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Positive allosteric modulation of GABA$_A$ receptors in the amygdala, hippocampus and thalamocortical circuits by XBD173, diazepam and allopregnanolone: a comparative VSDI study

Tatiana Engel

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3α,5α-THDOC   allotetrahydrodeoxycorticosterone, 3α,21-Dihydroxy-5α-pregnan-20-one

a   area [No of pixel]

ACSF   artificial cerebrospinal fluid

Allo   allopregnanolone, 5α-pregnan-3α-ol-20-one, 3α-5α-THP

ANT   adenine nucleotide translocase

CCD   charged-coupled device

CCK4   cholecystokinin tetrapeptide

Cl^- Chloride Ion

CNS   central nervous system

Diaz   diazepam

DMSO   dimethyl sulfoxide

eEPSC   electric evoked excitatory postsynaptic current

EPSC   excitatory postsynaptic current

F/XBD   finasteride-Experiments

FBS   fetal bovine serum

FDS   fast depolarization signal

Fina   finasteride

Fluma   flumazenil

GABA   γ-Aminobutyric acid

$GABA_A$-R $GABA_A$-Receptor

HR-CCD   high-resolution charge-coupled device

Int   Intensity [$\Delta F/F$]

Int_{max} maximal change of Intensity (layer analysis)

IPSC	inhibitorischer postsynaptic current
n	number of experiments performed
P450scc	Cholesterol side chain cleavage cytochrome P450
PAM	positive allosteric modulator
PK 11195	N-butan-2-yl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide, TSPO Antagonist
PSC	Postsynaptic current
RAS	reticular activating system
ROI	region of interest
SEM	standard error of the mean
SSRI	selective serotonin reuptake inhibitor
TC	thalamocortical neuron, also named relay neuron
TRN	Thalamic reticular neuron
TSPO	Translocator Protein (18 kDa (kilo Dalton)), formerly called: peripheral benzodiazepine receptor
VDAC	voltage-dependent anion channel
XBD	XBD-173, also named Emapunil or AC-5216, systematic name: N-benzyl-N-ethyl-2(7-methyl-8-oxo-2-phenylpurin-9-yl)acetamide

1 Introduction

1.1 Anxiety Disorders

Anxiety disorders are highly prevalent in today's society. They are amongst the most common psychiatric illnesses. Prevalence and lifetime risk differ considerably between countries. In Germany the projected lifetime risk is about 17%, whereas in the USA it is as high as 36% (Kessler et al. 2007). Anxiety disorders comprise a number of distinct diagnoses. They can further be categorized into panic disorders, specific phobias, social phobias, post-traumatic stress disorders, obsessive-compulsive disorders and generalized anxiety disorders amongst others (Baldwin et al. 2014). A multifactorial pathogenesis is assumed, including external factors such as traumatic or straining experiences as well as internal factors like genetics and neurobiological dysfunction (Höffler et al. 2003). While symptoms can be minor and temporarily, a lot of patients suffer from severe and recurring symptoms that impair their quality of life to an extent that requires treatment.

1.2 Pharmacological Treatment of Anxiety Disorders

Therapeutic approaches include psychotherapies and psychopharmacological drugs (Baldwin et al. 2014). However, current pharmacological treatment options are limited by pharmacodynamics and undesirable side effects. First line in treatment antidepressants such as selective serotonin reuptake inhibitors (SSRI) exert their anxiolytic effect only after several weeks of treatment (Bandelow et al. 2008). Though in general well-tolerated, potential initial adverse effects like nausea, jitteriness, insomnia and even increase of anxiety symptoms as well as weight gain and sexual dysfunction that may occur in long-term treatment are known to impair patient compliance (Bandelow et al. 2008). More rapidly onset drugs commonly used are benzodiazepines. Unfortunately they are not eligible either, due to their broad range of unfavorable adverse effects, development of tolerance and danger of addiction and withdrawal symptoms.

A new class of substances with a preferable pharmacological profile is needed. However, more suitable medication needs yet to be established. Drugs, which dispose of rapidly on setting anxiolytic effects like benzodiazepines, but lack a broad side effect profile, are much needed. Satisfying anxiolytic effects of endogenous neurosteroids such as allopregnanolone have been shown (Bitran et al. 1991, Wieland et al. 1991). Unfortunately, pharmacokinetic obstacles such as low bioavailability limit their medical use (Reddy 2010). However, there is a way to enhance natural neurosteroid synthesis: The Translocator Protein 18 kDa (TSPO) and its ligands provide a promising new option. TSPO is crucially involved in the synthesis of neurosteroids, for it is in charge

of the rate-determining step of neurosteroidogenesis (Papadopoulos et al. 1997a). Therefore ligands to TSPO, like XBD-173 (XBD; Emapunil), that enhance TSPO's effects and subsequently promote neurosteroid synthesis are currently under investigation as a potential new therapeutic alternative, that has already revealed great anxiolytic capabilities and a favorable side effect profile (Rupprecht et al. 2009).

1.3 Benzodiazepines

Besides in anxiety disorders Benzodiazepines are used in treatment of sleeping disorders, epilepsy and schizophrenia (Rote-Liste 2013, 2015). They are also commonly used as premedication before surgical interventions and for calming effects in emergencies as well as to treat muscle spasms. Even though they are very potent anxiolytic drugs and exhibit a quick onset of anxiolytic effects they are not suitable for long-term anxiety treatment due to a broad side effect profile, development of tolerance and danger of addiction and withdrawal symptoms (Bandelow et al. 2008) . Side effects include sedation, drowsiness, anterograde amnesia, respiratory depression and central diminution of tonicity (Rote-Liste 2013, 2015).

Benzodiazepines exert their effects by enhancing γ-Aminobutyric acid (GABA) impact in the central nervous system (CNS) (Rote-Liste 2013, 2015). Since GABA is the most important inhibitory transmitter in the CNS, they reduce neuronal activity (Bloom et al. 1971). Benzodiazepines bind to a specific compound of the $GABA_A$-receptor ($GABA_A$-R) between the α- and γ-subunit (Möhler 2006). Once connected to the receptor, they exert an allosteric effect that facilitates binding of GABA to the $GABA_A$-R and increases its opening frequency (see chapter 1.6), thus decreasing neuronal excitability, which causes anxiolytic effects amongst other things (Walters et al. 2000).

1.4 Neurosteroids

Neuroactive steroids are a specific kind of steroids that, in addition to genomic alternation, the common course of action of steroids, express the ability to modulate neurotransmitter receptors (Majewska et al. 1986, Paul et al. 1992, Rupprecht et al. 1999). While gene expression via steroid activated transcriptional factors take minutes to hours, effects by modulation of neurotransmitter receptors occur within seconds (McEwen 1991). One of the affected receptors is the $GABA_A$-R (Majewska et al. 1986).

Neurosteroids are predominantly produced in the brain and independent of peripheral steroid levels (Corpéchot et al. 1981). The precursor of all neurosteroids is pregnenolone, which is synthesized out of cholesterol inside the mitochondrion (Lacapère et al. 2003, Rupprecht et al. 2010). Synthesis of pregnenolone underlies regulation by TSPO (see chapter 1.5.2). Pregnenolone leaves the

mitochondrion and is processed further in the cytosol into a variety of other neurosteroids (see Fig. 1A).
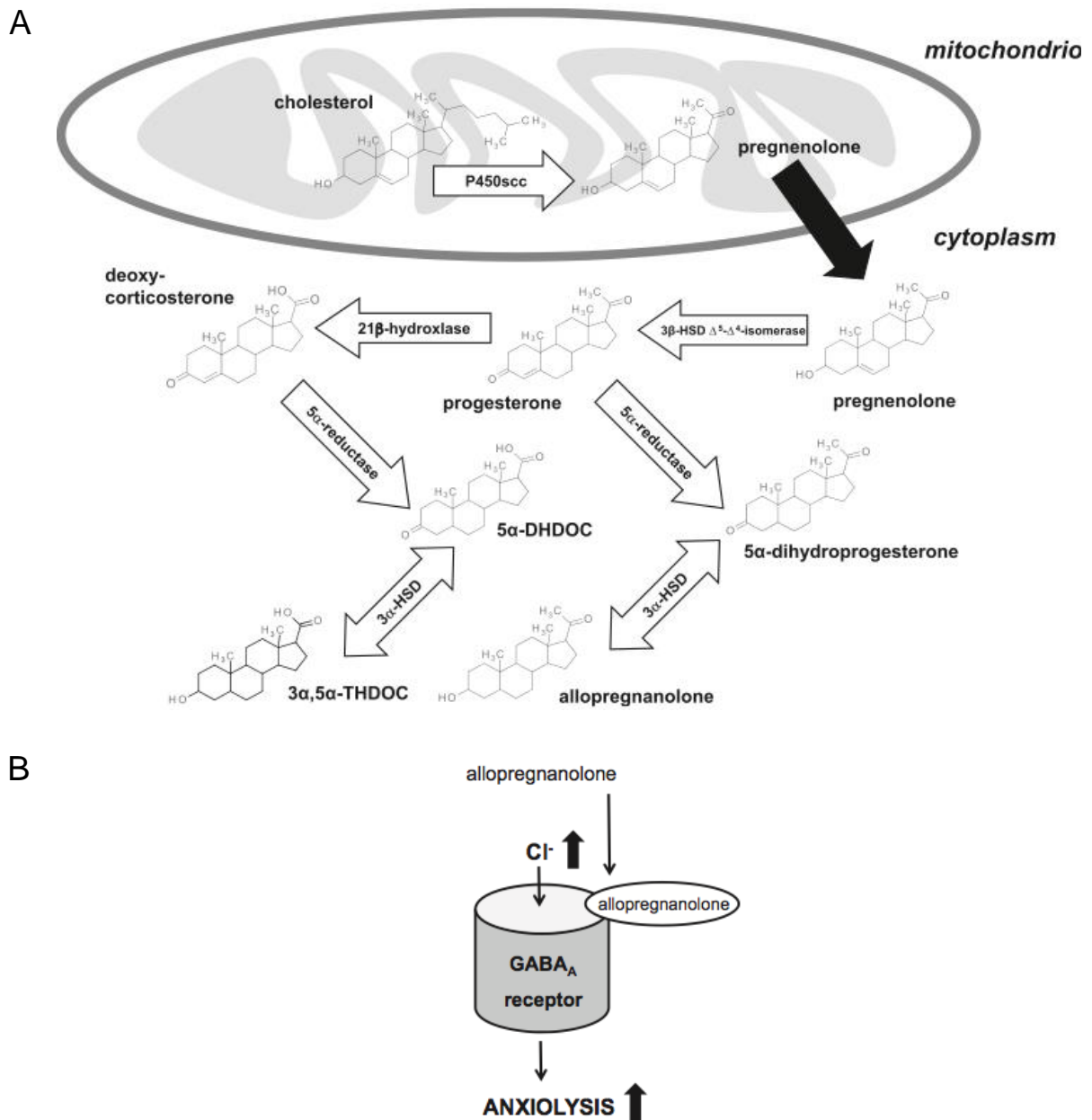
Fig. 1: Synthesis of neurosteroids and schematic sketch of allopregnanolone effect

A Scheme of synthesis of neurosteroids from (Schüle et al. 2014) according to (Nothdurfter et al. 2012a). Neurosteroids are formed out of cholesterol. First step, transformation to pregnenolone, the precursor of all neurosteroids, takes place in the mitochondrion. Further synthesis of diverse neurosteroids out of pregnenolone takes place in the cytoplasm. Utilized enzymes are depicted as arrows. **B** Scheme of allopregnanolone effect according to (Nothdurfter et al. 2012b). Allopregnanolone binds to the GABA$_A$ receptor where it exerts a modulating effect, enhancing GABA binding and subsequent chloride influx, thus potentiating its inhibitory impact on neuronal activity, which leads to Anxiolysis.

Neurosteroid biosynthesis is region and neuron specific, depending on the expression of the respective enzymes for neurosteroid formation (Rupprecht et al. 2010). Region-specific synthesis of neurosteroids is subsequently responsible for a distinct modulation of neurotransmitter receptors in certain brain areas, resulting in region-specific alteration of signal transduction. Some of the neurosteroids formed are 3α-reduced neurosteroids, such as allopregnanolone (Rupprecht 1997).

Certain 3α-reduced steroids, especially allopregnanolone, have been shown to feature anxiolytic properties in various stress-models in rodents (Bitran et al. 1991, Picazo et al. 1995, Wieland et al. 1995, Reddy et al. 1997). Furthermore they may play a role in pathology of affective diseases including anxiety disorders (Strohle et al. 2003, Reddy 2010). Similar to benzodiazepines, 3α-reduced steroids exercise their anxiolytic effects by potentiating inhibitory effects of GABA (see Fig. 1B) (Gee 1988, Bitran et al. 1991). However they modulate GABA$_A$-receptors using another binding site (see Fig. 3B) (Reddy et al. 1997, Hosie et al. 2006).

Allopregnanolone stands out with particular effectiveness in reducing stress and anxiety-like behavior in rodents. Increased allopregnanolone levels were detected in rodents (also adrenalectomized rodents) exposed to stressors or acute stress paradigms (Purdy et al. 1991, Barbaccia et al. 1997). Induced panic attacks in patients with panic disorders led to decreasing of allopregnanolone plasma concentrations (Strohle et al. 2003). While stressors raise allopregnanolone levels, presumably attempting to prevent anxiety or panic outbreak, panic attacks reduce allopregnanolone levels assumedly by consumption. Anti-anxiety behavior in rodents has been reduced by blockage of allopregnanolone formation (Frye et al. 2011, Koonce et al. 2012). Concordantly, anti-anxiety behavior was reinstated by allopregnanolone injection (Frye et al. 2011). These findings propose allopregnanolone to be a neurosteroid of particular importance concerning anxiety treatment (Schüle et al. 2014).

Unfortunately natural neurosteroids like allopregnanolone have a low bioavailability as a result of rapid inactivation and elimination by glucuronidation and sulfate conjugation, as well as possible oxidation to ketone (Reddy 2010). Hence, they are not viable for therapeutic use. Fortunately there are methods to enhance neurosteroid synthesis in the brain as described in the following chapters.

1.5 Translocator protein 18 kDa

The Translocator protein (18 kDa) (TSPO) was first recognized in 1977 in the kidney on the search for binding sites for benzodiazepines (Braestrup et al. 1977) and thus afterwards named peripheral benzodiazepine receptor. In consideration of its broad range of function, employment beyond its receptor capacities and due to the fact that not all benzodiazepines bind to it, but a number of other substances do and that its structure and function differ tremendously from the central

benzodiazepine receptor (part of the GABA$_A$ receptor complex), it was renamed Translocator protein (18 kDa) in 2006 (Papadopoulos et al. 2006a).

1.5.1 Location and structure

TSPO has since been detected in various tissues including the brain, though the highest levels of expression have been found in steroid producing tissues like adrenal and gonad (Anholt et al. 1985, Gavish et al. 1999). Inside the CNS it is primarily expressed in glia cells (Gavish et al. 1999).

At the subcellular level, TSPO is mainly – though not exceptionally (Olsen et al. 1988, Oke et al. 1992) – located at the outer mitochondrial membrane (Anholt et al. 1986), cumulating at contact sites of the outer and inner mitochondrial membrane (Culty et al. 1999). Topological analysis of TSPO revealed it to consist of 5 trans membrane α-helices (Joseph-Liauzun et al. 1998). 3D modeling of TSPO suggested that the helices form a channel, likely used for cholesterol transport (Bernassau et al. 1993, Culty et al. 1999). However to be accurate TSPO should be characterized as a cholesterol exchanger rather than a channel, for it actively transports cholesterol instead of just letting it pass (Lacapère et al. 2003).

TSPO interacts with other mitochondrial membrane proteins such as the 32 kDa voltage dependent anion channel (VDAC) and the 30 kDa adenine nucleotide translocase (ANT) (McEnery et al. 1992). They form a complex reaching from outer to inner mitochondrial membrane, whereupon TSPO is the minimal functional unit able to bind cholesterol and drug ligands (Lacapère et al. 2001). However VDAC is essential for maximizing benzodiazepine and endozepine (endogenous compounds with likewise effects as benzodiazepines) binding to TSPO (Garnier et al. 1994). The entire impact of these protein associations is not yet fully understood, but they are believed to alter drug binding properties and related functions (Golani et al. 2001). Furthermore, homopolymerization of TSPO influences pharmacological and physiological properties (Delavoie et al. 2003).

1.5.2 TSPO function

TSPO is expressed of a gene highly conserved throughout evolution, appearing in all sorts of organisms extending from bacteria to humans (Gavish et al. 1999). It has been found to participate in many cell regulation and signal transduction mechanism of the mitochondrion. Three main functions are thought to be primarily responsible for all TSPO-delegated effects (Papadopoulos et al. 2006a). These include cholesterol (Papadopoulos et al. 1997b, Li et al. 2001), protein (Wright et al. 1999, Hauet et al. 2005) and porphyrin (Synder et al. 1987, Taketani et al. 1994) binding and transport into the mitochondrion. Cholesterol translocation is essential for steroid and bile salt biosynthesis, protein import is necessary for membrane biogenesis and porphyrin import is a part of the heme biosynthesis pathway (Papadopoulos et al. 2006a). Associated functions might include

cellular proliferation and differentiation, immunomodulation, cellular respiration and involvement in oxidative processes, regulation of mitochondrial metabolism and apoptosis (Papadopoulos et al. 2006a, Rupprecht et al. 2010). The importance of TSPO for the organism is demonstrated by the fetal consequence of functional deactivation in an early embryotic phase in mice (Papadopoulos et al. 1997a).
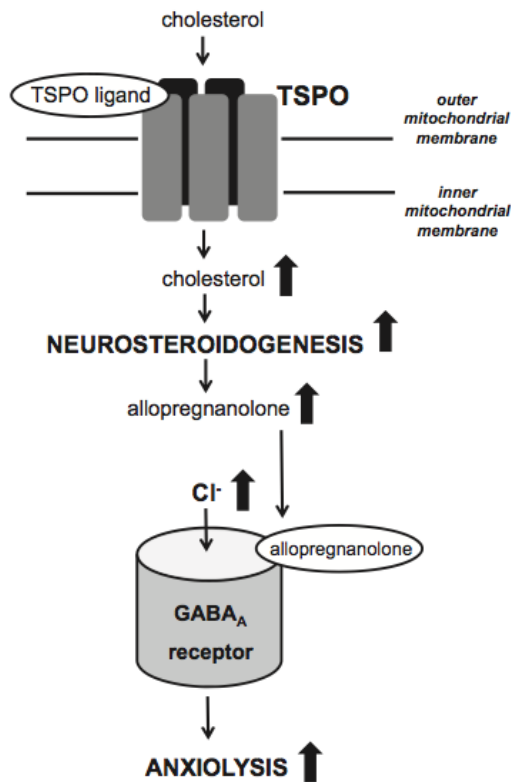
Fig. 2: TSPO relevance in anxiolysis

Illustration of TSPO function in anxiolysis according to (Nothdurfter et al. 2012b). Cholesterol is imported to the inside of the mitochondrion by TSPO. Here the first step of neurosteroidogenesis, pregnenolone (precursor of all neurosteroids) formation out of cholesterol, is performed. Subsequent, allopregnanolone is increasingly synthesized and ready to manipulate GABA$_A$-Receptors, resulting in anxiolysis.

As for TSPO involvement in neurosteroid genesis, cholesterol translocation from the cytosol to the inner mitochondrial membrane by TSPO (Papadopoulos et al. 1997a) is the rate-limiting step in neurosteroid genesis (Jefcoate 2002). Inside the Mitochondrion is the cholesterol side chain cleavage cytochrome enzyme P450 (P450scc), ready to metabolize cholesterol to pregnenolone, the precursor of all neurosteroids (Lacapère et al. 2003, Rupprecht et al. 2010). Positive allosteric neurosteroids such as allopregnanolone are subsequently formed and modulate GABA$_A$ receptors, thus exerting anxiolytic effects (Fig. 2) (Papadopoulos et al. 2006b). Since inside the CNS, TSPO is mainly expressed in glia cells, the acquired neurosteroids are believed to work in a paracrine fashion for the most part, operating at receptors located at surrounding neurons (Nothdurfter et al. 2012a).

In addition TSPO seems to be a considerable factor in a number of pathological conditions, including brain injury, peripheral neuropathy, neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, epilepsy, certain kinds of cancer and psychiatric disorders such as anxiety disorders (Papadopoulos et al. 2006a). As a matter of fact a number of studies demonstrated an increase in TSPO density in acute stress while under chronic stress as well as in anxiety disorders density is usually decreased, indicating TSPO to be a critical factor in the organism's way of coping with stress (Gavish et al. 1999). For instance, in patients suffering from generalized anxiety disorder, lower levels of ligand binding to TSPO have been measured (Weizman et al. 1987). Moreover, levels increased again

after treatment with diazepam whereupon relief of anxiety was stated (Weizman et al. 1987). Also in soldiers repeatedly exposed to stress exercises, reduced PBR density was monitored as well as in civilians during war (Dar et al. 1991, Weizman et al. 1994). Whereas in psychiatric residents completing their exams significant increase of platelet PBR density was shown, presumably as a way to cope with stress and prevent anxiety (Karp et al. 1989). Therefore, drugs acting at a mechanism that appears to be involved in pathology of anxiety formation propose to show great promise.

1.5.3   Ligands to TSPO

There are a number of substances capable of binding to TSPO. Within high affinity endogenous ligands are cholesterol and porphyrins. Also endozepines (derivatives of the common polypeptide precursor diazepam-binding inhibitor (DBI)) are to be mentioned, which are able to displace benzodiazepines from their binding site. While cholesterol is known to connect to the C-terminus of TSPO (Li et al. 2001), drug ligands bind to the amino terminus (Rupprecht et al. 2010). This is consistent with the observation that cholesterol is imported to the inner mitochondrial membrane by TSPO, while other ligands influence TSPO properties such as cholesterol translocation (Lacapère et al. 2003). Endogenous drugs are therefore likely to regulate TSPO function. Given the many TSPO functions and its involvement in pathologies, great therapeutic benefit is to be expected of synthetic TSPO ligands.

A number of synthetic ligands have been developed. A lot of them initially served as neuroimaging agents to determine TSPO distribution or for means of identification of TSPO function (Rupprecht et al. 2010). However certain ligands have been shown to promote neurosteroidogenesis in the brain and therefore exercise anxiolytic effects in rodent models (Serra et al. 1999, Bitran et al. 2000, Verleye et al. 2005). One of them is XBD173 (XBD) (Rupprecht et al. 2009).

1.5.4   XBD173

XBD's mode of action

XBD, a phenyl-purine acetamide with promising anxiolytic potential, is a selective ligand to TSPO with nanomolar affinity (Kita et al. 2004). Its affinity to neurotransmitter receptors including the GABA$_A$ receptor is negligible (Kita et al. 2004). A research group around Rupprecht (2009) demonstrated that XBD does not exert any direct effects at the GABA$_A$ receptor. However, appliance of XBD to neocortical slices potentiated GABAergic neurotransmission (Rupprecht et al. 2009). This was prevented by finasteride, a 5α-reductase inhibitor, indicating 5α-reduced neurosteroids to be responsible for GABA-mediated actions following XBD application (Rupprecht et al. 2009). Furthermore, significantly elevated levels of allopregnanolone in the brains of rats were measured after oral medication with XBD (Rupprecht et al. 2009). In addition, anxiolytic properties

of XBD during stress tests (social exploration and elevated plus maze test (Pellow et al. 1985)) in rats were prevented by the TSPO antagonist PK 11195, confirming XBD's mode of action to be TSPO-mediated up-regulation of neurosteroidogenesis (Fig. 3) (Rupprecht et al. 2009).

Effect and side-effect profile of XBD

Besides its anxiolytic abilities in stress tests, XBD also counteracted sodium lactate and cholecystokinin tetrapeptide (CCK4) induced panic attacks in rats (Rupprecht et al. 2009). Hereby no signs of sedation were detected, different to like-wise application of the benzodiazepine alprazolam. Moreover, there was no indication for decrease of XBD's anxiolytic efficiency (tolerance) in the social exploration stress test under subchronic administration, a phenomenon well known in benzodiazepine-treatment (Rupprecht et al. 2009, Rote-Liste 2013, 2015). This was also observed in anxiety models in mice by the research team around Kita (Kita et al. 2009). In addition, XBD unlike diazepam lacked withdrawal symptoms in special designed withdrawal experiments they performed on mice (Kita et al. 2009).

In humans, XBD revealed anxiolytic properties as well. Anxiolytic efficiency of XBD proved to be as potent as alprazolam's in the CCK-4 challenge (Rupprecht et al. 2009). Occurrence of side effects in XBD treated subjects was comparable to incidents in subjects treated with placebo. Alprazolam treated subjects however suffered a much higher incidence of adverse effects, especially dizziness and somnolence. The same was reported of withdrawal symptoms.

To summarize, the data presented by Rupprecht at al (2009) and Kita at al (2009) indicates that XBD possesses rapidly onset anxiolytic properties comparable to benzodiazepines, but with a distinct superior side effect profile. More precisely, as far as up to date studies detected, XBD lacks adverse effects like sedation, anterograde amnesia, tolerance development, addiction and withdrawal symptoms. Whereas all of the above have been observed in patients treated with benzodiazepines (Rote-Liste 2013, 2015). Therefore XBD may be a superior candidate in the treatment of anxiety disorders.

1.6 GABAA receptor

The diverse side effect profile of XBD and diazepam despite corresponding final modes of action could be due to different distribution of the various subtypes of $GABA_A$ receptors. Though both, benzodiazepines and certain neurosteroids (5α-reduced neurosteroids such as allopregnanolone and $3\alpha,5\alpha$-THDOC), act as positive allosteric modulators at the $GABA_A$ receptor, they occupy different binding sites (Fig. 3B), which do not always coexist in the same $GABA_A$ receptor (Olsen et al. 2008).
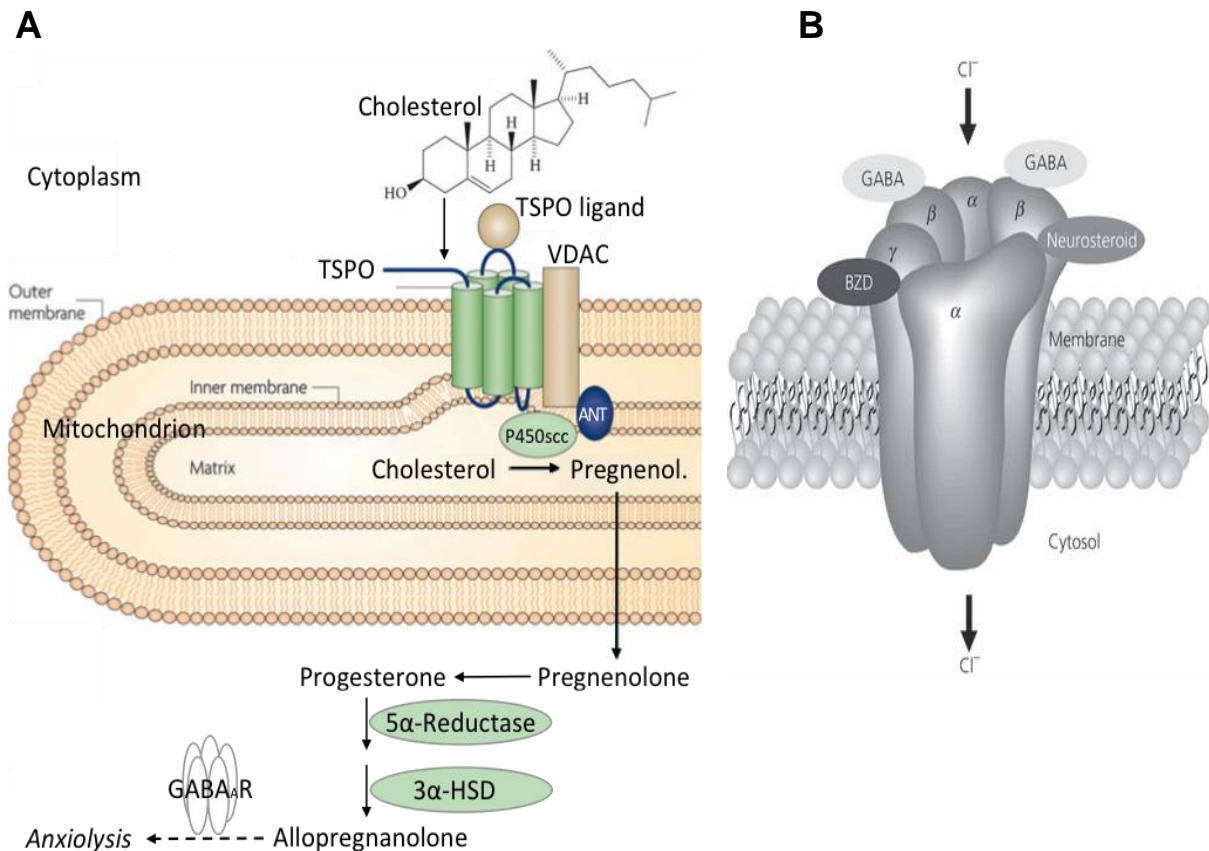
Fig. 3: Scheme of XBD's mode of action and display of GABAA receptor

A: TSPO ligand mediated neurosteroidogenesis; modified according to (Rupprecht et al. 2010). TSPO ligands, like XBD, enhance TSPO function, thus favoring translocation of Cholesterol from the cytosol to the inner mitochondrial membrane, the rate-limiting step in neurosteroid genesis. The cholesterol side-chain-cleaving cytochrome P450 enzyme (P450scc) metabolizes cholesterol to pregnenolone (Pregnenol.), the precursor of all neuroactive steroids. In the cytosol Pregnenolone is metabolized further. For allopregnanolone formation the enzymes 5α-Reductase and 3α-Hydroxysteroid dehydrogenase (3α-HSD) amongst others are necessary. Allopregnanolone then exerts anxiolytic effects by modulation of the GABAA receptor. **B:** systematic display of the GABAA receptor including various binding sites; according to (Nothdurfter et al. 2012a). The GABAA receptor is located at the cell membrane. It is build out of 5 subunits deriving out of 8 subunit classes. Different binding sides for GABA, diazepam (in between α- and γ-subunits) and allopregnanolone (in between α- and β- subunits) can be identified.

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter receptor in the CNS (Bloom et al. 1971). The GABAA receptor belongs to the category of ligand-gated ion channels. It is built of 5 subunits, each containing 4 transmembrane domains, forming a central pore (Bormann et al. 1987, Pirker et al. 2000). Upon binding of two GABA molecules, the pore becomes permeable for chloride ions (Cl^-) and bicarbonate (HCO_3^-) (Bormann et al. 1987, Macdonald et al. 1994). Influx of negative charged ions induces hyperpolarization, causing an inhibitory postsynaptic current to occur in the corresponding neuron, cancelling out potential excitatory postsynaptic currents emerging simultaneously from another synapse of the same neuron. In short: GABA has a diminishing impact on neuronal activity e.g. signal transduction.

The subunits forming the GABA$_A$ receptor can vary out of a range of 19 different subunits divided in 8 subunit-classes: α 1-6, β 1-3, γ 1-3, δ, ϵ, θ, π, ρ 1-3. (Barnard et al. 1998, Olsen et al. 2008). Depending on the subunit composition, the GABA$_A$ receptor is able to bind a variety of different drugs and endogenous ligands. Distribution of diverse GABA$_A$ receptor subtypes in the brain is heterogeneous (Pirker et al. 2000).

Benzodiazepines and certain neurosteroids (5α-reduced neurosteroids such as allopregnanolone and THDOC) both facilitate GABA binding by enhancing receptor affinity towards GABA mediated by allosteric changes of the GABA$_A$ receptor (Sigel et al. 1997, Belelli et al. 2005). While the binding site for benzodiazepines is formed by α- and γ-subunits, neurosteroids occupy another binding site located in between the α- and β- subunits (Fig. 3) (Hosie et al. 2006, Möhler 2006). Therefore, not only availability of benzodiazepines or neurosteroids is necessary for respective effects, but also the expression of corresponding GABA$_A$ receptor subtypes. This might be a significant factor, leading to the disparity of their side effect profiles.

1.7   Amygdala

The main focus of this dissertation is on the discordance between the side effect profile of XBD and diazepam. This discordance is most likely due to different effects that these drugs have on respective parts of the brain. Hence the influence of those substances on the amygdala, the hippocampus and the thalamocortical connections was measured in the course of this dissertation. Those cerebral areas will therefore be introduced in this and the following chapters.

1.7.1   Anatomy and Neurophysiology

The corpus amygdaloideum, more commonly known as the amygdala, is localized in the medial temporal lobe in each hemisphere. The amygdala consists of many distinct, however densely interconnected, nuclei (Amaral et al. 1992). They can be functionally and anatomically grouped into three core domains: the basolateral (composed of the lateral and the basal nuclei (Keifer et al. 2015)), the centromedial and the superficial nuclei group (Bzdok et al. 2013). The basolateral amygdala obtains its information from the sensory organs, the hippocampus and the cortex and is in charge of assigning emotional values to distinct stimuli (Sah et al. 2003). This will be the nuclei group this dissertation focuses on.

1.7.2   Function

The amygdala is a part of the limbic system and is known to play a crucial role in processing emotions and initiating fear and anxiety reactions (Trepel 2008). Functional loss of the amygdala results in a decrease of negative emotions such as anxiety and rage (Weiskrantz 1956, Zola-

Morgan et al. 1991). Concordantly, stimulation of the amygdala provokes anxiety and fear (Kaada 1951, Chapman et al. 1954). Furthermore, direct infusion of allopregnanolone into the amygdala resulted in a decrease of anxiety-patterned behavior (Akwa et al. 1999).

1.8 Hippocampus

The hippocampus is an evolutionary old and well-conserved brain structure. It too is located in the medial temporal lobe and belongs to the limbic system. It is a part of the hippocampal formation, a group of functionally and neuronally connected cerebric areas, which in addition to the hippocampus also includes the entorhinal cortex, the subiculum and the dentate gyrus. The actual hippocampus equates to the Cornu Ammonis and can be functionally and morphologically subdivided into 3 domains, the Cornu Ammonis 1 to 3 (CA1-CA3).

1.8.1 Neurophysiology

The hippocampus obtains information from several sensory systems. The entorhinal cortex, as the main input source of the hippocampus, hereby channels said information (Lomo 1971). The incoming data is then processed and forwarded back to the cortex. Within the neuronal network of the hippocampal formation, there are three major successive excitatory projections, which combined resemble the trisynaptic circuit (Doller et al. 1982, Yeckel et al. 1990). The starting point is formed by the perforant path, which is made up of fibers emerging from the entorhinal cortex leading to the dentate gyrus. From here the so-called mossy fibers innervate the CA3 region, which in turn connects via the Schaffer collaterals to the CA1 region (Amaral 1993). Axons from the CA1 region then project to the subiculum and back to the entorhinal cortex, thus completing the trisynaptic circuit (see Fig. 4). Within each region there are inhibitory GABAergic interneurons processing the data (Jones 1993, Pettit et al. 2000). These GABAergic fibers are the working point of GABA$_A$ receptor modulating drugs such as benzodiazepines.
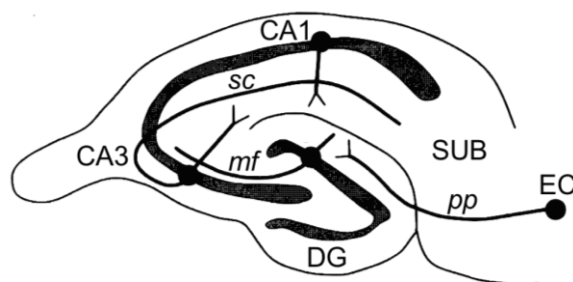
Fig. 4: Schematic display of hippocampal formation and the trisynaptic circuit

Modified according to (Amaral 1993). The hippocampal formation is made up out of the entorhinal cortex (EC), the dentate gyrus (DG), the hippocampus and the subiculum (SUB). The hippocampus can be subdivided into regions CA1-CA3. The trisynaptic circuit is a successive pathway that is made up of three major excitatory projections: the perforant path (pp) emerging from the EC to innervate the DG, the mossy fibers (mf) that connect the DG with the CA3 region of the hippocampus and the schaffer collaterals (sc) that excite the CA1 region

1.8.2 Function

A core function of the hippocampus is the consolidation of the declarative memory. Scoville and his team were the first to discover, that upon bilateral removal of the hippocampus, patients suffered from anterograde amnesia (Scoville et al. 1957). Afterwards it was repeatedly confirmed that the hippocampus is indeed crucial for forming new memories (Moss et al. 1981, Eichenbaum et al. 1992, Squire 1992). Close evaluation showed that hereby only the declarative memory was compromised, whilst the procedural memory was not impaired (Eichenbaum et al. 1992). Since the hippocampus plays such a leading role in memory formation it can be supposed, that drugs with amnestic side effects interfere within some point of hippocampal data processing. For instance, benzodiazepines might act at hippocampal $GABA_A$ receptors, enhancing inhibitory interneurons that subsequently reduce data transfer and therefore prevent memory consolidation.

1.9 Thalamocortical Connections

The thalamus is densely connected to the cortex and together with the latter shapes up to form the thalamocortical system (Guillery et al. 2002).

1.9.1 Anatomy and Neurophysiology

The thalamus is located in the diencephalon, can be anatomically split into a ventral and dorsal thalamus and consists of numerous nuclei. The nuclei can be divided according to their connections. While some nuclei (so called specific nuclei) send their efferent nerve fibers quite distinct to each respective corresponding area of the cerebral cortex and gather information from sensory and sensitive organs for the most part, others have rather fewer direct efferent nerve fibers to the cortex but connect mainly to the first mentioned sort of thalamic nuclei (Trepel 2008). The second kind of nuclei, also called unspecific nuclei, receive their afferent input from the cerebellum, the basal ganglia, the cortex and above all from the reticular formation. Their efferent fibers to specific nuclei serve to modulate the activity and therefore output of the specific nuclei (Trepel 2008).

A certain nucleus of the ventral thalamus needs to be emphasized: the thalamic reticular nucleus (TRN) that wraps around the remaining thalamus (Fig. 5A). As indicated by the name, it mostly consists of reticular cell that converse with diverse specific nuclei in an inhibitory manner using GABAergic connections (Lubke 1993). It is reached by collaterals of thalamocortical and corticothalamic fibers and regulates activity in the dorsal thalamus accordingly (Fig. 5B) (Jones 1975, Shosaku et al. 1989). The TRN is regulated itself by the reticular formation (located in the brainstem) via inhibitory afferent fibers, which enables activity of thalamocortical fibers by disinhibition, thus allowing efferent nerve fibers of specific thalamic nuclei to excite the cerebral

cortex (Fig. 5B) (McCormick et al. 1997, Trepel 2008). While the excitatory cortex afferent fibers quite distinguished and selective determine which specific nuclei will be increasingly inhibited by the TRN, the inhibitory reticular formation afferents inhibit the TRN in an undirected manner (Trepel 2008).

The cerebral cortex plays a vital role in perception, awareness, attention, thought, intended actions and language (Trepel 2008). The most part of the cortex is amounted by neocortex, the evolutionary youngest developed component of the brain (Trepel 2008). Due to distribution of different cellular types, it can be sectioned into six layers, also called laminas, which each play their role in neuronal processing (Mountcastle 1997). Layer 4 is known to be the main entrance of external information to the cerebral cortex, basically where specific thalamic nuclei efferent nerve fibers end (Trepel 2008). Neurons of the 5th layer project to diverse subcortical destinations except the thalamus (Molnár et al. 2006). Lamina 6 is made up out of many different cells, whereupon the pyramidal cells of this layer sent their axons to the specific nuclei of the thalamus (Trepel 2008). Layer 1 to 3 serve to modulate lower layers and to interconnect cortex areal units (Trepel 2008).
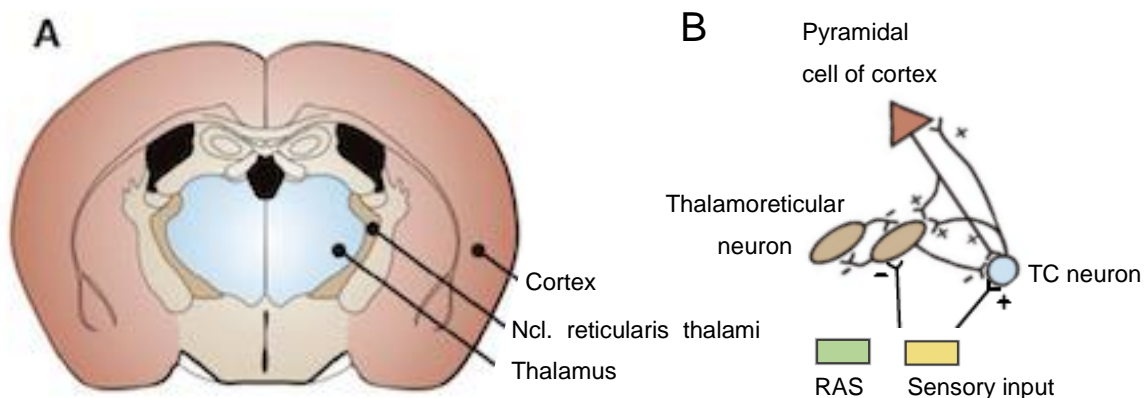
Fig. 5: Schematic sketch of the thalamocortical connections

A: Sketch of a coronary brain slice depicting the location of the thalamus and the cortex. Changed according to (Wahl-Schott et al. 2009). **B:** Simplified display of the thalamocortical connections. Hereby the + indicates glutamatergic fibers while the – represents GABAergic contacts. Ascending sensory information activates neurons of the specific thalamic nuclei, which then in turn excite the cortex. Thalamoreticular neurons modulate relay neuron function via GABAergic synapses. When the reticular formation as a part of the reticular activation system (RAS) is active, it inhibits inhibitory thalamoreticular neurons, disinhibiting negative influence of thalamoreticular neurons on thalamocortical (TC) neurons. Changed according to (Wahl-Schott et al. 2009, Mattusch 2012)

1.9.2 Function

In means of perception of one's environment the thalamus plays the role of an operator. All sensory information gathered, apart from olfaction, passes through the diverse thalamic nuclei, where it is sorted and selected before being rendered to the respective cortex areas (McCormick et al. 1997, Huguenard et al. 2007). This switchover serves as a filter, deciding which part of the incoming information flow is relevant for the individual at the moment and therefore passed on to the cortex, i.e. to consciousness, and vice versa. Thus did the thalamus acquire its reputation as the gate to consciousness. In addition to its gate-like function, the thalamus also portrays a regulatory function in alternating states of consciousness, such as sleeping and being awake (McCormick et al. 1997, Steriade 2005). Therefore benzodiazepine effect on the thalamocortical connections may be responsible for side effects such as drowsiness and sedation.

1.10 Aim of this dissertation

Benzodiazepines such as diazepam and the TSPO ligand XBD elicit similar anxiolytic properties but feature diverging side effect profiles. While diazepam exposes amnestic and sedative adverse effects as well as tolerance development and risk of addiction, XBD lacks any of those, as far as experiments on humans and rodents have surveyed (Kita et al. 2009, Rupprecht et al. 2009, Rote-Liste 2013, 2015). XBD enhances the synthesis of neurosteroids such as allopregnanolone, which are presum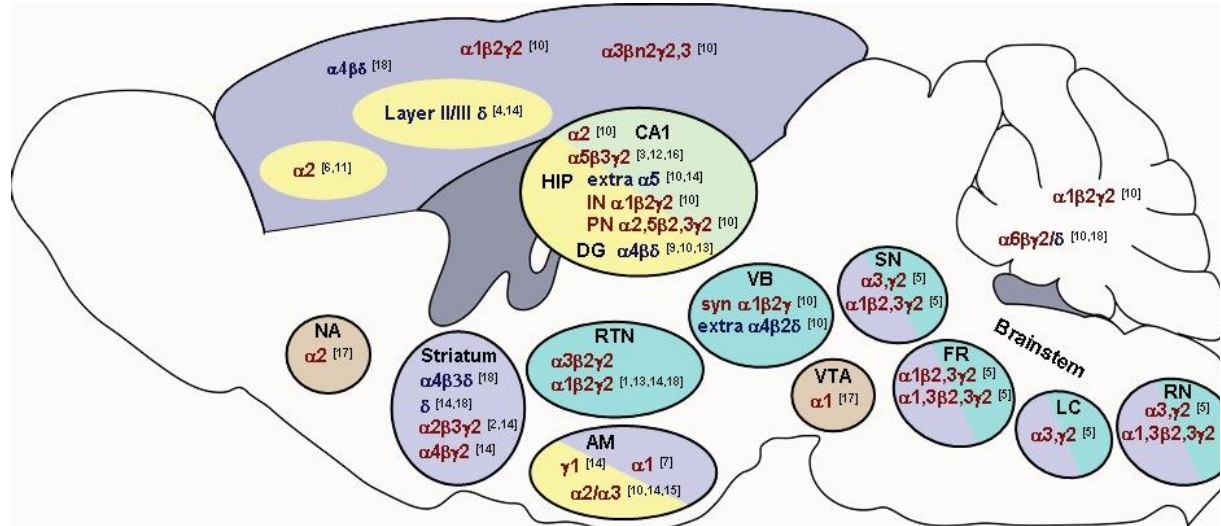ably responsible for XBD effects (Rupprecht et al. 2009). Both, allopregnanolone and diazepam are positive allosteric modulators (PAMs) at the $GABA_A$ receptor, though they occupy different binding sides (Hosie et al. 2006, Möhler 2006). Due to diverse $GABA_A$ receptor subunit expression, $GABA_A$ receptor binding abilities vary throughout different brain areas, probably accounting for the discrepancy of diazepam and XBD side effect profile (see Fig. 6) (Pirker et al. 2000).

The intention of this study is to investigate the diverging side effect profile of diazepam and XBD on a neurophysiological level using the imaging technique voltage sensitive dye imaging (VSDI). Therefore, a series of experiments was designed and conducted to determine neuronal activity propagation under influence of diazepam, as a representative of benzodiazepines, and XBD. According to the different side effect profile of these drugs, VSDI experiments in areas of the brain crucial for neuronal processing related to anxiety, memory and sedation, namely the amygdala, hippocampus and thalamocortical region respectively were performed. To verify diazepam and XBD effects, measurements with their antagonists (flumazenil and finasteride) were performed as well.

Additionally, experiments with allopregnanolone, a neurosteroid acting as a PAM at GABA$_A$ receptors and producing anxiolysis, were performed (Bitran et al. 1995). It has been shown that allopregnanolone synthesis increases upon XBD-induced TSPO activation (Rupprecht et al. 2009).

Fig. 6: Scheme of brain regions associated with benzodiazepine effects and adverse effects

According to (Nothdurfter et al. 2012b). Depicted are the brain areas relevant for the adverse effect profile of benzodiazepines and neurosteroids based on the specific GABA$_A$ receptor selectivity (red: receptive for benzodiazepines, blue: receptive for neurosteroids) and GABA$_A$ receptor subunit distribution in the brain. The color coded chart indicates in which brain region the side effects origin:

Anxiety: cerebral cortex, amygdala (AM), bed nucleus of stria terminalis (BNST), hippocampus (HIP) (Charney et al. 1996, Brandao et al. 2003)
Sedation: AM, formatio reticularis (FR) (Bonin et al. 2008)
Hypnosis: ventrobasal nucleus (VB), reticular nucleus (RTN), locus coeruleus (LC), Raphe nuclei (RN), substantia nigra (SN), ventrolateral preoptic nucleus (VLPO), tuberomamillary nucleus (TMN) (Bonin et al. 2008)
Abuse: N. accumbens (NA), ventral tegmental area (VTA) (Di Chiara et al. 1993)
Physical dependence: NA, VTA (Lingford-Hughes et al. 2003, Wafford 2005)
Tolerance: HIP, NA, AM (Wafford 2005)
Withdrawal: HIP, NA, AM (Wafford 2005)
Amnesia: cerebral cortex, HIP (Savic et al. 2005, Bonin et al. 2008)

Abbreviations: extra - extrasynaptical receptor, syn - synaptical receptor, IN – interneuron, PN - pyramidal neuron

The diagram is not suitable to compare the absolute levels of various subunits in either brain region. The distribution of GABA receptor mRNA is not indicated. (Nothdurfter et al. 2012b)

2 Material and Methods

To observe the effects of XBD, diazepam and allopregnanolone in the desired brain regions, brain slices of mice were prepared. The respective slices were subsequently stained with a voltage sensitive fluorescent dye, that allowed monitoring changes in signal transduction of neuronal networks due to substance application (enhancement of GABAergic inhibition) using VSDI.

A detailed description of all applied solutions is listed in the appendix.

2.1 Preparation of brain slices

All experiments were performed on 3 to 8 week old male C57Bl6 mice. The rodents were decapitated under sedation with the inhalation anesthetic Isofluran. All steps of the preparation henceforth were realized in ice-cold sucrose-based-ACSF (artificial cerebrospinal fluid) saturated with carbogen (95% oxygen / 5% carbon dioxide). Access to the brain was achieved after stripping the skull by two transversal cuts from caudal and a shallow guided cut along the crown's midline. The divided cranium halves were bent upwards with forceps, the Nervi optici torn and the brain carefully removed from the cranial cavity using a rounded spatula. Depending on the desired slice the brain was prepared further differently. The obtained plain surface was then glued to a metal block with a cyanoacrylate-glue (Histoacryl, B. Braun, Melsungen, Germany).

Coronary brain slices

In order to get a preparation of the amygdala, the cerebellum was cut off with a razor blade in a coronary manner.

Sagittal brain slices

For preparation of sagittal brain slices to reach the hippocampus the brain was divided into its hemispheres via a sagittal cut after removing the cerebellum.

Thalamocortical brain slices

The thalamocortical connections were obtained using a method developed by Agmon and Connors (Agmon et al. 1991). This preparation allows preservation of the ventrobasal nucleus of the thalamus, the barrel cortex (somatosensory cortex of rodents) and the fiber tract connecting them. To achieve intact connections the forebrain needs to be cut off at specific angles (55° sagittal, 10° horizontal).

Subsequently, regardless of the desired preparation, the metal block with the glued on brain was integrated into a vibratome (HM 650 V, Microm International, Walldorf, Germany). The brain has been cut into slices of 350 µm (amygdala, hippocampus) and of 550 µm thickness (thalamocortical slices; Fig. 9, p. 21). With the wide end of a Pasteur pipette the slices were transferred into a transportable chamber filled with general ACSF continuously saturated with carbogen. For the following 30 minutes they were incubated in a 34°C hot water bath, before being dyed, to allow the slices to rest and recover from the stress of slice preparation.

2.2 Voltage sensitive dye imaging

VSDI is an imaging technique that allows monitoring of neuronal electric networks in high temporal and spatial resolution imaging down to 20-50 µm and a millisecond (Chemla et al. 2010). Offering the great advantage of visualizing the processing of neuronal activity in real time, VSDI provides a new perspective in the field of neuroscience. Key element to this technique is the voltage sensitive dye (VSD), which infiltrates organic tissue and binds to the external surface of cell membranes without interrupting their usual function (Loew 2015). In addition to fluorescent properties, voltage-sensitive-dyes feature the ability to change its emission spectrum according to the surrounding charge. Therefore changes in membrane voltage are displayed as changes in fluorescence. This feature allows the fast monitoring of neuronal signal transduction. Validation and accuracy of VSDI has been confirmed by close correlation of the optical signal of VSDI recordings and changes in membrane potential measured via conventional electrophysiological means (Davila et al. 1973, Salzberg et al. 1973, Morad et al. 1979, Windisch et al. 1985, Chien et al. 1991). Since VSDI was first introduced in 1968 (Tasaki et al. 1968) it has further improved thanks to the development of new and superior dyes and progress in technology.

2.2.1 Staining of brain slices with Di-4-ANEPPS

In this dissertation brain slices were stained with Di-4-ANEPPS ((4-(2-(6-(Dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)pyridinium hydroxide inner salt; Sigma-Aldrich Chemie GmbH; Schnelldorf; Germany), a well-established and commonly used fast acting voltage sensitive dye (Fluhler et al. 1985, Thiele 2008, Stepan et al. 2012), which allows the monitoring of voltage changes within a millisecond range. When excited with light of wavelengths between 350 to 550 nm it emits light with longer wavelengths (about 500 to 800 nm) (stokes shift). If the surrounding tissue is electrically excited the fluorescence shifts to lower wavelength due to a shift of the absorption, excitation and emission spectrum caused by the dyes electrochromism characteristic (Loew et al. 1992). Therefore differences of tissue charge can be measured by assessing the intensity of emission equal and higher than a certain wavelength as demonstrated in the figure below (Fig. 7). In addition due to the change of excitation spectrum when the surrounding tissue is

depolarized, the dye will be less excited (presupposed the same spectrum of light enlightens the dye) and consequently a further reduction of emission of fluorescence occurs (see Fig. 7).
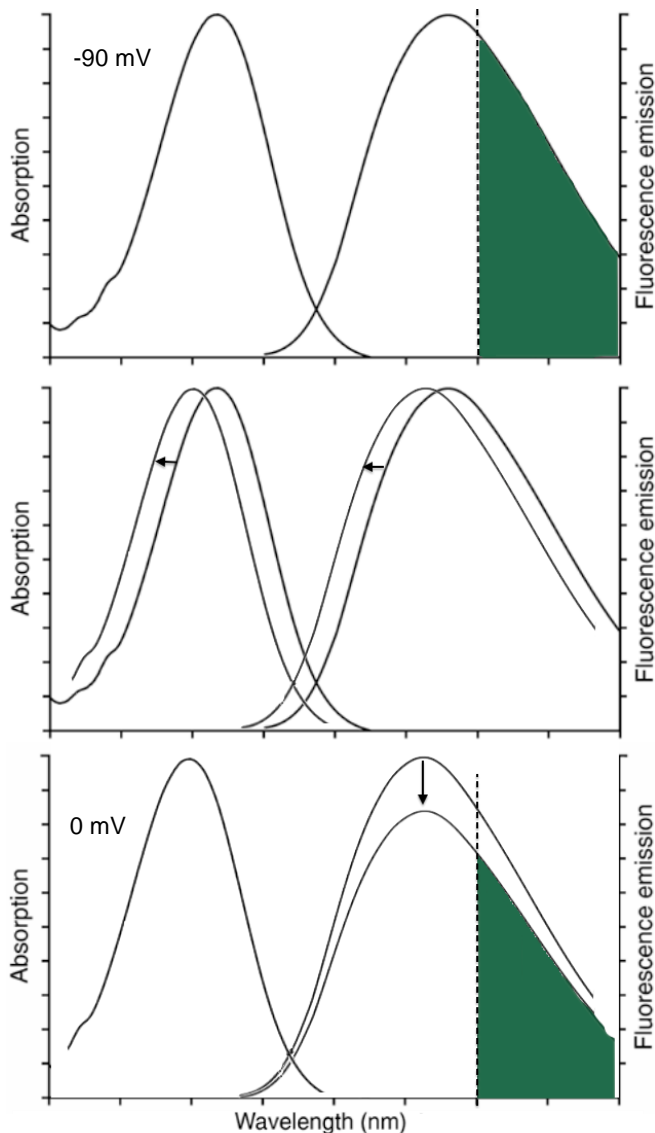
Fig. 7: Schematic sketch of the principle of voltage sensitive shift of emission

Modified according to (Loew et al. 1992). Depending on the voltage of the surrounding tissue the emission spectrum varies due to the electrochromism characteristic of voltage sensitive dyes. As depolarization advances the excitation and emission spectrum of the dye changes (depicted by the arrows in the middle diagram). In addition due to the change of excitation spectrum when the surrounding tissue is depolarized, the dye will be less excited (presupposed the same spectrum of light enlightens the dye) and consequently a further reduction of emission of fluorescence occurs (depicted by the arrow in the last diagram). Therefore changes in membrane voltage of the surrounding tissue can be calculated by considering the intensity of the fluorescence greater or equal of a certain wavelength (represented as the green area).

Depending on the investigated region of the brain and therefore variable necessity of tissue magnification, the setup was build up differently, with or without a Barlow lens. Since the Barlow lens also affected exposure to light, which in turn affected excitation and emission of the dye, two different dyeing methods were applied.

Staining procedure for slices of the amygdala and thalamocortical connections

Di-4-ANEPPS, aliquoted in DMSO (10 mg/ml) was stored at -20°C. Before staining the dye was incubated at 50° for 5 minutes and thoroughly vortexed. 7.5 µl were properly mixed in with 10 ml

ACSF (final concentration of about 0.0075 mg/ml) and filled into a petri dish continually aerated with carbogen. Here the slices were stained for 15 minutes in a shaded room.

Staining procedure for hippocampal slices

Initially 5 mg of Di-4-ANEPPS were aliquoted in 1 ml DMSO and 0.5 ml 10-%-El-Cremophor-solution and kept at -20°C. For staining 40 µl of the aliquot were blended in with 0.5 ml ACSF and 0.5 ml fetal bovine serum. The slices were dyed in the resulting staining solution under continuous aeration with carbogen for about 20 minutes in a darkened room.

After either staining procedure excessive dye was rinsed off with fresh ACSF. Following the slices were transferred to a shaded, ACSF-filled, continuously carbogen-aerated holding chamber where they were allowed to rest for at least 30 minutes before starting measurements. From this point on and forward Bicuculline (GABA$_A$-R-antagonist) was always added to the ACSF to reach a concentration of 0.6 µMolar. This step was necessary to assure an adequate signal for two reasons (Stepan et al. 2012). First, Di-4-ANEPPS potentiates GABA function at the GABA$_A$-receptor (Mennerick et al. 2010) and second due to their in comparison longer range, glutamatergic axons are cut to a greater extend than the usually relatively shorter ranged axons of GABAergic interneurons during brain slice preparation (Stepan et al. 2012).

2.2.2 VSDI Setup

The VSDI-Setup including recording chamber, microscope and electrodes was safely positioned on a vibration-cushioned table (via pressurized air). To avoid electric noise it was encased by a Faraday cage and earthed. The employed epifluorescence microscope (BX51 WI, Olympus, Hamburg, Germany) was equipped with a MiCAM02 HR-CCD camera and processor (BrainVision Inc., Tokyo, Japan) and an XLFluor4X/340 objective lens (NA 0.28; Olympus, Hamburg, Germany). A motorized micromanipulator (Luigs and Neumann, Ratingen, Germany) was enabled to manage movement of the microscope, making it possible to arrange it in all three dimensions without affecting the recording chamber.

Source of light was a halogen bulb (MHAB-150 W; Moritex corp., China). A software-triggered shutter controlled light exposure. A band-pass excitation filter ensured that only light of 480-550 nm wavelengths illuminated the slice. A dichroitic mirror (570 nm) reflected the light, projecting it onto the slice. The fluorescence however, being of longer wavelength, passes through the dichroitic mirror and through a following long-pass emission filter (590 nm). Thus ensuring that only the dye-emitted fluorescence is detected by the CCD sensor chip and that the resulting signal is unaltered by excitation radiation and potential other fluorescence (Fig. 8A). The recorded signal is then processed as normalized differences of fluorescence intensity [ΔF/F] in each pixel over time and presented as a color-coded sequence-film on the monitor (Fig. 8B).

A

B

dF/F$_{max}$
(%)
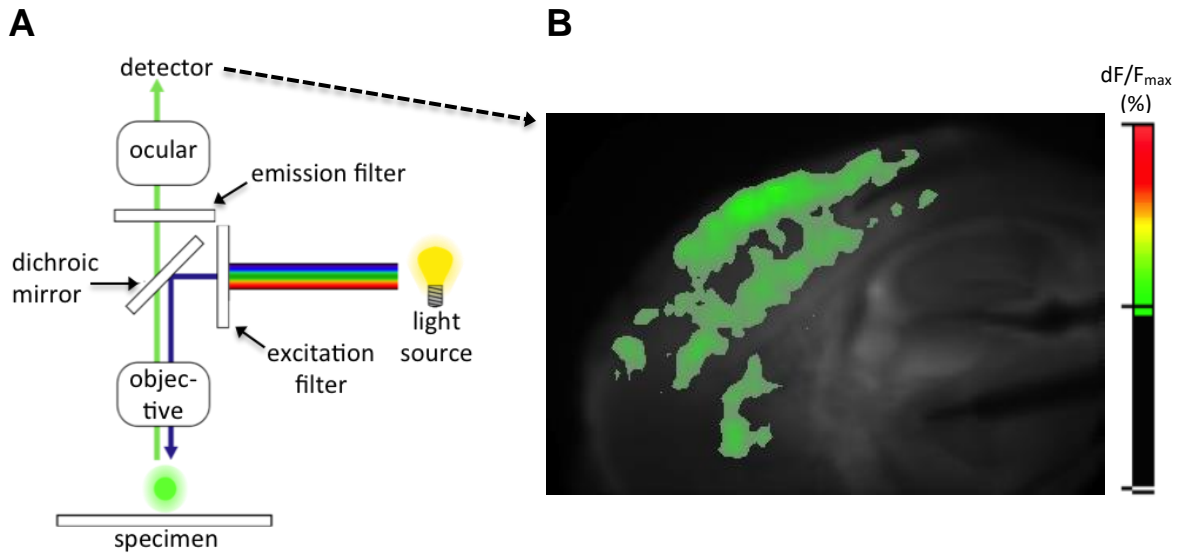
Fig. 8: Schematic sketch of the VSDI-setup and presentation of the processed signal

A: schematic sketch of the VSDI-setup, slightly modified according to (Mühlpfordt et al. 2008). Bulb light was filtered by a band-pass excitation filter and projected onto the slice by a dichroitic mirror. Fluorescence emerging from the stained slice passed through the dichroitic mirror and through a following long pass emission filter to the CCD-sensor (detector). **B**: Color-coded presentation in BrainVision of the processed CCD-camera-recorded signal projected onto a live-picture of the employed brain slice.

2.2.3   Recordings

The slices were carefully positioned in the recording chamber and fixed by a grid (thin nylon threads tensely attached to a platinum frame). Continuously carbonated ACSF perfused the recording chamber throughout the entire experiment at a flow rate of about 5-8 ml/min. Experiments were conducted at room temperature to increase tolerance of hypoxia. To monitor changes in signal transduction of neuronal networks by GABAergic inhibition under substance influence, the slices were artificially electrically excited. For excitation of the to examine brain regions a bipolar tungsten electrode was carefully placed visually. For analysis only the desired regions were considered. Therefore a ROI (region of interest) was placed to include the area under investigation.

Basolateral amygdala:

The electrode was cautiously positioned inside the amygdala right next to the branching point of the external capsule that encloses the amygdala (Fig. 9A). The ROI was put to include the basolateral amygdala.

CA1 region of the hippocampus:

In this preparation the Schaffer collateral was stimulated and the signal obtained from the CA1 region of the hippocampus innervated by it (Fig. 9B).

Thalamocortical preparation:

In this slice preparation two electrodes were positioned (Fig. 9C), one in the ventrobasal thalamus and another one where layer 4 of the cortex was suspected to be and a total of three areas were investigated, including the thalamus, the thalamocortical connections and the cortex itself to not only examine whether the substances have an effect but also to distinguish at which point the thalamocortical connectivity is being manipulated. Therefore the ROI was positioned to include the cortex in both stimulation techniques and the thalamus as a second area of interest when stimulated inside the thalamus.

Fig. 9: Display of the slice preparations and electrode and ROI placement

White arrows indicate electrode placement. **A:** Amygdala preparation, the electrode was placed next to the branching point of the external capsule to stimulate the basolateral and lateral amygdala (yellow frame). **B:** Hippocampus preparation, the electrode was placed to excite the Schaffer collateral, which stimulate the CA1 region of the hippocampus (green). **C:** Thalamocortical preparation, two electrodes were placed, one in the thalamus to stimulate thalamocortical connections and one in the cortex. Region of interests were the cortex (blue) stimulated both ways and the thalamus (red) stimulated by the electrode placed in the thalamus.

The intensity and duration of pulse stimulation was adjusted via isolated stimulators (type 2533, Devices Instruments, Welwyn Garden City, England). Single square pulses were applied to determine the necessary stimulus intensity (0.1-100 V) and length (0.05-0.2 ms). They were set for each experiment individually in order to generate a similar intense signal throughout all experiments.

Recording settings were adjusted by BrainVision software (BrainVision 13.04.20, BrainVision Inc., Tokyo, Japan). 512 frames were recorded with a sample rate of 2.2 ms per frame, allowing a spatial resolution of 88*60 pixel with a pixel size of 8.4*9.8 µm in the hippocampus preparations and 36.4*40.0 µm in the amygdala and thalamocortical slices. The resulting 512 images were put together as a sequence to a film of 1.126 seconds. All electric stimuli were applied in the 103rd Frame, except for the pulses set in the thalamus, which were released in the 48th Frame.

Recordings were set to record 16 sequences of 512 frames every 6.5 minutes. To improve signal to noise ratio 16 recorded sequences were averaged, resulting in one averaged sequence every

6.5 minutes. This recording protocol was altered for thalamocortical slice measurements, since there were two electrodes placed in this preparation. Alternating they stimulated the slice, producing an averaged sequence made of 16 recordings each in a time frame of 11.5 minutes. The protocol was controlled by WinLTP 1.11b software (Anderson and Collingridge, University Bristol, UK), which triggered the stimulators and the shutter (controlling light exposure) according to the set protocol. To spatially and temporally smooth ∆F/F values a 3x3x3 average filter was applied for all quantifications in addition to an interpolation function to reduce pixilation of images.

As soon as all settings were adjusted measurements started. Once there has been a steady signal in the desired region for at least 19 minutes (3 control movies), the investigated substance was applied (XBD: 3 µM, diazepam: 1 µM, allopregnanolone: 1 µM) and recordings continued for about an hour. Therefore, in addition to the 3 control recordings, 8 more recordings of substance influence were achieved in amygdala and hippocampal slices or in case of thalamocortical preparation 5 recordings of substance influence. In experiments with diazepam flumazenil (10 µM) was added afterwards to antagonize diazepam. In addition experiments with finasteride (10 µM) and XBD were performed, to inhibit XBD effects. Different from flumazenil, finasteride needed to be applied at the same time as XBD. XBD's mode of action is via enhancement of neurosteroid genesis, which then in turn carry out the actual impact. As a 5α-reductase-inhibitor, finasteride prevents neurosteroids from being synthesized, but cannot antagonize effects of already synthesized neurosteroids. To avoid data interference by possible effects of finasteride itself it was already added to the ACSF during control recordings. Finally, to avoid interference by potential (though unlikely, considering the small amounts) cell damage caused by the Alcohol (70%) used for XBD aliquotation, the same amount of Alcohol was also added to the ACSF during control recordings in all experiments containing XBD medication.

2.3   Data analysis

The measured data was presented in BrainVision as a color-coded signal in a sequence-film of 1.126 seconds. This signal can be broken down into three main components: Intensity, spread and duration of the excitation. Therefore BrainVision-data was processed with a custom programmed macro in Matlab (Matlab R2008b, The MathWorks, Natick, USA). Hereby the BrainVision acquired fluorescence inside the ROI representing the fast depolarization signal (FDS) was broken down into descriptive and comparable numerical values as described below:

The '*area*' is a numeric value counting all active pixel. An active pixel is defined as a pixel inside the ROI where the change of fluorescence (∆F/F) was at least three times higher than the standard deviation of random background noise at anytime within the 512 frames. It is a parameter to determine the spread of the excitation upon stimulation.

The '*FDS$_{maxInt}$*' [ΔF/F] is a value describing the mean of the maximal change of fluorescence throughout all frames of every active pixel within a ROI.

The value '*FDS$_{areaInt}$*' [ΔF/F] represents the sum of the intensities of every active pixel for each of the 512 frames within a defined ROI. It will be displayed as a graph of *FDS$_{areaInt}$* values over time. Since neuronal activity propagation upon stimulus only lasts for about 50-300 ms, depending on the brain area, and since the dye bleaches over time, only the first 228 frames (500 ms) will be presented in this dissertation.

The '*amplitude of FDS$_{areaInt}$*' is a parameter that amounts to the maximal *FDS$_{areaInt}$* value under substance influence normalized to the maximal *FDS$_{areaInt}$* value of the respective control recording, basically representing the maximal amplitude of the FDSareaInt graph (peak of neuronal activity) over the 550 frames. This parameter depicts the effect the substances have on propagation and intensity of neuronal activity upon stimulation.

The product '*FDS$_{AUC}$*' [ΔF/F] is a value designed to give an impression of a summarized overall effect. It includes the compounds propagation (*a*rea), Intensity (*Int*) and duration of excitation. It is calculated by adding the values of *'FDS$_{areaInt}$'* [ΔF/F] of all frames from pulse release till excitation fade-out, basically resembling the area under the curve (AUC) of the graph depicting the *FDS$_{areaInt}$*.

To allow comparability the data was normalized to its respective control (all values were divided by the mean value of the last 19.8 ms before electric stimulation of the respective three controls). The results are present by comparing the last recording of the substance to the last control recording. They will be featured as mean ± SEM (standard error of the mean). Diagrams were created in Excel (Excel 2003, Microsoft Corporation, Redmond, USA).

Statistic significance was investigated with either the Wilcoxon signed rank test or the Man-Whitney-U test depending on pairing. Level of significance was defined as α = 0.05 and is indicated by asterisks as followed: * for $p < 0.05$, ** for $p < 0.02$ and *** for $p < 0.01$.

3   Results

Several rows of experiments in the amygdala, the hippocampus and the thalamocortical connections were performed as described above. In the figure below an exemplary excitation transmission of neuronal activity in the cortex upon thalamic stimulation is depicted.
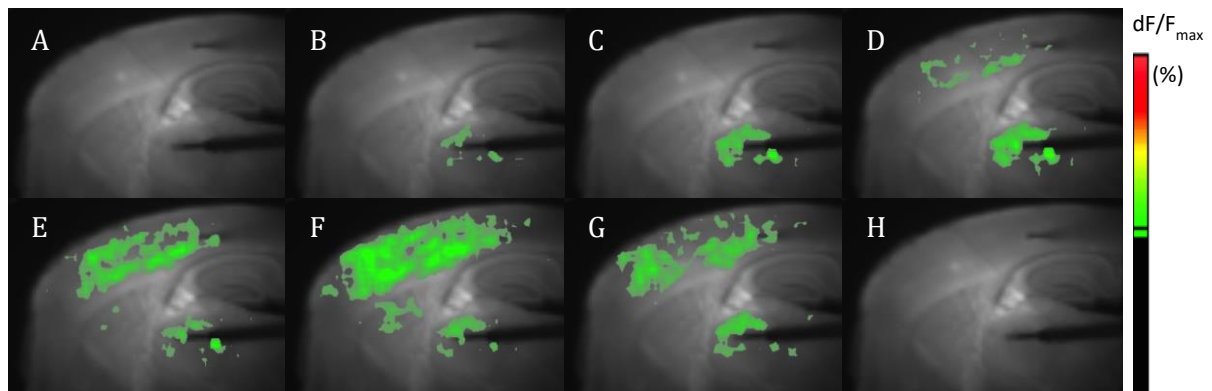
Fig. 10 Excitation transmission in the thalamocortical circuit

Depicted is the thalamocortical cut, the first picture is before stimulation (A), then stimulus is released by the electrode in the thalamus (B). The stimulus spreads throughout the thalamus (C) before exciting the thalamocortical connections and hitting the cortex (D) where it spreads (E-F) before fading away again (G-H).

3.1   Alteration of neuronal activity upon stimulus in the Amygdala by diazepam, XBD and allopregnanolone

Since the amygdala is the crucial area for those information processing related to fear and anxiety and hence, initiates referring actions (see ch. 1.7) a set of experiments was performed in this brain region.

3.1.1   Effect of diazepam on neuronal activity upon stimulus in the amygdala

Diazepam application to amygdala brain slices resulted in a decrease of the stimulated excitation transmission in the basolateral amygdala. As depicted in the graph below (Fig. 11A), $FDS_{areaInt}$ showed a decrease throughout stimulated excitation transmission by diazepam (*amplitude of $FDS_{areaInt}$* 81.0 ± 8.2 %, n=6). FDS_{AUC} showed a distinct though not significant decrease to 85.0 ± 12.8 % (n=6) in average. Hereby especially the FDS_{maxInt} of neuronal activity upon stimulus was reduced (82.2 ± 0.05 %, n=6, p<0.05) (Fig. 11B), while the *area* remained quite stable (98.2 ± 6.6 %, n=6) (Fig. 11C). The subsequent application of the benzodiazepine antagonist flumazenil

resulted in an increase of the diminished neuronal activity (FDS_{AUC} 100.6 ± 12.8 %, n=6), successfully though not significantly antagonizing diazepam effect.

Fig. 11: Diazepam impact on neuronal activity in the amygdala

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and diazepam (red) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of control and diazepam (diaz, 0.81 ± 0.08, n=6) and 10 µg flumazenil (fluma, 0.92 ± 0.08, n=6) recordings in the amygdala are depicted, representing the normalized amplitude of their peak values. **B**: Effect of 1 µM diazepam (red) on FDS_{maxInt} (ΔF/F) of the VSDI-signal in the amygdala. Control recordings are illustrated as black squares. The grey arrow indicates the average effect. Distinct and significant decrease of FDS_{maxInt} by diazepam 0.82 ± 0.05 (n=6, p<0.5). **C**: No significant alteration of *area* in the amygdala under 1 µM diazepam 0.98 ± 0.07 (n=6).

3.1.2   Effect of XBD on neuronal activity upon stimulus in the amygdala

XBD application to amygdala brain slices resulted in a decrease of the stimulated excitation transmission in the basolateral amygdala. As depicted in the graph below (Fig. 12A), $FDS_{areaInt}$ showed a decrease throughout stimulated excitation transmission by XBD (*amplitude of $FDS_{areaInt}$*

83.3 ± 4.8 %, n=6, p<0.05). FDS_{AUC} showed a distinct though not significant decrease to 86.3 ± 8.5 % (n=6) in average. Hereby especially the FDS_{maxInt} of neuronal activity upon stimulation was reduced (87.4 ± 2.3 %, n=6, p<0.05) (Fig. 12B), while the *area* remained quite stable (95.6 ± 2.9%, n=6) (Fig. 12C). XBD effect could not be significantly antagonized by joint application with finasteride (*amplitude of $FDS_{areaInt}$* 87.9 ± 7.0, n=6).

A

B

C
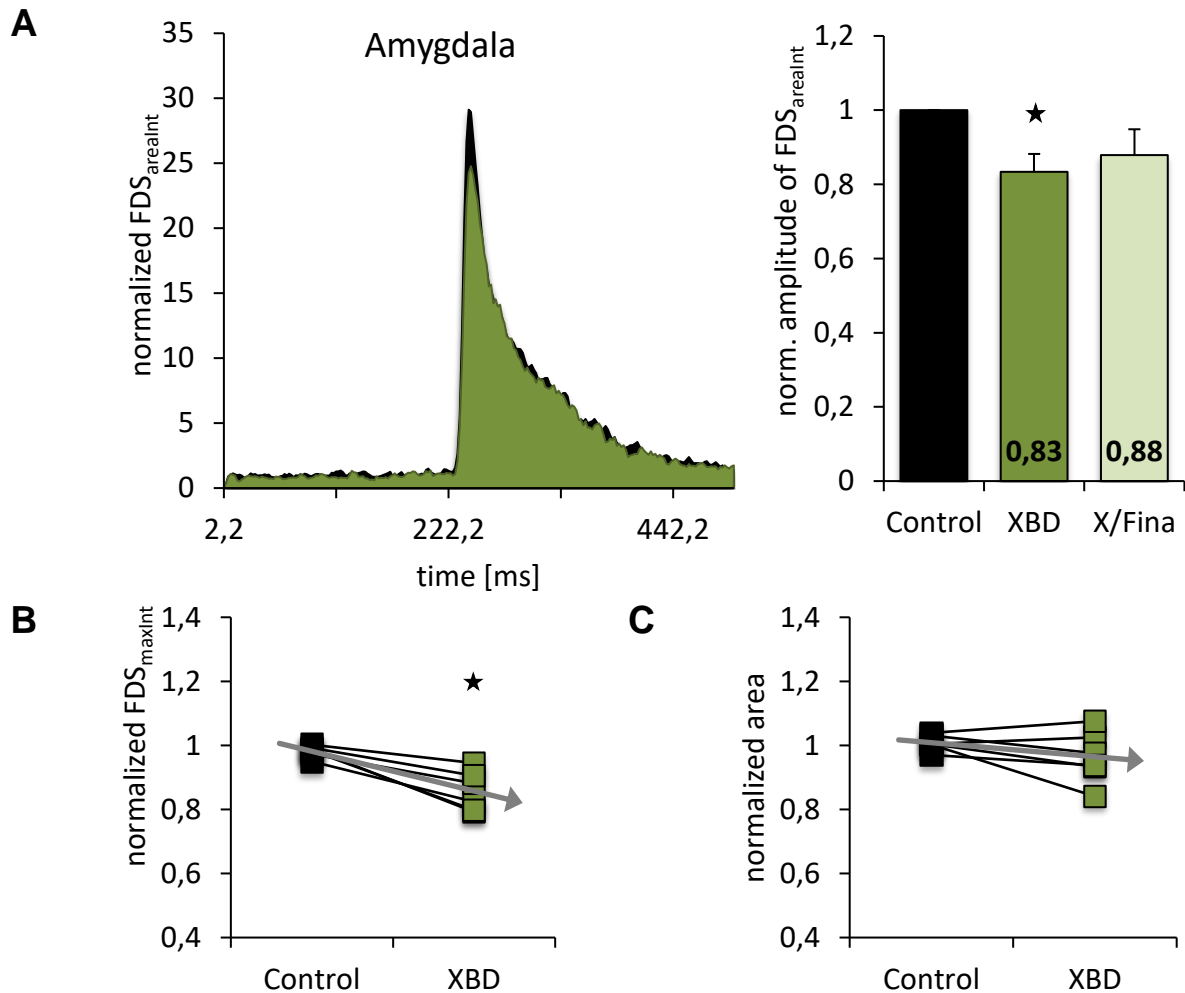
Fig. 12: XBD impact on neuronal activity in the amygdala

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and XBD (green) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of, XBD (0.83 ± 0.05, n=6, p<0.05) and XBD plus finasteride (x/fina, 0.88 ± 0.07, n=6) recordings in the amygdala are depicted, representing the normalized amplitude of peak values. The black bar represents both control recordings. **B**: Effect of 3 µM XBD on FDS_{maxInt} ($\Delta F/F$) of the VSDI-signal in the amygdala. Control recordings are illustrated as black squares. The grey arrow indicates the average effect. Distinct and significant decrease of FDS_{maxInt} by XBD 0.87 ± 0.03 (n=6, p<0.05). **C**: No significant alteration of *area* in the amygdala under 3 µM XBD influence 0.96 ± 0.03 (n=6).

3.1.3 Effect of allopregnanolone on neuronal activity upon stimulus in the amygdala

Allopregnanolone application to amygdala brain slices resulted in a decrease of the stimulated excitation transmission in the basolateral amygdala (Fig. 13A). As depicted in the graph below (Fig. 13A), $FDS_{areaInt}$ showed a decrease throughout stimulated excitation transmission by allopregnanolone (*amplitude of $FDS_{areaInt}$* 88.9 ± 3.8 %, n=6, p<0.05). FDS_{AUC} showed a distinct though not significant decrease to 80.5 ± 6.0 % (n=6) in average. Hereby especially the FDS_{maxInt} of neuronal activity upon stimulus was reduced (87.6 ± 4.3 %, n=6, p<0.05) (Fig. 13B), while the *area* remained quite stable (99.1 ± 5.1 %, n=6) (Fig. 13C).

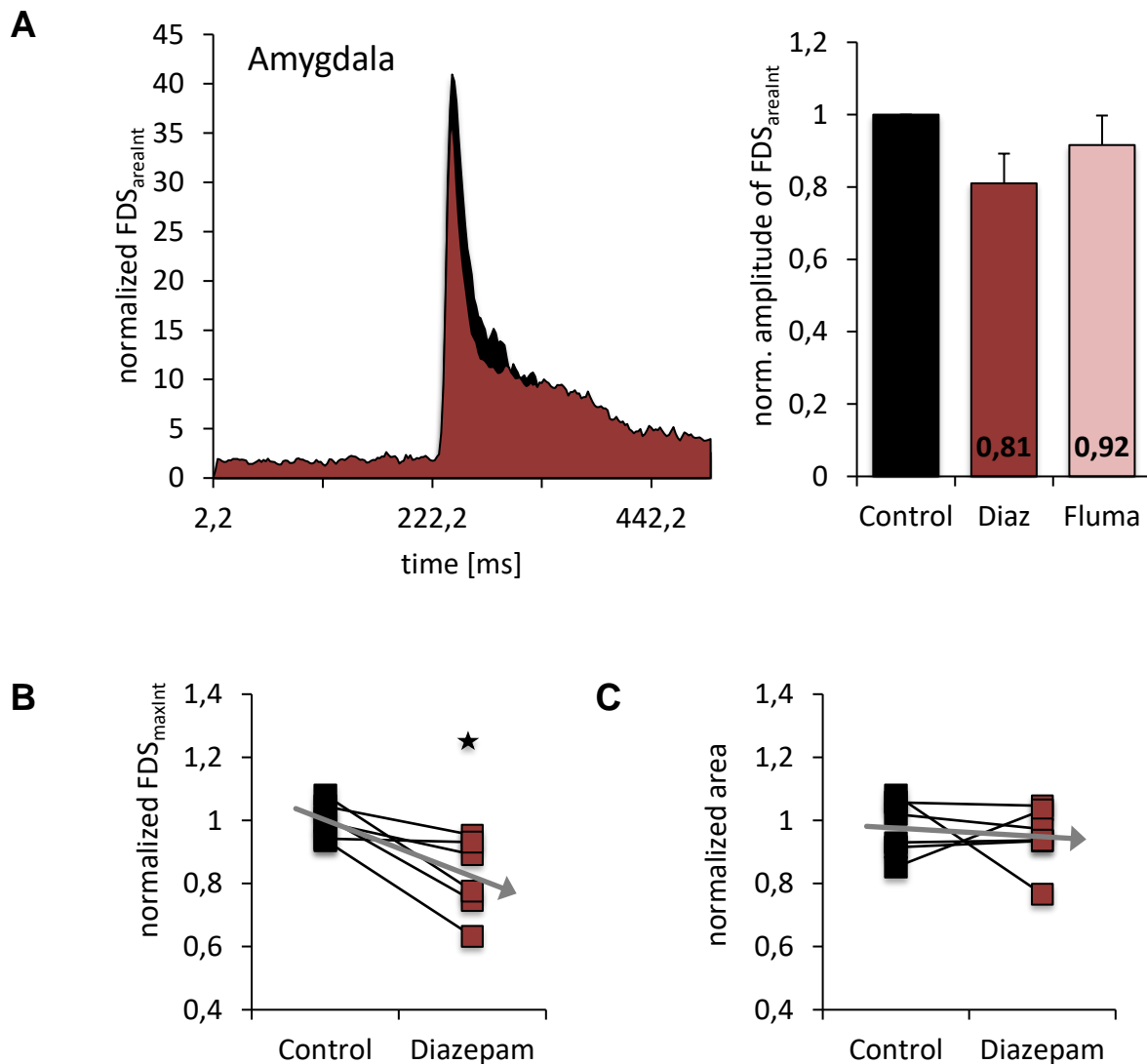**Fig. 13: allopregnanolone impact on neuronal activity in the amygdala**

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and allopregnanolone (yellow) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of control and allopregnanolone (allo, 0.89 ± 0.04, n=6, p<0.05) recordings in the amygdala are depicted, representing the normalized amplitude of peak values. **B**: Effect of 1 µM allopregnanolone on FDS_{maxInt} (ΔF/F) of the VSDI-signal in the amygdala. Control recordings are illustrated as black squares. The grey arrow indicates the average effect. Distinct and significant decrease of FDS_{maxInt} by allopregnanolone 0.88 ± 0.04 (n=6, p<0.05). **C**: No significant alteration of *area* in the amygdala under 1 µM allopregnanolone 0.99 ± 0.05 (n=6).

3.1.4 Comparison of diazepam, XBD and allopregnanolone effect on neuronal activity in the amygdala
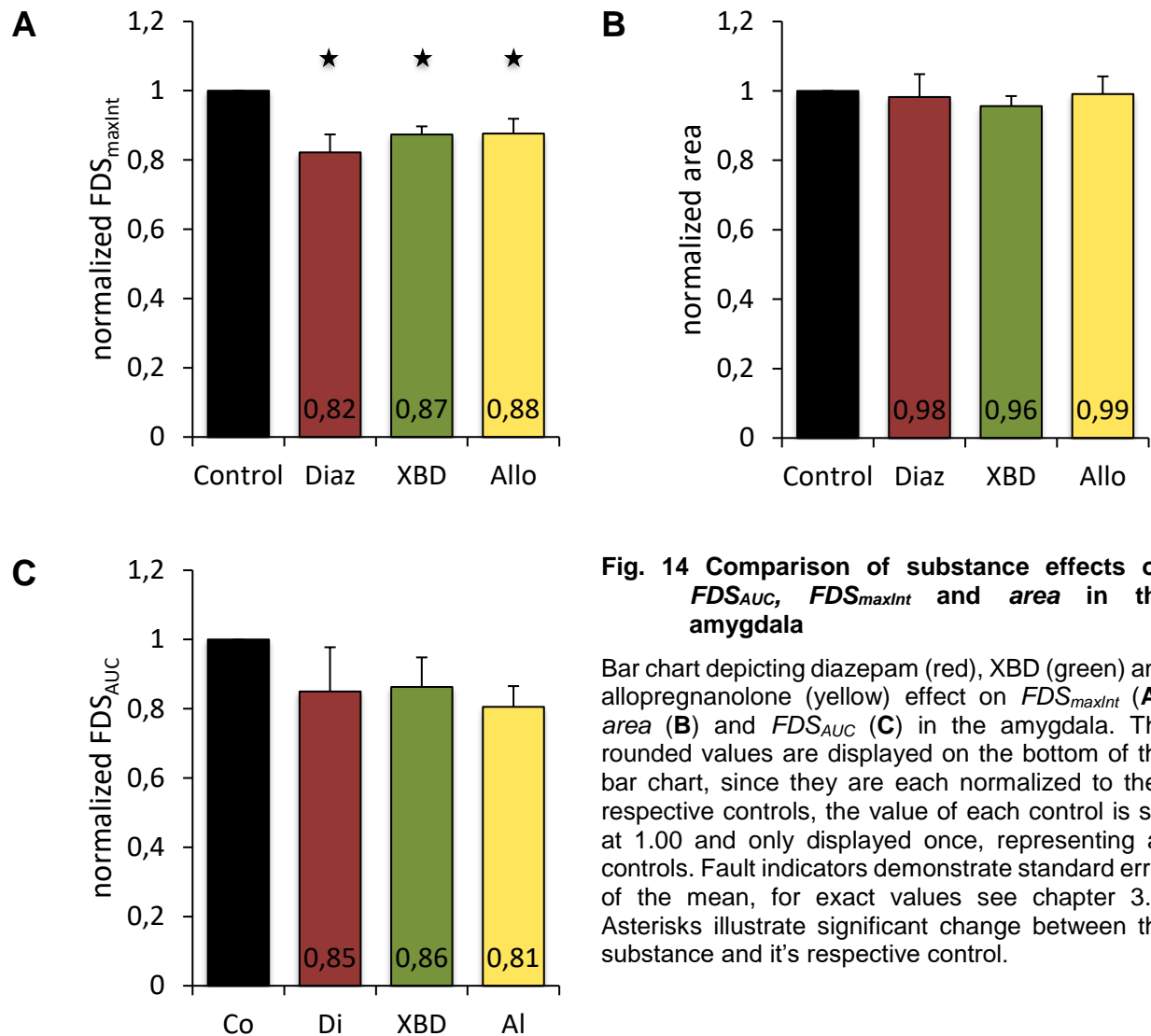
Fig. 14 Comparison of substance effects on FDS_{AUC}, FDS_{maxInt} and _area_ in the amygdala

Bar chart depicting diazepam (red), XBD (green) and allopregnanolone (yellow) effect on FDS_{maxInt} (**A**), _area_ (**B**) and FDS_{AUC} (**C**) in the amygdala. The rounded values are displayed on the bottom of the bar chart, since they are each normalized to their respective controls, the value of each control is set at 1.00 and only displayed once, representing all controls. Fault indicators demonstrate standard error of the mean, for exact values see chapter 3.1. Asterisks illustrate significant change between the substance and it's respective control.

3.2 Alternation of neuronal activity upon stimulus in the hippocampus by diazepam, XBD and allopregnanolone

As the brain area that is essentially involved in memory formation, the hippocampus portrays the brain area the next set of experiments was realized in.

3.2.1 Effect of diazepam on neuronal activity upon stimulus in the hippocampus

Diazepam application to hippocampal brain slices resulted in a decrease of the stimulated exci-tation transmission in the basolateral amygdala (FDS_{AUC} 82.2 ± 7.90 %, n=7). As depicted in the

graph below (Fig. 15A), $FDS_{areaInt}$ showed a distinct decrease throughout stimulated excitation transmission by diazepam (*amplitude of FDS_{areaInt}* 84.4 ± 4.5 %, n=7, p<0.02). Hereby intensity of neuronal activity upon stimulation was distinctly though not significantly reduced in average to 90.4 ± 4.2 % (n=7) (Fig. 15B). The decrease in *area* was significant and averaged to 88.0 ± 4.1 % (n=7, p<0.02) of initial stimulus propagation (Fig. 15C). Subsequent admission of benzodiazepine antagonist flumazenil resulted in significant increase of the diminished excitation transmission (*area* = 101.3 ± 2.7 %, n=7, p<0.05), however increase of iazepam-diminished FDS_{maxInt} (91.6 ± 6.4 %, n=7) and FDS_{AUC} (85.6 ± 8.0, n=7) by flumazenil was only slight and not significant.
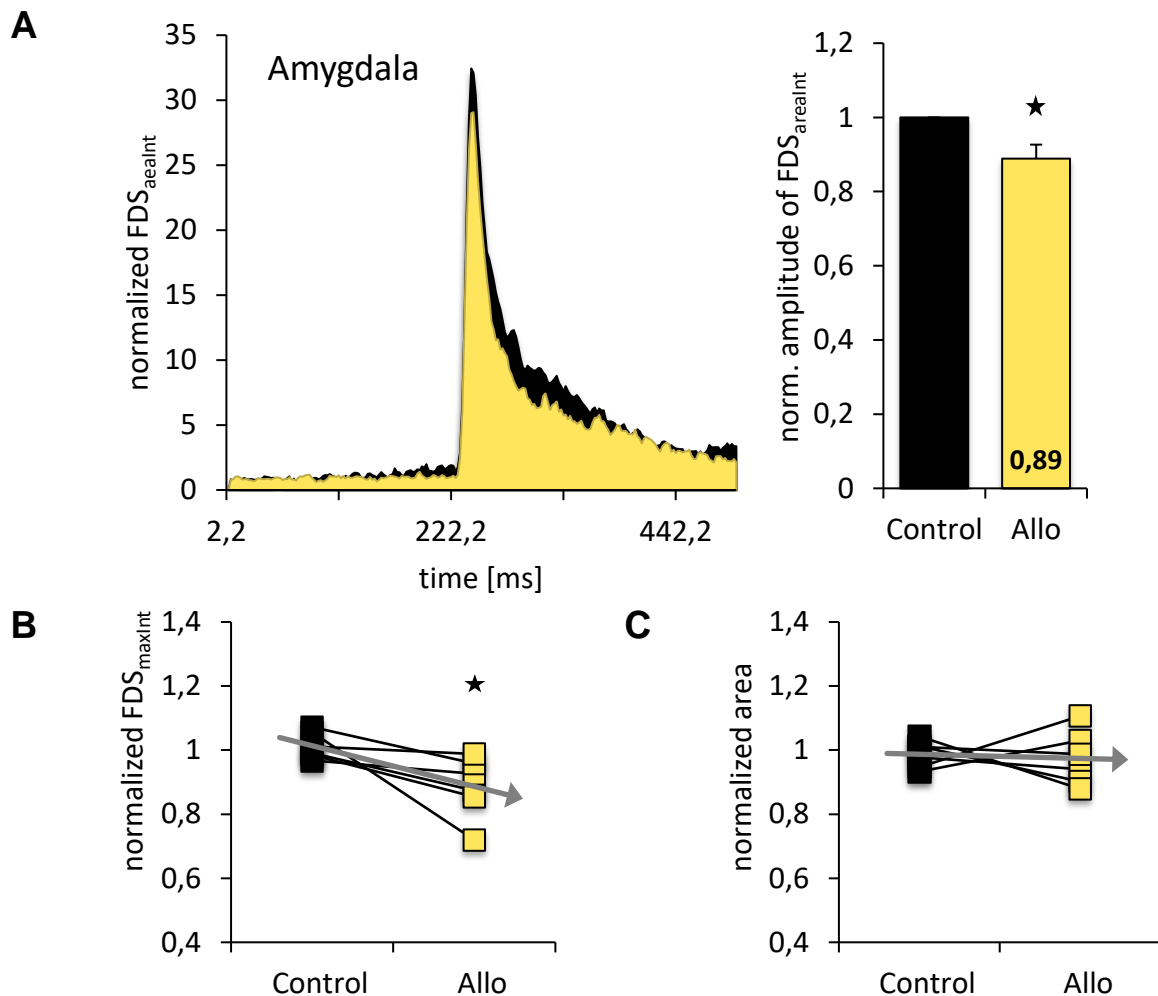

Fig. 15: diazepam impact on neuronal activity in the hippocampus

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and diazepam (red) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of control and diazepam (diaz, 0.84 ± 0.04, n=7, p<0.02) and 10 µg flumazenil (fluma, 0.91 ± 0.07, n=7) recordings in the hippocampus are depicted, representing the normalized amplitude of their peak values. **B**: Effect of 1 µM diazepam (red) on FDS_{maxInt} (ΔF/F) of the VSDI-signal in the hippocampus. Control recordings are illustrated as black squares. The grey arrow indicates the average effect. Distinct decrease of intensity by diazepam 0.90 ± 0.04 (n=7). **C**: Effect on the *area* in the hippocampus. Significant reduction of *area* under 1 µM diazepam 0.88 ± 0.04 (n=7, p<0.02).

3.2.2   Effect of XBD on neuronal activity upon stimulus in the hippocampus

XBD application to hippocampal brain slices did not result in a significant alteration of the stimulated excitation transmission in the basolateral amygdala (FDS_{AUC} = 96.1 ± 7.8 %, n=7) (Fig. 16A). Neither *amplitude of $FDS_{areaInt}$* (93.2 ± 4.2 %, n=7), FDS_{maxInt} (93.5 ± 4.8 %, n=7) nor the *area* (103.5 ± 4.8 %, n=7) of neuronal activity upon stimulation showed significant alteration under XBD influence (Fig. 16B,C).
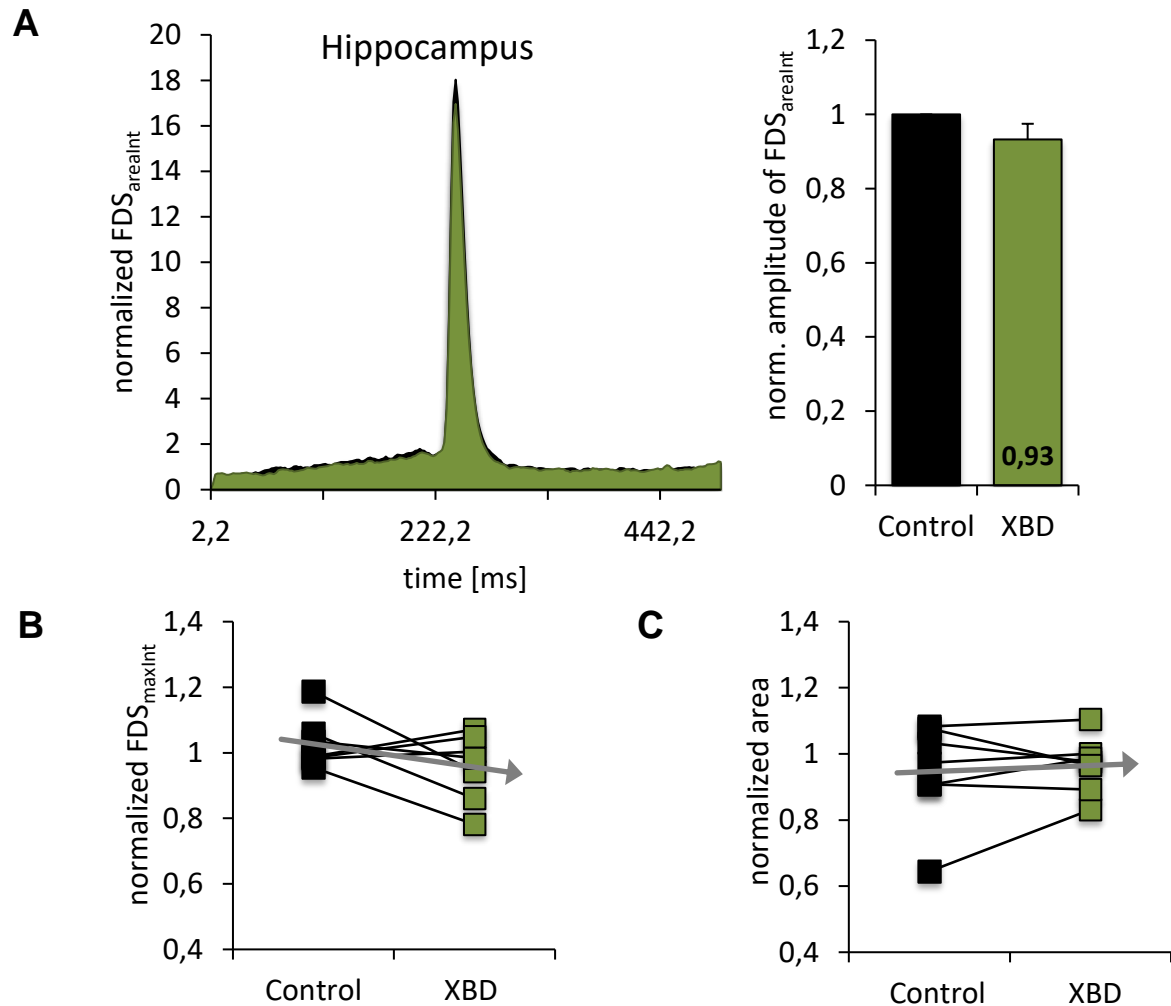
Fig. 16: XBD impact on neuronal activity in the hippocampus

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 frames of control (black) and XBD (green) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of control and XBD (0.93 ± 0.04, n=7) recordings in the hippocampus are depicted, representing the normalized amplitude of their peak values. **B**: Effect of 3 µM XBD (green) on FDS_{maxInt} (∆F/F) of the VSDI-signal in the hippocampus. Control recordings are illustrated as black squares. The grey arrow indicates the average effect. No significant decrease of FDS_{maxInt} by XBD 0.94 ± 0.05 (n=7). **C** No significant alteration of *area* under 3 µM XBD influence in the hippocampus 1.04 ± 0.05 (n=7).

3.2.3 Effect of allopregnanolone on neuronal activity upon stimulus in the hippocampus

Allopregnanolone application to hippocampal brain slices resulted in a decrease of the stimulated excitation transmission in the basolateral amygdala (Fig. 17A). As depicted in the graph below (Fig. 17A), $FDS_{areaInt}$ showed a distinct decrease throughout stimulated excitation transmission by allopregnanolone (*amplitude of $FDS_{areaInt}$* 79.5 ± 4.1 %, n=6, p<0.05). FDS_{AUC} showed a distinct and significant decrease to 77.2 ± 5.0 % (n=6, p<0.05) in average. Hereby only the intensity of neuronal activity upon stimulation was reduced (82.0 ± 3.0 %, n=6, p<0.05) (Fig. 17B), while the *area* of activity propagation remained quite stable (97.1 ± 2.2 %, n=6) (Fig. 17C).

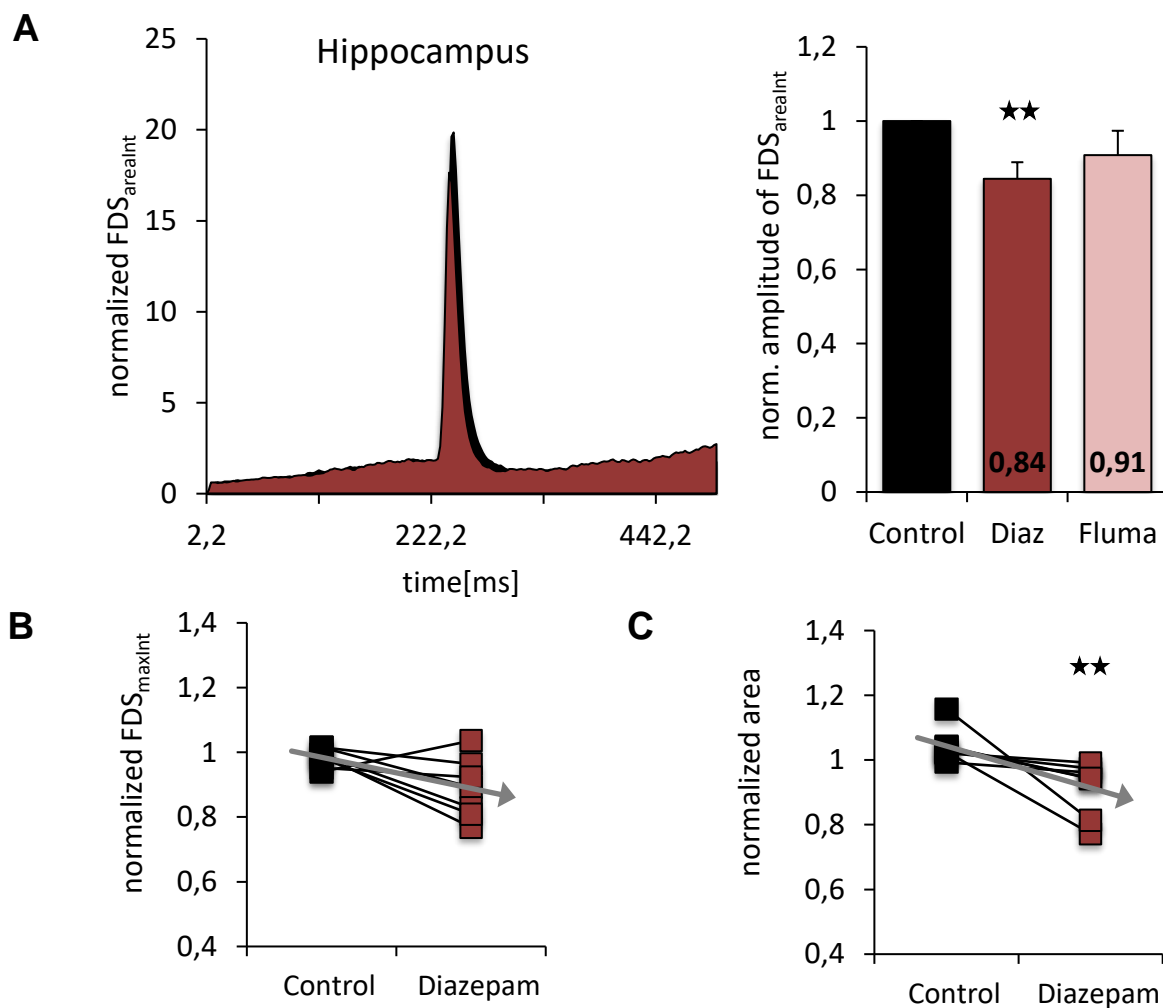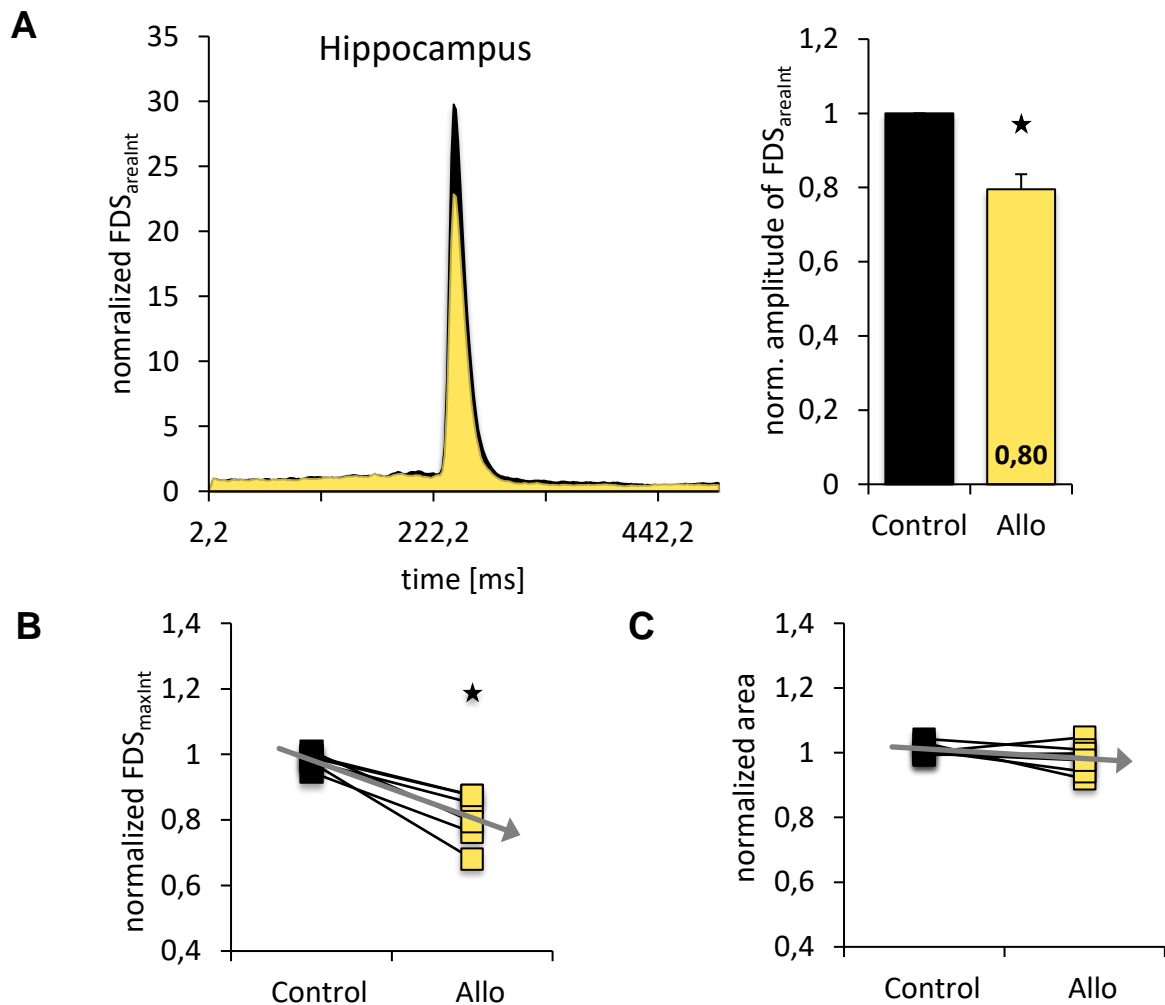**Fig. 17: allopregnanolone impact on neuronal activity in the hippocampus**

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 frames of control (black) and allopregnanolone (yellow) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of control and allopregnanolone (allo, 0.80 ± 0.04, n=6, p<0.05) recordings in the hippocampus are depicted, representing the normalized amplitude of their peak values. B: Effect of 1 µM allopregnanolone (yellow) on FDS_{maxInt} (ΔF/F) of the VSDI-signal in the hippocampus. Control recordings are illustrated as black squares. The grey arrow indicates the average effect. Distinct and significant decrease of FDS_{maxInt} by allopregnanolone 0.82 ± 0.03 (n=6, p<0.05). C: Effect of 1 µM allopregnanolone on *area* of stimulus propagation in the amygdala. No significant alteration of *area* under allopregnanolone 0.97 ± 0.02 (n=6).

3.2.4 Comparison of diazepam, XBD and allopregnanolone effect on neuronal activity in the hippocampus

A

B

C

Fig. 18 Comparison of substance effects on FDS_{AUC}, FDS_{maxInt} and *area* in the hippocampus

Bar chart depicting diazepam (red), XBD (green) and allopregnanolone (yellow) effect on FDS_{maxInt} (**A**), *area* (**B**) and FDS_{AUC} (**C**) in the hippocampus. The rounded values are displayed on the bottom of the bar chart, since they are each normalized to their respective controls, the value of each control is set at 1.00 and only displayed once, representing all controls. Fault indicators demonstrate standard error of the mean, for exact values see chapter 3.2. Asterisks illustrate significant change between the substance and it's respective control (one asterisks indicates p<0.05, double asterisks indicate p<0.02).

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone

3.3 Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam, XBD and allopregnanolone

Two questions were addressed with the measurement in the thalamocortical connections. First, is there an effect of the corresponding substances and second, if so, at which point within the thalamocortical system does the effect occur: right in the thalamus, the connecting TC neurons or within the cortex? Therefore, measurements were designed to detected neuronal activity in the thalamus (intrathalamic measurements) as well as in the cortex (thalamocortical measurements) upon stimulation in the ventrobasal thalamus. In addition FDS propagation in the cortex was measured upon intracortical stimulation (intracortical measurements). Therefore an electrode was additionally placed in layer 4 of the cortex.

3.3.1 Effect of diazepam on neuronal activity upon stimulus in the thalamocortical connections

Diazepam application to thalamocortical brain slices resulted in a decrease of the FDS propagation. As depicted in the graph below (Fig. 19A), $FDS_{areaInt}$ showed a distinct decrease of neuronal activity in the cortex upon thalamic stimulation (*amplitude of* $FDS_{areaInt}$ 79.5 ± 3.2 %, n=7, p<0.02; FDS_{AUC} = 76.3 ± 4.9, n=7, p<0.02). Hereby especially FDS_{maxInt} of neuronal activity upon stimulation was significantly reduced in average to 84.4 ± 3.11 % (n=7, p<0.02) (Fig. 19B). There was no significant alteration in *area* (94.6 ± 3.01 %, n=7) (Fig. 19C). Subsequent admission of benzodiazepine antagonist flumazenil resulted in distinct increase of the reduced *area* (FDS_{maxInt} = 96.0 ± 6.3 %, n=7; *area*= 98.9 ± 2.9 %, n=7) (Fig. 19A). Intrathalamic (*amplitude of* $FDS_{areaInt}$ 87.7 ± 2.4 %, n=7, p<0.02; FDS_{AUC} = 86.4 ± 4.0 %, n=7) as well as intracortical (*amplitude of* $FDS_{areaInt}$ 83.3 ± 3.6 %, n=6, p<0.05; FDS_{AUC} = 81.4 ± 4.6 %, n=6, p<0.05) experiments with diazepam showed similar results (Fig. 19B,C). Hereby diazepam showed equal impact on thalamocortical and intracortical excitation propagation, while diazepam effect on the intrathalamic connections was slighter in comparison. Intrathalamic diazepam impact could not be antagonized by subsequent flumazenil application, in contrast to thalamocortical and intracortical diazepam effect.

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone
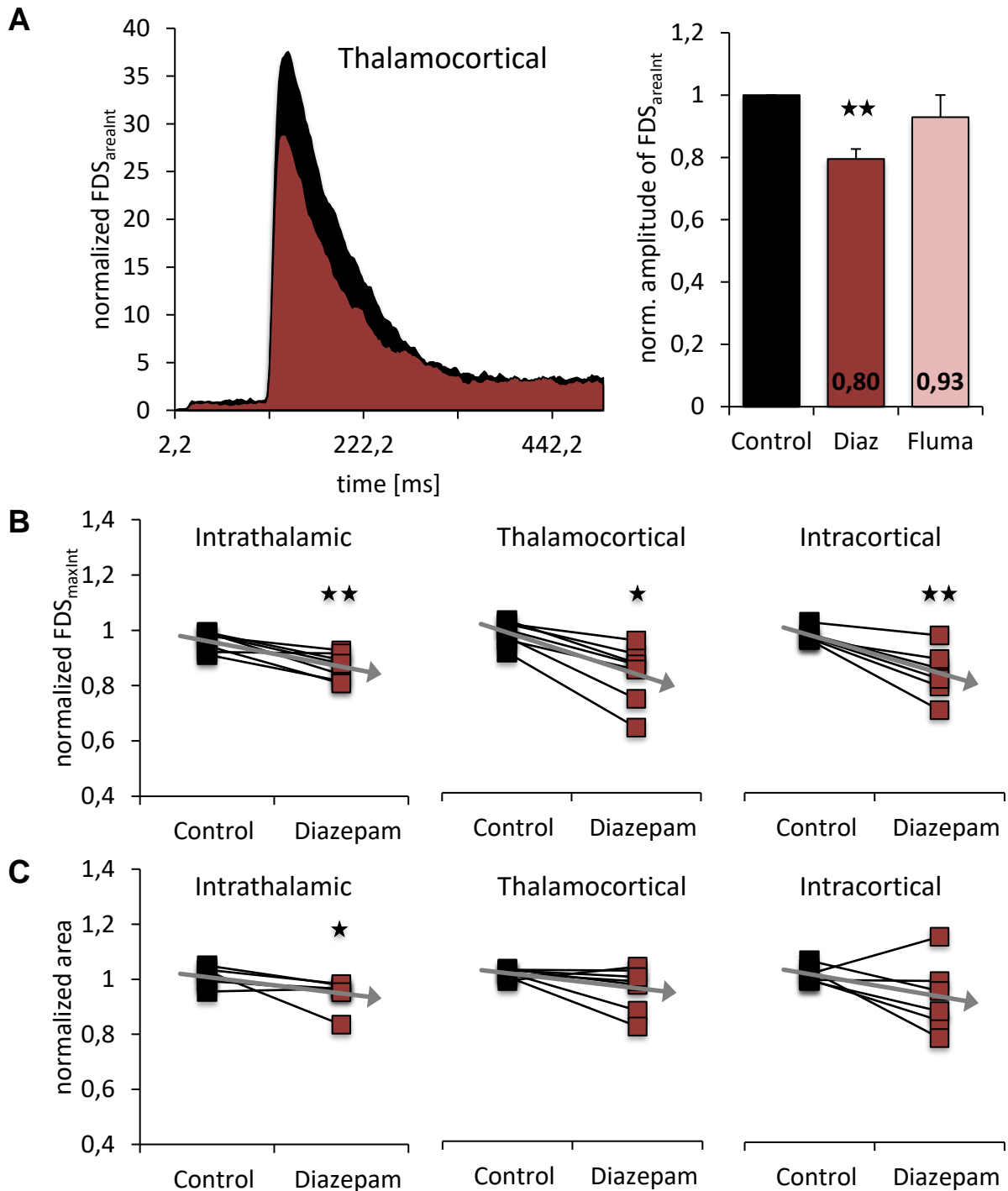
Fig. 19: diazepam impact on neuronal activity in the thalamocortical connections

A: On the left averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and diazepam (red) recordings are depicted. On the right, the maximal normalized $FDS_{areaInt}$ values of control and diazepam (diaz, 0.80 ± 0.03, n=7, p<0.02) and 10 µg flumazenil (fluma, 0.93 ± 0.07, n=7) recordings in the thalamocortical connections are depicted, representing the normalized amplitude of their peak values. **B**: Effect of 1 µM diazepam (red) on FDS_{maxInt} (ΔF/F) of the VSDI detected signal in the thalamus (0.90 ± 0.02, n=7, p<0.02), the thalamocortical connections (0.84 ± 0.03, n=7, p<0.02) and intracortical (0.86 ± 0.03, n=6, p<0.05). Control recordings are illustrated as black squares. The grey arrow indicates the average effect. **C**: Effect of 1 µM diazepam on the *area* of stimulus propagation in the thalamus (0.94 ± 0.02, n=7, p<0.05), the thalamocortical connections (0.95 ± 0.03, n=7) and intracortical (0.92 ± 0.05, n=6).

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Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone

3.3.2 Effect of XBD on neuronal activity upon stimulus in the thalamocortical connections

XBD application to thalamocortical brain slices resulted in a decrease of the stimulated excitation transmission. As depicted in the graph below (Fig. 20A), $FDS_{areaInt}$ showed a distinct decrease of neuronal activity in the cortex upon thalamic stimulation (*amplitude of $FDS_{areaInt}$* 83.5 ± 3.2 %, n=8, p<0.01; FDS_{AUC} 77.6 ± 8.1%, n=8, p<0.02). Hereby especially FDS_{maxInt} of neuronal activity upon stimulation was significantly reduced in average to 86.1 ± 3.3 % (n=8, p<0.01) (Fig. 20B), while there was no significant alteration in *area* (102.0 11.5 %, n=8) (Fig. 20C). In experiments with simultaneous admission of XBD and finasteride, no alteration of excitation propagation was detectable (FDS_{maxInt} 101.8 ± 5.3 %, n=7; *area* 98.9 ± 2.9 %, n=7). Intrathalamic (*amplitude of $FDS_{areaInt}$* 86.2 ± 3.0 %, n=8, p<0.01; FDS_{AUC} 84.2 ± 7.0 %, n=8) as well as intracortical experiments (*amplitude of $FDS_{areaInt}$* 87.4 ± 2.3 %, n=8, p<0.01; FDS_{AUC} 84.6 ± 3.3 %, n=8, p<0.01) with XBD showed similar results (Fig. 20B,C). Hereby XBD showed equal impact on thalamocortical and intrathalamic neuronal activity upon stimulation, while intracortical XBD effect was slighter in comparison. Intrathalamic and intracortical XBD impact could not be antagonized by additional finasteride application, in contrast to thalamocortical XBD effect.

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone

Fig. 20: XBD impact on neuronal activity upon stimulus in the thalamocortical circuit

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and XBD (green) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of XBD (0.83 ± 0.03, n=8, p<0.01) and XBD plus finasteride (X/Fina, 1.00 ± 0.05, n=7) recordings in the thalamocortical connections are depicted, representing the normalized amplitude of their peak values. The black bar represents both their respective control recordings. Additional finasteride application significantly antagonized XBD effects (p<0.05). **B**: Effect of 3 µM XBD (green) on FDS_{maxInt} of the VSDI-signal in the thalamus (0.89 ± 0.03, n=8, p<0.02), the thalamocortical connections (0.86 ± 0.03, n=8, p<0.01) and intracortical (0.93 ± 0.03, n=8). Control recordings are illustrated as black squares. The grey arrow indicates the average effect. **C**: Effect of 3 µM XBD on the *area* in the thalamus (0.94 ± 0.05, n=8), the thalamocortical connections (1.02 ± 0.11, n=8) and intracortical (0.96 ± 0.04, n=8).

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone

3.3.3 Effect of allopregnanolone on neuronal activity upon stimulus in the thalamocortical connections

As Fig. 21A demonstrates, allopregnanolone affected the duration of neuronal activity in the cortex upon thalamic stimulation, as quantified by the parameter FDS_{AUC} = 87.5 ± 9.4 % (n=5) in comparison to *amplitude of FDS$_{areaInt}$* (1.01 ± 6.5 %, n=5). Allopregnanolone application did not affect FDS_{maxInt} of intrathalamic (97.6 ± 4.7 %, n=4) and thalamocortical (97.5 ± 5.4 %, n=5) excitation transmission (Fig. 21B). However, allopregnanolone exertion resulted in decrease of FDS_{maxInt} of intracortical neuronal activity upon cortical stimulation to about 81.5 ± 6.7 % (n=5). Intrathalamic no alteration of *area* could be detected under allopregnanolone influence (93.8 ± 5.4 %, n=4). Allopregnanolone showed a tendency to increased thalamocortical (113.1 ± 13.3 %, n=5) and intracortical stimulus propagation (*area* = 118.6 ± 10.8 %, n=5) (Fig. 21C). None of the allopregnanolone experiments performed on thalamocortical slices provided significant results.

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone
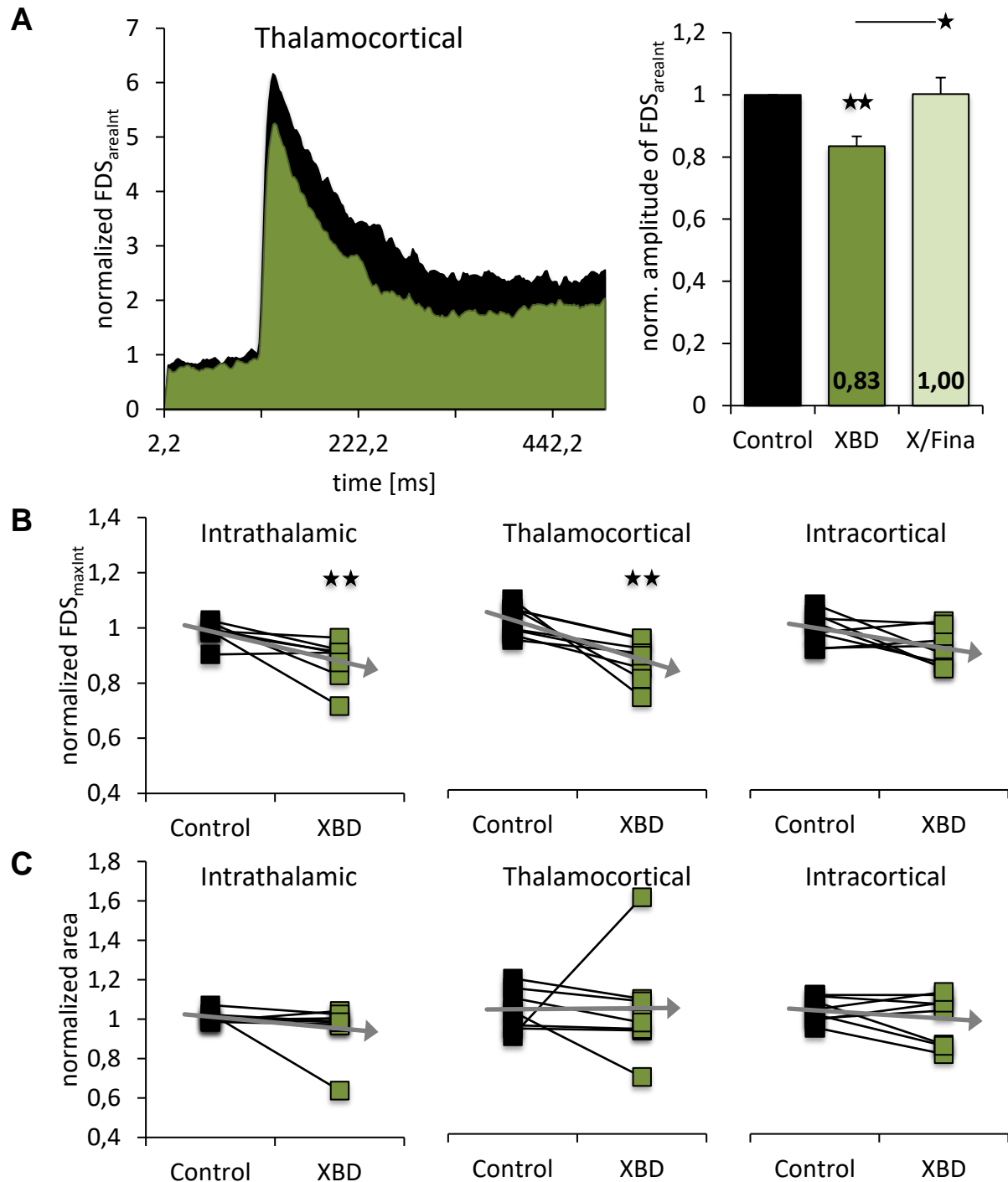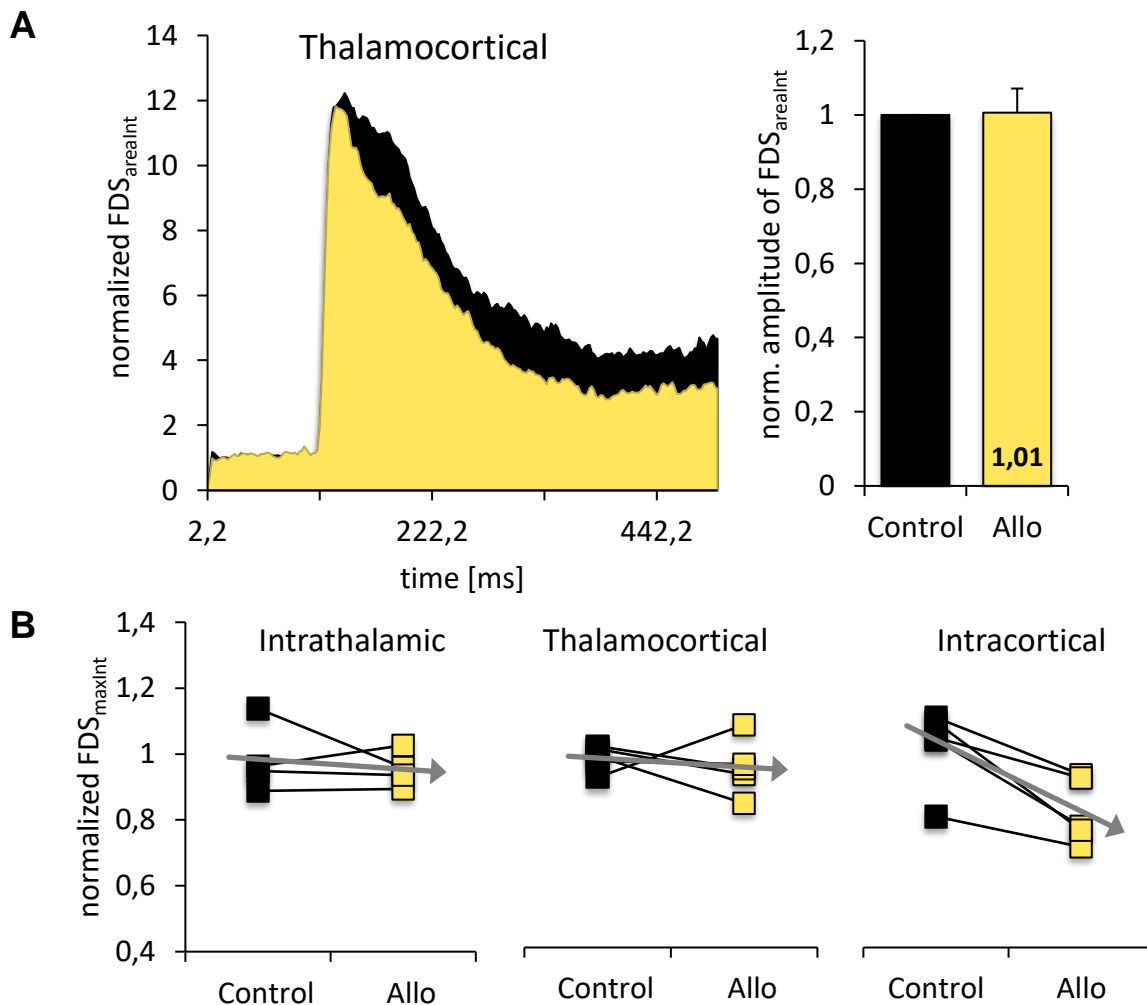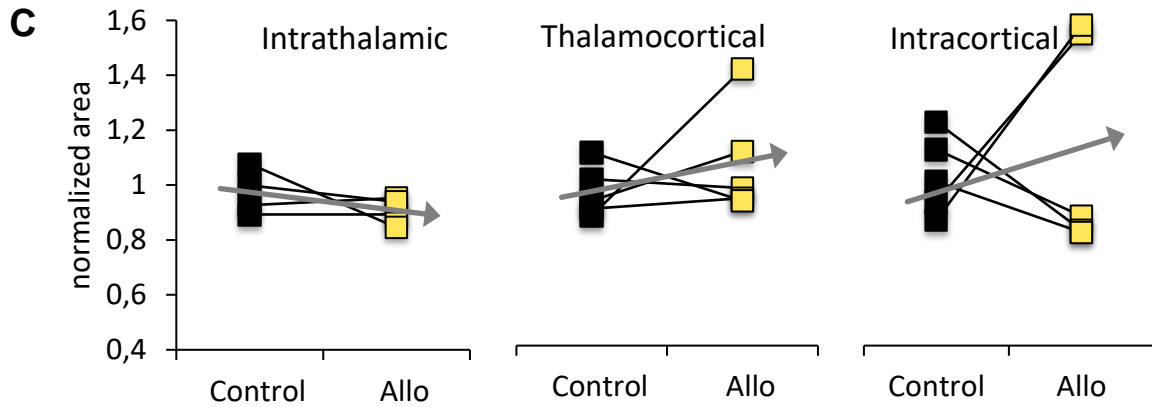
Fig. 21: allopregnanolone impact on neuronal activity upon stimulus in the thalamocortical connections

A: The diagram on the left shows averaged normalized $FDS_{areaInt}$ values of the first 500 ms of control (black) and allopregnanolone (yellow) recordings. On the right, the maximal normalized $FDS_{areaInt}$ values of control and allopregnanolone (allo, 1.01 ± 0.07, n=5) recordings in the thalamocortical connections are depicted, representing the normalized amplitude of their peak values. **B**: Effect of 1 µM allopregnanolone (yellow) on FDS_{maxInt} (ΔF/F) of the VSDI-signal in the intrathalamic (0.98 ± 0.05, n=4), the thalamocortical connections (0.97 ± 0.05, n=5) and intracortical (0.81 ± 0.07, n=5). Control recordings are illustrated as black squares. The grey arrow indicates the average effect **C**: Effect of 1 µM allopregnanolone on the *area* of the stimulus propagation in the thalamus (0.94 ± 0.05, n=4), the thalamocortical connections (1.13 ± 0.13, n=5) and intracortical (1.19 ± 0. 11, n=5).

3.3.4 Comparison of diazepam, XBD and allopregnanolone effect on neuronal activity in the thalamocortical connections

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone
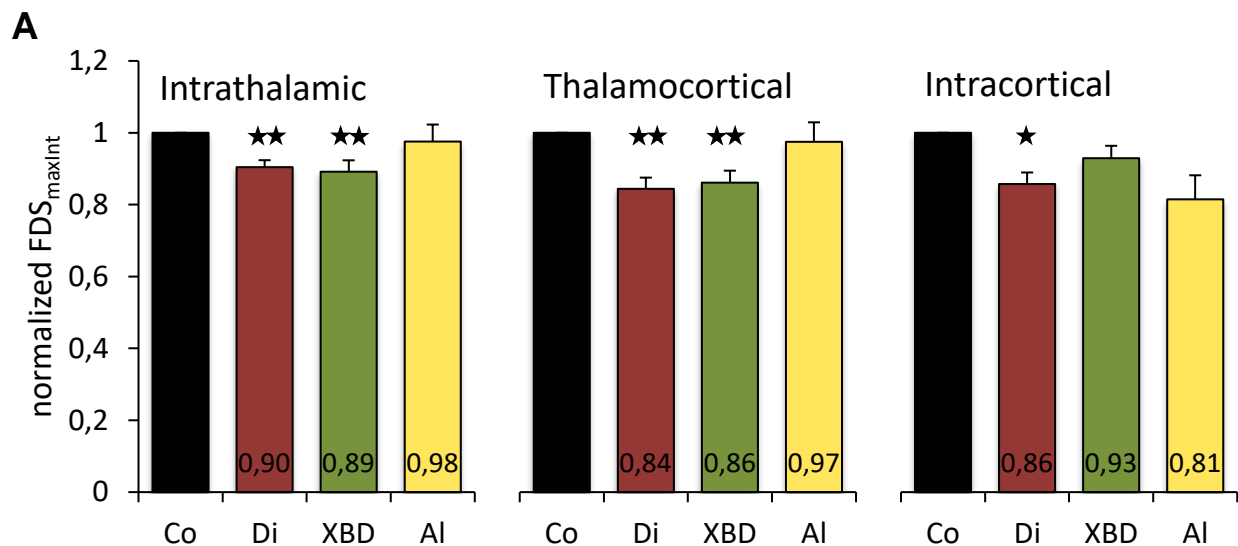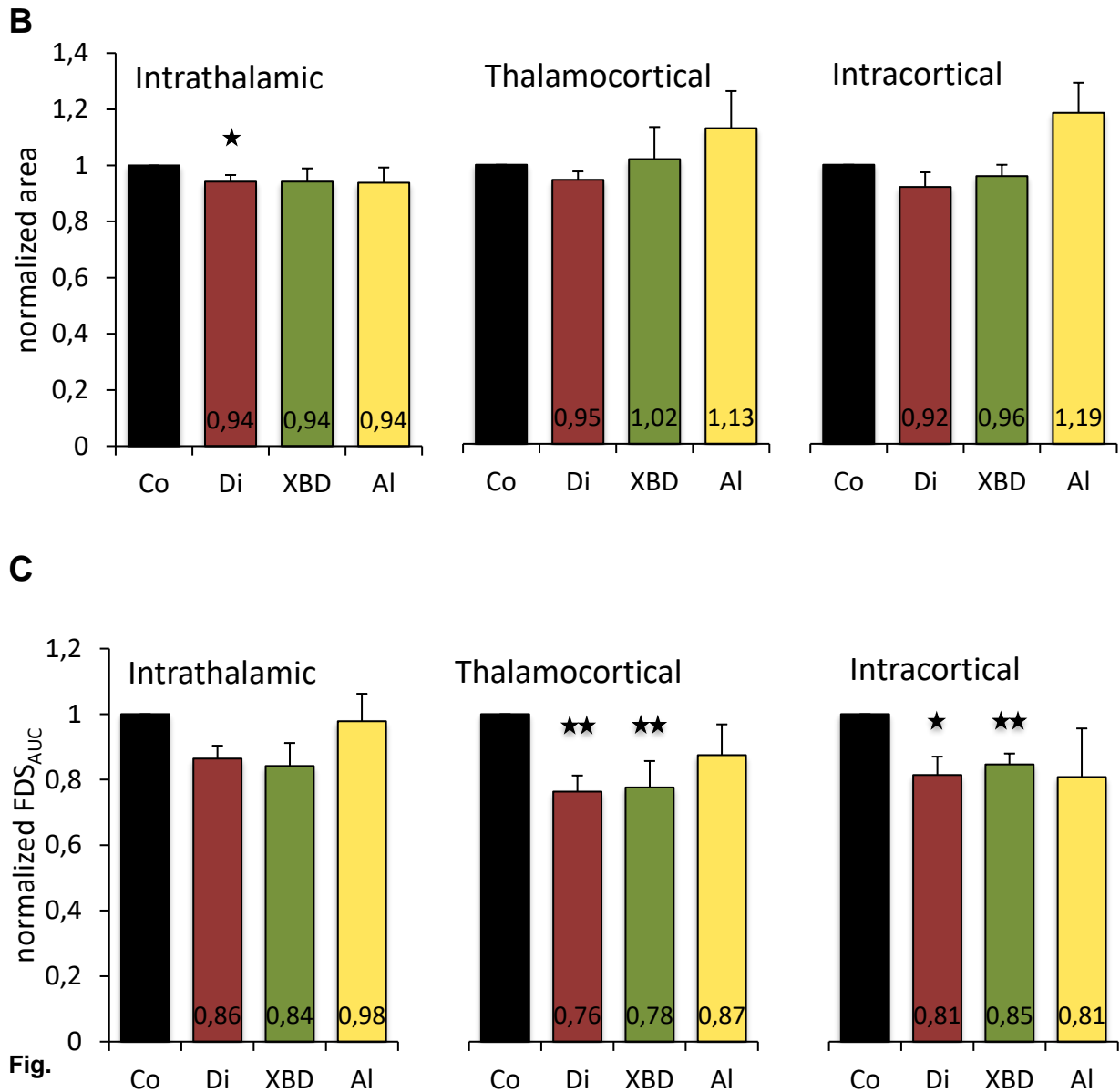
22 Comparison of substance effects on FDS_{AUC} FDS_{maxInt} and *area* in the thalamocortical connections

The bar charts serve to ease comparison of average effect of diazepam (red), XBD (green) and allopregnanolone (yellow) on FDS_{maxInt} (**A**), *area* (**B**) and FDS_{AUC} (**C**) in the thalamocortical connections. The rounded values are displayed on the bottom of the bar chart, since they are each normalized to their respective controls, the value of each control is set at 1.00 and only displayed once, representing all controls. Fault indicators demonstrate standard error of the mean, for exact values see chapter 3.3. Asterisks illustrate significant change between the substance and it's respective control (one asterisks indicates $p < 0.05$, double asterisks indicate $p < 0.02$).

3.3.5 Analyses of diazepam, XBD and allopregnanolone impact on the individual layers of the cortex

To get more insight into the diazepam, XBD and allopregnanolone mediated impact on thalamocortical excitation propagation upon stimulation, the change in neuronal activity of each layer of the cortex inside the ROI upon thalamic stimulation was investigated. For this purpose the following parameters were analyzed in each layer individually: the maximal change of intensity (Int_{max}) and the time till Int_{max} (*delay of Int_{max}*).

The reduction of Int_{max} by diazepam, XBD and allopregnanolone was distinct throughout all three substances, however different amongst them in regard of the extend with which each layer was affected (see

Fig. 24). Diazepam elicited most effect in layer 1 to 3 and was successfully and partially significantly antagonized by flumazenil. XBD affected layer 1,2,5 and 6 the most. Allopregnanolone seemed to diminish the maximal intensity of thalamocortical stimulus propagation least of all three substances and only showed consistent decrease in layer 3 and 4.

Diazepam significantly decreased the *delay of Int_{max}* in the 2nd layer (74.7 ± 7.2 %, n=7, p<0.05) and 4th layer (86.6 ± 4.8 %, n=7, p<0.05) and showed a tendency to decrease *delay of Int_{max}* in the 1st layer (89.5 ±7.7 %, n=7) without significance. Flumazenil did not significantly antagonize diazepam effect on the *delay of Int_{max}*. XBD significantly decreased the *delay of Int_{max}* in the 4th layer of the cortex (to 73.4 ± 13.4%, n=8, p<0.05). Simultaneous application of XBD and finasteride showed to the tendency to but did not significantly prevent XBD effect in the 4th layer of the cortex. Allopregnanolone application showed the same tendency in the 4th layer without significance (76.8 ± 27.9 %, n=5). Apart form the 4th layer and in the case of diazepam also in the 1st and 2nd layer of the cortex, the evaluation of *delay of Int_{max}* did not offer valuable information due to discrepancies in the results.
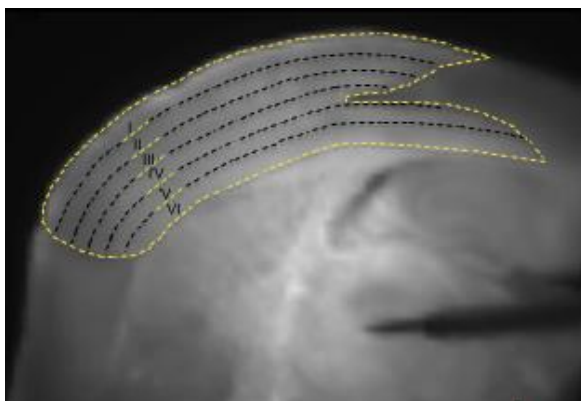
Fig. 23 Display of cortex division for layer analysis

Depiction of the roi (yellow outlining) and the 6 areas (I-VI) presenting the 6 lawyers of the cortex.

Results
Alternation on neuronal activity upon stimulus in the thalamocortical connections by diazepam,
XBD and allopregnanolone
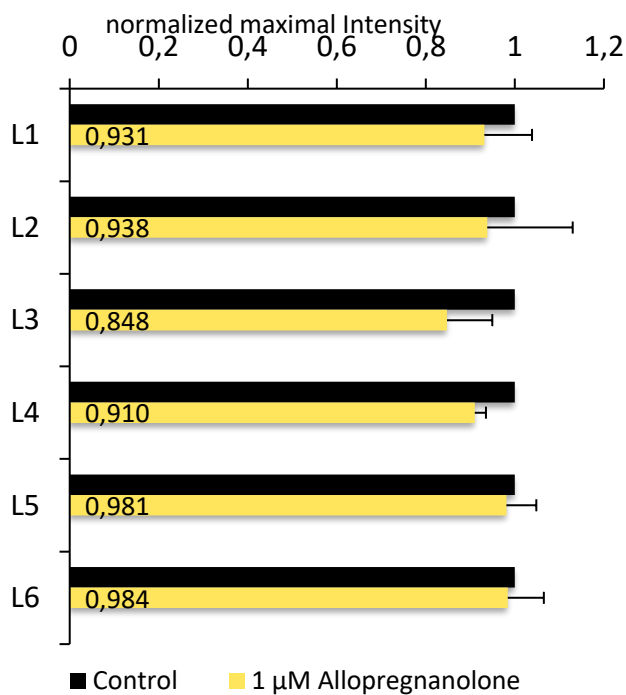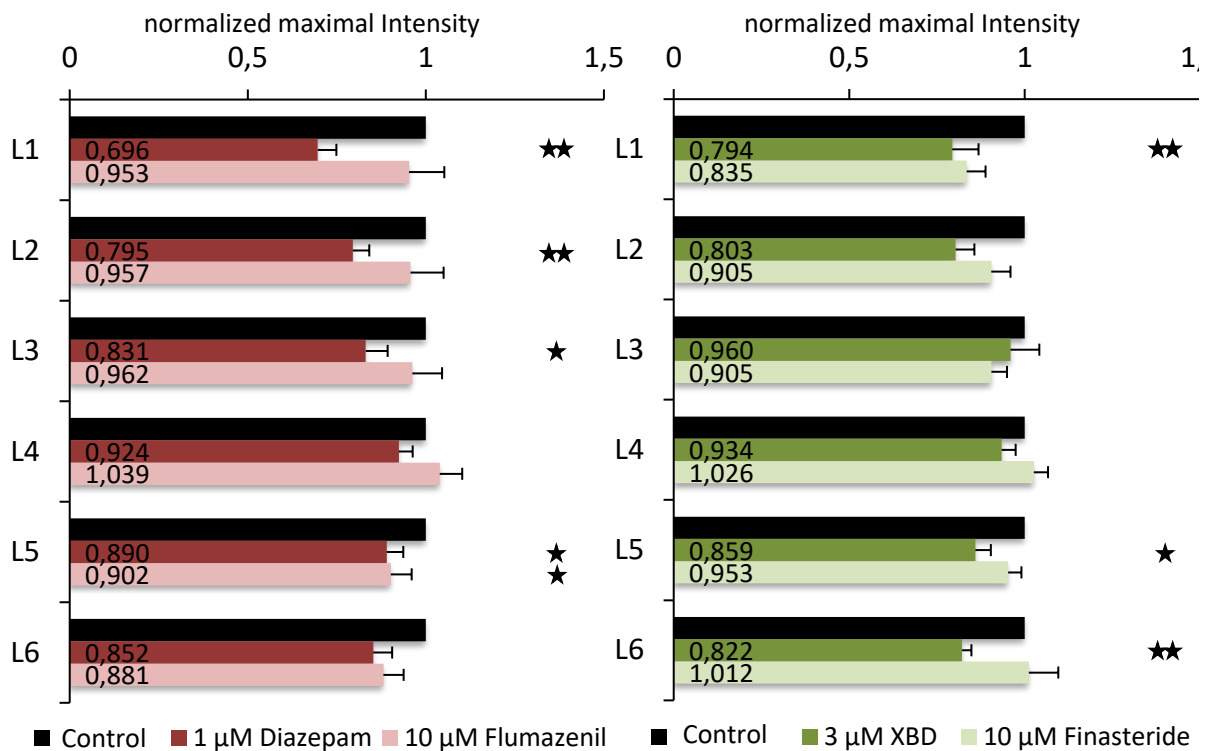
Fig. 24 Layer analysis of diazepam and XBD impact on normalized maximal Intensity

Depicted is a bar chart of the effect of diazepam (red), XBD (green) and allopregnanolone (yellow) on maximal Intensity normalized to their respective control recordings (black) in each layer (L1-6) of the cortex upon thalamic stimulation. Diazepam and XBD effects are depicted with their respective antagonists. The values are depicted inside the bars. Fault indicators demonstrate standard error of the mean. Asterisks represent significant results (one asterisks: $p < 0.05$, two asterisks: $p < 0.02$).

4   Discussion

4.1   Result interpretation

Benzodiazepines such as diazepam and XBD, a ligand of TSPO, elicit similar anxiolytic properties but dispose of diverging side effect profiles. While diazepam exposes amnestic and sedative adverse effects as well as tolerance development and risk of addiction, XBD lacks any of those, as far as experiments on humans and rodents have surveyed (Kita et al. 2009, Rupprecht et al. 2009, Rote-Liste 2013, 2015). XBD enhances neurosteroid genesis, e.g. allopregnanolone synthesis, which is responsible for XBD effects (Rupprecht et al. 2009). Both, allopregnanolone and diazepam are positive allosteric modulators at the $GABA_A$ receptor, though they occupy different binding sides (Hosie et al. 2006, Möhler 2006). Due to diverse $GABA_A$ receptor subunit expression, $GABA_A$ receptor binding abilities vary throughout different brain areas, probably accounting for the discrepancy of diazepam and XBD side effect profile (Pirker et al. 2000). To investigate diazepam, XBD and allopregnanolone impact on neuronal activity in brain areas relevant to their effects, alteration of excitation propagation upon stimulation under their influence was detected using VSDI in the course of this dissertation. Brain areas included in the experiments are the amygdala as the anxiety-center of the brain (see ch. 1.7), the hippocampus as the brain area where memory formation occurs (see ch. 1.8) and the thalamocortical system, indispensable for awareness (see ch. 1.9). Corresponding mice brain slices were prepared for VSDI and signal propagation upon stimulus under the influence of diazepam, allopregnanolone and XBD was determined.

4.1.1   Impact of diazepam, XBD and allopregnanolone on neuronal activity in the amygdala

The amygdala has been found to be crucially involved in processing emotions, especially anxiety (see chapter 1.7.2). Diazepam, XBD and allopregnanolone displayed significant negative impact on neuronal excitability in the amygdala. Hereby all three substances demonstrated similar inhibition of signal transduction in the amygdala, whereupon especially the Intensity (FDS_{maxInt}) rather than the *area* of signal propagation was affected, indicating that the amount of neurons activated upon stimulation remained the same but the level of their excitation was reduced by diazepam, XBD and allopregnanolone.

These results are in line with clinical observations and animal models determining their anxiolytic potential. Similar anxiolytic properties of XBD and benzodiazepines have been observed in rodents as well as in humans (Kita et al. 2009, Rupprecht et al. 2009). Comparable anxiolytic properties of allopregnanolone have been shown in rodents as well (Bitran et al. 1991, Picazo et al. 1995).

Taken together, the anxiolytic effect of all three substances probably origins in their diminishing effect on neuronal activity in the amygdala. In terms of similarity of XBD and allopregnanolone

results, it is likely that the impact of XBD on the amygdala is due to allopregnanolone formation. These results therefore support the data of Rupprecht at al (Rupprecht et al. 2009), which proposes that XBD effects are mediated by allopregnanolone.

4.1.2 Impact of diazepam, XBD and allopregnanolone on neuronal activity in the hippocampus

Diazepam displayed distinct impact on neuronal activity in the hippocampus, whereupon the *area* of the stimulation was significantly reduced and the intensity distinctly diminished, indicating that diazepam did not only affect the amount of neurons excited upon stimulation but also reduced the level of excitation. This appears to explain diazepam's known adverse effect on memory resulting in anterograde amnesia (Rote-Liste 2013, 2015). XBD however, did not significantly disturb neuronal activity upon stimulus in the hippocampus, indicating that XBD administration to subjects would not result in affection of memory. Just as no affection in memory upon XBD application has been observed in clinical studies so far (Rupprecht et al. 2009).

Interestingly, allopregnanolone had a significant negative effect on the intensity of signal transduction in the hippocampus even though XBD showed no significant or even relevant effect here. However, glia cells located in the hippocampus might lack expression of enzymes needed to form allopregnanolone out of pregnenolone. If this was the case, allopregnanolone may exert a negative impact on signal propagation in the hippocampus, but would not be increasingly synthesized in the hippocampus following XBD administration. Consequently, XBD would not result in inhibition of signal transduction in the hippocampus and thus not effect memory (assuming other neurosteroids that positive allosteric modulate at GABA$_A$ receptors are not promoted either, which could be the case, for XBD experiments in the amygdala did not result in suppression of neuronal activity). XBD would still enhance pregnenolone synthesis and following other neurosteroids in the hippocampus, but not allopregnanolone or any other positive allosteric GABA$_A$ receptor modulating neurosteroids or XBD promotes diverse neurosteroids, which cancel out each others effects. Supporting this theory, allopregnanolone has been observed to have a negative impact on memory (Mayo et al. 1993, Zimmerberg et al. 1995). Further affirming this theory, enzymes for synthesis of diverse neurosteroids have been determined to be region and neuron specific (Rupprecht et al. 2010). For instance, enzymes needed for formation of 5α-reduced neurosteroids have been found to be almost absent in GABAergic hippocampal interneurons (Agís-Balboa et al. 2006).

4.1.3 Impact of diazepam, XBD and allopregnanolone on neuronal activity in the thalamocortical connections

XBD impact on neuronal activity in the thalamocortical system was overall similar to diazepam effect, with some distinct differences though. While XBD's effect focused on merely diminishing the intensity of intrathalamic and thalamocortical activity propagation, diazepam additionally affected

the intensity in the intracortical experiment and the *area* of neuronal stimulus propagation in the intrathalamic neuronal network. Ergo diazepam impact on the thalamocortical connections exceeded XBD impact.

However, like diazepam, XBD clearly and significantly had an impact on intensity of signal propagation in the thalamocortical connections arising from the ventrobasal nucleus of the thalamus leading to the somatosensory cortex. Kita and his research group could not observe any signs of sedative adverse effects upon XBD application to rodents (Kita et al. 2009). Furthermore, subjects exposed to XBD intake did not report sedative effects (Rupprecht et al. 2009). In hindsight it can be debated that a decline in the signal transduction in the thalamocortical connections might be an additional reason for anxiety dissolution and not a cause for sedation. Hereby the train of thought is that impairment of the thalamocortical connections might lead to a decrease in awareness, but not on the physical condition of being awake. If this is the case, XBD might affect alertness but would not sedate subjects. Encouraging this theory is the fact that the reticular formation (as a part of the Reticular Activating System) is essentially involved in physical condition states such as wakefulness and sleep-wake transitions (Trepel 2008) and might therefore be the working point of the adverse effect of sedation upon diazepam application instead of the thalamocortical system. To get more insight in this matter it would be interesting to perform clinical studies investigating possible impact of XBD on awareness, concentration and reaction time. It also would be interesting to see if XBD and diazepam show an effect in the Ascending Reticular Activating System (RAS). Since up to date a slice preparation to properly inspect the RAS has not been developed, there was no way to include this brain area in this dissertation.

The experiments with allopregnanolone in the thalamocortical system showed quite different results. Allopregnanolone did not significantly affect the intrathalamic neuronal network. It did however increase the *area* in the thalamocortical connections. Additionally, allopregnanolone decreased the intensity of the stimulus propagation intracortical, however it did not in the thalamocortical connections. The tendency of allopregnanolone to increase *area* in the thalamocortical experiments might be considered as scattering of stimulus propagation, implying that the once accurate and specific projection of excitation from the thalamus to a certain area of the cortex is now affected and stimulus propagation left unguided.

The discrepancy in allopregnanolone and XBD results in the thalamocortical connections might indicate that allopregnanolone is not the only neurosteroid promoted upon XBD application in the thalamocortical connections. Since XBD rendered a quite different outcome, it is to be supposed that other neurosteroids besides allopregnanolone conducted its actions, which might be due to a different enzymatic equipment for NS synthesis in the thalamocortical system compared to the hippocampus.

For further insight into substance effect on the thalamocortical connections an analysis of alteration of neuronal activity in each cortex layer upon thalamic stimulation was additionally undertaken. While a few tendencies can be derived from this layer analysis (see chapter 4.1.3), due to discrepancies in the results no ascertained conclusions can be drawn.

4.2 Clinical relevance, sources of error and outlook

While XBD and diazepam concentrations correspond with clinically used dosage, allopregnanolone concentration was quite elevated in comparison to endogenous found concentrations. Little is known about the XBD-induced synthesis of other neurosteroids than allopregnanolone. However, the experiments with allopregnanolone served to determine general discrepancies to XBD. Even though allopregnanolone has been applied at low doses, the effects of allopregnanolone on neuronal activity might change remarkably when concentrations are adapted to physiological levels.

Possible source of interference could have been the voltage sensitive dye di-4-ANEPPS, which is known to have a modulating effect on GABA-receptors itself (Mennerick et al. 2010). However, these effects can be considered negligible, since control recordings were equally affected as recordings in the presence of substances.

Another TSPO ligand, alpidem, has already been approved for anxiety treatment in France in 1991 (Langer et al. 1990). Due to severe liver damage with profound and even lethal consequences it was withdrawn in 1994 (Barki et al. 1993, Baty et al. 1994, Ausset et al. 1995). However, different to XBD, alpidem also binds to $GABA_A$ receptors directly, which could also be the cause for liver dysfunction (Langer et al. 1990). Nevertheless, further assessment of possible adverse effects and tolerance in long-term clinical studies, including studies on diseased patients, are necessary to investigate long-term tolerability and confirm clinical safety and relevance before XBD can be introduced as a treatment option in anxiety disorders.

4.3 Conclusion

According to the data collected, XBD reduces neuronal activity in the amygdala, correspondingly counteracting anxiety, while not significantly modulating neuronal networks in the hippocampus, explaining the lack of anterograde amnesia. As for its alteration of neuronal activity in the thalamocortical system, further investigations such as clinical studies determining XBD effect on alertness need to be realized previous to final conclusions. To further elucidate the discrepancy concerning the adverse sedative effect, the neurophysiological impact of diazepam and XBD on

brainstem areas involved in sleep-wake rhythms such as the reticular formation are of considerable interest.

Similar to XBD, diazepam affects neuronal activity in the amygdala and the thalamocortical circuits, but unlike XBD, also demonstrated diminishing effects on neuronal activity in the hippocampus, which is in line with clinical observation of anterograde amnesia upon benzodiazepine application (Rote-Liste 2013, 2015). In comparison, the presented data provides further evidence for XBD as a promising anxiolytic drug to be superior to diazepam, regarding its side effect profile (Kita et al. 2009, Rupprecht et al. 2009).

The acquired data suggests that allopregnanolone is not the only neurosteroid formed upon XBD application. This can be drawn from the fact that XBD and allopregnanolone utilization in the thalamocortical connections resulted in different change of stimulation propagation. Furthermore, in contrast to XBD, allopregnanolone reduced stimulation propagation in the hippocampus. This indicates that, while allopregnanolone could affect neuronal networks in the amygdala, XBD application does not lead to allopregnanolone formation in the hippocampus. This is in line with the observation that neurosteroid biosynthesis is region and neuron specific and depends on TSPO abundance and expression of the respective enzymes, responsible for genesis of the diverse neurosteroids (Rupprecht et al. 2010). Region-specific synthesis of neurosteroids is subsequently responsible for distinct modulation of neurotransmitter receptors in certain brain areas, resulting in region-specific alteration of signal transduction. Additionally, distribution of $GABA_A$ receptor subtypes adds to region specific alteration of signal transduction, since they vary in binding capabilities (Hosie et al. 2006). Therefore, there are diverse parameters relevant for the pharmacological profile of XBD, including TSPO abundance, distribution of the respective enzymes needed for neurosteroid syntheses and $GABA_A$ receptor subtypes.

5 Abstract

Despite the high prevalence of anxiety disorders in today's society, suitable anxiolytic medication needs yet to be established. Frequently used benzodiazepines show rapidly on-setting anxiolytic effects. However, they elicit a broad range of adverse effects such as sedation and anterograde amnesia. XBD, a ligand to the Translocator Protein 18 kDa (TSPO), is a promising new option. XBD enhances TSPO mediated syntheses of neurosteroids, which have been shown to exert anxiolytic effects. Clinical studies suggest XBD to lack unfavorable side effects commonly known in benzodiazepine treatment. Like benzodiazepines, neurosteroids e.g. allopregnanolone act as positive allosteric modulators at the $GABA_A$ receptor, though they occupy another binding site. The discordance in the side effect profile is thought to origin in $GABA_A$ subtype distribution in the brain. This present study investigates effects of XBD and diazepam on diverse brain areas using an imaging technique, VSDI, that enables monitoring of signal propagation in the brain. The effects of XBD and diazepam in diverse brain areas were monitored and compared. While both substances showed considerable and similar impact on stimulus propagation in the amygdala, the anxiety center of the brain, only diazepam affected neuronal activity in the hippocampus, the essential brain area for memory consolidation. The collected data suggest similar effects on neuronal activity in the amygdala (development of anxiety), while XBD lacks the negative effect of benzodiazepines on neuronal activity in the hippocampus (known for memory formation). This is concordant with clinical studies where XBD lacks the adverse effect of anterograde amnesia much known in benzodiazepine treatment, while exerting similar anxiolytic properties. Like diazepam, XBD affected neuronal activity in the thalamocortical system, which might be an indicator that XBD could impair alertness and concentration. To investigate the mechanism of action of XBD, an additional set of experiments was performed with allopregnanolone as presumably the most important neurosteroid promoted upon XBD application. The discrepancies in neuronal activity observed after XBD or allopregnanolone application in different brain regions may be explained by the fact that it is highly probable that beside allopregnanolone, XBD enhances the syntheses of other neurosteroids which are also pharmacological active. Furthermore, neurosteroid synthesis upon TSPO activation depends on the specific enzymatic machinery needed for neurosteroid formation. Which, at least in the hippocampus, does not allow the synthesis of allopregnanolone. Altogether, the data collected provides further evidence that XBD promises to be superior to diazepam in pharmacological treatment of anxiety disorders.

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7 Appendix

Used Substances

Sucrose-based-ACSF: pH ≈ 7,37 (saturated with carbogen) by Ying und Goldberg (Ying et al. 2005)

Substance	Concentration (mM)	Manufacturer
$CaCl_2$	0,5	Sigma Aldrich
Glucose	11	Sigma Aldrich
Sucrose	234	Sigma Aldrich
KCl	2,5	Sigma Aldrich
$MgSO_4$	10	Sigma Aldrich
$NaHCO_3$	24	Sigma Aldrich
NaH_2PO_4	1,25	Sigma Aldrich

General ACSF: pH ≈ 7,37 (saturated with carbogen)

Substance	Concentration (mM)	Manufacturer
$CaCl_2$	2	Sigma Aldrich
D(+)-Glucose	25	Sigma Aldrich
KCl	2,5	Sigma Aldrich
$MgCl_2$	1	Sigma Aldrich
NaCl	125	Sigma Aldrich
$NaHCO_3$	25	Sigma Aldrich
NaH_2PO_4	1,25	Sigma Aldrich

Further Chemicals:

Substance	Concentrations used	Manufacturer
Allopregnanolone	1 µM (DMSO)	Sigma Aldrich
Bicucullin	0,6 µM (DD)	Sigma Aldrich
Di-4-Annepps	see ch. 2.2.1	Sigma Aldrich
Diazepam	1 µM (DMSO)	Sigma Aldrich
DMSO	-	Sigma Aldrich
Finasterid	10 µM (DMSO)	Sigma Aldrich
Flumazenil	10 µM (DMSO)	Sigma Aldrich
XBD 173	3 µM (70% Alcohol)	Novartis

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