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$GABA_A$ receptor subunit-related neuroanatomical substrates for attenuation of anxiety

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Abstract

GABAergic inhibition is crucial for regulation of neuronal excitability and hence for optimal function of the nervous system. The $GABA_A$ receptor forms a pentameric ligand-gated anion channel. The subunit combinations of the receptors define their pharmacological and electrophysiological properties. The individual subunits display a distinct but often widespread distribution throughout the nervous system. $GABA_A$ receptor-mediated inhibition can be divided to fast and transient synaptic inhibition and to background (tonic) inhibition that is mediated by extrasynaptic $GABA_A$ receptors. The synaptic inhibition regulates the rhythmic activities in neuronal networks. Benzodiazepines, interacting with an allosteric binding site of $GABA_A$ receptors typically concentrated at synaptic locations, are anxiolytic drugs, but their long-term use evokes severe side-effects. The functional role of extrasynaptic $GABA_A$ receptor-mediated inhibition is not yet well understood. Expanding the understanding of local role of $GABA_A$ receptors and neuronal circuits mediating anxiolysis is relevant for the development of more selective and safe treatment for anxiety disorders. Studying the properties of extrasynaptic $GABA_A$ receptors may help to understand their physiological relevance and role in psychiatric and neurological disorders.

The main objectives of this thesis were to study the local expression of benzodiazepine-sensitive $GABA_A$ receptors in human locus coeruleus (LC), the pharmacology of extrasynaptic $GABA_A$ receptors *in vivo* (in mice) and *in vitro* and the brain structures mediating acute anxiolytic responses in a transgenic mouse model as a consequence of enhanced tonic inhibition in specific forebrain structures. Firstly, the present *in situ* hybridization and receptor autoradiography studies in human LC revealed benzodiazepine binding sites indicating that LC may directly mediate the sedative and/or anxiolytic effects of benzodiazepines in humans. Previously, contradictory reports had been published in regard to the benzodiazepine binding sites in the rodent LC suggesting a potential species difference in the direct sites of action of benzodiazepines. Secondly, the behavioral tests showed that gaboxadol, a GABA site agonist, acts preferentially via extrasynaptic α6β/γ2 receptors *in vivo* as the transgenic mice overexpressing these receptors were significantly more sensitive to the anxiolytic and hypnotic effects of gaboxadol than the wild-type mice. Electrophysiological recordings on recombinant receptors revealed that GABA is a partial agonist as compared to gaboxadol at α6β3, α6β3δ and α4β3δ receptors but not at α6β3γ2 receptors. Gaboxadol and GABA displayed also different receptor desensitization and deactivation kinetics on these receptors. Thirdly, by increasing tonic inhibition in specific forebrain structures, the anxiolytic dose of gaboxadol increased c-Fos expression in the transgenic mouse model especially in the limbic areas, such as the cingulate cortex, septal nuclei, central extended amygdala and basolateral nucleus of

amygdala. In addition to demonstrating how neuronal excitability can be altered in different brain regions as a consequence of enhanced tonic inhibition, this result suggests that a widespread neuronal inhibition, as typically associated with benzodiazepines, may not be the exclusive mechanism of acute anxiolysis.

List of original publications

I Hellsten K. S., Sinkkonen S. T., Hyde T. M., Kleinman J. E., Särkioja T., Maksimow A., Uusi-Oukari M., Korpi E. R. (2010). Human locus coeruleus neurons express the $GABA_A$ receptor γ2 subunit gene and produce benzodiazepine binding. Neurosci. Lett. 477(2):77-81.

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II Saarelainen K. S., Ranna M., Rabe H., Sinkkonen S. T., Möykkynen T., Uusi-Oukari M., Linden A. M., Lüddens H., Korpi E. R. (2008). Enhanced behavioral sensitivity to the competitive GABA agonist, gaboxadol, in transgenic mice over-expressing hippocampal extrasynaptic alpha6beta $GABA_A$ receptors. J. Neurochem. 105(2):338-350.

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III Hellsten K. S., Linden A. M., Korpi E. R (2015). Paradoxical widespread c-Fos expression induced by a GABA agonist in the forebrain of transgenic mice with ectopic expression of the $GABA_A$ α6 subunit. Neuroscience 293:123-135.

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Abbreviations

ACTH	adrenocorticotropic hormone
AHN	anterior hypothalamic nucleus
AP2	clathrin adaptor protein 2
Best1	bestrophin 1 channel
BLA	basolateral nucleus of amygdala
BNST	bed nucleus of stria terminalis
CA	carbonic anhydrase
CAML	calcium-modulating cyclophilin ligand
CeA	central nucleus of amygdala
CRH	corticotropin-releasing hormone
CS	conditioned stimulus
dlPAG	dorsolateral part of periaqueductal gray
DMCM	methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate
dmPAG	dorsomedial part of periaqueductal grey
dmPMD	dorsomedial part of dorsal premammillary nucleus
dmVMH	dorsomedial part of ventromedial hypothalamic nucleus
E_{Cl^-}	reversal potential of chloride ions
$E_{GABA\text{-}A}$	reversal potential of $GABA_A$ receptor
ER	endoplasmic reticulum
fMRI	functional magnetic resonance imaging
$GABA_A$	γ-aminobutyric acid A
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase
GAD	generalized anxiety disorder
GAT	GABA transporter
GIS	GABA-insensitive
GnRH	gonadotropin releasing hormone
GODZ	Golgi-specific DHHC (aspartate-histidine-histidine-cysteine) zinc finger protein
GTPase	guanosine triphosphatase
HAP1	huntingtin-associated protein 1
HPA	hypothalamic-pituitary-adrenal
ICM	intercalated cell masses
IL	infralimbic cortex
KCC2	potassium-chloride co-transporter 2
KIF5	kinesin superfamily motor protein 5
LA	lateral nucleus of amygdala
LC	locus coeruleus
LORR	loss of righting reflex
MAOB	monoamine oxidase B
MDTB	mouse defense test battery
MEA	medial amygdala

mIPSC	miniature inhibitory postsynaptic current
mPFC	medial prefrontal cortex
MPN	medial preoptic nucleus
MRS	magnetic resonance spectroscopy
NKCC1	sodium-potassium-chloride co-transporter 1
NMDA	N-methyl-D-aspartate
pBMA	posterior part of basomedial amygdala
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
pdMEA	posterodorsal part of medial amygdala
PET	positron emission tomography
PFC	prefrontal cortex
PL	prelimbic cortex
PLIC	protein linking integrin associated protein with cytoskeleton
PMV	ventral premammilliary nucleus
pvMEA	posteroventral part of medial amygdala
rCBF	regional cerebral blood flow
SAD	social anxiety disorder
SPECT	single photon emission computed tomography
TBPS	*t*-butyl-bicyclo-phosphorothionate
TH	tyrosine hydroxylase
TM	transmembrane
US	unconditioned stimulus
vlPAG	ventrolateral periaqueductal gray
vlPMD	ventrolateral part of dorsal premammillary nucleus
vlVMH	the ventrolateral part of ventromedial hypothalamus
Vm	membrane potential
VMH	ventromedial hypothalamus

1 Introduction

$GABA_A$ (γ-aminobutyric acid A) receptors are ligand-gated anion channels. There are many different isoforms of $GABA_A$ receptors, each consisting of five subunits arranged around a central chloride and bicarbonate selective ion channel. The subunit arrangement of the receptor defines the physiological and pharmacological properties of the receptors. In adult mammalian brain, activated $GABA_A$ receptors typically hyperpolarize neurons, and therefore GABA is traditionally considered as the main inhibitory transmitter in mature brain. The agonist binding to the ligand-binding domain triggers a conformational change of the $GABA_A$ receptor, which results in the opening of the gate permitting chloride and bicarbonate ions to flow through the channel. The functional outcome of the $GABA_A$ receptor activation depends on the intra- and extracellular distribution of the chloride and bicarbonate anions and on the membrane potential of the neuron (reviewed in Farrant and Nusser, 2005).

$GABA_A$ receptor-mediated inhibition can be divided to fast and transient synaptic inhibition and to background (tonic) inhibition that is mediated by extrasynaptic $GABA_A$ receptors. An important function of phasic inhibition is the generation and maintaining of rhythmic activities in neuronal networks, such as theta and gamma frequency network oscillations associated with cognitive functions (Buzsáki and Chrobak, 1995). Benzodiazepine diazepam is an allosteric modulator of α1–3 (or 5) and γ2 subunits-containing $GABA_A$ receptors, *i.e.* receptors typically concentrated at synaptic locations. The α1 subunit-containing receptors mediate diazepam-induced sedation and anterograde amnesia (Rudolph et al., 1999; McKernan et al., 2000), whereas the α2 subunit-containing receptors mediate anxiolysis (Löw et al., 2000). Both α2 and α3 (and α5) subunit-containing receptors are associated with myorelaxant effects of diazepam (Crestani et al., 2001; Milić et al., 2012). Tonic GABAergic inhibition is usually mediated via receptors containing α4, α5, α6 or δ subunits making them highly sensitive to GABA and thus suitable for detection of low GABA concentrations (Brickley et al., 1996; Farrant and Nusser, 2005; Lee and Maguire, 2014). Although not yet well understood, tonic $GABA_A$ receptor-mediated inhibition may play an important role in physiological functions as well as in psychiatric and neurological disorders (Belelli et al., 2009; Egawa and Fukuda, 2013). The pharmacological investigations of extrasynaptic $GABA_A$ receptors may help to understand their physiological relevance and role in psychiatric and neurological disorders.

Anxiety disorders are the most frequent mental disorders in the contemporary EU countries, Switzerland, Norway and Iceland estimated to affect 14% of population across all age groups

(Wittchen et al., 2011). Anxiety disorders are pharmacologically treated by benzodiazepines but the adverse effects such as dependence, tolerance, drowsiness and impairment of cognition limit their use in chronic anxiety disorders (Millan, 2003; Farb and Ratner, 2014). Selective serotonin reuptake inhibitors and serotonin/noradrenaline reuptake inhibitors are currently used as the first-line pharmacological treatment of anxiety disorders, but many individuals are not responsive to these drugs and their adverse effects such as nausea, dizziness and sexual problems can also limit their use (Millan, 2003; Farb and Ratner, 2014). Expanding the understanding of the pharmacology and local roles of $GABA_A$ receptors and the neuronal circuits mediating anxiolysis is relevant for the development of more selective and safe treatment for anxiety disorders.

2 Review of the literature

2.1 GABA$_A$ receptors

2.1.1 Subunits and their assembly

GABA$_A$ receptors are ligand-gated anion channels. The GABA$_A$ receptors belong to the superfamily of the Cys-loop pentameric ligand-gated ion channels. Each subunit of the receptors in this superfamily consists of a long N-terminal extracellular hydrophilic region, followed by four transmembrane (TM) segments and ending with a relatively short extracellular C-terminal domain. The TM2 segment forms the lining of the ion channel, and there is a large intracellular loop between the TM3 and TM4 segments (Olsen and Sieghart, 2009). Two cysteine residues spaced by 13 amino acids form the cysteine loop at the N-terminal extracellular domain of the GABA$_A$ receptor (Korpi et al., 2002a) (Figure 1).

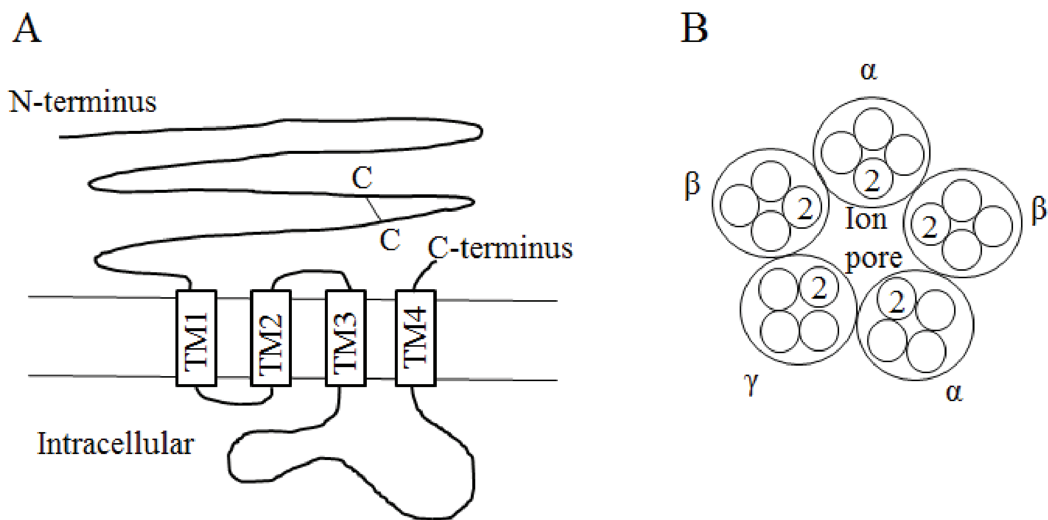

Figure 1. *A. Each GABA$_A$ receptor subunit contains an N-terminal extracellular hydrophilic region, four transmembrane segments (TM) and short extracellular C-terminal domain. The cysteine loop (C-C) locates at the N-terminal extracellular domain of each subunit. B. The TM2 domain (2) of each subunit of the pentameric receptor forms the wall of the ion channel. The majority of native GABA$_A$ receptors are composed of two α, two β and one γ subunit.*

There are many different isoforms of GABA$_A$ receptors, each consisting of five subunits surrounding a central chloride and bicarbonate selective ion channel. The subunit arrangement of the receptor defines its physiological and pharmacological properties. The individual subunits

display a distinct but often widespread distribution throughout the nervous system varying in their regional, cellular and sub-cellular localization. Therefore they have different roles in brain circuits and behaviors. Currently 19 subunits of $GABA_A$ receptor are known; α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3, and an alternative splicing contributes to an additional receptor diversity (reviewed in Uusi-Oukari and Korpi, 2010; Olsen and Sieghart, 2008; Rudolph and Knoflach, 2011). The homopentameric $GABA_A$ receptors consisting of ρ subunits have sometimes been called $GABA_C$ receptors because they are insensitive to bicuculline (blocks response to GABA in other $GABA_A$ receptors) and baclofen (stimulates metabotropic $GABA_B$ receptors), but the Nomenclature Committee of the International Union of Pharmacology (IUPHAR) has recommended not to use $GABA_C$ nomenclature for such receptors (Barnard et al., 1998; Bormann, 2000).

The receptor subunit assembly takes place in the endoplasmic reticulum (ER). Unassembled or improperly folded receptor subunits are subject to degradation and are not targeted to the cell surface (Luscher et al., 2011). Based on the current knowledge, it has been estimated that more than 800 distinct $GABA_A$ receptor subtypes may exist in the brain. Subunit-specific antibodies have revealed that receptors composed of α1β2γ2 subunits are the most abundant $GABA_A$ receptors in the brain (Olsen and Sieghart, 2009).

2.1.2 Binding sites

$GABA_A$ receptors contain different binding sites for different types of ligands. The GABA and benzodiazepine binding sites are located at extracellular regions of the $GABA_A$ receptors whereas barbiturates, neurosteroids and alcohol have been suggested to bind mostly to the TM regions of the receptors. Picrotoxin and TBPS (*t*-butyl-bicyclo-phosphorothionate) bind to the channel pore (Korpi et al., 2002a). Below is a more detailed review of the binding sites and the respective ligands that are the most relevant for this thesis.

GABA binding site. As revealed mostly by studies on recombinant receptors, the GABA binding site has been located at the interface of α and β subunits. The amino acid residue Phe64 of the α1 subunit forms part of the functional GABA binding site (Sigel et al., 1992; Smith and Olsen, 1994 and 1995; Boileau et al., 1999). This amino acid has been proposed to contribute to a low-affinity binding site for GABA (Baur and Sigel, 2003). Also other amino acids (Arg66, Ser68, Arg120, Val178, Val180 and Asp183) of the α1 subunit have been associated with the low-affinity GABA binding site at the extracellular N-terminus of the $GABA_A$ receptor (Boileau et al., 1999; Westh-Hansen et al., 1999; Newell and Czajkowski, 2003). A conformational variant of the low-affinity binding site has been proposed to form a high-affinity binding site for GABA (Smith and Olsen

1994; Newell et al. 2000; Baur and Sigel., 2003). Alternatively, the high-affinity GABA binding site has been proposed to locate at a different subunit interface, separate from the low-affinity binding site (Newell et al., 2000). Nevertheless, the affinity of GABA to the binding site is largely dependent on the type of α subunit in the receptor complex (Hevers and Lüddens, 1998). The α6 subunit-containing receptors are the most sensitive to GABA with EC_{50} values of 0.2-2 μM (Ducic et al., 1995; Knoflach et al., 1996; Saxena and Macdonald, 1996), whereas in α3 subunit-containing receptors the EC_{50} value for GABA is approximately ten or hundred times higher (Sigel et al., 1990; Ebert et al., 1994; Verdoorn, 1994). GABA has an intermediate affinity to the α1, α2, α4 or α5 containing $GABA_A$ receptors (Wafford et al., 1993; Ebert et al., 1994; Knoflach et al., 1996). $GABA_A$ receptors containing the δ subunit are ten times more sensitive to GABA than receptors containing the γ subunit (Saxena and Macdonald, 1996; Hevers et al., 2000; Brown et al., 2002). In addition, GABA has higher affinity to αβ receptors than to αβγ (Sigel et al., 1990; Horne et al., 1993).

Also several GABA analogs bind to the GABA binding site. Muscimol has been regarded as a substance that can activate all $GABA_A$ receptor subtypes (Krogsgaard-Larsen et al, 1979). In early autoradiographic experiments, $[^3H]$muscimol was used to label GABA binding sites (Olsen et al., 1990). However, low concentrations (10 nM) of $[^3H]$muscimol appeared to reveal only a fraction of all $GABA_A$ receptors as the binding signal was not as widely distributed as after radioactively labelled benzodiazepine or channel site ligands (Korpi et al., 2002a). This high-affinity $[^3H]$muscimol binding has been associated with α6 and δ subunits in cerebellum and with α4 and δ subunits in the forebrain, as the high-affinity $[^3H]$muscimol binding was reduced in the cerebellar and forebrain sections of the δ-deficient mice (Chandra et al., 2010; Korpi et al, 2002b; Mihalek et al., 1999), in the forebrain of α4-knock-out mice (Chandra et al., 2010) and in the cerebellar sections of the α6-knock-out mice (Mäkelä et al., 1997). Chandra et al. (2010) studied also the motor performance of several mutant mice by fixed speed rotarod after an *i.p.* injection of muscimol at doses estimated to become bioavailable at nanomolar concentrations in mouse brains. The α4- and δ-deficient mutant mice displayed a decreased behavioral sensitivity to muscimol as compared to their wild-type controls although recombinant receptors have been paradoxically reported to exhibit unaltered binding affinities to $[^3H]$muscimol in the presence or absence of the δ-subunit (Hevers et al., 2000). The α1-knock-out mice had an unaltered behavioral sensitivity to muscimol as well as an unaltered high-affinity $[^3H]$muscimol binding (Chandra et al., 2010). The transgenic Thy1α6 mice overexpressing the α6 subunit in the forebrain including hippocampus and cerebral cortex showed an increased high-affinity $[^3H]$muscimol binding in these brain regions and had an increased behavioral sensitivity to muscimol (Chandra et al., 2010).

Muscimol has been used as a model substance to design several other GABA analogs, such as gaboxadol (Krogsgaard-Larsen et al., 2004). Also gaboxadol is a GABA-site agonist (Karobath and Lippitsch 1979; Wafford et al., 1996; Ebert et al., 1997), and the regional distribution of high-affinity binding of [^{3}H]gaboxadol resembles that of [^{3}H]muscimol in the rat brain (Friemel et al., 2007) as it acts preferentially via α4 and δ subunit-containing $GABA_A$ receptors in the brain (Belelli et al. 2005; Cope et al. 2005; Jia et al. 2005; Chandra et al. 2006; Storustovu and Ebert 2006; Winsky-Sommerer et al. 2007, Meera et al., 2011). Although gaboxadol has been shown to be less potent (EC_{50} 13 μM) than GABA (EC_{50} 0.35 μM) or muscimol (EC_{50} 0.20 μM) at recombinant human α4β3δ receptors, it has been shown to be more efficient (E_{max} 224%) than GABA (E_{max} 98%) or muscimol (E_{max} 120%) at these receptors (Mortensen et al., 2010). Native α4 and δ subunit-containing $GABA_A$ receptors on thalamic neurons (Chandra et al. 2006; Herd et al. 2009) as well as α6 and δ subunit-containing $GABA_A$ receptors on cerebellar granule cells were shown to be responsive to sub-micromolar concentrations of gaboxadol, and such gaboxadol sensitivity required the δ subunit (Meera et al., 2011). In addition, gaboxadol has been reported to be a partial agonist at α1 subunit-containing receptors such as α1βγ2 receptors and a full agonist at α2 containing receptors as compared to GABA (Ebert et al., 1994; Ebert et al., 1997; Wafford and Ebert, 2006).

Ionophore binding site. The ionophore binding site of the $GABA_A$ receptor is also called a convulsant site or an ion channel site. Binding of compounds such as picrotoxinin and [^{35}S]TBPS to the ionophore binding site, block the chloride ion flow through the $GABA_A$ receptor ion channel and cause convulsions (reviewed in Korpi et al., 2002a). The exact binding sites of these compounds are not known. It has been suggested that although picrotoxinin and [^{35}S]TBPS possibly bind to the same site of the $GABA_A$ receptor, they may have different structural requirements. Electrophysiologically detected picrotoxinin block has been reported in homomeric receptors composed of α, β, γ, δ and ρ subunits and in heteromeric receptors of αβ, βγ, αγ and αβγ combinations. [^{35}S]TBPS binding has been detected in homomeric β receptors and in heteromeric αβ, βγ and αβγ combinations but not in homomeric α or γ receptors or in heteromeric receptors composed of αγ subunits (reviewed in Sieghart et al., 1995). Electrophysiological recordings have revealed that TBPS can block also homopentameric ρ receptors (Vale et al., 1999). Picrotoxin (an equimolar mixture of picrotoxinin and picrotin) has been proposed to interact with Val257 of ion channel lining TM2 residue of rat α1 subunits (Xu et al., 1995). In β3 subunits, Ala252 and Leu253 of TM2 have been proposed to be important for picrotoxin and TBPS affinity (Buhr et al., 2001), and Val251, Ala252 and Leu253 of TM2 have been proposed to be important for the formation of a high-affinity binding site for TBPS (Jursky et al., 2000).

Benzodiazepine binding site (zolpidem, flumazenil, diazepam, Ro 15-4513). Benzodiazepine site ligands modulate allosterically GABA-induced currents via $GABA_A$ receptors only in the presence of GABA. The high-affinity benzodiazepine binding site is located at the interface between α and γ subunits (Sigel and Buhr, 1997; Ernst et al., 2003). The type of β subunit present in these receptors is not likely to significantly modulate the benzodiazepine action. Benzodiazepine site ligands may also interact with receptors containing γ1 or γ3 instead of γ2 subunits with different pharmacological profiles (Wafford et al., 1993; Sieghart, 1995; Hevers and Luddens, 1998; Khom et al., 2006).

Ro 15-4513 exhibits high affinity to all $GABA_A$ receptors consisting of αβγ subunit combinations (reviewed in Sieghart, 1995). It usually acts as a partial inverse agonist at the benzodiazepine sites (Bonetti et al., 1988; Hadingham et al., 1993; Korpi et al., 2002a). However, Ro 15-4513 is an agonist in α4/6βγ2 receptors (Bonetti et al., 1988; Knoflach et al., 1996) and it has a slight positive modulatory effect in α2β1γ1 receptors (Wafford et al., 1993). Flumazenil (Ro 15-1788) is a benzodiazepine site competitive antagonist that binds to the same $GABA_A$ receptor subunit combinations as Ro 15-4513 (Sieghart, 1995).

Diazepam has a comparable affinity and efficacy at α1βγ2, α2βγ2, α3βγ2 and α5βγ2 subunit combinations (Sieghart, 1995; Hevers and Luddens, 1998), but α4βγ2 and α6βγ2 receptors are diazepam-insensitive (Wisden et al., 1991; Wieland et al., 1992; Luddens et al., 1995). It has been shown that the amino acid arginine at the position 99 and 100 of the α4 and α6 subunit, respectively, plays an important role in their insensitivity to diazepam (the equivalent amino acid is histidine in the other α variants) (Wieland et al., 1992; Korpi et al., 2002a). Substituting the arginine of the α6 subunit by histidine made the receptor sensitive to diazepam, whereas substituting the histidine at the homologous position 101 by arginine in the α1 subunit, made the α1 subunit-containing receptors insensitive to diazepam (Wieland et al., 1992). In a study by Benson et al. (1998), it was shown that the replacement of the conserved histidine residue of α1, 2, 3 and 5 by arginine (H101R, H101R, H126R and H105R, respectively) resulted in diazepam-insensitivity of the respective αβ2/3γ2-receptors. Furthermore, Ro 15-4513 acted as an agonist at these mutant receptors.

Diazepam-sensitive receptors contain zolpidem-sensitive and -insensitive receptors (Luddens et al., 1995). Zolpidem binds with high affinity to α1βxγ2 $GABA_A$ receptors and $GABA_A$ receptors containing γ1 or γ3 subunits exhibit little to no zolpidem sensitivity (Luddens et al., 1994; Sanna et al., 2002). Zolpidem has a low affinity to $GABA_A$ receptors containing α2 or α3 subunits, and

receptors containing the α5 subunit are nearly zolpidem-insensitive (Pritchett and Seeburg, 1990; Luddens et al., 1995; Sieghart, 1995).

Furosemide binding site. Furosemide does not cross the blood-brain barrier (Seelig et al., 1994), and therefore it does not bind to the brain $GABA_A$ receptors *in vivo*. *In vitro*, furosemide has been shown to antagonize the function of α6 and β2/3 subunits containing $GABA_A$ receptors, and the effect was demonstrated to be independent of the presence of γ or δ subunits (Korpi and Luddens, 1997). Furosemide sensitivity was shown to involve the amino acid isoleucine at the position 228 in TM 1 of the α6 subunit, but also other residues were likely to be involved (Thompson et al., 1999). The α4β3γ2 receptor was shown to be less sensitive to furosemide than the α6β3γ2 receptor, but it was more than 50-fold more sensitive than receptors containing other α subunits, *i.e.* α1β3γ2, α2β3γ2, α3β3γ2 or α5β3γ2 (Wafford et al., 1996). The action of furosemide has also been shown to be dependent on the β-subunit type, being weaker at β1-containing receptors than at β2- or β3-containing receptors (Korpi et al., 1995).

2.1.3 Hyperpolarizing action

In adult mammalian brain, activated $GABA_A$ receptors typically hyperpolarize neurons, and therefore GABA is traditionally considered as the main inhibitory transmitter in mature brain. The agonist binding to the ligand-binding domain triggers a conformational change of the $GABA_A$ receptor, which results in the opening of the gate permitting chloride and bicarbonate ions to flow through the channel. The functional outcome of the $GABA_A$ receptor activation depends on the intra- and extracellular distribution of the chloride and bicarbonate anions and on the membrane potential of the neuron (Farrant and Nusser, 2005). A hyperpolarizing GABA response might not be inhibitory if it indirectly triggers hyperpolarization-activated depolarizing mechanism (Kaila et al., 1997; Farrant and Nusser, 2005).

In most mature neurons, the activity of the potassium-chloride co-transporter KCC2 that extrudes chloride ions from the neuron to the extracellular space causes a chloride equilibrium potential that is more negative than the resting membrane potential (Figure 2). In contrast, the equilibrium potential for bicarbonate is more positive than the resting membrane potential (Farrant and Nusser, 2005). However, chloride ions are much more permeable than bicarbonate ions. It has been estimated that the relative bicarbonate/chloride permeability of $GABA_A$ receptors ranges between 0.18 and 0.6 (Fatima-Shad and Barry, 1993). Consequently, $GABA_A$ receptor activation typically results in a net influx of anions, causing hyperpolarizing inhibitory postsynaptic potential (IPSP) and decreasing the probability of an action potential initiation (Farrant and Nusser, 2005).

2.1.4 Depolarizing action

$GABA_A$ receptor-mediated currents may also be depolarizing. Depolarizing responses can be generated by activated $GABA_A$ receptors in an immature brain. At early stages of development GABAergic signals act as an important source of excitatory drive and modulate neuronal migration and circuit formation (reviewed in Ben-Ari and Spitzer, 2010). High intracellular chloride concentration leads to chloride ion efflux via the $GABA_A$ receptor channels once activated. During prenatal and early postnatal life, hippocampal and cortical neurons display positive reversal potential of chloride ions (E_{Cl^-}) relative to membrane potential (V_m) as a result of robust activity of NKCC1 (sodium-potassium-chloride co-transporter) but a minimal activity of KCC2 (potassium-chloride co-transporter) (Ben-Ari et al., 2012). NKCC1 is driven by sodium gradient and accumulates intracellular chloride ions whereas chloride ion efflux is mediated largely by KCC2 driven by potassium gradient (Figure 2). The spike threshold differs in different immature neurons and therefore GABA may depolarize some neurons and excite others. For instance, GABA has been reported to depolarize pyramidal neurons and interneurons in both deep and superficial layers of the immature neocortex, but to generate action potentials only in deep layers (L5/6) but not in superficial layers (L2/3) because L5/6 pyramidal cells have more depolarized resting potentials and more hyperpolarized threshold of action potential generation (Rheims et al., 2008). If the spike threshold is above the reversal potential of $GABA_A$ receptor (E_{GABA-A}), GABAergic depolarization can trigger activation of voltage-gated currents that may enable to reach the spike threshold (Ben-Ari et al., 2012). Depolarizing $GABA_A$ receptor-mediated currents have also been reported in embryonic rat spinal and olfactory bulb cells (Serafini et al., 1995).

A negative shift in the E_{Cl^-} relative to V_m is associated with a robust increase in KCC2 expression, a decrease in functional activity of NKCC1 and with hyperpolarizing $GABA_A$ currents (Ben-Ari, 2002, Ben-Ari and Spitzer, 2010; Ben-Ari et al., 2012). In addition to the hippocampus and cortex a significant increase in the activity of KCC2 has been reported in mature retina, cerebellum and dorsal horn of the spinal cord as compared to that at birth (Ben-Ari et al., 2012).

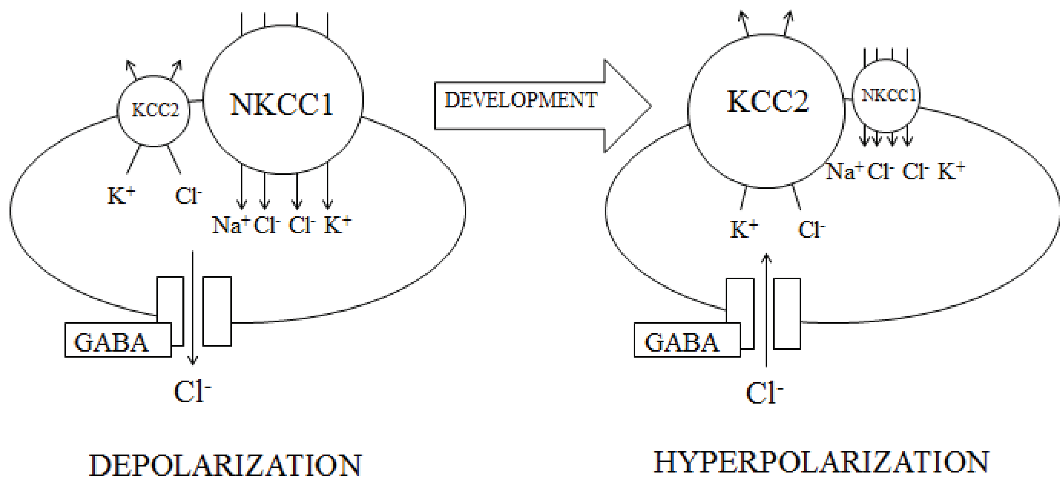

Figure 2. *The NKCC1 and KCC2 expression-dependent shift from $GABA_A$ receptor-mediated depolarization to hyperpolarization. In certain immature neurons, a robust activity of the chloride importer NKCC1 (sodium-potassium-chloride cotransporter) but a minimal activity of the chloride exporter KCC2 (potassium-chloride cotransporter) is present. In adult neurons the hyperpolarizing $GABA_A$ receptor mediated currents are associated with a robust increase in KCC2 expression and a decrease in functional activity of NKCC1 leading to a lower intracellular accumulation of chloride in mature neurons and to chloride ion influx as a result of $GABA_A$ receptor activation (modified from Ben-Ari et al., 2012).*

Depolarizing and excitatory responses can be generated by $GABA_A$ receptor activation also in a mature brain. Initially, depolarizing responses were recorded in hippocampal pyramidal cells when GABA or pentobarbitone was applied to their dendrites. $GABA_A$ receptors located at the dendritic tree were suggested to be depolarizing whereas those in the cell body or close to it were expected to be hyperpolarizing (Alger and Nicoll, 1979; Andersen et al., 1980). A few years later, Alger and Nicoll (1982) reported that GABA elicited hyperpolarizing responses in CA1 pyramidal cells when applied to the soma, but primarily depolarizing responses when applied to the apical dendrites, although hyperpolarizing responses were also recorded. They concluded that hyperpolarizing responses reflected the activation of synaptic receptors, which were highly concentrated on the pyramidal cell soma/initial segment, but that these receptors were also present on the dendrites. The depolarizing responses evoked in the dendrites were proposed to reflect the activation of extrasynaptic receptors (Alger and Nicoll, 1982). In adult hypothalamic gonadotropin releasing hormone (GnRH) neurons, $GABA_A$ receptors have been reported to exert both depolarizing and hyperpolarizing effects and the most recent studies indicate that the predominant action of

endogenously released GABA is excitation in slice preparations of adult hypothalamic GnRH neurons (Herbison and Moenter, 2011).

$GABA_A$ receptor-mediated depolarizing responses have been reported in several *in vitro* studies in adult guinea pig and rat hippocampus (Wong and Watkins, 1982; Michelson and Wong, 1991; Lambert et al., 1991; Xie and Smart 1993). The depolarizing postsynaptic response as a result of tetanic stimulation of the adult hippocampal GABAergic interneurons has been shown to be more effective in triggering spikes than the simultaneously activated glutamatergic inputs. In line with the finding, blockade of the $GABA_A$ receptors alleviated rather than promoted neuronal excitability (Taira et al., 1997). It was proposed that high-frequency stimulation mimicking the *in vivo* firing frequencies of GABA-releasing interneurons, and any other condition that increased the open time of the $GABA_A$ receptor, depolarized the postsynaptic membrane because the electrochemical gradient for chloride ions collapsed more significantly than that for bicarbonate ions (Staley et al., 1995). An intraneuronal carbonic anhydrase (CA) was proposed to compensate the bicarbonate efflux by regenerating bicarbonate of CO_2 that diffused across the membrane after being released from the extracellular bicarbonate ions. Intra- and extracellular pH buffers were binding and releasing, respectively, hydrogen ions for the process. It was shown that in rat hippocampal slices an intense stimulation of $GABA_A$ receptors on the distal dendrites produced a hyperpolarizing membrane potential response followed by a slow depolarizing potential that was 20 mV positive to the resting membrane potential (Staley et al., 1995). The phenomenon was proposed to occur because the intracellular chloride ion concentrations could change significantly in small structures such as in dendrites and therefore reduce the driving force for chloride whereas in larger structures the ionic gradients were more stable (Qian and Sejnowski, 1990). Also the voltage-dependent magnesium ion block of the N-methyl-D-aspartate (NMDA) receptor was proposed to be preferentially lessened in those dendrites with the most intense $GABA_A$ receptor activation resulting in higher membrane depolarization (Staley et al., 1995).

Later on, a new theory on the mechanism of GABA-mediated depolarization was presented (Figure 3). It involved GABAergic extracellular potassium ion transients ($[K^+]_o$) causing an inward potassium ion current that induced a long-lasting and a more positive membrane potential than E_{GABA-A} in spite of a positive shift in E_{GABA-A} (Kaila et al., 1997; Smirnov et al., 1999; Viitanen et al., 2010). It was proposed that in rat hippocampus, $GABA_A$ receptor-mediated excitation was dependent on bicarbonate efflux via $GABA_A$ receptor channels. Bicarbonate efflux caused depolarization that drove an equal influx of chloride ions via the $GABA_A$ receptor channel. This intracellular accumulation of chloride ions activated extrusion of chloride and potassium ions by

KCC2, thereby giving rise to a transient increase in the extracellular potassium concentration. The intracellular bicarbonate levels did not fall during its efflux phase because CA regenerated bicarbonate using CO_2 as a substrate. Bulk electroneutrality was maintained without a transmembrane cation influx since H^+ ions were produced in the hydration reaction of CO_2 at the same rate as bicarbonate ions. These H^+ ions were bound by cytoplasmic buffers to maintain the intracellular pH (Viitanen et al., 2010). It was also postulated that the bicarbonate-dependent increase in $[K^+]_o$ did not affect only the postsynaptic neurons but it also had a depolarizing influence on all nearby neurons, glial cells and presynaptic terminals, therefore having a non-synaptic nature (Smirnov et al., 1999).

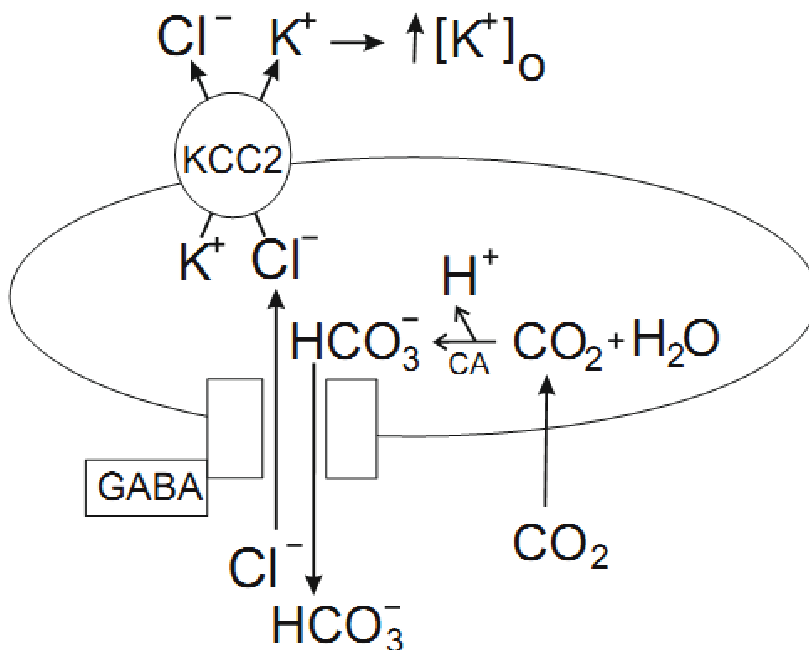

Figure 3. *Illustration of the ionic mechanism of the $GABA_A$ receptor-dependent increase in $[K^+]o$. The HCO_3^- efflux via $GABA_A$ receptors causes depolarization that drives uptake of Cl^- into the neuron. Carbonic anhydrase (CA) provides an intracellular source of HCO_3^- during its efflux from intracellular water and CO_2. The bulk electroneutrality of the neuron is maintained without a transmembrane cation flux because H^+ and HCO_3^- ions are produced at the same rate in the hydration reaction of CO_2. The intracellular pH is maintained because cytoplasmic buffers bind these H^+ ions. The HCO_3^--driven intraneuronal accumulation of Cl^- activates extrusion of Cl^- by KCC2, giving rise to a K^+ efflux and thereby to an increase in $[K^+]o$ (modified from Viitanen et al., 2010).*

More recently it has been proposed that the local intra- and extracellular chloride concentrations and hence the direction of chloride flux via $GABA_A$ receptors are determined by negatively charged impermeant intra- and extracellular macromolecules rather than by the function of KCC2 and NKCC1 (Glykys et al., 2014a). However, the cation-chloride transporters were considered critically important for restoring intracellular chloride concentrations and volume after signaling transients (Glykys et al., 2014b).

2.1.5 Synaptic $GABA_A$ receptors and phasic inhibition

Transient activation of synaptic $GABA_A$ receptors represents phasic type of GABAergic inhibition. An action potential at the nerve terminal triggers the presynaptic vesicles to fuse with the presynaptic membrane and to release GABA into the synaptic cleft (Figure 4). It has been estimated that each vesicle contains several thousand GABA molecules and an action potential generates high (a millimolar range) peak GABA concentration in a synaptic cleft. Miniature inhibitory postsynaptic currents (mIPSCs) are rapid and occur spontaneously when GABA is released from a single synaptic vesicle and activates nearly synchronously a proportion of the $GABA_A$ receptors in the underlying postsynaptic density. An action potential of the presynaptic neuron may trigger a synchronous or asynchronous release of multiple vesicles and an activation of $GABA_A$ receptors at adjacent postsynaptic densities within the same synaptic bouton. Currents that result from GABA spillover and activation of extrasynaptic receptors can be considered phasic if they are temporally linked to the GABA vesicle release event (Farrant and Nusser, 2005). An important function of phasic inhibition is the generation and maintaining of rhythmic activities in neuronal networks, such as theta and gamma frequency network oscillations associated with cognitive functions (Buzsáki and Chrobak, 1995).

It has been proposed that ten to a few hundred $GABA_A$ receptors are clustered to the postsynaptic membrane (Farrant and Nusser, 2005). Synaptic $GABA_A$ receptors contain typically the $\gamma 2$ subunit together the $\alpha 1$, $\alpha 2$, or $\alpha 3$ subunits and any β subunits (Brünig et al., 2002; Korpi and Sinkkonen, 2006) (Figure 4) although no $GABA_A$ receptor subunit is known to be exclusively located at synaptic membranes. The mechanism by which $GABA_A$ receptors are delivered to and maintained at synapses is poorly known and many proteins have been postulated to play a role in targeting and stabilizing $GABA_A$ receptors at the postsynaptic density (Farrant and Nusser 2005). Gephyrin has been proposed to play a role in stabilizing $GABA_A$ receptors at postsynaptic sites (Cherubini and Conti, 2001; Möhler et al., 2002; Lüscher and Keller, 2004), and this role may be receptor subtype-specific (Lüscher et al., 2011). Gephyrin interacts with microtubules and with several regulators of

microfilament dynamics (Kirsch and Betz, 1995; Mammoto et al., 1998). When $GABA_A$ receptors leave the area containing postsynaptic scaffold proteins, they become highly mobile and diffuse along the plane of the phospholipid bilayer (Lüscher et al., 2011).

The number of $GABA_A$ receptors in the postsynaptic membrane controls the efficacy of phasic inhibition. This is regulated by $GABA_A$ receptor subunit gene expression and subunit assembly, receptor exocytosis, diffusion, endocytosis, recycling and degradation (reviewed in Lüscher et al., 2011). $GABA_A$ receptor subunits assemble in ER involving an interaction with ER-associated chaperones (Lüscher et al., 2011). Unfolded or improperly folded subunits are ubiquitinated and degraded in proteasomes (Lüscher et al., 2011). Also chronic blockage of neural activity enhances ER-associated degradation (Lüscher et al., 2011). An interaction of α and β subunits with ubiquitin-like protein PLIC (protein linking integrin associated protein with cytoskeleton) inhibits their ubiquitination and promotes the exit of receptors from the ER to the Golgi (Lüscher et al., 2011). In the Golgi, the cytoplasmic cysteines of γ2 subunits are palmitoylated by the Golgi resident palmitoyltransferase [Golgi-specific DHHC zinc finger protein (GODZ)], which promotes the receptor translocation to the plasma membrane and synapses (Lüscher et al., 2011). Reduced expression of GODZ has been shown to reduce the number of $GABA_A$ receptors at synapses and the amplitude and frequency of mIPSCs and whole cell currents (Fang et al., 2006).

$GABA_A$ receptors are removed from the cell surface by endocytosis through clathrin-coated vesicles (Tehrani and Barnes, 1993; Kittler et al., 2000) that are specialized sites on the plasma membrane of eukaryotic cells responsible for the internalization of many cell-surface receptors and other membrane proteins (Gaidarov et al., 1999). The endocytosis occurs via interaction of $GABA_A$ receptor β and γ subunits with the clathrin adaptor protein AP2 after which the complex interacts with other binding partners including clathrin, guanosine triphosphatase (GTPase) dynamin and its binding partner amphiphysin (Kittler et al., 2000; Lüscher et al., 2011). Phosphorylation of β (S408/409 in β3) and γ (Y365/367 in γ2) subunits interferes with these interactions and stabilizes the receptors at plasma membrane. Endocytosed receptors that are ubiquitinated at γ2 subunits are targeted to lysosomal degradation whereas those interacting with a calcium-modulating cyclophilin ligand (CAML) and huntingtin-associated protein 1 (HAP1) at γ2 and β subunit, respectively, are recycled back to plasma membrane by kinesin superfamily motor protein 5 (KIF5) -dependent vesicular transport. (Lüscher et al., 2011).

The efficacy of synaptic GABAergic transmission is regulated also by changes in GABA release and uptake (Farrant and Nusser 2005). GABA release can be modulated by receptors for GABA or

other neurotansmitters on presynaptic terminals or on astrocytes (Cherubini and Conti, 2001; Kang et al., 1998). It has been proposed that GABA activates astrocytic $GABA_B$ receptors triggering glutamate release from astrocytes and increasing GABA release when glutamate activates the presynaptic glutamate receptors (Kang et al., 1998). GABA is cleared from the synaptic cleft by diffusion and by the action of plasma membrane GABA transporters (GATs). Diffusion is influenced by the viscosity of the synaptic cleft liquid, geometry of the cleft and the surrounding extrasynaptic space, and by the localization of receptors and GATs (Cherubini and Conti, 2001). High-affinity GATs are located in the presynaptic neurons and astrocytes, although neuronal uptake has been estimated to be three to six-fold more efficient than glial uptake (Madsen et al., 2008). GATs are capable of bidirectional neurotransmitter transport and their function is highly dependent on extracellular Na^+ and to a lower extent on the Cl^- ions. The driving force for GABA transport against its concentration gradient is supplied by the movement of Na^+ down its concentration gradient. Following the GABA uptake into the presynaptic neuron, the transmitter is preferentially recycled directly into synaptic vesicles being readily available for further release (Cherubini and Conti, 2001; Madsen et al., 2008). Alternatively, GABA taken up into axon terminals may enter a so called GABA shunt which is a closed-loop process producing and conserving the supply of GABA. First, GABA is metabolized by GABA-transaminase (GABA-T) to succinic semialdehyde that is further metabolized by succinic semialdehyde dehydrogenase to succinic acid entering the tricarboxylic acid cycle (Krebs cycle) for oxidative metabolism and resulting in α-ketoglutarate. The α-ketoglutarate is then transaminated into glutamate that is decarboxylated by glutamic acid decarboxylase (GAD) back to GABA that can then re-enter the process (Olsen and DeLorey 1999; Madsen et al., 2008) (Figure 4). In glia, GABA is metabolized to succinic semialdehyde by GABA-T but GABA cannot be resynthesized by GAD in this compartment since glia lack GAD. In glia GABA can be converted to glutamine, which is transferred back to the neuron, in which glutamine is converted to glutamate by glutaminase, which re-enters the GABA shunt (Figure 4). Therefore, it has been postulated that GABA that is taken up by glia is not immediately available for synaptic transmission (Olsen and DeLorey 1999; Cherubini and Conti, 2001).

2.1.6 Extrasynaptic $GABA_A$ receptors and tonic inhibition

Some $GABA_A$ receptors do not accumulate at postsynaptic membranes. These extrasynaptic receptors with a high affinity for GABA mediate tonic inhibition providing a persistent conductance (Stell and Mody, 2002, Semyanov et al., 2004; Farrant and Nusser, 2005; Glykys and Mody, 2007). The persistent hyperpolarizing current generated by extrasynaptic $GABA_A$ receptors makes the neuron less likely to generate an action potential (Pavlov et al., 2009). The α4, α5, α6, and δ

subunits are predominantly localized extrasynaptically or perisynaptically (Farrant and Nusser, 2005) (Figure 4), but their subcellular distribution may vary between cell types. The δ subunit has been localized in the cortex, hippocampus, thalamus, striatum, and cerebellum (Lee and Maguire 2014). In cerebellar granule cells as well as in hippocampal dentate gyrus granule cells, the δ subunit has been shown to be localized exclusively outside the postsynaptic density (Nusser et al., 1998; Wei et al., 2003). The δ subunit forms receptors specifically with the α6 and β2/3 subunits in cerebellar granule cells and with the α4 and βx subunits in the forebrain (Barnard et al., 1998). The α6 subunit expression is almost entirely limited to the cerebellum, whereas the α4 subunit is highly expressed in the thalamus, hippocampus, striatum, and cortex (Lee and Maguire 2014). In thalamus, extrasynaptic α4βδ $GABA_A$ receptors mediate tonic conductance in ventrobasal nuclei, medial geniculate body and dorsal lateral geniculate nucleus (Cope et al., 2005; Jia et al., 2005; Richardson et al., 2013). The α5 (and γ2) subunit-containing receptors are highly expressed in the hippocampus and to lower extent in the cortex, olfactory bulb, and hypothalamus (Olsen and Sieghart, 2009; Lee and Maguire 2014).

Tonic GABAergic currents have been recorded in the basolateral (BLA) and lateral (LA) nuclei of amygdala (Marowsky et al., 2012). The α3 subunit protein was moderately expressed in the LA and strongly in the BLA, mainly at extrasynaptic sites (Marowsky et al., 2012). In α3 knock-out mice, tonic currents were significantly reduced in BLA principal cells, but not in LA principal cells (Marowsky et al., 2012). An α3-selective $GABA_A$ receptor agonist (TP003) increased tonic currents and dampened excitability markedly in BLA principal cells of wild-type mice but not in those of α3 knock-out mice (Marowsky et al., 2012). Also interneurons of the LA and BLA expressed a tonic current, but the α3-selective $GABA_A$ receptor agonist potentiated these currents only in a small fraction of these neurons (Marowsky et al., 2012). In the central nucleus of amygdala (CeA), tonic GABAergic inhibition was mediated by α1 or δ subunit-containing receptors depending on the neuronal subpopulation (Herman et al., 2013).

In hypothalamus, GnRH neurons release GnRH to regulate fertility by controlling the release of follicle-stimulating hormone and luteinizing hormone from the anterior pituitary. In the brain slice preparation, half of the GnRH neurons displayed tonic $GABA_A$ receptor current that was associated with δ subunit-containing $GABA_A$ receptors (Bhattarai et al., 2011). These tonically active $GABA_A$ receptors may be important in controlling the reproductive function as in GnRH neuron-specific conditional γ2 subunit knock-out mice no effects on fertility, estrous cycles, puberty onset, or luteinizing hormone levels were observed although the amplitude and frequency of postsynaptic IPSCs were decreased (Lee et al., 2010a). A tonic GABAergic conductance that was enhanced by

blocking the activity of glial GATs has been reported also in the magnocellular neurosecretory cells within the supraoptic nucleus of the hypothalamus (Park et al., 2006). It has been proposed that this tonic conductance is mediated via α5βγ2 receptors and to a lesser extent by δ-containing receptors (Jo et al., 2011).

The extrasynaptic α4βδ $GABA_A$ receptors in ventrobasal nuclei have been associated with the hypnotic/sedative action of etomidate and gaboxadol (Belelli et al., 2005; Chandra et al., 2006; Herd et al., 2009), but also other drugs have been proposed to alter the tonic inhibition in the brain (Brickley and Mody, 2012) (Table 1).

Table 1. *Drugs altering the tonic GABAergic inhibition in the brain (modified from Brickley and Mody, 2012).*

Drug	**Mechanism of action**	**Current drug indications**
Vigabatrin	Irreversible block of GABA transaminase raising ambient GABA levels.	Refractory complex partial seizures and infantile spasms. Not favored due to visual field loss in some adults and children.
Tiagabine	Blockade of GABA transporters on nerve terminals (predominantly GAT-1) raising ambient GABA levels.	Partial seizures; generalized anxiety disorders/panic disorders.
Gaboxadol	Selective agonist at extrasynaptic $GABA_A$ receptors leading to specific enhancement of the tonic conductance.	Sleep enhancer, but withdrawn from phase III clinical trials due to poor risk-to-benefit ratio (hallucinations and disorientation in a subset of patients).
L-655,708	High-affinity negative allosteric modulator of α5 subunit-containing $GABA_A$ receptors reducing tonic conductance.	Cognitive enhancer but not suitable for human use due to anxiogenic properties.
Ganaxolone	Positive allosteric modulator of most $GABA_A$ receptors with greater potency/efficacy at δ subunit-containing $GABA_A$ receptors leading to selective enhancement of the tonic conductance.	Catamenial epilepsy.
Alphaxalone	Positive allosteric modulator of most $GABA_A$ receptors with greater potency/efficacy at δ subunit-containing $GABA_A$ receptors leading to selective enhancement of the tonic conductance.	Anesthetic and sedative in long-term intensive care patients that was withdrawn from clinical practice due to complications with the vehicle, Cremophor EL. Widely used as an anesthetic in veterinary surgery.
Propofol	Positive allosteric modulator of most $GABA_A$ receptors including α5 or δ subunit-containing $GABA_A$ receptors leading to enhanced tonic conductance.	Widely used as an intravenous anesthetic.

2.1.7 Sources of extrasynaptic GABA

Under physiological conditions, GABA spillover from the synaptic cleft after vesicular fusion is likely the main source of extrasynaptic GABA (Glykys and Mody, 2007) (Figure 4). Tonic inhibition increases when GABA transporter subtypes 1 and 3 (GAT-1 and GAT-3) are pharmacologically blocked (Rossi et al., 2003; Keros and Hablitz, 2005) and in GAT-1 knockout mice (Jensen et al., 2003). There are also additional non-vesicular sources of extrasynaptic GABA, as tonic inhibition occurs also if vesicular GABA release is blocked (Rossi et al., 2003; Wu et al., 2003). Under certain conditions, neuronal and glial plasma membrane transporters (GAT-1 – GAT-3) can reverse their action and release GABA from neurons or glial cells (Richerson and Wu 2003; Koch and Magnusson, 2009) (Figure 4). The direction of operation is dependent on the Na^+ ions, the difference in GABA concentration between the intra- and extracellular space and on the membrane potential (Koch and Magnusson, 2009). GABA can be released from astrocytes by the reverse action of glial GAT subtypes GAT-2 or GAT-3 induced by glutamate uptake (Héja et al. 2012). Recently it has been reported that in striatum and cerebellum GABA is synthesized in glia from putrescine via monoamine oxidation by monoamine oxidase B (MAOB) (Yoon et al., 2014). The glial GABA is released via bestrophin 1 channels (Best1) (Figure 4) and contributes to tonic inhibition in hippocampus, cerebellum and striatum (Le Meur et al., 2012; Lee et al., 2010b; Yoon et al., 2011; Yoon et al., 2014). Also a release from dying cells or non-vesicular leakage may provide extrasynaptic GABA (Glykys and Mody, 2007). Ca^{2+}-dependent retrograde release of GABA from dendritic structures has been reported in olfactory granule cells, interneurons in thalamic relay nuclei, layer 2/3 neocortical bitufted interneurons and in the principal neurons of the lateral superior olive (Koch and Magnusson, 2009).

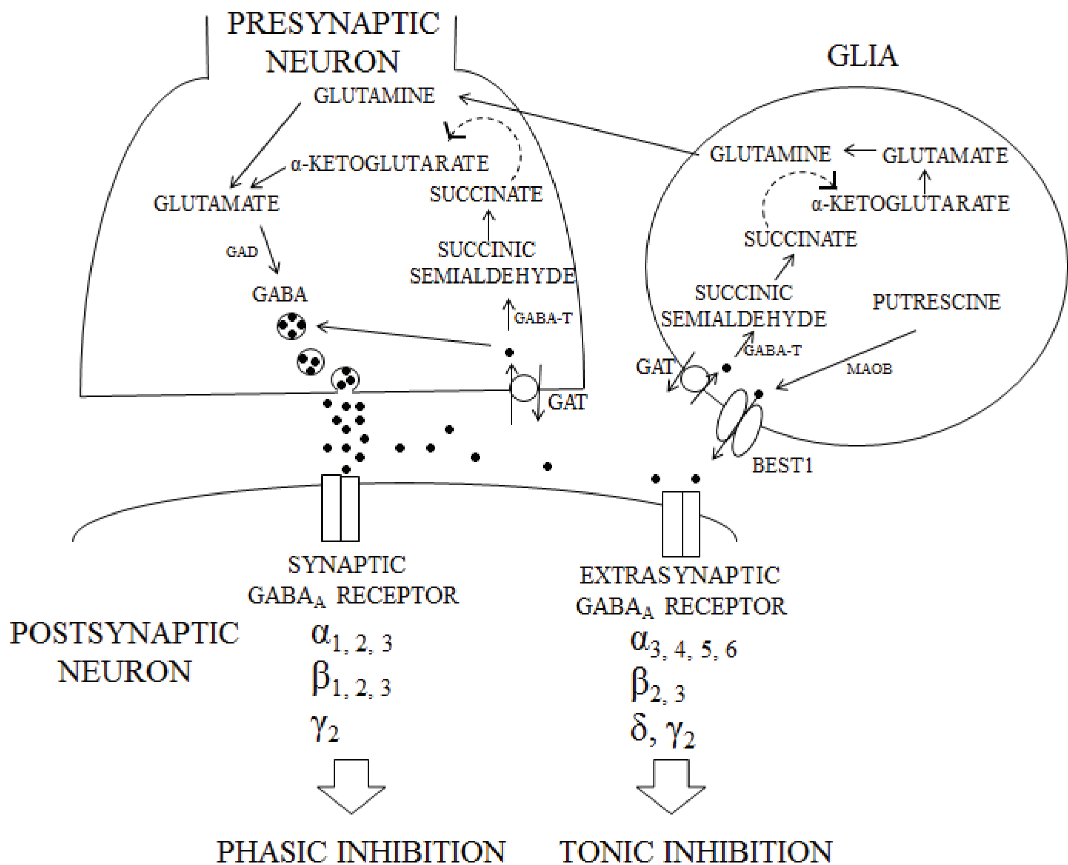

Figure 4. *Sources of synaptic and extrasynaptic GABA (black dots) activating synaptic and extrasynaptic GABA$_A$ receptors and contributing to phasic and tonic inhibition, respectively. Following the GABA uptake into the presynaptic neuron by GABA transporter (GAT), GABA may be recycled directly into synaptic vesicles being readily available for further release or enter the GABA shunt. In GABA shunt GABA is first metabolized by GABA-transaminase (GABA-T) to succinic semialdehyde that is further metabolized to succinate entering the tricarboxylic acid cycle (Krebs cycle) (dashed circular arrow) for oxidative metabolism and resulting in α-ketoglutarate. The α-ketoglutarate is then transaminated into glutamate that is decarboxylated by glutamic acid decarboxylase (GAD) back to GABA that can then re-enter the process. In glia, uptaken GABA can be first converted to glutamate that is then converted to glutamine. Glutamine is transferred back to the neuron, in which glutamine is converted to glutamate that re-enters the GABA shunt. The main source of extrasynaptic GABA is GABA spillover from the synaptic cleft after vesicular fusion. Under certain conditions, neuronal and glial GATs can reverse their action and release GABA from neurons or glial cells. GABA can also be synthesized in glia from putrescine by monoamine oxidase B (MAOB), and it is released via bestrophin 1 channels (Best1). Synaptic GABA$_A$ receptors contain typically the γ2 subunit with α1, α2, or α3 subunits and any β subunits, whereas the α4, α5, α6, and δ subunits are predominantly localized extra- or perisynaptically. The α5 subunits form extrasynaptic receptors with γ2 subunits but not with δ subunits.*

2.2 Anxiety

According to ICD-10 (International Statistical Classification of Diseases and Related Health Problems 10th Revision), anxiety disorders include agoraphobia, social phobia, specific phobias, panic disorder and generalized anxiety disorder (GAD) (Table 2). In 2010, anxiety disorders were the most frequent mental disorders in the contemporary EU countries, Switzerland, Norway and Iceland estimated to affect 69.1 million persons (14.0%) across all age groups (Wittchen et al., 2011). Anxiety disorders have typically an early onset, are often of chronic or recurrent nature, cause substantial personal distress, impair the ability to function socially and occupationally, increase the suicidal thoughts and behavior, and impose a substantial economic burden (Baldwin et al., 2013). Fear and anxiety overlap in anxiety disorders, but they are distinct phenomena (Grillon, 2007). Fear is an adaptive component of the acute stress response to potentially dangerous stimuli and is characterized by a flight or fight response (Millan, 2003; Grillon, 2007). In the sensation of anxiety, fear becomes disproportional in intensity, chronic and/or irreversible, or not associated with any genuine risk (Millan, 2003). Anxiety is characterized by tension, worry, negative affect and a feeling of insecurity and it is associated with avoidance and increases in overall sensory sensitivity (Grillon, 2007).

Table 2. *Main anxiety disorders and their description according to ICD-10 (International Statistical Classification of Diseases and Related Health Problems 10th Revision).*

<table>
<tr><td rowspan="3">Phobic anxiety disorders

Anxiety evoked in certain well-defined situations that are not currently dangerous. These situations are characteristically avoided or endured with dread. The patient's concern may be focused on individual symptoms like palpitations or feeling faint and is often associated with secondary fears of dying, losing control, or going mad. Contemplating entry to the phobic situation usually generates anticipatory anxiety.</td><td>Agoraphobia

A fairly well-defined cluster of phobias embracing fears of leaving home, entering shops, crowds and public places, or travelling alone in trains, buses or planes. Panic disorder is a frequent feature of both present and past episodes. Avoidance of the phobic situation is often prominent.</td></tr>
<tr><td>Social phobias

Fear of scrutiny by other people leading to avoidance of social situations. More pervasive social phobias are usually associated with low self-esteem and fear of criticism. They may present as a complaint of blushing, hand tremor, nausea, or urgency of micturition, the patient sometimes being convinced that one of these secondary manifestations of their anxiety is the primary problem. Symptoms may progress to panic attacks.</td></tr>
<tr><td>Specific (isolated) phobias

Phobias restricted to highly specific situations such as proximity to particular animals, heights, thunder, darkness, flying, closed spaces, urinating or defecating in public toilets, eating certain foods, dentistry, or the sight of blood or injury. Though the triggering situation is discrete, contact with it can evoke panic as in agoraphobia or social phobia.</td></tr>
<tr><td rowspan="2">Other anxiety disorders

Disorders in which manifestation of anxiety is the major symptom and is not restricted to any particular environmental situation. Depressive and obsessional symptoms, and even some elements of phobic anxiety, may also be present, provided that they are clearly secondary or less severe.</td><td>Panic disorder

The essential feature is recurrent attacks of severe anxiety (panic), which are not restricted to any particular situation or set of circumstances and are therefore unpredictable. As with other anxiety disorders, the dominant symptoms include sudden onset of palpitations, chest pain, choking sensations, dizziness, and feelings of unreality (depersonalization or derealization). There is often also a secondary fear of dying, losing control, or going mad.</td></tr>
<tr><td>Generalized anxiety disorder

Anxiety that is generalized and persistent but not restricted to, or even strongly predominating in, any particular environmental circumstances (i.e. it is "free-floating"). The dominant symptoms are variable but include complaints of persistent nervousness, trembling, muscular tensions, sweating, lightheadedness, palpitations, dizziness, and epigastric discomfort. Fears that the patient or a relative will shortly become ill or have an accident are often expressed.</td></tr>
</table>

The anxiety disorders are treated by benzodiazepines but the adverse effects limit their use in chronic anxiety disorders (Millan, 2003; Farb and Ratner, 2014). The short-term benefits of benzodiazepines are well known, but their long-term use causes dependence, daytime somnolence, blunted reflexes, memory impairment, and an increased risk of falls in older people (Mugunthan et al., 2011). Selective serotonin reuptake inhibitors and serotonin/noradrenaline reuptake inhibitors are currently used as the first-line pharmacological treatment of anxiety disorders but many individuals are not responsive to the treatment and the side effects such as nausea, dizziness and sexual problems of these drugs are often limiting their use (Millan, 2003; Farb and Ratner, 2014). Other pharmacological treatments of anxiety disorders include tricyclic antidepressants (inhibit serotonin and noradrenaline reuptake but have also affinity on different receptors), buspirone (serotonin$_{1A}$ receptor agonist), pregabalin (reduces the synaptic release of several neurotransmitters by binding to voltage-gated calcium channels), noradrenaline reuptake inhibitors and the α2-adrenergic agonist (clonidine) (Bandelow et al., 2014; Itoi and Sugimoto, 2010).

A better understanding of neurocircuitry and molecular pathways mediating anxiety is required for the development of more selective and safe treatment for anxiety disorders (Millan, 2003; Farb and Ratner, 2014). Brain-imaging methods in humans have revealed the involvement of the amygdala and prefrontal, insular, limbic, paralimbic, and occipital cortices as well as the basal ganglia in anxiety (Farb and Ratner, 2014). These imaging methods include the functional Magnetic Resonance Imaging (fMRI) that reveals changes in regional cerebral blood flow (rCBF) that is a marker of energy consumption by neurons. Both excitatory and inhibitory neurotransmission are energy-consuming processes and cause changes in rCBF (Detre and Floyd, 2001). Magnetic resonance spectroscopy (MRS) is a noninvasive technique monitoring the levels of neurochemicals in the brain (Farb and Ratner, 2014). The positron emission tomography (PET) involves a systemic exposure to a short-lived radioactive tracer. It has been used to study metabolism, receptor binding, and alterations in regional blood flow within certain brain areas, but it lacks a resolution for structural details and is a highly costly technique (Politis and Piccini, 2012). The single photon emission computed tomography (SPECT) is another emission tomography technique using a radionucleotide-labeled tracer but it has lower resolution than PET (Bailey and Willowson, 2013). To be complemented with clinical findings, animal tests of anxiety are crucial for biomedical research, including testing of anxiolytic drugs, assessment of behavioral phenotypes of mutant and transgenic animals, testing neurobiological hypotheses and finding candidate genes for anxiety disorders (Kalueff and Nutt, 2007).

2.2.1 Locus coeruleus and anxiety

The locus coeruleus (LC) is the largest noradrenergic nucleus producing half of the total noradrenaline in the brain (de Lecea et al., 2012). LC projects virtually to all brain regions with the exception of basal ganglia (Sara, 2009). The adrenergic receptors are G-protein-coupled receptors that modulate neuronal excitability via distinct G-protein-coupled signal cascades. There are three major categories of adrenergic receptors, α1, α2 and β, each eliciting different cellular responses. Each category is further divided into subtypes. It has been suggested that the effect of noradrenaline in any brain region is complex and depends on the intracellular concentration of noradrenaline and the receptor subtypes in the target region (Sara, 2009). LC mediates the alarm reaction during stress, and its over-activity increases the arousal level and amplifies emotional reactions to stress (Yamamoto et al., 2014).

Noradrenergic dysfunction has been associated with anxiety disorders (Yamamoto et al., 2014), although a causal relationship has not been demonstrated (Itoi, 2008). However, the activation of LC by direct electrical stimulation or by pharmacologic agents has elicited fear responses in primates whereas such responses have been reduced by decreasing LC firing (Southwick et al., 1999). In addition, noradrenaline reuptake inhibitors and the α2-adrenergic agonist (clonidine) are used as a pharmacological treatment for anxiety disorders, whereas the α2-adrenergic antagonist (yohimbine) has been shown to increase anxiety symptoms (Itoi and Sugimoto, 2010; Itoi, 2008).

LC-mediated responses to stress are linked to the neuroendocrine stress system by corticotropin-releasing hormone (CRH) (reviewed in Kovács, 2013). Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a well-known mediator of neuroendocrine stress responses, but in addition to stimulating the release of adrenocorticotropic hormone (ACTH), also known as corticotropin, and glucocorticoids from the pituitary gland and adrenal cortex, respectively, CRH from the paraventricular nucleus of hypothalamus increases the firing of TH-positive neurons in LC. LC receives CRH-positive afferents also from the brainstem, Barrington nucleus, CeA and bed nucleus of stria terminalis (BNST). Different stressors recruit different CRH pathways projecting to LC. As an example, psychosocial stress increases CRH mRNA levels in the CeA and BNST (Kovács, 2013).

2.2.2 Anxiety tests in rodents

Numerous preclinical anxiety tests have been developed for animals (Griebel and Holmes, 2013) (Table 3). One major difficulty confronted in animal studies on anxiety is the absence of concrete parameters reflecting the emotional component of anxiety. Surrogate markers, such as an increase in arterial pressure, tachycardia, hyperthermia and excessive secretion of glucocorticoids into the systemic circulation, can be instructive in the evaluation of anxiety, but behavioral end-points such as avoidance, escape, freezing are generally preferred although their precise relevance to different types of anxiety disorders in man is not easy to determine (Millan 2003). The translational value of an animal test in understanding pathological anxiety in humans can be assessed by three validity criteria; face, predictive and construct validity (Griebel and Holmes, 2013). Face validity determines if the test measures something analogous to one or more human anxiety symptoms. Predictive validity defines whether the test is reliably sensitive to clinically efficacious anxiolytics. Construct validity determines if the test measures something analogous to one or more mechanisms of human anxiety disorder (Griebel and Holmes, 2013). None of the currently available anxiety tests or models is considered unequivocally meet these criteria (Griebel and Holmes, 2013).

Table 3. *Commonly used anxiety tests in experimental rodents and the anxiety disorders they are proposed to model (Griebel and Holmes, 2013; Parsons and Ressler, 2013; Bourin et al., 2007). In bold are highlighted the most frequently used animal tests in anxiety research between 1990 and 2011 (Haller et al., 2013).*

Procedure	**Group**	**Principle**	**Test**	**Anxiety disoder**
Unconditioned	Approach-avoidance conflict tests	Generation of a conflict between a drive to approach novel areas and a simultaneous avoidance of potential threat therein.	**Open‑field test, elevated plus‑maze test, light/dark exploration test**	Generalized anxiety disorder, specific phobias
	Fear tests	Testing mice for their responses (fight, flight, freeze, vocalize or scan) to an approaching anaesthetized natural predator.	Mouse Defense Test Battery (MDTB)	Generalized anxiety disorder, panic disorder
	Interaction-based tests	Measuring the time spent by pairs of rodents in social interaction (e.g. sniffing, following or grooming the partner).	Active social interaction (unfamiliar rat pairs), resident intruder, ultrasonic vocalization (separation induced)	Generalized anxiety disorder, social anxiety disorder
Conditioned	Conditioned fear tests	Learning process in which a neutral stimulus as a result of pairing with fear-provoking stimulus becomes a conditioned stimulus (CS) that alone elicits fear. Fear extinction is learning that the CS no longer predicts the unconditioned stimulus.	Pavlovian fear conditioning	Specific phobias and other anxiety disorders
	Conflict‑based tests	Measuring anxiolytic‑like activity via the maintenance of a behavioral response despite the receipt of a punishment (mild electric shock).	Vogel conflict test, Geller‑Seifter test	Generalized anxiety disorder

Unconditioned anxiety tests, especially the elevated plus-maze, light/dark and open-field tests, are easy, quick and inexpensive to perform and analyze and are therefore popular in preclinical drug testing and genetic research (Blanchard et al., 2008). The elevated plus-maze, light/dark and open-field tests generate a conflict between the natural drive to approach novel areas and an avoidance of a potential threat there, and are considered to model especially specific phobias and GAD (Griebel

and Holmes, 2013). The social interaction test of anxiety is thought to mimic the state of anxiety in GAD and social anxiety disorder (SAD) (File and Seth, 2003; Griebel and Holmes, 2013). The test measures the time spent by pairs of rodents in social interaction (e.g. sniffing, following or grooming the partner) (File and Seth, 2003). The test conditions (the light intensity and the novelty of the test arena) can be manipulated to generate different levels of anxiety (File and Seth, 2003). The Mouse Defense Test Battery (MDTB) provides multiple measures related to fear and anxiety. In the test, mice are placed in an oval runway and tested for their fight, flight, freeze, vocalize or scan responses to a natural predator (Griebel and Holmes, 2013). Predator-elicited flight responses in mice have been suggested to serve as an experimental model of panic attacks (Griebel et al., 1996).

In fear conditioning an emotionally neutral stimulus is paired with an aversive unconditioned stimulus (US). As a result of the pairing, the neutral stimulus becomes a conditioned stimulus (CS) that alone elicits fear. If the CS is subsequently presented repeatedly in the absence of the US, it gradually loses the ability to elicit fear. This phenomenon, i.e. learning that the CS no longer predicts the US, is called extinction (LeDoux, 2000, Sotres-Bayon et al., 2004; Johansen et al., 2011). The Pavlovian fear conditioning test and its variations are considered as useful models to study the neural circuitry and the cellular and molecular mechanisms of fear-related disorders as anxiety disorders are thought result from a dysregulation of normal fear learning mechanisms (Dunsmoor and Paz, 2015). Early life experience and genetic background are important risk factors in the development of fear-related disorders (Parsons and Ressler, 2013). When an individual experiences a traumatic event, the person learns to fear the cues that are associated with the traumatic event, and this fear memory is transferred from short-term memory into long-term memory (Parsons and Ressler, 2013). The psychopathology of anxiety disorders is characterized by recruitment of cues not originally associated with the traumatic event (a phenomenon called generalization) and by sensitization to fear experiences when an exposure to fear cues occurs (Parsons and Ressler, 2013). It has been demonstrated that a similar brain activation pattern is observed in humans and rodents in a fear conditioning test, and that these areas are notably dysregulated in anxiety disorders suggesting a promising face and construct validity of the test (Parsons and Ressler, 2013).

Other types of conditioned procedure tests include the Vogel conflict and Geller-Seifter tests that measure anxiolytic-like activity as the maintenance of a behavioral response despite the receipt of a mild electric shock (Griebel and Holmes, 2013). In the Vogel conflict test water-deprived rodents are exposed to a mild and intermittent electric shock via a water bottle whereas in the Geller-Seifter

conflict test, rats that are trained to respond for a food reward are exposed to a modest electric shock during a “conflict” component of the procedure (Millan, 2003). Vogel conflict test and other fear-based conflict tests are postulated to be relevant for testing anxious states related to GAD, social and other phobias, but their validity in reflecting other anxious states, such as those related to panic attacks is less apparent (Millan, 2003).

Despite of a large number of existing preclinical anxiety tests, preferences for their use have not changed between the years 1990 and 2011 (Haller et al., 2013). The elevated plus-maze test was used in half of all anxiety studies identified in a thorough Medline search, and the elevated plus-maze, open-field, social interaction, and light/dark box tests accounted for about 80% of them (Haller et al., 2013). The most widely used animal tests of anxiety have been argued to predict only a specific type of anxiolytic activity (Haller et al., 2013). Actually, about 40% of compounds that have been promising in preclinical tests were ineffective in clinical trials. The important difference between the anxiety disorders and anxiety-like behavior monitored by classical preclinical animal tests is that anxiety disorders are chronic states with “excessive” or “unreasonable” fear whereas the animal tests often monitor acute manifestations of natural fears. The mechanisms mediating the non-pathological extreme forms of anxiety are essential for survival and likely to be highly conserved in different mammalian species (Steimer, 2001). As certain compounds have been shown to reduce both natural fear responses and symptoms of anxiety disorders, the mechanisms underlying these natural and “unreasonable” fears are probably overlapping. However, as some substances selectively reduce only the natural fear responses or the symptoms of anxiety disorders, their mechanisms are not identical (Haller et al., 2013).

An important component of anxiety disorders is linked to personality characteristics. An inhibited (fearful) temperament, *i.e.* trait anxiety, is supposed to be a predisposing factor for anxiety disorders (Steimer, 2011). Therefore trait anxiety animal models including genetic models, chronic exposure to fear-provoking stimuli, rodent strains displaying high or low anxiety and inter-individual differences within a defined strain model may be critical tools for scientists trying to unravel the mechanisms of these complex disorders (Steimer, 2011).

2.2.3 Neuroanatomy of conditioned fear

Amygdala has been implicated in the acquisition, expression and extinction of conditioned fear (LeDoux, 2000). During the acquisition phase, pathways transmitting the conditioned auditory stimulus and unconditioned nociceptive stimulus were proposed to be transmitted to the lateral nucleus of amygdala (LA) serving as a sensory input region (LeDoux, 2000). Expression of

conditioned fear was proposed to involve changes in the activity of the LA and in CeA to which LA is connected both directly and indirectly via the basal and accessory basal nuclei and intercalated cell masses (ICM) (LeDoux, 2000, Sotres-Bayon et al., 2004; Johansen et al., 2011). During contextual fear conditioning, the acquisition phase was proposed to involve the hippocampus and the communication between the ventral hippocampus (CA1 and subiculum) and the basal and accessory basal nuclei of amygdala that project further to the CeA (LeDoux, 2000). According to the theory by LeDoux, CeA serves as an output region of both acoustic and contextual CSs and controls the expression of fear responses by sending projections to the hypothalamus and brainstem (LeDoux, 2000, Sotres-Bayon et al., 2004; Johansen et al., 2011).

Indeed, many animal studies involving lesions, pharmacological manipulations and electrical stimulation support the central role of amygdala in the acquisition and expression of conditioned fear. Post-training and pre- or post-training lesions of CeA and BLA, respectively, blocked fear-potentiated startle to both auditory and visual CSs in rats (Campeau and Davis, 1995). Pharmacological inactivation of the prelimbic cortex (PL) reduced conditioned fear expression (Corcoran and Quirk, 2007). As PL projects to BLA, PL may augment conditioned fear responses via this pathway (Milad and Quirk 2002). Infusion of muscimol into BLA and LA either before conditioning training or before testing the responses to CS (tone or context) decreased the freezing-response to the conditioned stimuli (Muller et al., 1997). Pharmacological blockage of NMDA receptors in the rat BLA or CeA prior to fear conditioning training abolished the acquisition of both auditory and contextual fear conditioning (Goosens and Maren, 2003). Infusions of muscimol into CeA and LA resulted in an impairment of acquisition of conditioned fear further suggesting that also CeA is involved in fear learning and is not a simple output structure (Wilensky et al., 2006). As a result, the fear circuitry theory by LeDoux was updated to involve also CeA as an input station for CS and US resulting in memory formation of conditioned fear. However, it still remained unsolved whether plasticity and memory formation in CeA occurred in parallel to that in LA or if it relied on distributed plasticity throughout the amygdala that was initiated by LA neurons (Wilensky et al., 2006). Recent developments in methodologies have made it possible to study how fear memories are acquired at the circuitry level. An *in vivo* study in mice using implanted chronic recording electrodes and optogenetic and pharmacological approaches has shown that inactivation of lateral subdivision of the CeA during the acquisition phase of fear conditioning interfered with the learning process indicating that excitation of neurons in the lateral subdivision of the CeA is required for fear acquisition (Ciocchi et al., 2010). It was further shown that the conditioned freezing behavior was driven by excitation of output neurons in the medial subdivision of CeA (Ciocchi et al., 2010). In the lateral subdivision of CeA some neurons exhibited CS-evoked short-latency excitatory

responses, whereas some displayed a strong inhibitory response and the majority did not exhibit any CS-evoked responses (Ciocchi et al., 2010).

Many studies suggest that amygdala has a role also in fear extinction. Like other types of learning, extinction learning can be divided into acquisition, consolidation, and retrieval phases (Quirk and Mueller, 2008). Acquisition is the initial phase of extinction learning in which conditioned responses are declining within an extinction training session. In the consolidation phase, physiological and molecular processes stabilize a long-term extinction memory. In the retrieval phase of extinction, a presentation of the extinguished CS triggers a retrieval of extinction, characterized by low levels of conditioned responding.

Blockade of NMDA (Sotres-Bayon et al., 2007) or metabotropic glutamate subtype 1 (Kim et al., 2007) receptors within the BLA has been reported to impair the acquisition of fear extinction. Also the ventrolateral periaqueductal gray (vlPAG) has been proposed to mediate extinction acquisition as the acquisition of extinction was impaired in rats with naloxone-blocked μ-opioid receptors in the vlPAG (McNally et al., 2004, 2005). Noradrenergic action within the BLA has been proposed to enhance the consolidation of conditioned fear extinction (Berlau and McGaugh, 2005). Several molecular processes have been identified within the amygdala (BLA), hippocampus and prefrontal cortex (PFC) during the consolidation of extinction (Quirk and Mueller, 2008).

PFC is poorly developed in rodents as compared to humans, and it consists of the orbital frontal, anterior cingulate, infralimbic (IL) and prelimbic (PL) cortices in rodents (Sotres-Bayon et al., 2004). Selective stimulation of IL during a CS session facilitates fear extinction (Milad and Quirk, 2002; Vidal-Gonzalez et al., 2006). It has been shown that CS-evoked firing of IL neurons increases during fear extinction training and this firing is larger a day after the extinction training when rats are recalling extinction (Milad and Quirk 2002). Rats with lesions in IL extinguish freezing responses within a CS session but display excessive freezing to the CS the following day, suggesting impaired recall of extinction (Quirk et al. 2000; Lebron et al. 2004).

There are also marked deficits in fear extinction when intercalated cells of amygdala (ITC) are injured (Likhtik et al., 2008). The ITC cells receive a strong projection from IL, and potentiated activity of IL during extinction retrieval could inhibit amygdala output via activation of ITC cells (Quirk and Muller, 2008). This is supported by the finding that electrical stimulation of IL reduces the responsiveness of CeA output neurons to BLA stimulation (Quirk et al, 2003). In contrast to IL, stimulation of PL has been shown to slower the rate of fear extinction and to prevent extinction learning (Vidal-Gonzalez et al., 2006).

Hippocampus is proposed to play an important role in the fear extinction of contextual CS (Sotres-Bayon et al., 2004, Quirk and Mueller, 2008; Maren et al., 2013). Hippocampus sends mainly glutamatergic projections from CA1/subiculum to mPFC (primarily to IL and ventral PL), and may regulate CeA output via mPFC or via its connections with basal and accessory basal nuclei of amygdala (Sotres-Bayon et al., 2004). It has been proposed that the activation of CeA through the direct glutamatergic projection from the hippocampus to BLA as well as through the indirect BLA activation via the glutamatergic projection to PL may be crucial for the renewal of fear expression in response to an extinguished conditional stimulus (Figure 5) (Maren et al., 2013). In contrast, hippocampus may indirectly inhibit the CeA firing via its glutamatergic projection to IL that further sends glutamatergic projections to ITC and thus can activate the inhibitory GABAergic projections from ITC to CeA (Maren et al., 2013). This inhibition of CeA output is involved in suppressing the expression of fear in response to an extinguished conditional stimulus (Maren et al., 2013).

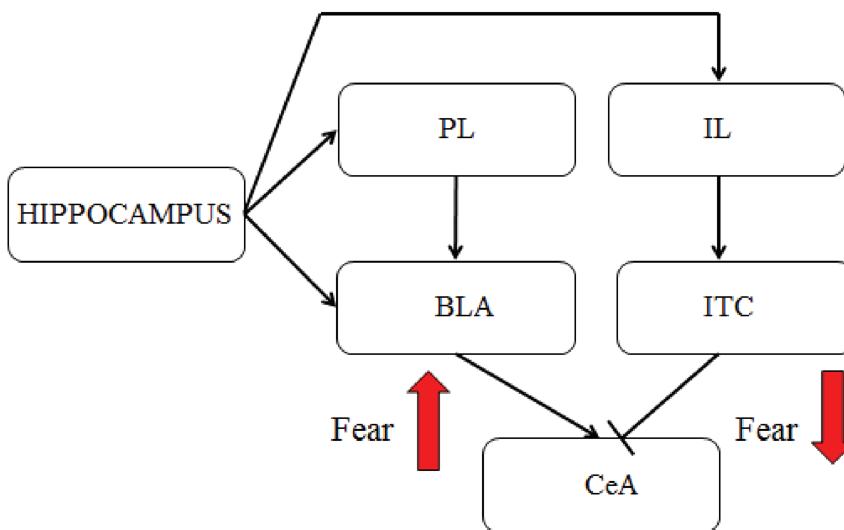

Figure 5. *The neural circuit involved in the context-dependent regulation of fear memory involves the hippocampus, infralimbic cortex (IL), prelimbic cortex (PL), basolateral amygdala (BLA), central nucleus of amygdala (CeA) and intercalated (ITC) cells of amygdala (modified from Maren et al., 2013). Black arrows represent glutamatergic excitatory projections, and the blunt line represents the GABAergic inhibitory projection. The hippocampus projects directly to BLA, and this projection may be crucial for the renewal of fear expression in response to an extinguished conditional stimulus. The red arrows represent whether the context-dependent fear renewal is stimulated or suppressed in response to an extinguished conditional stimulus as a result of GABAergic CeA output neuron activation or inhibition.*

2.2.4 Neuroanatomy of unconditioned (innate) fear

Responses to predators and to aggressive conspecifics are processed via independent neural pathways that include different subnuclei of amygdala, hypothalamus and periaqueductal gray (PAG) (Figure 6). The interconnected anterior hypothalamic nucleus (AHN), the dorsomedial part of ventromedial hypothalamic nucleus (dmVMH), and ventrolateral part of dorsal premammillary nucleus (vlPMD) form a so-called medial hypothalamic defensive circuit (Canteras, 2002) that is also called a predator-responsive circuit (Gross and Canteras, 2012). Lesions in PMD have been effective in reducing antipredator defensive responses (Canteras et al. 1997; Blanchard et al. 2003). The medial hypothalamic defensive system receives neurochemical signals from the posteroventral part of medial amygdala (pvMEA) upon olfactory stimulus representing a predator and from LA and the posterior part of basomedial amygdala (pBMA) upon auditory/visual stimulus representing a predator (Gross and Canteras, 2012). The vlPMD is the output nucleus of the predator-responsive circuit and it projects to the dorsolateral part of periaqueductal gray (dlPAG) (Gross and Canteras, 2012). Fear response to aggressive conspecifics involves the posterodorsal part of medial amygdala (pdMEA), the ventrolateral part of ventromedial hypothalamus (vlVMH) and its connections with the medial preoptic nucleus (MPN) and ventral premammilliary nucleus (PMV), the dorsomedial part of dorsal premammillary nucleus (dmPMD) and the dorsomedial part of periaqueductal grey (dmPAG) (Gross and Canteras, 2012).

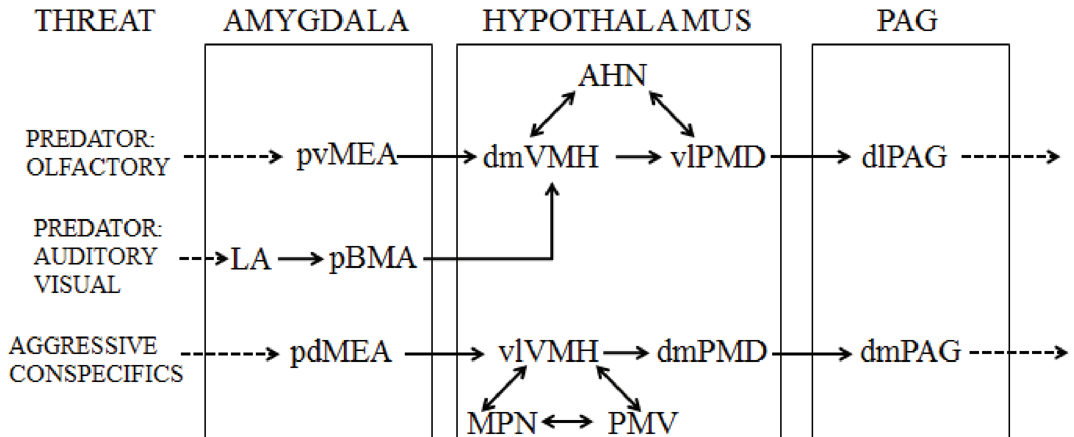

Figure 6. *The fear response to an olfactory or auditory/visual stimulus representing a predator is mediated by a neural pathway that involves the posteroventral part of medial amygdala (pvMEA) or the lateral amygdala (LA) and the posterior part of basomedial amygdala (pBMA), respectively. Both pvMEA and pBMA project to the dorsomedial part of ventromedial hypothalamus (dmVMH) that is connected to the ventrolateral part of dorsal premammillary nucleus (vlPMD) both directly and indirectly via the anterior hypothalamic nucleus (AHN). The vlPMD projects to the dorsolateral part of periaqueductal grey (dlPAG). The fear response to aggressive conspecifics involves a pathway including the posterodorsal part of medial amygdala (pdMEA), the ventrolateral part of ventromedial hypothalamus (vlVMH) and its connections with the medial preoptic nucleus (MPN) and ventral premammilliary nucleus (PMV), the dorsomedial part of dorsal premammillary nucleus (dmPMD) and the dorsomedial part of periaqueductal grey (dmPAG). Dashed arrows indicate stimulus input and behavioral output (modified from Gross and Canteras, 2012).*

Also BNST and BLA are likely to play a role in unconditioned fear responses to predators, as lesions in both structures have exhibited a significant reduction in unconditioned fear responses to a predator odor (Fendt et al., 2003; Li et al., 2004; Rosen, 2004; Vazdarjanova et al., 2001). However, lesions in CeA have not been shown to affect the natural defensive responses to a predator (de Oca and Fanselow 2004) or to its odor (Li et al. 2004). Exposure of mice to 2,5-dihydro-2,4,5-trimethylthiazoline, a synthetically derived component of fox feces, induced neuronal activation of the BNST, lateral septum (LS), paraventricular nucleus of hypothalamus, LC and PAG (Janitzky et al., 2015). Of these areas at least BNST and LS in addition to PAG receive direct innervations from the medial hypothalamic defensive system (Canteras, 2002).

As shown by increased c-Fos expression, the elevated plus-maze-induced anxiety has been shown to activate e.g. mPFC, nuclei of amygdala (the medial, cortical and lateral nucleus of amygdala, BLA, and to a low extent also CeA) and the crescentic LS, and it has been hypothesized that they are components of basic neural circuits related to the experience of anxiety or distress

(Duncan et al., 1996). Open-field exposure induced an increased c-Fos expression in the mPFC, cingulate, motor, somatosensory, insular, piriform and entorhinal cortices, LS, paraventricular thalamic nucleus, hypothalamus (anterior and lateral hypothalamic areas), amygdala (BLA, MEA), extended amygdala (BNST), hippocampus (CA1, subiculum) and LC (Hale et al., 2008). A local administration of a benzodiazepine site agonist midazolam into BLA showed an anxiolytic-like action by impairing the open arm avoidance in the plus-maze (Pesold and Treit, 1994, 1995). However, GABAergic drug action in the CeA did not have a similar effect as in the BLA because in the same protocol midazolam administration into CeA had no effect on the open arm activity in the plus-maze. A brightly lit test chamber has been shown to generate similar unconditioned fear in control animals and in animals with lesions in the CeA (Rosen, 2004).

3 Aims of the study

Expanding the understanding of local role of $GABA_A$ receptors and neuronal circuits mediating anxiolysis is relevant for the development of more selective and safe treatment for anxiety disorders. Studying the properties of extrasynaptic $GABA_A$ receptors may help to understand their physiological relevance and role in psychiatric and neurological disorders.

The main objectives of this thesis were as follows:

1. To investigate whether benzodiazepine-sensitive $GABA_A$ receptors are expressed in human LC neurons.

2. To study the pharmacology of extrasynaptic $GABA_A$ receptors *in vivo* in mice and *in vitro* in brain sections and recombinant receptors.

3. To study the neuronal networks mediating acute anxiolytic responses.

4 Materials and methods

4.1 Human brains (I)

Postmortem brain tissue was obtained from the Section on Neuropathology of the Clinical Brain Disorders Branch, Genes Cognition and Psychosis Program of the Intramural Research Program of the National Institute of Mental Health under a protocol approved by the Institutional Review Board (IRB).

The collection, screening, and analysis of subjects (2 females, 3 males, average age 30 ± 12.6 years) have been described in Lipska et al. (2006). The cause of death was determined and the toxicology profile was defined from brain or blood samples. The interval between death and tissue collection ranged from 16 to 50.5 h. The pons and cerebellar cortex were cut into 2–3 cm thick slabs, flash frozen in dry ice and isopentane, and stored at -80 °C. 14-µm-thick sections were cut from the slabs of pons and cerebellar cortex with a Leica CM 3050S cryostat (Leica Microsystems, Benheim, Germany) at -20 °C. The sections were thaw-mounted onto gelatin-coated or SuperFrost object classes (Menzel-Gläser) and stored frozen at -70 °C.

4.2 Experimental animals (II, III)

All animal experiments were approved by the Southern Finland Provincial Government and the Institutional Animal Use and Care committee of the University of Helsinki.

The Thy1α6 mice had been generated by microinjecting a transgene consisting of the Thy-1.2 pan-neuronal promoter, the mouse $GABA_A$ receptor α6 subunit cDNA and the Thy1 polyadenylation site into the genome of two-cell embryos of CBA/cba x C57BL/6 (Caroni, 1997; Wisden et al. 2002). The Thy1α6 founder line was expanded in C57BL/6 background for at least 10 generations (William Wisden, personal communication).

The experimental Thy1α6 mice were produced in the animal facilities of the Korpi research group by mating within a homozygous line (**II, III**). The control C57BL/6NHsd mice (Harlan Netherland, Horst, The Netherlands) were purchased at the age of 5 weeks (**II, III**).

To generate littermate wild-type controls for homozygous or heterozygous Thy1α6 mice (**II**), homozygous Thy1α6 mice were first mated with C57BL/6HNsd mice to produce heterozygous offspring. These heterozygous mice were then mated. Genotyping of the pups was performed from tail-tip samples by PCR (denaturing at 94°C for 3 min followed by 20 cycles, 94°C 30 s, 60°C 30 s, and 72°C 30 s) with primers (forward 5′-TCTTGCTTCTCCCCTGGCTCTTCA-3′ and reverse 5′-

AGACGGTTGTCATAGCCTTCCAGC-3′) designed to discriminate between the endogenous Gabra6 gene (362 bp) and the transgenic intronless Gabra6 cDNA (160 bp). The presence of the shorter PCR product was used to identify the transgenic, either homozygous or heterozygous, mice.

All experimental mice (**II, III**) were maintained until the experiments at the age of 3–6 months in groups of 2–6 in Makrolon cages (37 · 21 · 15 cm; Tecniplast, Buguggiate, Italy), under 12 : 12 h light : dark cycles (lights on 7 AM–7 PM), at 21–23°C and humidity of 50–60%. They received standard rodent pellets (Harlan BV., Horst, The Netherlands) and tap water *ad libitum*.

For behavioral tests (**II**), the mice were transferred to the experimental room at least 1 h before drug injections, which were performed 30 min before behavioral tests. In the light : dark exploration and elevated plus-maze tests, the mice received an *i.p.* injection of either saline or gaboxadol hydrochloride (3 mg/kg; H. Lundbeck A/S, Copenhagen, Denmark). In the loss of righting reflex (LORR) test, 25 mg/kg gaboxadol hydrochloride was injected.

For the immunohistochemical and autoradiography assays (**III**), the mice were pre-handled and accustomed to the test room and to injections for a week. On the test day, the mice were allowed to adapt to the test room at least 1 h before saline or gaboxadol (3 mg/kg, *i.p.*) injections. Immediately after the injections, the mice were returned in their home cages. Brain dissections were performed between 9 am and 1 pm, 2 h after the injections and the brains were frozen on dry ice and stored at -80 °C. 14-µm-thick coronal sections were cut with a cryostat (Leica CM 3050; Leica Microsystem, Nussloch, Germany), thaw-mounted onto gelatin-coated or SuperFrost object classes (Menzel Gläser) and stored frozen at -80 °C until used in the experiments. The mice were treated blindly for genotype and drug treatment.

4.3 Behavioral tests (II)

The light : dark exploration and elevated plus-maze tests have been described in Vekovischeva et al. (2004). All behavioral tests were performed between 9 AM and 1 PM.

4.3.1 The light : dark exploration test

A mouse was placed in the lit compartment of a two-compartment box (47 × 29 × 35 cm) divided into one dark (16 × 29 cm) and one lit (31 × 29 cm; about 450 lux) area separated by an open door (7 × 8 cm). During a 5-min testing period the time before the first crossing to the dark compartment (latency to the first entry), the number of crossings between the compartments and the time spent in

the lit compartment (minus the latency) were recorded and analyzed by EthoVision Color-Pro 3.0 software (Noldus Information Technology, Wageningen, The Netherlands).

4.3.2 The elevated plus-maze test

A mouse was placed on the central platform facing the open arm and was allowed a free exploration of the maze for 5 min. The time spent on the open arms, number of entries into the open and closed arms, and total movements were recorded and analyzed using the EthoVision video tracking system. The elevated plus-maze apparatus (gray plastic; elevated 54 cm from the floor level) consisted of a central platform (5 × 5 cm), from which two open arms (5 × 40 cm with a 0.7 cm marginal elevation) and two closed arms (5 × 40 × 20 cm) extended. The light intensity was set at 50 lux on the open arms, and 20 lux in the closed arms.

4.3.3 The LORR (loss of righting reflex) test

The LORR was determined every 4 min by turning a mouse in a supine position consecutively for 3 times on a V-shaped trough made of plastic. The mouse was considered to have lost its righting reflex when it was not able to right itself in any of three consecutive 5-s trials and gained the righting reflex when it was able to right itself at least once of the three trials.

4.4 Immunohistochemistry (I, III)

4.4.1 Tyrosine hydroxylase (TH) immunohistochemistry (I)

A rapid staining procedure as described in the instructions of Vectastain Elite ABC Kit (Vector laboratories) was applied. The human brain sections on gelatin-coated object classes were fixed in cold acetone for 5 min and washed briefly in 1× phosphate-buffered saline (PBS), pH 7.4. The first incubation was performed in the 1:25 dilution of primary rabbit anti-TH affinity-purified polyclonal antibody (AB152; Chemicon International, Temecula, USA), the second in the 1:50 dilution of secondary biotinylated anti-rabbit goat IgG (PK-6105; Vectastain Elite ABC Kit, Vector laboratories) and the third in the avidin/biotinylatedhorseradish peroxidase enzyme complex ABC (Vectastain Elite ABC Kit, Vector laboratories), each at room temperature for 4 min and with a brief rinsing with 1× PBS between each step. After the color development with diaminobenzidine (DAB) (Vector laboratories) for 60 s, the sections were rinsed in H_2O, and then dehydrated in 70%, 95% and 100% ethanol (30 s each) and xylene (twice for 5 min each). Finally, glass cover slips (Menzel-Gläser) were mounted on the sections. Negative controls were carried out in the absence of

the primary antibody. The LC sections were scanned (Epson expression 1680 Pro) with digital manipulation (shadow 179, gamma 2.89, highlight 215).

4.4.2 C-Fos immunohistochemistry (III)

The staining procedure has been described in Procaccini et al. (2011). The frozen slides on SuperFrost object classes were air-dried for 15 min, surrounded with a hydrophobic pen (Daigo Sangyo, Tokyo, Japan) and fixed for 10 min in ice-cold 4% paraformaldehyde in PBS (pH 7.4). The slides were washed in PBS containing 0.05% Tween 20 (PBST20) and blocked for endogenous peroxidases in methanol containing 0.3% hydrogen peroxide for 15 min. After washing in PBST20, the slides were incubated for 20 min in a blocking buffer consisting of 5% horse normal serum and 1% fatty acid free bovine serum albumin (BSA) in PBST20. After washing in PBST20, the goat polyclonal anti-c-Fos antibody (1:1000, SC-52G, Santa Cruz Biotechnology, Santa Cruz, CA) was applied in the incubation buffer (1% BSA in PBST20) overnight at 4 °C. Negative control sections were incubated without the primary antibody. On the next day, slides were washed with PBST20 and incubated in a solution containing biotinylated horse anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA) in 1% BSA in PBST20 for 1 h followed by an incubation in an avidin-horseradish peroxidase solution for 1 h (Vectastain Elite ABC, Vector). After washing in PBST20, the sections were incubated in a chromogen solution containing diaminobenzidine and nickel sulfate intensification (DAB Substrate kit, Vector) for 8 min. The reaction was stopped by PBST20. The sections were dehydrated in ethanol series, immersed in Histoclear (National Diagnostic, Atlanta, GA, USA) and coverslipped with Permount (Fisher Chemicals, Fair Lawn, NJ).

Photomicrographs from anatomically matched sections were captured blind to the treatment, genotype and sex using a light microscope (Leica DMR, Leica Microsystems, Wetzlar, Germany) and a CCD camera (Leica DC 300). Image Pro Plus digitizing software (Media Cybernetics, Silver Spring, ML) was used to set thresholds and to count c-Fos positive cells.

4.5 *In situ* hybridization (I, III)

The *in situ* hybridization procedures followed the protocol as described earlier by Sinkkonen et al. (2004). The air-dried sections on SuperFrost object classes (Menzel-Gläser) were fixed in ice-cold 4% paraformaldehyde for 5 min, washed in 1× PBS at room temperature for 5 min, dehydrated in 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until used (**I, III**).

Two different antisense DNA oligonucleotide probes complementary to the human γ2 subunit mRNA sequence (nucleotides 1442–1486 and 1695–1739; GenBank accession number NM_198904) and analogous sense probes (**I**) as well as one antisense DNA oligonucleotide probe complementary to the mouse $GABA_A$ receptor α6 subunit mRNA sequence (5'-CAG TCT CTC ATC AGT CCA AGT CAT-3') (**III**) were synthesized (Oligomer Oy, Helsinki, Finland). Poly[^{35}S]dA ([^{35}S]dATP from PerkinElmer Life and Analytical Sciences, Boston, MA, USA) tails were added to the 3′-ends of the probes by deoxynucleotidyl transferase (Promega Corporation, Madison, WI, USA) and unincorporated nucleotides were removed by Illustra ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, UK). Sufficient labelling efficiencies were confirmed by a scintillation counter (**I, III**).

The labelled probes were diluted to 0.06 fmol/µl with hybridization buffer (containing 50% formamide, 10% dextransulphate, 4× SSC) (**I, III**). Non-specific controls contained a 100-fold excess of unlabelled antisense probes. The hybridization occurred under glass cover slips (Menzel-Gläser) over-night at 42 °C. The next day, the slides were washed in 1× SSC at room temperature for 10 min, in 1× SSC at 55 °C for 30 min, and 1× SSC, 0.1× SSC, 70% EtOH and 95% EtOH at room temperature for 1 min each. After air-drying, the sections were exposed with plastic ^{14}C-radioactivity standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to BioMax MR film (Eastman Kodak Company, Rochester, NY, USA) for up to a week and scanned for images (Epson expression 1680 Pro). The binding densities were measured as optical density values (SCION IMAGE; Wayne Rasband, NIMH, Bethesda, MD) and converted to radioactivity levels in nCi/g of gray matter for [^{35}S]-labelled oligonucleotides. Non-specific signal was subtracted to obtain the specific signal.

For emulsion autoradiography (**I**), the sections were dipped in Kodak autoradiography emulsion (Eastman Kodak Company, Rochester, NY, USA) diluted in 600 mM ammonium acetate (1:1 volume) in the 42 °C water bath, air-dried over-night in the dark and exposed in a light-tight slide box at 4 °C for 6 weeks. The sections were developed at 15 °C in Kodak D-19 Developer for 3 min, water for 30 s, and Kodak fixer for 5 min, after that they were washed twice in water for 5 min and air-dried over-night at room temperature. The dry sections were counterstained by 0.125% thionin, rinsed in water, dehydrated in ethanol series (70%, 95%, 100%) and xylene and mounted by cover slips. Conventional transmitted and darkfield images were acquired using Olympus AX70 microscope with UPlanF1 20×/0.50 NA or PlanApo 60×/1.40 NA Oil objectives.

4.6 Autoradiographic binding assays (I, II, III)

4.6.1 The [^{3}H]Ro 15-4513 autoradiography (I, III)

The [^{3}H]Ro 15-4513 autoradiographic binding assays were modified from Mäkelä et al. (1997). Brain sections on gelatin-coated object classes were pre-incubated in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl for 15 min. The final incubation was performed in the pre-incubation buffer containing 15 nM [^{3}H]Ro 15-4513 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) without (basal binding) or with 1 nM to 10 µM zolpidem or 10 µM flumazenil (non-specific binding) for human brain sections (**I**) and without (basal binding) or with 10 µM diazepam or 10 µM flumazenil (non-specific binding) for brain sections of saline or gaboxadol-injected Thy1α6 or C57BL/6NHsd mice (**III**) at 4 °C for 1 h. The sections were washed in ice-cold pre-incubation buffer twice for 1 min, dipped in ice-cold distilled water, air-dried at room temperature and exposed with ^{3}H-plastic standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to BAS-TR 2040 imaging plate (Fujifilm Corp., Tokyo, Japan) for up to 2–3 days (**I**) or to Biomax MR films (Eastman Kodak, Rochester, NY, USA) (**III**).

The binding densities were measured as optical density values (SCION IMAGE; Wayne Rasband, NIMH, Bethesda, MD) from digitalized images scanned by the FLA-9000 Starion image scanner (Fujifilm) (**I**) or by Epson expression 1680 Pro scanner (**III**) and converted by co-exposed ^{3}H-standards to nCi/mg for [^{3}H]Ro 15-4513 binding.

4.6.2 The [^{35}S]TBPS autoradiography (II, III)

The [^{35}S]TBPS autoradiographic binding assay has been described in Sinkkonen et al. (2001). 14-µm-thick horizontal brain sections on gelatin-coated object glasses were pre-incubated in ice-cold buffer containing 50 mmol/L Tris–HCl (pH 7.4) and 120 mmol/L NaCl for 15 min. The final incubation was performed at 22 °C for 90 min in the buffer containing 6 nmol/L [^{35}S]TBPS (Perkin-Elmer, Boston, MA, USA) without (basal binding) or with 1 mmol/L gaboxadol hydrochloride and 10 mmol/L GABA (Sigma, St Louis, MO, USA), 1 µmol/L – 10 mmol/L GABA, 1 mmol/L gaboxadol hydrochloride (Sigma) or 100 µmol/L picrotoxin (Sigma) (non-specific binding) for naive adult Thy1α6 and control C57BL/6NHsd mouse brain sections (**II**) and in the buffer containing 2 nmol/L [^{35}S]TBPS without (basal binding) or with 10 mM GABA, 1 mM gaboxadol hydrochloride, combination of 1 mM gaboxadol hydrochloride and 10 mM GABA, combination of 10 mM GABA and 100 µM furosemide (Sigma) or 100 µM picrotoxinin (non-specific binding) for brain sections of saline or gaboxadol-injected Thy1α6 or C57BL/6NHsd mice (**III**). After incubations, the sections were washed 3× 30 min in ice-cold 10 mmol/L Tris–HCl (pH 7.4), dipped

into distilled H_2O, air-dried at 22 °C, and exposed with plastic ^{14}C radioactivity standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to Biomax MR films (Eastman Kodak, Rochester, NY, USA) for up to 6 weeks (**II, III**).

The binding densities were quantified on films by MCID M5 image analysis devices and programs (Imaging Research Inc., St. Catharines, ON, Canada) (**II**) or on scanned films by SCION IMAGE (**III**), and by using the simultaneously exposed ^{14}C-standards as the reference, the binding values were converted to radioactivity levels estimated for gray matter areas (nCi/g) for [^{35}S]TBPS binding.

4.7 Cell culture and transfection (II)

Human embryonic kidney cell line (HEK293) were passaged and replated on 12-mm glass coverslips located in 9.6-cm plastic dishes filled with 10 mL of Minimum Essential Medium supplemented with 158 mg/L sodium bicarbonate, 2 mmol/L glutamine, 100 U/mL penicillin–streptomycin, and 10% fetal calf serum (all from Invitrogen, Karlsruhe, Germany). Cultures were maintained at 37 °C in a humidified 95% O_2/5% CO_2 atmosphere for 2–3 days.

Transfections were carried out as described in Korpi and Lüddens (1993) and Lüddens and Korpi (1997) in double or triple subunit combinations using the phosphate precipitation method with rat $GABA_A$ receptor cDNAs in eukaryotic expression vectors. To identify transfected cells all subunit combinations except δ-internal ribosome entry site- enhanced green fluorescent protein (EGFP) were co-transfected with 1 μg of pN1-EGFP per plate.

4.8 Electrophysiology (II)

Two days after transfection single coverslips with human embryonic kidney cell line (HEK293) were placed in a recording chamber mounted on the movable stage of a fluorescence microscope (Olympus IX70; Olympus Optical CO. Europa GmbH, Hamburg, Germany) and perfused with a recording solution at 22°C. Transfected cells were identified by their eGFP fluorescence. Ligand-mediated membrane currents in these cells were studied in the whole-cell configuration of the patch-clamp technique (Rabe et al. 2007).

Dose-responses of GABA and gaboxadol and the kinetics of GABA and gaboxadol-induced peak currents were studied on HEK293 cells expressing α6β3, α6β3δ, α6β3γ2, or α4β3δ receptors. 0.01–1000 μmol/L of gaboxadol hydrochloride or GABA was applied to the cells with a fast application system (SF-77B, Perfusion Fast Step; Warner Instruments, LLC, Hamden, CT, USA). The binding

site competition by GABA and gaboxadol was tested on HEK cells expressing α6β3δ and α6β3γ2 receptors. 1 mmol/L GABA or gaboxadol or 1 mmol/L GABA or gaboxadol and 0.1, 1, 10, 100, and 1000 μmol/L gaboxadol or GABA, respectively, were applied to the cells.

Cell responses were recorded by a patch-clamp amplifier (EPC-8; HEKA-Electronic, Lambrecht, Germany) in conjunction with a standard computer and the pClamp 8.1 software package (Axon Instruments, Foster City, CA, USA). Data analysis was performed using the appropriate programs.

4.9 Statistical analyses (II, III)

Statistical analyses of the data were carried out using two- and three-way ANOVAs, non-parametric Mann–Whitney test, Wilcoxon matched pairs, Student's t-test, two-tailed Welch t-test and Newman-Keuls comparisons with the statistical software packages SPSS 10.0.7 or 14.0.1 for Windows (SPSS, Chicago, IL, USA) and the PRISM 3.0 or 5.0 program (GraphPad Software, San Diego, CA, USA).

5 Results and discussion

5.1 Human LC neurons express $GABA_A$ receptor γ2 subunit mRNA and benzodiazepine binding sites (I)

The current study was performed in order to investigate whether the human LC neurons express the $GABA_A$ receptor γ2 subunit mRNA and benzodiazepine binding sites. Benzodiazepines are well known anxiolytics, and could in theory directly decrease anxiety and the associated arousal and/or insomnia by increasing the activity of the γ2 subunit-containing $GABA_A$ receptors on the LC neurons thereby decreasing the firing of these cells. However, contradictory reports had been published in regard to the expression and participation of the γ2 subunit in the functional $GABA_A$ receptors in the mammalian LC. Some studies had suggested the presence of γ2 subunit mRNA or protein in the rat LC (Fritschy et al., 1992; Lüddens et al., 1995; Caldji et al., 1998 and 2000), whereas many studies could not detect the γ2 subunit gene expression in the rodent LC (Araki et al., 1992; Luque et al., 1994; Tohyama and Oyamada, 1994). Based on pharmacological investigations, Chen and colleagues (1999) concluded that the $GABA_A$ receptors of the rat LC neurons do not contain the γ subunit.

The localization of the LC in the human pons sections was determined by TH immunohistochemistry and by the endogenous neuromelanin pigment that gives the natural bluish-grey color for the LC. Neuromelanin is a waste product of catecholamine metabolism, derived from the oxidation of catecholamines and related compounds to quinones (German et al., 1988). Noradrenaline is synthesized from tyrosine (tyrosine → dopa → dopamine → noradrenaline), and the rate-limiting enzyme in this process is TH which can be used to label the noradrenergic neurons in the LC. However, although most TH expressing neurons in the human LC contain neuromelanin pigment, some larger neurons lack the pigmentation (Chan-Palay and Asan, 1989).

In this study, a clear γ2 mRNA signal was revealed in the human LC. The finding is reliable as consistent results were obtained by two antisense probes designed to hybridize to different locations of the human $GABA_A$ receptor γ2 subunit mRNA. The emulsion autoradiography, that gives information with a cellular level resolution, revealed that the γ2 subunit mRNA was expressed over the entire LC, including neuronal somas within the structure, covering both neuromelanin-pigmented and non-pigmented somas. In the negative control experiments with complementary sense probes and with competition hybridizations with an excess of unlabelled antisense probes only a very weak signal was detected on the films. The signal was completely absent in the negative controls after the emulsion autoradiography indicating that the faint signal on the film was an

artefact. The cerebellar granule cell layer served as a positive control for the γ2 subunit mRNA expression, and a strong expression was detected there, thus further increasing the reliability of the study.

As a clear γ2 subunit mRNA expression was detected in the human LC, it was further investigated whether the subunit is assembled in $GABA_A$ receptors within the structure. As Ro 15-4513 exhibits high affinity to all $GABA_A$ receptors consisting of αβγ subunit combinations (Sieghart, 1995), a [^{3}H]Ro 15-4513 binding autoradiography was performed. The [^{3}H]Ro 15-4513 binding was clearly detected in the LC and in the cerebellar granule cell layer that was used as a positive control for benzodiazepine binding (Albin and Gilman, 1990). The benzodiazepine binding-site antagonist flumazenil displaced all [^{3}H]Ro 15-4513 binding in the sections. These results indicate that the γ2 subunit is assembled in $GABA_A$ receptors in the human LC.

To further investigate the subunit combination in the benzodiazepine-binding receptors, we tested whether there were binding sites for zolpidem, an α1 subunit-preferring benzodiazepine-site agonist that has little or no sensitivity to γ1/γ3 subunit-containing receptors (Lüddens et al., 1994; Sanna et al., 2002). The cerebellar granule cell layer was used as a positive control as it expresses α1 subunits but not α2/3 subunits in rats (Wisden et al., 1992). Based on the IC_{50} values for zolpidem determined by displacement of [^{3}H]Ro 15-4513 binding, our results indicate that the $GABA_A$ receptors in the human cerebellar granule cell layer are 3-times more sensitive to zolpidem than those in the LC, but as zolpidem displays about 10-fold higher affinity to α1 subunit-containing receptors than to α2/3 subunit-containing receptors (Hadingham et al., 1993), it is likely that the α1 as well as α2/3 subunits are assembled in the $GABA_A$ receptors in the human LC. However, it cannot be fully excluded that the α2/3 subunits would be expressed in the human cerebellar granule cell layer and that the sensitivity difference between the granule cells and LC would have been due to some α1 expression in granule cells and none in LC. It is however most likely that also the α1 subunits assemble in receptors in the human LC as Waldvogel and colleagues (2010) reported α1 immunoreactivity in the human LC. In that study, the neuromelanin-pigmented neurons of LC were highly immunopositive for the $GABA_A$ receptor α3 and γ2 subunits and minimally labeled by α1, α2, or β2/3 subunit-antibodies. The non-pigmented neurons interspersed between the pigmented neurons and were immunopositive for the α1 and β2/3 subunits. The autoradiography films do not allow a cellular level analysis, and therefore it cannot be determined whether the α1 and α2/3 subunits in our LC samples were located in pigmented or non-pigmented neurons. In mice, the $GABA_A$ receptor α1 and α2 subunits have been associated with sedative and anxiolytic effects of benzodiazepines, respectively (McKernan et al., 2000; Löw et al., 2000). In the current study it was

shown that the $\gamma 2$ subunit is expressed in the neuromelanin-pigmented and non-pigmented human LC neurons and that the LC neurons express $\alpha 1$, $\alpha 2/\alpha 3$ and $\gamma 2$ subunit-containing $GABA_A$ receptors likely in the same neurons that express the $\gamma 2$ mRNA. These findings indicate that the human LC may directly mediate the sedative and/or anxiolytic effects of benzodiazepines. This study does not provide an answer to the inconsistent findings related to the $\gamma 2$ subunit expression in the rodent LC, but there is a possibility that there is a species difference in the expression profile of the $\gamma 2$ subunit in the LC. If the benzodiazepine binding sites are more abundant in the LC of man than rodents, the anxiolytic drugs in rodents may produce pronounced adverse sedative effects in man via the $\gamma 2$ subunit-containing benzodiazepine binding sites in the LC. However, any of the $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunits may assemble in human LC with the $\gamma 2$, and one of the reasons for the association of the mouse $\alpha 1$ subunit with the sedative effects of benzodiazepines may result from benzodiazepine action in other brain regions than LC.

5.2 The Thy1α6 mouse model expresses ectopically the $GABA_A$ receptor α6 subunit contributing to extrasynaptic receptors and tonic inhibition

The transgenic Thy1α6 mice express the $GABA_A$ receptor α6 subunit gene under the Thy-1.2 promoter, and the first study on the mouse line reported an ectopic mRNA expression of $GABA_A$ receptor α6 subunit in many brain regions including deep cerebellar nuclei, deep layers of neocortex, layer II of neocortex in the somatosensory area, hippocampal pyramidal cells and dentate granule cells, cells in the septum and olfactory bulb mitral cells (Wisden et al., 2002). Of the investigated Thy1α6 mouse brain areas, only some cells were α6-immunopositive and most often large cell types displayed strong α6 immunoreactivity. Strong α6 subunit immunoreactivity was reported in the globus pallidus, zona reticulata of the substantia nigra, nucleus ruber, large cells in the intermediate gray layer of superior colliculus, some cells of the pontine nuclei, the vestibular nuclei, deep cerebellar nuclei, gigantocellular reticular formation, trigeminal nucleus and lateral reticular nucleus. In α6 immunopositive cells, the transgene was always present in the cell body, and the dendrites were α6 immunopositive to different extent in different neurons (Wisden et al., 2002).

It was reported that in the Thy1α6 mouse forebrain, the α6 subunit-containing receptors contained also the $\gamma 2$ subunit or consisted of α6β receptors (Wisden et al., 2002; Sinkkonen et al., 2004), and that the α6 subunit-containing receptors were largely located extrasynaptically in the pyramidal cells of CA1 hippocampal area, a brain area that was chosen for more detailed microscopic investigation due to a strong α6 subunit protein expression on the entire somato-dendritic surface of

the neurons (Wisden et al., 2002). As compared to control C57BL/6 mice, the CA1 pyramidal neurons of the Thy1α6 mice had a five-fold increased tonic $GABA_A$ receptor-mediated current (Wisden et al., 2002), further supporting the extrasynaptic nature of the receptors containing the ectopically expressed subunit. This is also in line with the finding that in its natural environment in cerebellar granule cells, the α6 subunit forms extrasynaptic receptors with β2/3 and δ subunits (Barnard et al., 1998). In cerebellar granule cells, the α6 subunit assembles also in receptors containing the γ2 subunit. The majority of the α6βγ2 receptors are located synaptically, although some of these receptors have also been detected in the extrasynaptic membrane in cerebellar granule cells (Nusser et al., 1998).

5.3 Earlier behavioral characterization of consequences of ectopic expression of $GABA_A$ receptor α6 subunits

Thy1α6 mice are healthy, grow and breed as the control C57BL/6 mice. The initially performed basic characterization of their phenotype according to a standardized set of experimental procedures revealed no strain differences in most of the studied parameters (Sinkkonen et al., 2004). Only the tests on startle reflex and struggle-escape behavior showed that the Thy1α6 mice were more sensitive to the acoustic stimuli and had an enhanced struggle-escape behavior as compared to the control C57BL/6 mice. Therefore, a slightly increased behavioral excitability of the Thy1α6 mice was suggested by the authors, even though no changes in anxiety, exploration or working memory of the Thy1α6 mice were observed in the in the light–dark or T-maze test (Sinkkonen et al., 2004).

The first behavioral consequence of the increased tonic conductance in the Thy1α6 mice was observed when a low dose of a GABA uptake blocker, the antiepileptic drug tiagabine (*i.p.*, 30 min prior to *i.p.* picrotoxinin) delayed the picrotoxinin-induced generalized tonic-clonic convulsions in the Thy1α6 mice as compared to the wild-type C57BL/6 controls. However, higher tiagabine concentrations had a stronger antiepileptic effect in the control mice (Sinkkonen et al., 2004).

5.4 The Thy1α6 mice display an enhanced sensitivity to anxiolytic-like and sedative effects of gaboxadol (II)

In order to investigate further the behavioral consequences of the enriched extrasynaptic $GABA_A$ receptors, they were aimed to be selectively activated by gaboxadol in the present study. It had been shown in recombinant $GABA_A$ receptors that gaboxadol had higher potency and relative efficacy (as compared to GABA) in α6β3δ, 4β3δ and α4β3 receptors as compared to α1-6β3γ2 receptors of which the α2/5/6β3γ2 receptors displayed the highest sensitivity to gaboxadol and the efficacy of

GABA and gaboxadol were similar in these receptors. The α1/3/4β3γ2 had the lowest sensitivity to gaboxadol that was a partial agonist in them as compared to GABA (Ebert et al., 2001; Storustovu and Ebert, 2006). The α4βδ $GABA_A$ receptors have been reported to locate extrasynaptically mediating tonic conductance in thalamus (Cope et al., 2005; Jia et al., 2005; Richardson et al., 2013). In line with these findings, suggesting that gaboxadol acts preferentially via extrasynaptic α6β3δ, α4β3δ and α4β3 receptors, we expected that especially the extrasynaptic α6β receptors (but also α6βγ2 receptors) would be more sensitive to gaboxadol than the typical synaptically located $GABA_A$ receptors, and that this enhanced sensitivity would become visible in the behavior of the transgenic mice ectopically expressing the α6 subunit.

Indeed, gaboxadol induced strong anxiolytic-like response in the light : dark exploration and elevated plus-maze tests in Thy1α6 mice at 3 mg/kg, while the anxiolytic behavior was largely unaffected in the gaboxadol-treated wild-type C57BL/6 NHsd mice. In the light : dark exploration test, this anxiolytic-like behavior of the transgenic mice was shown as a shortened latency to the first dark entry, an increased number of crossings between the compartments, and in a doubled time the mice spent in the lit compartment. There was no difference between saline-treated C57BL/6NHsd control and Thy1α6 mice, except that there was a significant delay in the first entry into the dark compartment in the transgenic mice. In the elevated plus-maze test, gaboxadol was notably more effective in increasing the time on the open arms in the Thy1α6 mice than in the C57BL/6 NHsd mice, whereas the saline-treated mouse lines did not differ in any of the tested parameters. Neither test showed any sedative effect at this low dose of gaboxadol in either mouse line as determined by measuring crossings and total movements. The gaboxadol-treated female Thy1α6 mice had slightly, but significantly more crossings than the gaboxadol-treated Thy1α6 males, C57BL/6 NHsd males or C57BL/6NHsd females in the light : dark exploration test. In the elevated plus-maze test the saline-treated control males moved significantly more than the saline-treated females, the Thy1α6 males or females. However, there were no significant gender effects on the time in lit area or on time on open arms.

Differences in early nursing or genetic background could have had a role in the sensitivity difference between the Thy1α6 and C57BL/6NHsd mice to gaboxadol. The transgenic mice had been generated by injecting the Thy1α6 transgene into mouse eggs (strain CBA/cba×C57BL/6), and the Thy1α6 founder line was repeatedly expanded in C57BL/6 background for at least 10 generations. In the current study, the experimental Thy1α6 mice were produced by mating within a homozygous line, and the control C57BL/6NHsd mice were purchased at the age of 5 weeks. Thereafter, the mice were maintained until the experiments at the age of 3–6 months. In order to test

the potential confounding effects of genetic background and early nursing on the result, the Thy1α6 mice were backcrossed into C57BL/6HNsd mouse line to produce heterozygous offspring that were then mated to generate transgenic Thy1α6 mice (heterozygous and homozygous) and littermate wild-type controls. The behavioral difference after 3 mg/kg of gaboxadol was confirmed in these backcrossed transgenic and wild-type littermate mice. In the backcrossed Thy1α6 mice, gaboxadol increased significantly the time spent in the lit area and the number of crossings between the compartments in the light : dark exploration test. In general the latencies to the first dark entry were much shorter in the backcrossed mice than in the original Thy1α6 and C57BL/6HNsd control mice. The saline-treated backcrossed transgenic mice had a similar latency as the wild-type littermates deviating from the result obtained in the saline-treated original Thy1α6 and C57BL/6HNsd control mice. Even though gaboxadol prolonged the latency to the first dark entry of the wild-type littermates and had an opposite effect on the backcrossed Thy1α6 mice, the time in the lit area, *i.e.* the main indicator for the anxiolytic-like response, showed very similar results between the littermate and non-littermate transgenic and wild-type mice. Therefore, the initial differences in the latencies unlikely affected the observed anxiolytic-like response of the Thy1α6 mice to gaboxadol.

The Thy1α6 mice were also more sensitive to gaboxadol-induced hypnosis as assessed by the latency to and the duration of the LORR after administration of gaboxadol hydrochloride (25 mg/kg, *i.p.*). As compared to the wild-type C57BL/6NHsd mice, the Thy1α6 mice lost quicker and for longer time their righting reflex after the gaboxadol treatment. Furthermore, three out of eight wild-type mice did not lose their righting reflex and they were excluded from the data.

As gaboxadol did not induce any consistent effects on the locomotor activation, it is concluded that the response of transgenic mice to gaboxadol was anxiolytic-like rather than motor stimulant. $GABA_A$ receptor-mediated anxiolysis has been traditionally associated with α2βγ2 receptors based on studies with benzodiazepines (Löw et al., 2000), but in the Thy1α6 mice the strong anxiolytic response to gaboxadol was most likely mediated via the α6 subunit-containing $GABA_A$ receptors. Considering the enhanced behavioral sensitivity of the Thy1α6 mice to both anxiolytic and hypnotic effect of gaboxadol as compared to the wild-type mice, it seems likely that the α6 subunit-containing receptors formed extrasynaptic receptors with enhanced pharmacological sensitivity to gaboxadol.

5.5 The enhanced behavioral sensitivity to gaboxadol is likely mediated via α6β3 and α6β3γ2 $GABA_A$ receptors

The α6β receptors are about four times more sensitive to gaboxadol than α6βγ2 receptors (see the later sections of results) suggesting that gaboxadol produces the anxiolytic-like responses in Thy1α6 mice by activating the ectopic α6β receptors. Pharmacokinetic studies on rats by Cremers and Ebert (2007) showed that subcutaneously injected gaboxadol entered rapidly the brain with peak CNS concentrations in the range of 0.7 to 3 µM after one dose of gaboxadol at 2.5, 5 and 10 mg/kg. A very short half-life (28 min) of gaboxadol was observed in both plasma and CNS (Cremers and Ebert, 2007). Assuming that similar pharmacokinetics of gaboxadol applies also to mice and fitting our data on recombinant receptors to the Hill equation ($I = Imax/(1 + (EC_{50}/ [C])^n)$, where Imax is the maximal current density induced by 1 mmol/L gaboxadol, [C] is 1 µM (the estimated CNS concentration after one dose of gaboxadol at 3 mg/kg) and n is the apparent Hill coefficient (approximately equal to 1)) separately for α6β3γ2 and α6β3 receptors, the current densities (I) at 1 µM gaboxadol concentration in the brain are equivalent (9.2 pA/pF) via both α6β3γ2 and α6β3 receptors. In other words, based on this calculation, the sensitivity difference between these receptors to 1 µM gaboxadol is offset by the greater gaboxadol-induced current density in α6β3γ2 receptors than in α6β3 receptors. Therefore, it is likely that the anxiolytic dose of gaboxadol activates both the α6β3 and α6β3γ2 receptors in the Thy1α6 mice. When fitting our data on recombinant receptors into the Hill equation with [C] of 7.5 µM (the estimated CNS concentration after one hypnotic dose of gaboxadol at 25 mg/kg), the current density (I) is 44.9 and 60.4 pA/pF via the α6β3 and α6β3γ2 receptors, respectively. This suggests that at such high gaboxadol concentration, the current density is higher via α6β3γ2 despite of the lower sensitivity.

It is also unlikely that these pronounced behavioral effects of gaboxadol were mediated via any compensatory increase in the function of synaptic $GABA_A$ receptors in the transgenic mice. In Mortensen et al. (2010) the potencies and efficacies of gaboxadol were studied in recombinant α1β3γ2 and α4β3δ receptors. It was shown that in α4β3δ receptors, gaboxadol was more than twice as efficient as in α1β3γ2 receptors, but in addition to that, the α4β3δ receptors were eight times more sensitive to gaboxadol than the α1β3γ2 receptors. In the present study, α1β3γ2 receptors were not investigated but based on this information and our electrophysiological data, it can be assumed that the overexpressed extrasynaptic $GABA_A$ receptors selectively mediate the enhanced behavioral sensitivity of Thy1α6 mice to gaboxadol. According to Wafford and Ebert (2006), gaboxadol has no effect on synaptic currents at concentrations of up to 3 µM. In addition, the frequency of spontaneous inhibitory postsynaptic currents and amplitudes of mIPSCs were decreased in the CA1

pyramidal neurons of the Thy1α6 as compared to wild-type mice (Wisden et al., 2002), suggesting a reduced synaptic $GABA_A$ receptor function. However, the Thy1α6 mice were also less sensitive to electroshock-induced convulsions and had decreased amplitudes in the AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in patch clamp recordings of CA1 pyramidal neurons in hippocampal slices (Möykkynen et al., 2007). It is likely that this decrease in the postsynaptic excitability was a compensatory response to the reduction in the postsynaptic GABAergic inhibition, but it is not possible to fully exclude the possibility that in the transgenic mice the function of the postsynaptic excitatory AMPA receptor system would have been disabled more than the synaptic $GABA_A$ receptor system and therefore, or partly therefore, less gaboxadol would have been sufficient to induce the anxiolytic and hypnotic effects in the Thy1α6 mice. On the other hand, the non-treated Thy1α6 mice were more sensitive to the acoustic stimuli and had an enhanced struggle-escape behavior as compared to the control C57BL/6 mice (Sinkkonen et al., 2004), suggesting that the function of the neuronal excitatory system is not decreased at least in regions mediating the acute fear responses.

An enhanced behavioral sensitivity to ataxic, sedative, and analgesic effects of gaboxadol at 10 mg/kg has been associated with the α4 subunits that are expressed at high levels in the dentate gyrus and thalamus and contribute to extrasynaptic $GABA_A$ receptor-mediated tonic inhibition (Chandra et al. 2006). Although the Thy1α6 mouse model is expressing the α6 subunit in neurons where it is normally absent, the model allows examining the function of the added α6 subunit-containing extrasynaptic $GABA_A$ receptors and it provides *in vivo* evidence that anxiolytic and hypnotic doses of gaboxadol act preferentially via α6 subunit-containing $GABA_A$ receptors.

5.6 GABA-insensitive [^{35}S]TBPS autoradiographic signal reflects partial agonism of GABA (II)

TBPS blocks the of ion flux through the $GABA_A$ receptor by binding to the ionophore/convulsant site (Korpi et al., 2002a). Basal [^{35}S]TBPS binding represents non-conducting $GABA_A$ receptors and dissociation of [^{35}S]TBPS by agonists, serves as a biochemical measure of receptor activation (Im and Blakeman, 1991, Sinkkonen et al., 2001). It has been shown that the displacement of [^{35}S]TBPS binding by saturating concentration of GABA partially fails in the cerebellar granule cell layer and thalamus in contrast to the majority of other brain regions (Sinkkonen et al. 2001). These regions are enriched by the expression of α6, α4, and δ subunits (Wisden et al., 1992; Pirker et al., 2000). By expressing different subunit combinations in HEK cells, it has been shown that α6βx (but not α6βxγ2) receptors retain about 50% of the [^{35}S]TBPS binding in the presence of millimolar

GABA concentrations (Korpi and Lüddens, 1993). As previous electrophysiological data on recombinant receptors have suggested that GABA is a partial agonist as compared to gaboxadol in extrasynaptic α6β3δ, 4β3δ and α4β3 receptors but not in α1-6β3γ2 receptors (Adkins et al., 2001; Ebert et al., 2001; Storustovu and Ebert, 2006) and as GABA-insensitive (GIS)-[^{35}S]TBPS binding has been associated with α6β2/3 and α4/6β2/3δ receptors (Korpi and Lüddens, 1997; Mäkelä et al., 1997; Korpi et al., 2002b; Sinkkonen et al., 2001, 2004a and 2004b), our hypothesis was that as a stronger agonist than GABA, gaboxadol at high concentration would displace the GIS-[^{35}S]TBPS binding from α6β2/3 and α4/6β2/3δ receptors and if so, GIS-[^{35}S]TBPS binding would indeed represent partial agonism of GABA on α6β2/3 and α4/6β2/3δ receptors.

In line with the results of Sinkkonen et al. (2004b), the hippocampus of the Thy1α6 mice retained significantly more GIS-[^{35}S]TBPS binding (13.5 ± 1.0% of the basal [^{35}S]TBPS binding) than that of the control C57BL/6NHsd mice (1.7 ± 0.2% of the basal [^{35}S]TBPS binding) revealing the α6βx receptors. Gaboxadol (1mM) displaced the [^{35}S]TBPS binding more than GABA (10 mM) in the hippocampus of the transgenic mice whereas the efficacies of GABA and gaboxadol were similar in the hippocampus of the wild-type control mice indicating that GABA is a partial agonist in comparison to gaboxadol in the α6βx receptors. As a characteristic of partial agonists is that they can reverse the effects of full agonists acting as competitive antagonists, we co-applied different doses of GABA with gaboxadol (1mM) and measured the remaining [^{35}S]TBPS binding. Already 0.1 mM GABA significantly increased [^{35}S]TBPS binding as compared to gaboxadol alone, and 1 mM GABA increased the [^{35}S]TBPS binding to the same level as saturating GABA concentration (10 mM) alone, demonstrating a competitive displacement of full agonist gaboxadol from the agonist binding site by the partial agonist GABA.

The next question was whether a similar fingerprint for partial agonism of GABA could be found in the cerebellar granule cell layer and thalamus that are naturally expressing α6, α4, and δ subunits. In the Thy1α6 mice and control C57BL/6NHsd mice, a saturating concentration of GABA (10 mmol/L) revealed a GIS-[^{35}S]TBPS binding in the thalamus and cerebellar granule cell layer as expected. In both brain regions and mouse strains, gaboxadol displaced the [^{35}S]TBPS binding more than GABA, and when increasing concentrations of GABA were co-applied with 1 mmol/L gaboxadol, GABA at millimolar concentrations increased [^{35}S]TBPS binding in the transgenic and wild-type mice. However, in the thalamus and cerebellar granule cell layer, the difference between GIS-[^{35}S]TBPS and gaboxadol-insensitive [^{35}S]TBPS binding at high agonist concentrations was not as great as in the hippocampus of the transgenic mice, indicating that the efficacy difference between GABA and gaboxadol is greater in α6β3 receptors than in α6β3δ or α4β3δ receptors. These

results demonstrate that GIS-[^{35}S]TBPS binding reflects partial agonism of GABA also in the native brain regions containing α6β3δ or α4β3δ receptors in which gaboxadol acts with higher efficacy.

The collicular region was used as a control region because it expresses mainly α1βxγ2 receptors (Wisden et al., 1992; Pirker et al., 2000). These receptors represent the majority of $GABA_A$ receptors in the brain (Fritschy et al., 1992) and in these receptors gaboxadol acts as a partial agonist (Ebert et al., 1994; Ebert et al., 1997). In accordance with this, GABA was more efficient than gaboxadol in the collicular region of both mouse lines leaving no GIS[^{35}S]TBPS binding at saturating GABA concentrations.

Altogether, these results indicate that GIS-[^{35}S]TBPS binding can be used as a fingerprint for partial agonism of GABA.

5.7 Highly potent GABA is a partial agonist as compared to gaboxadol in α6β3, α6β3δ and α4β3δ receptors but not in α6β3γ2 receptors (II)

The aim of this study was to investigate further the pharmacology of gaboxadol and GABA on different extrasynaptic $GABA_A$ receptors, namely on α6β3, α6β3δ, α4β3δ and α6β3γ2 receptors and to confirm that the GIS[^{35}S]TBPS binding reflects the partial agonism of GABA as compared to gaboxadol in α6β3, α6β3δ and α4β3δ receptors. Gaboxadol had been earlier reported to act as a superagonist in recombinant α4β3δ receptors (Adkins et al., 2001). Later on, it was also shown that GABA is a partial agonist as compared to gaboxadol in human α4β3δ and α6β3δ receptors expressed on Xenopus oocytes and when GABA was co-applied with a fixed concentration of gaboxadol at α4β3δ receptors, GABA reduced concentration-dependently the response to gaboxadol until the response corresponded to the maximum response to GABA (Storustovu and Ebert 2006). In the present study the relative efficacy and other kinetic parameters of high concentrations of GABA (1 mmol/L) and gaboxadol (1 mmol/L) were studied in α6β3, α6β3δ, α6β3γ2 and α4β3δ receptors.

Our electrophysiological results showed that gaboxadol had the highest relative efficacy as compared to GABA in α6β3 receptors, but also in α6β3δ and α4β3δ receptors the efficacy of gaboxadol was about twofold higher than that of GABA. This is in line with the previous electrophysiological data on recombinant receptors on α6β3δ and 4β3δ receptors (Adkins et al., 2001; Ebert et al., 2001; Storustovu and Ebert, 2006). In α6β3γ2 receptors, the efficacy of GABA and gaboxadol were similar. However, the maximal GABA and gaboxadol-mediated currents via these receptors were much larger than those via α6β3, α6β3δ or α4β3δ receptors. As partial

agonists reverse the effects of full agonists acting as competitive antagonists, we compared the effects of increasing competing concentrations of GABA on the maximal responses evoked by 1 mmol/L gaboxadol and the effects of competing concentrations of gaboxadol on the maximal responses evoked by 1 mmol/L GABA in α6β3γ2 and α6β3δ receptors. As expected, in α6β3γ2 receptors the efficacy of GABA and gaboxadol were identical, and the co-application of the competitor did not change the maximal response evoked by either GABA or gaboxadol. However, in α6β3δ receptors GABA reduced the 1 mmol/L gaboxadol response and gaboxadol increased the 1 mmol/L GABA response. This interaction proves the partial agonism of GABA in the α6β3δ receptors.

The weighted time constant for desensitization of α6β3γ2 receptors was similar for GABA (1 mmol/L) and gaboxadol (1 mmol/L), and although α4β3δ receptors desensitized somewhat faster after GABA than after gaboxadol, both α6β3γ2and α4β3δ receptors showed largest extent of desensitization relative to the peak current at 4 s of both GABA and gaboxadol application. GABA and gaboxadol displayed most dissimilar kinetics in α6β3 and α6β3δ receptors which were desensitizing significantly faster after gaboxadol than after GABA application. Also the level of desensitization relative to the peak current at 4 s was greater after gaboxadol than after GABA in these receptors. In α6 subunit-containing receptors the deactivation after GABA was slowest in α6β3 receptors, followed in order by α6β3δ and α6β3γ2 receptors. The order of deactivation was the same after gaboxadol, and in all α6 subunit-containing receptors the deactivation occurred faster after gaboxadol than after GABA exposure. The relatively short deactivation time was similar after GABA and gaboxadol in α4β3δ receptors.

The effects of high agonist concentrations reported here may not be physiologically important for the extrasynaptic currents, but they may explain the different effects of the agonists on the [^{35}S]TBPS binding to native receptors. As the GIS-[^{35}S]TBPS binding is imaged in the presence of high concentrations of GABA in α6β and α4/6βδ receptors (Sinkkonen et al., 2001), the partial agonism of GABA at these receptors correlates with GIS-[^{35}S]TBPS binding. The partial agonism of GABA and superagonism of gaboxadol correlates also with the GIS-[^{35}S]TBPS binding that was dissociated to large extent by gaboxadol in the hippocampus of the Thy1α6 mice as well as in the thalamus and cerebellar granule cell layer of the wild-type mice, the regions known to express α6β, α4βδ or α6βδ receptors, respectively.

Also the desensitization characteristics of the receptor complex may regulate the remaining agonist-insensitive [^{35}S]TBPS binding component. In the present study on recombinant α6β3, α4βδ3, α6β3δ

and α6β3γ2 receptors, GABA and gaboxadol did not differ in their speed of desensitization (measured as weighted time constant of desensitization) in α6β3γ2 receptors whereas it was always faster after gaboxadol exposure than after GABA exposure in the α6β3, α4β3δ, α6β3δ receptors. The extent of desensitization (relative to the peak current of the tested agonist) or weighted time constant of deactivation did not provide evidence of different effect between GABA and gaboxadol at the α6β3γ2 receptors as compared to the α6β3, α4β3δ, α6β3δ receptors. If the speed of desensitization played a role in agonist-insensitive [^{35}S]TBPS binding, logically thinking GABA would displace [^{35}S]TBPS more than gaboxadol from α6β3, α4β3δ, α6β3δ receptors (opposite to what has been observed) due to its slower desensitization speed at these receptors. As the desensitization and deactivation kinetics were tested after a 4 s incubation, there is no data on how these kinetic parameters may change within the 90-minute incubation time in the [^{35}S]TBPS binding studies. As a conclusion, more data is needed to reveal whether the desensitization kinetics of the receptor complex regulate the agonist-insensitive [^{35}S]TBPS binding.

5.8 The α6 subunit-containing GABA$_A$ receptors are widely distributed in the Thy1α6 mice (III)

The α6 subunit mRNA expression was restricted to the cerebellar granule cell layer in the wild-type mice, in line with its known distribution (Laurie et al., 1992; Wisden et al., 1992), whereas in the Thy1α6 mice the α6 expression was widely distributed, including deeper layers of the isocortex (e.g. Cg1, motor, primary somatosensory, auditory and association areas), the olfactory areas (piriform area, anterior olfactory nucleus), hippocampal formation [CA1 and CA3 pyramidal cell layers, dentate gyrus (DG)] and BLA, being highest in the BLA, hippocampus and cortical regions. The receptor autoradiography revealed α6β and/or α6βδ receptors in the BLA and CA1 of the Thy1α6 mice as gaboxadol dissociated the [^{35}S]TBPS binding significantly more than GABA. The diazepam-insensitive [^{3}H]Ro 15-4513 binding, illustrating the localization of the ectopic α6βγ2 receptors, was strong in the middle and deep layers of isocortex, olfactory areas (piriform area, anterior olfactory nucleus), hippocampal formation (CA1 and DG) and BLA in the Thy1α6 mouse brain. Similar levels of the diazepam-insensitive [^{3}H]Ro 15-4513 binding were present in the cerebellar granule cell layer of both wild-type and transgenic mice containing native α6βγ2 receptors.

5.9 Widespread neuronal activation associated with gaboxadol-induced anxiolysis (III)

The induction of c-Fos, a protein product of a c-fos immediate early gene, is used as a tool to identify brain cells that become activated in response to various stimuli (Kovács, 1998). The expression of c-Fos is rather low in the brain of naive animals (Ryabinin et al., 1999). However, injection and handling stress has been shown to significantly increase c-Fos expression in C57BL/6 mice and a prehandling has been suggested as necessary to habituate the mice to the injection and handling stress and to decrease the c-Fos expression closer to the basal levels (Ryabinin et al., 1999). In the present study c-Fos expression was studied two hours after saline of gaboxadol injection (3 mg/kg, *i.p.*) in the transgenic Thy1α6 mice and wild-type C57BL/6HNsd control mice. The tested gaboxadol dose had been shown to be highly anxiolytic in Thy1α6 mice whereas only slight anxiolytic-like behavior was observed in the C57BL/6HNsd control mice. It was assumed that the anxiolytic dose of gaboxadol mediated the enhanced behavioral response in Thy1α6 mice by activating the artificially located α6β and α6βγ2 receptors in the forebrain. We prehandled the mice to avoid the injection and handling stress-induced c-Fos expression before gaboxadol fully entered the brain (30-60 min) (Cremers and Ebert, 2007) in order to investigate possible sites of action of low anxiolytic gaboxadol dose in the Thy1α6 mice as compared to basal c-Fos levels. We expected that gaboxadol would decrease any basal c-Fos expression due to its inhibitory GABAergic mode of action and because the anxiolytic effect of benzodiazepines has been generally associated with a decreased neuronal activity (Beck and Fibiger, 1995; Panhelainen and Korpi, 2012; Lkhagvasuren et al., 2014). It may be argued that our conditions with low level of stress/novel stimuli favored observations of only increases in neuronal activity whereas a decrease in already low basal c-Fos expression would have been difficult to detect. However, after saline injections, c-Fos was expressed at such levels in many regions that it should have been possible to detect increased inhibition (decreased c-Fos expression). Furthermore, in Lkhagvasuren et al. (2014), in many brain regions of non-stressed rats, including cerebral cortices (orbital, primary somatosensory, visual, secondary cingulate, retrosplenial and layer II piriform cortices and anterior olfactory nucleus), hippocampal formation, LS, claustrum, cortical and medial amygdaloid nuclei, posterior hypothalamic area, dorsal and medial premammillary nuclei, rhomboid nucleus of thalamus, superior colliculus and median raphe nucleus, diazepam was shown to decrease c-Fos expression approximately to half of the expression in the vehicle-treated rats, and similarly, diazepam decreased c-Fos expression in these regions in stress-exposed rats as compared to stress-exposed vehicle-treated rats.

In contrast to our hypothesis, we observed an increased c-Fos expression in gaboxadol-treated Thy1α6 mice especially in the limbic areas, such as the cingulate cortex, septal nuclei and in the central extended amygdala areas, including BNST and CeA as well as in BLA. C-Fos was also increased in dorsal tenia tecta, medial anterior olfactory nucleus, and dorsal endopiriform nucleus of the gaboxadol-treated Thy1α6 mice. Interestingly, many of these regions have previously shown decreased c-Fos expression in non-stress-exposed as well as in stress-exposed rats after diazepam injection (Lkhagvasuren et al., 2014). In addition, several studies suggest that different stressors activate the medial prefrontal cortex (mPFC), cingulate cortex, BLA, BNST and LS, and it has been suggested that the threshold for c-Fos induction in these areas is low (reviewed in Kovács, 1998). As we anyway had earlier observed a reduced anxiety at the tested dose of gaboxadol (**II**), our findings on widely increased c-Fos expression was surprising. Gaboxadol had only limited effects on the c-Fos expression in the wild-type mice. In the lateral part of the mediodorsal thalamic nucleus (MDL), there was a significant increase in c-Fos expression only in the gaboxadol-treated wild-type mice, but not in the transgenic mice. In some brain regions, including hippocampal CA1 and CA3 areas as well as layers 2-4 of the primary motor cortex, gaboxadol induced c-Fos expression regardless of the genotype.

5.10 Potential mechanisms of paradoxical gaboxadol-induced widespread neuronal activation

The gaboxadol-induced c-Fos expression may result from disinhibition of selected neuronal networks, although also depolarizing responses have been generated by $GABA_A$ receptor activation in a mature brain slices, and these depolarizing responses were originally proposed to be mediated via the activation of extrasynaptic $GABA_A$ receptors (Alger and Nicoll, 1979; Andersen et al., 1980; Alger and Nicoll, 1982). In rat hippocampal slices an intense stimulation of $GABA_A$ receptors on distal dendrites produced a hyperpolarizing membrane potential response followed by a slow depolarizing potential (Staley et al., 1995). It was proposed that this depolarization was a result of activity-dependent collapse of the opposing concentration gradients of chloride and bicarbonate, and that this consequently diminished the voltage-dependent block of the NMDA receptors thereby further depolarizing the cell membrane (Staley et al., 1995). It has also been postulated that the KCC2-dependent increase in extracellular potassium ion transients ($[K^+]_o$) cause an inward potassium ion current that induces a long-lasting and a more positive membrane potential than $E_{GABA\text{-}A}$ in CA1 pyramidal cells (Kaila et al., 1997; Smirnov et al., 1999; Viitanen et al., 2010). However, these depolarizing actions of $GABA_A$ receptors may well not be exclusive or even inclusive properties of extrasynaptic $GABA_A$ receptors and if such effects were occurring *in vivo*, it

would be likely that also diazepam would induce increased c-Fos expression. In addition, acute effects of systemic drugs with preferential action on extrasynaptic $GABA_A$ receptors, including gaboxadol, were not affected in mice lacking 80-85% of KCC2, although the responses to the synaptic $GABA_A$ receptor agonist benzodiazepine diazepam were strongly blunted (Tornberg et al., 2007), suggesting that KCC2 is predominantly involved in the phasic but not in the tonic component of GABAergic neurotransmission.

Methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM) is a potent inverse agonist in α1 subunit-containing receptors (Crestani et al., 2002), and a partial agonist in α6 subunit-containing receptors (Knoflach et al., 1996; Saxena and Macdonald, 1996). Interestingly, the Thy1α6 mice have been shown to be more sensitive to DMCM-induced convulsions than the wild-type mice (shorter latency to writhing clonus) (Sinkkonen et al., 2004b) indicating that Thy1α6 mice are more susceptible to convulsions induced by the inverse agonism of DMCM on α1 subunit-containing receptors and/or that agonism of DMCM on α6 subunit-containing receptors generates directly or indirectly shortened latency to neuronal excitation. Reduced synaptic $GABA_A$ receptor currents have been reported in the hippocampal CA1 neurons of Thy1α6 mice (Wisden et al., 2002), and therefore it seems likely that the Thy1α6 mice would be more sensitive to the convulsive effect of DMCM by decreasing the inhibitory effect of the receptors with an already reduced synaptic inhibition. It would be still an open question whether the agonism of DMCM on α6 subunit-containing receptors could generate directly or indirectly neuronal depolarization or whether the increased extrasynaptic inhibition was just not sufficient to balance the decreased synaptic inhibition.

Also the non-selective $GABA_A$ receptor blocker picrotoxinin shortened the latency of Thy1α6 mice to generalized convulsions as compared to wild-type mice (Sinkkonen et al., 2004b), which most likely resulted from reduced synaptic $GABA_A$ receptor responses. Picrotoxinin blocks also α6 subunit-containing receptors, but since these receptors were not present in the forebrain of the wild-type mice it is likely that the increased sensitivity to convulsions was due to blockage of already reduced synaptic $GABA_A$ responses in the transgenic mice. The result also suggests that physiological GABA concentrations are not sufficient to depolarize neurons via extrasynaptic α6 subunit-containing receptors as blocking this response would have had an opposite effect on the convulsion sensitivity of the Thy1α6 mice.

Tiagabine, an antiepileptic GABA uptake inhibitor increasing brain GABA levels, delayed picrotoxinin-induced convulsions at a low dose of 3.2 mg/kg in Thy1α6 mice but not in the wild-

type mice, but higher tiagabine doses shortened the convulsion latency in the Thy1α6 mice as compared to the wild-type mice (Sinkkonen et al., 2004b). This switch in the sensitivity of Thy1α6 mice to the tiagabine-reduced convulsions may be due to the function of highly potent but low-efficacy α6β3 receptors that at lower GABA concentrations would mediate the enhanced sensitivity of the Thy1α6 mice to tiagabine. At higher tiagabine concentrations causing higher extracellular GABA concentrations, the fully functioning synaptic $GABA_A$ receptors of wild-type mice with lower GABA sensitivity but higher efficacy would overcome the power of low efficacy α6β3 receptors and reduced synaptic inhibition in the Thy1α6 mice, thereby mediating the more enhanced antiepileptic response to high-dose tiagabine in the wild-type mice. An alternative explanation could be that at higher tiagabine concentrations an intense activation of extrasynaptic $GABA_A$ receptors by GABA resulted in depolarizing responses in the transgenic mice. In support of the latter, enhanced tonic $GABA_A$ receptor conductance via the knock-down of GABA reuptake has led to ataxia, tremor and increased nervousness (Chiu et al., 2005) suggesting increased neuronal excitation following increased extrasynaptic $GABA_A$ receptor function, but this neuronal excitation could also be an indirect effect of increased inhibition of inhibitory interneurons. Some reports have suggested that increased tonic inhibition of cortical interneurons by gaboxadol can lead to increased cortical network excitability (Krook-Magnuson and Huntsman 2005, Drasbek and Jensen 2005).

Overall, the direct gaboxadol-induced depolarization is an unlikely mechanism for the paradoxical c-Fos induction in the Thy1α6 mice because the hippocampal principal neurons were not increasingly activated by gaboxadol in the transgenic Thy1α6 mice, although the CA1 neurons express high level of ectopic and functionally active α6β/γ2 receptors (Wisden et al., 2002; Sinkkonen et al., 2004b). Furthermore, c-Fos induction by gaboxadol treatment in Thy1α6 brain was not restricted to areas highly expressing the α6-containing $GABA_A$ receptors (such as BLA and medial anterior olfactory nucleus) suggesting that indirect pathways lead to the paradoxically widespread activation. For example in the CeA and septum, gaboxadol induced strong c-Fos expression in spite of the lack of ectopic α6 subunit expression.

Altogether these results demonstrate that the anxiolytic dose of gaboxadol, preferring α4, α6 or δ subunit-containing extrasynaptic receptors, paradoxically activates multiple neurons in the limbic brain regions via α6 subunit-containing $GABA_A$ receptors in the Thy1α6 mice. This suggests that the widespread neuronal inhibition as typically associated with benzodiazepines is not the exclusive mechanism of anxiolysis. Instead, the selective activation of certain neuronal populations in the amygdala and BNST, might specifically lead to strong anxiolytic responses in the Thy1α6 mice.

5.11 Gaboxadol activated neurons in mediodorsal thalamic nucleus only in wild-type mice

MDL differed from the other brain regions, because there a significant increase in c-Fos expression was found only in the gaboxadol-treated wild-type mice, but not in the transgenic mice. MDL is interconnected with the mPFC and amygdala, being a part of the limbic circuitry (Vertes, 2006), and involved in fear-related information processing (Lee et al., 2012; Padilla-Coreano et al., 2012) and anxiety (Farb and Ratner, 2014). It has been shown recently, that a direct gaboxadol injection into the MDL, naturally containing gaboxadol-sensitive extrasynaptic α4βδ $GABA_A$ receptors (Cope et al., 2005; Jia et al., 2005; Richardson et al., 2013), increased fear freezing and attenuated fear extinction (Paydar et al., 2014). This suggests that local inhibition of MDL neurons mediate anxiety rather than anxiolysis.

As in our behavioral study (**II**), the low dose of gaboxadol was anxiolytic in Thy1α6 mice but not in the wild-type mice, it is tempting to speculate that in the transgenic mice, gaboxadol counteracted the anxiety mediating local action in MDL via α6 subunit-containing receptors outside MDL as no ectopic α6 subunit-containing $GABA_A$ receptors were observed in the MDL. However, as a result it would be expected that c-Fos was rather decreased than increased in the wild-type mice as compared to the Thy1α6 mice if gaboxadol did not induce direct α4βδ $GABA_A$ receptor-mediated depolarization. Histologically two populations of neurons, small inhibitory GABAergic interneurons projecting restrictedly to specific thalamic nuclei and larger excitatory relay neurons projecting to the cerebral cortex, have been described for the mediodorsal thalamic nucleus (Damgaard Nielsen et al., 2008). More detailed information on the activated neuronal MDL subpopulations and their association with gaboxadol-sensitive receptors would be necessary in order to understand the role of MDL in the anxiolytic action of gaboxadol in Thy1α6 mice. As a strong expression of ectopic α6 subunit-containing $GABA_A$ receptors was observed in the BLA as well as in the deep layers of PFC, both of which are abundantly projecting to the MDL (Vertes, 2006), these projections in the Thy1α6 mice may have inhibited the neuronal activation in the MDL that was observed in the wild-type mice after gaboxadol treatment.

6 Conclusions

$GABA_A$ receptors and neuroanatomy of anxiety have been widely studied over decades. By utilizing human brain tissue and taking the advantage of a unique mouse model, this thesis work provides new information on $GABA_A$ receptor pharmacology and on brain structures and mechanisms that may mediate anxiolytic responses via specific types of GABAegic inhibition:

1. Previously, contradictory reports had been published in regard to the expression and participation of the γ2 subunit in the functional $GABA_A$ receptors in the rodent LC. (Fritschy et al., 1992; Lüddens et al., 1995; Caldji et al., 1998 and 2000; Araki et al., 1992; Luque et al., 1995; Tohyama and Oyamada, 1994; Chen et al., 1999). Our results show that in human, the γ2, α1 and α2/α3 subunits are expressed in the LC neurons. These findings indicate that the human LC may directly mediate the sedative and anxiolytic effects of benzodiazepines.

2. Systemic gaboxadol acts preferentially via ectopically expressed α6β/γ2 receptors as compared to naturally occurring $GABA_A$ receptor subunit combinations in the forebrain, as the transgenic mice overexpressing these receptors were significantly more sensitive to the anxiolytic and hypnotic effects of 3 and 25 mg/kg of gaboxadol, respectively. This transgenic mouse model provides unique information on the pharmacology of gaboxadol *in vivo*.

3. GABA-insensitive (GIS) [^{35}S]TBPS autoradiographic signal reflects partial agonism of GABA because gaboxadol as a stronger agonist than GABA displaced the GIS-[^{35}S]TBPS binding from α6β2/3 and α4/6β2/3δ receptors at saturated concentrations. As a result, GIS-[^{35}S]TBPS binding can be used as a fingerprint for partial agonism of GABA.

4. GABA is a partial agonist as compared to gaboxadol in typically extrasynaptic α6β3, α6β3δ and α4β3δ receptors but not in α6β3γ2 receptors. Gaboxadol and GABA display also different receptor desensitization and deactivation kinetics in these receptors suggesting a different transduction pathway leading from agonist binding to gating of the extrasynaptic ion channel.

5. Gaboxadol acting preferentially via extrasynaptic $GABA_A$ receptors induced a widespread neuronal activation rather than inhibition in many brain areas of the transgenic Thy1α6

mice overexpressing the $GABA_A$ receptor α6 subunit. Although the α6 subunit is expressed outside its natural environment in the Thy1α6 mouse model, the model demonstrates how neuronal excitability can be altered in different brain regions as a consequence of enhanced tonic inhibition in specific forebrain structures. In many areas this neuronal activation was likely to be an indirect network effect, but in some areas (e.g. BLA, anterior olfactory nucleus) the neuronal activation occurred in the areas containing extrasynaptic $GABA_A$ receptors. Slow depolarizing potentials have been reported after an intense stimulation of $GABA_A$ receptors on distal dendrites of rat hippocampal slices and after an initial hyperpolarizing membrane potential (Staley et al., 1995). The transgenic Thy1α6 mouse model could be an interesting tool to study whether the extrasynaptic α6 subunit-containing $GABA_A$ receptors may function also as excitatory rather than only inhibitory receptors in adult mice.

6. The widespread neuronal inhibition as typically associated with benzodiazepines may not be the exclusive mechanism of anxiolysis. We observed an increased neuronal activation especially in the limbic areas of Thy1α6 mice after an anxiolytic dose of gaboxadol. However, future experiments are needed to demonstrate the actual causality between the neuronal activation and anxiolysis. In addition, as brain areas are built of different neuronal subpopulations that are connected in a complex network, it is also important to study the neuronal activation/inactivation at a cellular and sub-cellular level to understand the mechanism of anxiolysis in more detail and to further be able to develop more selective and safe treatments to anxiety disorders.

7. Unconditioned approach-avoidance conflict tests such as the elevated plus-maze and light/dark exploration test are considered to model some components of human anxiety disorders. However, the fear conditioning tests are likely to be more valid, as regards especially the construct validity, in studying the neuroanatomy and mechanisms of anxiety disorders as they involve the fear learning component that is dysregulated in anxiety disorders.

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9 Original publications