2$, 25 D-glucose and 1.2 NaH_2PO_4, saturated with carbogen gas (95% O_2/5% CO_2) with a final pH of 7.3. The brain was cut into two hemispheres and the cerebellum was disregarded using a razor blade. The hemispheres were glued to a metal platform with a tissue adhesive (Histoacryl®, B. Braun, Germany) and positioned into the cutting bowl, which was also filled with ice-cold Ringer and saturated with carbogen gas. Up to 8 sagittal hippocampal slices with a thickness of 350µm each were obtained using a microtome (HM 650 V; Microm International, Germany) and were placed in a submerged chamber with artificial cerebrospinal fluid (aCSF) containing (concentration in mM) 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 25 D-glucose and 1.2 NaH_2PO_4, also bubbled with carbogen. The hippocampal brain slices were initially recovering at 34°C for 30min and then at room temperature (21-23°C) for a further 60min. After this recovery time, the slices were transferred to the recording chamber of the extracellular recordings or patch-clamp setup.

2.3. Electrophysiology: extracellular recordings

2.3.1. Experimental setup

The extracellular recordings were obtained from two different setups, one located on the left side and the other on the right side. The setup on the left side consisted of a Wild M3Z Heerbrugg microscope (Wild Heerbrugg, Switzerland), two manual manipulators holding a stimulating electrode each, one electronic manipulator (Record-microcontrole, France) for the recording electrode and a light source (Euromex microscopes Holland, The Netherlands), all placed on a vibration-cushioned table via pressurised air (Spindler&Hoyer, Germany). Outside this table, there was the PM500-20 Piezomanipulator (Frankenberg, Germany) electric source for the electronic manipulator, together with the Ismatec ISM 852 pumping system (Ismatec, Switzerland). The two stimulating electrodes were connected to two ISO-STIM 01M stimulators (npi electronic GmbH, Germany) and to an BA-2S amplifier (npi electronic GmbH, Germany) where the signal was also filtered and recorded directly to the computer.

On the right-side setup, the Axiovert 35M microscope (Zeiss, Germany), the two stimulating electrodes (Narishige, Japan and SFB220 MU München, Germany) and the manual recording electrode (Narishige, Japan) were kept inside an anti-vibration table. There was also an Ismatec pumping system (Ismatec, Switzerland) and an EA-PS 3032-10B light source (EA Elektro-Automatik, Germany). The two stimulating electrodes were connected to two ISO-STIM 01M stimulators (npi electronic GmbH, Germany) and then to the EXT 10-2F amplifier (npi electronic GmbH, Germany), where the filtration was already integrated and directed to the computer through a BNC-2090A interface connection (National instruments, USA).

For measuring the field excitatory postsynaptic potentials (fEPSPs) and population spikes (PSs) signals, a glass pipette filled with aCSF was needed for the recording electrode. Borosilicate glass capillaries (inner diameter: 1.1mm; outer diameter: 1.5mm; BF150-110-10, Sutter Instrument, USA) were used to produce the pipettes for these recordings. These capillaries were pulled out with an open tip resistance of 1-2MΩ (when filled with aCSF) with the help of a micropipette puller (Hugo Sachs Elektronik-Harvard Apparatus, Germany).

Once the slice was placed in the recording chamber of the setup for the extracellular measurements, a house-made platinum ring with two nylon filaments was used to fix it at the floor of the recording chamber, immobilising it against the aCSF flow (5 ml/min)

pulsated by an Ismatec ISM 852 pumping system (Ismatec, Switzerland). All extracellular experiments were performed at room temperature.

2.3.2. fEPSPs recordings for LTP experiments

To monitor changes in the neuronal potentiation under drug influence, the slices were artificially electrically excited. fEPSPs were evoked using two bipolar tungsten self-made electrodes (50µm tip diameter), which were carefully positioned on the hippocampal Schaffer collateral-commissural pathway (SCCP). The electrodes stimulate non-overlapping populations of fibres of the SCCP with a stimulus frequency of 0.033Hz per electrode. A recording electrode covered with an aCSF-filled pipette was placed between the two stimulating electrodes to record the fEPSP signal.

Once the recording electrode reached the tissue, test stimuli were applied to establish a correct fEPSP and ensure a quality experiment. For example, the absence of PSs, the presence of a fibre volley, a low-noise signal and a growing response when stimulating intensity increases. Stimulation intensity was adjusted to a fEPSP of around -0.5 and 1mV when the signal became stable, and it remained constant throughout the whole experiment.

Stimuli were applied every 15s alternating the two stimulating electrodes. Once the fEPSP slope reached a stable plateau, a baseline recording of at least 20min was measured. Afterwards, LTP was induced by delivering a high-frequency stimulation (HFS) train (100 pulses delivered at 100Hz) through one of the two stimulating electrodes. The fEPSP response was monitored for 60min after the tetanic stimulus, maintaining the same settings used for the baseline recordings. Then, the drug of interest was applied in the aCSF solution for 60min to ensure slice incubation with the chosen drug. After this time, HFS was applied via the second electrode and the response was monitored for 60min. The use of both stimulating electrodes enabled the measurement of an internal control within the same slice (Fig. 5).

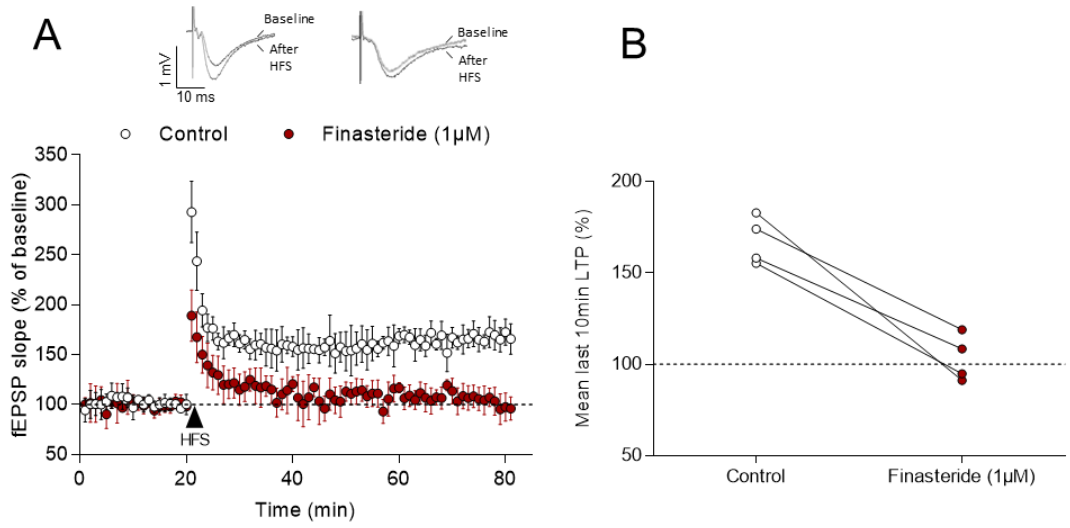

Fig. 5: Example of a LTP experiment.

(A) Each symbol represents the averaged fEPSP slopes (mean±SD) and all responses were normalised to the baseline recorded for 20min before delivering the tetanic stimulus (arrow). The insets show representative fEPSP traces before and after HFS for control and drug conditions (finasteride 1µM; Merck, Germany). **(B)** Connected scatter plot of the fEPSP slope from the min 50 to 60 after HFS. LTP was blocked but not significantly reduced after the application of finasteride 1µM.

Utilising the WinLTP software (Anderson & Collingridge, 2001), fEPSP slopes were recorded throughout the whole experiment and calculated between 20-80% of the peak amplitude. To determine an LTP, the potentiation of the fEPSP slope should be at least 20% higher in comparison to the slope in the baseline recordings (100%) when compared the slopes from min 50 to 60 (range of the last 10min) after the HFS delivery. Values were reanalysed offline and then normalised to the 20min baseline before HFS. Control experiments supported that the extent of LTP was not dependent on the time that slices were in the recording chamber, at least for the maximum duration of the present studies (up to 5h).

2.3.3. fEPSPs recordings for hypoxia/hypoglycaemia (H/H) experiments

In order to mimic an excitotoxicity situation in the brain, we measured how oxygen and glucose deprivation (OGD) affects the CA1 hippocampal structure by monitoring the fEPSP's slope with the WinLTP program. The same protocol as in the LTP experiments was used to establish a proper fEPSP. Although both stimulating electrodes were positioned on the slice, only the one producing the most stable measurement was chosen for posterior data analysis. With every mouse used, a control experiment (drug-free conditions) and an experiment with a prospective neuroprotective drug were performed.

Once the fEPSP slope reached a stable plateau for at least 20min for the control experiments or after 1h with the drug application, a 25min H/H period was induced (Fig. 6). At this point, the normal aCSF was substituted for a D-Mannitol-based aCSF (instead of glucose) and the carbogen gas source was stopped, while a 95% N_2/5% CO_2 was introduced to the modified aCSF. All these changes were necessary to eliminate all the glucose and oxygen traces from the brain slices and induce like this a state of hypoxia and hypoglycaemia via an OGD model. After this time, normoxic conditions were restored and the aCSF was replaced with the initial one, starting the 60min recovery phase and monitoring the possible neuroprotective effects of the drugs used with the fEPSP slope.

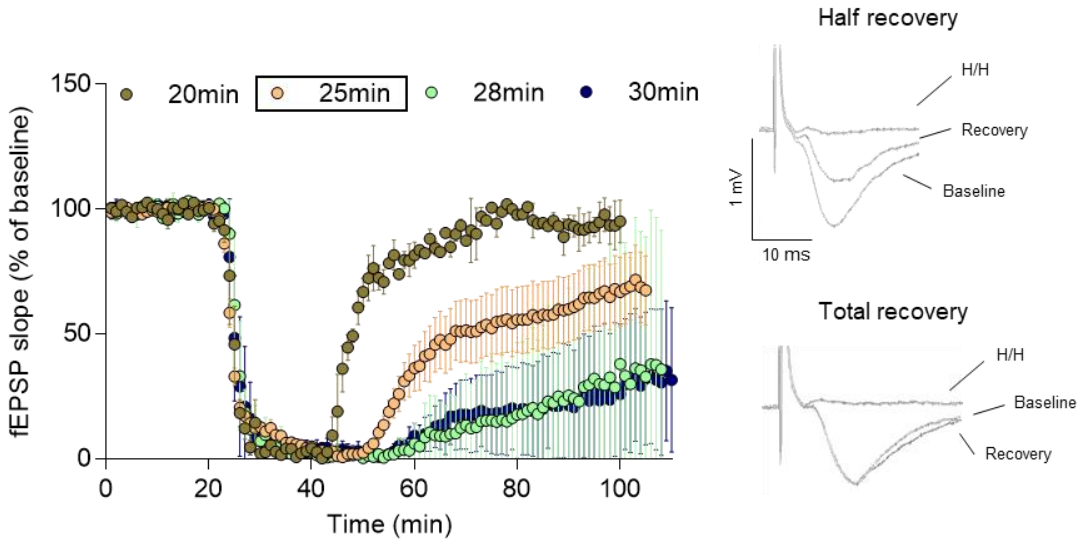

Fig. 6: Plotting of the fine-tuning of the H/H experiments.

Each symbol represents the averaged fEPSP slopes (mean±SD) and all responses were normalised to the baseline recorded for 20min prior to the OGD period started. The insets show representative fEPSP traces during the baseline, the H/H and the recovery periods in control conditions for a total (e.g., 20min) and half recovery (e.g., 25min). Different OGD periods were assessed (from 7 to 50min), delimiting the time window from 20 to 30min, where we determined that 25min was the correct period. 25min of H/H caused some detrimental effects on the slices without either killing them or let them completely recover.

fEPSP's slope values were reanalysed offline with WinLTP software and then normalised to the 20min baseline before H/H.

2.3.4. PS recordings for paired-pulse inhibition (PPI) experiments

The input and output of the excitatory networks in the hippocampus are controlled by various types of inhibitory GABAergic interneurons (Fricker & Miles, 2001; McBain, 2001; Somogyi & Klausberger, 2005). To study the GABAergic inhibition in the CA1 interneuron circuit, a PPI paradigm adapted from Petrides et al., (2007), was designed in which two artificially electrical paired orthodromic stimuli were used to evoke two PSs and record

the amplitude of the second one. A PS was evoked by a usually used self-made bipolar electrode located in the SCCP (orthodromic stimulus) and recorded with the recording electrode positioned in the soma of the CA1 pyramidal cells. The other electrode, in this case a cluster bipolar microelectrode (FHC INC, USA), was positioned in the distal fibres of the *Stratum lacunosum moleculare* (SLM) where it was possible to stimulate a population of interneurons (Fig. 7A and B). The stimulation in the SLM was paired 10ms later with the stimulation in the SCCP because 10ms is the delay time between the two stimuli when the maximum inhibition was reached. Consequently, the activation of the interneurons from the SLM could modulate the PS response in the SCCP's neurons.

Once about the 90% of the maximal PS from CA1 neurons was obtained, the intensity of the second electrode in the SLM was increased until its own PS appeared and diminished the PS from the SCCP by 30-70%.

Furthermore, an altered version of the initial protocol (PPI2) was introduced by following Cayla and colleagues (2019), who proposed an increased delay time between the pulses to differentiate amongst the different properties of GABAergic inhibition. In our PPI2 protocol, only one stimulating electrode in the Schaffer collaterals was needed, while the recording electrode stayed in the soma of the CA1 pyramidal neurons. The main difference between PPI and PPI2 is that in the latter, a 100ms delay time was set between two pulses from the same stimulating electrode. This delay time was optimised to 100ms for the purpose of discriminating between $GABA_AR$-fast-mediated inhibition (1st PS) and $GABA_AR$-slow-mediated inhibition (2nd PS). The recording started once the maximum PS amplitude was reached.

In both cases, the experiment begun with a baseline monitoring of at least 20min, followed by the drug application (60min) whilst monitoring the PS amplitudes (Fig. 7C and D), which indicates an increase or decrease in the SLM interneuron inhibition.

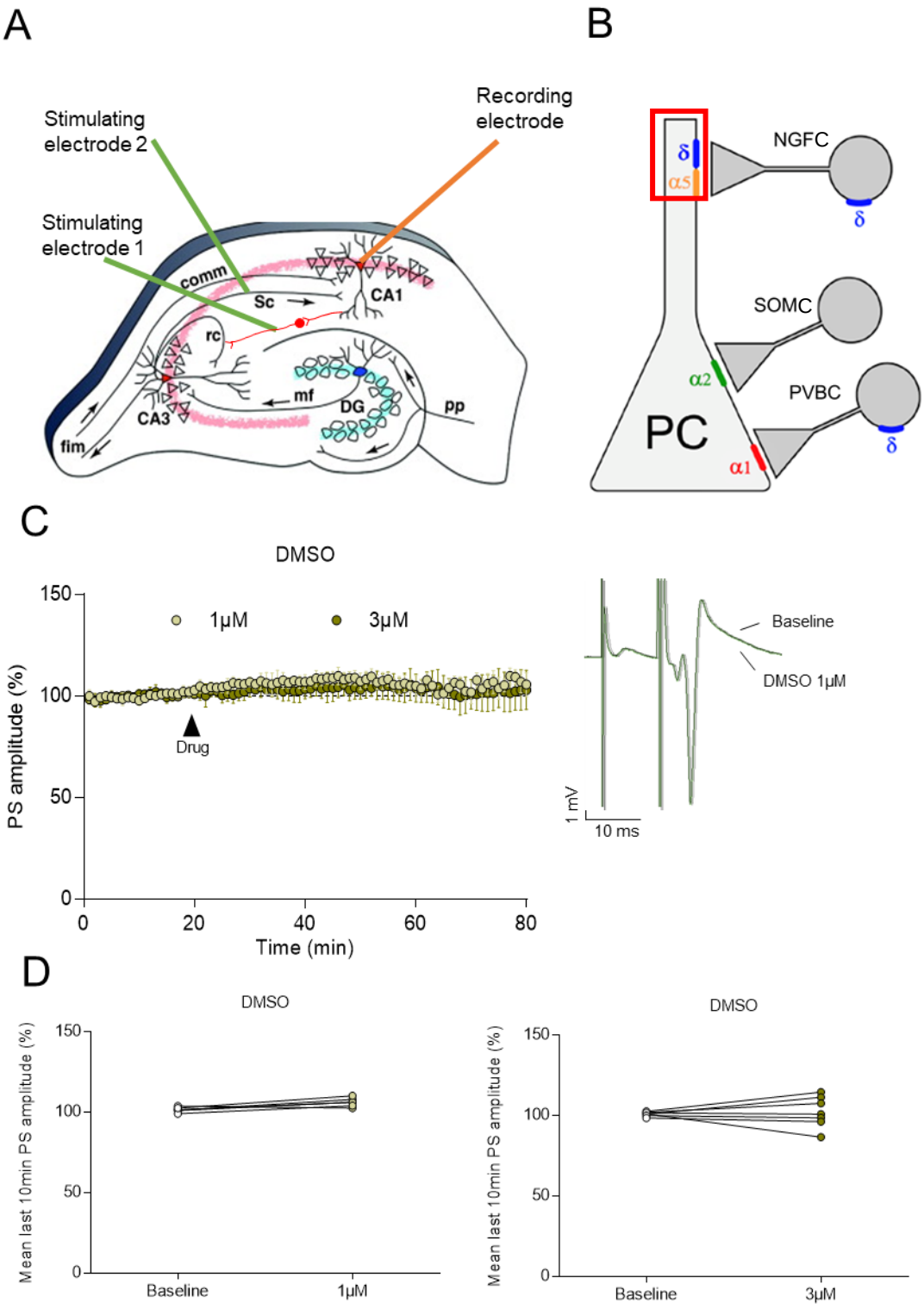

Fig. 7: Example of a PPI experiment.

(A) Schematic diagram of a PPI experiment, with the position of the stimulating electrode 1 at the SLM (neuron in red), while the stimulating electrode 2 excite the SCCP fibres. The recording electrode is located at the somata of the CA1 pyramidal neurons, recording PS amplitudes; adapted from (Yassa & Stark, 2011). **(B)** Diversity of GABAergic inhibition of hippocampal pyramidal cells (PCs) with the representation of parvalbumin-expressing basket cells (PVBC), neurogliaform cells (NGFC) and somatostatin-positive cells (SOMC). The $GABA_AR$ subunits α_1 (red), α_2 (green), α_5 (orange) and δ (blue) are also shown, and the red box highlights the specifically targeted subunits. **(C)** Time course of an experiment. Each symbol represents

the averaged PS amplitude (mean±SD) and all responses were normalised to the baseline recorded for 20min before the drug administration. The insets show representative PS traces during the baseline and in the last 10min of dimethyl sulfoxide (DMSO; Merck, Germany) application. **(D)** Connected scatter plots of the PS amplitude from the min 50 to 60 after DMSO 1µM (left) and 3µM (right) application. This solvent does not modulate the PS amplitude significantly neither at 1µM nor at 3µM.

The amplitude of the PS was reanalysed offline with WinLTP software and then normalised to the 20min baseline before adding the drug.

2.4. Electrophysiology: patch-clamp recordings

The slice was positioned in the recording chamber of the patch-clamp setup and fixed with a house-made platinum ring with two nylon filaments to immobilise it against the aCSF flow (10ml/min) pulsated by an MV-CA/4 pump (Ismatec, Switzerland). All patch-clamp experiments were performed at room temperature.

2.4.1. Experimental setup

The patch-clamp setup including recording chamber, microscope, camera, light source, and electrodes was safely positioned on a vibration-cushioned table via pressurised air (TMC syst: 63-560, Harvard Apparatus, Canada). To avoid electrical noise, it was encased by a Faraday cage and earthed. The epifluorescence microscope used (BX51Wl Olympus, Germany) was equipped with a TH4-200 lamp (Olympus, Japan) and with XLFLuor4x/340 (NA 0.28; Olympus, Germany) and ACHROPLAN 63x/0.90w as objective lens. A SM5 motorised micromanipulator (Luigs and Neumman, Germany) enabled the movement of the microscope and the stimulating electrode independently of each other, making it possible to arrange one in all three dimensions, without moving the other. Moreover, a SM1 motorised micromanipulator (Luigs and Neumman, Germany) was used to position the recording electrode in the desired position.

All required devices were located in a rack (Stemmer GmbH, Germany), such as the TBS 1032B oscilloscope (Tektronix, USA) and a HEKA InstruTECH LIH 8+8 high resolution, low-noise scientific data acquisition system (AutoMate Scientific, Inc., USA). Furthermore, the amplifier SEC-10L and the external stimulator ISO-stim 01M were used for these recordings (npi electronic GmbH, Germany).

Borosilicate glass capillaries (inner diameter: 1.1mm; outer diameter: 1.5mm; BF150-110-10, Sutter Instrument, USA) were utilised to produce the pipettes for the patch-clamp recordings in the whole-cell mode. Via the micropipette puller (Hugo Sachs Elektronik-Harvard Apparatus, Germany), glass pipettes were obtained with, in this case, a final open tip resistance of 4-6MΩ when filled with a liquid called intracellular solution (IS). The composition of the IS was accordingly adjusted for optimal measurements

conditions. To measure inhibitory $GABA_AR$-mediated currents, a high-chloride IS was used with the following composition (concentrations in mM): 140 KCl, 5 NaCl, 0.1 EGTA, 10 HEPES, 2 Mg^{2+}-ATP, 0.3 Na_2-GTP and 10 Phosphocreatine TRIS. The pH was adjusted at 7.2 with KOH. Lidocaine (5mM, Merck, Germany) was added to the IS to block the voltage-gated sodium channels from the patched cell, which are crucial for the generation and propagation of neuronal action potentials (Cummins, 2007).

2.4.2. Whole-cell patch-clamp recordings

To monitor changes in synaptic neuronal transmission before and after drug application, spontaneous IPSCs (sIPSCs) were measured with a recording electrode covered by the glass pipette filled with IS. As soon as the CA1 pyramidal region was recognised under the microscope, both recording electrode and microscope were manually adapted to reach the surface of the slice. The Hokawo imaging software version 3.0 (Hamamatsu Photonics, Japan) for the camera imaging and PatchMaster v2x90.3 (HEKA Elektronik GmbH, Germany) for the patch-clamp recordings were utilised.

Once a candidate cell was localised, the recording pipette was carefully approached and the cell membrane was reached, while the amplifier was in bridge mode, and a soft and steady negative-pressure air was applied. Then, the holding current decreased, achieving the attached mode. As a confirmation, the amplifier was switched to voltage-clamp mode, making sure that the holding current was at 0.01pA, indicating a tight seal. Thereafter, an abrupt and short negative-pressure air was applied to open the cell.

When the cell was finally open, a time of 6-10min was given for the IS from the cell and the pipette to exchange and an equilibrium could be reached. Subsequently, an IV test was performed to check the quality of the cell, and after ensuring its good condition, the monitoring of the cell was initiated.

2.4.3. $GABA_AR$-mediated currents: sIPSCs

$GABA_AR$-mediated currents were measured in voltage-clamp mode, holding the cell membrane potential constant at -70mV. To assess the effect of the several drugs on the phasic component of $GABA_AR$-mediated inhibition, the sIPSCs were measured. sIPSCs are inhibitory postsynaptic currents that arise through spontaneous exocytosis of individual GABAergic vesicles and by the occurrence of spontaneous presynaptic action potentials in the neuronal circuit (Ropert et al., 1990). For example, an increase in the decay time of IPSCs is regulated by the efficacy of GABA uptake and therefore the probability of the $GABA_AR$ channel to be open during more or less time, and the postsynaptic $GABA_AR$ subunit composition (Roepstorff & Lambert, 1994; Draguhn &

Heinemann, 1996). On the contrary, an increase in the IPSC amplitude is most likely due to an increased density or conductivity of postsynaptic receptors (O'Brien et al. 1998).

$GABA_AR$-mediated sIPSCs were measured under control conditions and after application of our drug of interest. Several parameters can be measured from a sIPSC and in our case, peak amplitude and decay time were quantified. A monoexponential function was not a good fit for the recollected data; therefore, a bi-exponential fit was applied and the decay time in this case was decomposed into fast (τ_1) and slow (τ_2) decay time (Fig. 8 and see section 2.5.2).

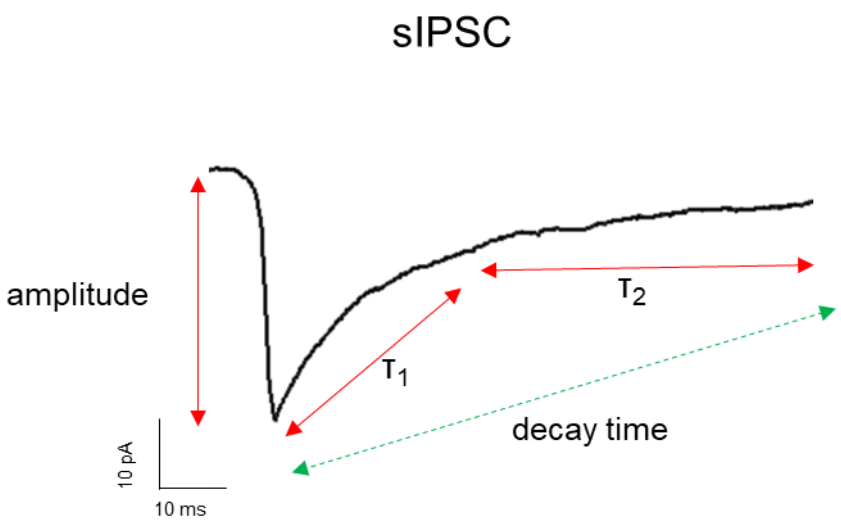

Fig. 8: Example of a sIPSC trace.

Peak amplitude and decay time were measured. However, after a bi-exponential fitting, τ_1 and τ_2 values were obtained from the decay time parameter. This is because the sIPSC presents two different decay times, and they must be singularly identified. τ_1 corresponds to the $GABA_AR$-fast-mediated inhibition, while τ_2 indicates $GABA_AR$-slow-mediated inhibition.

All recordings were conducted in presence of the specific NMDAR and AMPAR antagonists D-AP5 (50µM; Tocris, Germany) and NBQX (5µM; Tocris, Germany) respectively, in order to block the glutamatergic currents. Moreover, the specific $GABA_BR$ antagonist CGP55845 (5µM; Tocris, Germany) was also applied. At the end of the sIPSCs recording after drug application, the $GABA_AR$ antagonist bicuculline (10µM; Merck, Germany) was administered and the occurrence of sIPSCs was completely eliminated. This verified that the measurements were pharmacologically isolated $GABA_AR$-mediated currents.

2.5. Evaluation and statistical analysis

2.5.1. Extracellular recordings

For LTP experiments, after normalising the last 10min post-HFS (from min 50 to 60) with the baseline recordings, the control experiments were compared to the experiments with the drug of interest. This comparison allowed us to see if the different substances were able to modulate LTP.

Concerning the H/H experiments, fEPSP slope was firstly normalised to the baseline values before inducing the OGD period. Then, the last 10min of the 60min recovery time (control vs drug experiments) were compared. This comparison enabled us to see if the drugs assessed have a significant neuroprotective effect after a 25min period of H/H.

Similar to the previous described experiments, for the PPI and PPI2 reanalysis a comparison between the last 10min of the drug wash-in (from min 50 to 60) and the baseline values was performed. Before starting to apply the drugs of interest, control experiments (no drug) and administration of the solvent DMSO alone (Fig. 7C and D) were evaluated to ensure that these substances per se did not modulate the PS amplitude.

2.5.2. Patch-clamp recordings

Data were firstly replayed and exported from the PatchMaster v2x90.3 (HEKA Elektronik GmbH, Germany) software and secondly evaluated with the Minianalysis software (Synaptosoft, USA). Individual sIPSCs were recognised by the automatic detection protocol of the Minianalysis program and then revised manually. $GABA_AR$-IPSCs suggested detection parameter and a detection threshold of 5 times higher than the SD of the baseline current in absence of IPSCs were applied. Only uncontaminated events were selected, resulting in data collection on decay time and peak amplitude. The compiled sIPSCs were averaged graphically with a peak alignment and a bi-exponential function ($y(t)=A_1e^{(-t/\tau 1)} + A_2e^{(-t/\tau 2)}$), where y(t) corresponds to the current amplitude at any given time t, A is the current amplitude at time zero and τ is the decay time constant. Here, the values for the IPSC's $GABA_{A,fast}$ (τ_1) and $GABA_{A,slow}$ (τ_2) components were extracted, as an alternative of a singular decay time value which is no longer accurate due to the bi-exponential time course. The best fit was determined by eye and when the R^2 value was close to 1.

2.5.3. Statistics

No specific randomisation or blinding method was used for the designation of individual animals to experimental conditions. The n value is shown as x slices out of y animals as, e.g., n=8/4; being the first value the number of slices and the second value the total number of animals used at those conditions. The sample size was determined based on previous experience and a maximum of two slices per animal were used, based on the assumption that these slices were independent within animals.

Statistical analysis, as well as graph elaboration, was performed using GraphPad Prism 6.01 (GraphPad Software, USA). Due to the small sample size and therefore the impossibility to check for normality, data were not considered eligible for parametric testing. Thus, the pertinent non-parametric tests were applied. When the control and post-administration of the drug recordings were monitored in the same brain slice (linked samples), the paired Wilcoxon test was used. This includes whole-cell patch-clamp, LTP and PPI experiments. In contrast, when the control and post-drug application were measured in different slices leading to independent or unrelated samples (e.g., H/H experiments), the unpaired Mann-Whitney test was performed. All data from LTP, PPI and patch-clamp experiments are shown as mean±SD in percentage. For H/H experiments, time course values are also shown as mean±SD in percentage, but data from the last 10min of the experiments are reported as median(IQR) in percentage, being IQR the interquartile range obtained from the difference between the third and the first quartile ($IQR=Q_3-Q_1$). This is because these values are independent samples, and this data reporting is more precise. Statistical significance is indicated in the plots with an asterisk (*) when $p<0.05$.

3. Results

3.1. Modulation of LTP by BZD-binding site acting drugs and neurosteroids

3.1.1. Midazolam inhibits LTP

Midazolam (B. Braun) application clearly blocked the LTP after 60min of exposure. As depicted in the graph below (Fig. 9), midazolam at the low concentration of 10nM (98±11% vs control 151±15%, n=14/8, p<0.001) could significantly block the LTP in a similar level as 1µM (94±17% vs control 167±23%, n=6/5, p=0.031) in WT mice.

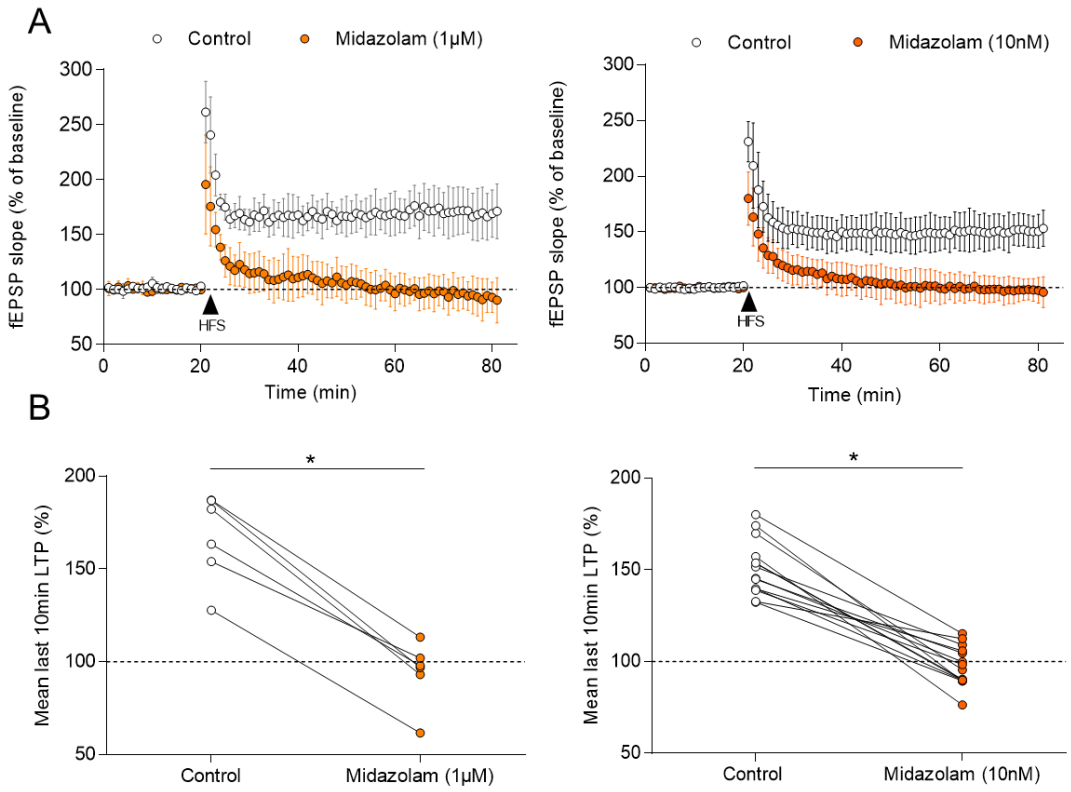

Fig. 9: Midazolam (10nM-1µM) significantly blocks LTP in WT mice.

(A) Normalised fEPSP slope time course following an HFS under control conditions and after 60min of midazolam 1µM (left) and 10nM (right) exposure. **(B)** Connected scatter plots summarising min 50 to 60 after HFS. LTP was significantly blocked after midazolam 1µM (left; 94±17% vs control 167±23%, n=6/5, p=0.031) and 10nM (right; 98±11% vs control 151±15%, n=14/8, p<0.001) when compared to their respective controls in WT animals. Adapted from Puig-Bosch et al., (2022).

To explore the role of the different $GABA_AR$ subunits in the presence of midazolam, several transgenic mouse lines were used, where one or three α-subunits carry a H to R mutation. The action of midazolam on evoked LTP was investigated in brain slices in which α_1-, α_2-, α_5-, $\alpha_{1/2/3}$-, $\alpha_{1/3/5}$- and $\alpha_{2/3/5}$-subunit-containing $GABA_ARs$ are insensitive to

midazolam. Due to the low expression of the α_3 in the hippocampus (Fritschy & Panzanelli, 2014), the focus was not on this subunit.

When HFS was administered to α_1KI mice after exposure to 10nM midazolam (Fig. 10A), LTP was not significantly altered (136±9% vs control 141±9%, n=18/13, p=0.174), whereas a significant blockage of the LTP was observed in slices of $\alpha_{2/3/5}$KI (Fig. 10B) (106±12% vs control 167±27%, n=12/6, p<0.001), α_5KI (Fig. 10C) (106±17% vs control 143±12%, n=20/15, p<0.001) and $\alpha_{1/2/3}$KI mouse lines (Fig. 10D) (101±21% vs control 148±10%, n=13/11, p<0.001). On the contrary, LTP on slices from $\alpha_{1/3/5}$KI mice (Fig. 10E) (141±10% vs control 139±12%, n=12/6, p=0.547) was not significantly modulated after the application of midazolam 10nM.

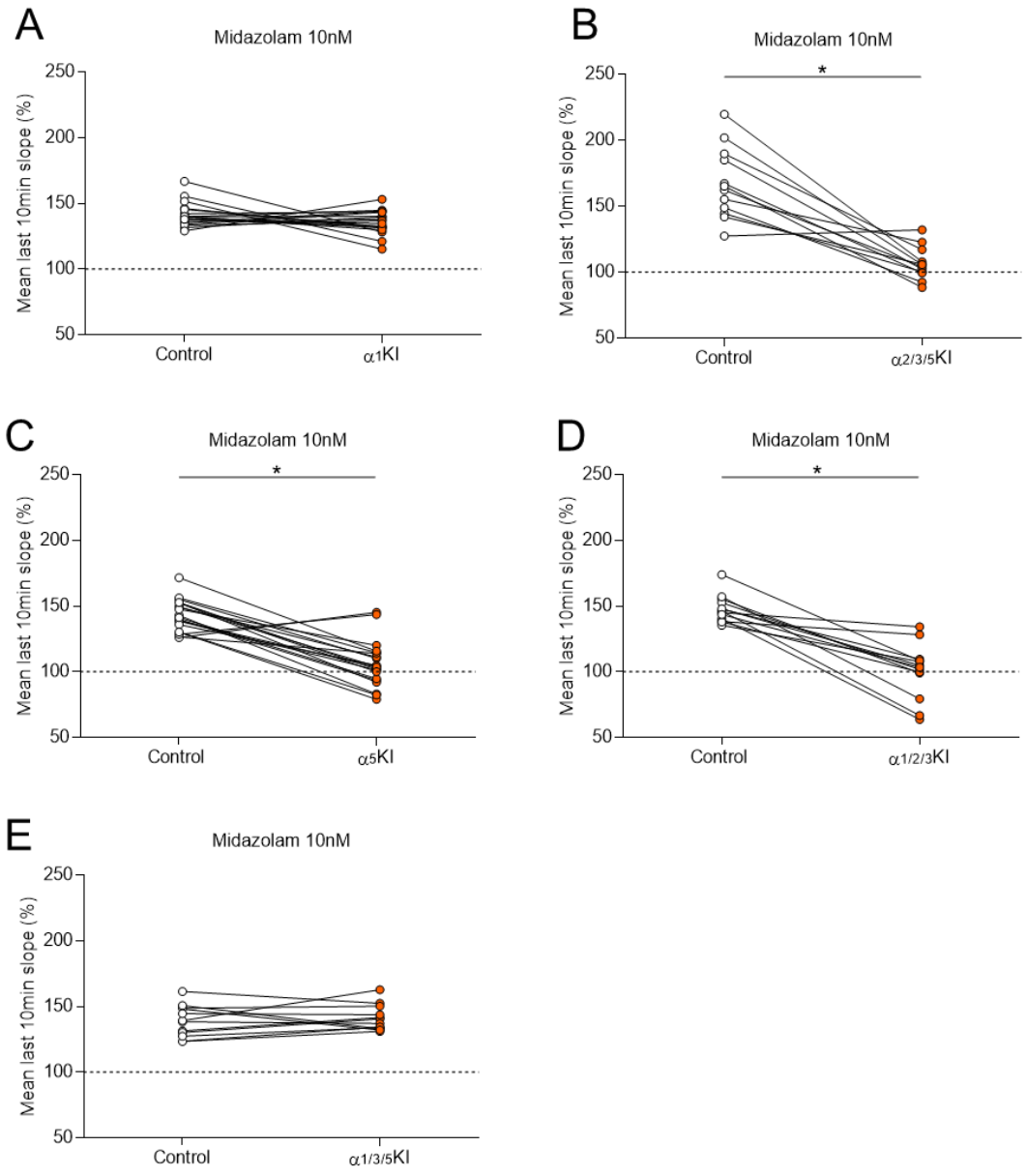

Fig. 10: Effect of midazolam on LTP in α_1KI, $\alpha_{2/3/5}$KI, α_5KI, $\alpha_{1/2/3}$KI and $\alpha_{1/3/5}$KI genotypes.

Connected scatter plots summarising min 50 to 60 after HFS for control and after midazolam 10nM exposure in the different KI transgenic mouse lines. **(A)** In α_1KI genotype, LTP is not altered when compared to control (136±9% vs control 141±9%, n=18/13, p=0.174). **(B)** In $\alpha_{2/3/5}$KI (106±12% vs control 167±27%, n=12/6, p<0.001), **(C)** α_5KI (106±17% vs control 143±12%, n=20/15, p<0.001) and **(D)** $\alpha_{1/2/3}$KI genotypes (101±21% vs control 148±10%, n=13/11, p<0.001) LTP was significantly blocked after 10nM midazolam. **(E)** LTP on $\alpha_{1/3/5}$KI mice (141±10% vs control 139±12%, n=12/6, p=0.547) was not significantly altered. Adapted from Puig-Bosch et al., (2022).

These results revealed that the effect of midazolam at small concentrations in WT mice is mainly mediated by α_1-subunits (Fig. 10A and B). Nevertheless, when these subunits are insensitive to BZD due to a KI point mutation, the combined action of midazolam on α_2- or α_5-subunits is unable to block LTP and in $\alpha_{1/2/3}$KI genotype (α_5 is intact) LTP resulted midazolam-sensitive (Fig. 10D). As represented in Fig. 11, a possible explanation to put these results together might be that α_2-subunit-dependent modulation of α_5-GABA$_A$Rs in controlling LTP when α_5 subunits are insensitive. This hypothesis emerges from the comparison between Fig. 10A (α_1KI becomes LTP midazolam-insensitive with α_2, α_3, and α_5 subunits intact) and Fig. 10D ($\alpha_{1/2/3}$KI renders LTP midazolam-sensitive with only α_5 intact).

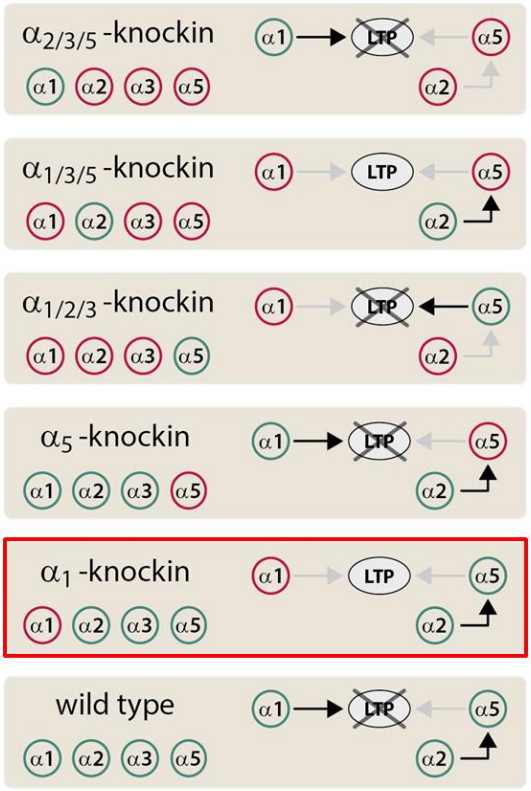

Fig. 11: Schematic model of midazolam on LTP in six different genotypes.

A proposed model assumes that midazolam can inhibit LTP via α_1-GABA$_A$Rs in WT mice, and α_5 but not through α_2 subunits in α_1KI mice. Additionally, the model assumes an inhibitory action of α_2-GABA$_A$Rs onto α_5-GABA$_A$Rs, as indicated with arrows. Marked with a red box is the critical mechanism of our assumption. Red circles indicate receptor subtypes that are resistant to midazolam, and green circles denote responsiveness to the drug. Figure and text adapted from Puig-Bosch et al., (2022).

3.1.2. Flumazenil antagonises the effect of midazolam on LTP

After demonstrating that midazolam 10nM blocked LTP, it was important to confirm that this effect was uniquely mediated through the classical BZD site. Hence, the specific BZD-binding site antagonist flumazenil (Votey et al., 1991) was used for this validation.

Firstly, it was assessed whether flumazenil (Merck, Germany) per se alters LTP, but it did not display any intrinsic effect when it was applied alone at 30nM (154±24% vs control 158±23%, n=8/4, p=0.945) (Fig. 12A and B). Thus, then proceeded with a co-application (Fig. 12C), in which after the preapplication of midazolam, flumazenil was able to significantly reverse LTP inhibition caused by the BZD when applied at a final 3:1 stoichiometric excess to flumazenil (midazolam 113±8% vs midazolam+flumazenil 145±17%, n=10/5, p=0.002). It was also observed that a preapplication of flumazenil (Fig. 12D) prevents the consequent blockage of LTP after midazolam exposure (flumazenil 157±25% vs flumazenil+midazolam 131±18%, n=8/4, p=0.109) at the same stoichiometry.

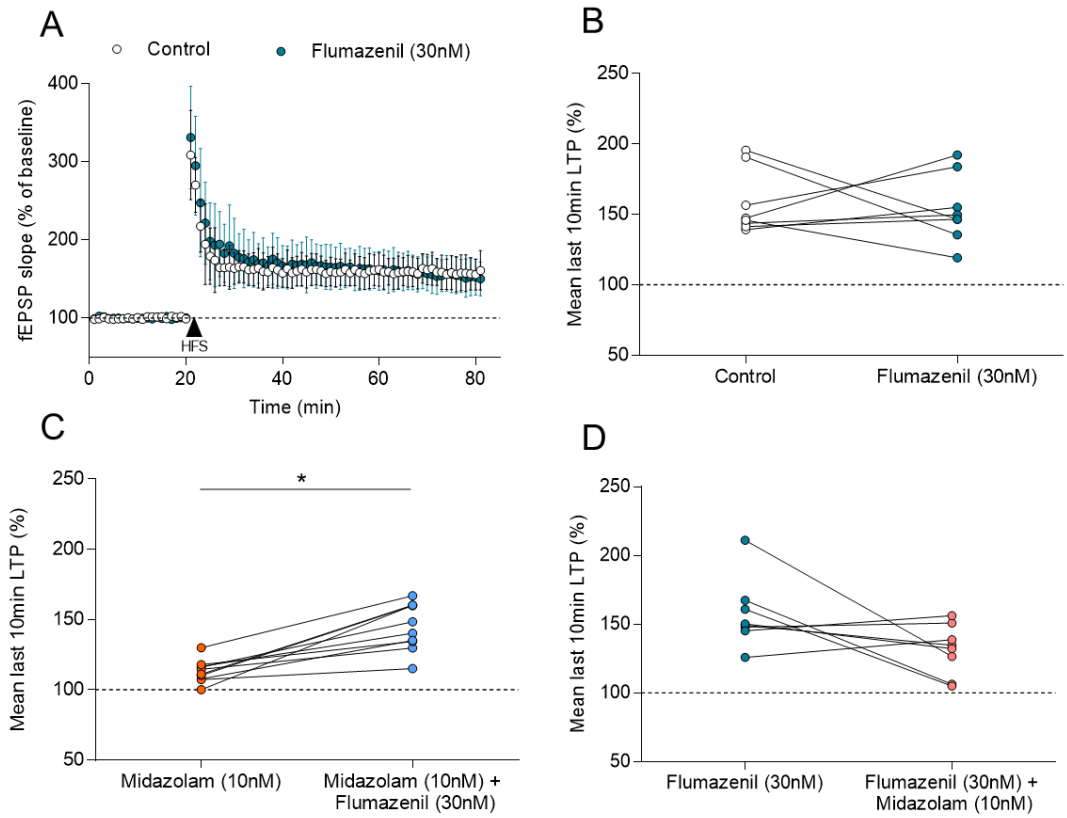

Fig. 12: LTP modulation by flumazenil.

(A) Normalised fEPSP slope time course following an HFS under control conditions and after 60min of flumazenil 30nM application. (**B)** Connected scatter plot summarising min 50 to 60 after HFS in control and drug conditions. LTP was not significantly altered after flumazenil 30nM exposure when compared to control

(154±24% vs control 158±23%, n=8/4, p=0.945). **(C, D)** Connected scatter plots summarising min 50 to 60 after HFS in midazolam and flumazenil co-application. **(C)** LTP blockage caused by midazolam 10nM (113±8%, n=10/5) was reversed by flumazenil 30nM (145±17%, n=10/5, p=0.002) and **(D)** when midazolam 10nM was administered after flumazenil 30nM, flumazenil could prevent the LTP blockage of midazolam 10nM (flumazenil 157±25% vs flumazenil+midazolam 131±18%, n=8/4, p=0.109). Adapted from Puig-Bosch et al., (2022).

3.1.3. MRK-016 modulates LTP

To assess the involvement of α_5-$GABA_A$Rs subtype on LTP modulation, MRK-016 was applied because of its high selectivity for this subunit. In our experiments, LTP was significantly increased in WT mice (Fig. 13A and B) after MRK-016 100nM (Tocris, Germany) application in comparison to control (158±19% vs control 147±6%, n=10/5, p=0.032), whereas in α_5KI animals (Fig. 13C), LTP was significantly blocked (106±10% vs control 146±13%, n=7/7, p=0.016). Nevertheless, no significant changes were seen in α_1KI (143±8% vs control 149±16%, n=8/8, p=0.250) and $\alpha_{1/2/3}$KI (127±39% vs control 148±8%, n=9/9, p=0.074) genotypes after LTP induction (Fig. 13D and E).

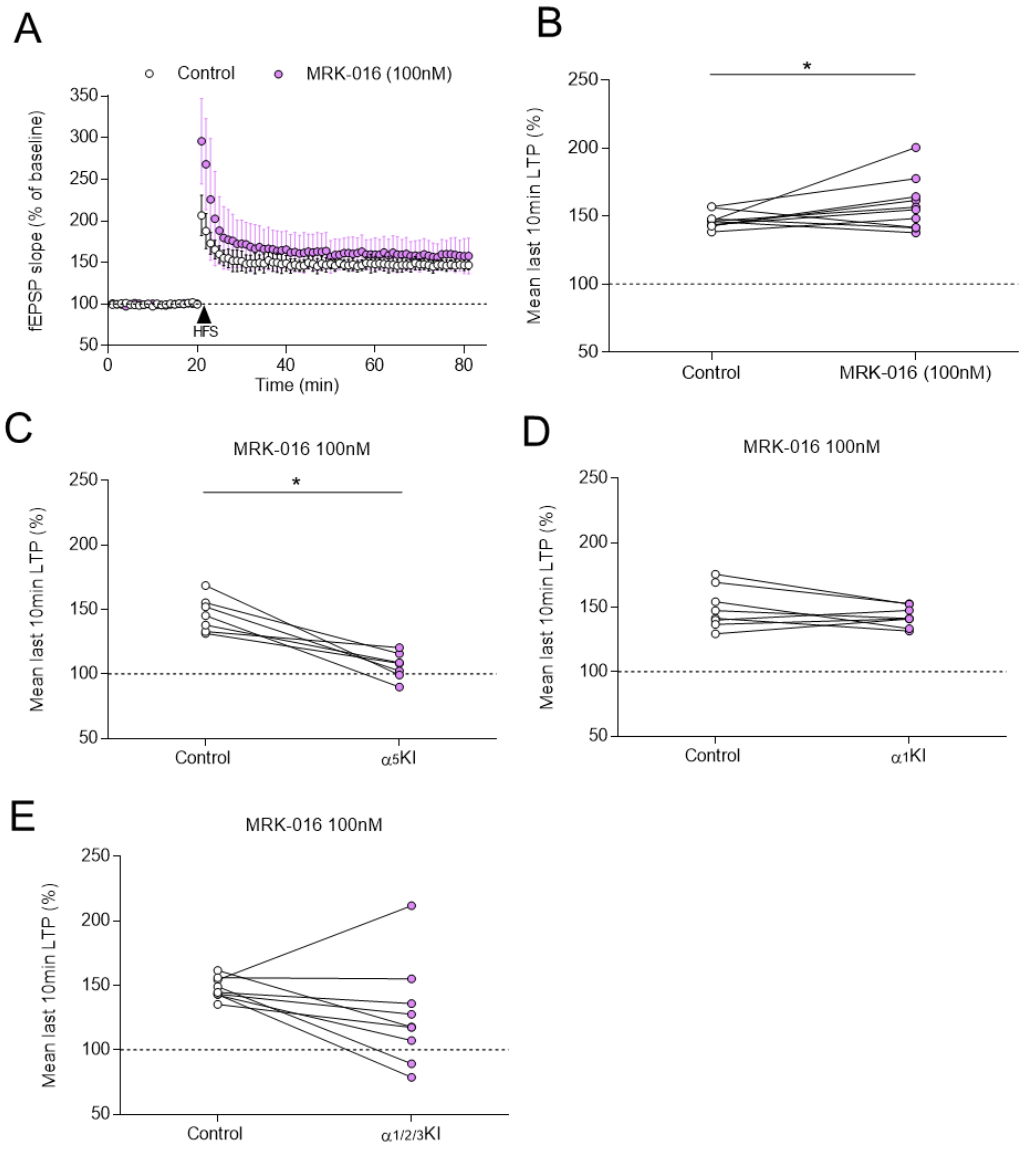

Fig. 13: LTP modulation of MRK-016 in WT and α_5KI, α_1KI and $\alpha_{1/2/3}$KI mouse lines.

(A) Normalised fEPSP slope time course following an HFS under control conditions and after 60min of MRK-016 100nM application in WT mice. **(B-E)** Connected scatter plots summarising min 50 to 60 after HFS in control and MRK-016 100nM. **(B)** In WT animals, LTP was significantly increased after MRK-016 100nM (158±19% vs control 147±6%, n=10/5, p=0.032). **(C)** In α_5KI mice, LTP was significantly blocked (106±10% vs control 146±13%, n=7/7, p=0.016), but in α_1KI **(D)** (143±8% vs control 149±16%, n=8/8, p=0.250), and $\alpha_{1/2/3}$KI animals **(E)** (127±39% vs control 148±8%, n=9/9, p=0.074), no significant LTP alteration. Adapted from Puig-Bosch et al., (2022).

3.1.4. Zolpidem can modulate LTP

Since it was possible to elucidate that α_1-GABA$_A$Rs are highly involved in LTP modulation in the experiments with midazolam, we aimed to confirm this fact by employing the drug zolpidem due to its high selectivity towards the α_1 subunit. Zolpidem (Merck, Germany) application at 100nM had no effect on LTP induction (136±14% vs control 148±13%, n=8/7, p=0.148). However, when the concentration was raised to 1µM, LTP was then

significantly inhibited (101±15% vs control 160±26%, n=8/8, p=0.008) (Fig. 14A and B). Interestingly, when zolpidem 1µM was administered in α_1KI brain slices (Fig. 14C), LTP returned to baseline levels (136±12% vs control 136±10%, n=8/8, p=0.945). With these results, it is possible to determine that zolpidem effectively modulates LTP at a concentration of 1µM via α_1-GABA$_A$R subunits.

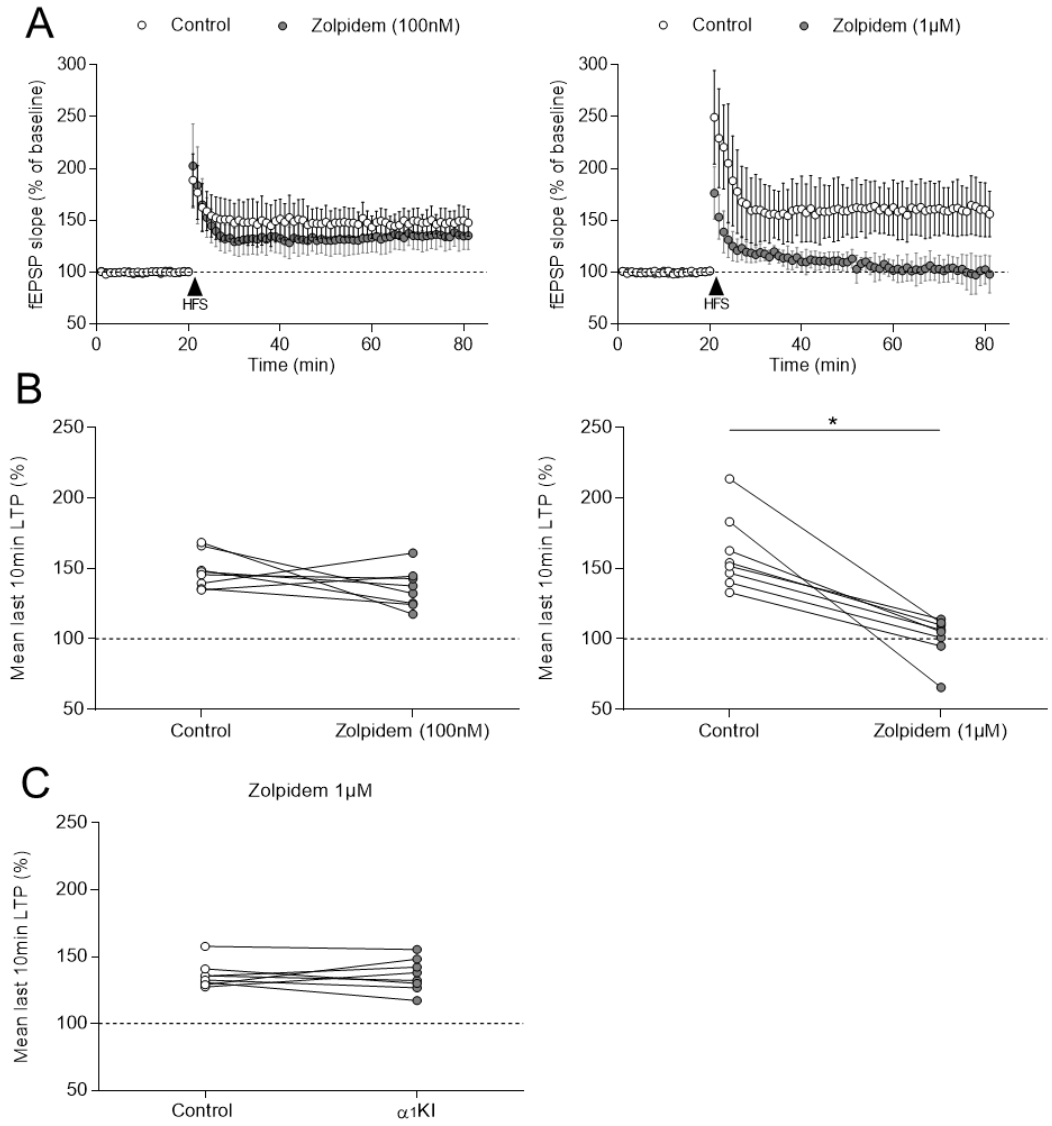

Fig. 14: Effect of zolpidem on LTP in WT and α_1KI mice.

(A) Normalised fEPSP slope time course following an HFS under control condition and after 60min of zolpidem 100nM (left) and 1µM (right) in WT mice. **(B)** Connected scatter plots summarising min 50 to 60 after HFS in control and drug conditions in WT mice. After application of zolpidem 100nM, LTP was not altered (left; 136±14% vs control 148±13%, n=8/7, p=0.148), while a significant LTP blockage occurred at 1µM (right; 101±15% vs control 160±26%, n=8/8, p=0.008). **(C)** Connected scatter plot summarising min 50

to 60 after HFS in control and zolpidem 1μM in α_1KI mice, showing a non-alteration of LTP after zolpidem when compared to control (136±12% vs control 136±10%, n=8/8, p=0.945).

3.1.5. Diazepam inhibits LTP

Diazepam is a widely used BZD with a long-lasting effect. We aimed to compare diazepam's mechanism of action with the one shown after midazolam exposure on LTP. Diazepam (Merck, Germany) application at 1μM in WT mice (Fig. 15A and B) resulted in a significant LTP blockage (115±22% vs control 148±15%, n=9/6, p=0.019). Nonetheless, when diazepam was tested in α_1KI (141±28% vs control 144±13%, n=8/8, p=0.312) and $\alpha_{1/2/3}$KI (141±11% vs control 149±22%, n=9/9, p=0.496) brain slices, LTP was not modified in comparison to the control (Fig. 15C and D, respectively). Interestingly, when the drug was washed-in in slices from the α_5KI genotype (Fig. 15E), LTP was significantly reduced (121±11% vs control 140±7%, n=10/10, p=0.006). With these results, it is feasible to determine that diazepam effectively blocks LTP at a concentration of 1μM via α_1-$GABA_AR$ subunits.

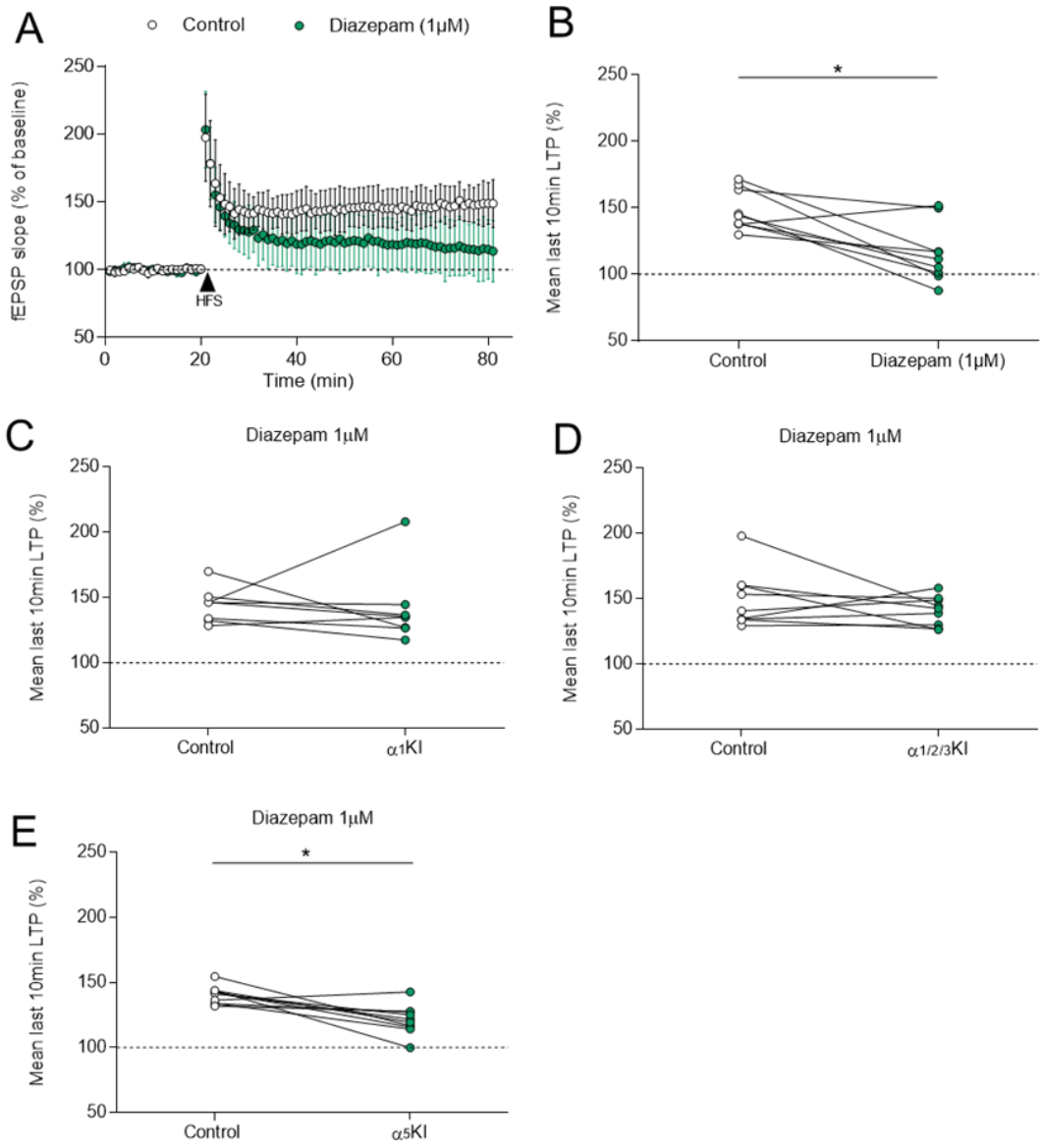

Fig. 15: Effect of diazepam on LTP in WT and α_1KI, $\alpha_{1/2/3}$KI and α_5KI mice.

(A) Normalised fEPSP slope time course following an HFS under control condition and after 60min of diazepam 1µM in WT mice. **(B)** Connected scatter plot summarising min 50 to 60 after HFS in control and diazepam 1µM administration, showing a significant LTP blockage (115±22% vs control 148±15%, n=9/6, p=0.019) in WT animals. The same concentration tested in **(C)** α_1KI (141±28% vs control 144±13%, n=8/8, p=0.312) and in **(D)** $\alpha_{1/2/3}$KI (141±11% vs control 149±22%, n=9/9, p=0.496) mice did not modulate LTP. However, when applied it to **(E)** α_5KI mice, LTP was significantly reduced (121±11% vs control 140±7%, n=10/10, p=0.006).

3.1.6. XBD173 has no effect on LTP

XBD173 is a TSPO ligand which binding activates the neurosteroidogenesis cascade, resulting in a release of neurosteroids, such as THDOC and allopregnanolone. Consequently, these compounds bind to $GABA_A$Rs and enhance their action, resulting in anxiolytic effects (Kita et al., 2009; Rupprecht et al., 2009).

Exposure of XBD173 (Merck, Germany) at 300nM in WT mice (143±11% vs control 149±10%, n=8/8, p=0.148) did not alter LTP induction when compared to its control but when a higher concentration of 1µM was applied (118±20% vs control 147±13%, n=9/9, p=0.012), LTP was significantly blocked (Fig. 16).

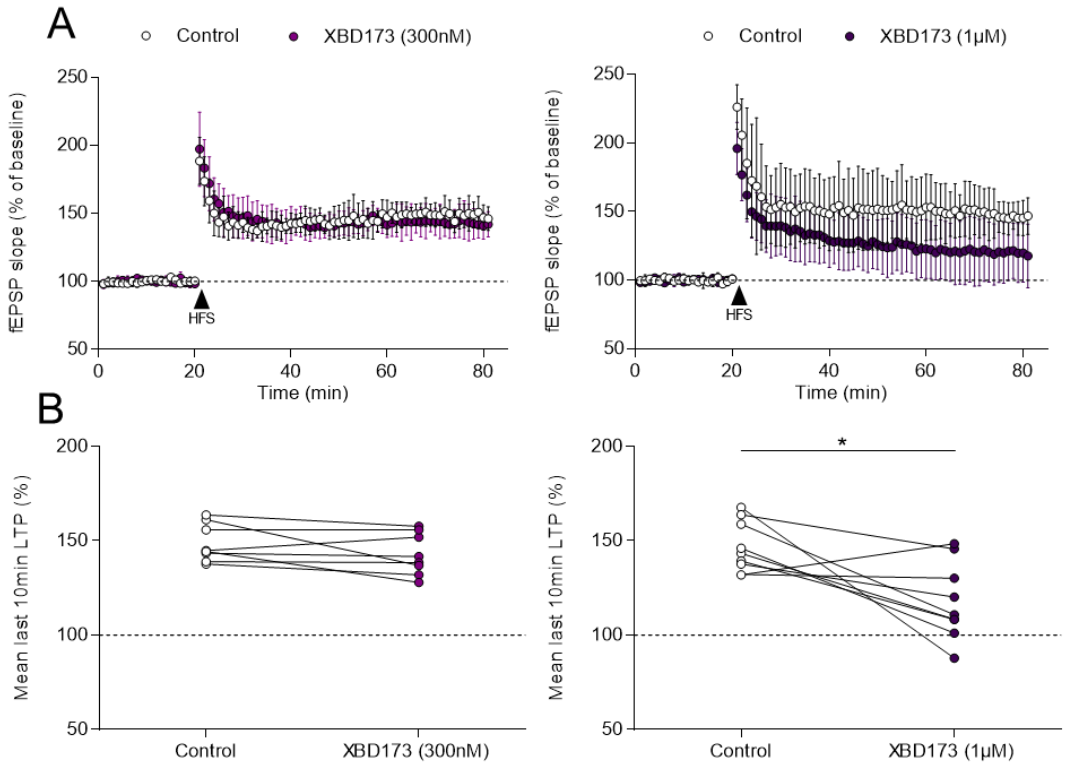

Fig. 16: Effect of XBD173 on LTP in WT mice.

(A) Normalised fEPSP slope time course following an HFS under control and after 60min of XBD173 300nM (left) and 1µM (right) in WT mice. **(B)** Connected scatter plots summarising min 50 to 60 after HFS in control and XBD173 300nM (left) and 1µM (right). XBD173 300nM (left; 143±11% vs control 149±10%, n=8/8, p=0.148) did not significantly alter LTP in comparison to control. However, at 1µM (right; 118±20% vs control 147±13%, n=9/9, p=0.012), it resulted in a significant LTP blockage.

XBD173 was also tested in different transgenic mouse lines (Fig. 17). In α_5KI genotype, neither 300nM (159±27% vs control 147±20%, n=8/8, p=0.742) nor 1µM (137±11% vs control 149±10%, n=7/6, p=0.078) modified LTP when compared to their respective controls (Fig. 17A and B). Similarly, when applied at 300nM in GABAδKO mice (147±21% vs control 140±10%, n=9/9, p=0.496), no LTP alteration was seen within genotype (Fig. 17C).

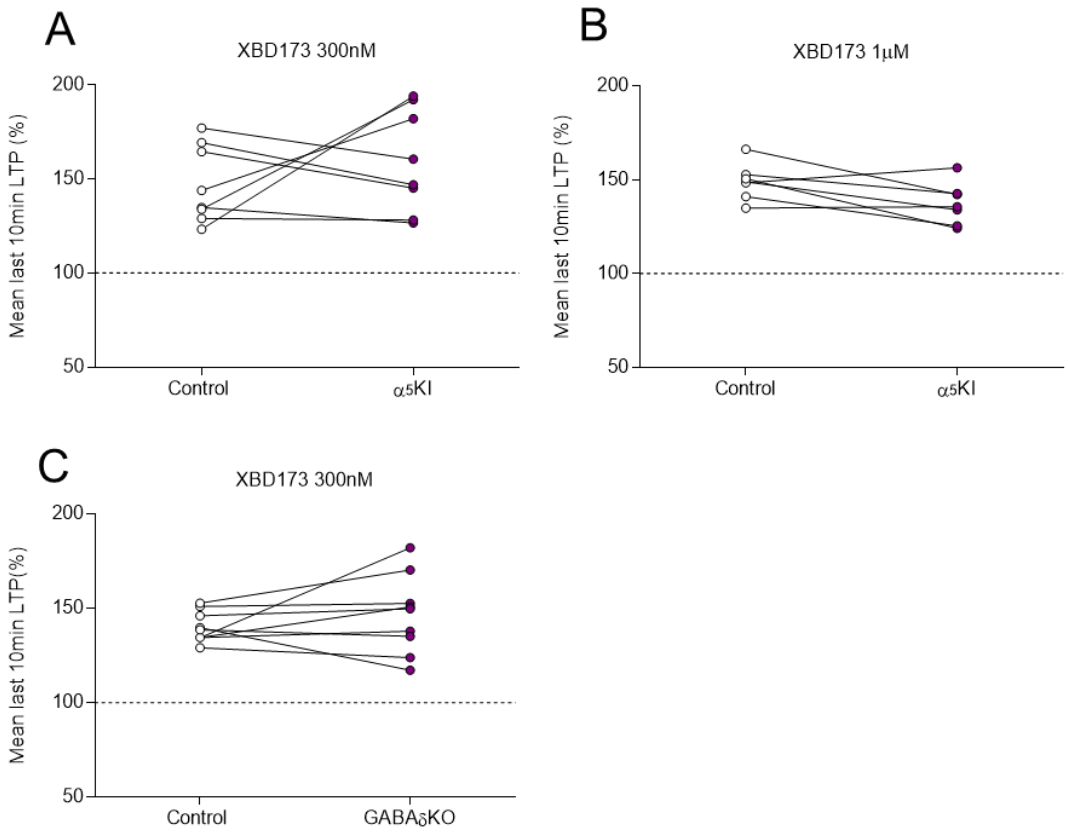

Fig. 17: Effect of XBD173 on LTP in α_5KI and GABAδKO mice.

Connected scatter plots summarising min 50 to 60 after HFS in control and drug conditions. **(A)** XBD173 300nM and **(B)** 1µM did not significantly change LTP before and after drug application at concentrations of 300nM (159±27% vs control 147±20%, n=8/8, p=0.742) or 1µM (137±11% vs control 149±10%, n=7/6, p=0.078) in α_5KI. **(C)** When XBD173 300nM was applied in GABAδKO brain slices, LTP was not modified (147±21% vs control 140±10%, n=9/9, p=0.496).

To go a bit further, we aimed to explore whether a pre-application of XBD173 and, therefore the consequent neurosteroids release, could prevent the detriment effect of midazolam on LTP. As depicted in Fig. 18A, midazolam 10nM applied in WT brain slices after the pre-application of XBD173 at 300nM did not modify LTP induction compared to XBD173 alone (133±19% vs XBD173 149±19%, n=4/4, p=0.250). Nevertheless, when these drugs were applied in α_5KI mice (Fig. 18B), midazolam 10nM after XBD173 300nM exposure blocked LTP (113±13% vs XBD173 150±14%, n=5/5, p=0.062). We could show that administration of XBD173 prior to midazolam exposure prevented the blockage of LTP caused by the BZD.

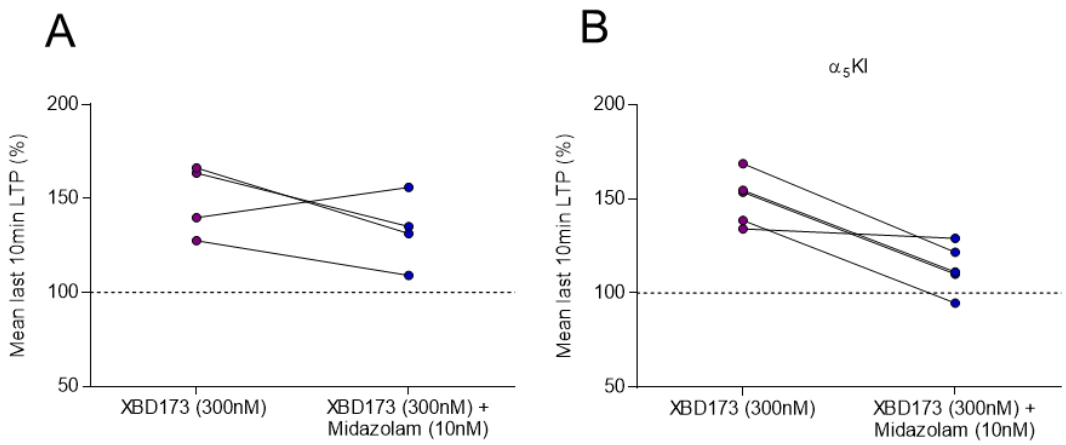

Fig. 18: Effect of XBD173 co-applied with midazolam in WT and α_5KI mice.

Connected scatter plots summarising min 50 to 60 after HFS in XBD173 alone and co-applied with midazolam 10nM. **(A)** Midazolam 10nM after the pre-application of XBD173 300nM did not alter the LTP in WT animals when compared to XBD173 alone (133±19% vs XBD173 149±19%, n=4/4, p=0.250). **(B)** When midazolam 10nM was applied after XBD173 300nM in α_5KI mice, LTP was blocked (113±13% vs XBD173 150±14%, n=5/5, p=0.062), although not statistically significantly.

3.1.7. Allopregnanolone has no effect on LTP

To elucidate the different neurosteroids released upon XBD173-induced neurosteroidogenesis, we first examined the effect of the neurosteroid allopregnanolone. Exposure to allopregnanolone (Tocris, Germany) 300nM in WT mice (Fig. 19A and B) resulted in a non-altered LTP induction when compared to control (162±37% vs control 158±16%, n=10/6, p=0.695). Similarly, LTP was not modified when allopregnanolone at 300nM was applied to α_1KI (158±26% vs control 145±12%, n=8/8, p=0.461) (Fig. 19C), $\alpha_{1/2/3}$KI (153±24% vs control 143±4%, n=9/9, p=0.359) (Fig. 19D) or GABAδKO mice (154±18% vs control 140±9%, n=9/9, p=0.055) (Fig. 19E). However, in α_5KI mice, LTP was significantly reduced (121±14% vs control 139±8%, n=10/10, p=0.027) (Fig. 19F).

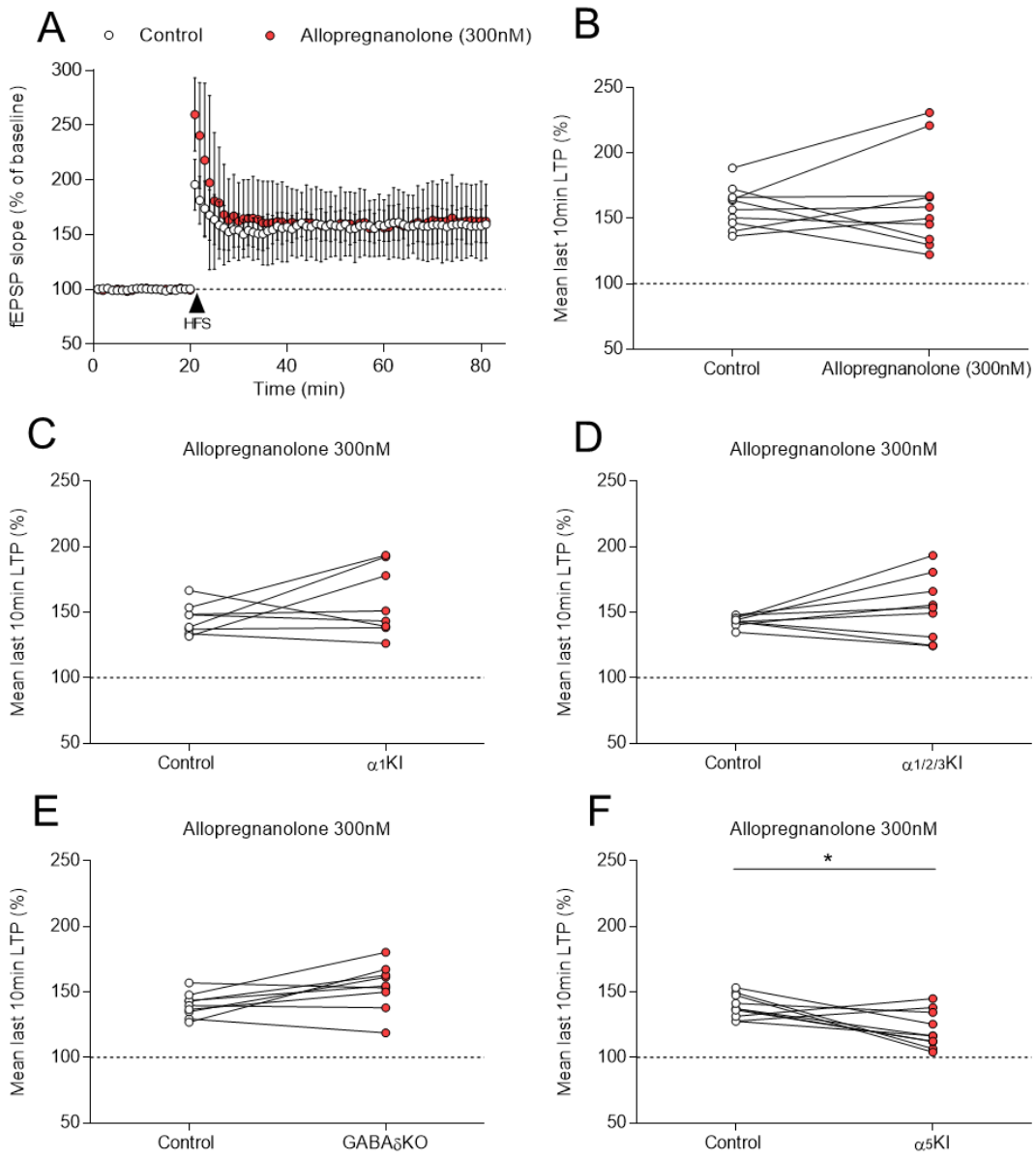

Fig. 19: Effect of allopregnanolone on LTP in WT and α_1KI, $\alpha_{1/2/3}$KI, GABAδKO and α_5KI mice.

(A) Normalised fEPSP slope time course following an HFS under control condition and after 60min of allopregnanolone 300nM in WT mice. **(B-F)** Connected scatter plots summarising min 50 to 60 after HFS in control and allopregnanolone 300nM administration, **(B)** showing how this drug does not significantly alter LTP (162±37% vs control 158±16%, n=10/6, p=0.695) in WT animals. The same concentration of allopregnanolone displayed the same results in **(C)** α_1KI (158±26% vs control 145±12%, n=8/8, p=0.461), **(D)** $\alpha_{1/2/3}$KI (153±24% vs control 143±4%, n=9/9, p=0.359) and **(E)** GABAδKO mice (154±18% vs control 140±9%, n=9/9, p=0.055). Although, in α_5KI **(F)**, the LTP resulted in a significant reduction (121±14% vs control 139±8%, n=10/10, p=0.027).

3.1.8. THDOC has no effect on LTP

The neurosteroid THDOC was also chosen to be tested as a potential biosynthesised neurosteroid after XBD173 application. THDOC (Tocris, Germany) 100nM administration in slices from WT animals (146±24% vs control 145±9%, n=6/6, p=0.562) did not modify

LTP when compared to control, but when the concentration was increased to 1µM (128±9% vs control 146±14%, n=8/6, p=0.008), LTP was significantly reduced (Fig. 20).

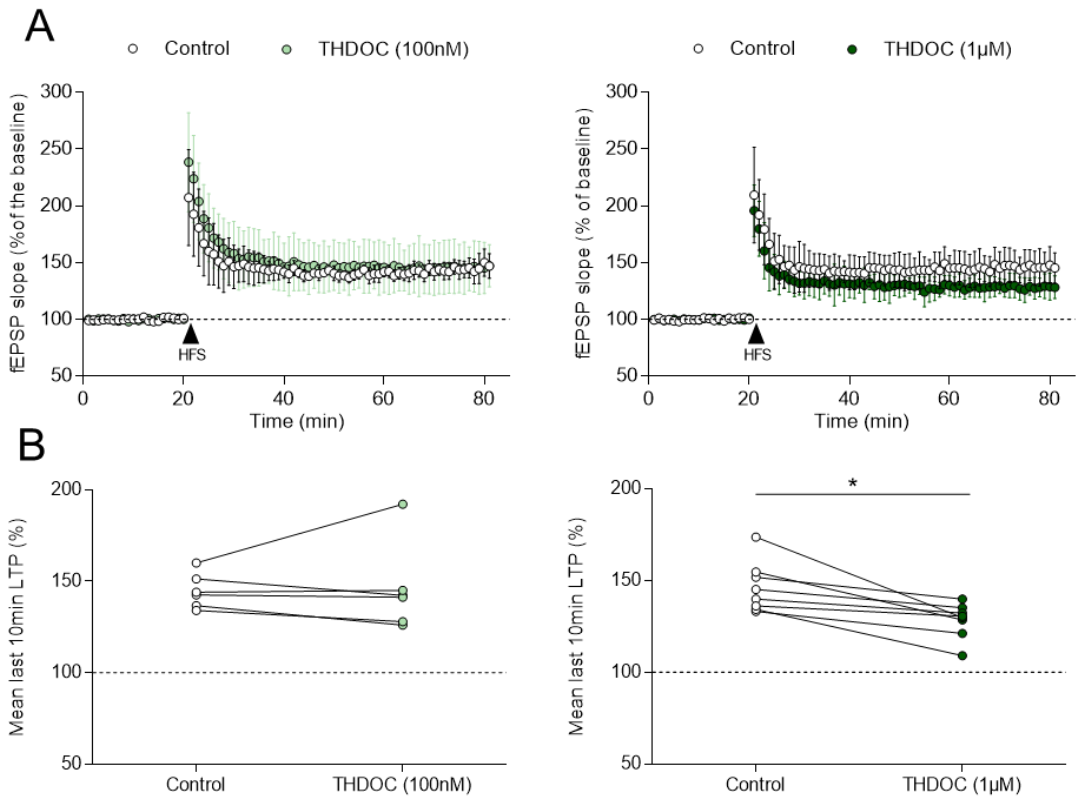

Fig. 20: Effect of THDOC on LTP in WT mice.

(A) Normalised fEPSP slope time course following an HFS under control and after 60min of THDOC 100nM (left) and 1µM (right) in WT mice. **(B)** Connected scatter plots summarising min 50 to 60 after HFS in control and drug conditions in WT genotype. THDOC 100nM did not modify LTP (left; 146±24% vs control 145±9%, n=6/6, p=0.562) whereas LTP was significantly reduced after 1µM (right; 128±9% vs control 146±14%, n=8/6, p=0.008).

Moreover, transgenic mouse lines were used to confirm that the point mutations in the BZD binding site did not affect the binding of the neurosteroid THDOC to $GABA_A$Rs and to test whether δ subunits are involved in LTP modulation (Fig. 21). When THDOC was applied to $\alpha_{1/2/3}$KI mice, neither 100nM (Fig. 21A left) (144±32% vs control 158±17%, n=8/8, p=0.312) nor 1µM (Fig. 21A right) (131±32% vs control 144±16%, n=8/8, p=0.311) altered LTP. In the same way, LTP was not modified after the administration of THDOC 100nM (Fig. 21B left) (141±14% vs control 139±9%, n=9/6, p=0.652) or 1µM (Fig. 21B right) (144±13% vs control 138±10%, n=8/5, p=0.641) in α_5KI mice. When THDOC 100nM was applied in GABAδKO animals, no significant LTP changes were apparent (144±11% vs control 142±11%, n=9/8, p=0.820) (Fig. 21C).

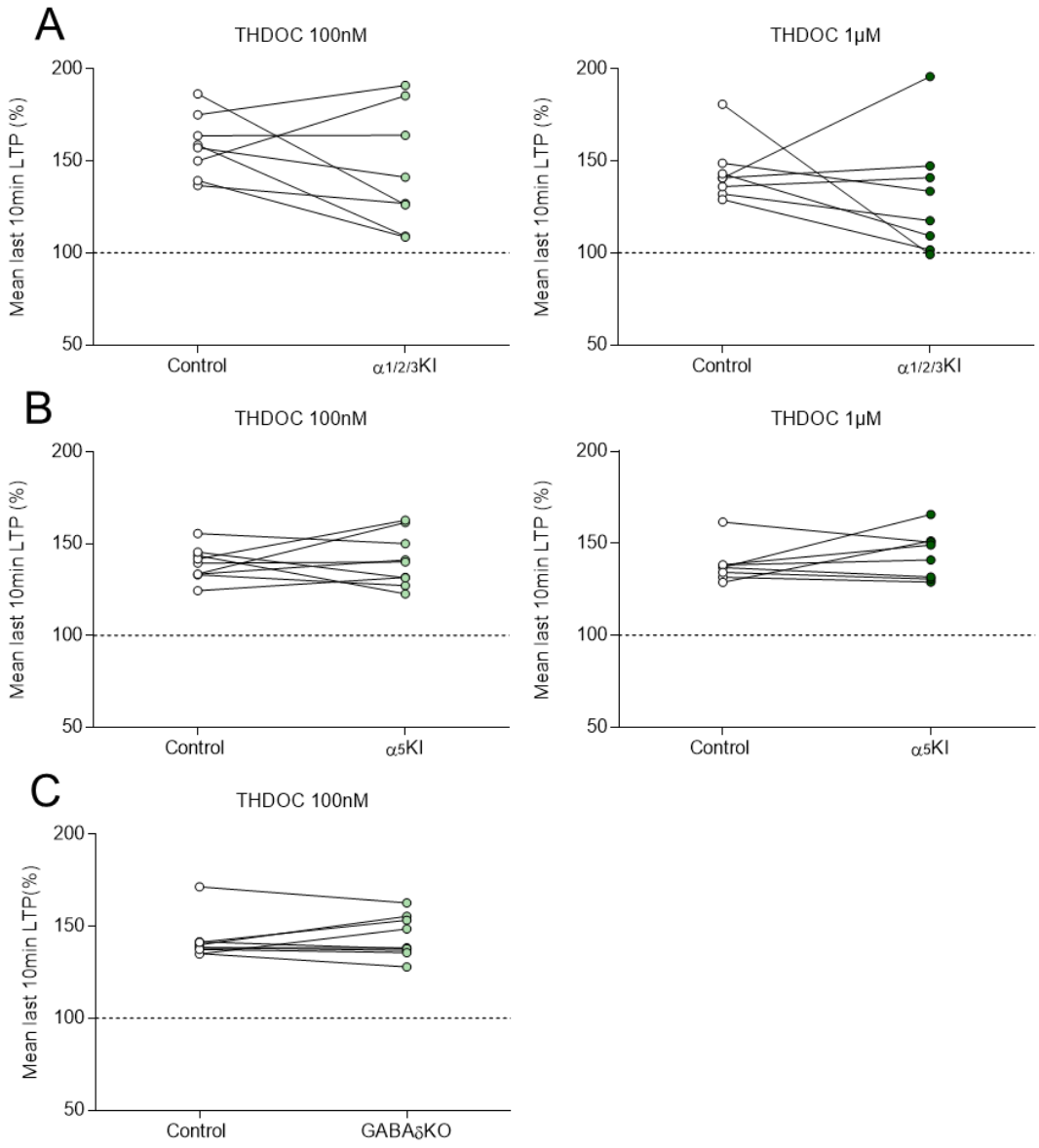

Fig. 21: Effect of THDOC on LTP in $\alpha_{1/2/3}$KI, α_5KI and GABAδKO mice.

Connected scatter plots summarising min 50 to 60 after HFS in control and drug conditions in **(A)** $\alpha_{1/2/3}$KI, **(B)** α_5KI and **(C)** GABAδKO genotype. **(A)** In $\alpha_{1/2/3}$KI mice, neither THDOC 100nM (left; 144±32% vs control 158±17%, n=8/8, p=0.312) nor 1µM (right; 131±32% vs control 144±16%, n=8/8, p=0.311) did modulate LTP. **(B)** In α_5KI genotype, THDOC 100nM (left; 141±14% vs control 139±9%, n=9/6, p=0.652) and 1µM (right; 144±13% vs control 138±10%, n=8/5, p=0.641) did not modify LTP. **(C)** When THDOC 100nM was

tested in GABAδKO animals, no significant LTP alteration was seen (144±11% vs control 142±11%, n=9/8, p=0.820).

3.2. Modulation of synaptic transmission by midazolam and neurosteroids

3.2.1. Midazolam modulates synaptic transmission at 100nM and 1µM

As midazolam 10nM produced a strong LTP inhibition in WT mice, this concentration was also tested whilst monitoring synaptic transmission. Nonetheless, 10nM did not alter sIPSC's amplitude (61±31% vs control 51±10%, n=7/4, p=0.375) (Fig. 22B), τ_1 (9±2% vs control 8±2%, n=7/4, p=0.938) (Fig. 22C) or τ_2 (44±16% vs control 49±20%, n=7/4, p=0.297) (Fig. 22D).

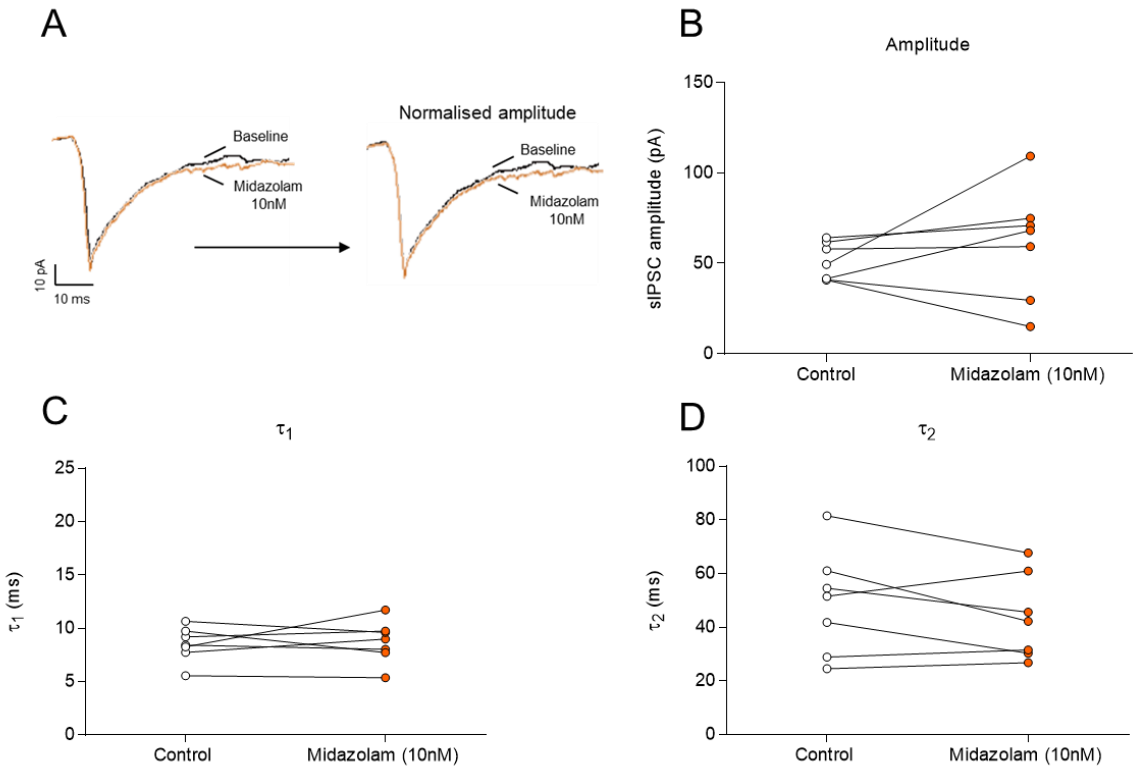

Fig. 22: Effect of midazolam 10nM on sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of midazolam 10nM during 35min. **(B)** Amplitude was not altered after midazolam application (61±31% vs control 51±10%, n=7/4, p=0.375). **(C)** Neither τ_1 (9±2% vs control 8±2%, n=7/4, p=0.938) nor **(D)** τ_2 (44±16% vs control 49±20%, n=7/4, p=0.297) were modified after midazolam exposure.

Midazolam at 100nM in WT mice could statistically increase the sIPSC's amplitude after 35min of exposure (49±16% vs control 34±11%, n=7/4, p=0.016) (Fig. 23A and B). On

one hand, τ_1 did not display changes when compared to control (13±4% vs control 10±3%, n=7/4, p=0.109) (Fig. 23C), but on the other hand, τ_2 did show significant differences after application of midazolam 100nM (55±8% vs control 35±12%, n=7/4, p=0.016) (Fig. 23D).

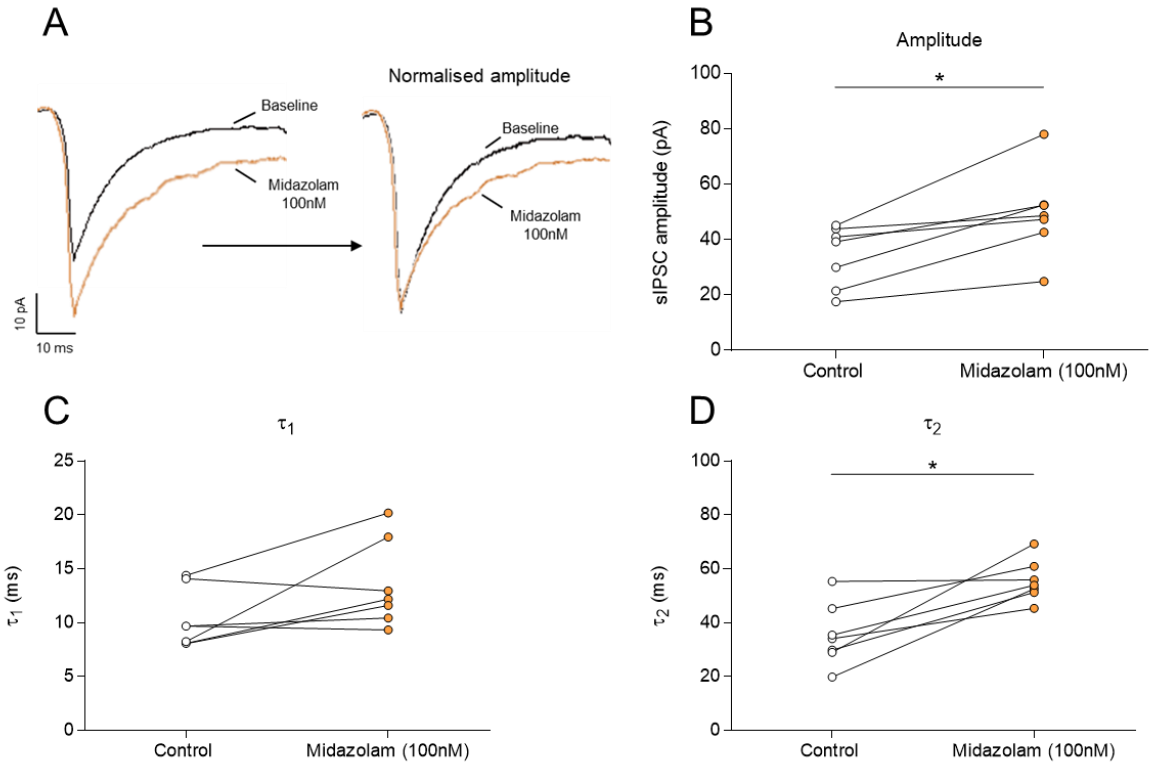

Fig. 23: Effect of midazolam 100nM on sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of midazolam 100nM during 35min. **(B)** Amplitude was increased after midazolam (49±16% vs control 34±11%, n=7/4, p=0.016). **(C)** τ_1 was not modified after midazolam (13±4% vs control 10±3%, n=7/4, p=0.109), but **(D)** τ_2 was augmented (55±8% vs control 35±12%, n=7/4, p=0.016). Adapted from Puig-Bosch et al., (2022).

Application of midazolam 100nM during 35min in α_1KI mice resulted in an increase of sIPSC's amplitude (54±17% vs control 39±9%, n=8/5, p=0.016) and τ_1 (35±2% vs control 8±2%, n=8/5, p=0.008) (Fig. 24A and B and C, respectively). τ_2 was not changed after midazolam exposure (57±11% vs control 38±14%, n=8/5, p=0.078) (Fig. 24D). These results indicate that when α_1-subunits are insensitive for midazolam, sIPSC amplitude is still increased as also seen in WT mice at the same concentration (Fig. 23), demonstrating that sIPSC's amplitude modulation is independent of α_1-subunits. However, τ_2 is α_1-sensitive because it was no longer increased after midazolam application.

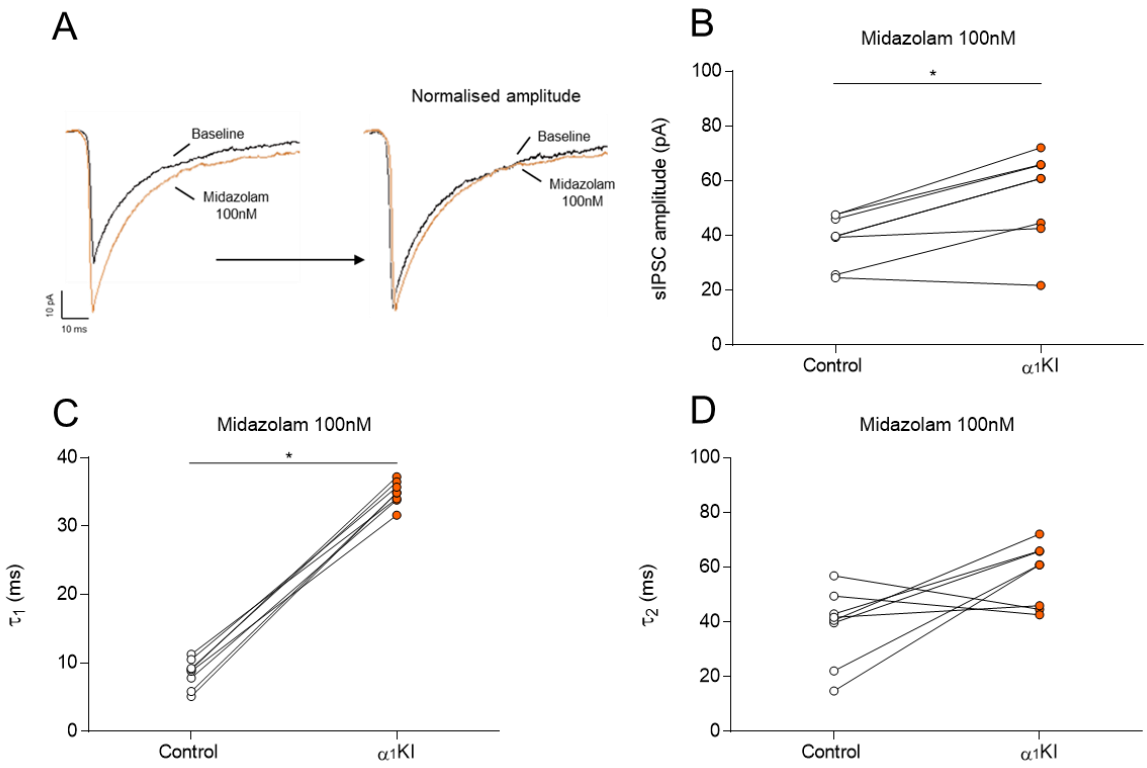

Fig. 24: Effect of midazolam 100nM on sIPSCs of α_1KI mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of midazolam 100nM during 35min. Both **(B)** amplitude (54±17% vs control 39±9%, n=8/5, p=0.016) and **(C)** τ_1 (35±2% vs control 8±2%, n=8/5, p=0.008) were statistically increased when compared to control. However, **(D)** τ_2 was not significantly changed after midazolam administration (57±11% vs control 38±14%, n=8/5, p=0.078).

After 35min of midazolam 1µM exposure, $GABA_AR$-synaptic transmission was raised by specifically increasing sIPSC's amplitude (53±20% vs control 43±13%, n=9/5, p=0.027) (Fig. 25A and B) and both τ_1 (14±4% vs control 10±3%, n=9/5, p=0.008) (Fig. 25C) and τ_2 (63±14% vs control 48±10%, n=9/5, p=0.008) (Fig. 25D).

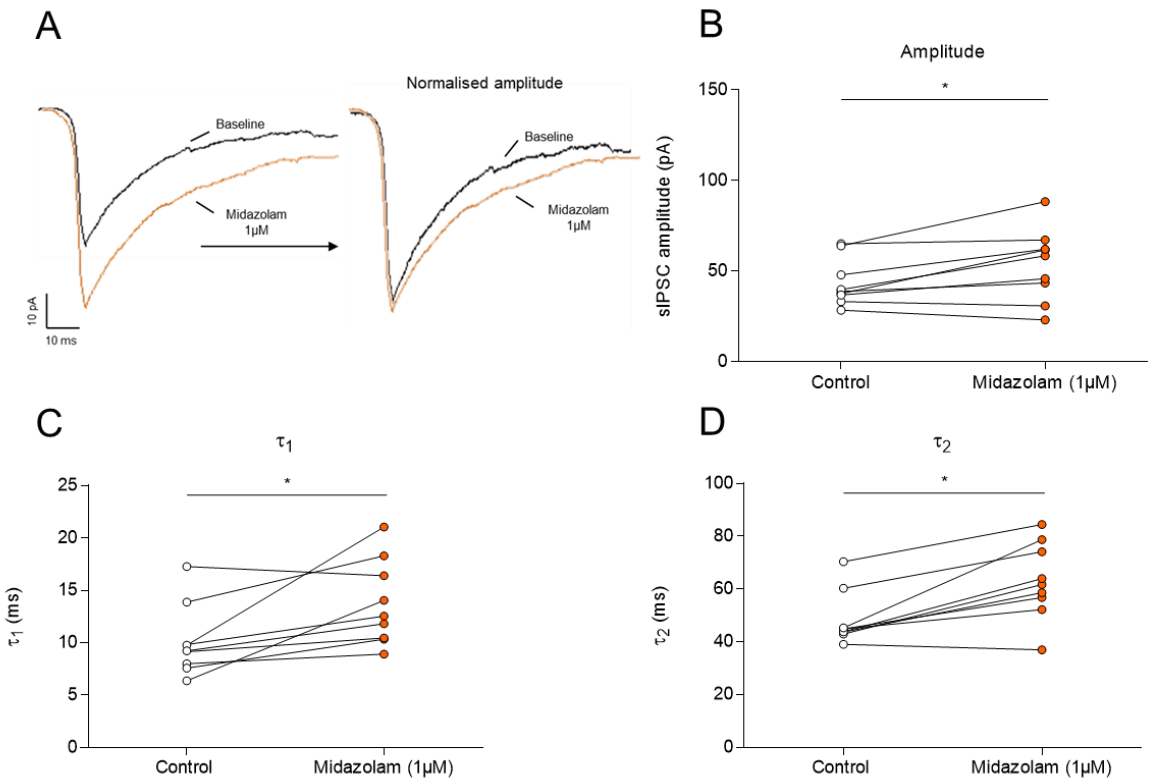

Fig. 25: Effect of midazolam 1µM on sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of midazolam 1µM during 35min. **(B)** sIPSC's amplitude (53±20% vs control 43±13%, n=9/5, p=0.027) was significantly increased after 35min of midazolam 1µM exposure. And both **(C)** τ_1 (14±4% vs control 10±3%, n=9/5, p=0.008) and **(D)** τ_2 (63±14% vs control 48±10%, n=9/5, p=0.008) were significantly enlarged after midazolam 1µM administration.

3.2.2. XBD173 increases synaptic transmission

sIPSCs were recorded before and after 30min of XBD173 300nM exposure, which resulted in an increase of sIPSC's amplitude (49±16% vs control 39±10%, n=10/5, p=0.049) (Fig. 26A and B). Nevertheless, τ_1 was not altered after XBD173 administration when compared to control (8±1% vs control 8±1%, n=10/5, p=0.232) (Fig. 26C). For τ_2, a significant increase was seen after XBD173 exposure (64±11% vs control 40±15%, n=10/5, p=0.004) (Fig. 26D).

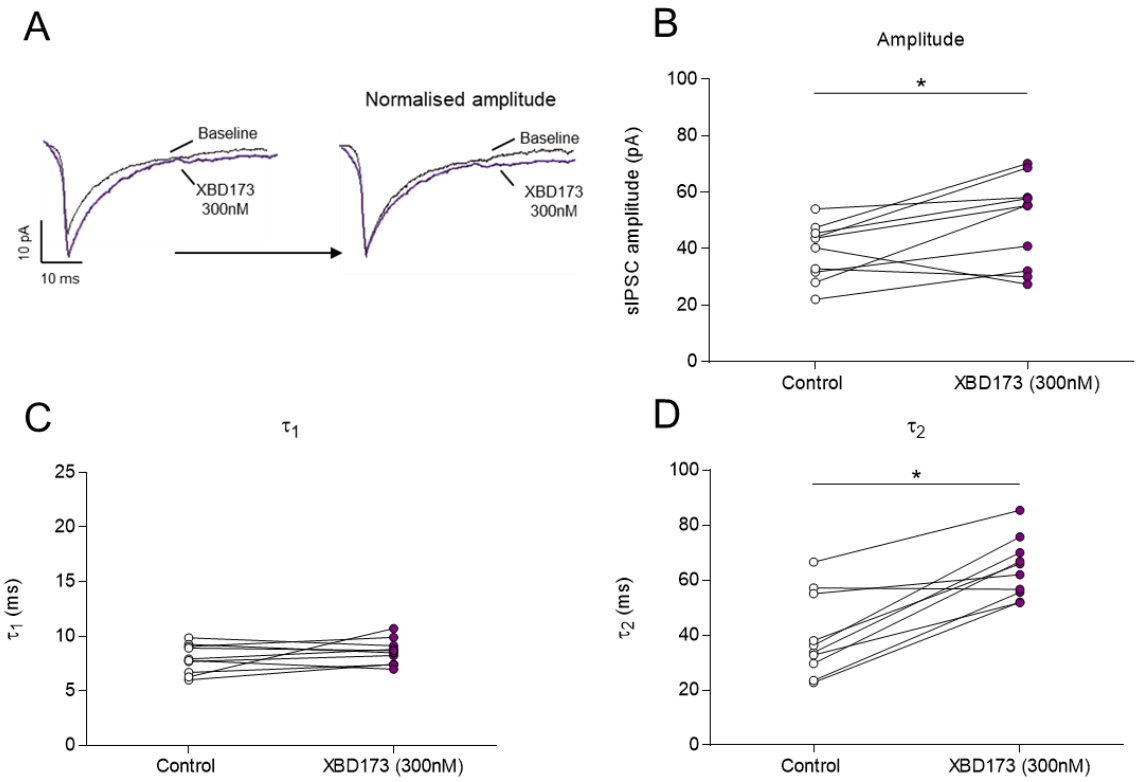

Fig. 26: Effect of XBD173 300nM on sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of XBD173 300nM during 30min. **(B)** sIPSC's amplitude (49±16% vs control 39±10%, n=10/5, p=0.049) was increased after XBD173 exposure. In contrary, **(C)** τ_1 was not modified (8±1% vs control 8±1%, n=10/5, p=0.232), but **(D)** τ_2 was augmented after XBD173 administration (64±11% vs control 40±15%, n=10/5, p=0.004).

3.2.3. Allopregnanolone modulates synaptic transmission

Recording sIPSCs after 40min of allopregnanolone application at 100nM resulted in a non-modulation of sIPSC's amplitude (40±17% vs control 35±11%, n=8/4, p=0.250) (Fig. 27A and B) or τ_1 (9±1% vs control 7±1%, n=8/4, p=0.078) when compared to control (Fig. 27C). However, τ_2 was significantly increased after allopregnanolone administration (67±16% vs control 30±11%, n=8/4, p=0.008) (Fig. 27D).

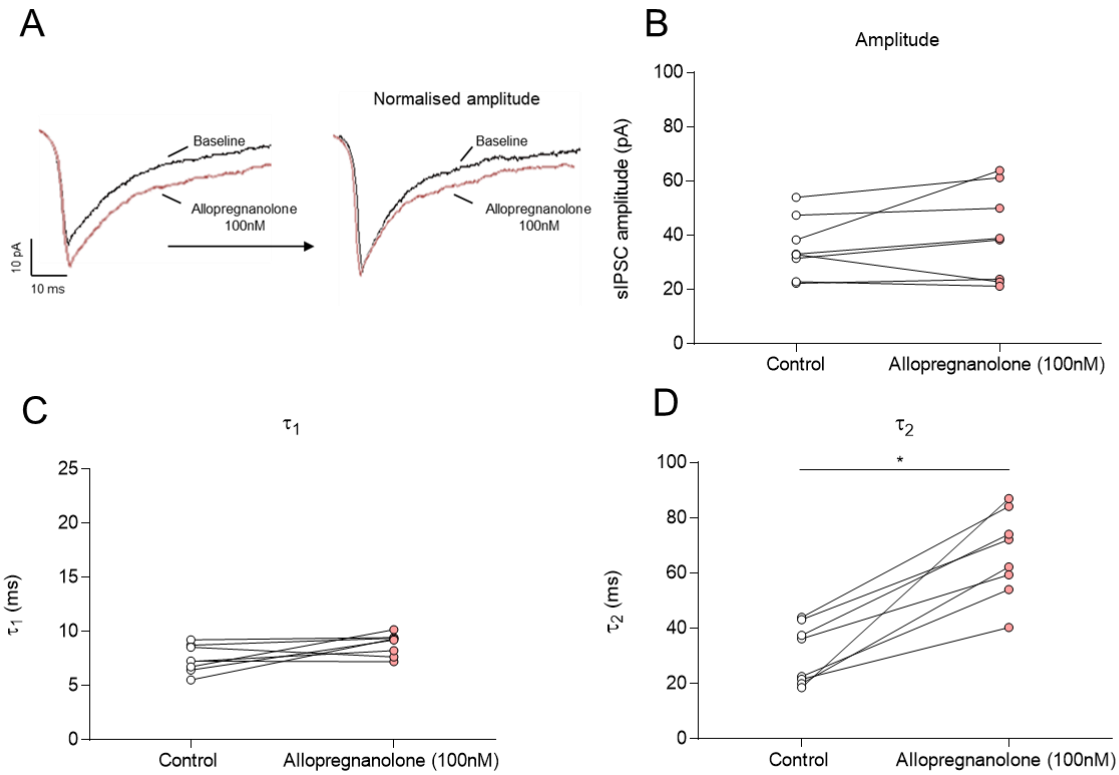

Fig. 27: Effect of allopregnanolone 100nM on sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of allopregnanolone 100nM during 40min. Both **(B)** amplitude (40±17% vs control 35±11%, n=8/4, p=0.250) and **(C)** τ_1 (9±1% vs control 7±1%, n=8/4, p=0.078) did not show a significant modification. Nevertheless, **(D)** τ_2 was significantly increased after allopregnanolone administration (67±16% vs control 30±11%, n=8/4, p=0.008).

3.2.4. THDOC increases synaptic transmission

THDOC 100nM was applied in WT mice during 30min and synaptic events were recorded before and after drug application, resulting in an increased synaptic transmission after THDOC wash-in. In particular, the amplitude of sIPSCs was significantly increased after THDOC exposure (54±11% vs control 39±10%, n=12/8, p=0.002) (Fig. 28A and B). No changes were displayed for τ_1 after THDOC administration (8±2% vs control 7±2%, n=12/8, p=0.301) (Fig. 28C), however τ_2 was significantly augmented after the wash-in of the drug (89±16% vs control 44±17%, n=12/8, p<0.001) (Fig. 28D).

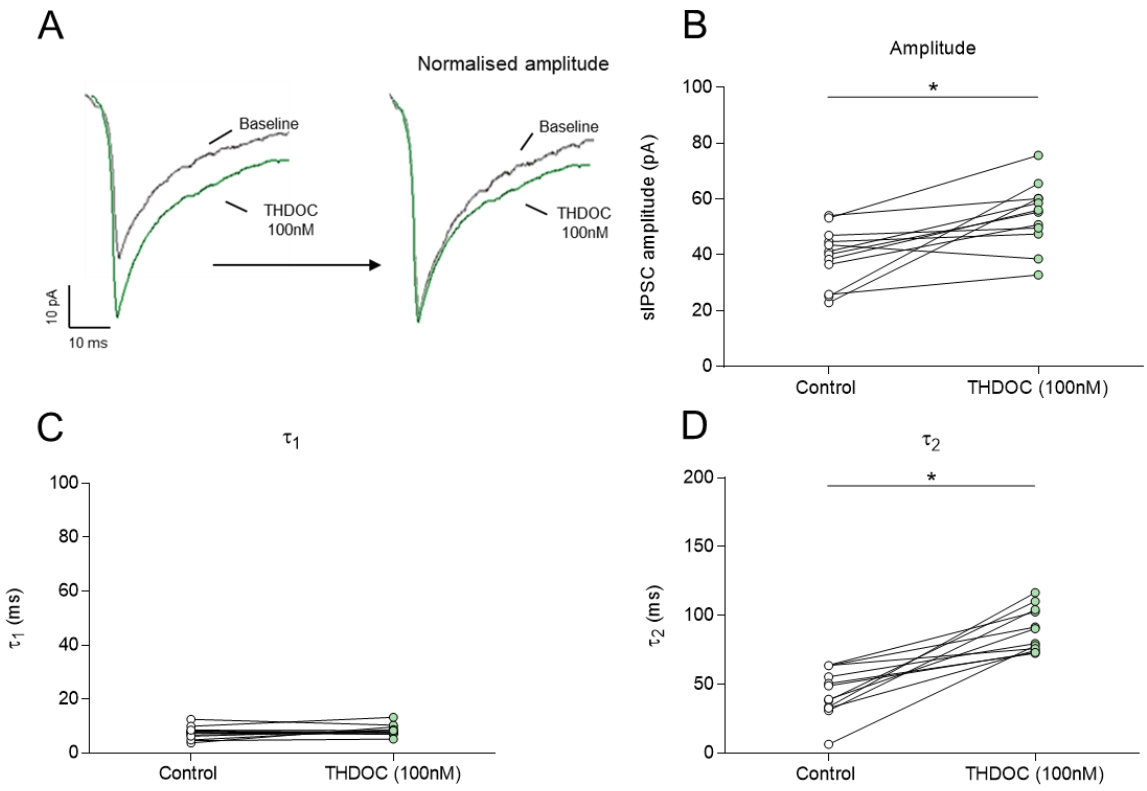

Fig. 28: Effect of THDOC 100nM on sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of THDOC 100nM during 30min in WT mice. **(B)** amplitude (54±11% vs control 39±10%, n=12/8, p=0.002) was raised after THDOC exposure. **(C)** τ_1 was not altered after THDOC (8±2% vs control 7±2%, n=12/8, p=0.301) but **(D)** τ_2 was significantly increased (89±16% vs control 44±17%, n=12/8, p<0.001).

It seems that 100nM concentration was high enough for THDOC to exert its effects because when 1µM was applied, the tendency of the results was similar to the ones with 100nM. Moreover, 100nM is relevant because it is within the physiological range of neurosteroids, while 1µM is too high to be found in a physiological state and this concentration could most likely result in unspecific binding of THDOC.

After 30min of THDOC 1µM exposure, the amplitude of sIPSCs (60±15% vs control 40±9%, n=12/8, p<0.001) (Fig. 29A and B) was significantly increased. Similarly, τ_1 (51±26% vs control 7±2%, n=12/8, p<0.001) (Fig. 29C) and τ_2 (123±34% vs control 43±14%, n=12/8, p<0.001) (Fig. 29D) were significantly augmented after THDOC administration.

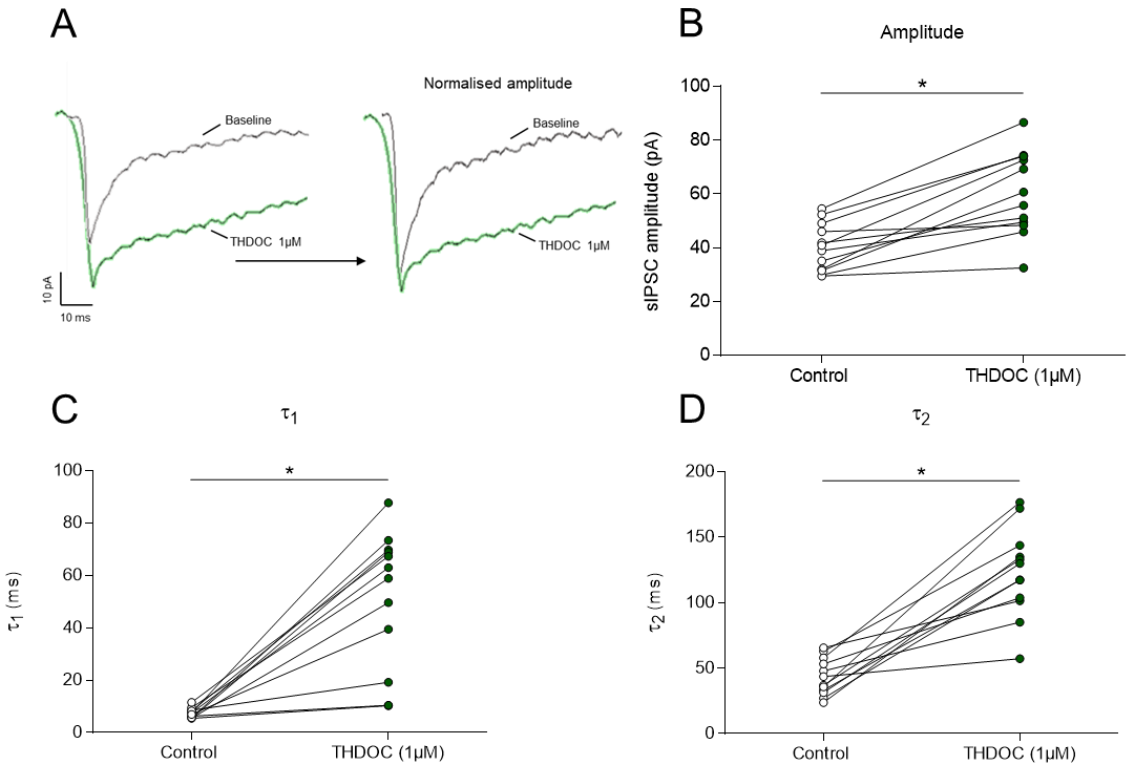

Fig. 29: Effect of THDOC 1µM in sIPSCs of WT mice.

(A) The insets show representative sIPSC traces for control and drug conditions, showing original overlapped traces (left) and overlapped traces with a normalised amplitude (right) for emphasising when τ_1 and τ_2 are increased. **(B-D)** Connected scatter plots showing the sIPSC's **(B)** amplitude, **(C)** τ_1 and **(D)** τ_2 before and after the application of THDOC 1µM during 30min. A significant increase was evident in the sIPSC components **(B)** amplitude (60±15% vs control 40±9%, n=12/8, p<0.001), **(C)** τ_1 (51±26% vs control 7±2%, n=12/8, p<0.001) and **(D)** τ_2 (123±34% vs control 43±14%, n=12/8, p<0.001) after THDOC 1µM application.

3.3. Neuroprotective properties against H/H-induced excitotoxicity in the hippocampal CA1 region

3.3.1. Midazolam protects against H/H-induced excitotoxicity

After 25min of hypoxia and hypoglycaemia, midazolam 10nM showed a significantly increased fEPSP slope compared to control levels in WT (100(29)% vs control 59(15)%, n=8/7, p=0.001) (Fig. 30A) and in $\alpha_{1/2/3}$KI mice (95(44)% vs control 59(53)%, n=8/8, p=0.028) (Fig. 30B), presenting neuroprotection at this low concentration. In the α_5KI genotype (53(6)% vs control 45(8)%, n=7/7, p=0.002), only a small although significant recovery was seen (Fig. 30C). These results demonstrate that α_5 subunits are essential for midazolam to exert neuroprotection after a period of H/H at a concentration of only 10nM.

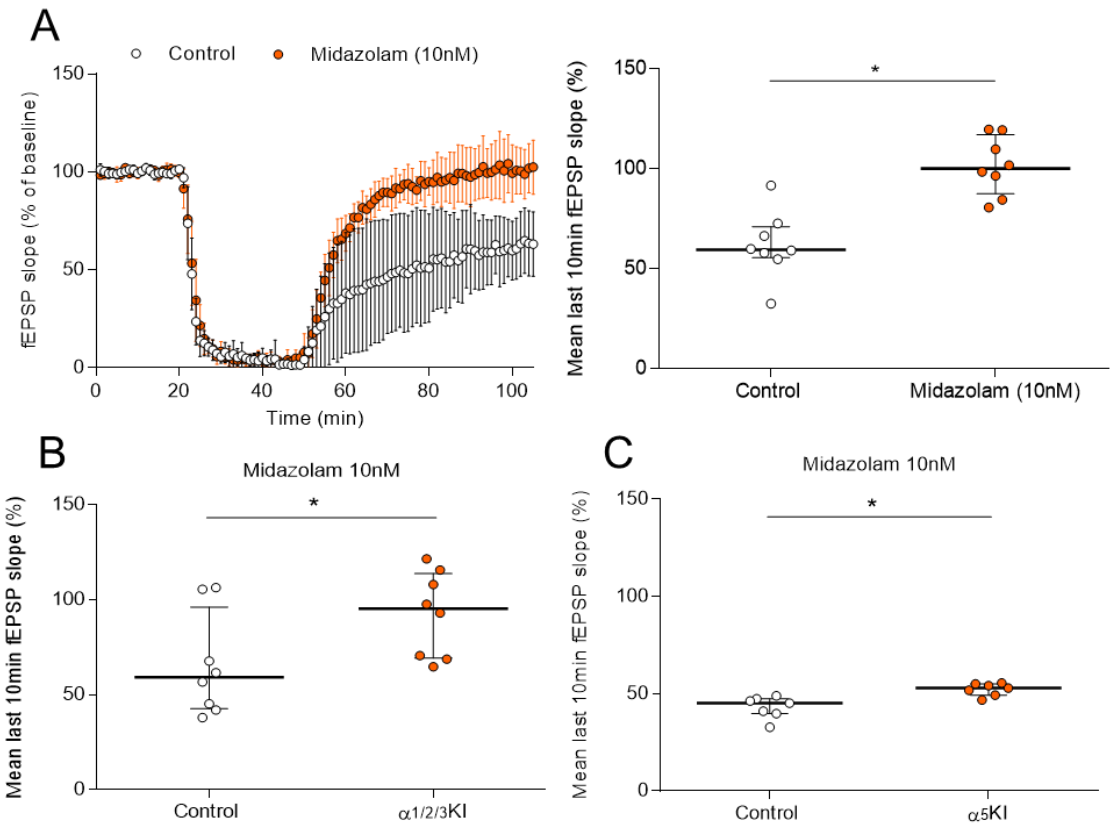

Fig. 30: Neuroprotective effects of midazolam in WT, $\alpha_{1/2/3}$KI and α_5KI mice.

(A, left) Normalised fEPSP slope time course following a H/H period under control and after 60min of midazolam 10nM in WT mice. Scatter plots summarising min 50 to 60 after H/H time in control and in the presence of midazolam 10nM in **(A, right)** WT, **(B)** $\alpha_{1/2/3}$KI and **(C)** α_5KI genotypes. Midazolam 10nM showed a significant fEPSP increase during the recovery time in WT (100(29)% vs control 59(15)%, n=8/7, p=0.001) and in $\alpha_{1/2/3}$KI animals (95(44)% vs control 59(53)%, n=8/8, p=0.028), but just a small recovery in α_5KI, although significant (53(6)% vs control 45(8)%, n=7/7, p=0.002).

3.3.2. XBD173 protects against H/H-induced excitotoxicity

XBD173 administration at 300nM triggered neuroprotection via neurosteroid release in WT mice (108(28)% vs control 55(18)%, n=9/9, p<0.001) (Fig. 31A and B) because recovered fEPSP slope was augmented when compared to control. Likewise, the same concentration applied in α_5KI mouse line showed a significant fEPSP slope increase in the recovery phase (86(38)% vs control 53(13)%, n=6/6, p=0.009) (Fig. 31C). In contrast, after XBD173 300nM exposure in GABAδKO mice (23(13)% vs control 22(15)%, n=8/8, p=0.629), no significant recovery was seen (Fig. 31D). These results suggest that δ-$GABA_AR$ subunits are essential for mediating XBD173-induced neuroprotection after the formation of neurosteroids in the hippocampal brain slices.

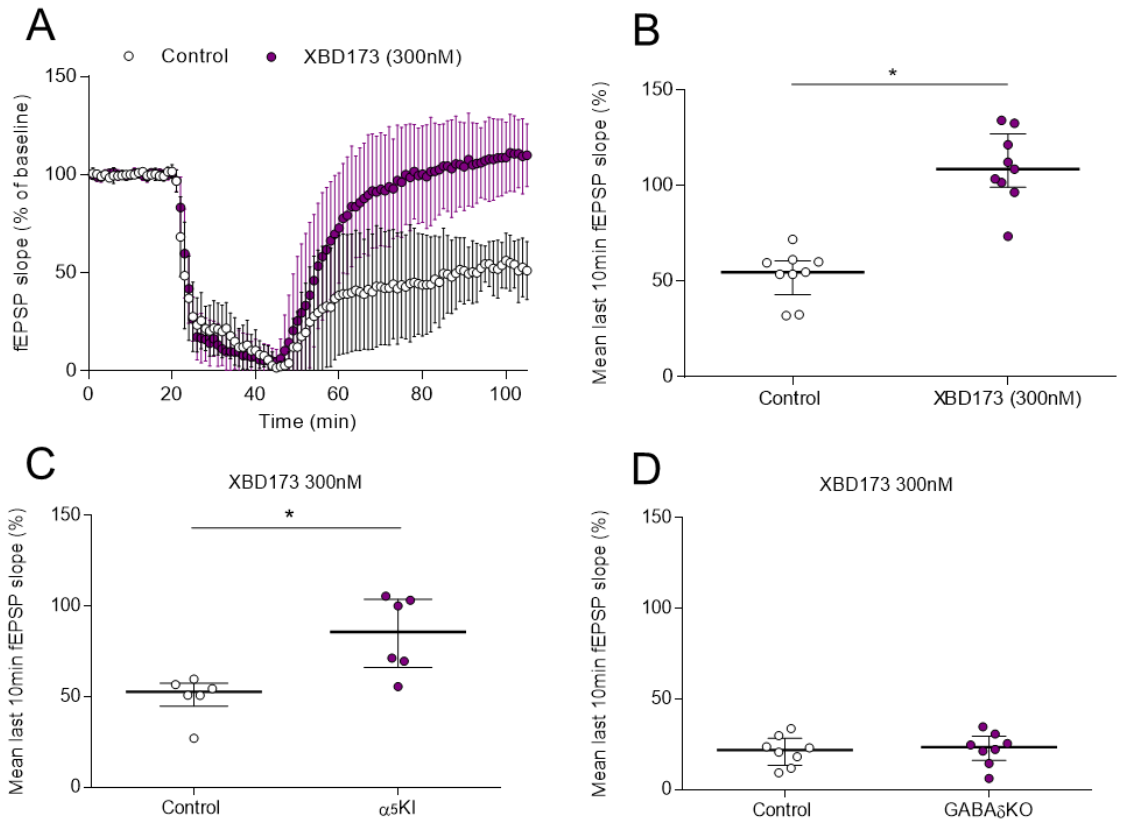

Fig. 31: Neuroprotective effects of XBD173 in WT, α_5KI and GABAδKO mice.

(A) Normalised fEPSP slope time course following a H/H period under control and after 60min of XBD173 300nM in WT animals. Scatter plots summarising min 50 to 60 after H/H time in control and in the presence of XBD173 300nM in **(B)** WT, **(C)** α_5KI and **(D)** GABAδKO genotypes. XBD173 300nM significantly increased the fEPSP during the recovery period in **(B)** WT (108(28)% vs control 55(18)%, n=9/9, p<0.001) and **(C)** α_5KI (86(38)% vs control 53(13)%, n=6/6, p=0.009) animals. But no fEPSP increase when applied in **(D)** GABAδKO mice (23(13)% vs control 22(15)%, n=8/8, p=0.629).

3.3.3. Allopregnanolone protects against H/H-induced excitotoxicity

Allopregnanolone displayed neuroprotection after its application at 100nM (111(15)% vs control 42(22)%, n=6/6, p=0.002) (Fig. 32). Due to several difficulties while performing these H/H experiments in the presence of allopregnanolone at 100nM, we did not investigate further this neurosteroid either at higher concentrations (300nM) as in LTP monitoring or in the transgenic mouse lines.

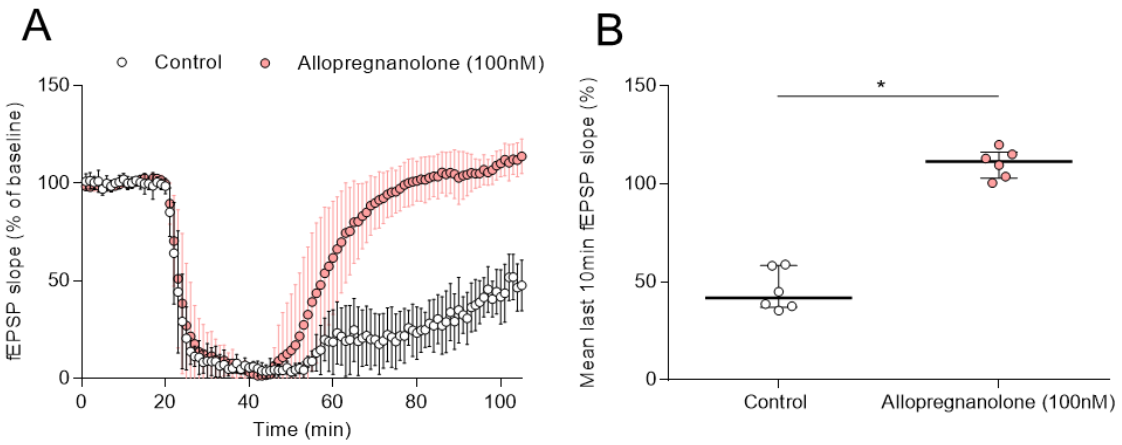

Fig. 32: Neuroprotective effects of allopregnanolone in WT mice.

(A) Normalised fEPSP slope time course following a H/H period under control and after 60min of allopregnanolone 100nM. **(B)** Scatter plot summarising min 50 to 60 after H/H time in control and in the presence of allopregnanolone at 100nM (111(15)% vs control 42(22)%, n=6/6, p=0.002) exerting neuroprotective effects. Markus Ballmann helped with the execution of these experiments.

3.3.4. THDOC protects against H/H-induced excitotoxicity

THDOC showed a significant increase in the fEPSP slope during the recovery period, exerting neuroprotection at either 100nM (103(20)% vs control 60(16)%, n=6/6, p=0.004) or 1µM (112(32)% vs control 60(24)%, n=9/9, p<0.001) (Fig. 33A and B respectively) in WT brain slices.

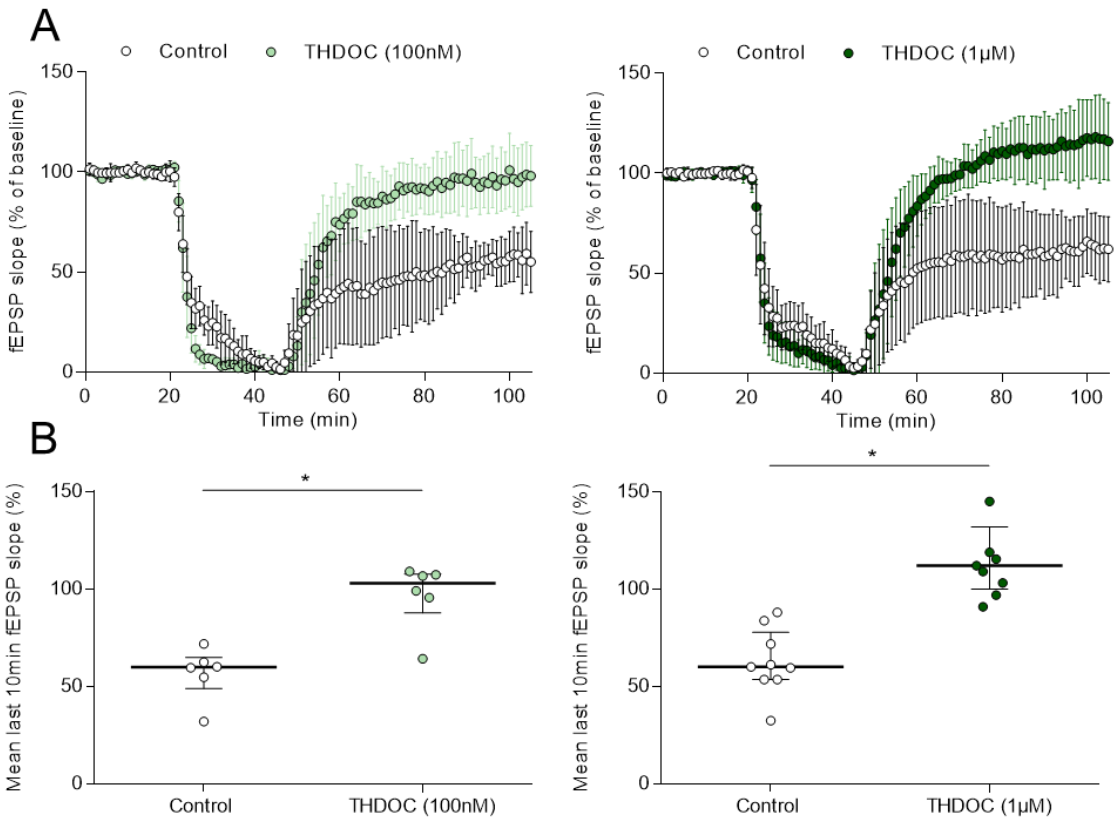

Fig. 33: Neuroprotective effects of THDOC in WT mice.

(A) Normalised fEPSP slope time course following a H/H period under control and after 60min of THDOC 100nM (left) and 1µM (right). **(B)** Scatter plots summarising min 50 to 60 after H/H time in control and in the presence of THDOC 100nM (left) and 1µM (right), where both at 100nM (103(20)% vs control 60(16)%, n=6/6, p=0.004) and at 1µM (112(32)% vs control 60(24)%, n=9/9, p<0.001) showed a significant recovery.

To specifically target the $GABA_AR$ subunits responsible for THDOC's neuroprotective effects, brain slices from $\alpha_{1/2/3}$KI, α_5KI and GABAδKO mice were utilised. In $\alpha_{1/2/3}$KI mice (Fig. 34A), THDOC 100nM (88(16)% vs control 62(24)%, n=7/7, p=0.017) and 1µM (103(32)% vs control 50(39)%, n=8/8, p=0.003) exhibited a significant increase in the fEPSP slope, as well as at 100nM (83(10)% vs control 46(7)%, n=8/8, p=0.001) and 1µM (93(14)% vs control 46(6)%, n=7/7, p=0.001) in α_5KI genotype (Fig. 34B). However, in GABAδKO animals, neither 100nM (21(16)% vs control 19(11)%, n=8/8, p=0.375) nor 1µM (27(23)% vs control 19(11)%, n=8/6, p=0.323) showed any neuroprotective effects (Fig. 34C). These results suggest that δ-$GABA_AR$ subunits play a crucial role for THDOC to exert neuroprotection after a 25min period of H/H.

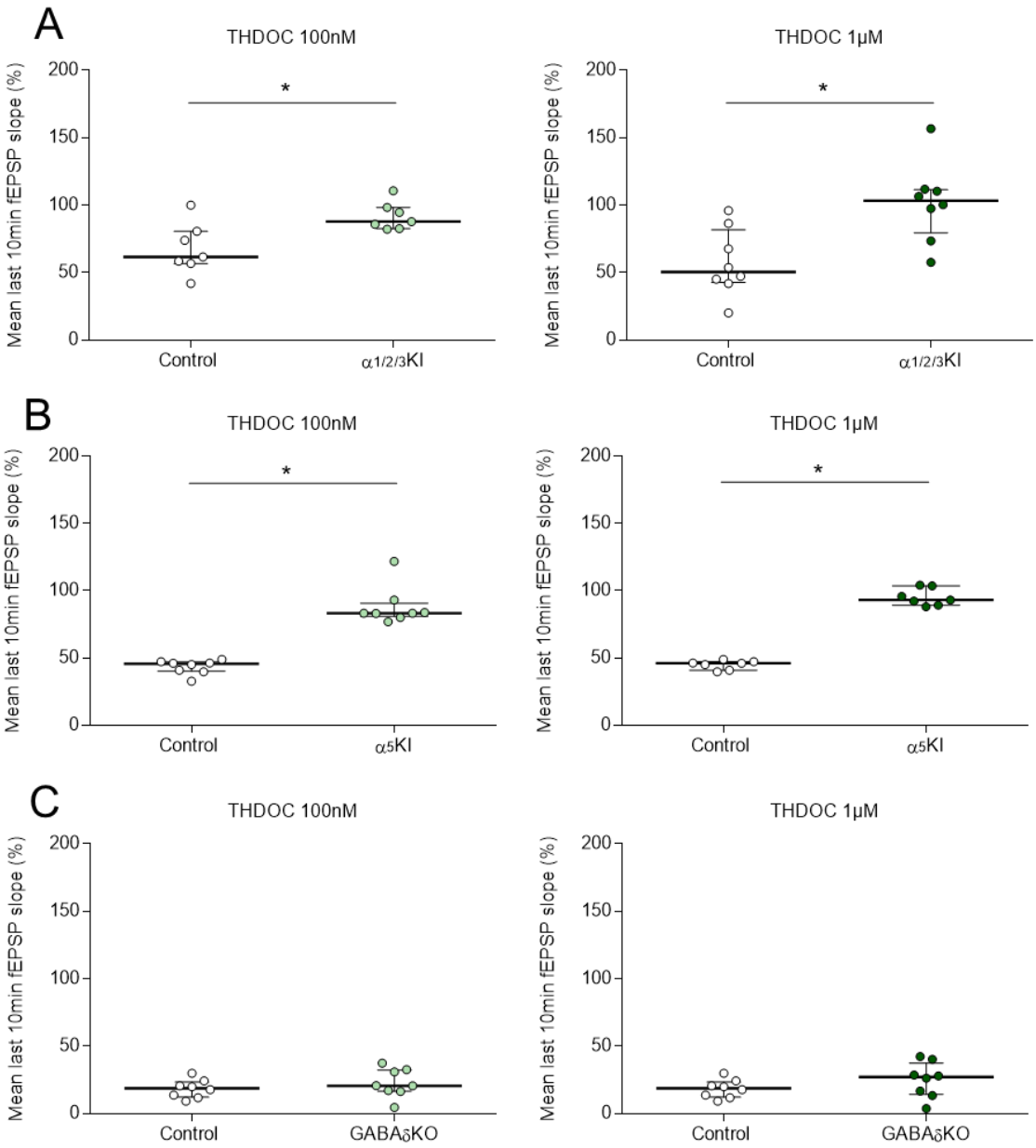

Fig. 34: Neuroprotective effects of THDOC in $\alpha_{1/2/3}$KI, α_5KI and GABAδKO mice.

Scatter plots summarising min 50 to 60 after H/H time in control and in the presence of THDOC 100nM (left side) and 1µM (right side) in **(A)** $\alpha_{1/2/3}$KI, **(B)** α_5KI and **(C)** GABAδKO genotypes. **(A)** In $\alpha_{1/2/3}$KI mice, either 100nM (left; 88(16)% vs control 62(24)%, n=7/7, p=0.017) or 1µM (right; 103(32)% vs control 50(39)%, n=8/8, p=0.003) significantly increased fEPSP during recovery. **(B)** In α_5KI, both at 100nM (left; 83(10)% vs control 46(7)%, n=8/8, p=0.001) and at 1µM (right; 93(14)% vs control 46(6)%, n=7/7, p=0.001) presented a significant increased recovery. **(C)** On the contrary, when THDOC 100nM (left; 21(16)% vs control 19(11)%, n=8/8, p=0.375) and 1µM (right; 27(23)% vs control 19(11)%, n=8/6, p=0.323) were applied to GABAδKO, the fEPSP after recovery was back to baseline levels.

3.4. Modulation of GABAergic CA1-SLM interneuron inhibition

3.4.1. MRK-016 decreases interneuronal connectivity

The modulation of SLM interneuron inhibition was monitored by recording PSs. On one hand, the amplitude of the PS decreased after a drug application when this compound enhanced GABAergic inhibition and therefore, neuronal inhibition was increased. On the other hand, when the amplitude of the PS augmented after a drug administration, GABAergic inhibition is reduced, resulting in a decrease in neuronal inhibition.

It is known that high expression of α_5-$GABA_AR$ subtype can be found at the distal dendrites of CA1 pyramidal neurons (Fig. 7B). Thus, we aimed to confirm this strong α_5 expression by employing MRK-016 because of the high affinity towards this subunit. PS amplitude was significantly augmented after MRK-016 exposure at 100nM in WT mice (141±39% vs baseline 102±1%, n=8/4, p=0.039) (Fig. 35A and B), but no modification was seen in α_5KI animals (101±13% vs baseline 101±2%, n=4/4, p=0.875) (Fig. 35C). On the contrary, PS amplitude was increased in both α_1KI (124±16% vs baseline 101±2%, n=8/8, p=0.016) and $\alpha_{1/2/3}$KI genotypes (133±31% vs baseline 101±1%, n=5/5, p=0.031) (Fig. 35D and E, respectively). These results show that α_5-subunits are expressed in this specific inhibitory circuit.

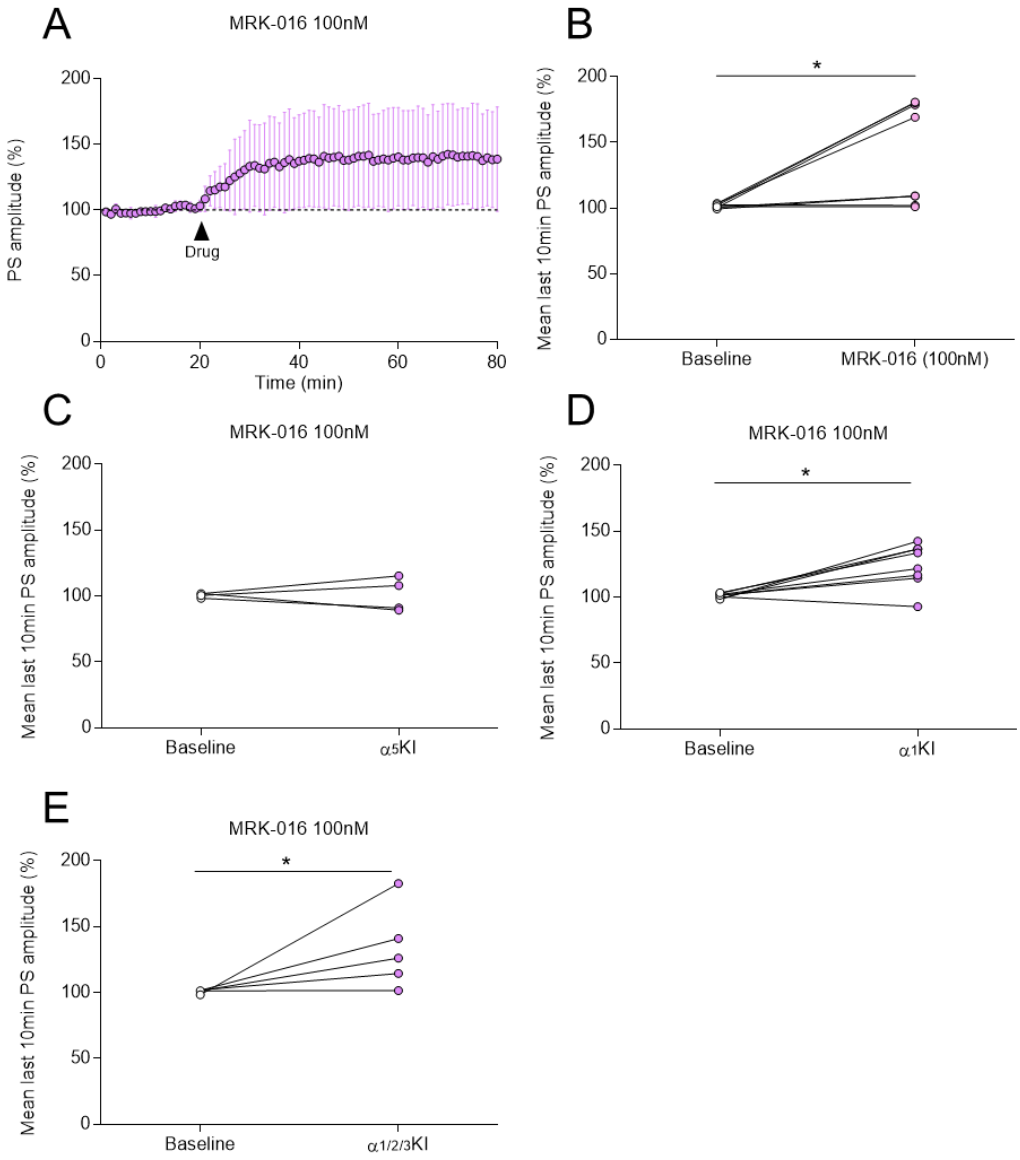

Fig. 35: Effects of MRK-016 on PPI in WT mice.

(A) Normalised PS amplitude time course under control conditions and after 60min of MRK-016 administration at 100nM administration. Connected scatter plots summarising min 50 to 60 after exposure of MRK-016 (with 10ms delay time) in **(B)** WT, **(C)** α_5KI, **(D)** α_1KI and **(E)** $\alpha_{1/2/3}$KI genotypes. PS amplitude was increased after MRK-016 in **(B)** WT mice (141±39% vs baseline 102±1%, n=8/4, p=0.039), but it was not altered in **(C)** α_5KI brain slices (101±13% vs baseline 101±2%, n=4/4, p=0.875). PS amplitude was increased in both **(D)** α_1KI (124±16% vs baseline 101±2%, n=8/8, p=0.016) and (**E)** $\alpha_{1/2/3}$KI (133±31% vs baseline 101±1%, n=5/5, p=0.031) genotypes.

3.4.2. Midazolam modulates interneuronal connectivity

Different concentrations of midazolam in WT animals were tested (Fig. 36A), showing that 10nM could not modulate the PS amplitude (107±11% vs baseline 100±1%, n=7/5, p=0.219) (Fig. 36B). However, 100nM (78±21% vs baseline 101±2%, n=13/7, p=0.001) and 1µM (77±29% vs baseline 100±1%, n=10/7, p=0.037) (Fig. 36C and D respectively) significantly reduced PS amplitude when compared to baseline values.

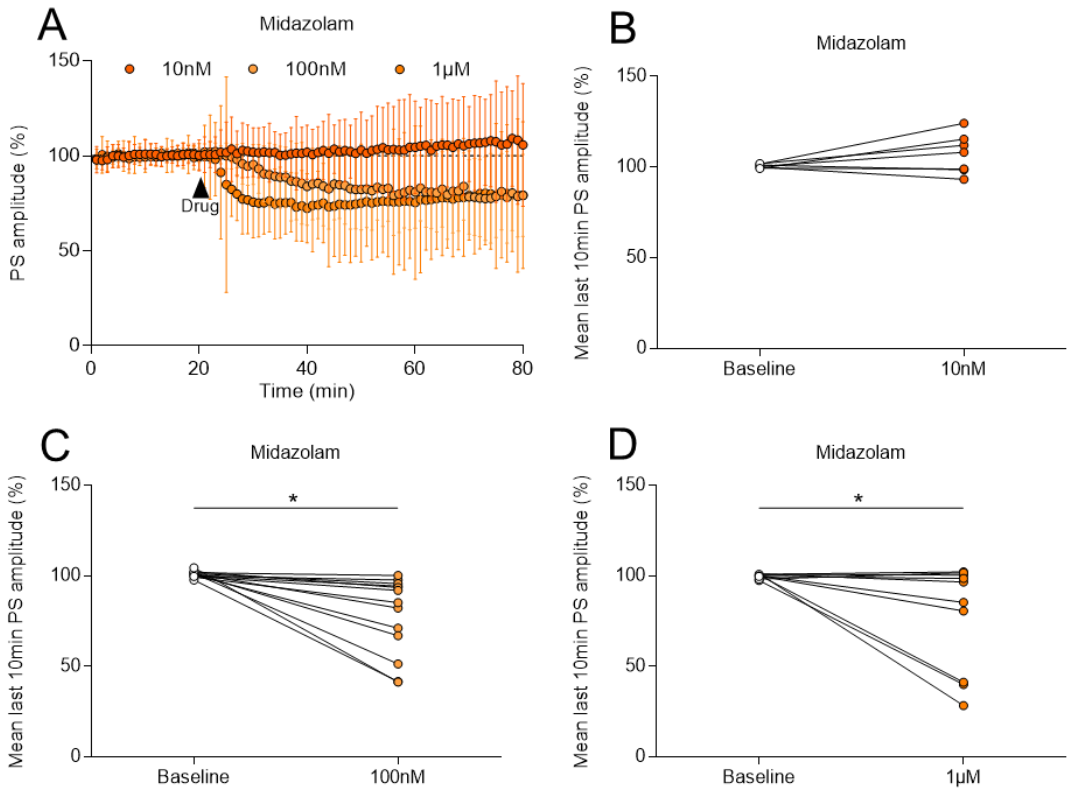

Fig. 36: Effects of midazolam on PPI in WT mice.

(A) Normalised PS amplitude time course under control conditions and after 60min of midazolam administration at 10nM, 100nM and 1µM. Connected scatter plots summarising min 50 to 60 after exposure of midazolam at **(B)** 10nM, **(C)** 100nM and **(D)** 1µM. PS amplitude was not altered after midazolam **(B)** 10nM (107±11% vs baseline 100±1%, n=7/5, p=0.219), but it was significantly reduced after **(C)** 100nM (78±21% vs baseline 101±2%, n=13/7, p=0.001) and **(D)** 1µM exposure (77±29% vs baseline 100±1%, n=10/7, p=0.037).

In addition, midazolam was tested at 10nM and 1µM in different transgenic mouse lines. In α_5KI mice, no PS amplitude alteration was seen after 10nM (107±17% vs baseline 99±2%, n=7/6, p=0.219), but when the concentration was raised up to 1µM (80±25% vs baseline 101±1%, n=11/8, p=0.032), the PS amplitude was significantly reduced (Fig. 37A). In $\alpha_{1/2/3}$KI animals, neither 10nM (108±21% vs baseline 102±1%, n=6/6, p=0.437) nor 1µM (113±12% vs baseline 101±1%, n=4/4, p=0.250) were able to significantly modulate PS amplitude (Fig. 37B). Finally, in α_1KI mice, no changes were seen when 10nM was applied (112±11% vs baseline 101±1%, n=8/8, p=0.055), but 1µM (74±28% vs baseline 100±2%, n=10/9, p=0.027) significantly reduced PS amplitude in comparison to baseline (Fig. 37C). These results show that 10nM is probably too low to affect this circuit, but midazolam at 1µM could be sensitive for α_2-subunits or for a α_1/α_2-subunits combination in our specifically studied inhibitory interneuron circuit.

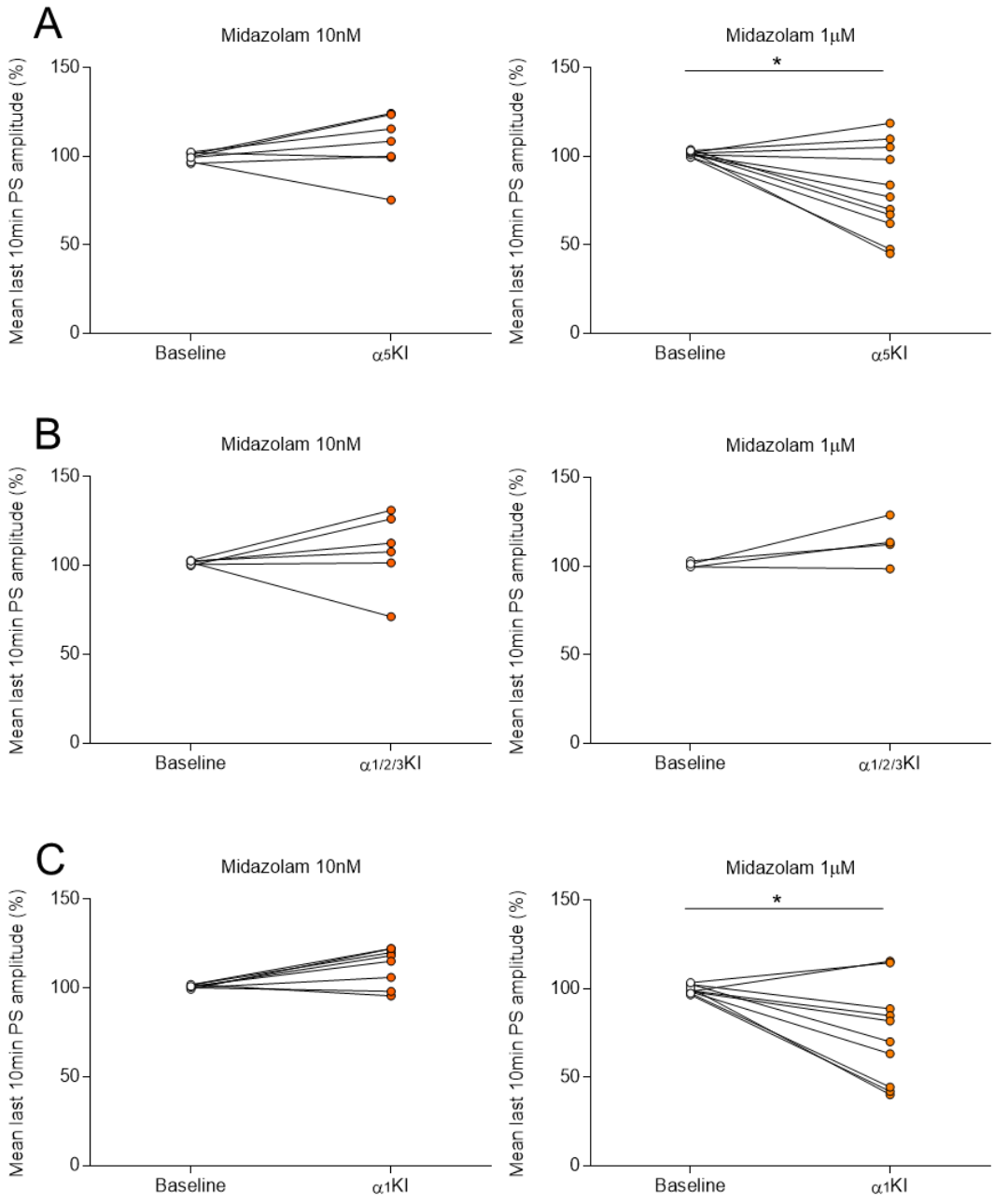

Fig. 37: Effects of midazolam on PPI in α_5KI, $\alpha_{1/2/3}$KI and α_1KI mice.

Connected scatter plots summarising min 50 to 60 after exposure of midazolam at 10nM (left) and 1µM (right) in **(A)** α_5KI, **(B)** $\alpha_{1/2/3}$KI and **(D)** α_1KI genotypes. **(A)** In slices from α_5KI animals, PS amplitude was not modified after 10nM (left; 107±17% vs baseline 99±2%, n=7/6, p=0.219), but it was significantly decreased at 1µM (right; 80±25% vs baseline 101±1%, n=11/8, p=0.032). **(B)** In $\alpha_{1/2/3}$KI slices, neither 10nM (left; 108±21% vs baseline 102±1%, n=6/6, p=0.437) nor 1µM (right; 113±12% vs baseline 101±1%, n=4/4, p=0.250) could significantly modulate PS amplitude. And in **(C)** α_1KI mice, 10nM (left; 112±11% vs baseline 101±1%, n=8/8, p=0.055) did not modify PS amplitude but 1µM (right; 74±28% vs baseline 100±2%, n=10/9, p=0.027) significantly decreased it when compared to baseline.

3.4.3. Flumazenil antagonises the effects of midazolam on interneuronal connectivity

Flumazenil 100nM did not exert any intrinsic effects when tested on PPI (103±6% vs baseline 100±1%, n=10/5, p=0.275) (Fig. 38A). As displayed in Fig. 36C, midazolam 100nM could decrease PS amplitude in WT mice and, for this reason, it was relevant to see if midazolam mediates its depressing effects in this inhibitory interneuron circuit through the BZD binding site. Therefore, midazolam 100nM was administered after a pre-application of flumazenil at a 3:1 stoichiometry excess (300nM), unveiling that midazolam 100nM (101±8% vs flumazenil 300nM 99±6%, n=6/6, p=0.844) could no longer reduce the PS amplitude because flumazenil prevented it (Fig. 38B). Here, it is demonstrated that midazolam 100nM is acting via the BZD binding site, also in this particular interneuron circuit.

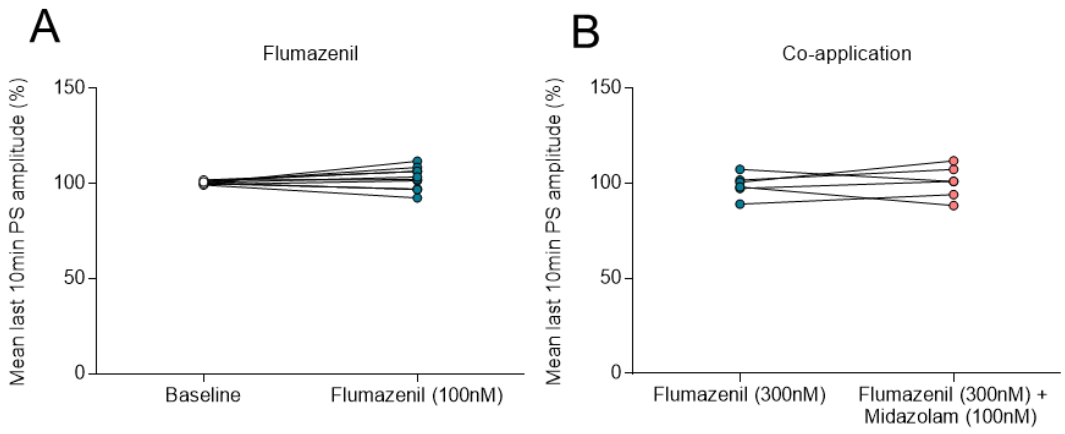

Fig. 38: Effect of flumazenil alone and co-applied with midazolam on PPI in WT mice.

Connected scatter plots summarising min 50 to 60 after exposure of **(A)** flumazenil 100nM and **(B)** co-application of midazolam 100nM after prior flumazenil 300nM exposure. **(A)** Flumazenil 100nM did not modify PS amplitude per se (103±6% vs baseline 100±1%, n=10/5, p=0.275). **(B)** Co-application of midazolam 100nM after a prior administration of flumazenil 300nM, did not exert significant changes (101±8% vs flumazenil 300nM 99±6%, n=6/6, p=0.844) when compared to flumazenil 300nM alone.

3.4.4. Zolpidem has no effect on interneuronal connectivity

Zolpidem exposure on PPI experiments in WT genotype resulted in a non-modulation of the PS amplitude, neither at 100nM (90±34% vs baseline 101±1%, n=7/5, p=0.812) (Fig. 39A) nor at 1µM (100±29% vs baseline 100±1%, n=6/5, p=0.562) (Fig. 39B). Thus, zolpidem does not alter the interneurons in the studied circuit at these concentrations.

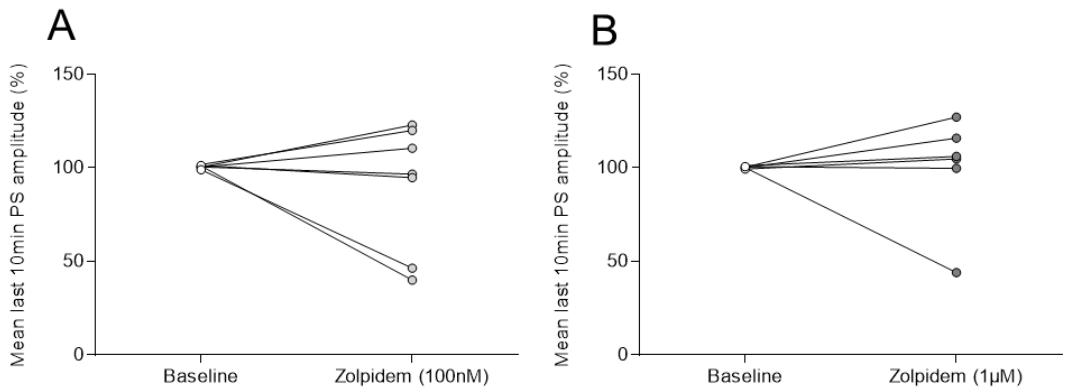

Fig. 39: Effect of zolpidem on PPI in WT mice.

Connected scatter plots summarising min 50 to 60 after exposure of zolpidem **(A)** 100nM and **(B)** 1µM in WT animals. Neither **(A)** 100nM (90±34% vs baseline 101±1%, n=7/5, p=0.812) nor **(B)** 1µM (100±29% vs baseline 100±1%, n=6/5, p=0.562) exhibited any PS amplitude alterations when compared to the baseline.

3.4.5. Small effect of diazepam on interneuronal connectivity

Diazepam 1µM administration in WT mice resulted in a marginal yet significant PS amplitude reduction when compared to baseline values (89±11% vs baseline 99±1%, n=12/12, p=0.021) (Fig. 40A and B). However, when administered to α_5KI (96±29% vs baseline 102±2%, n=6/6, p=0.844) (Fig. 40C), α_1KI (99±29% vs baseline 101±3%, n=9/9, p=0.652) (Fig. 40D) or $\alpha_{1/2/3}$KI mice (91±29% vs baseline 101±2%, n=7/7, p=0.469) (Fig. 40E), PS amplitude was not modified.

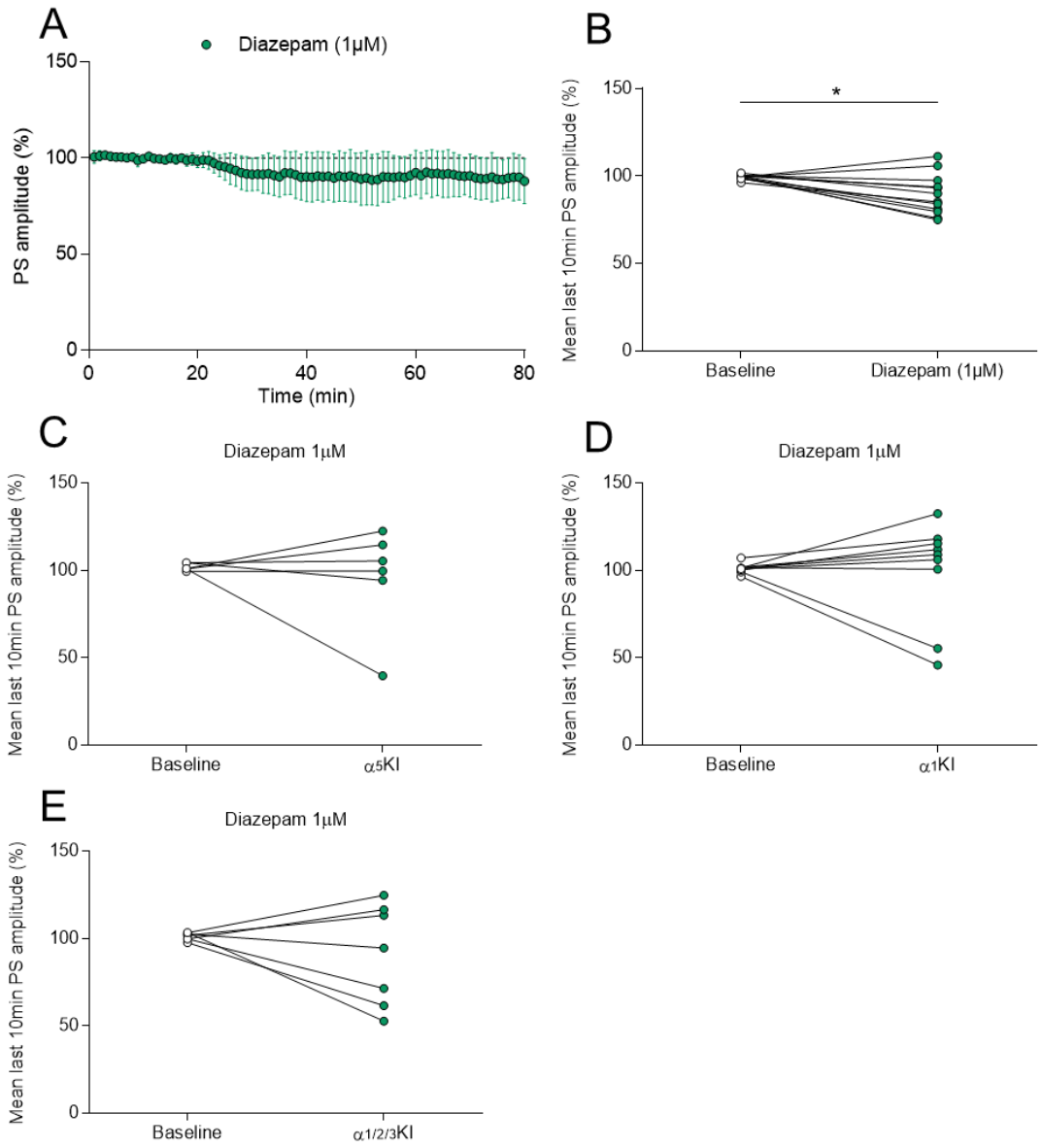

Fig. 40: Effect of diazepam on PPI in WT, α_5KI, α_1KI and $\alpha_{1/2/3}$KI mice.

(A) Normalised PS amplitude time course under control conditions and after 60min of diazepam administration at 1µM. Connected scatter plots summarising min 50 to 60 after exposure to diazepam in **(B)** WT, **(C)** α_5KI, **(D)** α_1KI and **(E)** $\alpha_{1/2/3}$KI genotypes. **(B)** In WT animals, PS amplitude was significantly reduced after diazepam 1µM (89±11% vs baseline 99±1%, n=12/12, p=0.021), but it was not altered in **(C)** α_5KI (96±29% vs baseline 102±2%, n=6/6, p=0.844), **(D)** α_1KI (99±29% vs baseline 101±3%, n=9/9, p=0.652) or **(E)** $\alpha_{1/2/3}$KI (91±29% vs baseline 101±2%, n=7/7, p=0.469) animals.

3.4.6. XBD173 has no effect on interneuronal connectivity

XBD173 was tested at different concentrations in WT mice, but neither 100nM (106±7% vs baseline 101±1%, n=8/5, p=0.055), 1µM (102±14% vs baseline 100±2%, n=7/6, p=0.687) nor 3µM (109±15% vs baseline 100±2%, n=5/5, p=0.312) did produce any significant alterations on PS amplitude when compared to baseline levels (Fig. 41).

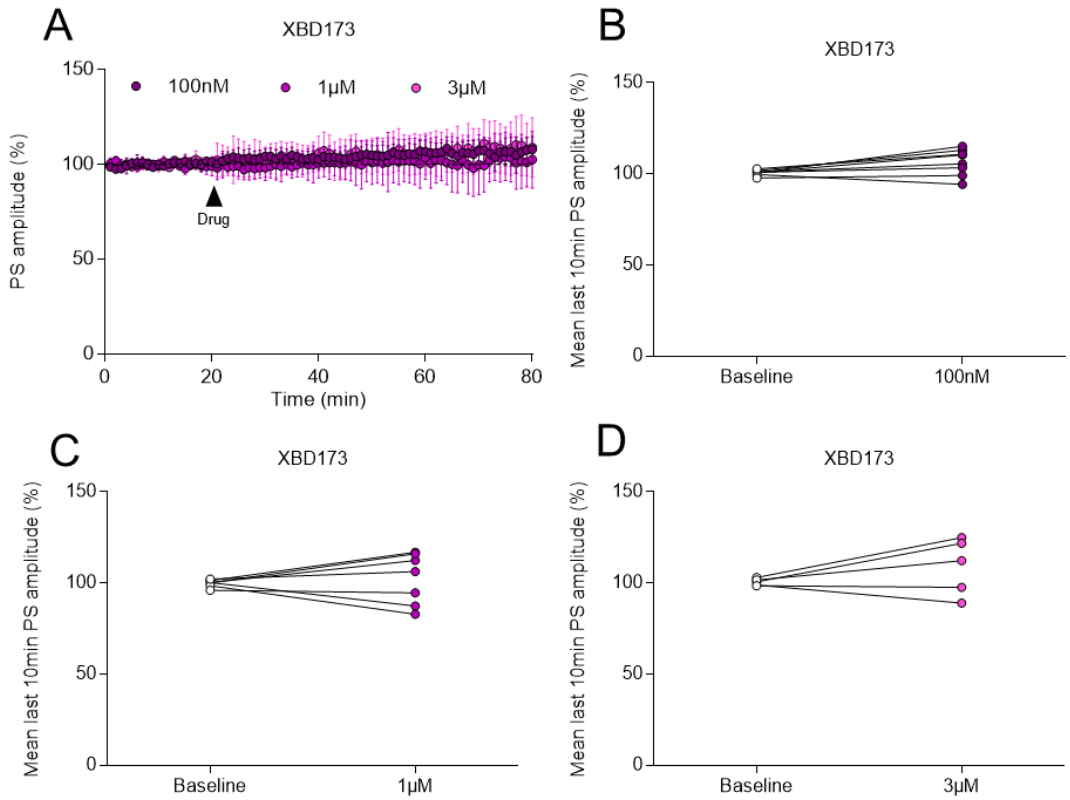

Fig. 41: Effects of XBD173 on PPI in WT mice.

(A) Normalised PS amplitude time course under control conditions and after 60min of XBD173 administration at 100nM, 1µM and 3µM. Connected scatter plots summarising min 50 to 60 after exposure of XBD173 **(B)** 100nM, **(C)** 1µM and **(D)** 3µM. PS amplitude was not altered after **(B)** 100nM (106±7% vs baseline 101±1%, n=8/5, p=0.055), **(C)** 1µM (102±14% vs baseline 100±2%, n=7/6, p=0.687) or **(D)** 3µM (109±15% vs baseline 100±2%, n=5/5, p=0.312).

XBD173's effect on $GABA_{A,fast}$ (1st PS) and $GABA_{A,slow}$ (2nd PS) inhibitory components was also examined. In WT mice, XBD173 at 300nM did not display any alterations in the 1st (100±10% vs baseline 101±2%, n=6/6, p>0.999) or 2nd PS amplitude (103±9% vs baseline 101±2%, n=6/6, p=0.562) (Fig. 42A). Similarly, in α_5KI mice, 1st (99±9% vs baseline 99±2%, n=4/4, p>0.999) and 2nd PS amplitudes (94±24% vs baseline 99±2%, n=4/4, p>0.999) stayed unchanged (Fig. 42B).

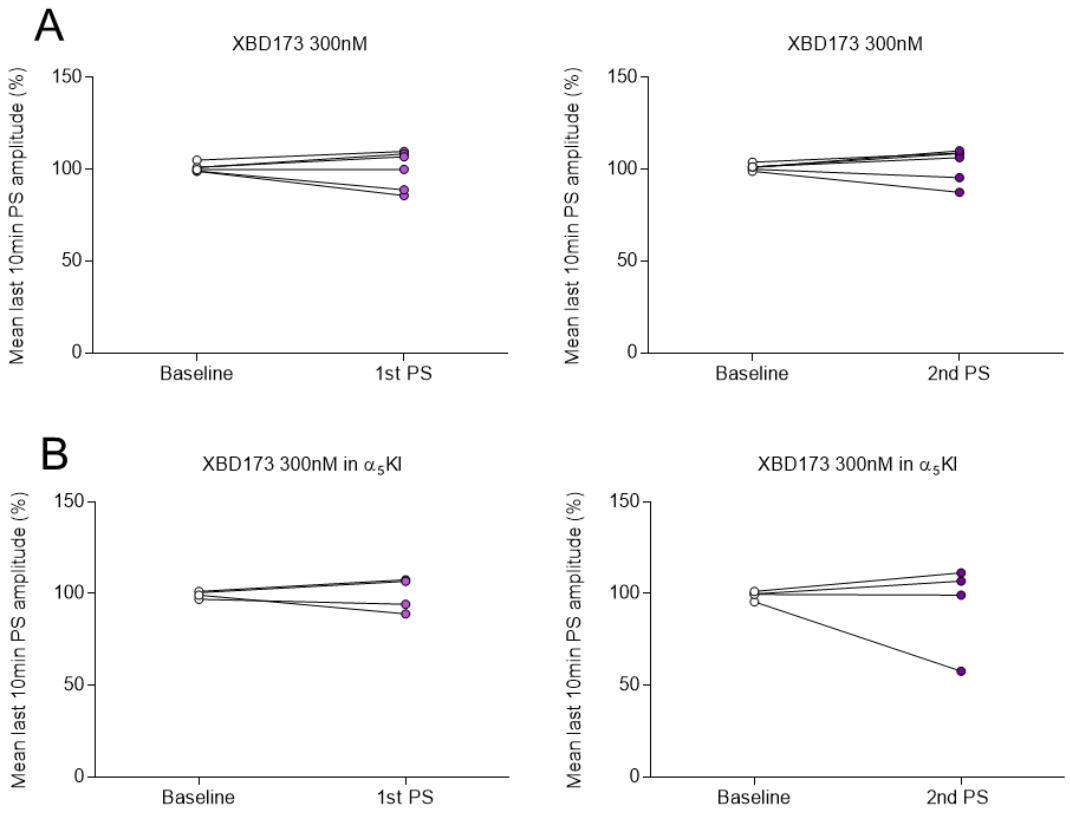

Fig. 42: Effects of XBD173 on PPI2 in WT and α_5KI mice.

Connected scatter plots summarising min 50 to 60 after exposure of XBD173 300nM in **(A)** WT and **(B)** α_5KI genotypes. **(A)** In WT animals, XBD173 300nM did not modify 1st (left; 100±10% vs baseline 101±2%, n=6/6, p>0.999) or 2nd PS amplitude (right; 103±9% vs baseline 101±2%, n=6/6, p=0.562). **(B) I**n α_5KI mice, both 1st PS (left; 99±9% vs baseline 99±2%, n=4/4, p>0.999) and 2nd PS amplitudes (right; 94±24% vs baseline 99±2%, n=4/4, p>0.999) remained unchanged.

3.4.7. Allopregnanolone has no effect on interneuronal connectivity

Exposure of allopregnanolone in WT slices did not alter PS amplitude at either 100nM (104±5% vs baseline 100±1%, n=8/4, p=0.078) or 300nM (103±4% vs baseline 100±1%, n=8/7, p=0.078) (Fig. 43A and B, respectively). Similarly, allopregnanolone 300nM did not show any changes after its application in α_5KI (104±14% vs baseline 101±1%, n=7/7, p=0.687) (Fig. 43C) or in $\alpha_{1/2/3}$KI genotypes (98±14% vs baseline 101±2%, n=5/5, p>0.999) (Fig. 43D). These results show that allopregnanolone does not modulate the interneuron circuit via any of the α-BZDs binding sites (α_1, α_2 or α_5).

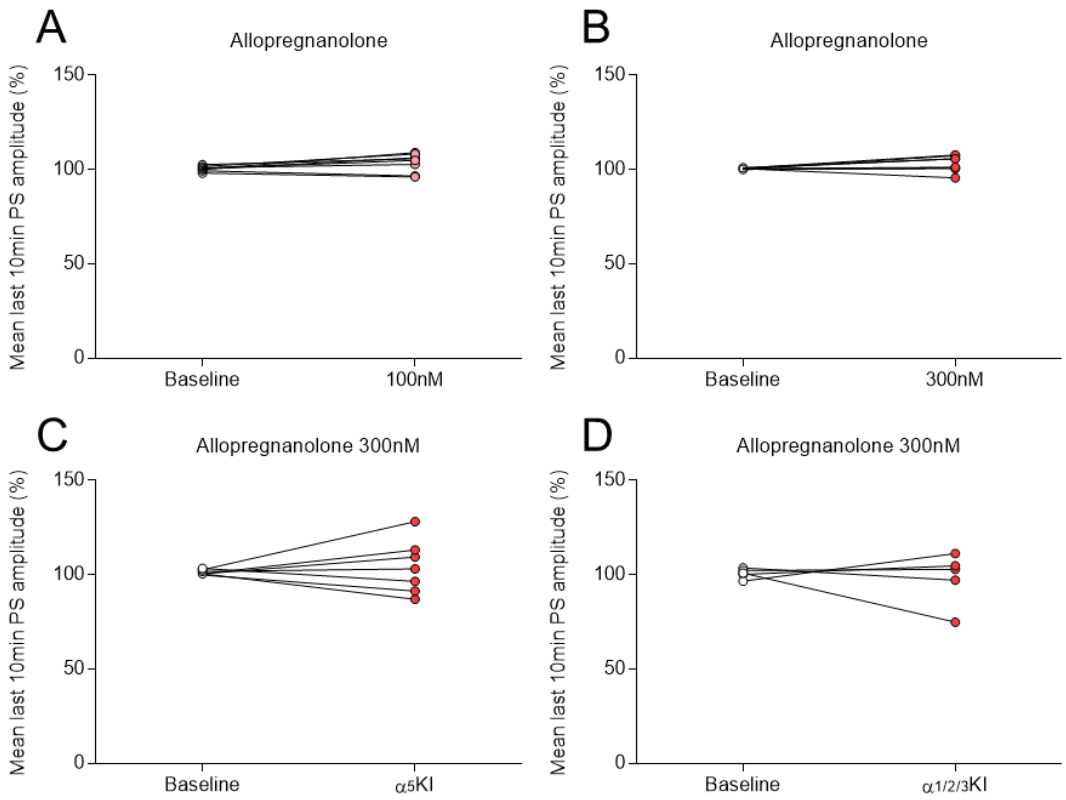

Fig. 43: Effect of allopregnanolone on PPI in WT, α_5KI and $\alpha_{1/2/3}$KI mice.

Connected scatter plots summarising min 50 to 60 after exposure of allopregnanolone **(A)** 100nM and **(B)** 300nM in WT and 300nM in **(C)** α_5KI and **(D)** $\alpha_{1/2/3}$KI genotypes. In WT animals, PS amplitude was modified neither after **(A)** 100nM (104±5% vs baseline 100±1%, n=8/4, p=0.078) nor **(B)** 300nM (103±4% vs baseline 100±1%, n=8/7, p=0.078). Allopregnanolone 300nM did not alter the PS amplitude in **(C)** α_5KI (104±14% vs baseline 101±1%, n=7/7, p=0.687) **(D)** or in $\alpha_{1/2/3}$KI genotypes (98±14% vs baseline 101±2%, n=5/5, p>0.999).

The modulation of $GABA_{A,fast}$ (1st PS) and $GABA_{A,slow}$ (2nd PS) inhibitory components was also studied with allopregnanolone application in WT mice (Fig. 44A). No changes in the PS amplitude were seen after allopregnanolone administration at 100nM neither in the 1st (102±6% vs baseline 101±1%, n=6/6, p=0.844), nor in the 2nd PS (99±7% vs baseline 101±1%, n=6/6, p=0.562) (Fig. 44B). Similarly, at 300nM, 1st (94±3% vs baseline 99±2%, n=5/5, p=0.062) and 2nd PS amplitudes (91±6% vs baseline 99±1%, n=5/5, p=0.062) remained unaltered (Fig. 44C).

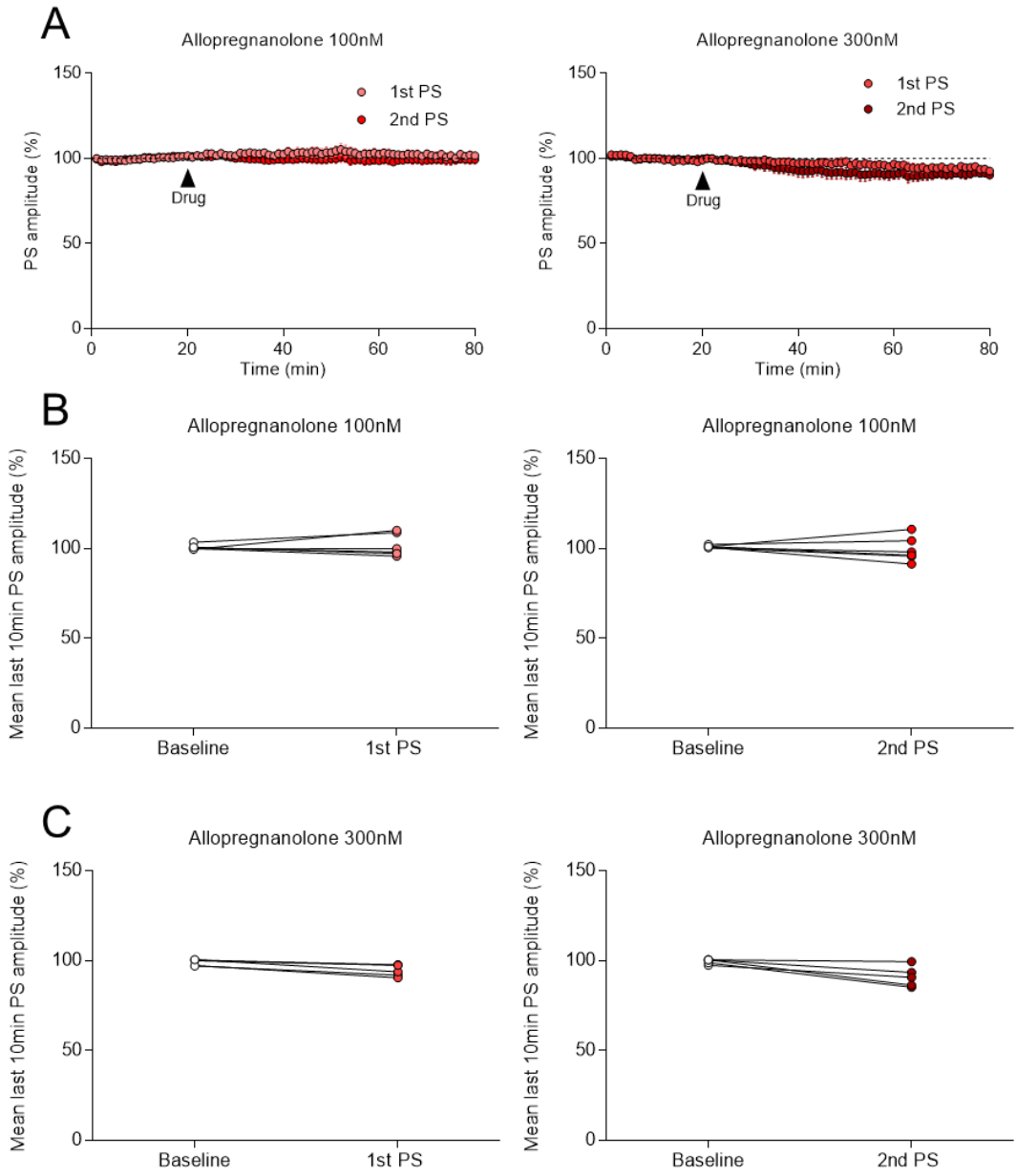

Fig. 44: Effects of allopregnanolone on PPI2 in WT mice.

(A) Normalised 1st and 2nd PS amplitude time course under control conditions and after 60min of allopregnanolone administration at 100nM (left) and 300nM (right). **(B, C)** Connected scatter plots summarising min 50 to 60 after exposure of allopregnanolone at **(B)** 100nM and **(C)** 300nM in WT genotype. **(B)** After allopregnanolone exposure of 100nM, neither the 1st (left; 102±6% vs baseline 101±1%, n=6/6, p=0.844) nor the 2nd PS amplitudes (right; 99±7% vs baseline 101±1%, n=6/6, p=0.562) were altered when compared to baseline. **(C)** After 300nM administration, 1st (left; 94±3% vs baseline 99±2%, n=5/5, p=0.062) and 2nd PS amplitudes (right; 91±6% vs baseline 99±1%, n=5/5, p=0.062) were not modified.

$GABA_{A,fast}$ (1st PS) and $GABA_{A,slow}$ (2nd PS) inhibitory components were also investigated in transgenic mouse lines. Application of allopregnanolone 100nM in α_5KI mice did not modulate the 1st PS (102±19% vs baseline 101±2%, n=3/3, p>0.999) or the 2nd PS amplitudes (87±14% vs baseline 101±3%, n=3/3, p=0.250) (Fig. 45A). No changes were seen when the concentration was increased to 300nM in either the 1st

(97±13% vs baseline 101±2%, n=5/4, p=0.625) or the 2nd PS amplitude (93±8% vs baseline 100±1%, n=5/4, p=0.125) (Fig. 45B). Likewise, 1st PS (left; 101±7% vs baseline 100±1%, n=7/4, p=0.937) and 2nd PS amplitudes (right; 98±8% vs baseline 101±1%, n=7/4, p=0.469) remained unchanged after 300nM exposure in GABAδKO animals (Fig. 45C).

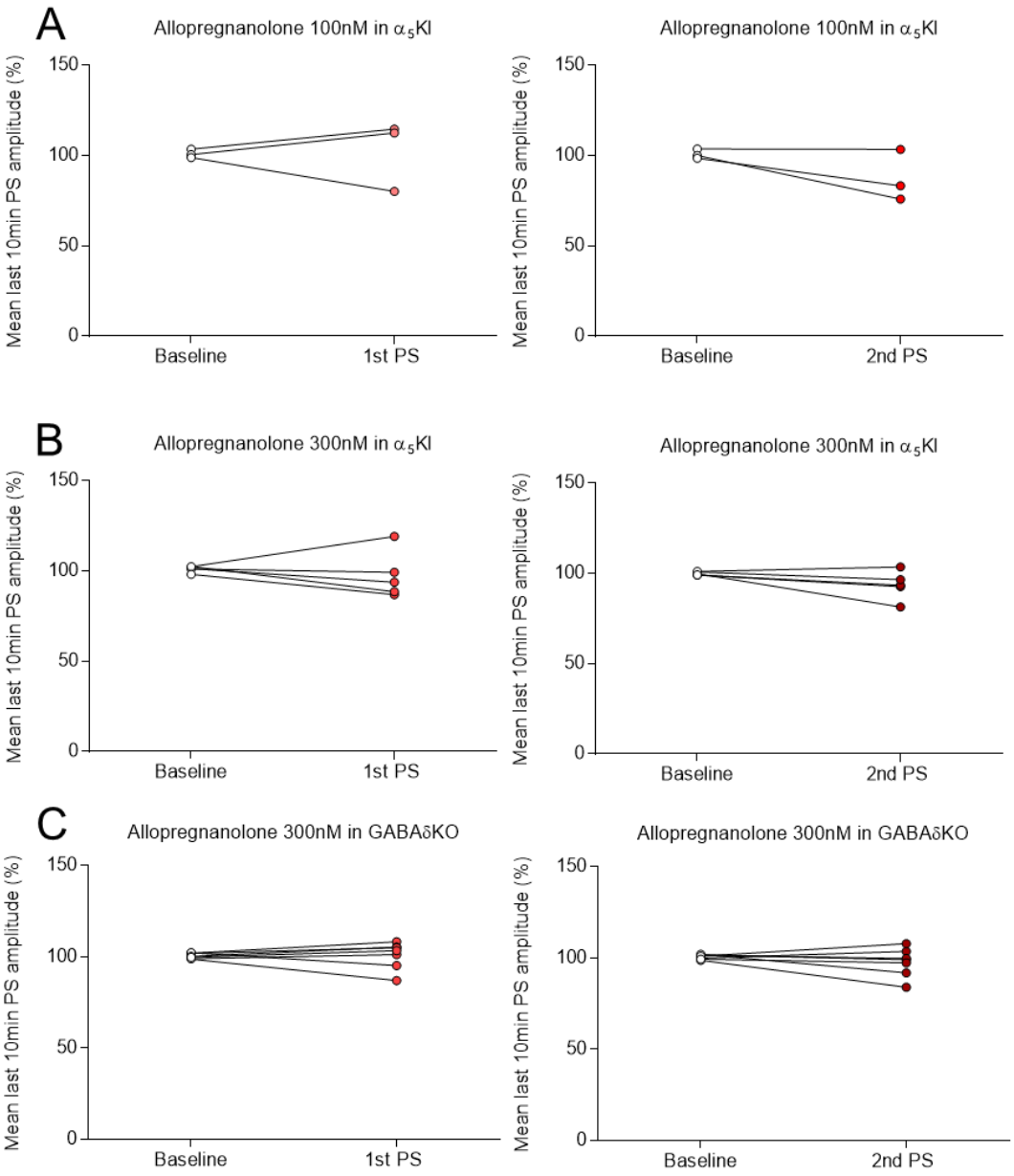

Fig. 45: Effects of allopregnanolone on PPI2 in α5KI and GABAδKO mice.

Connected scatter plots summarising min 50 to 60 after exposure of allopregnanolone **(A)** 100nM in α5KI animals and **(B)** 300nM in α5KI and **(C)** GABAδKO genotypes. **(A)** After 100nM exposure in α5KI mice, 1st (left; 102±19% vs baseline 101±2%, n=3/3, p>0.999) and 2nd PS amplitudes (right; 87±14% vs baseline 101±3%, n=3/3, p=0.250) were not altered. **(B)** Even after 300nM, 1st (left; 97±13% vs baseline 101±2%, n=5/4, p=0.625) and 2nd PS amplitudes remained unchanged (right; 93±8% vs baseline 100±1%, n=5/4, p=0.125). **(C)** In GABAδKO animals, 1st (left; 101±7% vs baseline 100±1%, n=7/4, p=0.937) and 2nd PS

amplitudes (right; 98±8% vs baseline 101±1%, n=7/4, p=0.469) were not modified after 300nM administration.

3.4.8. THDOC has no effect on interneuronal connectivity

Exposure of THDOC in WT brain slices did not significantly alter PS amplitude at 100nM (99±6% vs baseline 101±1%, n=7/5, p=0.812) or 1µM (104±7% vs baseline 100±1%, n=11/6, p=0.083) (Fig. 46), suggesting that this neurosteroid has no effect on the investigated interneuron circuit.

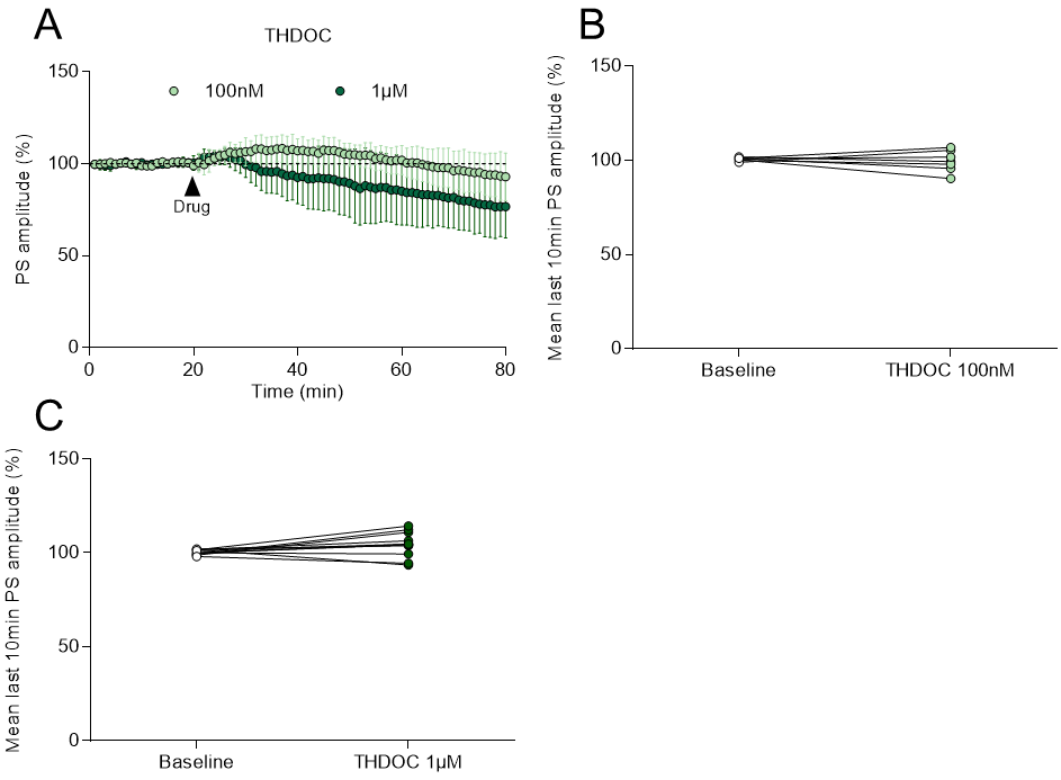

Fig. 46: Effect of THDOC on PPI in WT mice.

(A) Normalised PS amplitude time course under control conditions and after 60min of THDOC administration at 100nM and 1µM. **(B, C)** Connected scatter plots summarising min 50 to 60 after exposure of THDOC **(B)** 100nM and **(C)** 1µM. PS amplitude was not altered either after THDOC at **(B)** 100nM (99±6% vs baseline 101±1%, n=7/5, p=0.812) or at **(C)** 1µM (104±7% vs baseline 100±1%, n=11/6, p=0.083).

4. Discussion

Since the discovery of BZDs in the 1950s, they have been extensively used in the operation room, as well as in the treatment of anxiety-related disorders. Due to potentiation of $GABA_AR$s (Whiting et al., 1995; Sigel & Buhr, 1997), they induce a wide range of actions, such as anxiolysis, sedation, seizure suppression, and muscle relaxation (Rudolph & Knoflach, 2011). However, it has been shown that following the administration of BZDs e.g., midazolam or diazepam, anterograde amnesia and cognitive impairment may occur (Hennessy et al., 1991; del Cerro et al., 1992; Mejo, 1992; Thomas-Antérion et al., 1999; Suri, 2000). Thus, current research in anaesthesiology is focusing on finding alternative drugs exerting a similar anxiolytic profile, yet without adverse side effects. XBD173 is a TSPO ligand that induces neurosteroidogenesis (Rupprecht et al., 2009) and evidence supports that neurosteroids like allopregnanolone and THDOC are also PAMs of $GABA_AR$s at nanomolar concentrations (Majewska et al., 1986; Paul & Purdy, 1992). In rodent and human models, these compounds exert BZD-similar anxiolytic effects, with the lack of sedation, tolerance development, addiction, or anterograde amnesia (Kita et al., 2009; Rupprecht et al., 2009). Hence, the detailing of the acting mechanism of XBD173, and the neurosteroids released upon its administration, may open a new window of opportunity for reducing the side effects in perioperative anaesthesia.

The topic of the present doctoral project was to investigate whether XBD173 provides a beneficial mechanism of action in comparison to BZDs in perioperative care. We studied the influence of BZDs and neurosteroids on LTP, which is a cellular correlate for memory and learning processes (Evans & Viola-McCabe, 1996) and we aimed to elucidate the $GABA_AR$ subunits responsible for its alteration. Moreover, we evaluated the modulation of synaptic transmission by focusing on the cellular level and the role of a concrete circuit of interneurons in the CA1 area. As it would be beneficial that neuroprotection can be guaranteed when anaesthetics are applied, we also examined the $GABA_AR$s subtypes that mediate neuroprotective effects after mimicking an excitotoxic situation in the presence of midazolam or neurosteroids. All investigations were performed employing different electrophysiological techniques in acute brain slices from mice. The hippocampus was the targeted brain area because it is the main structure for learning and memory (Scoville & Milner, 1957).

4.1. Impact of BZDs, BZD-binding site compounds and neurosteroids after LTP induction

In this study, we extensively investigated the action of midazolam on neuronal circuits in the CA1 area of the hippocampus. This drug depicts a shorter acting profile when compared to the typical BZD diazepam (Cole et al., 1983; Suri, 2000). Therefore, midazolam is usually preferred in perioperative anaesthesia. Despite this, it has been widely reported that midazolam administration causes memory impairment in *in vivo* studies and LTP blockage in *in vitro* investigations (Evans & Viola-McCabe, 1996; Veselis et al., 2009). In previous studies, midazolam inhibited CA1 hippocampal LTP at concentrations of 100nM (Tokuda et al., 2010) and 500nM (Evans & Viola-McCabe, 1996). At the CA3 hippocampal region of guinea pigs, midazolam at just 1nM blocked LTP (Satoh et al., 1986). Although this experiment was performed in a different brain region and animal model, this finding already suggested the potency of midazolam against LTP at low nanomolar concentrations. Apart from these investigations and based on our knowledge, low nanomolar concentrations of midazolam were not tested in the CA1 region of the hippocampus so far. In the present results, midazolam at the low concentration of 10nM could inhibit LTP in WT mice in a similar strength as 1µM. However, a dose-response curve was not conducted because it was beyond the scope of the present thesis. When midazolam 10nM was tested in genetically modified mice with KI point mutations at the BZD binding site of $GABA_ARs$, turning them insensitive to bind at this site (Rudolph et al., 1999; Ralvenius et al., 2015), we could identify the subtypes responsible for midazolam's action on LTP. In this study, we found that midazolam inhibits LTP predominantly via acting at α_1-$GABA_ARs$. Moreover, when midazolam was applied in α_1KI mice (α_1 subunits were insensitive for BZD binding), we hypothesise that midazolam targeting α_2-$GABA_ARs$ could not directly modulate LTP, yet dampened α_5-$GABA_ARs$ activity. Thus, these findings provide a hint that there might be a circuit in which α_2 subtypes modulate α_5 subunits. A possible explanation is that neurons expressing postsynaptic α_2-containing $GABA_ARs$ are modulating, through GABA release, the activity of a downstream neuron expressing postsynaptic α_5 subunits. At first sight, the importance of α_1 subunits was an unexpected result since previous investigations concluded that specifically α_5-$GABA_ARs$ control hippocampal-dependent LTP, including learning and memory processes (Collinson et al., 2002; Pofantis & Papatheodoropoulos, 2014). Nevertheless, evidence in rhesus monkeys showed that although both α_1- and α_5-$GABA_ARs$ are involved in cognition, only α_1-$GABA_ARs$ modulation by BZDs was sufficient to exert cognitive impairment (Makaron et al., 2013).

In another study, by employing techniques of *in situ* hybridization, diminished mRNA levels of α_1- and α_5-$GABA_AR$ subunits in the CA1 hippocampal region were found in patients with Alzheimer's disease-like symptoms (Rissman et al., 2004). These mentioned investigations support the result from the present thesis, indicating that α_1-$GABA_AR$ subunits are essential for midazolam's action on memory and learning-involving mechanisms. Moreover, studies showed that effective concentrations of midazolam in humans and rodents are equivalent (Veselis et al., 1997; Laurijssens & Greenblatt, 2002). According to published calculations, a free concentration of midazolam between 10 and 23nM is estimated for causing amnesia and moderate sedation in humans (Veselis et al., 1997). Thus, the studied concentration of 10nM fits very well into the clinically relevant concentration range of midazolam for mild sedative effects.

It is widely known that flumazenil antagonises the sedative effects of different BZDs such as midazolam and diazepam at the $GABA_AR$ without displaying an intrinsic effect. It binds with high affinity to the BZD binding site containing the subunits α_1, α_2, α_3 or α_5 (Ki ~1nM), and with less affinity to the receptors containing α_4 or α_6 subunits (Ki ~150nM), for both human and rat models (Sieghart, 1995; Möhler et al., 2002; Pym et al., 2005). Due to this high selectivity for the BZD binding site in $GABA_ARs$, competitive binding with midazolam is of interest to confirm the binding site of this BZD. The results in this study showed that in the presence of flumazenil, the inhibitory action of midazolam through $GABA_ARs$ after LTP induction was prevented, at least when flumazenil was applied at a 3:1 stoichiometric excess. Our findings are in accordance with previous reports in which flumazenil prevented the detrimental LTP inhibition caused by BZDs (Evans & Viola-McCabe, 1996; Tokuda et al., 2010). Therefore, we confirmed that LTP inhibition after midazolam administration is entirely mediated by the modulation of the BZD binding site at $GABA_ARs$.

The widely used non-BZD drug zolpidem produces hypnotic effects, acting at the BZDs binding site of $GABA_ARs$, but exerting less adverse side effects when compared to BZDs (Arbilla et al., 1985; Balkin et al., 1992). Extensive evidence supports that zolpidem acts mainly via α_1-$GABA_ARs$ (Macdonald, 1994; Rudolph et al., 2001) and that its sedative effects are fully mediated through this subunit in experiments performed *in vivo* (Crestani et al., 2000). Hence, we used zolpidem as a pharmacological tool to specifically target α_1 subunits on LTP regulation. At low nanomolar concentrations, zolpidem shows high affinity for $\alpha_1\beta\gamma_2$ receptors, whereas an intermediate affinity for α_2 and α_3 subtypes at higher nanomolar concentrations has been reported. Nonetheless, the affinity for α_5-containing receptors is extremely low (Korpi et al., 2002a; Olsen & Sieghart, 2008).

Zolpidem is a good candidate to replace BZDs for insomnia medication because reduced tolerance and withdrawal occurrence after long-term treatments have been reported. Albeit lower incidence, discrepancies about tolerance and withdrawal effects between zolpidem and BZDs are obvious (Wright, 2016). Zolpidem at sedative doses exerted no detrimental effects on LTP in the human cortex (Lücke et al., 2014), and the same effect was reported in rat hippocampal slices at a concentration of 1µM, while 10µM inhibited LTP (Higashima et al., 1998). In contrast to these findings, zolpidem at 1µM, but not 100nM, inhibited LTP in our mouse hippocampal slices via α_1-$GABA_A$Rs. Therefore, with these results we can confirm that α_1-$GABA_A$R subtype is highly involved in LTP regulation. It is worth noting that BZDs have non-preferential binding to α_1-, α_2-, α_3-, and α_5-containing $GABA_A$Rs, but zolpidem displays preferential binding and efficacy at $GABA_A$Rs containing α_1 subtype, thereby producing therapeutic sedative effects (Wright, 2016).

In mammals, hippocampal-dependent memory formation is disrupted when $GABA_A$Rs action is increased by diazepam's binding (Seabrook et al., 1997). Indeed, prior studies already noted the important relationship between diazepam and LTP blockage (del Cerro et al., 1992; Wayner et al., 1993; Higashima et al., 1998; Hu et al., 2006), even though Taube and Schwartzkroin (1986) were the only ones to found contradictory results when analysing PS amplitude after HFS. We can show in this study that diazepam did inhibit LTP induction mainly through the α_1-$GABA_A$R subtype at a concentration of 1µM. It is known that diazepam, a classical non-selective BZD, binds with high affinity to $GABA_A$Rs containing α_1, α_2, α_3 or α_5 subunits (Hadingham et al., 1996). Nonetheless, the predisposition for diazepam action through α_1 subunits in modulating LTP could be explained by the fact that 91% of the $GABA_A$Rs sensitive for diazepam in the brain bear one α_1 subunit, in combination of either α_1, α_2 or α_3 subunits (Benke et al., 2004; Müller Herde et al., 2017). Hence, as showed in the experiments mentioned above with midazolam and zolpidem, α_1 subunits can be crucial for regulating LTP in the presence of BZDs or BZD-binding site drugs.

MRK-016 is known for being a selective NAM for α_5-$GABA_A$Rs, therefore facilitating neuronal excitation (Atack et al., 2009; Eimerbrink et al., 2018). Atack and colleagues (2009) demonstrated that MRK-016 has a greater intrinsic efficacy at human recombinant $GABA_A$Rs containing α_5-subunits (-55%) in comparison to α_1- (-16%), α_2- (+6%) and α_3- (-9%) subunits, describing the ability to attenuate (negative values=NAM) or potentiate (positive values=PAM) the current that was produced by an EC_{20}-equivalent concentration of GABA measured with whole-cell patch-clamp recordings. Because of the high affinity for the α_5-$GABA_A$R subtype, we applied MRK-016 to examine the

involvement of α_5 subunits in LTP. The results of our study indicate that α_5-$GABA_AR$ subunits are involved in LTP regulation, since after the application of MRK-016 in WT mice, LTP was increased yet inhibited in α_5KI mice. Our results suggest that when we directly target α_5 subunits with a specific α_5-NAM, LTP can be modulated. However, when we consider the results stated above, we can conclude that α_5 contribute to LTP modulation, but in a physiological receptor when non-specific BZDs are used, the main modulatory LTP effect is mediated via α_1-$GABA_AR$ subunits, as already demonstrated with the administration of midazolam and diazepam.

Due to the adverse side effects of BZDs, we propose the use of potential anxiolytic agents with a neurosteroid-similar action profile. For instance, XBD173 induces neurosteroidogenesis, resulting in anxiolysis via $GABA_ARs$ potentiation. Moreover, XBD173 does not display sedation, tolerance development, withdrawal effects or anterograde amnesia, neither when tested in rodents nor in humans (Majewska et al., 1986; Kita et al., 2009; Rupprecht et al., 2009). Interestingly, a clinical study to assess XBD173's efficacy, safety, and tolerability for treating patients with generalised anxiety disorder has already been conducted by Novartis (ClinicalTrials.gov identifier: NCT00108836). We therefore used XBD173 to induce neurosteroidogenesis and to study the impact of these newly formed neurosteroids on LTP regulation. Furthermore, we tested allopregnanolone and THDOC as potential neurosteroids released upon XBD173 application (Rupprecht et al., 2010; Paul et al., 2020). Generally speaking, XBD173 application for a posterior neurosteroids biosynthesis or direct administration of neurosteroids did not influence LTP and may provide evidence that potential learning and memory processes are not disturbed. We showed that XBD173 administration did not alter LTP upon XBD173-induced neurosteroidogenesis in WT mice. Moreover, by employing the different KI lines targeting the BZD binding site, we could show that these point mutations do not affect neurosteroid binding at the $GABA_AR$. At a concentration of 300nM, XBD173 did not alter LTP, but it was inhibited at 1µM. Even though 1µM is beyond the relevant and physiological neurosteroids concentration since unspecific pathways could be activated, a detailed analysis regarding the effects of neurosteroids at micromolar concentrations was not conducted in this project. Hence, further investigations should consider the involvement of several receptors and activation of other processes at this high and unphysiological concentration.

During the individual evaluation of the selected neurosteroids, our results demonstrated that allopregnanolone at 300nM does not alter LTP in WT animals. Compatibly with these findings, previous investigations reported that allopregnanolone at 100nM did not modify LTP (Izumi et al., 2007; Tokuda et al., 2010); thus, in native conditions, allopregnanolone

does not have detrimental effects on LTP. As for THDOC, 100nM did not influence LTP, but 1µM reduced it. This latter concentration is quite above the physiological range of THDOC (10-500nM) (Wohlfarth et al., 2002; Carver & Reddy, 2013); hence, this LTP reduction could be because of unspecific binding to other receptors. It is worth mentioning that to the best of our knowledge, LTP modulation was not previously tested after the administration of THDOC in CA1 hippocampal neurons. Therefore, we here provide an unprecedented outcome in which this neurosteroid at the relevant physiological concentration of 100nM does not produce detrimental effects on LTP induction.

To mimic the physiological situation in the brain where neurosteroids are commonly present at nanomolar concentrations in the CNS (Purdy et al., 1991), the compound XBD173 was applied before administering the anaesthetic midazolam. Interestingly, the results from this thesis show that the detrimental effects of midazolam at 10nM on LTP can be prevented with a prior administration of XBD173 at 300nM. The assumption about beneficial interaction between neurosteroids and BZDs was already found by Reddy and Kulkarni (1997). Moreover, our results are in line with a recent published work by Lumley and colleagues (2019), where pregnanolone together with diazepam, prevented memory impairment in behavioural tests. Our results could explain that the presence of naturally occurring levels of neurosteroids before administering a BZD anaesthetic may prevent cognitive detrimental effects yet producing anxiolytic effects. From our understanding, this kind of co-application could be an advantage in perioperative anaesthesia.

To sum up, detrimental effects on LTP after nanomolar concentrations of XBD173 were not detected in the present study. Furthermore, the administration of presumably released neurosteroids after XBD173 administration, such as of allopregnanolone and THDOC, did not inhibit LTP. A detailed analysis of the diversity of neurosteroids that are released upon XBD173 application is needed to better understand the acting mechanism of this TSPO ligand.

4.2. Midazolam and neurosteroids enhance $GABA_AR$-mediated synaptic transmission

In this study, midazolam 100nM and 1µM similarly increased synaptic inhibition when sIPSCs were recorded, while 10nM had no effect. These results support the evidence from previous observations in acute brain slices from hippocampal pyramidal neurons (Bai et al., 2001) and from neurons of the auditory cortex (Verbny et al., 2005). Moreover, application of 100nM in slices where α_1 is insensitive for midazolam's action, IPSC's

$GABA_{A,slow}$ constant (τ_2) was not augmented any more, suggesting that the $GABA_{A,slow}$ kinetic may be α_1-subunits dependent. This fact is consistent with an earlier observation which described that the modulation of IPSC's decay time can be related to the varying expression of α-$GABA_A$R subtypes, particularly highlighting the involvement of α_1 and α_3 subunits (Eyre et al., 2012).

In accordance with the present results, published studies have demonstrated that the triggered local production of neurosteroids (via TSPO ligand XBD173) or the direct neurosteroid application (e.g., THDOC and allopregnanolone) resulted in an augmented decay time of the IPSCs through $GABA_A$Rs modulation (Harrison et al., 1987; Cooper et al., 1999; Rupprecht et al., 2009). The literature regarding administration of allopregnanolone and XBD173 resulting in an increased synaptic transmission supports the results of this thesis (Fritschy & Brünig, 2003; Belelli & Lambert, 2005; Rupprecht et al., 2009; Carver & Reddy, 2013; Bukanova et al., 2021). In the present experiments, application of XBD173 at 300nM resulted in a potentiation of the amplitude and the $GABA_{A,slow}$ current of sIPSCs when synaptic transmission was analysed via sIPSCs monitoring. On one hand, allopregnanolone at 100nM increased IPSC's decay time in neocortical mouse neurons (Drexler et al., 2016) and in rat cerebellar and hippocampal neurons (Harney et al., 2003; Bukanova et al., 2021). In the present experiments, only the $GABA_{A,slow}$ component of sIPSCs was increased after allopregnanolone administration. However, the results were consistent with the fact that sIPSC's amplitude was not altered after allopregnanolone exposure. On the other hand, conflicted outcomes have been found after THDOC application. Several reports showed that at low concentrations (10nM), THDOC could not induce IPSC modulation in the phasic component in both thalamocortical and DG granulate cells. Yet, tonic conductance was enhanced (Stell et al., 2003; Cope et al., 2005; Sarkar et al., 2011). In accordance with the present results, previous studies have demonstrated that THDOC at 100nM can increment the decay time of IPSCs in acute and cultured slices (Vicini et al., 2002; Cope et al., 2005; Wu et al., 2012). Although some authors did not even detect this potentiation at 100nM while recording IPSCs in CA1 pyramidal neurons (Stell et al., 2003; Glykys & Mody, 2006). After considering all results, some published reports support the outcome from the present thesis when IPSCs were recorded after 100nM THDOC application, showing an increase of the IPSC's amplitude and $GABA_{A,slow}$ mediated inhibition. Furthermore, we desplay in the present study that THDOC at 1µM increments both decay time constants and amplitude of the evaluated sIPSCs, although this concentration may not be reliable for $GABA_A$R subunit investigations since published data evidenced that at concentrations higher than 500nM, neurosteroids can directly activate $GABA_A$Rs

without GABA. 1µM is not of high interest since this concentration is beyond the physiological range that neurosteroids normally will be released (Wohlfarth et al., 2002; Carver & Reddy, 2013).

The evaluation of synaptic transmission from our results points towards the fact that at least some of the neurosteroids released upon XBD173 administration might be THDOC and allopregnanolone. This assumption could be partially explained because both $GABA_{A,slow}$ decay time constant and amplitude of sIPSCs were enhanced after XBD173 and THDOC application, however allopregnanolone only augmented the amplitude of sIPSCs. To be certain, further investigations involving the monitoring of tonic inhibition and the analysis of the specific neurosteroids released upon XBD173 administration should be conducted.

When δ-subunits of $GABA_A$Rs are missing as in the GABAδKO mouse line, the anxiolytic effect of THDOC was attenuated in mice presenting this alteration. The specificity for this subunit may explain the ability of neurosteroids to induce anxiolysis in rodents (Mihalek et al., 1999; Covey et al., 2000). Taken together, when comparing our results with the published studies, it is possible to estimate that different concentrations of neurosteroids required to achieve a certain effect are dependent on $GABA_A$R subunit composition and are neuron-specific, since enzymes necessary for their synthesis are differentially expressed throughout the CNS (Zorumski et al., 1998; Lambert et al., 2003). Moreover, the phosphorylation state of the receptors, as well as the rodent age appears to also contribute to the distinct modulation of $GABA_A$Rs by neurosteroids (Cooper et al., 1999; Harney et al., 2003).

A possible shortcoming from this project is that it is devoid of tonic conductance measurements. The evaluation of tonic inhibition could corroborate our assumptions concerning the release of THDOC and allopregnanolone after XBD173 administration. Therefore, obtaining phasic and tonic inhibition information should be taken into account for further investigations to specifically detail the $GABA_A$R subunits that are modulated by neurosteroids.

4.3. Neuroprotective properties of midazolam and neurosteroids

The need for improved anaesthetics in perioperative anaesthesia is indisputable since currently used agents like BZDs exert sedation and anxiolysis, but they are directly associated with adverse side effects such as delirium, anxiety, addiction, tolerance

development, anterograde amnesia, and withdrawal symptoms (Curran, 1986; Rudolph et al., 1999; Buffett-Jerrott & Stewart, 2002). Hence, a refined compound without undesired side effects is required for perioperative anaesthesia.

In this study, we extensively investigated the detrimental effects of midazolam on CA1-LTP induction. Nonetheless, we were also interested in studying the potential of the different substances to confer neuroprotection under an excitotoxic scenario. An OGD model was utilised for mimicking an ischaemic situation in the brain which results after a stroke. During this event, brain vessels are being destroyed and the supply of oxygen and glucose is disrupted, causing hypoxia and hypoglycaemia, respectively. This excitotoxicity derives from an enhancement of glutamate levels in the synaptic cleft (Benveniste et al., 1984), leading to an excessive potentiation of NMDARs and producing neuronal death in the brain (Schwartz et al., 1995). For instance, it has been described that NMDAR antagonists are effective neuroprotectants against ischaemic damage in the hippocampus (Gill et al., 1988), but unfortunately, none of the tested compounds in animals reached final clinical phases because of the impossibility to administer these drugs at doses high enough to offer neuroprotection. Furthermore, there is evidence supporting that an acute increase in GABA function could lead to neuroprotection, not only because GABA synthesis and release seems to be decreased during an ischaemic situation, but also because a potentiation in GABAergic activity should reduce the glutamatergic activity that is generating cell death (Green et al., 2000). The neuroprotective effects against ischaemia models had been proven for compounds such as muscimol, but not extensively studied in the presence of BZDs. However, diazepam application before an ischaemic event in the CA1 hippocampal region of gerbils exerted neuroprotective properties related to diazepam exposure before (Sternau et al., 1989) and after the ischaemic period (Schwartz et al., 1994, 1995). More recent investigations described that acute increase in tonic inhibition produced neuroprotective properties, whereas a chronic enhancement of tonic inhibition caused detrimental effects by increasing neuronal death (Clarkson et al., 2010; Brickley & Mody, 2012).

Xue et al., (2004) incubated midazolam at 0.5, 1 and 10μM with rat cortical slices before and after an OGD period, revealing an attenuation of ischaemic damages when high concentrations (10μM) of midazolam were administered. Moreover, it has been suggested that midazolam could mitigate excitotoxicity by modulating the excessive glutamate and neuronal apoptosis after a hypoxia injury in the brain (Yu et al., 2019; Tang et al., 2020). Nevertheless, midazolam concentrations from the mentioned studies are within the high nanomolar and micromolar range. In the present work, midazolam was tested at 10nM because it is the minimum concentration in which a detrimental effect

on LTP was observed in the present study. Strikingly, midazolam at this low concentration exerted neuroprotective effects after H/H induction and as a result of investigating its effect in transgenic mice, it was possible to reveal that α_5-containing $GABA_ARs$ are indispensable for midazolam to produce its neuroprotective action against this model of excitotoxicity. The literature proposes that BZDs are not able to modulate typical $GABA_ARs$ expressed in the extrasynaptic site containing α_4, α_6 or δ subunits (Barnard et al., 1998). However, BZDs can bind to α_5-$GABA_ARs$, and specifically to the $\alpha_5\beta\gamma_2$ subtype, which is predominantly expressed in the CA1 hippocampal extrasynaptic region (Brünig et al., 2002; Crestani et al., 2002), although expression in the synaptic cleft has also been reported (Farrant & Nusser, 2005; Serwanski et al., 2006). Wang et al., (2022) showed that midazolam could increase currents mediated by $GABA_ARs$ assembled of $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$ and $\alpha_5\beta_2\gamma_2$ subunit combinations, and that flumazenil was able to abolish those effects. Prior studies have noticed the importance of extrasynaptic α_5 subunits in tonic conductance modulation, hence attributing to this subtype a vital role in hippocampal-related cognitive processes (Caraiscos et al., 2004). It is therefore proposed that midazolam exerts neuroprotection by acting at α_5-containing $GABA_ARs$ mainly located in the extrasynaptic site.

In the present study, allopregnanolone provided neuroprotection in the presence of H/H-induced excitotoxic processes when applied at 100nM. This outcome is in line with previous investigations, which indicated that allopregnanolone promoted neuroprotection long after ischaemia, despite not preventing pyramidal neuron loss in CA1 hippocampal area (Moralí et al., 2011). After TBI in rats, allopregnanolone administration reduced apoptotic markers and improved behavioural outcome in animals (Djebaili et al., 2004, 2005). Moreover, this neurosteroid provided neuroprotection in experimental models of neurodegenerative diseases (see review Guennoun, 2020), and after ischaemic injury due to artery occlusion (Sayeed et al., 2006). Here, we present an additional scenario where allopregnanolone at nanomolar concentrations exerts neuroprotective effects after resembling a neuronal excitotoxic situation *in vitro*. Unfortunately, the $GABA_AR$ binding site of allopregnanolone is still not fully identified, and further investigations are needed to elucidate it. A suggested approach would be to use mice with a genetic modification at the α-β subunit interface, which is the proposed binding site for allopregnanolone (Hosie et al., 2007; 2009) to prove its efficacy because in the present study, the KI mutations specifically targeted the BZD binding site, and not the proposed $GABA_AR$ binding site for allopregnanolone.

As for THDOC, this neurosteroid conferred neuroprotection after H/H-induced excitotoxicity at a relevant physiological concentration of 100nM, the same concentration

in which detrimental effects on LTP were proven to be absent. Here, it was possible to uncover the importance of the δ-$GABA_AR$ subtype for neuroprotection upon a 25min of OGD period. In H/H experiments with GABAδKO mice, control slices could not recover as much as the slices from WT mice, and posterior application of THDOC was unable to enhance the recovery. The fact that in GABAδKO mice the recovery was lower than in WT mice shows that δ-$GABA_AR$ subunits are critical for neuronal survival. It is worth mentioning that immunochemical staining revealed a rather low expression of δ-$GABA_ARs$ in the CA1 region, whilst it is abundant in the DG granulate cells (Pirker et al., 2000; Peng et al., 2002), and present in the interneurons from the *stratum radiatum* in the CA1 area (Lee & Maguire, 2013). Nonetheless, $GABA_ARs$ containing δ subunits have a significant impact on neuroprotection, as seen in the present experiments with GABAδKO mice, and in previous studies when THDOC was applied (Scimemi et al., 2005). Neumann and colleagues (2019) reported that modulation of δ-$GABA_ARs$ produced neuroprotective effects after stroke and regulated inflammation in the mouse model. Several investigations describe that extrasynaptic receptors ($GABA_ARs$ containing either δ or α_5 subunits) provide tonic conductance under low concentrations of ambient GABA (Glykys & Mody, 2007), and this inhibition is associated with neuroprotection (Brickley et al., 2001; Stell et al., 2003; Cope et al., 2005; Clarkson et al., 2019). Moreover, suppression of δ subunits consequently decrease α_4 subunits while increasing γ_2 expression (Korpi et al., 2002b; Peng et al., 2002), since α_4 subunits are usually expressed together with δ and δ/γ_2 subunits are mutually exclusive. This change in subunit expression may help to explain that mice lacking functional δ subunits presented a lower sensitivity to the anxiolytic effects of neurosteroids (Mihalek et al., 1999), due to extensive evidence supporting the fact that sensitivity to neurosteroids is strongly conferred by δ subunits (Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002).

Nanomolar concentrations of allopregnanolone and THDOC were reported to have different effects on network inhibition at neocortical cultured cells (Puia et al., 2012). Perhaps there are slight distinctions in their action *in vivo* that account for this discrepancy. These two neurosteroids might bind with different affinities to distinct $GABA_AR$ subtypes, they may cause different effects on non-$GABA_ARs$ targets, or they could activate intracellular pathways in certain cells. Moreover, in previous studies, THDOC showed a higher effectiveness than allopregnanolone at inducing tonic conductance (Locci & Pinna, 2017). Hence, further research should be undertaken to investigate the concrete targeted subunits of these neurosteroids to fully understand their effects.

Our findings regarding the neuroprotective effects after XBD173 administration are in line with previous investigations, in which XBD173-induced neuroprotection after temporal ischaemia in the retina has been proven, reducing neuronal cell loss in mice (Mages et al., 2019). Furthermore, a similar effect to THDOC was seen when XBD173 was tested in the same GABAδKO mice after H/H-induced excitotoxicity. In our results, when δ subunits are lacking in the receptor, not even the application of XBD173 triggering neurosteroidogenesis could produce beneficial effects after H/H, indicating that $GABA_AR$s containing δ subunits are essential for a well-functioning brain. Thus, it is proposed that XBD173 induces the synthesis of neurosteroids such as THDOC and allopregnanolone, providing neuroprotection in the CA1 hippocampal region when applied at concentrations within the physiological range. Here, I would like to emphasise that for a detailed assessment of XBD173's mechanism of action, a thorough analysis of the specific neurosteroids released upon XBD173 application is urgently needed.

A potential limitation must be taken into consideration when attempting to transfer the results from this study into the clinical practice. In the OGD model used to mimic excitotoxicity in the brain, the neurosteroids and drugs of interest were applied before and after induction of the H/H period. This design is not suitable for novel ischaemia treatment because in this case, the interest relays on using a drug that confers neuroprotection immediately after suffering a stroke. However, in this study the focus was on determining the neuroprotective action profile during and after the application of an anaesthetic or neurosteroid, and the proposed experimental design is well-chosen for this kind of assessment.

4.4. Alteration of GABAergic-mediated inhibition via stimulating SLM interneurons

The hippocampal CA1 structure is one of the most studied regions in the mammalian brain. This area contains pyramidal neurons, which are quite homogenous and interneurons, which are extremely heterogeneous. In the specific CA1 area, after decades of extensive research, 23 classes of GABAergic interneurons have been described (Harris et al., 2018). With the PPI configuration used in this study, the interneurons from the SLM were stimulated and the response at the soma of the pyramidal neurons was recorded. The principal purpose of this approach was to analyse the influence of α_5 and δ subunits on GABAergic interneuron modulation in this chosen circuit, since these $GABA_AR$ subtypes are expressed at the distal dendrites of pyramidal cells (see Fig. 7B). The interneurons that innervate these pyramidal cells from the SLM

layer are the axo-axonic cells (AAC), parvalbumin-expressing basket cells (PVBC) and a specific type of cholecystokinin-expressing basket cells (CCKBC) (Pelkey et al., 2017).

This investigation demonstrated that midazolam at 100nM and 1μM increased GABAergic inhibition in the hippocampus mediated by the stimulated CA1 interneurons, but not at 10nM. A possible explanation for the lack of effect at 10nM might be that the specific synapse studied with the PPI paradigm was not directly affected by this low concentration of midazolam; perhaps a higher concentration is needed for activating this particular CA1 interneuron circuit. Although no extensive research was found in which interneuron GABAergic-mediated inhibition was studied with PPI paradigms, a publication in 2010 reported similar effects for midazolam at 100nM when PS amplitude was monitored (Tokuda et al., 2010). In the present work, midazolam at 1μM enhanced GABAergic inhibition via CA1 interneurons at the studied circuit. After investigating this concentration in transgenic mice, we suggest that this particular interneuron inhibition could be regulated by an interplay of different $GABA_A$Rs subtypes, since midazolam could enhance GABAergic inhibition by modulating α_1 and α_2 subunits. Even though α_1 subunits are highly expressed at the somata of CA1 pyramidal cells (Klausberger et al., 2002), the PVBC and AAC interneurons innervating the dendrites of those pyramidal cells from the SLM region also express these subunits (Christie et al., 2002). Moreover, the results in this study showed that in the presence of BZD-binding site antagonist flumazenil, the inhibitory action of midazolam through $GABA_A$Rs was prevented in the studied interneuron circuit, at least when flumazenil was applied at a 3:1 stoichiometric excess. Comparison of these findings with those of other studies confirms that flumazenil can reverse the effect of midazolam under different settings, including the PPI paradigm (Imperato et al., 1993; Evans & Viola-McCabe, 1996; Bai et al., 2001; Tokuda et al., 2010; Reddy et al., 2015). Hence, these results support that midazolam in this studied SLM interneuron circuit also modulates $GABA_A$Rs via targeting the BZD binding site.

MRK-016 was used as a pharmacological tool to specifically investigate the role of α_5-$GABA_A$R subunits in the SLM interneuron circuit. Its application decreased the interneuron-mediated inhibition, thereby demonstrating that α_5-containing $GABA_A$Rs are expressed in this specific interneuron circuit. In fact, this result is in accordance with previous reports in which this subunit was found extensively expressed in distal dendrites of pyramidal cells (Fritschy & Mohler, 1995; Sperk et al., 1997). It is worth noting that multiple genetic and pharmacological studies have demonstrated the importance of α_5 in CA1-mediated tonic inhibition (Glykys et al. 2008) and therefore the involvement of these subunits in the regulation of learning and memory. With this experiment using MRK-016, it is proposed that at least the tonic conductance dependent on the specific SLM

interneurons investigated here can be mediated via α_5-containing $GABA_A$Rs. It has been shown that α_5 subunit expression is more intense in the dendrites of the CA1 pyramidal cells, rather than in the somata (Fritschy & Mohler, 1995; Sperk et al., 1997; Christie et al., 2002), and recently findings described the expression of α_5 subunits in certain CA1 interneurons including CCK-expressing interneurons (Petrache et al., 2020), which are stimulated in the PPI paradigm used in the present work. Altogether, the results from this project are consistent with previously published findings, suggesting that interneurons highly modulate GABAergic synaptic inhibition when α_5 subunits are targeted.

In the present work, the non-BZD drug zolpidem did not increase the GABAergic inhibition in the interneuron circuit neither at 100nM nor at 1µM, albeit the latter concentration inhibited LTP induction, as already displayed in this thesis. This absence of modulation in GABAergic inhibition was also seen at concentrations of 400nM (Ali & Thomson, 2008; Petrache et al., 2020) and 10µM (Higashima et al., 1998). Hence, the main findings propose that zolpidem even at micromolar concentrations is not potent enough to modulate interneuron inhibition in the studied CA1 hippocampal circuit.

We showed in the current study that after diazepam exposure, GABAergic-mediated interneuron inhibition was slightly increased in WT animals. Since only a marginal effect was seen in the WT model, it is not feasible to conclude the subunits modulated by diazepam. However, it could be possible that an interplay including both α_1 and α_5 subunits might be involved in the regulation of the examined CA1 interneuron circuit. In agreement with this suggestion, other authors reported that diazepam increased GABAergic inhibition (Lee et al., 1979) in the neocortical interneurons by targeting dendritic α_5-containing $GABA_A$Rs, either with two α_5 subunits or together with another α subtype (Ali & Thomson, 2008). Here, it is suggested that diazepam may slightly increase GABAergic inhibition in the studied SLM interneuron circuit at a concentration of 1µM.

The interneuron circuit is composed of several types of neurons, expressing different $GABA_A$R subunits and hence contributing to various implications. However, the synchronisation of all these neurons is vital for understanding how GABAergic-mediated synaptic inhibition occurs (Pelkey et al., 2017). A plausible limitation in this work is that the approach used for studying the SLM interneuron inhibition did not fully allow to discriminate the subunits responsible for mediating this type of inhibition in all the different BZDs or BZD-binding site drugs tested. It is therefore suggested that further investigations should define an improved experimental design to elucidate the exact subunits implicated in this process for the different compounds.

The specific $GABA_AR$ binding sites for the neurosteroids allopregnanolone and THDOC are still not fully described. Nevertheless, evidence show that they modulate extrasynaptic δ-containing $GABA_ARs$ (Stell et al., 2003), yet these subunits are relatively insensitive for BZD regulation (Maguire et al., 2005; Maguire & Mody, 2008). Regarding α subunit, lower concentrations of allopregnanolone were more effective at α_1 subtype, rather than at α_2, α_4 or α_5 subunits, but β subtype of $GABA_ARs$ has low influence on allopregnanolone modulation (Belelli et al., 2002; Bracamontes et al., 2011). Moreover, when δ subunits were incorporated instead of γ, $GABA_AR$ modulation via allopregnanolone was increased (Belelli et al., 2002; Bianchi & Macdonald, 2003), although early studies described that $GABA_ARs$ containing $\alpha_1\beta_x\gamma_{2/3}$ subunits are highly specific for this neurosteroid's binding at physiological nanomolar concentrations (Maitra & Reynolds, 1999). By using the PPI2 configuration, we intended to separate $GABA_{A,fast}$ and $GABA_{A,slow}$ mediated inhibition since it is still not clear which component contributes to the undesired side effects of anaesthetic agents (Cayla et al., 2019).

Previous investigations showed that allopregnanolone at 100nM did not influence PS amplitude (Izumi et al., 2007; Tokuda et al., 2010). This finding is in line with the results of the present study, in which allopregnanolone even at a higher concentration of 300nM could not modulate PS amplitude. In fact, neither allopregnanolone, THDOC (100nM or 1μM) nor neurosteroids release upon XBD173 administration (300nM) altered PS amplitude in any of the PPI configurations tested (pulses separated 10 or 100ms). Interestingly, published data showed that essential enzymes for 5α-reduced neurosteroids formation (like THDOC and allopregnanolone) are nearly absent in GABAergic hippocampal interneurons (Agís-Balboa et al. 2006); therefore, neurosteroidogenesis might not take place directly in those interneurons. However, 5α-reductase is expressed in astrocytes and excitatory neurons, indicating that neurosteroids could influence interneurons through paracrine release. Unfortunately, at the specific SLM interneuron circuit studied in this project, the regulation of GABAergic interneuron inhibition was not detected after the administration of XBD173, suggesting that the expression of 5α-reductase enzymes might not be high enough. Some studies argued a low expression of δ subunits also in interneurons (Sperk et al., 1997; Peng et al., 2002), implying that perhaps the expression of these subunits is not enough for neurosteroids to modulate interneuron inhibition in the specific studied circuit. A critical point in this project may be that in the slice model used, the expression of these subunits in the interneurons was not high enough and thus neurosteroids were unable to reach the sufficient inhibition threshold in order to modulate the amplitudes of PSs. Another explanation could be that due to the distal location and the decay of the signal along the

dendrite, the effect of δ-$GABA_AR$ subunits may be too weak to be able to modulate the PS activity at the soma, or perhaps because the designed PPI paradigms were not the best fit for measuring the effects of neurosteroids in this particular interneuron circuit.

Interneurons located close to the somata of pyramidal neurons are possible candidates for presynaptically modulating $GABA_{A,fast}$ mediated-inhibition, whilst interneurons projecting to dendrites of the pyramidal neurons could be responsible for $GABA_{A,slow}$ mediated-inhibition (Pearce, 1993). It is probable that $GABA_{A,fast}$ and $GABA_{A,slow}$ emerge from activation of different $GABA_AR$ subtypes. Unfortunately, this matter could not be resolved with the neurosteroids applied in this study because none of them distinctly modulate these two components. Hence, more emphasis should be given to elucidate the targeted $GABA_AR$ subunits of neurosteroids and to understand how they modulate GABAergic inhibition in CA1 interneurons.

4.5. Conclusions

The data acquired in the present study suggest that α_1-$GABA_AR$ subunits play an essential role for LTP modulation when BZDs such as midazolam and diazepam are used. Initially, this finding was not expected since various reports described the importance of α_5-$GABA_AR$ subunits for regulating these processes (Collinson et al., 2002; Pofantis & Papatheodoropoulos, 2014). When specifically targeting α_5 subtypes by employing MRK-016, LTP was potentiated, indicating that these subunits are involved in modulating LTP. However, the usage of midazolam which affects all $GABA_AR$ subunits that are BZD sensitive in combination with KI mice, evidenced that α_1 plays a crucial role for LTP modulation, revealing the importance of α_1-$GABA_AR$ subunits in mechanisms of synaptic plasticity. This finding is worthwhile for the design of novel anaesthetics, not only for relating α_1 subunits to sedation as it is already known, but to cognition as well.

The experiments highlighted the importance of extrasynaptic $GABA_AR$ subtypes (containing α_5 and δ subunits) for neuroprotection after H/H-induced excitotoxicity. We showed that midazolam, via enhancement of α_5-$GABA_AR$ subunits, and the neurosteroids released after XBD173 application and THDOC via δ-$GABA_AR$ subunits, conferred neuroprotection in the CA1 region of the hippocampus. Moreover, administration of XBD173 and the neurosteroids allopregnanolone and THDOC did not inhibit LTP. The presented data provide evidence for XBD173 as a promising anxiolytic agent being superior to BZDs since no detrimental effects on LTP were seen, yet neuroprotective properties were described when applied within the physiological concentration range. Moreover, the suggested co-administration of XBD173 and

midazolam resulting in anxiolysis without inhibiting LTP may indicate the path towards a feasible improved adverse side effect profile for perioperative anaesthesia.

5. References

Adaa.org. 2021. Facts & Statistics | *Anxiety and Depression Association of America, ADAA.* [online] Available at: https://adaa.org/understanding-anxiety/facts-statistics

Agís-Balboa, R. C., Pinna, G., Zhubi, A., Maloku, E., Veldic, M., Costa, E., & Guidotti, A. (2006). Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. *PNAS*, *103*(39), 14602–14607. https://doi.org/10.1073/pnas.0606544103

Ali, A. B., & Thomson, A. M. (2008). Synaptic α5 subunit-containing GABAA receptors mediate ipsps elicited by dendrite-preferring cells in rat neocortex. *Cerebral Cortex*, *18*(6), 1260–1271. https://doi.org/10.1093/cercor/bhm160

Amaral, D. G., & Witter, M. P. (1989). The tree-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience*, *31*(3), 571–591.

Anand, K., & Dhikav, V. (2012). Hippocampus in health and disease: An overview. *Annals of Indian Academy of Neurology*, *15*(4), 239–246. https://doi.org/10.4103/0972-2327.104323

Andersen, P., Morris, R., Amaral, D., Bliss, T., & O'Keefe, J. (2007). *The Hippocampus Book*.

Anderson, W. W., & Collingridge, G. L. (2001). The LTP Program: A data acquisition program for on-line analysis of long-term potentiation and other synaptic events. *Journal of Neuroscience Methods,* 108(1), 71–83. https://doi.org/10.1016/S0165-0270(01)00374-0

Anholt, R. R. H., Pedersen, P. L., De Souza, E. B., & Snyder, S. H. (1986). The peripheral-type benzodiazepine receptor. Localization to the mitochondrial outer membrane. *Journal of Biological Chemistry*, *261*(2), 576–583. https://doi.org/10.1016/s0021-9258(17)36131-8

Antkowiak, B., & Rammes, G. (2019). GABA(A) receptor-targeted drug development-new perspectives in perioperative anesthesia. *Expert Opinion on Drug Discovery*. Taylor and Francis Ltd. https://doi.org/10.1080/17460441.2019.1599356

Anzini, M., Cappelli, A., Vomero, S., Seeber, M., Menziani, M. C., Langer, T., … Bourguignon, J. J. (2001). Mapping and fitting the peripheral benzodiazepine receptor binding site by carboxamide derivatives. Comparison of different approaches to quantitative ligand-receptor interaction modeling. *Journal of Medicinal Chemistry*, *44*(8), 1134–1150. https://doi.org/10.1021/jm0009742

Arbilla, S., Depoortere, H., George, P., & Langer, S. Z. (1985). Pharmacological profile of the imidazopyridine zolpidem at benzodiazepine receptors and electrocorticogram in rats. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *330*(3), 248–251. https://doi.org/10.1007/BF00572441

Atack, J. R., Maubach, K. A., Wafford, K. A., O'Connor, D., Rodrigues, A. D., Evans, D. C., … Petty, K. J. (2009). In vitro and in vivo properties of 3-tert-butyl-7-(5-

methylisoxazol-3-yl)-2-(1-methyl-1H-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-d]-[1,2,4]triazine (MRK-016), a GABAA receptor α5 subtype-selective inverse agonist. *Journal of Pharmacology and Experimental Therapeutics*, *331*(2), 470–484. https://doi.org/10.1124/jpet.109.157636

Bai, D., Zhu, G., Pennefather, P., Jackson, M. F., MacDonald, J. F., & Orser, B. A. (2001). Distinct Functional and Pharmacological Properties of Tonic and Quantal Inhibitory Postsynaptic Currents Mediated by γ-Aminobutyric Acid $_A$ Receptors in Hippocampal Neurons. *Molecular Pharmacology*, *59*(4), 814–824. https://doi.org/10.1124/mol.59.4.814

Balkin, T. J., O'Donnell, V. M., Wesensten, N., McCann, U., & Belenky, G. (1992). Comparison of the daytime sleep and performance effects of zolpidem versus triazolam. *Psychopharmacology*, *107*(1), 83–88. https://doi.org/10.1007/BF02244970

Balon, R., & Starcevic, V. (2020). Role of Benzodiazepines in Anxiety Disorders. In K. Yong-Ku (Ed.), *Anxiety Disorders. Advances in Experimental Medicine and Biology* (Vol. 1191, pp. 375–427). Springer. https://doi.org/10.1007/978-981-32-9705-0_20

Bandelow, B., & Michaelis, S. (2015). Epidemiology of anxiety disorders in the 21st century. *Dialogues in Clinical Neuroscience*, *17*(3). https://doi.org/10.31887/DCNS.2015.17.3/bbandelow

Barnard, E. A., Skolnick, P., Olsen, R. W., Mohler, H., Sieghart, W., Biggio, G., … Langer, S. Z. (1998). International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric acidA receptors: Classification on the basis of subunit composition, pharmacology, and function. *Pharmacological Reviews*, *50*(2), 291–313. https://doi.org/10.1124/pr.108.00505

Bartesaghi, R., & Gessi, T. (2003). Activation of perforant path neurons to field CA1 by hippocampal projections. *Hippocampus*, *13*(2), 235–249. https://doi.org/10.1002/hipo.10074

Baulieu, E. E., & Robel, P. (1990). Neurosteroids: A new brain function? *Journal of Steroid Biochemistry and Molecular Biology*, *37*(3), 395–403. https://doi.org/10.1016/0960-0760(90)90490-C

Belelli, D. (2005). Extrasynaptic GABAA Receptors of Thalamocortical Neurons: A Molecular Target for Hypnotics. *Journal of Neuroscience*, *25*(50), 11513–11520. https://doi.org/10.1523/JNEUROSCI.2679-05.2005

Belelli, D., & Lambert, J. J. (2005). Neurosteroids: Endogenous regulators of the GABAAreceptor. *Nature Reviews Neuroscience*, *6*(7), 565–575. https://doi.org/10.1038/nrn1703

Belelli, D., Casula, A., Ling, A., & Lambert, J. J. (2002). The influence of subunit composition on the interaction of neurosteroids with GABAAreceptors. *Neuropharmacology*, *43*(4), 651–661. https://doi.org/10.1016/S0028-3908(02)00172-7

Belelli, D., Harrison, N. L., Maguire, J., Macdonald, R. L., Walker, M. C., & Cope, D. W. (2009). Extrasynaptic GABAa receptors: Form, pharmacology, and function. In

Journal of Neuroscience (Vol. 29, pp. 12757–12763). https://doi.org/10.1523/JNEUROSCI.3340-09.2009

Benke, D., Fakitsas, P., Roggenmoser, C., Michel, C., Rudolph, U., & Mohler, H. (2004). Analysis of the presence and abundance of GABAA receptors containing two different types of α subunits in murine brain using point-mutated α subunits. *Journal of Biological Chemistry*, *279*(42), 43654–43660. https://doi.org/10.1074/jbc.M407154200

Benson, J. A., Löw, K., Keist, R., Mohler, H., & Rudolph, U. (1998). Pharmacology of recombinant γ-aminobutyric acida receptors rendered diazepam-insensitive by point-mutated α-subunits. *FEBS Letters*, *431*, 400–404. https://doi.org/10.1016/S0014-5793(98)00803-5

Benveniste, H., Drejer, J., Schousboe, A., & Diemer, N. H. (1984). Elevation of the Extracellular Concentrations of Glutamate and Aspartate in Rat Hippocampus During Transient Cerebral Ischemia Monitored by Intracerebral Microdialysis. *Journal of Neurochemistry*, *43*(5), 1369–1374. https://doi.org/10.1111/j.1471-4159.1984.tb05396.x

Bezaire, M. J., & Soltesz, I. (2013). Quantitative assessment of CA1 local circuits: Knowledge base for interneuron-pyramidal cell connectivity. *Hippocampus*, *23*(9), 751–785. https://doi.org/10.1002/hipo.22141

Bianchi, M. T., & Macdonald, R. L. (2003). Neurosteroids shift partial agonist activation of GABAA receptor channels from low- to high-efficacy gating patterns. *The Journal of Neuroscience*, *23*(34), 10934–10943. https://doi.org/23/34/10934

Bixo, M., Andersson, A., Winblad, B., Purdy, R. H., & Bäckström, T. (1997). Progesterone, 5 α-pregnane-3,20-dione and 3 α-hydroxy-5 α-pregnane- 20-one in specific regions of the human female brain in different endocrine states. *Brain Research*, *764*(1–2), 173–178. https://doi.org/10.1016/S0006-8993(97)00455-1

Bloom, F. E., & Iversen, L. L. (1971). Localizing 3H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. *Nature*, *229*(5287), 628–630. https://doi.org/10.1038/229628a0

Boehm, S. L., Homanics, G. E., Blednov, Y. A., & Harris, R. A. (2006). δ-Subunit containing GABAA receptor knockout mice are less sensitive to the actions of 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol. *European Journal of Pharmacology*, 541(3), 158–162. https://doi.org/10.1016/j.ejphar.2006.02.054

Booker, S. A., & Vida, I. (2018). Morphological diversity and connectivity of hippocampal interneurons. *Cell and Tissue Research*, *373*(3), 619–641. https://doi.org/10.1007/s00441-018-2882-2

Bormann, J. (2000). The “ABC” of GABA receptors. *Trends in Pharmacological Sciences*, *21*, 16–19. https://doi.org/10.1016/S0165-6147(99)01413-3

Borowicz, K. K., Piskorska, B., Banach, M., & Czuczwar, S. J. (2011). Neuroprotective actions of neurosteroids. *Frontiers in Endocrinology*, *2*(OCT), 1–10. https://doi.org/10.3389/fendo.2011.00050

Bracamontes, J., McCollum, M., Esch, C., Li, P., Ann, J., Steinbach, J. H., & Akk, G. (2011). Occupation of either site for the neurosteroid allopregnanolone potentiates the opening of the GABAA receptor induced from either transmitter binding site. *Molecular Pharmacology*, *80*(1), 79–86. https://doi.org/10.1124/mol.111.071662

Braestrup, C., & Squires, R. F. (1977). Specific benzodiazepine receptors in rat brain characterized by high-affinity [3H]diazepam binding. *PNAS*, *74*(9), 3805–3809. https://doi.org/10.1073/pnas.74.9.3805

Brickley, S. G., & Mody, I. (2012). Extrasynaptic GABA A Receptors: Their Function in the CNS and Implications for Disease. *Neuron*. https://doi.org/10.1016/j.neuron.2011.12.012

Brickley, S. G., Revilla, V., Cull-Candy, S. G., Wisden, W., & Farrant, M. (2001). Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. *Nature*, *409*(6816), 88–92. https://doi.org/10.1038/35051086

Brohan, J., & Goudra, B. G. (2017). The Role of GABA Receptor Agonists in Anesthesia and Sedation. *CNS Drugs*, *31*(10), 845–856. https://doi.org/10.1007/s40263-017-0463-7

Brown, N., Kerby, J., Bonnert, T. P., Whiting, P. J., & Wafford, K. A. (2002). Pharmacological characterization of a novel cell line expressing human alpha(4)beta(3)delta GABA(A) receptors. *British Journal of Pharmacology*, *136*(7), 965–974. https://doi.org/10.1038/sj.bjp.0704795

Brünig, I., Scotti, E., Sidler, C., & Fritschy, J. M. (2002). Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *Journal of Comparative Neurology*, *443*(1), 43–55. https://doi.org/10.1002/cne.10102

Buffett-Jerrott, S., & Stewart, S. (2002). Cognitive and Sedative Effects of Benzodiazepine Use. *Current Pharmaceutical Design*, *8*(1), 45–58. https://doi.org/10.2174/1381612023396654

Bukanova, J., Solntseva, E., Kondratenko, R., & Kudova, E. (2021). Epipregnanolone as a positive modulator of gabaa receptor in rat cerebellar and hippocampus neurons. *Biomolecules*, *11*(6), 1–9. https://doi.org/10.3390/biom11060791

Campagna, J. A., Miller, K. W., & Forman, S. A. (2003). Mechanisms of Actions of Inhaled Anesthetics. *New England Journal of Medicine*, *349*(9), 909–910. https://doi.org/10.1056/nejm200308283490917

Capogna, M., & Pearce, R. A. (2011). GABAA,slow: Causes and consequences. *Trends in Neurosciences*. https://doi.org/10.1016/j.tins.2010.10.005

Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., … Orser, B. A. (2004). Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. *PNAS*, *101*(10), 3662–3667. https://doi.org/10.1073/pnas.0307231101

Carver, C. M., & Reddy, D. S. (2013). Neurosteroid interactions with synaptic and extrasynaptic GABAA receptors: regulation of subunit plasticity, phasic and tonic

inhibition, and neuronal network excitability. *Psychopharmacology*, *230*(2). https://doi.org/10.1007/s00213-013-3276-5

Carver, C. M., & Reddy, D. S. (2016). Neurosteroid structure-activity relationships for functional activation of extrasynaptic δGABAA receptors. *Journal of Pharmacology and Experimental Therapeutics*, *357*(1), 188–204. https://doi.org/10.1124/jpet.115.229302

Cayla, N. S., Dagne, B. A., Wu, Y., Lu, Y., Rodriguez, L., Davies, D. L., … Bertaccini, E. J. (2019). A newly developed anesthetic based on a unique chemical core. *PNAS*, 116(31), 15706–15715. https://doi.org/10.1073/pnas.1822076116

Charalampopoulos, I., Remboutsika, E., Margioris, A. N., & Gravanis, A. (2008). Neurosteroids as modulators of neurogenesis and neuronal survival. *Trends in Endocrinology and Metabolism*, *19*(8), 300–307. https://doi.org/10.1016/j.tem.2008.07.004

Chauveau, F., Boutin, H., Van Camp, N., Dollé, F., & Tavitian, B. (2008). Nuclear imaging of neuroinflammation: A comprehensive review of [11C]PK11195 challengers. *European Journal of Nuclear Medicine and Molecular Imaging*, *35*(12), 2304–2319. https://doi.org/10.1007/s00259-008-0908-9

Chen, Z. W., Bracamontes, J. R., Budelier, M. M., Germann, A. L., Shin, D. J., Kathiresan, K., … Evers, A. S. (2019). Multiple Functional Neurosteroid Binding Sites on GABAA Receptors. *PLOS Biology*, 1–27. https://doi.org/10.1101/357574

Cheng, V. Y., Martin, L. J., Elliott, E. M., Kim, J. H., Mount, H. T. J., Taverna, F. A., … Orser, B. A. (2006). α5GABAA receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. *Journal of Neuroscience*, *26*(14), 3713–3720. https://doi.org/10.1523/JNEUROSCI.5024-05.2006

Christie, S. B., Miralles, C. P., & Blas, A. L. De. (2002). GABAergic Innervation Organizes Synaptic and Extrasynaptic GABAA Receptor Clustering in Cultured Hippocampal Neurons. *The Journal of Neuroscience*, *22*(3), 684–697.

Clarkson, A. N., Boothman-Burrell, L., Dósa, Z., Nagaraja, R. Y., Jin, L., Parker, K., … Chebib, M. (2019). The flavonoid, 2′-methoxy-6-methylflavone, affords neuroprotection following focal cerebral ischaemia. *Journal of Cerebral Blood Flow and Metabolism*, *39*(7), 1266–1282. https://doi.org/10.1177/0271678X18755628

Clarkson, A. N., Huang, B. S., Macisaac, S. E., Mody, I., & Carmichael, S. T. (2010). Reducing excessive GABAergic tonic inhibition promotes post- stroke functional recovery. *Nature*, *468*(7321), 305–309. https://doi.org/10.1038/nature09511

Colciago, A., Bonalume, V., Melfi, V., & Magnaghi, V. (2020). Genomic and Non-genomic Action of Neurosteroids in the Peripheral Nervous System. *Frontiers in Neuroscience*, *14*(July). https://doi.org/10.3389/fnins.2020.00796

Cole, S. G., Brozinsky, S., & Isenberg, J. I. (1983). Midazolam, a new more potent benzodiazepine, compared with diazepam: a randomized, double-blind study of preendoscopic sedatives. *Gastrointestinal Endoscopy*, *29*(3), 219–222. https://doi.org/10.1016/S0016-5107(83)72588-5

Collinson, N., Kuenzi, F. M., Jarolimek, W., Maubach, K. a, Cothliff, R., Sur, C., … Rosahl, T. W. (2002). Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(13), 5572–5580. https://doi.org/20026436

Cooper, E. J., Johnston, G. A. R., & Edwards, F. A. (1999). Effects of a naturally occurring neurosteroid on GABA(A) IPSCs during development in rat hippocampal or cerebellar slices. *Journal of Physiology*, *521*(2), 437–449. https://doi.org/10.1111/j.1469-7793.1999.00437.x

Cope, D. W., Hughes, S. W., & Crunelli, V. (2005). GABAA receptor-mediated tonic inhibition in thalamic neurons. *Journal of Neuroscience*, *25*(50), 11553–11563. https://doi.org/10.1523/JNEUROSCI.3362-05.2005

Costa, E., & Guidotti, A. (1985). Endogenous ligands for benzodiazepine recognition sites. *Biochemical Pharmacology*, *34*(19), 3399–3403. https://doi.org/10.1016/0006-2952(85)90709-9

Covey, D. F., Nathan, D., Kalkbrenner, M., Nilsson, K. R., Hu, Y., Zorumski, C. F., & Evers, A. S. (2000). Enantioselectivity of pregnanolone-induced γ-aminobutyric acid(A) receptor modulation and anesthesia. *Journal of Pharmacology and Experimental Therapeutics*, *293*(3), 1009–1116.

Crestani, F., Keist, R., Fritschy, J.-M., Benke, D., Vogt, K., Prut, L., … Rudolph, U. (2002). Trace fear conditioning involves hippocampal alpha5 GABAA receptors. *PNAS*, *99*(13), 8980–8985. https://doi.org/10.1016/j.dynatmoce.2012.05.001

Crestani, F., Martin, J. R., Möhler, H., & Rudolph, U. (2000). Mechanism of action of the hypnotic zolpidem in vivo. *British Journal of Pharmacology*, *131*(7), 1251–1254. https://doi.org/10.1038/sj.bjp.0703717

Cummins, T. R. (2007). Setting up for the block: The mechanism underlying lidocaine's use-dependent inhibition of sodium channels. *Journal of Physiology*, 582(1), 11. https://doi.org/10.1113/jphysiol.2007.136671

Curran, H. V. (1986). Tranquillising memories: A review of the effects of benzodiazepines on human memory. *Biological Psychology*, *23*(2), 179–213. https://doi.org/10.1016/0301-0511(86)90081-5

Damgaard, T., Plath, N., Neill, J. C., & Hansen, S. L. (2011). Extrasynaptic GABAA receptor activation reverses recognition memory deficits in an animal model of schizophrenia. *Psychopharmacology*, *214*(2), 403–413. https://doi.org/10.1007/s00213-010-2039-9

del Cerro, S., Jung, M., & Lynch, G. (1992). Benzodiazepines block long-term potentiation in slices of hippocampus and piriform cortex. *Neuroscience*, *49*(1), 1–6. https://doi.org/10.1016/0306-4522(92)90071-9

Diaz Brinton, R. (1994). The neurosteroid 3α-hydroxy-5α-pregnan-20-one induces cytoarchitectural regression in cultured fetal hippocampal neurons. *Journal of Neuroscience*, *14*(5 I), 2763–2774. https://doi.org/10.1523/jneurosci.14-05-02763.1994

Diaz Brinton, R., & Ming Wang, J. (2006). Therapeutic Potential of Neurogenesis for Prevention and Recovery from Alzheimers Disease: Allopregnanolone as a Proof of Concept Neurogenic Agent. *Current Alzheimer Research*, *3*(3), 185–190. https://doi.org/10.2174/156720506777632817

Djebaili, M., Guo, Q., Pettus, E. H., Hoffman, S. W., & Stein, D. G. (2005). The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. *Journal of Neurotrauma*, *22*(1), 106–118. https://doi.org/10.1089/neu.2005.22.106

Djebaili, M., Hoffman, S. W., & Stein, D. G. (2004). Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. *Neuroscience*, *123*(2), 349–359. https://doi.org/10.1016/j.neuroscience.2003.09.023

Doller, H. J., & Weight, F. F. (1982). Perforant pathway activation of hippocampal CA1 stratum pyramidale neurons: Electrophysiological evidence for a direct pathway. *Brain Research*, *237*(1), 1–13. https://doi.org/10.1016/0006-8993(82)90553-4

Doyle, K. P., Quach, L. N., Solé, M., Axtell, R. C., Nguyen, T. V. V., Soler-Llavina, G. J., … Buckwalter, M. S. (2015). B-lymphocyte-mediated delayed cognitive impairment following stroke. *Journal of Neuroscience*, *35*(5), 2133–2145. https://doi.org/10.1523/JNEUROSCI.4098-14.2015

Draguhn, A., & Heinemann, U. (1996). Different mechanisms regulate IPSC kinetics in early postnatal and juvenile hippocampal granule cells. *Journal of Neurophysiology*, *76*(6), 3983–3993. https://doi.org/10.1152/jn.1996.76.6.3983

Drexler, B., Balk, M., & Antkowiak, B. (2016). Synergistic Modulation of γ-Aminobutyric Acid Type A Receptor-Mediated Synaptic Inhibition in Cortical Networks by Allopregnanolone and Propofol. *Anesthesia and Analgesia*, *123*(4), 877–883. https://doi.org/10.1213/ANE.0000000000001429

Droogleever Fortuyn, H. A., Van Broekhoven, F., Span, P. N., Bäckström, T., Zitman, F. G., & Verkes, R. J. (2004). Effects of PhD examination stress on allopregnanolone and cortisol plasma levels and peripheral benzodiazepine receptor density. *Psychoneuroendocrinology*, *29*(10), 1341–1344. https://doi.org/10.1016/j.psyneuen.2004.02.003

Dundee, J. W., Halliday, N. J., Harper, K. W., & Brogden, R. N. (1984). Midazolam A Review of its Pharmacological Properties and Therapeutic Use. *Drugs*, *28*, 519–543. https://doi.org/10.2165/00003495-198428060-00002

Eichenbaum, H., Otto, T., & Cohen, N. J. (1992). The hippocampus-what does it do? *Behavioral and Neural Biology*, *57*(1), 2–36. https://doi.org/10.1016/0163-1047(92)90724-I

Eimerbrink, M. J., Pendry, R. J., Hodges, S. L., Wiles, J. D., Peterman, J. L., White, J. D., … Boehm, G. W. (2018). The α5-GABAAR inverse agonist MRK-016 upregulates hippocampal BDNF expression and prevents cognitive deficits in LPS-treated mice, despite elevations in hippocampal Aβ. *Behavioural Brain Research,* 359, 871–877. https://doi.org/10.1016/j.bbr.2018.07.013

Eom, W., Lee, J. M., Park, J., Choi, K., Jung, S. J., & Kim, H. S. (2011). The effects of midazolam and sevoflurane on the GABAA receptors with alternatively spliced variants of the γ2 subunit. *Korean Journal of Anesthesiology*, *60*(2), 109–118. https://doi.org/10.4097/kjae.2011.60.2.109

Evans, M. S., & Viola-McCabe, K. E. (1996). Midazolam inhibits long-term potentiation through modulation of GABAA receptors. *Neuropharmacology*, *35*(3), 347–357. https://doi.org/10.1016/0028-3908(95)00182-4

Eyre, M. D., Renzi, M., Farrant, M., & Nusser, Z. (2012). Setting the time course of inhibitory synaptic currents by mixing multiple GABAA receptor α subunit isoforms. *Journal of Neuroscience*, *32*(17), 5853–5867. https://doi.org/10.1523/JNEUROSCI.6495-11.2012

Farges, R., Joseph-Liauzun, E., Shire, D., Caput, D., Le Fur, G., & Ferrara, P. (1994). Site-directed mutagenesis of the peripheral benzodiazepine receptor: identification of amino acids implicated in the binding site of Ro5-4864. *Molecular pharmacology*, 46(6), 1160–1167.

Farrant, M., & Nusser, Z. (2005). Variations on an inhibitory theme: Phasic and tonic activation of GABA A receptors. *Nature Reviews Neuroscience*. https://doi.org/10.1038/nrn1625

Feng, A. Y., Kaye, A. D., Kaye, R. J., Belani, K., & Urman, R. D. (2017). Novel propofol derivatives and implications for anesthesia practice. *Journal of Anaesthesiology Clinical Pharmacology*, *33*(1), 9–15. https://doi.org/10.4103/0970-9185.202205

Fricker, D., & Miles, R. (2001). Interneurons, spike timing, and perception. *Neuron*, *32*(5), 771–774. https://doi.org/10.1016/S0896-6273(01)00528-1

Fritschy, J. M., & Brünig, I. (2003). Formation and plasticity of GABAergic synapses: Physiological mechanisms and pathophysiological implications. *Pharmacology and Therapeutics*, *98*(3), 299–323. https://doi.org/10.1016/S0163-7258(03)00037-8

Fritschy, J. M., & Mohler, H. (1995). GABAA-receptor heterogeneity in the adult rat brain: Differential regional and cellular distribution of seven major subunits. *Journal of Comparative Neurology*, *359*(1), 154–194. https://doi.org/10.1002/cne.903590111

Fritschy, J. M., & Panzanelli, P. (2014). GABAA receptors and plasticity of inhibitory neurotransmission in the central nervous system. E*uropean Journal of Neuroscience*, 39(11), 1845–1865. https://doi.org/10.1111/ejn.12534

Garcia, P., Kolesky, S., & Jenkins, A. (2010). General Anesthetic Actions on GABAA Receptors. *Current Neuropharmacology*, 8(1), 2–9. https://doi.org/10.2174/157015910790909502

Gavish, M., Bachman, I., Shoukrun, R., Katz, Y., Veenman, L., Weisinger, G., & Weizman, A. (1999). Enigma of the peripheral benzodiazepine receptor. *Pharmacological reviews*, 51(4), 629–650.

Gill, R., Foster, A. C., & Woodruff, G. N. (1988). MK-801 is neuroprotective in gerbils when administered during the post-ischaemic period. *Neuroscience*, *25*(3), 847–855. https://doi.org/10.1016/0306-4522(88)90040-1

Glykys, J., & Mody, I. (2006). Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABAA receptor α5 subunit-deficient mice. *Journal of Neurophysiology*, *95*(5), 2796–2807. https://doi.org/10.1152/jn.01122.2005

Glykys, J., & Mody, I. (2007). The main source of ambient GABA responsible for tonic inhibition in the mouse hippocampus. *Journal of Physiology*, *582*(3), 1163–1178. https://doi.org/10.1113/jphysiol.2007.134460

Glykys, J., Mann, E. O., & Mody, I. (2008). Which GABAA Receptor Subunits Are Necessary for Tonic Inhibition in the Hippocampus? *Journal of Neuroscience*, *28*(6), 1421–1426. https://doi.org/10.1523/JNEUROSCI.4751-07.2008

Goetz, T., Arslan, A., Wisden, W., & Wulff, P. (2007). GABAA receptors: structure and function in the basal ganglia. *Progress in Brain Research*, *160*, 21–41. https://doi.org/10.1016/S0079-6123(06)60003-4.GABA

Grasshoff, C., Drexler, B., Rudolph, U., & Antkowiak, B. (2006). Anaesthetic Drugs: Linking Molecular Actions to Clinical Effects. *Current Pharmaceutical Design*, *12*(28), 3665–3679. https://doi.org/10.2174/138161206778522038

Green, A. R., Hainsworth, A. H., & Jackson, D. M. (2000). GABA potentiation: A logical pharmacological approach for the treatment of acute ischaemic stroke. *Neuropharmacology*, *39*(9), 1483–1494. https://doi.org/10.1016/S0028-3908(99)00233-6

Greifzu, F., Schmidt, S., Schmidt, K. F., Kreikemeier, K., Witte, O. W., & Löwel, S. (2011). Global impairment and therapeutic restoration of visual plasticity mechanisms after a localized cortical stroke. *PNAS*, *108*(37), 15450–15455. https://doi.org/10.1073/pnas.1016458108

Guennoun, R. (2020). Progesterone in the brain: Hormone, neurosteroid and neuroprotectant. *International Journal of Molecular Sciences*, *21*(15), 1–21. https://doi.org/10.3390/ijms21155271

Gunn, B. G., Cunningham, L., Mitchell, S. G., Swinny, J. D., Lambert, J. J., & Belelli, D. (2015). GABAA receptor-acting neurosteroids: A role in the development and regulation of the stress response. *Frontiers in Neuroendocrinology*, *36*, 28–48. https://doi.org/10.1016/j.yfrne.2014.06.001

Hadingham, K. L., Garrett, E. M., Wafford, K. A., Bain, C., Heavens, R. P., Sirinathsinghji, D. J. S., & Whiting, P. J. (1996). Cloning of cDNAs encoding the human γ-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6-containing receptors. *Molecular Pharmacology*, *49*(2), 253–259.

Haefely, W. (1984). Benzodiazepine Intercation with GABA Receptors. *Neuroscience Letters*, *47*(3), 201–206.

Harney, S. C., Frenguelli, B. G., & Lambert, J. J. (2003). Phosphorylation influences neurosteroid modulation of synaptic GABA A receptors in rat CA1 and dentate gyrus neurones. *Neuropharmacology*, *45*(6), 873–883. https://doi.org/10.1016/S0028-3908(03)00251-X

Harrah, S., 2022. *Medical Milestones: Discovery of Anesthesia & Timeline*. [online] Umhs-sk.org. Available at: https://www.umhs-sk.org/blog/medical-milestones-discovery-anesthesia-timeline

Harris, K. D., Hochgerner, H., Skene, N. G., Magno, L., Katona, L., Bengtsson Gonzales, C., … Hjerling-Leffler, J. (2018). Classes and continua of hippocampal CA1 inhibitory neurons revealed by single-cell transcriptomics. *PLoS Biology*, *16*(6), 1–37. https://doi.org/10.1371/journal.pbio.2006387

Harrison, N. L., Vicini, S., & Barker, J. L. (1987). A steroid anesthetic prolongs inhibitory postsynaptic currents in cultured rat hippocampal neurons. *Journal of Neuroscience*, *7*(2), 604–609. https://doi.org/10.1523/jneurosci.07-02-00604.1987

He, J., Evans, C. O., Hoffman, S. W., Oyesiku, N. M., & Stein, D. G. (2004). Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. *Experimental Neurology*, *189*(2), 404–412. https://doi.org/10.1016/j.expneurol.2004.06.008

Hennessy, M. J., Kirkby, K. C., & Montgomery, I. M. (1991). Comparison of the amnesic effects of midazolam and diazepam. *Psychopharmacology*, *103*(4), 545–550. https://doi.org/10.1007/BF02244257

Higashima, M., Kinoshita, H., & Koshino, Y. (1998). Differences in the effects of zolpidem and diazepam on recurrent inhibition and long-term potentiation in rat hippocampal slices. *Neuroscience Letters*, *245*, 77–80. https://doi.org/10.1016/S0304-3940(98)00178-5

Holm, M. M., Nieto-Gonzalez, J. L., Vardya, I., Henningsen, K., Jayatissa, M. N., Wiborg, O., & Jensen, K. (2011). Hippocampal GABAergic dysfunction in a rat chronic mild stress model of depression. *Hippocampus*, *21*(4), 422–433. https://doi.org/10.1002/hipo.20758

Hörtnagl, H., Tasan, R. O., Wieselthaler, A., Kirchmair, E., Sieghart, W., & Sperk, G. (2013). Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. *Neuroscience*, *236*, 345–372. https://doi.org/10.1016/j.neuroscience.2013.01.008

Hosie, A. M., Clarke, L., da Silva, H., & Smart, T. G. (2009). Conserved site for neurosteroid modulation of GABAA receptors. *Neuropharmacology*, *56*(1), 149–154. https://doi.org/10.1016/j.neuropharm.2008.07.050

Hosie, A. M., Wilkins, M. E., & Smart, T. G. (2007). Neurosteroid binding sites on GABAA receptors. *Pharmacology and Therapeutics*, *116*(1), 7–19. https://doi.org/10.1016/j.pharmthera.2007.03.011

Hu, X.-D., Ge, Y.-X., Hu, N.-W., Zhang, H.-M., Zhou, L.-J., Zhang, T., … Liu, X.-G. (2006). Diazepam inhibits the induction and maintenance of LTP of C-fiber evoked field potentials in spinal dorsal horn of rats. *Neuropharmacology*, *50*, 238–244. https://doi.org/10.1016/j.neuropharm.2005.09.010

Imperato, A., Dazzi, L., Carmen Obinu, M., Luigi Gessa, G., & Biggio, G. (1993). Inhibition of hippocampal acetylcholine release by benzodiazepines: antagonism by flumazenil. *European Journal of Pharmacology*, *238*, 135–137.

Ip, H. Y. V., Abrishami, A., Peng, P. W. H., Wong, J., & Chung, F. (2009). Predictors of postoperative pain and analgesic consumption: A qualitative systematic review. *Anesthesiology*, *111*(3), 657–677. https://doi.org/10.1097/ALN.0b013e3181aae87a

Izumi, Y., Murayama, K., Tokuda, K., Krishnan, K., Covey, D. F., & Zorumski, C. F. (2007). GABAergic neurosteroids mediate the effects of ethanol on long-term potentiation in rat hippocampal slices. *European Journal of Neuroscience*, *26*(7), 1881–1888. https://doi.org/10.1111/j.1460-9568.2007.05809.x

Jacob, T. C. (2019). Neurobiology and Therapeutic Potential of α5-GABA Type A Receptors. *Frontiers in Molecular Neuroscience*, *12*, 1–10. https://doi.org/10.3389/fnmol.2019.00179

Jones, P., Atack, J. R., Braun, M. P., Cato, B. P., Chambers, M. S., O'Connor, D., … MacLeod, A. M. (2006). Pharmacokinetics and metabolism studies on (3-tert-butyl-7-(5- methylisoxazol-3-yl)-2-(1-methyl-1H-1,2,4-triazol-5-ylmethoxy) pyrazolo[1,5-d][1,2,4]triazine, a functionally selective GABAA α5 inverse agonist for cognitive dysfunction. *Bioorganic and Medicinal Chemistry Letters*, *16*(4), 872–875. https://doi.org/10.1016/j.bmcl.2005.11.012

Jones, R. S. G. (1993). Entorhinal-hippocampal connections: a speculative view of their function. *Trends in Neurosciences*, *16*(2), 58–64. https://doi.org/10.1016/0166-2236(93)90018-H

Joseph-Liauzun, E., Delmas, P., Shire, D., & Ferrara, P. (1998). Topological analysis of the peripheral benzodiazepine receptor in yeast mitochondrial membranes supports a five-transmembrane structure. *J Biol Chem*, *273*(4), 2146–2152.

Kaplan, S. A., Jack, M. L., Alexander, K., & Weinfeld, R. E. (1973). Pharmacokinetic Profile of Diazepam in Man following Single Intravenous and Oral and Chronic Oral Administrations. *Journal of Pharmacological Sciences*, *62*, 1789–1796.

Kelly, M. D., Smith, A., Banks, G., Wingrove, P., Whiting, P. W., Atack, J., … Maubach, K. A. (2002). Role of the histidine residue at position 105 in the human α5 containing GABAa receptor on the affinity and efficacy of benzodiazepine site ligands. *British Journal of Pharmacology*, *135*(1), 248–256. https://doi.org/10.1038/sj.bjp.0704459

Kim, T. H., & Pae, A. N. (2016). Translocator protein (TSPO) ligands for the diagnosis or treatment of neurodegenerative diseases: a patent review (2010–2015; part 1). *Expert Opinion on Therapeutic Patents*, *26*(11), 1325–1351. https://doi.org/10.1080/13543776.2016.1230606

Kita, A., Kinoshita, T., Kohayakawa, H., Furukawa, K., & Akaike, A. (2009). Lack of tolerance to anxiolysis and withdrawal symptoms in mice repeatedly treated with AC-5216, a selective TSPO ligand. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, (6), 1040–1045. https://doi.org/10.1016/j.pnpbp.2009.05.018

Kita, A., Kohayakawa, H., Kinoshita, T., Ochi, Y., Nakamichi, K., Kurumiya, S., … Oka, M. (2004). Antianxiety and antidepressant-like effects of AC-5216, a novel mitochondrial benzodiazepine receptor ligand. *British Journal of Pharmacology*, *142*(7), 1059–1072. https://doi.org/10.1038/sj.bjp.0705681

Klausberger, T., Roberts, J. D. B., & Somogyi, P. (2002). Cell Type- and Input-Specific

Differences in the Number and Subtypes of Synaptic GABAA Receptors in the Hippocampus. *Journal of Neuroscience*, *22*(7), 2513–2521. https://doi.org/10.1523/jneurosci.22-07-02513.2002

Knabl, J., Witschi, R., Hösl, K., Reinold, H., Zeilhofer, U. B., Ahmadi, S., … Zeilhofer, H. U. (2008). Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature*, *451*(7176), 330–334. https://doi.org/10.1038/nature06493

Kok, L., Slooter, A. J., Hillegers, M. H., Van Dijk, D., & Veldhuijzen, D. S. (2018). Benzodiazepine use and neuropsychiatric outcomes in the ICU: A systematic review. *Critical Care Medicine*, *46*(10), 1673–1680. https://doi.org/10.1097/CCM.0000000000003300

Korpi, E. R., Gründer, G., & Lüddens, H. (2002a). Drug interactions at GABAA receptors. *Progress in Neurobiology*, *67*(2), 113–159. https://doi.org/10.1016/S0301-0082(02)00013-8

Korpi, E. R., Mihalek, R. M., Sinkkonen, S. T., Hauer, B., Hevers, W., Homanics, G. E., … Lüddens, H. (2002b). Altered receptor subtypes in the forebrain of GABAA receptor δ subunit-deficient mice: Recruitment of γ2 subunits. *Neuroscience*, *109*(4), 733–743. https://doi.org/10.1016/S0306-4522(01)00527-9

Krueger, K. E., & Papadopoulos, V. (1990). Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. *Journal of Biological Chemistry*, *265*(25), 15015–15022. Retrieved from http://www.jbc.org/content/265/25/15015

Lacapère, J. J., & Papadopoulos, V. (2003). Peripheral-type benzodiazepine receptor: Structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. *Steroids*, *68*, 569–585. https://doi.org/10.1016/S0039-128X(03)00101-6

Lambert, J. J., Belelli, D., Hill-Venning, C., & Peters, J. A. (1995). Neurosteroids and GABAA receptor function. *Trends in Pharmacological Sciences*, *16*(9), 295–303. https://doi.org/10.1016/S0165-6147(00)89058-6

Lambert, J. J., Belelli, D., Peden, D. R., Vardy, A. W., & Peters, J. A. (2003). Neurosteroid modulation of GABAA receptors. *Progress in Neurobiology*, *71*(1), 67–80. https://doi.org/10.1016/j.pneurobio.2003.09.001

Laurie, D. J., Seeburg, P. H., & Wisden, W. (1992). The distribution of 13 GABA(A) receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *Journal of Neuroscience*, *12*(3), 1063–1076. https://doi.org/10.1523/jneurosci.12-03-01063.1992

Laurijssens, B. E., & Greenblatt, D. J. (2002). Effect of 7-day exposure to midazolam on electroencephalogram pharmacodynamics in rats: a model to study multiple pharmacokinetic-pharmacodynamic relationships in individual animals. *Journal of Pharmacy and Pharmacology*, *54*(1), 77–86. https://doi.org/10.1211/0022357021771959

Lee, H. K., Dunwiddie, T. V., & Hoffer, B. J. (1979). Interaction of Diazepam with Synaptic Transmission in the in vitro Rat Hippocampus. *NaunynSchmiedebergs Archives of*

Pharmacology, *309*, 131–136.

Lee, V., & Maguire, J. (2013). Impact of inhibitory constraint of interneurons on neuronal excitability. *Journal of Neurophysiology*, *110*(11), 2520–2535. https://doi.org/10.1152/jn.00047.2013

Lein, P. J., Barnhart, C. D., & Pessah, I. N. (2011). Acute hippocampal slice preparation and hippocampal slice cultures. *Methods in Molecular Biology*, *758*, 115–134. https://doi.org/10.1007/978-1-61779-170-3_8

Lheureux, P., Debailleul, G., de Witte, O., & Askenasi, R. (1990). Zolpidem Intoxication Mimicking Narcotic Overdose: Response to Flumazenil. *Human & Experimental Toxicology*, *9*(2), 105–107. https://doi.org/10.1177/096032719000900209

Li, H., Yao, Z. X., Degenhardt, B., Teper, G., & Papadopoulos, V. (2001). Cholesterol binding at the cholesterol recognition/interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. *PNAS*, *98*(3), 1267–1272. https://doi.org/10.1073/pnas.98.3.1267

Liguz-Lecznar, M., & Kossut, M. (2013). Influence of inflammation on poststroke plasticity. *Neural Plasticity*, *2013*, 258582. https://doi.org/10.1155/2013/258582

Lister, R. G. (1985). The amnesic action of benzodiazepines in man. *Neuroscience and Biobehavioral Reviews*, *9*, 87–94.

Liu, A., Margaill, I., Zhang, S., Labombarda, F., Coqueran, B., Delespierre, B., … Guennoun, R. (2012). Progesterone receptors: A key for neuroprotection in experimental stroke. *Endocrinology*, *153*(8), 3747–3757. https://doi.org/10.1210/en.2012-1138

Locci, A., & Pinna, G. (2017). Neurosteroid biosynthesis down-regulation and changes in GABA A receptor subunit composition: a biomarker axis in stress-induced cognitive and emotional impairment. *British Journal of Pharmacology*, *174*, 3226–3241. https://doi.org/10.1111/bph.13843

Longone, P., di Michele, F., D'Agati, E., Romeo, E., Pasini, A., & Rupprecht, R. (2011). Neurosteroids as neuromodulators in the treatment of anxiety disorders. *Frontiers in Endocrinology*, *2*(OCT), 1–9. https://doi.org/10.3389/fendo.2011.00055

Löw, K., Crestani, F., Keist, R., Benke, D., Brunig, I., Benson, J. A., … Rudolph, U. (2000). Molecular and neuronal substrate for the selective attenuation of anxiety. *Science*, *290*(5489), 131–134. https://doi.org/10.1126/science.290.5489.131

Lücke, C., Heidegger, T., Röhner, M., Toennes, S. W., Krivanekova, L., Müller-Dahlhaus, F., & Ziemann, U. (2014). Deleterious effects of a low amount of ethanol on LTP-like plasticity in human cortex. *Neuropsychopharmacology*, *39*(6), 1508–1518. https://doi.org/10.1038/npp.2013.350

Lumley, L., Miller, D., Muse, W. T., Marrero-Rosado, B., de Araujo Furtado, M., Stone, M., … Whalley, C. (2019). Neurosteroid and benzodiazepine combination therapy reduces status epilepticus and long-term effects of whole-body sarin exposure in rats. *Epilepsia Open*, *4*(3), 382–396. https://doi.org/10.1002/epi4.12344

Macdonald, R. L. (1994). GABAA Receptor Channels. *Annual Review of Neuroscience*, *17*, 569–602.

MacIver, M. B. (2014). Anesthetic agent-specific effects on synaptic inhibition. *Anesthesia and Analgesia*, *119*(3), 558–569. https://doi.org/10.1213/ANE.0000000000000321

MacKenzie, G., & Maguire, J. (2013). Neurosteroids and GABAergic signaling in health and disease. *Biomol Concepts*, *4*(1), 29–42. https://doi.org/10.1515/bmc-2012-0033

Maeda, J., Higuchi, M., Inaji, M., Ji, B., Haneda, E., Okauchi, T., … Suhara, T. (2007). Phase-dependent roles of reactive microglia and astrocytes in nervous system injury as delineated by imaging of peripheral benzodiazepine receptor. *Brain Research*, *1157*(1), 100–111. https://doi.org/10.1016/j.brainres.2007.04.054

Mages, K., Grassmann, F., Jägle, H., Rupprecht, R., Weber, B. H. F., Hauck, S. M., & Grosche, A. (2019). The agonistic TSPO ligand XBD173 attenuates the glial response thereby protecting inner retinal neurons in a murine model of retinal ischemia. *Journal of Neuroinflammation*, *16*(1), 1–16. https://doi.org/10.1186/s12974-019-1424-5

Maguire, J. L., Stell, B. M., Rafizadeh, M., & Mody, I. (2005). Ovarian cycle-linked changes in GABAA receptors mediating tonic inhibition alter seizure susceptibility and anxiety. *Nature Neuroscience*, *8*(6), 797–804. https://doi.org/10.1038/nn1469

Maguire, J., & Mody, I. (2008). GABAA R Plasticity during Pregnancy: Relevance to Postpartum Depression. *Neuron*, *59*(2), 207–213. https://doi.org/10.1016/j.neuron.2008.06.019.

Maitra, R., & Reynolds, J. N. (1999). Subunit dependent modulation of GABAA receptor function by neuroactive steroids. *Brain Research*, *819*(1–2), 75–82. https://doi.org/10.1016/S0006-8993(98)01316-X

Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L., & Paul, S. M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science*, *232*(4753), 1004–1007. https://doi.org/10.1126/science.2422758

Makaron, L., Moran, C. A., Namjoshi, O., Rallapalli, S., Cook, J. M., & Rowlett, J. K. (2013). Cognition-impairing effects of benzodiazepine-type drugs: Role of GABA A receptor subtypes in an executive function task in rhesus monkeys. *Pharmacology Biochemistry and Behavior*, *104*(1), 62–68. https://doi.org/10.1016/j.pbb.2012.12.018

Malapero, R. J., Zaccagnino, M. P., Brovman, E. Y., Kaye, A. D., & Urman, R. D. (2017). Etomidate derivatives: Novel pharmaceutical agents in anesthesia. *Journal of Anaesthesiology Clinical Pharmacology*, *33*(4), 429–431. https://doi.org/10.4103/0970-9185.222521

Martin, L. J., Oh, G. H. T., & Orser, B. A. (2009). Etomidate targets α5 γ-aminobutyric acid subtype a receptors to regulate synaptic plasticity and memory blockade. *Anesthesiology*, *111*(5), 1025–1035. https://doi.org/10.1097/ALN.0b013e3181bbc961

Martin, L. J., Zurek, A. A., MacDonald, J. F., Roder, J. C., Jackson, M. F., & Orser, B. A. (2010). α5GABAa receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. *Journal of Neuroscience*, *30*(15), 5269–5282. https://doi.org/10.1523/JNEUROSCI.4209-09.2010

McBain, C. J. (2001). Interneurons unbound. *Nature Reviews Neuroscience*, *2*(1), 11–23. https://doi.org/10.1038/35049047

McEnery, M. W., Snowman, A. M., Trifiletti, R. R., & Snyder, S. H. (1992). Isolation of the mitochondrial benzodiazepine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier. *PNAS*, *89*, 3170–3174.

Mejo, S. L. (1992). Anterograde amnesia linked to benzodiazepines. *Nurse Practitioner*. https://doi.org/10.1097/00006205-199210000-00013

Mihalek, R. M., Banerjee, P. K., Korpi, E. R., Quinlan, J. J., Firestone, L. L., Mi, Z.-P., … Homanics, G. E. (1999). Attenuated sensitivity to neuroactive steroids in gamma -aminobutyrate type A receptor delta subunit knockout mice. *PNAS*, 96(22), 12905–12910. https://doi.org/10.1073/pnas.96.22.12905

Minier, F., & Sigel, E. (2004). Positioning of the α-subunit isoforms confers a signature to γ-aminobutyric acid type A receptors. *PNAS*, *101*(20), 7769–7774. https://doi.org/10.1073/pnas.0400220101

Mody, I., & Pearce, R. A. (2004). Diversity of inhibitory neurotransmission through GABA A receptors. *Trends in Neurosciences*, *27*(9), 569–575. https://doi.org/10.1016/j.tins.2004.07.002

Möhler, H., Fritschy, J. M., & Rudolph, U. (2002). A New Benzodiazepine Pharmacology Synaptic Action of Benzodiazepines. *The Journal of Pharmacology and Experimental Therapeutics*, *300*(1). https://doi.org/10.1124/jpet.300.1.2

Moralí, G., Montes, P., Hernández-Morales, L., Monfil, T., Espinosa-García, C., & Cervantes, M. (2011). Neuroprotective effects of progesterone and allopregnanolone on long-term cognitive outcome after global cerebral ischemia. *Restorative Neurology and Neuroscience*, *29*(1), 1–15. https://doi.org/10.3233/RNN-2011-0571

Moss, M., Mahut, H., & Zola-Morgan, S. (1981). Concurrent discrimination learning of monkeys after hippocampal, entorhinal, or fornix lesions. *Journal of Neuroscience*, *1*(3), 227–240. https://doi.org/10.1523/jneurosci.01-03-00227.1981

Müller Herde, A., Benke, D., Ralvenius, W. T., Mu, L., Schibli, R., Zeilhofer, H. U., & Krämer, S. D. (2017). GABAA receptor subtypes in the mouse brain: Regional mapping and diazepam receptor occupancy by in vivo [18F]flumazenil PET. *NeuroImage*, *150*, 279–291. https://doi.org/10.1016/j.neuroimage.2017.02.022

Neumann, S., Boothman-Burrell, L., Gowing, E. K., Jacobsen, T. A., Ahring, P. K., Young, S. L., … Clarkson, A. N. (2019). The Delta-Subunit Selective GABAA Receptor Modulator, DS2, Improves Stroke Recovery via an Anti-inflammatory Mechanism. *Frontiers in Neuroscience*, *13*. https://doi.org/10.3389/fnins.2019.01133

Nothdurfter, C., Rupprecht, R., & Rammes, G. (2012). Recent Developments in Potential Anxiolytic Agents Targeting GABAA/BzR Complex or the Translocator Protein (18kDa) (TSPO). *Current Topics in Medicinal Chemistry*, *12*(4), 360–370. https://doi.org/10.2174/156802612799078748

O'Brien, R. J., Kamboj, S., Ehlers, M. D., Rosen, K. R., Fischbach, G. D., & Huganir, R. L. (1998). Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron*, 21(5), 1067–1078. https://doi.org/10.1016/s0896-6273(00)80624-8

Olsen, R. W., & Sieghart, W. (2008). International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric acidA receptors: Classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacological Reviews*, *60*(3), 243–260. https://doi.org/10.1124/pr.108.00505

Olsen, R. W., & Sieghart, W. (2009). GABAA receptors: Subtypes provide diversity of function and pharmacology. *Neuropharmacology*. https://doi.org/10.1016/j.neuropharm.2008.07.045

Papadopoulos, V., Baraldi, M., Guilarte, T. R., Knudsen, T. B., Lacapère, J. J., Lindemann, P., … Gavish, M. (2006). Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends in Pharmacological Sciences*, *27*(8), 402–409. https://doi.org/10.1016/j.tips.2006.06.005

Papadopoulos, V., & Lecanu, L. (2009). Translocator protein (18 kDa) TSPO: an emerging therapeutic target in neurotrauma. *Experimental Neurology*, *219*(1), 53–57. https://doi.org/10.1016/j.expneurol.2009.04.016

Papouin, T., & Haydon, P. G. (2018). Obtaining Acute Brain Slices. *Bio-protocol,* 8(2), e2699. https://doi.org/10.21769/BioProtoc.2699

Park, H. P., Jeong, E. J., Kim, M. H., Hwang, J. W., Lim, Y. J., Min, S. W., … Jeon, Y. T. (2011). Effects of sevoflurane on neuronal cell damage after severe cerebral ischemia in rats. *Korean Journal of Anesthesiology*, *61*(4), 327–331. https://doi.org/10.4097/kjae.2011.61.4.327

Patat, A., Naef, M. M., Gessel, E. Van, Forster, A., Dubruc, C., & Rosenzweig, P. (1994). Flumazenil antagonizes the central effects of zolpidem, an imidazopyridine hypnotic. *Clinical Pharmacology & Therapeutics*, *56*(4), 430–436. https://doi.org/10.1038/clpt.1994.157

Paul, S. M., & Purdy, R. H. (1992). Neuroactive steroids. *FASEB Journal*, *6*(6), 2311–2322. https://doi.org/10.1096/fasebj.6.6.1347506

Paul, S. M., Pinna, G., & Guidotti, A. (2020). Allopregnanolone: From molecular pathophysiology to therapeutics. A historical perspective. *Neurobiology of Stress*, *12*. https://doi.org/10.1016/j.ynstr.2020.100215

Pearce, R. A. (1993). Physiological evidence for two distinct GABAA responses in rat hippocampus. *Neuron*, *10*(2), 189–200. https://doi.org/10.1016/0896-6273(93)90310-N

Pelkey, K. A., Chittajallu, R., Craig, M. T., Tricoire, L., Wester, J. C., & McBain, C. J.

(2017). Hippocampal GABAergic Inhibitory Interneurons. *Physiological Reviews*, *97*, 1619–1747. https://doi.org/10.1152/physrev.00007.2017

Peng, Z., Hauer, B., Mihalek, R. M., Homanics, G. E., Sieghart, W., Olsen, R. W., & Houser, C. R. (2002). GABAA receptor changes in δ subunit-deficient mice: Altered expression of α4 and γ2 subunits in the forebrain. *Journal of Comparative Neurology*, *446*(2), 179–197. https://doi.org/10.1002/cne.10210

Petrache, A. L., Khan, A. A., Nicholson, M. W., Monaco, A., Kuta-Siejkowska, M., Haider, S., … Ali, A. B. (2020). Selective Modulation of α5 GABAA Receptors Exacerbates Aberrant Inhibition at Key Hippocampal Neuronal Circuits in APP Mouse Model of Alzheimer's Disease. *Frontiers in Cellular Neuroscience*, *14*, 1–18. https://doi.org/10.3389/fncel.2020.568194

Petrides, T., Georgopoulos, P., Kostopoulos, G., & Papatheodoropoulos, C. (2007). The GABAA receptor-mediated recurrent inhibition in ventral compared with dorsal CA1 hippocampal region is weaker, decays faster and lasts less. *Experimental Brain Research*, *177*(3), 370–383. https://doi.org/10.1007/s00221-006-0681-6

Pettit, D. L., & Augustine, G. J. (2000). Distribution of functional glutamate and GABA receptors on hippocampal pyramidal cells and interneurons. *Journal of Neurophysiology*, *84*(1), 28–38. https://doi.org/10.1152/jn.2000.84.1.28

Pinna, G. (2020). Allopregnanolone, the Neuromodulator Turned Therapeutic Agent: Thank You, Next? *Frontiers in Endocrinology*, *11*(236). https://doi.org/10.3389/fendo.2020.00236

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., & Sperk, G. (2000). GABAA receptors: Immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience*, *101*(4), 815–850. https://doi.org/10.1016/s0306-4522(00)00442-5

Pofantis, H., & Papatheodoropoulos, C. (2014). The α5GABAA receptor modulates the induction of long-term potentiation at ventral but not dorsal CA1 hippocampal synapses. *Synapse*, *68*(9), 394–401. https://doi.org/10.1002/syn.21751

Prasad, V. V. K., Raju Vegesna, S., Welch, M., & Lieberman, S. (1994). Precursors of the neurosteroids. *PNAS*, *91*(8), 3220–3223. https://doi.org/10.1073/pnas.91.8.3220

Puia, G., Gullo, F., Dossi, E., Lecchi, M., & Wanke Dr., E. (2012). Novel modulatory effects of neurosteroids and benzodiazepines on excitatory and inhibitory neurons excitability: A multi-electrode array (MEA) recording study. *Frontiers in Neural Circuits*, *6*, 1–17. https://doi.org/10.3389/fncir.2012.00094

Puia, G., Santi, M., Vicini, S., Pritchett, D. B., Purdy, R. H., Paul, S. M., … Costa, E. (1990). Neurosteroids Act on Recombinant GABAA receptors. *Neuron*, *4*, 759–765.

Puig-Bosch, X., Bieletzki, S., Zeilhofer, H. U., Rudolph, U., Antkowiak, B., & Rammes, G. (2022). Midazolam at low nanomolar concentrations affects long-term potentiation and synaptic transmission predominantly via the α1-GABAA receptor subunit in mice. *Anesthesiology*, 10.1097/ALN.0000000000004202. Advance online publication. https://doi.org/10.1097/ALN.0000000000004202

Purdy, R. H., Morrow, A. L., Moore, P. H., & Paul, S. M. (1991). Stress-induced elevations of γ-aminobutyric acid type a receptor-active steroids in the rat brain. *PNAS*, *88*(10), 4553–4557. https://doi.org/10.1073/pnas.88.10.4553

Pym, L. J., Cook, S. M., Rosahl, T., McKernan, R. M., & Atack, J. R. (2005). Selective labelling of diazepam-insensitive GABA A receptors in vivo using [3H]Ro 15-4513. *British Journal of Pharmacology*, *146*(6), 817–825. https://doi.org/10.1038/sj.bjp.0706392

Rajmohan, V., & Mohandas, E. (2007). The limbic system. *Indian journal of psychiatry*, 49(2), 132–139. https://doi.org/10.4103/0019-5545.33264

Ralvenius, W. T., Benke, D., Acuña, M. A., Rudolph, U., & Zeilhofer, H. U. (2015). Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABAA receptor subtype. *Nature Communications*, 6. https://doi.org/10.1038/ncomms7803

Reddy, D. S., & Estes, W. A. (2016). Clinical Potential of Neurosteroids for CNS Disorders. *Trends in Pharmacological Sciences*, *37*(7), 543–561. https://doi.org/10.1016/j.tips.2016.04.003

Reddy, D. S., & Kulkarni, S. K. (1997). Chronic neurosteroid treatment prevents the development of morphine tolerance and attenuates abstinence behavior in mice. *European Journal of Pharmacology*, *337*(1), 19–25. https://doi.org/10.1016/S0014-2999(97)01294-6

Reddy, D. S., Younus, I., Clossen, B. L., & Reddy, D. S. (2015). Antiseizure activity of midazolam in mice lacking δ-Subunit extrasynaptic GABAA receptors. *Journal of Pharmacology and Experimental Therapeutics*, *353*(3), 517–528. https://doi.org/10.1124/jpet.114.222075

Rissman, R. A., Bennett, D. A., & Armstrong, D. M. (2004). Subregional analysis of GABAA receptor subunit mRNAs in the hippocampus of older persons with and without cognitive impairment. *Journal of Chemical Neuroanatomy*, *28*(1–2), 17–25. https://doi.org/10.1016/j.jchemneu.2004.05.003

Rodgers, F. C., Zarnowska, E. D., Laha, K. T., Engin, E., Zeller, A., Keist, R., … Pearce, R. A. (2015). Etomidate Impairs Long-Term Potentiation In Vitro by Targeting 5-Subunit Containing GABAA Receptors on Nonpyramidal Cells. *Journal of Neuroscience*, *35*(26), 9707–9716. https://doi.org/10.1523/JNEUROSCI.0315-15.2015

Roepstorff, A., & Lambert, J. D. C. (1994). Factors contributing to the decay of the stimulus-evoked IPSC in rat hippocampal CA1 neurons. *Journal of Neurophysiology*, *72*(6), 2911–2926. https://doi.org/10.1152/jn.1994.72.6.2911

Ropert, N., Miles, R., & Korn, H. (1990). Characteristics of miniature inhibitory postsynaptic currents in CA1 pyramidal neurones of rat hippocampus. *The Journal of Physiology*, 428(1), 707–722. https://doi.org/10.1113/jphysiol.1990.sp018236

Rudolph, U., & Antkowiak, B. (2004). Molecular and neuronal substrates for general anaesthetics. *Nature Reviews Neuroscience*, 5(9), 709–720. https://doi.org/10.1038/nrn1496

Rudolph, U., Crestani, F., Benke, D., Brünig, I., Benson, J. A., Fritschy, J.-M., … Möhler, H. (1999). Benzodiazepine actions mediated by specific gamma-aminobutyric acidA receptor subtypes. *Nature*, *401*, 796–800.

Rudolph, U., Crestani, F., & Möhler, H. (2001). GABAA receptor subtypes: Dissecting their pharmacological functions. *Trends in Pharmacological Sciences*, *22*(4), 188–194. https://doi.org/10.1016/S0165-6147(00)01646-1

Rudolph, U., & Knoflach, F. (2011). Beyond classical benzodiazepines: Novel therapeutic potential of GABA A receptor subtypes. *Nature Reviews Drug Discovery*. https://doi.org/10.1038/nrd3502

Rupprecht, R. (2003). Neuroactive steroids: Mechanisms of action and neuropsychopharmacological properties. *Psychoneuroendocrinology*, *28*(2), 139–168. https://doi.org/10.1016/S0306-4530(02)00064-1

Rupprecht, R., & Holsboer, F. (1999). Neuroactive steroids: Mechanisms of action and neuropsychopharmacological perspectives. *Trends in Neurosciences*, *22*(9), 410–416. https://doi.org/10.1016/S0166-2236(99)01399-5

Rupprecht, R., Papadopoulos, V., Rammes, G., Baghai, T. C., Fan, J., Akula, N., … Schumacher, M. (2010). Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. *Nature Reviews Drug Discovery*, *9*(12), 971–988. https://doi.org/10.1038/nrd3295

Rupprecht, R., Rammes, G., Eser, D., Baghai, T. C., Schule, C., Nothdurfter, C., … Kucher, K. (2009). Translocator protein (18 kD) as target for anxiolytics without benzodiazepine-like side effects. *Science*, *325*, 490–493. https://doi.org/10.1126/science.1175055

Safavynia, S. A., & Goldstein, P. A. (2019). The role of neuroinflammation in postoperative cognitive dysfunction: Moving from hypothesis to treatment. *Frontiers in Psychiatry*, *9*(January). https://doi.org/10.3389/fpsyt.2018.00752

Sanger, D. J., & Depoortere, H. (1998). The pharmacology and mechanism of action of zolpidem. *CNS Drug Reviews*, *4*(4), 323–340. https://doi.org/10.1111/j.1527-3458.1998.tb00074.x

Sarkar, J., Wakefield, S., MacKenzie, G., Moss, S. J., & Maguire, J. (2011). Neurosteroidogenesis is required for the physiological response to stress: Role of neurosteroid-sensitive GABA A receptors. *Journal of Neuroscience*, *31*(50), 18198–18210. https://doi.org/10.1523/JNEUROSCI.2560-11.2011

Satoh, M., Ishihara, K., Iwama, T., & Takagi, H. (1986). Aniracetam augments, and midazolam inhibits, the long-term potentiation in guinea-pig hippocampal slices. *Neuroscience Letters*, *68*(2), 216–220. https://doi.org/10.1016/0304-3940(86)90145-X

Sayeed, I., Guo, Q., Hoffman, S. W., & Stein, D. G. (2006). Allopregnanolone, a progesterone metabolite, is more effective than progesterone in reducing cortical infarct volume after transient middle cerebral artery occlusion. *Annals of Emergency Medicine*, *47*(4), 381–389. https://doi.org/10.1016/j.annemergmed.2005.12.011

Schonborn, J. L., & Anderson, H. (2019). Perioperative medicine: a changing model of care. *BJA Education*, *19*(1), 27–33. https://doi.org/10.1016/j.bjae.2018.09.007

Schultz, C., & Engelhardt, M. (2014). Anatomy of the hippocampal formation. *The Hippocampus in Clinical Neuroscience*, *34*, 6–17. https://doi.org/10.1159/000360925

Schultz, S., Panzeri, S., Treves, A., & Rolls, E. T. (1999). Analogue resolution in a model of the schaffer collaterals. *Lecture Notes in Computer Science (Including Subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*, *1327*, 61–66. https://doi.org/10.1007/bfb0020133

Schwartz, R. D., Huff, R. A., Yu, X., Carter, M. L., & Bishop, M. (1994). Postischemic diazepam is neuroprotective in the gerbil hippocampus. *Brain Research*, *647*(1), 153–160. https://doi.org/10.1016/0006-8993(94)91411-7

Schwartz, R. D., Yu, X., Katzman, M. R., Hayden-Hixson, D. M., & Perry, J. M. (1995). Diazepam, given postischemia, protects selectively vulnerable neurons in the rat hippocampus and striatum. *Journal of Neuroscience*, *15*(1 II), 529–539. https://doi.org/10.1523/jneurosci.15-01-00529.1995

Scimemi, A., Semyanov, A., Sperk, G., Kullmann, D. M., & Walker, M. C. (2005). Multiple and plastic receptors mediate tonic GABAA receptor currents in the hippocampus. *Journal of Neuroscience*, *25*(43), 10016–10024. https://doi.org/10.1523/JNEUROSCI.2520-05.2005

Scoville, W. B., & Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of Neurology, Neurosurgery, and Psychiatry*, *20*(1), 11–21. https://doi.org/10.1136/jnnp.20.1.11

Seabrook, G. R., Easter, A., Dawson, G. R., & Bowery, B. J. (1997). Modulation of long-term potentiation in CA1 region of mouse hippocampal brain slices by GABA(a) receptor benzodiazepine site ligands. *Neuropharmacology*, *36*(6), 823–830. https://doi.org/10.1016/S0028-3908(97)00040-3

Serra, M., Madau, P., Chessa, M. F., Caddeo, M., Sanna, E., Trapani, G., … Biggio, G. (1999). 2-Phenyl-imidazo[1,2-alpyridine derivatives as ligands for peripheral benzodiazepine receptors: Stimulation of neurosteroid synthesis and anticonflict action in rats. *British Journal of Pharmacology*, *127*(1), 177–187. https://doi.org/10.1038/sj.bjp.0702530

Serwanski, D. R., Miralles, C. P., Christie, S. B., Mehta, A. K., Li, X., & De Blas, A. L. (2006). Synaptic and nonsynaptic localization of GABAA receptors containing the α5 subunit in the rat brain. *Journal of Comparative Neurology*, *499*(3), 458–470. https://doi.org/10.1002/cne.21115

Shen, H., Kenney, L., & Smith, S. S. (2020). Increased Dendritic Branching of and Reduced δ-GABAA Receptor Expression on Parvalbumin-Positive Interneurons Increase Inhibitory Currents and Reduce Synaptic Plasticity at Puberty in Female Mouse CA1 Hippocampus. *Frontiers in Cellular Neuroscience*, *14*(203). https://doi.org/10.3389/fncel.2020.00203

Sieghart W. (1995). Structure and pharmacology of gamma-aminobutyric acidA receptor

subtypes. *Pharmacological reviews*, 47(2), 181–234.

Sieghart, W., & Sperk, G. (2002). Subunit Composition, Distribution and Function of GABAA Receptor Subtypes. *Current Topics in Medicinal Chemistry*, *2*, 795–816.

Sigel, E., & Buhr, A. (1997). The benzodiazepine binding site of GABAA receptors. *Trends in Pharmacological Sciences*, *18*(11), 425–429. https://doi.org/10.1145/2107556.2107557

Slater, E. P., Hesse, H., & Beato, M. (1994). Regulation of Transcription by Steroid Hormones. *Ann N Y Acad Sci*, *733*, 103–112. https://doi.org/10.1111/j.1749-6632.1994.tb17260.x

Smith, S. S., She, H., Gong, Q. H., & Zhou, X. (2007). Neurosteroid regulation of GABA A receptors: Focus on the alpha4 and delta subunits. *Pharmacological Therapies*, *116*(1), 58–76. https://doi.org/10.1016/j.pharmthera.2007.03.008

Somogyi, P., & Klausberger, T. (2005). Defined types of cortical interneurone structure space and spike timing in the hippocampus. *Journal of Physiology*. https://doi.org/10.1113/jphysiol.2004.078915

Sperk, G., Schwarzer, C., Tsunashima, K., Fuchs, K., & Sieghart, W. (1997). GABA(A) receptor subunits in the rat hippocampus I: Immunocytochemical distribution of 13 subunits. *Neuroscience*, *80*(4), 987–1000. https://doi.org/10.1016/S0306-4522(97)00146-2

Squire, L. R. (1992). Declarative and nondeclarative memory: Multiple brain systems supporting learning and memory. *Journal of Cognitive Neuroscience*, *4*(3), 232–243. https://doi.org/10.1162/jocn.1992.4.3.232

Stell, B. M., Brickley, S. G., Tang, C. Y., Farrant, M., & Mody, I. (2003). Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing GABAA receptors. *PNAS*, *100*(24), 14439–14444. https://doi.org/10.1073/pnas.2435457100

Sternau, L. L., Lust, W. D., Ricci, A. J., & Ratcheson, R. (1989). Role for gamma-aminobutyric acid in selective vulnerability in gerbils. *Stroke*, *20*, 281–287.

Sugasawa, Y., Cheng, W. W. L., Bracamontes, J. R., Chen, Z. W., Wang, L., Germann, A. L., … Evers, A. S. (2020). Site-specific effects of neurosteroids on gabaa receptor activation and desensitization. *ELife*, *9*, 1–32. https://doi.org/10.7554/ELIFE.55331

Sur, C., Wafford, K. A., Reynolds, D. S., Hadingham, K. L., Bromidge, F., Macaulay, A., … Rosahl, T. W. (2001). Loss of the major GABAa receptor subtype in the brain is not lethal in mice. *Journal of Neuroscience*, *21*(10), 3409–3418. https://doi.org/10.1523/jneurosci.21-10-03409.2001

Suri, Y. (2000). Evaluation of midazolam and diazepam for pre-operative sedation. *Medical Journal Armed Forces India*, *56*(4), 287–292. https://doi.org/10.1016/s0377-1237(17)30209-5

Taliani, S., Da Settimo, F., Da Pozzo, E., Chelli, B., & Martini, C. (2009). Translocator Protein Ligands as Promising Therapeutic Tools for Anxiety Disorders. *Current Medicinal Chemistry*, *16*(26), 3359–3380.

https://doi.org/10.2174/092986709789057653

Tang, Z., Yang, F., Dong, Y., Ma, C., Sun, S., Shan, Y., … Liu, H. (2020). Midazolam contributes to neuroprotection against hypoxia/reoxygenation-induced brain injury in neonatal rats via regulation of EAAT2. *Brain Research Bulletin*, *161*(36), 136–146. https://doi.org/10.1016/j.brainresbull.2020.04.016

Taube, J. S., & Schwartzkroin, P. A. (1986). Ineffectiveness of organic calcium channel blockers in antagonizing long-term potentiation. *Brain Research*, *379*(2), 275–285. https://doi.org/10.1016/0006-8993(86)90781-X

The Royal College of Anaesthetists (2022). *The History of Anaesthesia*. [online] Available at: https://rcoa.ac.uk/about-college/heritage/history-anaesthesia

Thomas-Antérion, C., Koenig, O., Navez, M., & Laurent, B. (1999). Midazolam effects on implicit and explicit memory processes in healthy subjects. *Psychopharmacology*, *145*(2), 139–143. https://doi.org/10.1007/s002130051042

Tokuda, K., O'Dell, K. A., Izumi, Y., & Zorumski, C. F. (2010). Midazolam inhibits hippocampal long-term potentiation and learning through dual central and peripheral benzodiazepine receptor activation and neurosteroidogenesis. *Journal of Neuroscience*, *30*(50), 16788–16795. https://doi.org/10.1523/JNEUROSCI.4101-10.2010

Uusi-Oukari, M., & Korpi, E. R. (2010). Regulation of GABAA receptor subunit expression by pharmacological agents. *Pharmacological Reviews*. https://doi.org/10.1124/pr.109.002063

Van Rijnsoever, C., Täuber, M., Choulli, M. K., Keist, R., Rudolph, U., Mohler, H., … Crestani, F. (2004). Requirement of α5-GABAA receptors for the development of tolerance to the sedative action of diazepam in mice. *Journal of Neuroscience*, *24*(30), 6785–6790. https://doi.org/10.1523/JNEUROSCI.1067-04.2004

Verbny, Y. I., Merriam, E. B., & Banks, M. I. (2005). Modulation of γ-aminobutyric acid type A receptor-mediated spontaneous inhibitory postsynaptic currents in auditory cortex by midazolam and isoflurane. *Anesthesiology*, *102*(5), 962–969. https://doi.org/10.1097/00000542-200505000-00015

Verleye, M., Akwa, Y., Liere, P., Ladurelle, N., Pianos, A., Eychenne, B., … Gillardin, J. M. (2005). The anxiolytic etifoxine activates the peripheral benzodiazepine receptor and increases the neurosteroid levels in rat brain. *Pharmacology Biochemistry and Behavior*, *82*(4), 712–720. https://doi.org/10.1016/j.pbb.2005.11.013

Veselis, R. A., Pryor, K. O., Reinsel, R. A., Li, Y., Mehta, M., & Johnson, R. (2009). Propofol and midazolam inhibit conscious memory processes very soon after encoding: An event-related potential study of familiarity and recollection in volunteers. *Anesthesiology*, *110*(2), 295–312. https://doi.org/10.1097/ALN.0b013e3181942ef0

Veselis, R. A., Reinsel, R. A., Feshchenko, V. A., & Wronski, M. (1997). The comparative amnestic effects of midazolam, propofol, thiopental, and fentanyl at equisedative concentrations. *Anesthesiology*, *81*(4), 749–764. https://doi.org/10.1097/00000542-199710000-00007

Vicini, S., Losi, G., & Homanics, G. E. (2002). GABAAreceptor δ subunit deletion prevents neurosteroid modulation of inhibitory synaptic currents in cerebellar neurons. *Neuropharmacology*, *43*(4), 646–650. https://doi.org/10.1016/S0028-3908(02)00126-0

Vinkers, C. H., & Olivier, B. (2012). Mechanisms underlying tolerance after long-term benzodiazepine use: A future for subtype-selective GABAA receptor modulators? *Advances in Pharmacological Sciences*, (416864). https://doi.org/10.1155/2012/416864

Votey, S. R., Bosse, G. M., Bayer, M. J., & Hoffman, H. R. (1991). Flumazenil: A new benzodiazepine antagonist. *Annals of Emergency Medicine*, 20(2), 181–188.

Wang, N., Lian, J., Cao, Y., Muheyati, A., Yuan, S., Ma, Y., … Su, R. (2022). High-dose benzodiazepines positively modulate GABAA receptors via a flumazenil-insensitive mechanism. *International Journal of Molecular Sciences*, *23*(1). https://doi.org/10.3390/ijms23010042

Wayner, M. J., Armstrong, D. L., Polan-Curtain, J. L., & Denny, J. B. (1993). Role of angiotensin II and AT1 receptors in hippocampal LTP. *Pharmacology, Biochemistry and Behavior*, *45*(2), 455–464. https://doi.org/10.1016/0091-3057(93)90265-U

Weiner, J. L., Gu, C., & Dunwiddie, T. V. (1997). Differential ethanol sensitivity of subpopulations of GABAA synapses onto rat hippocampal CA1 pyramidal neurons. *Journal of Neurophysiology*, *77*(3), 1306–1312. https://doi.org/10.1152/jn.1997.77.3.1306

Weir, C. J., Mitchell, S. J., & Lambert, J. J. (2017). Role of GABA A receptor subtypes in the behavioural effects of intravenous general anaesthetics. *British Journal of Anaesthesia*, *119*, i167–i175. https://doi.org/10.1093/bja/aex369

Whiting, P. J., McKernan, R. M., & Wafford, K. A. (1995). Structure and Pharmacology of Vertebrate GABAA Receptor Subtypes. *International Review of Neurobiology*, *38*, 95–138. https://doi.org/10.1016/S0074-7742(08)60525-5

Wieland, H. A., Luddens, H., & Seeburg, P. H. (1992). A single histidine in GABA(A) receptors is essential for benzodiazepine agonist binding. *Journal of Biological Chemistry*, *267*(3), 1426–1429. https://doi.org/10.1016/s0021-9258(18)45961-3

Wisden, W., Laurie, D. J., Monyer, H., & Seeburg, P. H. (1992). The Distribution of 13 GABA, Receptor Subunit mRNAs in the Rat Brain. I. Telencephalon, Diencephalon, Mesencephalon. *The Journal of Neuroscience*, *12*(3), 1040–1062.

Wohlfarth, K. M., Bianchi, M. T., & Macdonald, R. L. (2002). Enhanced neurosteroid potentiation of ternary GABAA receptors containing the delta subunit. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(5), 1541–1549. https://doi.org/22/5/1541

Wright, B. T. (2016). *Repeated Zolpidem Treatment Effects on Sedative Tolerance, Withdrawal, mRNA Levels, and Protein Expression*. *Theses and Dissertations (ETD)*. Retrieved from https://manchester.idm.oclc.org/login?url=https://search.proquest.com/docview/1817021207?accountid=12253%0Ahttp://man-fe.hosted.exlibrisgroup.com/openurl/44MAN/44MAN_services_page?genre=dissertatio

ns+%26+theses&atitle=&author=Wright%2C+Brittany+T.&volume=

Wu, X., Wu, Z., Ning, G., Guo, Y., Ali, R., Macdonald, R. L., … Chen, G. (2012). γ-aminobutyric acid type A (GABA A) receptor α subunits play a direct role in synaptic Versus extrasynaptic targeting. *Journal of Biological Chemistry*, *287*(33), 27417–27430. https://doi.org/10.1074/jbc.M112.360461

Xue, Q.-S., Yu, B.-W., Wang, Z.-J., & Chen, H.-Z. (2004). Effects of ketamine, midazolam, thiopental, and propofol on brain ischemia injury in rat cerebral cortical slices. *Acta Pharmacologica Sinica*, *25*(1), 115–120. https://doi.org/10.1016/j.gloplacha.2004.12.001

Yassa, M. A., & Stark, C. E. L. (2011). Pattern separation in the hippocampus. *Trends in Neuroscience*, *34*(10), 515–525. https://doi.org/10.1016/j.tins.2011.06.006.

Yeckel, M. F., & Berger, T. W. (1990). Feedforward excitation of the hippocampus by afferents from the entorhinal cortex: Redefinition of the role of the trisynaptic pathway. *PNAS*, *87*(15), 5832–5836. https://doi.org/10.1073/pnas.87.15.5832

Yu, H., Wang, X., Kang, F., Chen, Z., Meng, Y., & Dai, M. (2019). Neuroprotective effects of midazolam on focal cerebral ischemia in rats through anti-apoptotic mechanisms. *International Journal of Molecular Medicine*, *43*(1), 443–451. https://doi.org/10.3892/ijmm.2018.3973

Zorumski, C. F., Mennerick, S. J., & Covey, D. F. (1998). Enantioselective modulation of GABAergic synaptic transmission by steroids and benz[e]indenes in hippocampal microcultures. *Synapse*, *29*(2), 162–171. https://doi.org/10.1002/(SICI)1098-2396(199806)29:2<162::AID-SYN7>3.0.CO;2-5

Zorumski, C. F., Paul, S. M., Izumi, Y., Covey, D. F., & Mennerick, S. (2013). Neurosteroids, stress and depression: Potential therapeutic opportunities. *Neurosci Biobehav Rev.*, *37*(1), 109–122. https://doi.org/10.1016/j.neubiorev.2012.10.005

Scientific papers resulting from this thesis

- Puig-Bosch, X., Bieletzki, S., Zeilhofer, H. U., Rudolph, U., Antkowiak, B., & Rammes, G. (2022). Midazolam at low nanomolar concentrations affects long-term potentiation and synaptic transmission predominantly via the α1-GABAA receptor subunit in mice. *Anesthesiology*, 10.1097/ALN.0000000000004202. Advance online publication. https://doi.org/10.1097/ALN.0000000000004202

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