Study on the regulation of mRNA expression of GABA transporters (GAT-1 and GAT-3) in rat brain

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Contents

1 Introduction

The brain receives, processes, and interprets multiple types of stimuli. It controls motor output, records experiences in form of memory, carries out complicated cognitive tasks, including understanding and producing language, and it is responsible for mood, behaviour and for consciousness itself. All of the brain functions are carried out by nerve cells that communicate with each other in a network of remarkably complex but precise connections. In addition to neurones, the brain contains glial cells. Though the glial cell mass is bigger than that of neurones, glial cells are not thought to play a primary role in information processing. They seem likely to play a more supportive and permissive role in neuronal function.

The primary elements of information processing are not neurones, but rather the individual connections that they make with each other, called the synapses. Neurones in the brain can form a thousand, sometimes several thousand, synapses with other neurones. At synapses neurones communicate with each other by releasing a chemical substance called a neurotransmitter.

Chemical transmission is the major means by which nerves communicate with one another in the nervous system. The pre- and postsynaptic events are highly regulated and subjected to use-dependent changes that are the basis for plasticity and learning in the central nervous system (CNS). Chemical transmission requires the following steps: 1. Synthesis of the neurotransmitter in the presynaptic nerve terminal; 2. Storage of the neurotransmitter in secretory vesicles; 3. Regulated release of neurotransmitter into the synaptic space between pre- and postsynaptic neurones; 4. The presence of specific receptors for the released neurotransmitter on the postsynaptic membrane; 5. A means for termination of the action of the released neurotransmitter.

Neurotransmitter binding to the receptor activates the receptor and thus elicits an increase or decrease in either the formation of second messengers, in the opening or closing of ion channels, or in the recruitment of certain cytoplasmatic proteins. The next phase involves the activation of enzymes, typically protein

kinases or phosphatases, which mediate the biological response. Thus the initial interaction of the neurotransmitter with its receptor results in amplification of the signal by means of cascade responses.
Certain neurotransmitter receptors contain intrinsic ion channels in addition to their neurotransmitter recognition site. Neurotransmitter receptors that allow the entry of positively charged ions into neurones make the local membrane potential less negative with respect to the outside; that is, they produce a depolarisation of the membrane. Neurotransmitters that produce depolarisation are excitatory, because they bring a neurone toward its threshold for firing, an action potential. Inhibitory neurotransmitters cause hyperpolarisation of the membrane, generally by admitting anions. Many different types of substances function as neurotransmitters, including amines (e.g. dopamine, serotonin, norepinephrine), amino acids (e.g. glutamate, γ-aminobutyric acid (GABA), glycine), and acetylcholine. The submitted work was focused on the study of GABAergic system.

1.1 GABA

γ-Aminobutyric acid is the major inhibitory neurotransmitter in the mammalian central nervous system (Sivilotti and Nistri, 1991). It is a small amino acid derived from glutamate by the action of glutamic acid decarboxylase (GAD). It exerts its synaptic action via binding to three types of receptor classes: $GABA_A$, $GABA_B$ and $GABA_C$ receptors. Released GABA is actively taken up into GABAergic nerve terminals or glia cells by GABA transporters (GATs), which terminate its synaptic actions. GABA is degraded by the enzyme GABA transaminase that is localised in the mitochondria.

1.2 GABA receptors

1.2.1 $GABA_B$ receptors

$GABA_B$ receptors are heterodimeric complexes made up of two closely related transmembrane proteins (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). They are coupled via G proteins to membrane potassium channels, on which they exert their action by means of second messengers. When activated, these receptors can decrease calcium conductance and inhibit cyclic AMP (cAMP) production via intracellular mechanisms mediated by G proteins. $GABA_B$ receptors are localised not only postsynaptically but also on presynaptic terminals, where they are believed to participate in the modulation of neurotransmitter release. They are activated by GABA and baclofen and inhibited by phaclofen and saclofen (Bowery and Pratt, 1992; Marshall et al., 1999). Unlike $GABA_A$ receptors, $GABA_B$ receptors are insensitive to bicuculline.

1.2.2 $GABA_C$ receptors

$GABA_C$ receptors are GABA-gated chloride ion channels and belong to the same superfamily as $GABA_A$ receptors. They are homooligomeric protein complexes derived from various isoforms of ρ subunit. $GABA_C$ receptors are insensitive to the $GABA_A$ antagonist bicuculline (neutralising modulator) as well as the $GABA_B$ agonist baclofen (positive modulator). They are activated by GABA and certain analogues of GABA, such as CACA and TACA (cis- and trans-4-aminocrotonic acid). $GABA_c$ receptors are inhibited by picrotoxin and other Cl^- channel blockers (Johnston, 1996).

1.2.3 $GABA_A$ receptors

$GABA_A$ receptors are transmembrane heterooligomeric proteins expressed in the peripheral and the central nervous system. They are members of the fast-acting ligand-gated ion channel superfamily that also includes the nicotinic acethyl-choline- (nAchR), the strychnine-sensitive glycine-, and the $5\text{-}HT_3$-receptors (Schofield et al., 1987). These receptors combine the ligand binding site as well as

the ion-permeating pore within the same homo- or heterooligomeric complex. Each receptor subunit comprises a large extracellular N-terminal domain that includes the ligand binding site, four hydrophobic membrane spanning domains labelled TM1-TM4, and a small extracellular C-terminus. About 10 to 20% homology exists among $GABA_A$ receptor subunits and those of other members of the ligand-gated ion channel gene superfamily (review in Mehta and Ticku, 1999).

$GABA_A$ receptors are pentameric protein complexes derived from a combination of various subunits (Nayeem et al., 1994). These subunits have been classified according to their degree of amino acid identity as α, β, γ, δ, ρ, and ε. The amino acid identity between these groups is 30 - 40 %, whereas subunit variants share 70 - 80 % identity (Olsen and Tobin, 1990; Lüddens et al., 1995). To date, 17 different subunit variants are known (figure 1.1). Each subunit has a molecular mass between 40 - 60 kDa (reviewed in Mehta and Ticku, 1999). It is assumed that subunit combination $\alpha_1\beta_2\gamma_2$ represents the major adult isoform of $GABA_A$ receptor (McKerman and Whiting, 1996) and that the stoichiometry of the three subunits in the presumed pentamer is 2×α, 2×β and 1×γ (Nayeem et al., 1994).

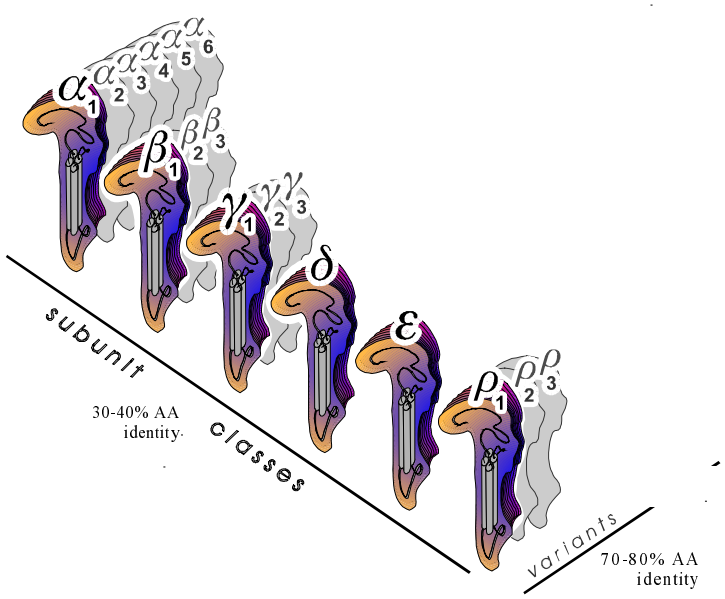

Figure 1.1

Schematic representation of $GABA_A$ receptor subunits: To date 17 $GABA_A$ receptor subunits are identified. The grouping into six classes α, β, γ, δ, ε and ρ is based on a sequence identity of 30 - 40%. Within each class between one and six variants exist that share 70 - 80% amino acid identity (from Hevers and Lüddens, 1998).

1.2.3.1 $GABA_A$ receptor pharmacology

A number of psychoactive drugs interact with $GABA_A$ receptors so as to elicit their pharmacological effects (figure 1.2). Aside from the natural agonist GABA, these drugs include benzodiazepines, barbiturates, general anaesthetics and some steroids. Additional binding sites for a number of substances exist (reviewed in Hevers and Lüddens, 1998)

GABA: Each receptor subunit is able to form channels that contain a functional binding site for GABA. This has been shown by *in vitro* expression of single subunits in *Xenopus* oocytes (Blair et al., 1988). Electrophysiological studies suggest that there are two GABA recognition sites per $GABA_A$ receptor complex (White, 1992). Additionally to GABA, $GABA_A$ receptors are stimulated by muscimol and isoguvacine and inhibited by bicuculline in a competitive manner. Further competitive antagonists at the GABA binding site of $GABA_A$ receptors are the amidine steroid Ru 5135 (Hunt and Jewery, 1981) and the arylaminopyridazines SR95103 and SR95531 (Chambon et al., 1985; Heaulme et al., 1987).

Benzodiazepines: Benzodiazepines (BZ) have gained major clinical relevance among the pharmacological agents that modulate $GABA_A$ receptors. Present evidence suggests $GABA_A$ receptors to represent the only effector site of BZ in the CNS. Benzodiazepines exert their effects by modulating the effect of GABA. BZ do not effect Cl^- currents in the absence of GABA (Polc, 1988) and BZ binding does not compete with GABA (Schofield et al., 1987). Benzodiazepines bind to an additional site within the $GABA_A$ receptor complex and allosterically modulate $GABA_A$ receptor currents. Benzodiazepines shift the GABA dose response curve to other concentrations. Additionally, a wide variety of nonbenzodiazepines, such as the β-carbolines, cyclopyrrolones and imidazopyridines, also bind to the benzodiazepine site (reviewed in Teubner et al., 1999).

Steroids: The so-called neurosteroids exert part of their actions through the $GABA_A$ receptors (Peters et al., 1988). They are synthesised in various tissues including glial cells of the brain and are found in a higher concentration in the brain than in the plasma. Neurosteroids include metabolites of progesterone (e.g. 3α-hydroxylated or 3α,5α-reduced progesterones), metabolites of deoxy-

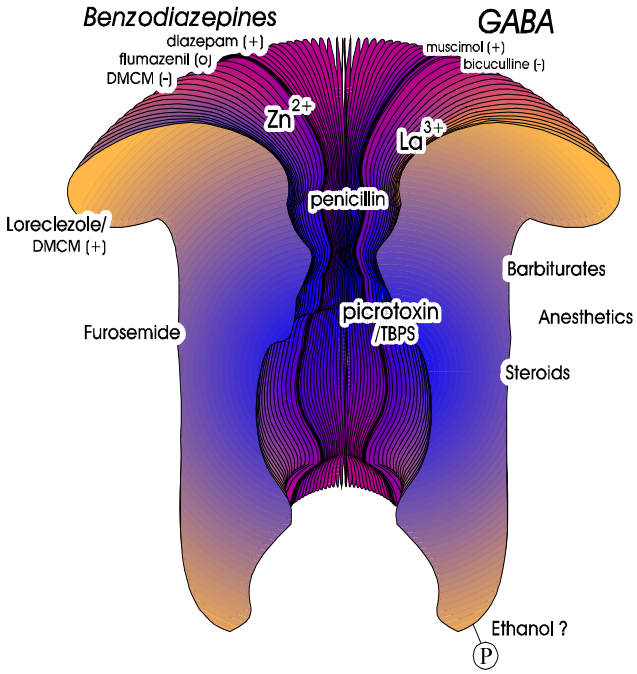

Figure 1.2

The $GABA_A$ receptor as a drug target: Variety of drugs, aside from GABA and its agonists (e.g. muscimol) or antagonists (e.g. bicuculline), exert their action by modulating $GABA_A$ receptor. These include clinically relevant benzodiazepines, which can be subdivided into positive modulators (e.g. diazepam), null modulators (e.g. Ro15-1788) or negative modulators (e.g. DMCM). Further binding sites have been identified for loreclezole, furosemide and picrotoxin. Sedative barbiturates and steroids as well as various anaesthetics also work via $GABA_A$ receptors. Binding sites for the polycationic ions Zn^{2+} and La^{3+} exist (from Hevers and Lüddens, 1998).

corticosterone (Majewska et al., 1986; Kokate et al., 1994; Frye, 1995). They have sedative, hypnotic and anaesthetic effects.

Barbiturates: Barbiturates exert an influence on receptor function similar to that of benzodiazepines. They increase the affinity of the receptor for GABA and thereby increase the ability of GABA to activate receptor Cl^--channels. They bind to $GABA_A$ receptors, but at a physically separate site from benzodiazepines. They have sedative, hypnotic and anaesthetic effects. Unlike the benzodiazepines, barbiturates at higher concentrations (> 50 µM) can directly gate the intrinsic ion channel (Macdonald and Baker, 1978b; Franks and Lieb, 1994).

Anaesthetics: In addition to the anaesthetic steroids and the barbiturates, different anaesthetics of various chemical classes modulate the $GABA_A$ receptors. They enhance the GABA-gated Cl^--currents (Concas et al., 1991).

Channel blockers: One chemically inhomogeneous group of compounds mainly comprises to allosteric antagonists. They all antagonise GABA evoked Cl^--currents and therefore are convulsants. Substances of this class are picrotoxin, TBPS (t-butylbicyclophosphorothionate), pentylentetrazole and a variety of insecticides such as dieldrin and lindane. These compounds are thought to bind at a single site on $GABA_A$ receptor that differs from the GABA, BZ, barbiturate, or steroid binding sites (Inomata et al., 1988; Gurley et al., 1995) and is localised at or near the Cl^--channel itself.

Substances recognising additional binding sites: The anticonvulsant loreclezole (Ashton et al., 1992), developed for the treatment of epilepsy, also modulates $GABA_A$ receptor function. Its action is not affected by BZ, barbiturates, steroids or other anaesthetics, suggesting that it binds to a distinct, novel site.

A direct effect of ethanol on $GABA_A$ receptors was originally suggested based on the similarities of the effects of ethanol, BZ and barbiturates. Despite the general indication that $GABA_A$ receptors mediate some effects of ethanol, no direct evidence is reported for an ethanol specific binding site on the $GABA_A$ receptor.

1.2.3.2 Benzodiazepine binding site

As already mentioned, the GABA response of $GABA_A$ receptors can be allosterically modulated by ligands acting at the benzodiazepine binding site (Macdonald and Baker, 1978). The ligands are often referred to as agonists, antagonists and inverse agonists (negative modulators). The agonists potentiate the inhibitory response to GABA, whereas the inverse agonists decrease the GABA-response. The actions of both agonists and inverse agonists are inhibited by antagonists.

Benzodiazepine binding site is believed to be located on the interface of the α and γ subunits of $GABA_A$ receptors (Bateson et al., 1991; Macdonald and Olsen, 1994). The γ_2 subunit is essential for the conservation of full BZ site pharmacology

(Pritchett et al., 1989; Herb et al., 1992; Günther et al., 1995). Point mutation studies have shown that specific amino acid residues in γ_2 (threonine^{142}) (Mihic et al., 1994; Buhr et al., 1997) and several amino acid residues in the α subunit (Pritchett and Seeburg, 1991; Duncalfe et al., 1996) are directly involved in benzodiazepine binding. $GABA_A$ receptors containing α_4 or α_6 subunits are generally considered to be insensitive to classical benzodiazepines (reviewed in Teubner et al., 1999).

Shortly after the identification of the BZ binding site on the $GABA_A$ receptor complex it became clear that the population of binding sites is heterogeneous. Depending on the sensitivity to diazepam, $GABA_A$ receptors are designated as diazepam sensitive (DS) and diazepam insensitive (DI). DI receptors show high affinity to imidazobenzodiazepine Ro 15-4513. The diuretic furosemide is reported to be a low affinity but highly selective ligand for the DI receptors (Mäkelä et al., 1996). The DI receptors consist of $GABA_A$ receptor subtypes carrying α_4 and/or α_6 subunits. The DS receptors are divided, based upon their pharmacology, into two subtypes designated BZ1 and BZ2. From work with recombinant receptors, it is evident that the subdivision of BZ receptors emerges from the identity of the α subunit (Lüddens et al., 1994). The pharmacological and electrophysiological properties of $GABA_A$ receptors of BZ1 type can be mimicked only by receptors containing the α_1 subunit (Pritchett et al., 1989; Pritchett and Seeburg, 1990). The BZ1 sites show high affinity for classical benzodiazepines. They constitute the majority of BZ binding sites throughout the brain (Doble and Martin, 1992; Bureau and Olsen, 1993). Selective non-classical ligands like the imidazopyridine zolpidem, triazolopyridazine CL218872 and the β-carboline β-CCE show preference for the BZ1 sites. The BZ2 sites show slightly lower affinity for benzodiazepines and are enriched only in a few brain areas such as the hippocampus, the striatum and especially the spinal cord (Doble and Martin, 1992; Bureau and Olsen, 1993). Selective ligands for the BZ2 site are not available. $GABA_A$ receptors containing α_2, α_3 or α_5 are of BZ2 type.

1.3 GABA transporters

1.3.1 Function of neurotransmitter transporters

Transporters serve virtually in all cells as the conduit by which essential nutrients such as glucose and amino acids gain access to the cell interior (reviewed in Silverman, 1991), and also allow cells to adapt to alternations in osmolarity of the extracellular fluid. Within the nervous system certain transporters have taken on a specialised role related to the synaptic transmission, namely, regulation of the concentration and duration of the neurotransmitter in the synapse.

The function most frequently ascribed to neurotransmitter transporters is the termination of synaptic transmission. The termination process is catalysed by a sodium-coupled neurotransmitter transport system located in the plasma membranes of nerve endings and glial cells.

Another critical function of neurotransmitter transporters is that they prevent the spread of neurotransmitter to neighbouring synapses, thereby assuring the fidelity of synaptic transmission (reviewed in Krogsgaard-Larsen et al., 1987).

Moreover, neurotransmitter transporters allow the neurotransmitter to be reutilised by sequestering it. The reutilization can be direct, as when transport is directly in the presynaptic terminal, or indirect, as when transport occurs into glial cells, which then convey the transmitter or its metabolite into the presynaptic neurone (Hertz, 1979).

Finally, some neurotransmitter transporters may also work in reverse, releasing the neurotransmitter in the synapse in a calcium independent manner (Levi and Raiteri, 1993; Attwell et al., 1993).

1.3.2 Mechanism of GABA transport

Transporters, like channels and ATPase pumps, serve as conduit through which molecules can cross the bilipid plasma membrane. Mechanistically, transporters function as molecular shuttles, transferring substrates across the membrane, one molecule at a time, then going back for more (reviewed in Stein, 1990). GABA transporters belong to the family of Na^+- and Cl^--coupled transporters, because

these ions are co-transported with the substrate. The substrate binding site of the transporter in its resting state faces the extracellular space. Upon binding substrate, Na^+ and Cl^- ions, the transporter undergoes conformational changes such that these substances face the intracellular space, where they are released. The GABA transporter 1 transports within one cycle two Na^+, one Cl^-, and one zwitterionic GABA molecule (reviewed in Mager et al., 1996). The "empty" transporter then reverts to the resting state where it can bind additional substrate molecules and thus repeat the cycle.

1.3.3 Structure of Na^+- Cl^--dependent transporters

The first neurotransmitter transporter to be cloned was a GABA transporter, named GAT-1 (Guatestella et al., 1990). Injection of GAT-1 RNA into *Xenopus* oocytes resulted in a 50 - 100 fold increase in [^{3}H]GABA uptake which was saturable, of high affinity (K_M = 7 µM), and dependent on Na^+ and Cl^- in the external medium (Guatestella et al., 1990). Data base searches revealed little or no homology to any known protein, suggesting that GAT-1 belongs to a novel family of proteins. One year later, the cDNA encoding a Na^+- and Cl^--dependent norepinephrine transporter was cloned (Pacholczyk et al., 1991), which displayed a high degree of amino acid identity (45%) with GAT-1. The use of functional cDNA expression assays and amplification of related sequences by polymerase chain reaction (PCR) resulted in the cloning of additional transporters. These newly emerging family of Na^+- and Cl^--dependent transporters includes, in addition to those for GABA and norepinephrine, transporters for the neurotransmitters glycine (Smith et al., 1992a; Liu et al., 1992b), serotonin (Blakely et al., 1991; Hoffman et al., 1991) and dopamine (Shimada et al., 1991; Kilty et al., 1991), as well as the neuromodulatory substances taurine (Smith et al., 1992b; Liu et al., 1992a) and proline (Fremenau et al., 1992) and two "orphan" transporters (Uhl et al., 1992; Liu et al., 1993). The deduced amino acid sequences of these proteins exhibit 30 - 65 % identity between different members of the family. Based on these differences in homology the family has been divided into four subgroups: **A.** Transporters of biogenic amines (norepinephrine, dopamine and serotonin);

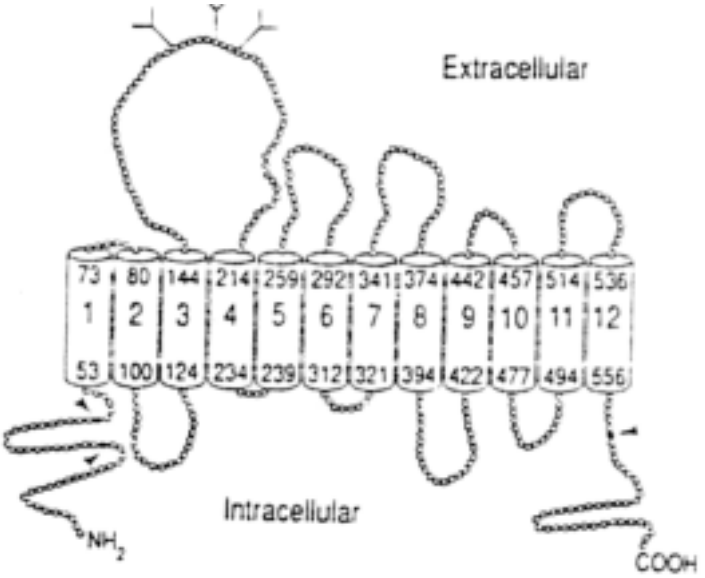

Figure 1.3

Hypothetical secondary structure of the GAT-1 protein: Schematic diagram of possible membrane orientation of GAT-1. Individual amino acid residues are shown as circles. Putative transmembrane segments are depicted as cylinders. Three putative protein kinase C phosphorylation sites are marked with arrowheads. Three of the four putative glycosylation sites are located on the large extracellular loop connecting segments 3 and 4 and are depicted as branched lines; the remaining glycosylation site is located within membrane segment 9 (from Guatestella et al., 1990).

B. various GABA transporters (GAT-1, -2, -3 and Betaine GABA transporter (BGT-1)) as well as transporter of taurine; **C.** transporters of proline and glycine and **D.** "orphan" transporters. These proteins share some features of a common secondary structure. Hydropathy analysis predicts proteins with 12 hydrophobic putative transmembrane α-helices (figure 1.3). Both amino- and carboxy-termini face the cytoplasm. These regions contain putative phosphorylation sites that may be involved in the regulation of the transport process. The second extracellular loop between transmembrane domains 3 and 4 (3/4 loop) is the largest one and three putative glycosylation sites are located on it. Alignment of the deduced amino acid sequences of 13 different members of this superfamily, whose substrates are known (subgroups A. - C.) revealed that some segments within these proteins share a higher degree of homology than others. The most highly conserved region (>50% homology) are helix 1 together with the extracellular loop

connecting it with helix 2, and helix 5 together with a short intracellular loop connecting it with helix 4 and a large extracellular loop connecting it with helix 6. These regions may be involved in stabilising a tertiary structure, which is essential for the function of all these transporters. Alternatively, they may be related to a common function of these transporters, such as the translocation of sodium ions. The region stretching from helix 9 onwards is far less conserved than the segment containing the first 8 helices. Possibly, this domain contains some residues that are involved in translocating the different substrates (Kanner, 1996). The least conserved segments are the amino- and carboxy-termini. These areas may be involved in regulation of the transport process.

1.3.4 GABA transporter heterogeneity

The most abundant and well-characterised uptake systems in rat brain are those for GABA and L-glutamate, the two major neurotransmitters in the central nervous system. The application of molecular biology to the study of neurotransmitter transporters has greatly expanded the knowledge of these molecules and has revealed a far greater complexity of GABA transporters than previously imagined. To date, genes for five distinct high-affinity GABA transporters have been cloned (GAT-1, GAT-2, GAT-3, BGT-1 and vesicular GAT (VGAT)), as has the gene for a related member of this family, the taurine transporter.

Rat GAT-1 mRNA was found to be present in all brain regions examined, but was not detected in liver, suggesting that it is restricted to the central nervous system (Borden et al., 1992). Rat GAT-1 cDNA is 4.2 kb long and has a predicted open reading frame of 1797 nucleotides which could encode a protein of 599 amino acids with a molecular weight of 67 kDa (Guatestella et al., 1990). This is in agreement with the size of the deglycosylated, purified transporter (Kanner et al., 1989).

Rat GAT-2 cDNA contains a 2.0 kb sequence with an open reading frame of 1806 base pairs which could encode a protein of 602 amino acids (Borden et al., 1992). The GAT-2 is expressed in brain and some peripheral tissues (liver, kidney, heart) (Ikegaki et al., 1994).

Rat GAT-3 cDNA contains a 2.1 kb sequence with an open reading frame of 1881 base pairs, encoding a protein of 627 amino acids (Borden et al., 1992). The GAT-3 mRNA expression is restricted to the brain as shown by Northern blot analysis (Durkin et al., 1995).

GAT-2 and GAT-3 display ≅ 52 % amino acid identity with GAT-1, and only slightly less identity (45 %) with the norepinephrine transporter. The highest degree of identity of GAT-2 and GAT-3 are one with another (67 % amino acid identity) and with the fourth GABA transporter, BGT-1 (68 and 65 % identity for GAT-2 and GAT-3, respectively). Interestingly, they also show high degree of identity with the taurine transporter (61%) which lacks high affinity for GABA (Borden 1996).

BGT-1: The identification of the fourth GABA transporter resulted from studies of renal physiology. Renal medulla is usually hypertonic and medullar epithelial cells cope with this hypertonicity via accumulation of the osmolyte glycine betaine ("betaine"). In 1992, Yamauchi and colleagues (Yamauchi et al., 1992) isolated from dog a clone encoding Na^+- and Cl^--dependent betaine transporter. Additionally, they have shown that this transporter is able to utilise both betaine and GABA as substrates. Accordingly, the clone was termed BGT-1 (for Betaine GABA Transporter). Cloned canine BGT-1 cDNA is 2938 bases long and encodes a protein of 614 amino acids. The rat BGT-1 mRNA is expressed in brain and some peripheral tissues (Borden et al., 1995).

VGAT: The last of the GABA transporters was identified in 1997 by McIntire and co-workers (1997). It is a vesicular GABA transporter, which is necessary for GABA transfer from cytoplasm into synaptic vesicles. The rat VGAT cDNA is 2.5 kb long and encodes a protein of 525 amino acids. From this amino acid sequence, a protein with ten transmembrane domains is predicted, suggesting that VGAT is a member of another neurotransmitter transporter family as other high affinity GABA transporters. The rat VGAT mRNA expression is restricted to the brain and it co-localises with expression of one isoform of the GAD (GAD-67) in the GABAergic neurones (McIntire et al., 1997).

Homologous sequences for the first four transporters were cloned from human and mouse. For the human homologues the same nomenclature was used as for the rat with addition of the "h" in front of the abbreviation, e.g. hBGT-1. For the GABA transporters from mouse, a different nomenclature was used: mouse GAT1 is the

homologue of human and rat GAT-1; mouse GAT2 is the homologue of BGT-1; mouse GAT3 is the homologue of GAT-2; mouse GAT4 is the homologue of GAT-3. A comparison of the amino acid sequences of different transporters is shown in table 1.1.

Table 1.1

Amino acid sequence identities of human, rat and mouse GABA transporters (from Smith et al., 1996).

Amino acid identities	GAT-1	GAT-2	GAT-3	BGT-1
human vs. rat	97%	90%	95%	86%
human vs. mouse	96%	91%	95%	87%

1.3.5 GABA transporter ligands

Neurotransmitter transporters mediate the uptake of amino acids and biogenic amine transmitters into presynaptic terminals and surrounding glial cells. Since inhibition of the uptake by pharmacological agents increases the levels of neurotransmitter in the synapse and thus enhances the synaptic transmission, neurotransmitter transporters provide important targets for therapeutic intervention. Specifically, inhibition of GABA uptake provides a novel therapeutic approach to enhance inhibitory GABAergic transmission in the central nervous system. This is because, in this case, a major enhancement of GABAergic neurotransmission takes place under conditions where GABA is already being released physiologically (Andersen et al., 1993). It may be useful in the treatment of various neuropsychiatric disorders (e.g. epilepsy). Some years ago, a number of lipophilic GABA transport inhibitors have been designed and synthesised. They are capable of crossing the blood brain barrier and display anticonvulsive activity. The potencies of some of these compounds were determined with each of the four cloned GABA transporters (table1.2). These tested substances were found to be highly selective for GAT-1. These data suggest that the anticonvulsant activity of

the compounds such as tiagabine is mediated via inhibition of the uptake by GAT-1 (Borden et al., 1994). The availability of specific inhibitors allowed studies on function and regulation of GAT-1. Unlike GAT-1, little is known about the functional role(s) of GAT-2, GAT-3, and BGT-1, partly due to the lack of selective inhibitors.

Table 1.2

Affinities of transport inhibitors for cloned GABA transporters. Data show the IC_{50} [µM] values for inhibition of [^{3}H] GABA uptake and represent means and SEMs (from Borden et al., 1994).

Clone / compound	rGAT-1	rGAT-2	rGAT-3	hBGT-1
GABA	30 ± 8	5 ± 2	33 ± 10	36 ± 3
(±)- Nipecotic acid	24 ± 6	39 ± 4	159 ± 30	2370 ± 617
Tiagabine	0.64 ± 0.15	1410 ± 250	2040 ± 107	1670 ± 722
NNC-711	0.38 ± 73	171 ± 53	349 ± 151	622 ± 265
SKF 89976-A	0.64 ± 0.19	550 ± 225	4390 ± 1420	7210 ± 3630

1.4 Physiological and pathophysiological consequences of alternations in GABAergic transmission

A growing body of evidence suggests a role for altered GABAergic function in human neurological and psychiatric disorders, including epilepsy, anxiety, sleep disorders, alcoholism, schizophrenia and Parkinson's disease (Shekhar et al., 1993; Gerlach et al., 1996; Woo et al., 1998; Obenaus et al., 1993). The attempts to treat this diseases led to the production of a variety of substances acting on different sites in the CNS. The continued search for new more effective compounds that are without adverse side effects has led to proliferation of potential agents. This has reinforced the need for efficient, pre-clinical animal screening tests. Animal screening tests are used initially to predict safety and efficacy of any novel compound. Another application of animal tests is as biological test systems wherein the neural mechanisms of drug action can be studied. At this stage, the animal test is called "model" and it is used as a biobehavioural analogue of an underlying mechanism. Knowledge of the

mechanisms of drug action can help to synthesise drugs that act more safely and selectively. Furthermore, by understanding the mechanisms by which the drugs produce their effects, one can understand important aspects of the biological basis of different diseases. In the present work, the animal models of epilepsy and of anxiety related behaviour were used.

1.4.1 Epilepsy

"Epilepsy" refers to an etiologically and clinically diverse group of neurological disorders characterised by disordered, synchronised firing of populations of neurones in CNS, called seizures (reviewed in Dichter et al., 1997). On the basis of clinical criteria, more than 40 distinct forms of epilepsy have been defined in humans (CCTILE, 1989). Epilepsy is commonly a delayed consequence of head injury. Some forms of epilepsy in humans appear to have a genetic basis. Moreover, many inborn errors of metabolism and the major developmental disorders are associated with seizures. Epilepsy is a disorder of recurrent, spontaneous episodes of aberrant synchronisation of electrical activity in a pocket of active neurones, which then form a focus for an epileptic seizure. The seizure can remain local, spread to other sites or engage all cortical region simultaneously. Neocortical or hippocampal circuits are always activated during an epileptic seizure (Noebels, 1996; Starr, 1996).
Only limited progress has been made in understanding the molecular and cellular basis of either the proness to epileptic seizures or their acute manifestation.

Animal models of epilepsy: To study the basis of epilepsy, some strains of rodent have been selected and bred for seizure susceptibility. These strains serve as models for epilepsy. Some of the strains exhibit spontaneous seizures, while the majority have "evoked" seizures as a response to a specific stimulus, such as sound or vestibular stimulation (Puranam and McNamara, 1999). For example, DBA/2 mice, EL mice and genetically epilepsy prone rats rank among this group.
Another possibility to study electrophysiological and biochemical bases of seizures is to use animals in which the seizures are evoked. The seizures can be triggered

by short electrical pulse or by injection of proconvulsant substances such as kainic acid, pentylentetrazole (PTZ), picrotoxin or DMCM (methy-6,7-dimethoxy-4-ethyl-β-carboline).
Furthermore, some of these substances are used to "model" epilepsy-like syndromes in animals. This model is based on a brief burst of nerve stimulation sufficient to induce a local discharge but not sufficient to trigger a seizure. However, this stimulus triggers a seizure if repeated frequently. This phenomenon is referred to as kindling. It can be most readily induced by stimulating the structures that comprise the limbic system, such as hippocampus or amygdala. This stimulation is performed electrically (implantation of electrodes). Kindling effect can be obtained also following chemical stimulation (repeated intraperitoneal injections of PTZ). The changes responsible for the lowered seizure threshold occur diffusely in the brain and are permanent. They appear to involve voltage sensitive ion channels and several neurotransmitter systems, including GABA, acetylcholine and glutamate.

The most prevalent seizure disorder in human adults is temporal lobe epilepsy. Electrophysiological studies of human temporal lobe epilepsy suggest that a loss of hippocampal GABA-mediated inhibition may underlie the neuronal hyperexcitability generating this condition (Isokawa et al., 1991; Knowles et al., 1992). The hypothesis is supported by the fact that some substances strengthening the GABAergic transmission are anticonvulsant and are used for treatment of epilepsy, e.g. tiagabin, vigabatrin, diazepam. In animal models of epilepsy, a substantial loss of glutamic acid decarboxylase containing neurones in hippocampus was observed (Obenaus et al., 1993). Therefore, diminished GABA release might mediate the decrease of the inhibition. There are two modes of GABA release from neurones the major one, vesicular calcium-dependent release and additional transporter reversal calcium-independent release. During and colleagues (1995) have shown that calcium-dependent GABA release after potassium-stimulation was 2-fold higher in human epileptogenic hippocampi than in controls. Furthermore, they have shown that calcium-independent, glutamate-induced, GABA release significantly increases GABA concentration in the

extracellular fluid only in the controls and not in the epileptogenic hippocampi. Moreover, they detected a decreased nipecotic acid binding (GAT-1 inhibitor) in the hippocampus of kindled rats, an animal model of epilepsy (During et al., 1995). They hypothesised that reversal of GABA transport is necessary in addition to the vesicular GABA release to dampen the seizures. Furthermore, the decrease of the number of GABA transporters in the epilepsy-like hippocampus may underlie the insufficient neuronal inhibition and therefore spread of seizures. Here arisen the question, whether decreased binding activity of GABA transporters is due to changes in transcription, translation or other regulation mechanisms (reviewed in Olsen and DeLorey, 1999).

1.4.2 Anxiety disorders

Anxiety is apprehension of danger or something unpleasant. Whereas fear is a response to current threats, anxiety occurs in anticipation of a threat not yet present and often not clearly defined. Anxiety is a part of every day human life, and something anxiety-like, or conditional fear, occurs in most vertebrate species. It protects an individual from dangerous or unknown situations and has clear adaptive value. In some humans, anxiety reaches a level that is counterproductive or even incapacitating leading to anxiety disorders that are among the most common of all psychiatric conditions. In addition, anxiety is a prominent symptom in many psychiatric disorders. There are distinct forms of anxiety disorders. They are distinguished according to their characteristic clustering of symptoms as panic attacks, obsessive-compulsive disorder, post-traumatic stress disorder and generalised anxiety disorder.

Animal models of anxiety: The evidence used to determine the neurochemical basis of anxiety has come from studying the action of anxiety-reducing, or anxiolytic, drugs. This has been aided by the existence of several animal models of anxiety. The basic phenomena that were used as foundations for models of anxiolytic effects of drugs can be grouped into three categories: **A.** Models based on the effects of proven anxiolytics on simple or "unconditioned" animal reactions,

B. Models based on the effects of anxiolytics in traditional animal learning paradigms, and **C.** Models based on the effects of anxiolytics on phylogenetically "prepared" forms of aversive learning (reviewed in Treit, 1985).
In the present work, two tests were used, the open field and the elevated plus-maze tests. Both tests rank into the group of tests detecting effects on unconditioned behaviour (A.). In addition, the employed tests record effects on exploratory behaviour of rats (Treit, 1985). They are based on rodents` natural tendency to avoid brightly lit areas. In these tests, the rats are placed into the arena and the number of transitions into and the time spent in the light parts are electronically recorded. In addition, other exploratory activities, like head dips and rears, can be recorded. It has been shown that a variety of anxiolytics produce dose-dependent increase in transitions (Crawley, 1981), whereas nonanxiolytic agents do not have this facilitatory effect. Evidence was also provided that anxiolytic agents selectively increased exploration, rather than general activity (Crawley and Goodwin, 1980).

The current work dealing with anxiety concentrated on the GABA mechanisms. Pharmacological manipulation of GABAergic transmission is an effective approach for the treatment of anxiety (Woods et al., 1987b). The benzodiazepines are highly effective for the relief of anxiety. They have a lower potential for addiction than other drugs used earlier and are less likely to cause death when taken in overdoses. The clinical effects of benzodiazepines are anxiolytic, sedative, anticonvulsant and muscle relaxant. There are now several dozen of benzo-diazepine drugs in clinical use worldwide, although use of them has become less popular because of side effects, including development of tolerance and dependence.

1.4.3 Tolerance and dependence to benzodiazepines

Benzodiazepines were considered to be devoid of adverse consequences. However, concern has increased regarding an abuse and potential dependence liability of these drugs. Long-term use may lead to tolerance to their effects (File, 1985) and withdrawal syndromes can follow abrupt discontinuation of benzodiazepine treatment (Woods et al., 1987a; File, 1990).

"Drug tolerance" has been defined as the process by which the effects of the same dose of a drug decreases with repeated administration, resulting in the need to increase the dose to obtain the same therapeutic effect (Miller and Gold, 1990; Rall, 1991). Drug tolerance can be due to metabolic change or adaptive changes within the central nervous system in response to repeated drug exposure. One of the effects of tolerance is to restore homeostasis, keeping physiological functioning within a normal range. In some cases, tolerance can be partly pharmacokinetic and partly pharmacodynamic. A large body of evidence from animal studies suggests that the tolerance observed with benzodiazepines is largely pharmacodynamic in nature. Therefore, a large component of benzodiazepine tolerance must be due to CNS plasticity (reviewed in Hutchinson et al., 1996).

"Drug dependence" is best defined as the circumstance in which the termination of drug administration leads to the onset of withdrawal symptoms (Miller and Gold, 1990; Rall, 1991). It has been suggested that the development of drug tolerance and dependence may be linked. Pharmacodynamic tolerance has been defined as the process by which drug receptors in the brain adapt to, or compensate for, the continued presence of the drug. Thus, when receptors adapt to the presence of the drug, then, if the drug treatment is terminated, abnormal neural activity may occur which causes withdrawal symptoms indicative for dependence. When the drug is no longer present, the receptors must re-adapt before neural function can return to normal (Miller and Gold, 1990).

There is a large body of evidence which documents the development of tolerance to the sedative (Matsubara and Matsushita, 1982; Harro et al., 1990), muscle relaxant (Matsubara and Matsushita, 1982), anticonvulsant (Gallager et al., 1985;

Gosalves and Gallager, 1988) and anxiolytic (Treit, 1985; Ishihara et al., 1993) effects of benzodiazepines within their long-term continuous administration. The development of tolerance is not a unitary process, but a rather stratified process in which tolerance to the different behavioural effects of benzodiazepines develops at different rates and to different extents. The specific withdrawal symptoms which occur may depend on the particular forms of tolerance which have developed at the time benzodiazepine treatment is terminated (reviewed in Hutchinson et al., 1996).

The molecular mechanisms underlying development of tolerance and dependence are still not clearly understood. A large number of studies have attempted to elucidate the neurochemical changes associated with repeated benzodiazepine administration, using a wide range of biochemical techniques. Many of these studies demonstrated a decrease in the maximal number of the benzodiazepine binding sites when treated animals were compared to control animals (Allan et al., 1992; Byrnes et al., 1993; Wu et al., 1994). Regional differences were found with down-regulation of BZ binding sites, most of them found in the cortex, hippocampus and amygdala (Wu et al, 1994; Wu et al., 1995). However, large number of studies failed to find down-regulation of BZ binding sites (Roca et al., 1990; Brett and Pratt 1992a, 1992b; Ishihara et al., 1993; Ramsey-Williams et al., 1994). No study indicated an increase in the number of BZ binding sites. Moreover, chronic benzodiazepine treatment did not alter the affinity of BZ binding. Several studies using TBPS, $GABA_A$ receptor Cl^- channel blocker, have shown that repeated BZ administration does not lead to a change in the number of Cl^- channels (Heninger and Gallager, 1988; Miller et al., 1988). Also several studies have been aimed on expression of different subunits of $GABA_A$ receptors (Holt et al., 1997; Impagnatiello et al., 1996; Zhao et al., 1994).

There has been no study carried out on GABA transporters. Neither function nor expression of GATs has been studied under the long-time increase of GABAergic inhibition due to the benzodiazepine treatment.

1.5 The aim of the study

The present work has been focused on the study of GABA transporters in rat brain. The study has been directed on GAT-1 and GAT-3, the only GABA transporters specifically expressed in the central nervous system, and their possible role in the plasticity of the CNS. The experiments were aimed on detection and analysis of mRNA expression of the chosen GABA transporters under various pathophysiological and control conditions, in which altered GABAergic transmission is thought to play a role. One of the aims of the present work has been to establish molecular methods capable to detect and quantify GABA transporter mRNAs. The RNase protection assay and competitive RT-PCR have been employed. The established methods have been used to quantify the basal expression of mRNAs for GAT-1 and GAT-3 in the rat brain. Furthermore, the hypothesis has been tested whether mRNA levels of GAT-1 and/or GAT-3 are altered in kindled rats, an animal model of epilepsy. The last aim was to analyse the mRNA expression of GATs after chronic treatment with drugs enhancing the GABA mediated inhibition via their binding on $GABA_A$ receptors. The effect of diazepam has been compared to effect of zolpidem. Diazepam is a classical benzodiazepine known to cause tolerance and dependence, when administered chronically. Zolpidem is an imidazopyridine that also binds to benzodiazepine binding site on $GABA_A$ receptors, however, unlike diazepam it binds with high affinity only to $\alpha 1$ subunit containing receptors. Zolpidem is suggested not to develop the tolerance in rats.

2 Materials and Methods

2.1 Drugs

- 1,5-Pentamethylentetrazole (Sigma-Aldrich; Deisenhofen; Germany)
- Diazepam (Valiquid® 0.3, Roche, Germany)
- Zolpidem (donated by Synthelabo, Paris, France)
- Halothane (Fluothane®, Zeneca, Plankstadt, Germany)

2.2 Chemicals

In all molecular biology experiments double-distilled water (H_2O_{bidest}) was used. For the water processing, the Milli Q system was used.

Standard chemicals were of molecular biology or analytical grade. Additionally used chemicals were:

- 10 × PCR buffer (Perkin Elmer/Applied Biosystems; Branchburg; New Jersey; USA)
- 10 × Transcription buffer (Boehringer Mannheim; Mannheim; Germany)
- 5 × Reverse transcriptase buffer (Promega; Madison; WI; USA)
- Agarose; RNA/DNA grade (Roth; Karlsruhe; Germany)
- Dithiothreitol (DTT) (Promega; Madison; WI; USA)
- DNA Molecular weight marker (Boehringer Mannheim; Mannheim; Germany)
- DNA sequencing kit (Dye Terminator Cycle Sequencing Ready Reaction; Perkin Elmer/ Applied Biosystems; Foster City; CA; USA)
- DNase RNase-free (Boehringer Mannheim; Mannheim; Germany)
- dNTPs (Boehringer Mannheim; Mannheim; Germany)
- Film (Reflection™; NEN® Research Products; DuPont)
- Film (X-Omat;Xar-2; Ready Pack; Kodak)
- HybSpeed™ RPA kit (Ambion; Austin; Texas; USA)
- Murine Moloney Leukemia Virus Reverse Transcriptase (MMLV-RT)(Promega; Madison; WI; USA)
- NTPs (Boehringer Mannheim; Mannheim; Germany)

- Oligonucleotides (Pharmacia; Freiburg; Germany)
- Radioactive labelled nucleotides [α-^{32}P] UTP and [α-^{33}P] dATP (ICN Biomedicals GmbH; Meckenheim; Germany)
- Recombinant RNase Inhibitor (RNasin®; Promega; Madison; WI; USA)
- RNaid kit (Bio 101; Vista; CA; USA)
- T3 RNA Polymerase (Boehringer Mannheim; Mannheim; Germany)
- T4 DNA Ligase (Boehringer Mannheim; Mannheim; Germany)
- Taq DNA Polymerase (Perkin Elmer/Applied Biosystems; Branchburg; New Jersey; USA)
- Template Suppression Reagent (Perkin Elmer/ Applied Biosystems; Foster City; CA; USA)
- Wizzard™ Plus Maxipreps and Wizzard™ Plus Minipreps kits (Promega; Madison; WI; USA)

2.3 Equipment

- Centrifuge Sorvall® RC28S (Du Pont; Sorvall® Products; Newtown; Connecticut; USA)
- Centrifuge Sorvall® RMC14 (Du Pont; Sorvall® Products; Newtown; Connecticut; USA)
- Gel Dryer 583 (BioRad Laboratories; Munich; Germany)
- Heat block; (for 1.5 ml tubes) (Thermomixer 5436; Eppendorf Netheler Hinz; Hamburg; Germany)
- Homogeniser Type T8 (Janke&Kunkel; Staufen; Germany)
- Milli Q water processing system (Millipore; Eschborn; Germany)
- Power Supply 200/2.0 (BioRad Laboratories; Munich; Germany)
- Power Pac 3000 (BioRad Laboratories; Munich; Germany)
- Sequencer (ABI PRISM™ 310 Genetic Analyser; Perkin Elmer/ Applied Biosystems; Foster City; CA; USA)
- Sequi-Gen GT Sequencing Cell (BioRad Laboratories; Munich; Germany)
- Spectrophotometer (Type Uvikon; Tegimenta; Rotkreuz; Schwitzerland)

- Thermal cycler (Thermal cycler 4800; Perkin Elmer/ Applied Biosystems; Foster City; CA; USA)
- Video image analysis system GelDoc 1000 (BioRad Laboratories; Munich; Germany)

2.4 Software

- EthoVision® (Noldus Information Technology; Utrecht; Netherlands)
- GraphPad Prism 2.0 (GraphPad Software Inc.; USA)
- HUSAR (Heidelberg Unix Sequence Analysis Resources of German Cancer Research Center; Germany)
- Molecular Analyst® 1.5 (Windows Software for GelDoc1000 image analysis system; BioRad Laboratories; Munich; Germany)
- STATISTICA for Windows 4.5 (StatSoft Inc.; USA)

2.5 Buffers

- TBE buffer (1×): 90 mM Tris base; 90 mM boric acid; 2 mM EDTA
- TE buffer (1×): 10 mM Tris base; 1 mM EDTA
- TTE buffer (1×): 90 mM Tris base; 28.5 mM taurine; 0.5 mM EDTA
- DNA gel loading buffer (5×): 30% glycerol, 0.025% xylene cyanole and 0.025% bromphenol blue
- RNA gel loading buffer (5×): 95% formamide, 0.025% SDS, 0.025% xylene cyanole and 0.025% bromphenol blue
- Denaturing solution (solution D) (1×): 4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol
- MEN buffer (1×): 0.02 M MOPS; 8 mM sodium acetate; 1 mM EDTA
- Probe elution buffer (1×): 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS

2.6 Media

- LB Medium: 10g NaCl, 10g tryptone and 5g yeast extract per 1 litre (pH 7.0)
- LB agar: 10g NaCl, 10g tryptone, 5g yeast extract and 20g agar per 1 litre (pH 7.0)
- X-gal agar: 10g NaCl, 10g tryptone, 5g yeast extract, 20g agar, 1 ml IPTG (stock 0.1 M) and 2 ml X-gal (stock 40 mg/ml) per 1 litre (pH 7.0)

2.7 Primers

- GAT1-RT: 5`-TTCCATGTGTCC (Tm=38.7°C; position 281 - 292 of rat GAT1 cDNA; EMBL database code: rngabat)
- GAT3-RT: 5`-CCACAGCAGATGA (Tm=40.3°C; position 284 - 296 of rat GAT3 cDNA; EMBL database code: rn3gat)
- GAT1-fw: 5`-CTCTACCGAGGTCAGCGAGG (Tm=66.2°C; position 188 - 207 of rat GAT1 cDNA)
- GAT1-rev 5`-TTCCATGTGTCCCGGTCAGG (Tm=70.5°C; position 273 - 292 of rat GAT1 cDNA)
- GAT3-fw: 5`-CGACAAGGCGGTCCACG (Tm=69.3; position 126 - 142 of rat GAT3 cDNA)
- GAT3-rev: 5`-CCACAGCAGATGAAAAACACCAC (Tm=66.3; position 274 - 296 of rat GAT3 cDNA)
- GAT1-fw-T3: 5`-TGATCAATTACCCTCACTAAAGGGAGCTCTACCGAGGTCAGCGAGG
- GAT1-rev-T7: 5`-AAGCTTTAATACGACTCACTATAGGGAATTCCATGTGTCCCGGTCAGG
- GAT3-fw-T3 5`-TGATCAATTACCCTCACTAAAGGGCGACAAGGCGGTCCACG
- GAT1-fw-mut: 5`-CTCTACCGAGGTCAGCGAGGACCATGATTACGAATTCG
- GAT1-rev-βac: 5`-TTCCATGTGTCCCGGTCAGGAGCATCGTCGCCCGCG
- GAT3-fw-mut: 5`-CGACAAGGCGGTCCACGACCATGATTACGAATTCG
- GAT3-rev-βac: 5`-CCACAGCAGATGAAAAACACCACCCCACCATCACACCCTG

- pUC18-fw: 5`-GTAAAACGACGGCCAGT (Tm=56.4°C)
- pUC18-rev: 5`-GCGGATAACAATTTCACACAGGAA (Tm=66.4°C)
- D1-exon: 5`-ATGCTGCCTCTTTTTCTG (Tm=61.1°C; position 2915-2896 of rat DRD1 gene; GenBank accession number S46131)
- D1-intron: 5`-GGAGATGATGCGGTGGG (Tm=65.8°C; position 2746-2762 of rat DRD1 gene)
- βactin-fw: 5`-GATGACGATATCGCTGCGCTC (Tm=65.4C; position 1247-1267 of rat β-actin cDNA)
- βactin-rev: 5`-GCTACGTACATGGCTGGG (Tm=59.6C; position 1630-1647 of rat β-actin cDNA)

2.8 Animals

Kindling experiments: Male rats, strain Wistar - Shoe:Wist (Shoe), age 12 weeks, weight 350 - 400g, obtained from Schönwalde GmbH (Schönwalde, Germany)
All other experiments: Male rats, strain PVG/OlaHsd (PVG), age 9 weeks, weight 180 - 200g, obtained from Harlan-Winkelmann (Borchen, Germany)
Animals were kept under standard conditions, housed 3 per cage with free access to food and water, 12 hours light/dark cycle, 22°C and a relative humidity of 60%.

2.9 Treatment of animals

Kindling: To kindle, animals (n=8) were treated with 1,5-pentamethylentetrazole (PTZ) for 28 days. During this period each treated animal received 13 intraperitoneal injections of 40 mg/kg PTZ (on each Monday, Wednesday and Friday). Control animals (n=8) received NaCl injections. All PTZ treated animals showed at least during the last three stimulations with PTZ tonic-clonic seizures. The kindling of animals was carried out by colleagues in the laboratory of Prof. Dr. G. Grecksch (Department of Pharmacology and Toxicology; University of Magdeburg; Germany).
Chronic administration of drugs: For chronic treatment drugs were administered in the drinking water. Adjusted doses were 10 mg/kg/day of diazepam and

5mg/kg/day of zolpidem. The amount of consumed water was determined daily. Body weight was recorded every third day. The body weight and the volume of consumed water were used to calculate the drug concentration in the drinking water to reach desired doses for each consecutive day. Controls received only tap water. Animals were treated in this way for 23 days. Since the rats sometimes drunk more or less than expected, the real amount of ingested drug was determined at the end of study on the basis of the body weight and water consumption (Schmitt et al., 1999).

Acute administration of drugs: For acute treatment, diazepam (2 mg/kg) or zolpidem (3 mg/kg) was injected intraperitoneally 30 minutes before the test started.

2.10 Behavioural analysis

2.10.1 Experimental design and testing routine

Fifty-six rats were divided into three groups receiving water, zolpidem or diazepam. Control and diazepam treated animals were divided into subgroups depending on the acute treatment that followed chronic administration. Control animals were divided into three subgroups receiving injection of either NaCl (n=10), diazepam (n=8) or zolpidem (n=8). Diazepam treated animals were divided into two subgroups receiving either diazepam (n=10) or NaCl (n=10). Zolpidem treated animals received only NaCl injection (n=10). Double treatment with zolpidem was left out, because zolpidem was not expected to cause tolerance. All animals received habituation handling for one week 5 min daily before the tests started (Schmitt and Hiemke; 1998). In a testing period each rat was tested twice. The tests started on day 22 of the treatment using the open field arena (10 min) continued on day 24 with the elevated plus-maze (7.5 min). All tests were carried out between 9.00 a.m. and 1.00 p.m.. Rats were transported within their home cages to the test room 1h before testing. During all tests the path of each rat was registered by computerised image analysis system. The hardware consisted of an IBM-type AT computer combined with a video-digitizer and a CCD video camera. The software used for the data acquisition and

analysis was EthoVision®. After removal of each animal, the test arena was cleaned with wet cloth and wiped dry.

2.10.2 Open field

The open field arena consisted of dark grey PVC with the bottom painted in ochre. It was divided into 25 squares (A1 to E5) by grey lines. The arena measured 100 × 100 × 35 cm and was indirectly illuminated. The following data were recorded "total distance moved" (cm), "time moving" (percent of recording time) and "number of entries into the centre" (n).

2.10.3 Elevated plus-maze

The plus-maze arena was also made of dark grey PVC. It consisted of two open arms 42.5 × 15 cm and two enclosed arms 42.5 × 15 × 14 cm. The arms extended from a central platform, 15 × 15 cm. The maze was attached on a metal frame 70 cm above the floor. The parameters recorded for elevated plus-maze were "total arm entries" (n), "closed arm entries" (n), "open arm entries" (n), "total time spent in open arm" (percent of recording time) and "percent open arm entries" (percent of total arm entries).

2.11 Tissue preparation

The rats were anaesthetised with Fluothane® and decapitated. The brains were removed. The brains were kept intact or dissected. By dissection, the samples from cerebellum, cortex frontalis, cortex occipitalis, hippocampus, hypothalamus, medulla oblongata, striatum and thalamus were taken. All tissue samples (dissected and whole brains) were immediately frozen in liquid nitrogen and stored at -80°C until needed.

Trunk blood was collected for serum preparation. The blood was centrifuged at 2500 × g for 10 minutes and serum was separated. The serum was stored at -20°C until needed. Drug concentration was determined in the serum by Dr. U. Schmitt using high performance liquid chromatography with ultraviolet or

fluorescence detection (Neurochem. Laboratory; Dept. of Psychiatry; University of Mainz).

2.12 Cloning

PCR fragments were cloned into a plasmid vector pUC18. A blunt end cloning into the *Sma*I restriction site was used. The linearised (*Sma*I) and dephosphorylated plasmid DNA was purchased from Pharmacia-Biotech. First, the protruding ends of the PCR fragments to be cloned were filled up using Klenow fragment.

Blunting of PCR products: The reaction mix (20 µl) contained 0.5 - 1 µg fragment DNA, 1 × reaction buffer, 0.5 mM dNTPs and 1 unit of Klenow fragment. The polishing was carried out for 30 minutes at 37°C. Then, the solution was heated up to 65°C for 5 minutes in order to inactivate the enzyme. Subsequently, the DNA was precipitated with three volumes of ethanol at -20°C for 2 hours. The DNA was pelleted by centrifugation for 15 minutes at 13000 × g at 4°C, the pellet was air-dried and the DNA was dissolved in 10 µl of H_2O_{bidest}. The obtained DNA was used in ligation reaction.

Ligation: The ligation mixture (20 µl) contained fragment DNA, 1 x reaction buffer, plasmid DNA (125 - 250 ng) and 3 units of T4 DNA ligase. The ligation reaction was carried out for 15 hours at 4°C. Then, the DNA was precipitated with ethanol, washed and dissolved in deionised water. Ligated plasmid was used for transformation of competent *E. coli* cells.

Transformation and selection: The competent *E. coli* cells (strain RRIδM15; (Rüther, 1982)) were transformated by electroporation method. The aliquot of the ligation mixture (1 µl) was added to the 50 µl of competent cells and incubated on ice for 10 minutes. Sequentially, the cells were submitted to electroshock (1500 - 2000 V; 5 msec). The treated cells were transferred to 1 ml of the LB medium. Then, 100 µl were spread on the agar selection medium (X-gal agar containing 25mg/l ampicillin) and left over night by 37°C. The positive colonies were used for inoculation of 1 ml of LB medium (containing 25 mg/l ampicillin) and incubated over night at 37°C and 220 rpm in Celloshaker. The obtained cells were used for analytical plasmid DNA isolation (2.13). The selection of positive clones

was done by restriction analysis. The purified plasmid DNAs carrying insert of the approximately right size were chosen. These DNAs were partially sequenced (2.14), using plasmid specific primers (pUC18-fw; pUC18-rev), in order to verify the identity of the cloned inserts.

2.13 DNA isolation and purification

Plasmid DNA was isolated and purified using commercially available kits following the kit protocols. Wizzard™ Plus Minipreps kit was used for analytical and Wizzard™ Plus Maxipreps kit for preparative plasmid DNA isolation. Obtained DNA was dissolved in H_2O_{bidest}, the DNA concentration was determined by spectrophotometry and the solution was stored at -20°C until needed.

PCR products were separated by electrophoresis in low-melting agarose (1 - 2%) and subsequently isolated out of the gel by so called "QN^+ extraction". It is a modification of the method firstly described by Langridge (Langridge et al., 1980). Hexadecyl-trimethyl ammonium bromide (QN^+-salt) was recrystalised from water, washed with acetone, dried and stored at -20°C until needed. For DNA extraction, 1% solution of QN^+-salt was prepared in mixture of water-saturated n-butanol and butanol-saturated water (volume ratio 1:1). The solution was vortexed for 2 minutes thoroughly and then centrifuged at 13000 × g for 10 minutes. The water and butanol phases were separated and placed in the heat block (pre-heated to 56°C). DNA fragments separated in low-melt agarose were cut out and the pieces of agarose were melted at 65°C for 10 minutes. The volume of melted agarose was estimated and mixed with 1 volume of QN^+-salt/butanol solution and 1 volume of QN^+-salt/water solution (both pre-heated to 56°C). The mixture was vortexed for 30 seconds and the phases were separated by following centrifugation at 13000 × g for 2 minutes. The butanol phase was collected and 1 volume of fresh butanol solution was added to the agarose/water mixture. The vortex, centrifugation and collection steps were repeated once more. In order to separate the last traces of water from butanol, the collected butanol samples were pooled together, placed on ice for 5 minutes and then centrifuged for 2 minutes. The butanol was separated and DNA was

re-extracted from butanol by addition of 10 - 20 µl of 0.2 M NaCl, vortexing for 30 seconds, centrifugation and separation. The traces of butanol in DNA containing NaCl solutions were removed by repeating the chloroform extraction up to 3 times. The chloroform rests were evaporated from sample by incubation at 65°C for 30 minutes. DNA was precipitated by addition of 3 volumes of ethanol and incubation at -20°C for at least 1 hour. The precipitated DNA was pelleted by centrifugation and the pellet was dissolved in H_2O_{bidest}. The concentration of DNA was determined by spectrophotometry and the solution was stored at -20°C until needed.

2.14 Sequencing

DNA sequencing was performed by cycle sequencing, which is a combination of the dideoxy-chain-termination method (Sanger et al., 1977) and the linear PCR (Mullis and Faloona, 1987). In this case, the PCR reaction is carried out in the presence of only one type of primer and four dideoxynucleotides. The advantage of this method is the need of only very small amount of template DNA and the possibility to use all the four dideoxynucleotides at once in a sequencing reaction. The reaction products can be distinguished, since the dideoxynucleotides are labelled with different fluorescent dyes, which enable later detection and analysis of synthesised fragments by laser scan. The amplified DNA was separated and analysed on ABI PRISM 310 sequencer.

Probe preparation: DNA to be sequenced has to be purified prior to sequencing reaction in order to be free of primers or small DNA fragments. These small DNA fragments can unspecifically prime the reaction during cycle sequencing and so increase the undesirable background signal. Because of this, DNA was precipitated prior to sequencing. The DNA/water solution was mixed with an equal volume of 4 M ammonium acetate and a double volume of isopropanol. The solution was mixed and centrifuged by 13000 × g and 22°C for 45 minutes in Sorvall® RMC14. The pellet was washed with 70% ethanol, air dried and dissolved in H_2O_{bidest}.

Sequencing: Each sequencing reaction mixture (20µl) contained primer (10 pmol), template DNA (60 -360 ng), 2 µl of premix solution and H_2O_{bidest}. The premix solution was provided in DNA sequencing kit and it contained reaction buffer, DNA polymerase (AmpliTaq®FS), deoxynucleotides and fluorescent labelled dideoxynucleotides. All compounds were mixed together and reaction mixture was covered with a thin layer of mineral oil. Sequencing reaction was carried out in a thermal cycler under conditions optimised for the used primer.

Product purification: DNA solution was separated from mineral oil. To the solution 2 µl of 3 M sodium acetate (pH 4.6) and 55 µl of 100% ethanol were added, well mixed and centrifuged at 13000 × g in Sorvall® RMC14 for 20 minutes at 22°C. The pellet was air dried. Dried DNA was dissolved in 25 µl of TSR solution (Template Suppression Reagent), heated up to 90°C for 2 minutes, then chilled on ice. Subsequently, the DNA solution was transferred into the sequencer and analysed.

2.15 Work with RNA

To avoid contamination of RNA with RNases, a number of precautions were used. Only sterile, disposable plastic ware free from DNases and RNases was used. Glassware prior to use was decontaminated by baking at 180 °C for 10 hours. All solutions were prepared using RNase-free glassware, water and chemicals reserved for work with RNA. Water and solutions prior to use were treated with 0.1% DEPC (diethyl pyrocarbonate) for at least 12 hours at 37 °C and then heated to 100 °C for 15 minutes.

2.16 RNA isolation

RNA was isolated following a modified protocol of guanidinium-thiocyanate method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). First, the weight of the tissue was determined. Then the tissue was treated as described below (description for 100 mg of tissue). The tissue was homogenised in a glass tube together with 2.5 ml of ice-cold denaturing solution (solution D). All tubes and solutions were kept on ice. Sequentially, 0.25 ml of 2 M sodium

acetate, 2 ml of phenol (TE saturated) and 1 ml of phenol/chloroform (TE saturated) were added to the homogenate and mixed thoroughly. The final suspension was transferred into the centrifugation tubes. The tubes were placed on ice for 15 minutes and were shaken vigorously once every minute. The samples were centrifuged at 11000 × g in Sorvall® RC28S for 20 minutes at 4°C. After centrifugation, the aqueous phase (containing RNA) was withdrawn, mixed with equal volume of isopropanol and placed at -20°C for 1.5 hour to precipitate the RNA. The RNA was sedimented at 25000 × g in Sorvall® RC28S for 20 minutes at 4°C and the resulting pellet was dissolved in 0.6 ml of ice-cold solution D, transferred into 1.5 ml Eppendorf tube and precipitated with 0.6 ml of isopropanol at -20°C for 1.5 hour. Isolated RNA was sedimented at 13000 × g in Sorvall® RMC14 for 20 minutes at 4°C. The RNA pellet was dissolved in DEPC-treated double-distilled water (H_2O_{DEPC}) and purified by additional phenol/chloroform extraction. Subsequently, the RNA was precipitated with an equal volume of isopropanol in the presence of 0.2 M sodium acetate at -20°C for at least one hour.

2.17 RNA purification and control

Precipitated RNA was pelleted, and then the pellet was air-dried and dissolved in H_2O_{DEPC}. The remaining traces of DNA were removed by digestion with DNase I (RNase-free). The digestion mixture (120 µl) contained 80 µl of the RNA solution in the presence of 80 units of DNase, 1 x transcription buffer, 160 units RNasin® and 0.5 mM $MnCl_2$. The mixture was incubated at 37°C for 10 min. Afterwards, the RNA was extracted with phenol/chloroform and precipitated with isopropanol. The precipitated RNA was dissolved in H_2O_{DEPC} .

To check for DNA contaminations, PCR was performed with all RNA samples using the primers D1-exon and D1-intron. These primers are complementary to the dopamine receptor D1 gene and give rise to a 170 bp product. One primer is localised in the non-coding region of the receptor gene (Zhou et al., 1992), so that the PCR product can only be obtained in the presence of the DNA contaminations. The reaction mix contained 1 - 2 µl of RNA solution, 1 × reaction

buffer, 0.2 mM deoxynucleotides, 50 pmol from each primer and 2.5 units of Taq DNA polymerase. The PCR conditions were two minutes of denaturation at 94°C followed by 40 cycles of 30 seconds at 94°C, 1 minute at 60°C, 40 seconds at 72°C and the final extension time of 5 minutes at 72°C. After the end of the reaction, the aliquots of the solutions were separated by electrophoresis in 2% agarose gels that were stained with ethidium bromide and analysed under the UV light. If a distinct 170 bp band had been observed, the whole purification procedure was repeated.

The integrity of the isolated RNA was controlled by electrophoresis in agarose gels containing formaldehyde following a standard protocol (Sambrook et al., 1989). The gels were stained for 20 minutes in ethidium bromide bath (10 µg EtBr/ 100 ml MEN buffer), de-stained in MEN buffer for 15 hours and analysed under the UV light.

The purity and the concentration of RNA were determined by spectrophotometry at 260, 280 and 240 nm. All RNA samples should have similar values of 260/240 and 260/280 ratios. Some RNA samples had to be extracted with phenol/chloroform twice because of protein impurities as evidenced by 260/280 ratios below 1.8. The RNA was stored at -80°C until needed.

2.18 RNase Protection Assay

The Ribonuclease Protection Assay (RPA) is a sensitive procedure for the detection and quantification of RNA species in a complex sample mixture of total cellular RNA (Zinn et al., 1983). For the RPA, a labelled RNA probe is synthesised that is complementary to the part of the target RNA to be analysed. The labelled probe is mixed with the sample RNA and incubated under conditions that favour hybridisation of complementary transcripts. After hybridisation, the mixture is treated with ribonuclease to degrade single-stranded, unhybridised probe. Labelled probe that hybridises to complementary RNA in the sample mixture is protected from ribonuclease digestion, can be separated with polyacrylamide gel electrophoresis (PAGE) and visualised by autoradiography. When the probe is present in molar excess over the target fragment in the hybridisation reaction,

the intensity of the protected fragment is directly proportional to the amount of complementary RNA in the sample mixture.

2.18.1 Probe synthesis

For RNase Protection Assay we used radioactive labelled antisense RNA probes for GAT-1 and β-actin mRNAs. Both probes were obtained by in vitro transcription. The transcription reaction was carried out using 100 - 200 ng of template DNA. Additionally, the reaction mixture contained transcription buffer, 20 units T7 RNA polymerase, 30 units RNasin®, 1 µg BSA, 1mM NTPs (ATP, CTP and GTP) and 13,2 µM UTP. UTP, the limiting nucleotide, was added in the reaction as a mixture of non-labelled UTP and radioactive-labelled ^{32}P αUTP. The concentration ratio of non- and radioactive-labelled UTP was 1:1 and 3:1 for synthesis of GAT1 and β-actin probes, respectively. The reaction was carried out at 30°C for 2 hours. The obtained GAT-1 probe was 163 and that one of β-actin was 464 bases in length.

2.18.2 Probe purification

The synthesised RNA was purified in two steps. Firstly, DNA template was digested by DNase I. Secondly, the obtained RNA was separated through PAGE in order to isolate full-length transcripts.

Twenty units of DNase I (RNase-free) were added to reaction mixture and incubated at 37°C for 30 minutes. After DNase digestion equal volume of RNA gel loading buffer was added to RNA sample. The tube was heated for 3 minutes at 95°C and then chilled on ice. Subsequently, the sample was loaded on a polyacrylamide gel (5%, 8 M urea, 0.75 mm thick) and separated for 1 hour at 20 mA. After electrophoresis the gel was covered with plastic wrap and an X-ray film (Reflection™) was exposed to it for 10 minutes. The localisation of the film on the gel was labelled. After the exposure, the film was developed and used to precisely localise the area of the gel that contained the full-length transcripts. The indicated area was excised with a scalpel, transferred to a microfuge tube and submerged in 300 µl of probe elution buffer. To elute the probe out of the gel, the

tube was shaken and incubated at 37°C for 4 hours. The probe was stored at -20°C over night or maximally for two days.

2.18.3 RPA assay

Predetermined volumes of sample RNA (10 µg in 10 - 15 µl), yeast RNA (8 µl = 40 µg) and labelled probe (25 µl) were mixed in 0.5 ml microcentrifuge tube. For each probe two different control tubes were included one with 10 µg of rat total RNA and 45 µg of yeast RNA and second only with 50 µg of yeast RNA. Ammonium acetate (5 M) was added, so that its resulting concentration was 0.5 M. Additionally, 300 µl of ethanol was added and solution was mixed thoroughly. The tubes were placed in -20°C freezer for 30 minutes. Subsequently, the RNAs were pelleted by centrifugation at 13000 × g for 15 minutes. The ethanol supernatant was removed and to the pellets 10 µl of HybSpeed hybridisation buffer (pre-heated to 95°C) was added. The tubes were immediately placed in a heating block (thermal cycler pre-heated to 95°C). After hybridisation buffer was added to all samples, each one was vortexed thoroughly and placed back in the hot block. The vortexing step was repeated once more. Then the tubes were incubated at 95°C for 3 minutes in order to dissolve and denature RNAs. To allow hybridisation of probe RNA and complementary mRNA in the sample, the incubation temperature was decreased to 68°C for 10 minutes. After hybridisation, 100 µl of RNase digestion buffer (HybSpeed kit), containing 100 units of RNase T1 and 1.25 unit of RNase A, were added to each tube except of two control tubes. To these controls, one for GAT-1 and one for β-actin probe (containing only probe and yeast RNA), 100 µl of digestion buffer without RNase were added. The incubation temperature was decreased to 37°C and each sample was vortexed thoroughly. To digest unprotected single-stranded RNA, the samples were incubated at 37°C for 2 hours. All samples were re-vortexed after 15 and 60 minutes of incubation. After digestion, 150 µl of Inactivation/Precipitation mix (HybSpeed kit) and 100 µl of ethanol was added to each sample. Tubes were vortexed briefly and transferred to -20°C for 1 hour in order to precipitate the undigested RNA.

2.18.4 Product separation, detection and analysis

The precipitated products of RNase digest were pelleted by centrifugation at 13000 × g for 15 minutes. Supernatant was removed. To remove the last traces of supernatant the tubes were re-centrifuged for about 5 seconds and then the residual fluid was withdrawn. To the pellets, 8 µl of RNA gel loading buffer was added. In order to re-suspend the pellets, the tubes were placed in the heat block (heated up to 95°C) and vortexed twice for 30 seconds. Then the tubes were briefly centrifuged and incubated at 95°C for 3 minutes. The samples were vortexed, centrifuged again briefly and chilled on ice. The samples were loaded on denaturing polyacryamide gel (5%; 0.75 mm thick; 8 M urea). From control samples, those without RNase digestion, only 1 µl was loaded. To resolve the protected fragments, the gel was run at 10 mA for 10 minutes and at 20 mA for 1 hour. Afterwards, the gel was transferred onto gel-blotting paper, covered with plastic wrap and dried in gel dryer at 80°C for 1 hour. The X-ray film (Reflection™) was exposed to the gel for 4 hours. The autoradiogram was then scanned with a video analysis system and analysed with Molecular Analyst® Software. The band intensities of GAT-1 and β-actin protected bands were measured and the intensity ratios GAT-1/β-actin of tested samples were compared.

2.19 Competitive RT-PCR

A method for quantification of specific RNA species by the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). In the assay, reverse transcription of the template RNA is followed by PCR. PCR provides for this assay sensitivity for detection and amplification of specific nucleic acid sequences. The method utilises a synthetic internal control (competitor) that is co-amplified with the target. An important condition of the method is that the target and the competitor have the same amplification efficiencies. Because of this, the competitor differs slightly from the target (in size and/or sequence) and shares the same primer sequences. To quantify the target, identical (but unknown) amounts of the target template are amplified in replicate reactions with known increasing amount of internal control template. This results in the titration of the unknown copy number of target

template. The amount of the target template present prior to the amplification is determined as the titration equivalence point.

2.19.1 Construction of competitor templates

2.19.1.1 Construction of GAT1-mut competitor

To construct the GAT1-mut competitor template, a 93 bp long GAT-1 cDNA fragment cloned into pUC18 was used. This fragment was identical to GAT-1 wild type cDNA fragment except of missing the first 12 bases. The loss of these bases destroyed the GAT1-fw primer recognition sequence. In order to be able to amplify the competitor with same primers as the GAT-1 cDNA fragment with resulting in the different PCR product that GAT-1 cDNA, the GAT1-fw sequence was added to the cloned fragment by PCR with GAT1-rev and GAT1-fw-mut primers. GAT1-fw-mut primer was a plasmid specific primer that carried GAT1-fw sequence coupled to its 5´end. This reaction attached the GAT1-fw sequence to the cloned fragment and at the same time inserted plasmid sequence of 30 base pairs behind the forward primer sequence (figure 2.1A). The amplification conditions were: 2 minutes denaturation at 94°C, 35 amplifi-cation cycles of 30 seconds at 94°C, 30 seconds at 55°C, 60 seconds at 72°C and the last extension of 5 minutes at 72°C. The amplified GAT-1 fragment was 143 bp long. This mutated PCR product was purified and the T3 promoter sequence was added to it by re-amplification with GAT1-fw-T3 and GAT1-rev primers. This modified DNA fragment was separated by agarose gel electro-phoresis, purified by QN^+ extraction method, precipitated with ethanol and dissolved in H_2O_{DEPC}. The identity of the constructed fragment was verified by dideoxy-chain-termination sequencing using GAT1-rev primer. Purified DNA was used for in vitro transcription (2.19.2) of GAT1-mut competitor.

2.19.1.2 Construction of GAT3-βac competitor

For construction of the GAT-3 competitor template, the β-actin fragment (400bp) cloned into pUC18, already used in the RPA assay, was employed. For the part of the template between the primer sequences, a sequence was chosen that was 27 bases longer and had a similar CG content as the amplified GAT-3 cDNA fragment (58% in wild type; 66% in competitor). It consisted of 30 bp plasmid sequence and 128 bp of ß-actin sequence (position 1253 - 1380 of ß-actin cDNA). The chosen sequence was amplified with one plasmid (GAT3-fw-mut) and one ß-actin specific (GAT3-rev-βac) primer that had coupled GAT3-fw and GAT3-rev primer sequences, respectively, to their 5`ends (figure 2.1C). The DNA was amplified under the following conditions: 2 minutes denaturation at 94°C, 30 amplification cycles of 30 seconds at 94°C, 30 seconds at 55°C, 60 seconds

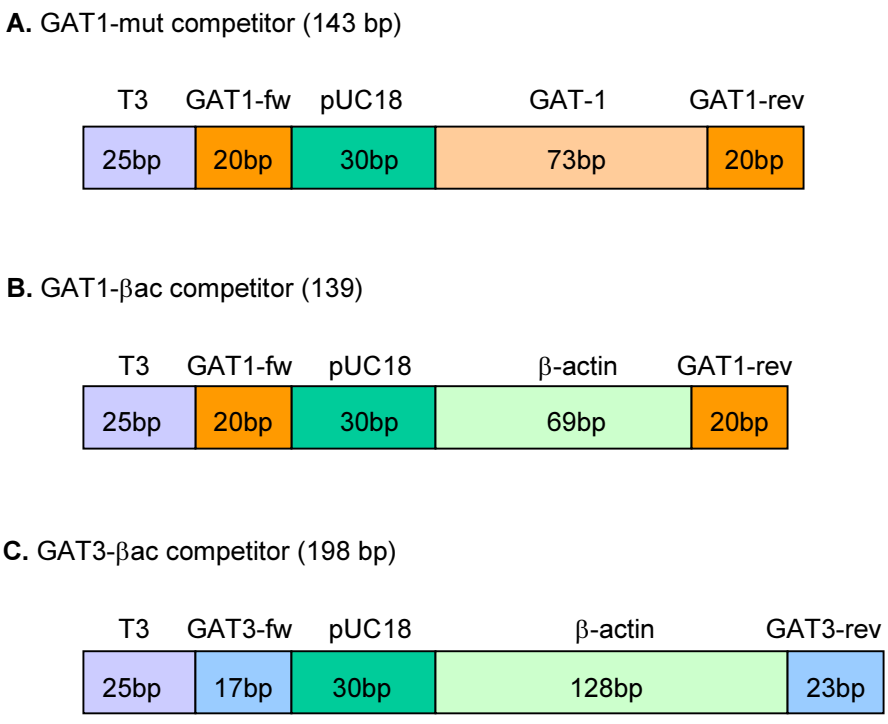

Figure 2.1

Competitors: Schematic presentation of various DNA constructs used as templates for in vitro transcription of RNA competitors. Displayed are the sequences of origin and fragment size.

at 72°C and the last extension of 5 minutes at 72°C. The obtained 198 bp sfragment was purified and the recognition sequence for T3 RNA polymerase was added to it by re-amplification with GAT3-rev and GAT3-fw-T3 primers under GAT-3 optimised PCR conditions (2.19.3.2). Re-amplified GAT3-βac product was isolated, purified and its sequence was verified by sequencing with dideoxy-chain termination method. The purified DNA fragment was used as a template for in vitro transcription of GAT3-βac competitor (2.19.2).

2.19.1.3 Construction of GAT1-βac competitor

Also, DNA template for GAT1-βac competitor was constructed. It carried a part of plasmid DNA and a part of the β-actin cDNA flanked with GAT1-fw and GAT1-rev primer sequences. The chosen sequence was 35 bp longer and had similar CG-content as GAT-1 wild type sequence (61% in competitor; 55% in wild type). The desired fragment was obtained from cloned β-actin fragment by PCR amplification with one plasmid specific (GAT1-fw-mut) and one β-actin specific (GAT1-rev-βac) primers which had GAT1-fw and GAT1-rev primer sequences coupled to their 5´ends, respectively (figure 2.1B). The amplification conditions were: 2 minutes denaturation at 94°C, 35 amplification cycles of 30 seconds at 94°C, 30 seconds at 55°C, 60 seconds at 72°C and the last extension of 5 minutes at 72°C. Then the T3 promoter sequence was added to it by re-amplification with GAT1-rev and GAT1-fw-T3 primers under GAT-1 specific PCR conditions (2.19.3.2). The DNA fragment was purified (2.13) and used as a template for the competitor RNA synthesis (2.19.2).

2.19.2 Competitor synthesis and purification

To synthesise the competitor RNA, an in vitro transcription was performed for 90 min at 37°C. The reaction mixture (100 µl) contained 600 ng DNA template, 1 x transcription buffer, 10 mM DTT, 160 units RNasin, 0.5 mM NTPs, and 30 units of T3 RNA polymerase. In order to remove the DNA template after the reaction, the mix was treated with 10 units of DNase I (RNase-free) for 20 min at 37°C. Then, the RNA was precipitated with 3 volumes of ethanol and dissolved

in 20 μl of RNA loading buffer. The RNA was dissolved and denatured at 60°C for 10 minutes and subsequently chilled on ice.

For purification of full-length transcripts, the RNA was separated with denaturing PAGE (5%; 0.75 mm thick; 6M urea). The gel area containing full-length transcripts was cut out and the RNA was eluted following a RNaid kit protocol. The RNA was precipitated with ethanol, washed with 75 % ethanol, the pellet air dried and re-dissolved in H_2O_{DEPC}. To control for impurities and to quantify the RNA, its absorbance at 260, 240 and 280 nm was measured.

To further check for remaining DNA contamination 40 PCR cycles were carried out with 1 ml of RNA solution and appropriate primers. Direct PCR did not yield any product. As a positive control, an aliquot of the RNA (1 μl) was reverse-transcribed and amplified with the same primers to check whether the full-length amplification product was obtained (143 bp GAT1-mut; 139 bp GAT1-βac or 198 bp GAT3-βac).

2.19.3 Competitive RT-PCR assay

Total RNA (100ng) was reverse-transcribed together with different amounts of competitor RNA (e.g. 10^5, $2{,}5\times10^5$; 5×10^5; $7{,}5\times10^5$; 10^6 GAT3-βac molecules) with RT-primer under the reverse transcription conditions described bellow (2.19.3.1). An aliquot (1 μl) from each obtained cDNA sample was used for PCR amplification with the appropriate forward and reverse primers (conditions described bellow 2.19.3.2). This amplification gave rise to two PCR products, corresponding to GABA transporter (wild type) and competitor cDNAs. Aliquots of PCR products were separated by PAGE (5% gel). Product bands were visualised by ethidium bromide staining of the gel. The band intensities were determined. The ratio of the GAT- wild type band to the competitor band, corrected for the size difference of the two fragments, was calculated. The ratio of band intensities was plotted against the logarithm of the amount of RNA competitor. The amount of target mRNA equals the amount of competitor RNA at the point ratio=1.

2.19.3.1 Reverse transcription

Total RNA (100 ng) was reverse-transcribed using the specific primer (RT-primer). RNA and RT-primer (in H_2O_{DEPC}) were mixed (total volume 8.5 μl). In order to denature RNA, the mixture was heated up at 70°C for 5 minutes and chilled on ice. Afterwards, all other components of the reaction mixture were added. The reaction mix contained 1 × reverse transcription buffer, 0.5 mM dNTPs, 10 mM DTT, 2.5 μM primer, 20 units of RNasin, and 200 units of MMLV reverse transcriptase. The mixture (total volume 20 μl) was left at room temperature for 8 minutes. Then, the reaction was carried out for 90 minutes at 37°C. The reverse transcription was terminated by a denaturing step of 5 minutes at 95 °C. Obtained cDNA solutions were stored at -20°C.

2.19.3.2 PCR

The amplification conditions were optimised for the 105 bp GAT1 cDNA fragment. The chosen programme for the GAT-1 forward and reverse primers was: denaturation 2 minutes at 94°C, PCR cycles of 30 seconds at 94°C, 20 seconds at 59°C, 40 seconds at 72°C, and the final elongation for 5 minutes at 72°C.

To amplify the 171 bp GAT-3 cDNA fragment, the following PCR profile was found to be optimal: denaturation 2 minutes at 94°C, amplification cycles of 30 seconds at 94°C, 60 seconds at 65°C, 60 seconds at 72°C, and finally, the extension step for 5 minutes at 72°C. The optimisation of cycle number is described in results (3.1.2.1). In both cases, the PCR reaction mixture (50 μl) contained DNA template (1μl), 1 μM GAT-fw, 1 μM GAT-rev, 0.2 mM dNTPs, 1 × reaction buffer, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase.

2.20 Differential Display

Differential Display is the method for identification of differentially expressed genes firstly described by Liang and Pardee (1992). It is based on the assumption that every individual mRNA molecule can be reverse-transcribed and amplified by the PCR. The general strategy of differential display is to amplify partial cDNA sequences from the set of mRNAs. The method consists of two steps: the first, reverse transcription (RT) and the second, PCR amplification of cDNA fragments. The reverse transcription uses a set of anchored primers, such as $T_{12}MN$ where M = G, C or A and N = G, C, A or T. The use of these primers takes advantage of the polyA sequence present in most eukaryotic mRNA species and anchors the primer at the 3`end of the mRNA. Addition of two bases on the 3`end of the oligo-dT primer gives the specificity to it and allows only a part of the mRNA set to be reverse-transcribed. There are twelve combinations of the last two bases in the anchor primer possible and because of this reverse transcription with this set of primers subdivides the total cDNA into twelve fractions with nearly equal number of represented mRNA species. The obtained cDNAs are amplified using downstream anchored primer (identical to the one used for the RT) and upstream arbitrary primer in the presence of a radioactive nucleotide. Decamer-oligonucleotides are used as the upstream primers. Decamers were chosen, because they anneal fairly frequently near the 5`end of the cDNA strand and at the same time give specific DNA amplification. The amplified cDNA fragments are resolved by the polyacrylamide gel electrophoresis and subjected to the autoradiographic analysis.

2.20.1 Reverse transcription

Total RNA (2 µl), 50 pmol (2 µl) of downstream primer and 4 µl of H_2O_{DEPC} were mixed together. In order to denature RNA and allow the primer annealing the solution was heated up to 70°C for 10 minutes and then chilled on ice. Subsequently, all reaction components except of enzyme were added. The reaction mix contained 200 ng total RNA, 50 pmol downstream primer, 1 × reverse transcriptase buffer, 10 mM DTT, 20 units of RNasin® and 20 µM

dNTPs. The mixture was left at room temperature for 3 minutes. Then, 300 units of MMLV reverse transcriptase were added and the mixture was again left at room temperature for 8 minutes. The reverse transcription (final volume 20 µl) was carried out at 35°C for 1 hour. The reaction was stopped by enzyme inactivation for 5 minutes at 95°C and chilling on ice. To the solution, 10 µl of H_2O_{DEPC} were added. The obtained cDNA was stored at -20°C.

2.20.2 PCR

The synthesised cDNA was amplified by PCR. Reaction mixture (20 µl) contained 1 µl cDNA, 50 pmol downstream primer, 10 pmol upstream primer, 1 × reaction buffer, 1.5 mM $MgCl_2$, 2 µM dNTPs, 0.33 µM α-[^{33}P]dATP and 1 unit Taq polymerase. The reaction conditions were: 2 minutes denaturation at 94°C, 40 amplification cycles of 30 seconds at 94°C, 60 seconds at 40°C, 30 seconds at 72°C and the last extension of 5 minutes at 72°C. The primers used for the differential display analysis had been chosen following the nomenclature described by Bauer and colleagues (1993). All possible combinations of the displayed primers were tested.

Table 2.1

Primer sequences used in Differential display

Upstream primers	**Downstream primers**
U1: 5`-TACAACGAGG	**D1:** 5`-TTTTTTTTTTTCA
U2: 5`-TGGATTGGTC	**D2:** 5`-TTTTTTTTTTTCG
U3: 5`-CTTTCTACCC	**D3:** 5`-TTTTTTTTTTTCT
U4: 5`-TTTTGGCTCC	**D4:** 5`-TTTTTTTTTTTCC
U5: 5`-GGAACCAATC	
U6: 5`-AAACTCCGTC	
U7: 5`-TCGATACAGG	
U8: 5`-TGGTAAAGGG	
U10: 5`-GGTACTAAGG	

2.20.3 Product determination and analysis

The amplified fragments were resolved by polyacrylamide gel electrophoresis. An aliquot of the PCR product (6 µl) together with 2 µl of DNA loading buffer were loaded on 6% PA gel (0.5 mm thick) separated in 1 × TTE buffer for 20 minutes at 15 mA and for 4 hours at 40 mA using Sequi-Gen apparatus. After the electrophoresis, the gel was transferred onto Gel-blotting paper and dried at 80°C for 1 hour in Gel Dryer. Finally, a film (X-Omat) was exposed to the gel overnight. Autoradiograms were analysed with Molecular Analyst® software.

3 Results

3.1 Establishment and validation of methods for quantification of GABA transporter mRNAs

3.1.1 RNase Protection Assay

To detect and measure changes in mRNA expression of GAT-1 and GAT-3, it was necessary to establish assays for the quantification of mRNAs. The first method chosen to be established in our laboratory was the RNase Protection Assay (RPA).

To measure GAT-1 mRNA levels in different samples, β-actin mRNA was used as an internal control. Prior to the quantification, DNA templates for the antisense probe synthesis had to be constructed. Both, GAT-1 and β-actin fragments were cloned into a plasmid vector. First, GAT-1 fragment was amplified from oligo-dT primed cDNA using GAT1-fw and GAT1-rev primers under the optimised PCR conditions (2.19.3.2). The obtained PCR product was 105 bp in length (position 188 - 292 on GAT-1 cDNA). Also, the β-actin fragment (position 1247 - 1647 on β-actin cDNA, 400 bp) was amplified from oligo-dT primed cDNA with β-actin forward and reverse primers under the appropriate PCR conditions. Then, the recognition sequence for T7 RNA polymerase was attached to both fragments by reamplification with the appropriate forward primer and reverse primer with T7 promoter coupled to its 5`end. The obtained products were separated by agarose gel electrophoresis and purified. Sequentially, both fragments were cloned into the *Sma*I restriction site of the pUC18 plasmid. The positive clones were selected and the identity of the cloned fragments was verified by sequencing. Both inserted fragments were reamplified using the plasmid specific primers, pUC18-fw and pUC18-rev. The synthesised PCR products were purified and used as templates for run-off transcription of the antisense probes. The probe synthesis and purification were carried out as described in chapters 2.18.1 and 2.18.2. The synthesised probes consisted of either GAT-1 or β-actin antisense sequence

with 58 and 64 bases of plasmid sequence attached to their 3`end, respectively. The plasmid sequence was not homologous to the target sequences detected by the RPA. It did not hybridise with the target mRNAs and because of this it was cleaved away by RNase. This allowed to distinguish the protected (partly digested) from undigested probe and served as a control of the digestion conditions (enzyme concentration and incubation time) by establishing the method. The RNase protection assay and autoradiogram analyses were carried out as described in Materials and Methods (2.18.3, 2.18.4). Furthermore, it was necessary to determine the amount of sample RNA needed for the experiments. The amount of total RNA tested in the assay should be small in order to keep the consumption of the sample material as small as possible. On the other hand, the amount of RNA should be sufficient for detection of the desired mRNAs within a maximum of 2 days under the adjusted conditions. One condition for the assay was that the probe has to be present in excess to the target mRNA. To estimate the amount of sample RNA needed and to test whether the amount of the added probe was sufficient, the course of saturation of the hybridisation reaction was determined. Different amounts of whole brain RNA, 5; 15; 20 and 5; 10; 15; 20 µg, were analysed together with GAT-1 and β-actin probe, respectively. Within the whole range of used RNA amounts, both probes detected appropriate mRNAs under the established conditions and gave visible signals on the autoradiogram (figure 3.1). However, GAT-1 probe with the 5 µg of total RNA gave a very weak signal. The band intensities of protected probes were detected. Both probes gave signals linearly increasing with increasing amounts of RNA tested (figure 3.1). For the further quantification experiments, 10 µg of sample RNA was used. There were two reasons for choosing this amount of RNA. First, the reduction of tested RNA to 50% (from 10 to 5 µg) gave still detectable signal. Therefore, the use of 10 µg sample enabled the detection of the GAT-1 mRNA also by its possible downregulation with expression reduced to 50%. Second, both probes were present in all tested samples in excess and gave a signal linearly dependent from the amount of sample RNA (up to 20 µg). Therefore, in the 10 µg of sample RNA, both probes were able to detect increases (up to 200%) in the levels of appropriate mRNAs under the established conditions.

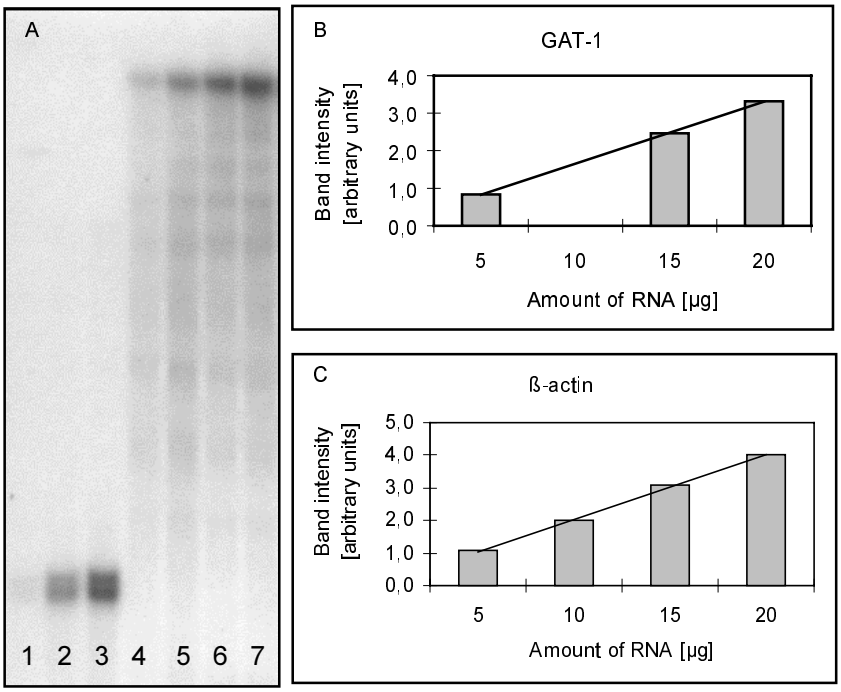

Figure 3.1

Ribonuclease protection assay: The resulting band intensities of protected GAT-1 and β-actin probes depending on the used amount of total RNA.

A: The autoradiogram. Lanes 1-3: GAT-1 probe tested together with 5, 15 and 20 µg of total RNA. Lanes 4-7: β-actin probe tested together with 5, 10, 15 and 20 µg of total RNA.

B: Relative band intensity of protected GAT-1 probe depending on the amount of the tested total RNA.

C: Relative band intensity of protected β-actin probe depending on the amount of the tested total RNA.

3.1.1.1 Validation of Ribonuclease Protection Assay

For the validation of the RPA, intra- and inter-assay variabilities were determined. To estimate the intra-assay variability of RPA, one total RNA sample was analysed in four different test tubes within one experiment. Ten µg of control whole brain RNA was tested (figure 3.2). The ratio GAT-1/β-actin of the band intensities was calculated. The intra-assay variation was determined as the standard deviation of the ratio values. The inter-assay variation was determined

as the standard deviation of the ratio values obtained in three different experiments. The intra-assay coefficient of variation (CV) of the RPA was 8% (figure 3.2) and the inter-assay coefficient of variation was 23%.

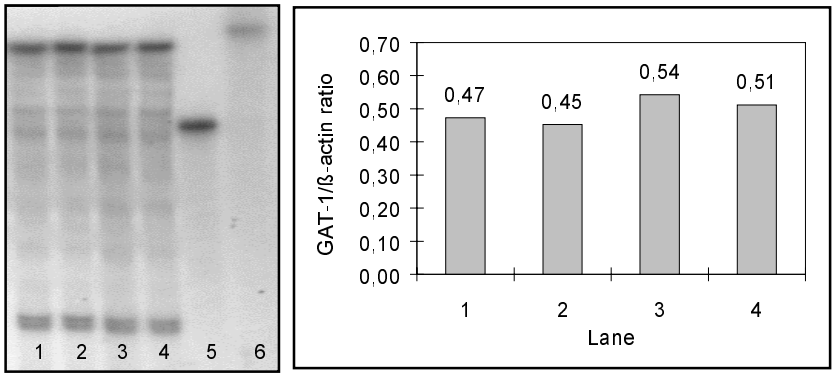

Figure 3.2

Determination of the intra-assay coefficient of variation of ribonuclease protection assay:

A: Autoradiogram. Lanes 1 - 4: protected GAT-1 (down) and ß-actin (up) probes after RPA with 10 µg of total RNA. Lane 5: Undigested GAT1 probe. Lane 6: Undigested ß-actin probe.

B: Band intensity ratios GAT-1/ß-actin from lanes 1 - 4. The mean standard deviation of the calculated ratios was 8%.

Sample	1	2	3
Measurement 1	0.41	1.13	0.90
Measurement 2	0.54	0.87	1.45
Measurement 3	0.79	1.02	1.25
Standard deviation	0.19	0.13	0.28
Standard deviation in %	33	13.2	23

Table 3.1

Inter-assay coefficient of variation of RNase protection assay: The ratios of relative intensities of protected GAT-1 and β-actin bands. The mean inter-assay standard deviation was 23%.

3.1.2 Competitive RT-PCR

3.1.2.1 Amplification conditions

The competitive RT-PCR was established for quantification of specific mRNAs from distinct brain areas like rat hippocampus or cortex frontalis. For the assay, the specific GAT-1 and GAT-3 primers were designed and the PCR conditions were adjusted to obtain the amplification of desired fragments from oligo-dT primed cDNA (2.19.3.2). The GAT-1 amplification gave rise to a 105 bp product (the same as in RPA) and the GAT-3 PCR reaction resulted in the synthesis of a 171 bp product (position 126 - 296 on GAT-3 cDNA). For quantification by means of competitive PCR, not only specificity of the product synthesis is important but also the discontinuation moment of the reaction. It is important to stop the PCR before the reaction reaches its plateau phase to avoid possible irregularities in the accumulation of PCR products. Because of this, the courses of GAT-1 and GAT-3 cDNA products amplification depending on the cycle number were detected under the adjusted PCR conditions. In this experiment, 100 ng of total RNA was reverse transcribed using either GAT1-RT or GAT3-RT primer following the described protocol (2.19.3.1). Each cDNA (12 µl) was used for set up of 12 identical PCR reactions using appropriate forward and reverse primers. The reactions were carried out under the optimised PCR conditions (2.19.3.2). For each cDNA, reactions were stopped after 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42 and 44 cycles by chilling on ice. Aliquots of the amplified PCR products were separated by polyacrylamide gel electrophoresis. The gels were stained with ethidium bromide and the band intensities measured by computerised video analysis. The obtained values are shown on figures 3.3 and 3.4. In both cases, the early postexponential phase of the reaction was chosen for further mRNA quantification of GABA transporters. The expression analysis was carried out with 36 cycles for GAT-1 and 33 cycles for GAT-3 cDNA fragments.

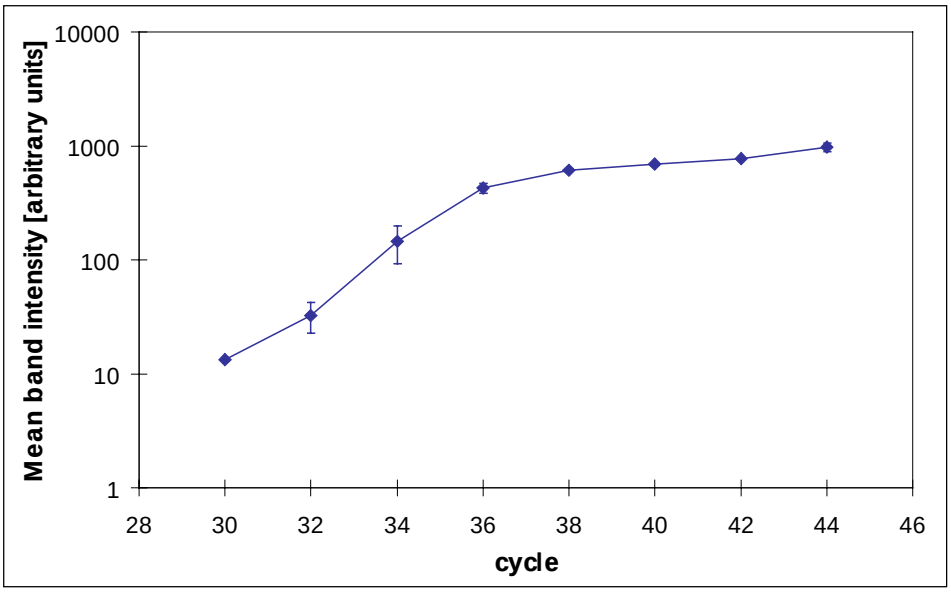

Figure 3.3

Reverse transcription and amplification efficiency of GAT-1 mRNA: 100 ng of total RNA was reverse transcribed and amplified using GAT-1 primers. Until cycle 30, the amount of PCR product was below the threshold level of the fluorescence detection system. The mean band intensity values from three experiments and standard deviations are shown.

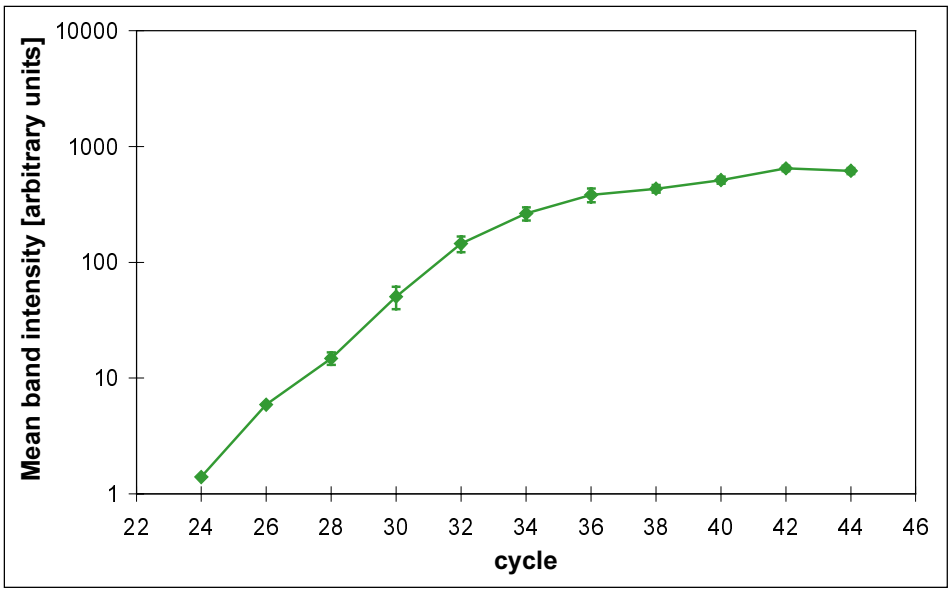

Figure 3.4

Reverse transcription and amplification efficiency GAT-3 mRNA: Total RNA (100 ng) was reverse transcribed and amplified using GAT-3 primers. Using the fluorescence detection system, the amount of amplified product was detectable after 24 cycles. The mean band intensity values from three experiments and standard deviations are shown.

3.1.2.2 Competitors

As an internal control, RNA competitor was used in the RT-PCR assay. As the competitor template can be used; **A.** a mutated target sequence with e.g. small deletion or insertion, in order to be able to distinguish competitor from wild type PCR product; **B**. a sequence unrelated to the target sequence flanked with the target specific primer sequences. For both cases, it is important that the competitor is reverse transcribed and amplified with the same efficiency as the target sequence.

3.1.2.2.1 GAT1-mut competitor

The most effective way to get equal amplification efficiency, is to use the sequence homologous to the target for competitor construction. Because of this, DNA template with GAT-1 wild type sequence was at first used for GAT-1 competitor synthesis.

Construction and synthesis of GAT1-mut competitor are described in Materials and Methods (2.19.1.1.). Prior to the quantification with the GAT1-mut competitor, it was necessary to show that the competitor RNA is reverse transcribed and amplified with the same efficiency as the GAT-1 wild type sequence. The real target of the assay, the GAT-1 mRNA, was not available in defined concentrations. Therefore, for this comparison, an additional GAT-1 fragment had to be constructed from which GAT-1 RNA (wild type) was transcribed and used as a model for the GAT-1 mRNA. To obtain this construct, the 105 bp GAT-1 cDNA fragment was reamplified with the GAT1-fw-T3 and GAT1-rev primers. From this construct, GAT-1 wild type RNA was generated as already described for the competitor RNA (2.19.2).

In this control experiment, equal amounts (0.01 pg) of wild type and competitor RNAs were reverse transcribed using the GAT1-RT primer and following the described protocol (2.19.3.1). Each obtained cDNA (11 µl) was used for set up of 11 identical PCR reactions using GAT1-fw and GAT1-rev primers. The reactions were carried out under the PCR conditions optimised for GAT-1 (2.19.3.2). For each cDNA, the reactions were stopped after 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,

and 40 cycles by chilling on ice. Aliquots of the amplified PCR products were separated by polyacrylamide gel electrophoresis. The gels were stained with ethidium bromide and the band intensity measured by video analysis system. As shown on figure 3.5, the time courses of reactions for both PCR amplicons were very similar. The products accumulate at the same rate and to the same relative intensity. Moreover, both reactions reach their plateau after the same cycle number. These data show that GAT-1 wild type and GAT1-mut sequences were reverse transcribed and amplified with similar efficiencies. This allowed the detection of the GAT-1 mRNA with the GAT1-mut competitor. GAT1-mut competitor was used for quantification of GAT-1 mRNA in hippocampus and whole brain samples from kindled and control animals (3.2.1.1).

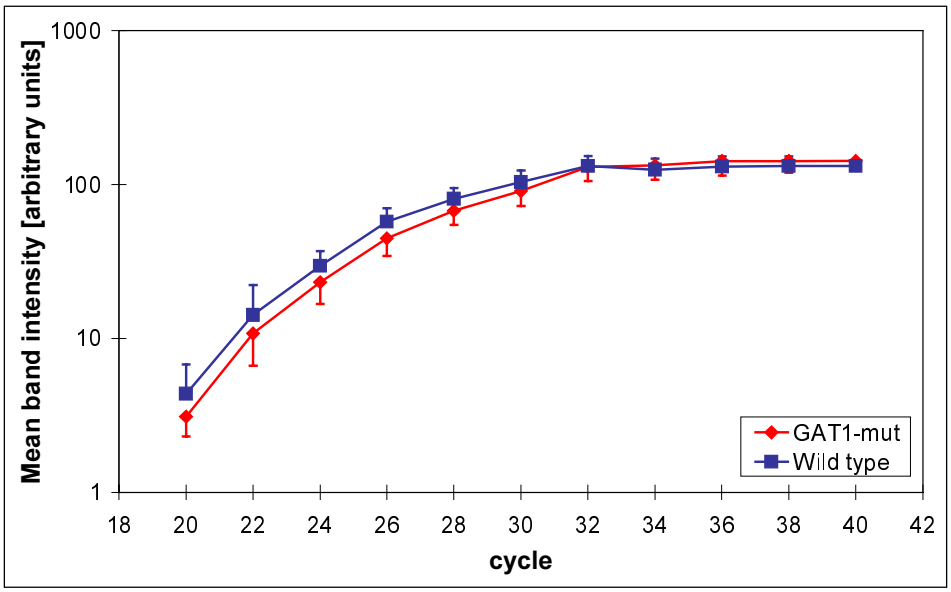

Figure 3.5

GAT1-mut competitor amplification efficiency: The reverse transcription and amplification efficiencies of GAT1-mut competitor and GAT-1 wild type sequences were compared. From each RNA, equal amounts were reverse transcribed and amplified in 11 separate reactions. From each reaction set, one reaction was stopped after every two cycles. The obtained PCR products were separated by PAGE and stained with ethidium bromide. The mean band intensities values from three experiments and standard deviations are shown.

By quantification with GAT1-mut competitor, very weak heteroduplex bands were observed in some gels (figure 3.6). Heteroduplex formation occurs only by amplification of related sequences in the postexponential and plateau phase of the reaction. Constant heteroduplex formation may shift the band intensity values and so the obtained data.

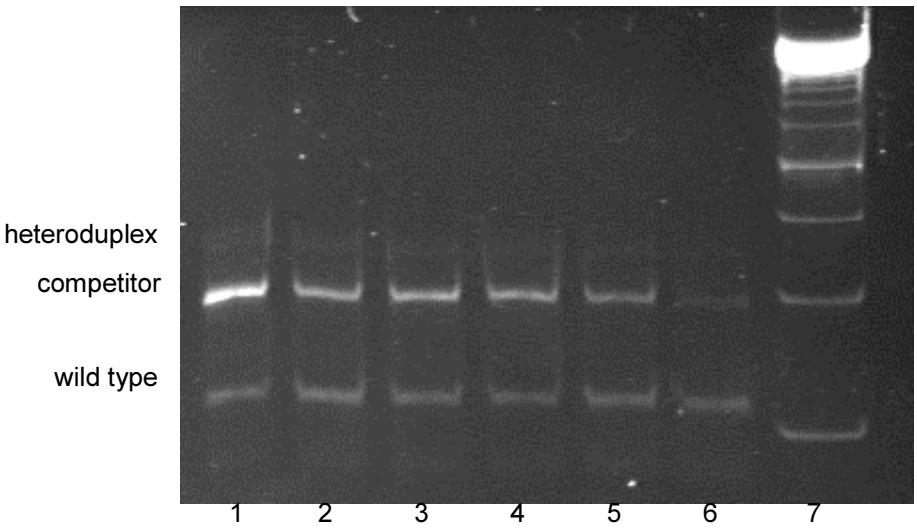

Figure 3.6

Amplification using GAT1-mut competitor: Total RNA was amplified with various amounts of GAT1-mut competitor. The use of competitor with the similar sequence to that one of GAT-1 cDNA resulted in a weak heteroduplex formation during the PCR in addition to competitor and GAT-1 wild type fragments synthesis. Lanes 1-6: Amplification of 100 ng of total RNA together with 2×10^8, 10^8, 8×10^7, 6×10^7, 4×10^7 and 2×10^7 GAT1-mut RNA molecules. Lane7: DNA molecular weight marker (50 bp ladder)

3.1.2.2.2 GAT1-βac competitor

Another strategy was used to design the GAT-1 competitor template for the following mRNA quantification. This DNA template carried sequence unrelated to the amplified GAT-1 cDNA fragment in order to keep the amplification free from heteroduplex formation. The GAT1-βac competitor consisted of β-actin related sequence flanked with GAT-1 primer sequences.

For the comparison of the amplification efficiencies of the GAT-1 mRNA (wild type) and the competitor sequences, GAT-1 wild type RNA sequence in defined concentration was needed. Therefore, the 105 bp GAT-1 cDNA fragment was amplified and T3 promoter was added to it by reamplification with GAT1-rev and GAT1-fw-T3 primers under the GAT-1 specific PCR conditions (2.19.3.2). The resulting DNA fragment was purified by agarose gel electrophoresis and QN extraction (2.13). Subsequently, the wild type RNA was synthesised and purified as already described for the competitor RNA (2.19.2).

Then, the reverse transcription and amplification efficiencies of the GAT1-βac competitor and the GAT-1 wild type RNAs were compared. The same amounts (0,1 pg) of competitor and target RNAs were reverse transcribed. Three different reverse transcription reactions were carried out for each RNA with GAT1-RT primer under the aforementioned conditions (2.19.3.1). The obtained cDNAs were used in subsequent GAT-1 PCR amplifications. One reaction mixture was used for preparing 11 identical reactions for each cDNA. Always one GAT-1 wild type and one GAT1-βac competitor amplification set ran parallel in the same PCR block (PCR conditions already described). For each cDNA, always one reaction was stopped by chilling on ice after every two cycles from cycle 22 to 44. Amplified PCR products were separated by PAGE, stained with ethidium bromide and the band intensities were determined by computerised image analysis system. The mean band intensity values depending on the cycle number for each sequence are plotted on figure 3.7. There was no significant difference between the courses of the accumulation of the GAT-1 wild type and GAT1-βac competitor amplicons. The reverse transcription and amplification efficiencies of the GAT-1 mRNA and competitor sequences were almost identical.

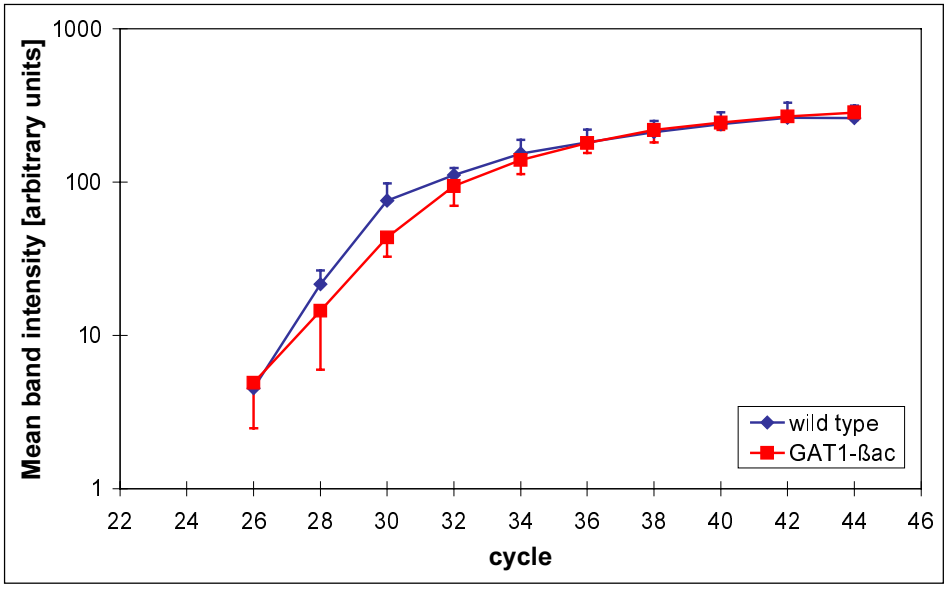

Figure 3.7

GAT1-βac competitor amplification efficiency: Comparison of the reverse transcription and amplification efficiencies of GAT1-βac competitor and GAT-1 wild type sequence. The mean band intensities values and standard deviations from three experiments are shown.

3.1.2.2.3 GAT3-βac competitor

As mentioned above, β-actin derived competitor was also used for GAT-3 mRNA quantification in further experiments. The reverse transcription and amplification efficiencies of the GAT3-βac competitor and the GAT-3 wild type sequences were also compared. Similarly, three reverse transcription reactions were set up for each RNA (all contained equal amounts of RNA) and then 11 identical PCR reactions were carried out for each obtained cDNA. For each cDNA, one reaction was stopped after every two cycles from cycle 20 to 40. The mean band intensity values of the obtained PCR product bands are shown on figure 3.8. The displayed data demonstrate that GAT3-βac competitor and GAT-3 wild type sequences were reverse transcribed and amplified with equal efficiencies.

The co-amplification of the GAT-1 and GAT-3 cDNAs with unrelated sequence (β-actin) flanked with the appropriate primer sequences gave rise to two different PCR products without any heteroduplex formation (figure 3.9:A). Therefore, the use of the competitor carrying the sequence unrelated to the target was favoured.

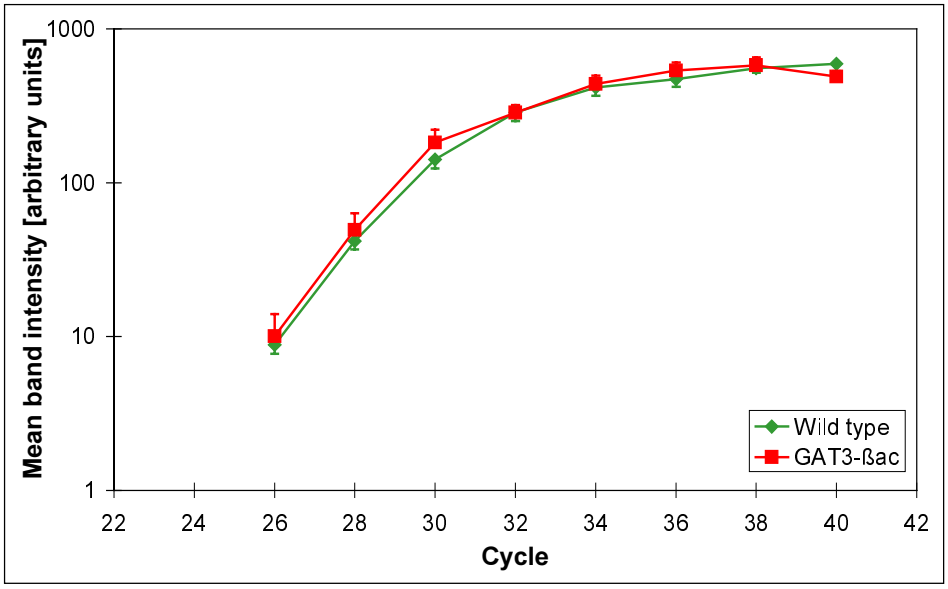

Figure 3.8

GAT3-βac competitor amplification efficiency: Comparison of amplification efficiencies of GAT3-βac competitor and GAT-3 wild type sequences. The equal amounts of competitor and wild type RNAs were reverse transcribed and amplified with GAT-3 primers. The mean band intensities values from three experiments and standard deviations are shown.

3.1.2.3 Quantification by means of competitive RT-PCR

The band intensity ratios wild type/competitor were plotted against logarithm of the competitor amount added to the sample. The amount of the present transporter mRNA was determined as the titration equivalence point (figure 3.9).

3.1.2.4 Validation of competitive RT-PCR

For the validation of the competitive RT-PCR, intra- and inter-assay variabilities were estimated.

To assess the intra-assay variability, the abundance of GAT-3 mRNA in cerebellar total RNA preparations was analysed. The intra-assay variation (table 3.2) was determined as the standard deviation of the values obtained for one sample

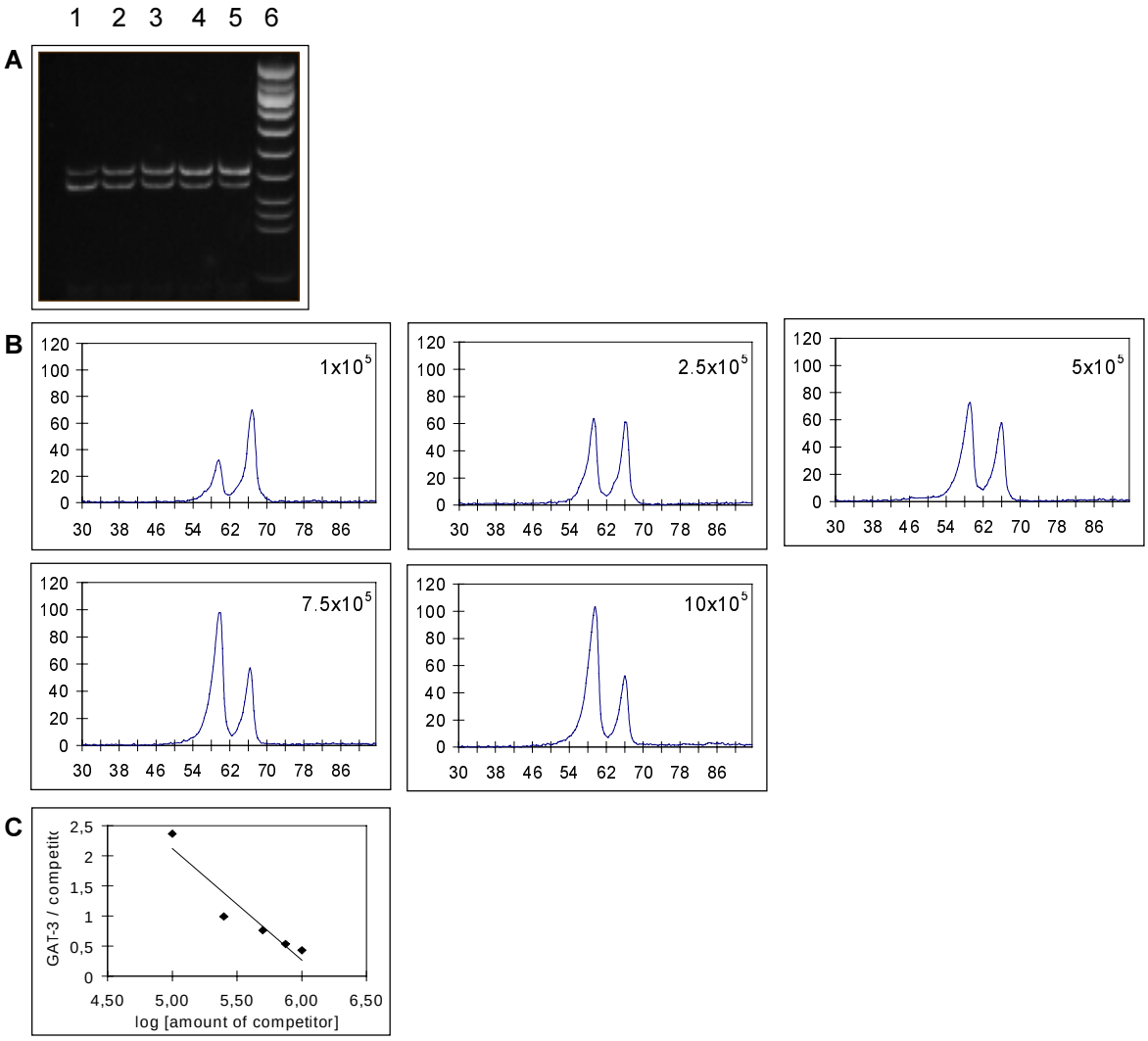

Figure 3.9

Quantification by means of RT-PCR: Representative data analysis after competitive RT-PCR: **A:** GAT-3 mRNA level was measured in hippocampus sample of control animal. Total RNA (100 ng) was amplified together with increasing amount of GAT3-βac competitor RNA (Lanes 1-5: 1×10^5, 2.5×10^5, 5×10^5, 7.5×10^5 and 10^6 competitor molecules) using GAT-3 primers. Obtained PCR products were separated by PAGE. Lane 6: DNA molecular weight marker (No VIII Boehringer)

B: The band intensity was measured using video analysis system. The fluorescence intensity (Y axis, [arbitrary units]) in each lane was measured depending on the distance from the start of the gel (X axis, [mm]). The first peak represents always the competitor band and the second represents the wild type product. The band intensity was calculated by integration of the area under the peak.

C: The ratios of band intensities (wild type / competitor) were calculated. Linear regression analysis (ratio against amount of competitor used) was carried out. The amount of competitor at point ratio=1 was calculated.

multiply measured within one experiment. The intra-assay coefficient of variation was 6%.

The inter-assay variation (table 3.3) was determined as the standard deviation of the GAT-3 mRNA level values of each brain region sample measured in 3 different assays. The resulting inter-assay coefficient of variation was 15%.

Table 3.2

Intra-assay variability of competitive RT-PCR: The results from triplicate measurements of one sample from three different experiments are shown.

	Experiment 1	Experiment 2	Experiment 3
No of GAT-3 molecules / 100 ng total RNA	88920 104231 96605	91161 88511 94841	80353 76736 88105
Standard deviation	7656	3179	5808
Standard deviation in %	7,9	3,5	7,1

Table 3.3

Inter-assay variability of competitive RT-PCR: For each sample, mean values and standard deviations are shown.

Brain region	**No of GAT-3 molecules/ 100 ng total RNA**	**Standard deviation**	**Standard deviation in %**
Cerebellum	88029	8324	9,5
Cortex frontalis	151893	24178	15,9
Cortex occipitalis	654153	50369	7,8
Hippocampus	179260	19119	10,7
Hypothalamus	541808	165289	30,5
Medulla oblongata	1044176	265743	25,4
Striatum	969948	131783	13,6
Thalamus	677411	38582	5,7

3.2 Expression experiments

3.2.1 Effects of kindling on expression of GAT-1 and GAT-3 mRNAs

The established methods were used to study effects on the expression of GABA transporter mRNAs in different tissue samples. As the first step, the hypothesis was tested, whether a decrease in GABA transporter ligand binding in hippocampus in an animal model of epilepsy (During et al., 1995) is caused by diminished expression of GABA transporter mRNAs. Therefore, the GAT-1 and GAT-3 mRNAs levels were measured in hippocampus and whole brain samples of kindled and control animals.

Chemical kindling (repeated injections of PTZ) was used as a model of epilepsy. After the 28 days kindling period, the animals were decapitated in deep anaesthesia and brains removed. Brains from 16 animals, 8 kindled and 8 controls, were analysed. From each treatment group 4 brains were left intact and 4 were dissected and the hippocampus was withdrawn. From all tissue samples, total RNA was isolated using the guanidinium thiocyanate method (2.16).

3.2.1.1 GAT-1 mRNA expression in kindled animals

Using the RPA, the mRNA expression of GAT-1 in kindled and control animals was measured. The band intensities of protected GAT-1 and β-actin probes were detected by video analysis system. The ratio of the band intensities GAT-1/β-actin provided the basis of the quantification.

As shown on the figure 3.10**,** there was no significant difference in the relative GAT-1 mRNA expression between the whole brain samples of the kindled (98 ± 28 %) and control (100 ± 20 %) animals.

Furthermore, expression of GABA transporter mRNAs in the hippocampus had to be compared. However, there were only about 35 µg of RNA available from each hippocampus sample. This was not enough for triplicate experiments necessary for GAT-1 and GAT-3 mRNA quantification by means of RPA.

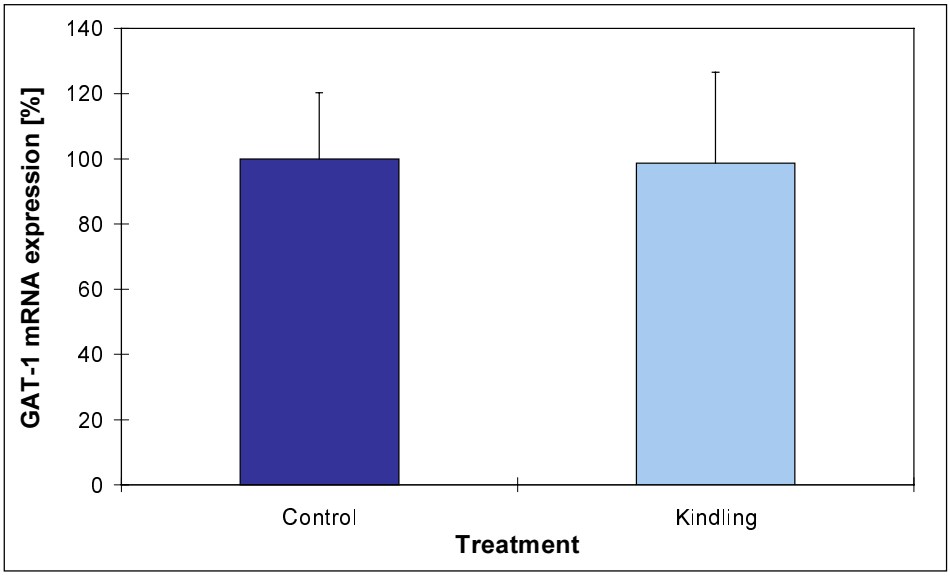

Figure 3.10

GAT-1 mRNA expression in kindled animals: GAT-1 mRNA levels were determined by Ribonuclease protection assay in whole brain samples from kindled and control animals using GAT-1 and β-actin antisense RNA probes. Samples from four animals per each group were analysed in triplicate experiments. The mean values and the standard deviations are shown.

By means of competitive RT-PCR, it was possible to quantify GAT-1 mRNA in whole brain and hippocampus samples of kindled and control animals. GAT1-mut competitor was employed in the assay as an internal control. Using competitive RT-PCR, 500 ng of total RNA (divided in five samples of 100 ng) was analysed together with increasing amounts of competitor RNA (2 $x10^7$; $4x10^7$; 6 $x10^7$; 8 $x10^7$ and 10^8 molecules). The amplified cDNA fragments were separated by PAGE, stained with ethidium bromide and the fluorescence intensity of the bands was determined. The ratio of band intensities, GAT-1 wild type/GAT1-mut, provided the basis of the quantification. Kindled animals reached 83±36 % and 95±28 % of control GAT-1 mRNA expression in whole brain and hippocampus, respectively (figure 3.11). There was no significant difference in GAT-1 mRNA levels between control and kindled animals neither in whole brain nor in hippocampus total RNA samples.

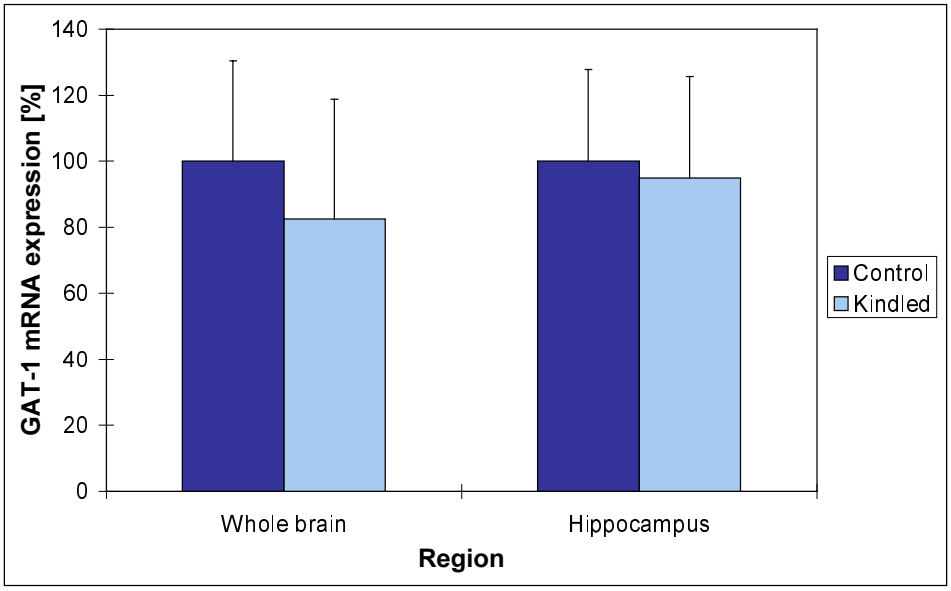

Figure 3.11

GAT-1 mRNA expression in kindled rats: GAT-1 mRNA levels were determined by competitive RT-PCR method in whole brain and hippocampus samples from kindled and control animals. Samples from four animals per each group were analysed in triplicate experiments. The mean values from four animals and the standard deviations are shown.

3.2.1.2 GAT-3 mRNA expression in kindled animals

GAT-3 mRNA expression in hippocampus and whole brain RNA samples of kindled and control animals was measured by competitive RT-PCR using GAT3-βac competitor (2.19). Five hundred ng of total RNA (divided in 5 x 100 ng) from each sample were analysed together with increasing amounts of the GAT3-βac competitor (10^5; $2.5x10^5$; $5x10^5$; $7.5x10^5$ and 10^6 molecules). The obtained PCR products were separated by PAGE, stained with ethidium bromide and the band intensities were detected . The amount of GAT-3 mRNA in tested samples was calculated using linear regression analysis of the band intensity ratios (GAT-3 wild type/ GAT3-βac competitor). There was no significant difference in GAT-3 mRNA expression in hippocampus, 99 ± 19 % and 100 ± 10 %, and in whole brain samples, 99 ± 26 % and 100 ± 22 %, of kindled and control animals, respectively (figure 3.12).

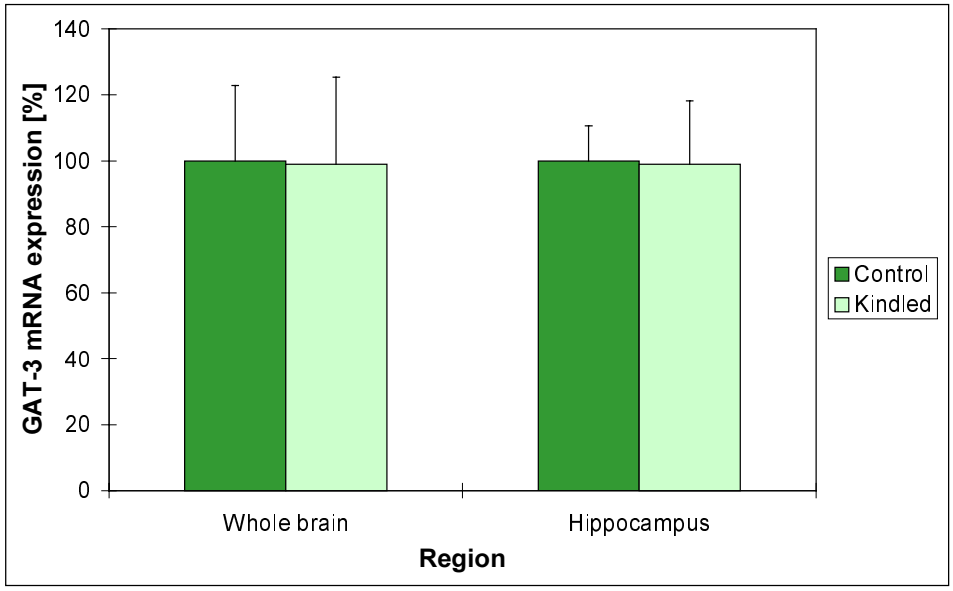

Figure 3.12

GAT-3 mRNA expression in kindled animals: Competitive RT-PCR method was used for detection of GAT-3 mRNA levels in whole brain and hippocampus samples from kindled and control animals. Each sample was analysed by triplicate measurements. The mean values and the standard deviations are shown from four animals per each group.

3.2.2 GAT-1 and GAT-3 mRNA expression profile in the rat brain

To compare the abundance of GAT-1 and GAT-3 and their distribution in the brain, the basal mRNA expression of both transporters was investigated. The mRNA expression patterns of both transporters were determined in various rat brain regions by using the β-actin derived competitors. Messenger RNA levels of both GABA transporters were quantified by RT-PCR triplicate measurements in samples from cerebellum, cortex frontalis, cortex occipitalis, hippocampus, hypothalamus, medulla oblongata, striatum and thalamus.

To detect the GAT-1 mRNA, GAT1-βac competitor was used in the range of 10^7; $5x10^7$; 10^8 and $5x10^8$ molecules per sample together with 100 ng of total RNA to be analysed. The GAT-1 mRNA expression was found to be in a comparable range of 500 - 1000 molecules per 1 pg of total RNA in all evaluated brain regions

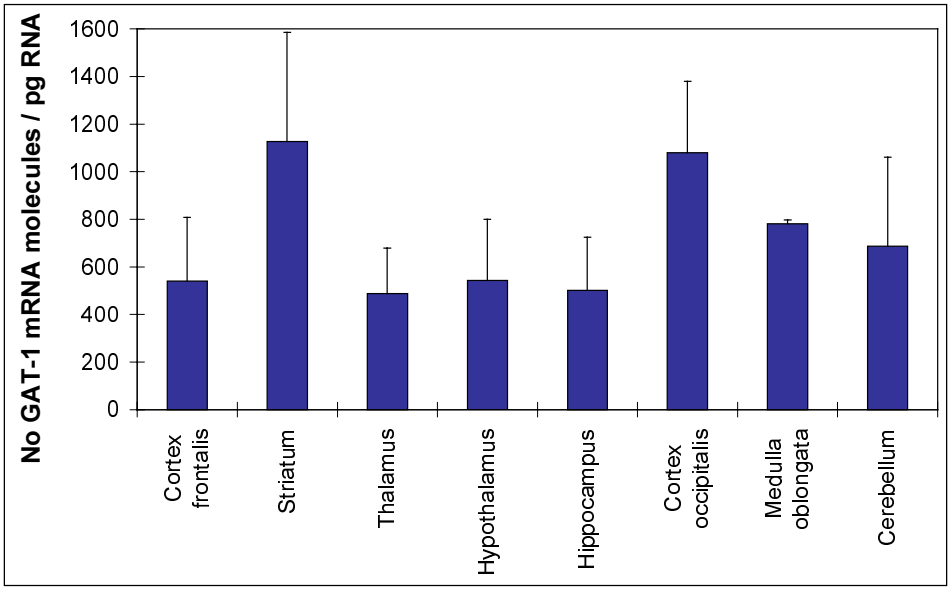

Figure 3.13

GAT-1 mRNA expression profile: GAT-1 mRNA levels were measured in various brain regions by competitive RT-PCR method. Each sample was analysed by triplicate measurements. The mean values and the standard deviations are shown.

(figure 3.13). The highest expression of GAT-1 mRNA was detected in striatum and the lowest in thalamus and hippocampus.

In order to determine GAT-3 mRNA expression pattern always 100 ng of total RNA were analysed together with 10^5; $2.5x10^5$; $5x10^5$; $7.5x10^5$ and 10^6 GAT3-βac RNA competitor molecules. GAT-3 mRNA expression in two brain regions had to be analysed in lower range of the competitor dilution scale. The cortex frontalis RNA was tested together with $5x10^4$; $7{,}5x10^4$; $1x10^5$, $2{,}5x10^5$; $5x10^5$ and the cerebellum RNA with $1x10^4$; $2{,}5x10^4$; $5x10^4$; $7{,}5x10^4$; $1x10^5$ competitor molecules. The results are shown on figure 3.14. The detected GAT-3 mRNA levels differed between tested brain regions. The GAT-3 mRNA expression was much lower than that of GAT-1 mRNA. The detected GAT-3 mRNA levels were in the range of 1 to 10 molecules per pg of total RNA, which is roughly 100 less that the detected levels of GAT-1 mRNA. In addition, the distribution of GAT-3 mRNA seemed to be more region-specific than GAT-1 mRNA.

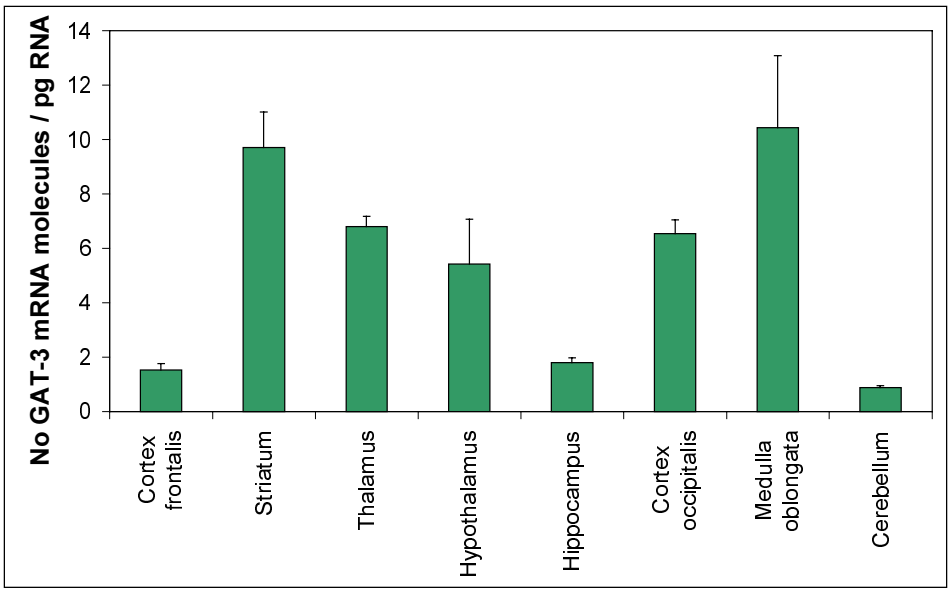

Figure 3.14

GAT-3 mRNA expression profile: GAT-3 mRNA levels have been measured in different brain regions by competitive RT-PCR method. The mean values and the standard deviations from triplicate measurements are shown.

3.2.3 Chronic diazepam and zolpidem treatment

The next aim was to test the influence of long-time treatment with drugs, acting on the GABA receptor, on animal behaviour and gene expression. The analysis was aimed to test the hypothesis whether the chronic benzodiazepine treatment influences the expression of neuronal GABA transporters and other genes. The effects on GABA transporters were studied by means of competitive RT-PCR. The effects on the expression of other genes were analysed by means of Differential Display method.

3.2.3.1 Chronic drug administration

Diazepam and zolpidem, GABA receptor ligands, were chosen for chronic treatment of rats. Both drugs were administered via drinking water. One group of rats received diazepam in the dose of 10 mg/kg/day. The second group was treated with 5 mg/kg/day of zolpidem. Control group received tap water. The concentrations of the administered drugs in the drinking water were adjusted daily depending on the amount of consumed water on the previous day. On the first day of treatment, the diazepam-treated animals drank much less than the controls. This may be due to the change of the liquid taste after addition of diazepam. However, the animals adapted to it and reached control values within a few days. Over the rest of the observed period, the animals drank constantly (figure 3.15). Each animal consumed in average 19.5 ml of water (solution) per day.

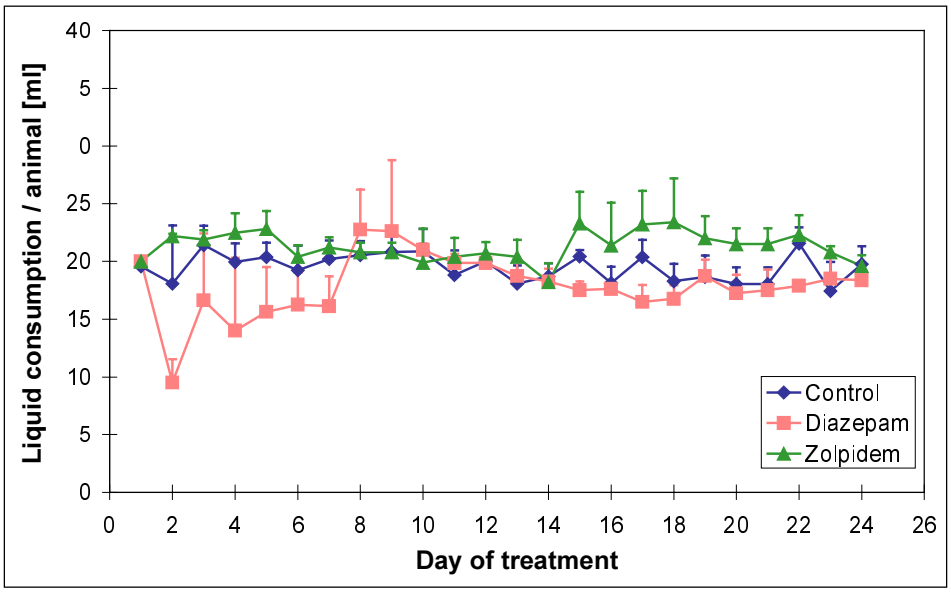

Figure 3.15

Liquid consumption: The amount of consumed liquid was recorded daily. During the 23 days of treatment controls received only tap water. Treated animals received via drinking water either zolpidem or diazepam. Plotted are the mean values and the standard deviations of ingested volume of liquid per animal and day. The values were calculated from 10 animals per each treatment group.

The next parameter necessary for the adjustment of the drug concentration was the animal body weight. It was recorded every third day. Within the treatment period the animals showed an average increase in the weight of 38 g (figure 3.16). There was neither any significant difference in the water consumption nor in the increase of the weight between the three treatment groups.

The body weight and the volume of consumed water were used to calculate the drug concentration in the drinking water to reach desired doses for each consecutive day. The real amount of the ingested drug was calculated at the end of the study on the basis of the body weight and liquid consumption. The rats ingested in average 10.3 ± 1.2 mg/kg of diazepam and 5.0 ± 0.4 mg/kg of zolpidem per day (figure 3.17).

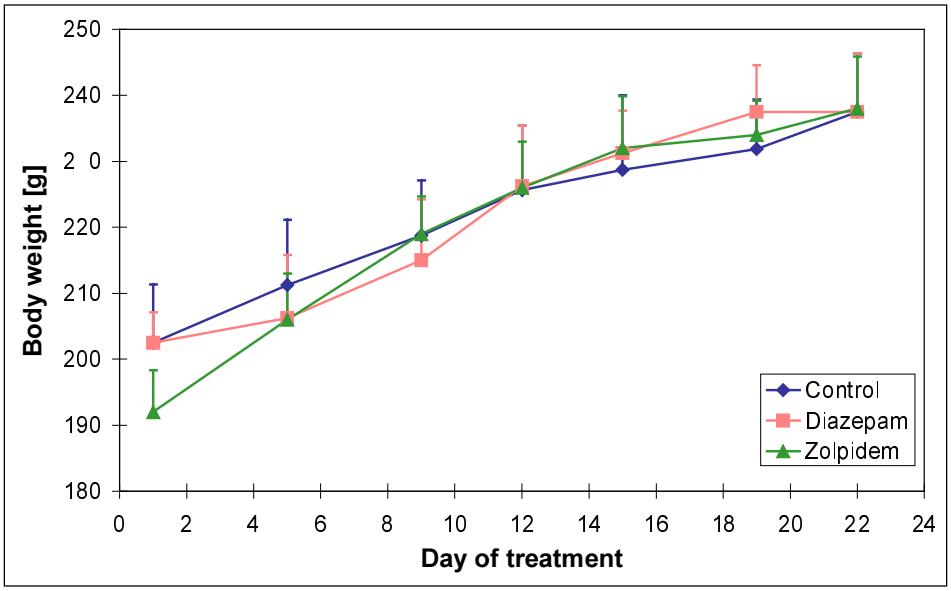

Figure 3.16

Body weight: The body weight of each animal was recorded every third day. During the 23 days of treatment, the tested animals were divided into three groups: controls, zolpidem- and diazepam-treated animals. The mean values and the standard deviations of detected body weight per animal are plotted. The values were calculated from 10 animals per each treatment group.

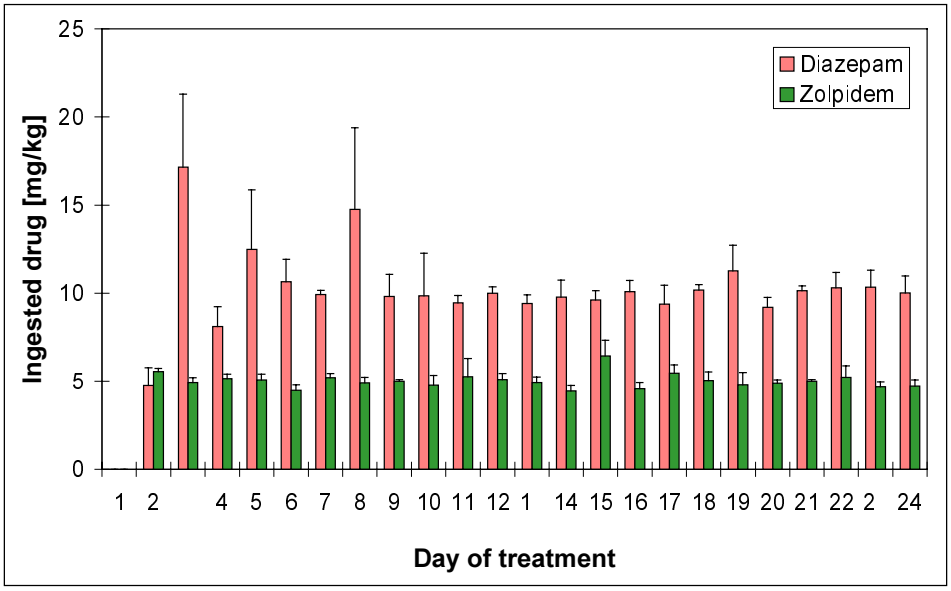

Figure 3.17

Dose of drug: The amount of ingested drug was calculated from recorded body weight and daily liquid consumption. During the 23 days of treatment, the drug concentration in the drinking water of tested animals was adjusted daily depending on the liquid consumption from previous day. The desired drug dose was either 10 mg/kg/day of diazepam or 5 mg/kg/day of zolpidem. The mean values and the standard deviations of ingested drug per animal and day are plotted. The values were calculated from data obtained from 10 animals per each treatment group.

3.2.3.2 Behavioural analyses

At the end of the chronic treatment period, the animals were submitted to behavioural analysis. To detect benzodiazepine-dependent modulation in the behaviour of the differently treated animals, they were tested in an open field (day 22) and in an elevated plus-maze (day 24). In addition to chronic treated animals, two groups of acute treated animals were tested in order to detect the specific drug effects caused by the chronic treatment. The acute treated animals were injected with either diazepam (2 mg/kg) or zolpidem (3 mg/kg) 30 minutes before the tests started. Moreover, one group of double treated animals, chronic and acute diazepam treatment, was also included. Within the behavioural tests, the respective parameters (2.10) characterising the basal activity, exploration- and anxiety-related behaviour were recorded and analysed.

3.2.3.2.1 Basal activity

The basal activity of the animals is described by the total "distance moved" in the open field arena. As it is shown in table 3.4, all tested animals moved with the comparable path lengths. In the plus-maze test, the basal activity of the animals is characterised by parameter "number of closed arm entries" (table 3.4). One-way analysis of variance (ANOVA) revealed no significant difference neither in the parameter "distance moved" ($F_{(5.50)}$=1.56, n.s.) nor in the "number of closed arm entries" ($F_{(5.50)}$=1.23, n.s.) of diazepam- and zolpidem-treated animals compared to controls. There was no significant difference in the basal activity of animals between the treatment groups.

Table 3.4

Basal activity: The basal activity parameters analysed in open field arena and elevated plus-maze. Six different treatment groups were tested. The present data represent means and SEMs from

8 - 10 animals per each treatment group.

Treatment / Behavioural parameter	Control	Diazepam acutely	Diazepam chronically	Diazepam acutely + chronically	Zolpidem acutely	Zolpidem chronically
Distance moved	1174 ± 232	1517 ± 262	923 ± 204	1014 ± 226	1387 ± 125	1521 ± 157
No of closed arm entries	2.5 ± 0.4	3.9 ± 0.8	2.2 ± 0.4	4.0 ± 1	3.6 ± 0.9	2.8 ± 0.5

3.2.3.2.2 Exploration

The exploration-related behaviour was analysed using an open field test. The exploration activity of the animals is characterised by two parameters: "entries into the centre" and "time moving". One-way ANOVA revealed significant changes in "entries into the centre" parameter ($F_{(5.50)}$=4.44, p=0.002). Post hoc t-test confirmed a significantly increased number of entries into the central zone ($p \leq 0.05$) of zolpidem-treated compared to control animals. There was no significant difference between the acutely and chronically zolpidem-treated animals. Furthermore, post hoc test revealed no significant change in number of entries into the central zone after diazepam treatment.

One-way ANOVA revealed also significant changes in the parameter "time moving" ($F_{(5.50)}$=5.79; p=0.0003). Post hoc t-test confirmed that acutely diazepam-treated animals spent more time with movement within the test arena than controls (p≤0.05). Other diazepam-treated groups did not differ from control group. On the other hand, both zolpidem-treated groups spent less time with movement than controls (p≤0.05) (figure 3.18).

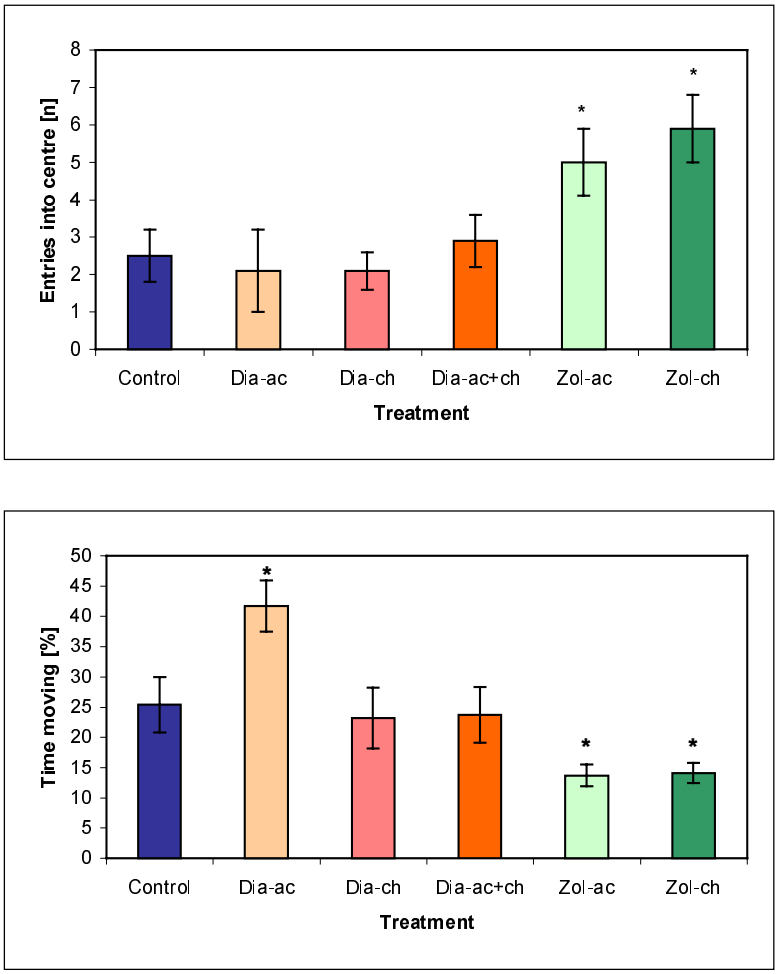

Figure 3.18

Exploration behaviour of treated animals: The animals were tested in an open field arena. The exploration behaviour was characterised by “number of entries into the centre” and “time moving”. “Time moving” is the time within the animals move described as % of total test time. For both parameters, the mean values and SEMs from 8-10 animals are shown for each treatment group. There were 6 treatment groups of animals analysed: controls, animal treated acutely with diazepam (Dia-ac), chronically with diazepam (Dia-ch), acutely and chronically with diazepam (Dia-ac+ch), acutely with zolpidem (Zol-ac) and chronically with zolpidem (Zol-ch). Significant difference label: *

3.2.3.2.3 Anxiety

The elevated plus-maze was used for the detection of anxiety-related behaviour. One-way ANOVA of anxiety related parameters: "number of open arm entries", "time on the open arms" and "% of open arm entries" from sum of all arm entries, revealed significant changes in two parameters and in the third one showed the tendency towards the significance ($F_{(5.50)}$=3.31, p=0.01; $F_{(5.50)}$=12.99, p=0.0001 and $F_{(5.50)}$=2.09, p=0.08). Post hoc t-test confirmed that acutely diazepam-treated animals, in average, entered more often the open arms than controls ($p \leq 0.05$), spent more time on the open arms ($p \leq 0.05$) and entered the open arms more often than the closed arms ($p \leq 0.05$) (figure 3.19). However, neither chronically nor double diazepam-treated animals showed any significant difference in any of these parameters compared to controls. Also zolpidem treatment caused no significant changes in the anxiety-related behaviours of tested animals.

3.2.3.2.4 Development of tolerance

Development of tolerance was tested by comparing chronically treated animals to acutely treated and control animals. There was no difference in behaviour between acutely and chronically zolpidem-treated animals (Figure 3.19). Chronic zolpidem treatment caused the same effect as acute zolpidem administration compared to the controls. Chronic diazepam administration caused no significant change in the analysed parameters compared to controls, whereas acute diazepam administration decreased the anxiety-related parameters. Moreover, the same acute dose of diazepam, when administered to animals chronically pre-treated with diazepam, lost the ability to decrease the anxiety of these double treated animals (Figure 3.19).

This loss of the anxiolytic effect of diazepam indicated tolerance development during chronic administration of diazepam. Zolpidem caused the same effects after acute and chronic administration and there were no hints suggesting development of tolerance to zolpidem.

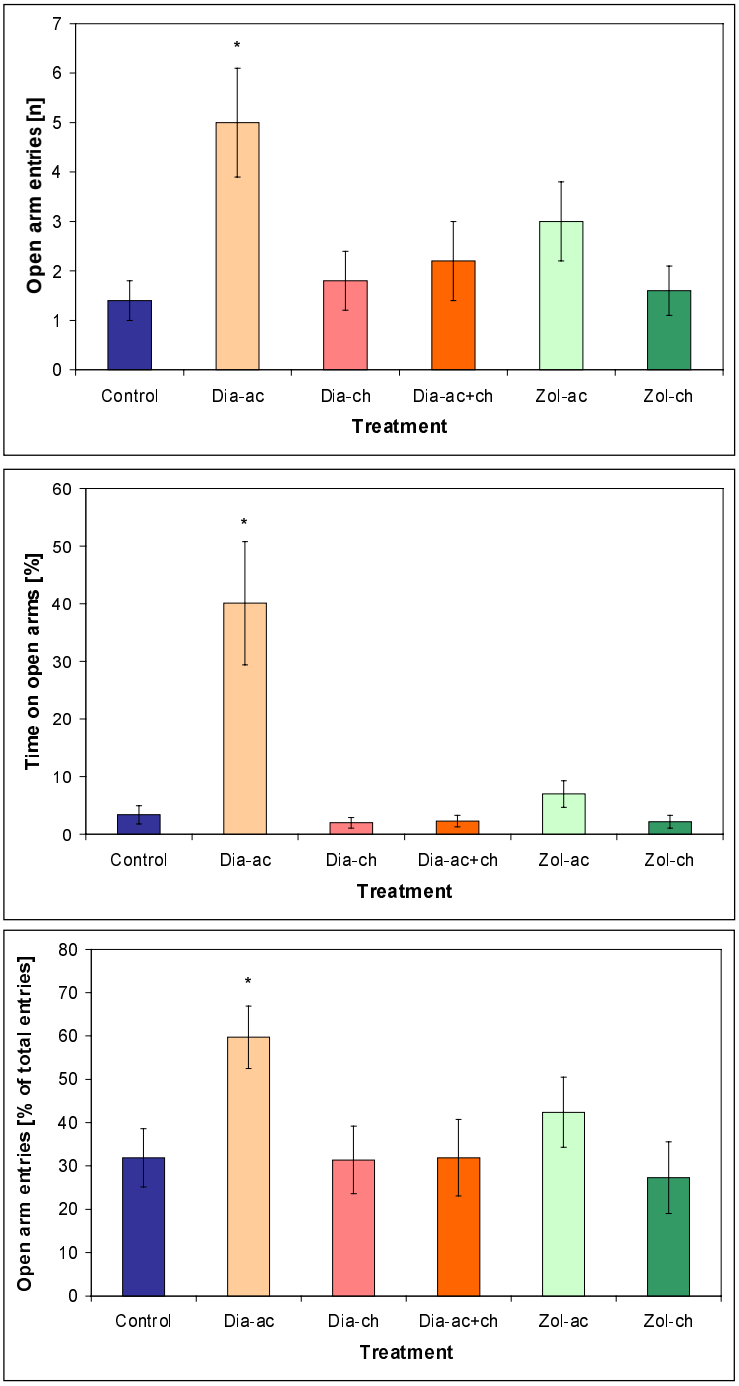

Figure 3.19

Anxiety-related behaviour: The anxiety-like behaviour was tested in the elevated plus-maze. The following three parameters were analysed: number of open arm entries, time spent on open arms and open arm entries as % of all arm entries. For both parameters, the mean values and SEMs from 8-10 animals are shown for each treatment group. There were 6 treatment groups of animals analysed: controls, animal treated acutely with diazepam (Dia-ac), chronically with diazepam (Dia-ch), acutely and chronically with diazepam (Dia-ac+ch), acutely with zolpidem (Zol-ac) and chronically with zolpidem (Zol-ch). Significant difference compared to controls is labelled: *

3.2.3.3 Gene expression analyses

3.2.3.3.1 Competitive RT-PCR analysis of GABA transporters mRNA levels

After the behavioural tests, the animals were decapitated and the brains were removed. Three brains from each treatment group were dissected and the cortex frontalis, hippocampus, and cerebellum were removed. From dissected brain samples and also from three whole brains from each treatment group, the total RNA was isolated (2.16) and the mRNA levels of the GAT-1 and GAT-3 were determined by competitive RT-PCR using β-actin derived competitors, GAT1-βac and GAT3-βac (2.19).

For the GAT-1 mRNA expression analysis, 100 ng of total RNA were tested together with either 10^7; $2.5x10^7$; $5x10^7$; $7.5x10^7$ or 10^8 molecules of RNA competitor. The detected GAT-1 mRNA levels were in cortex frontalis 114 ± 15 % and 109 ± 21 %, in hippocampus 109 ± 3 % and 103 ± 11 %, in cerebellum 131 ± 22 % and 105 ± 18 %, in whole brain samples 144 ± 30 % and 112 ± 50 %, of control after diazepam and zolpidem treatment, respectively (figure 3.20). In all tested regions, similar expression patterns were observed with GAT-1 mRNA expression after zolpidem treatment at the same level as controls and with increased GAT-1 mRNA levels after diazepam treatment. However, the detected GAT-1 mRNA levels in three chosen regions and in whole brain, after chronic diazepam and zolpidem treatment, revealed no significant differences between the three treatment groups.

Furthermore, the analysis of GAT-3 mRNA expression in tested samples was carried out. The hippocampus and whole brain RNA samples (100 ng) were analysed together with 10^5; $2.5x10^5$; $5x10^5$; $7.5x10^5$ and 10^6 molecules. The cerebellum RNA was tested with the competitor in the range 10^4; $2.5x10^4$; $5x10^4$; $7.5x10^4$; 10^5, and the cortex frontalis RNA in the range of $5x10^4$; $7.5x10^4$; 10^5; $2.5x10^5$; $5x10^5$ molecules. The detected GAT-3 mRNA values reached in cortex frontalis 112 ± 38 % and 83 ± 36 %, hippocampus 105 ± 21 % and 96 ± 15 %, cerebellum 102 ± 33 % and 98 ± 28 %, whole brain 105 ± 10 % and 90 ± 4 % of the control level after diazepam and zolpidem administration, respectively

(figure 3.21). The analysis of the GAT-3 mRNA expression revealed no differences after chronic diazepam and zolpidem treatment in tested brain regions.

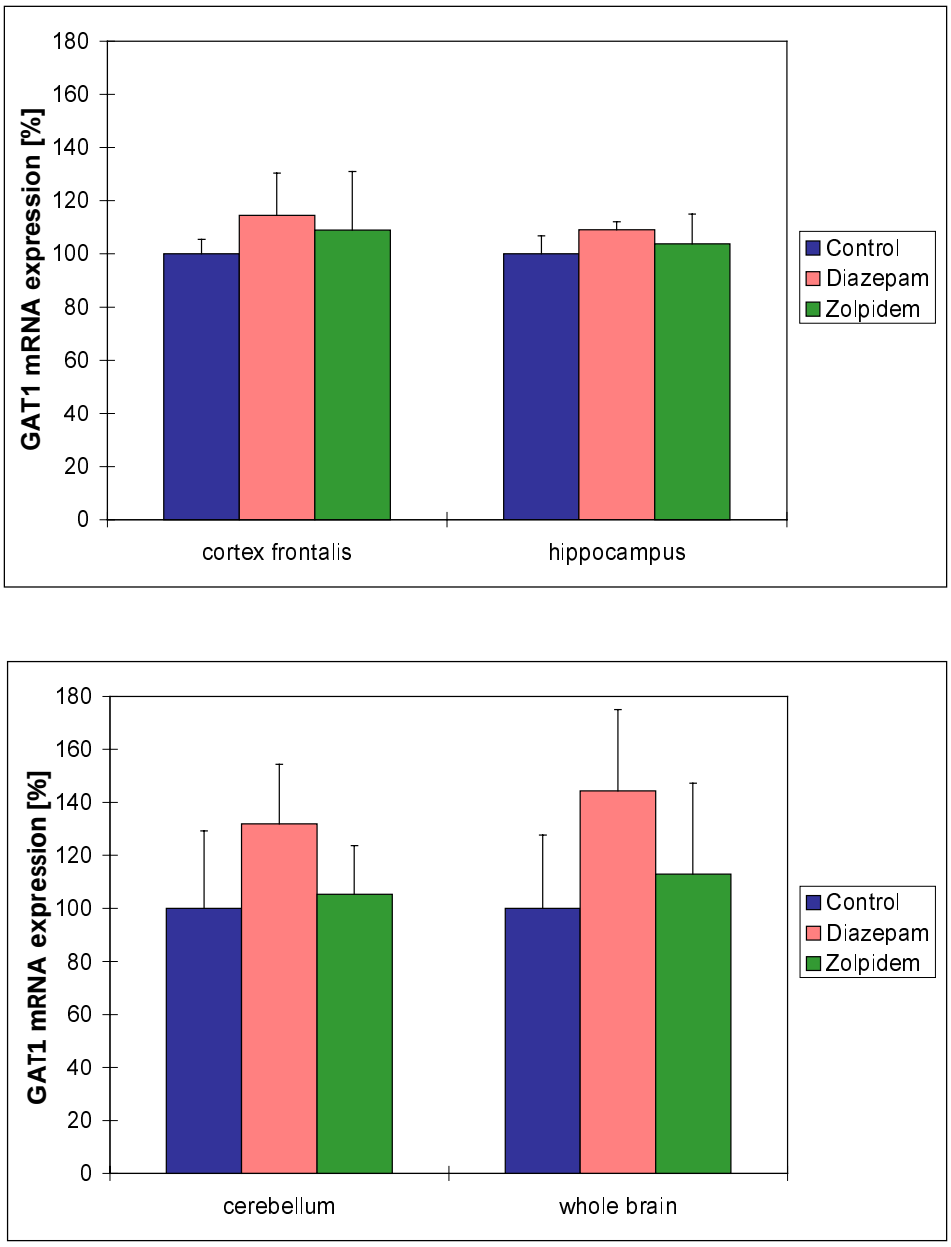

Figure 3.20

GAT-1 mRNA expression after chronic treatment with either diazepam or zolpidem: The GAT-1 mRNA levels were measured by competitive RT-PCR in cortex frontalis, hippocampus, cerebellum and whole brain samples from control, diazepam- and zolpidem-treated rats. Each sample was examined in triplicate measurements. The mean values and the standard deviations from three animals are plotted.

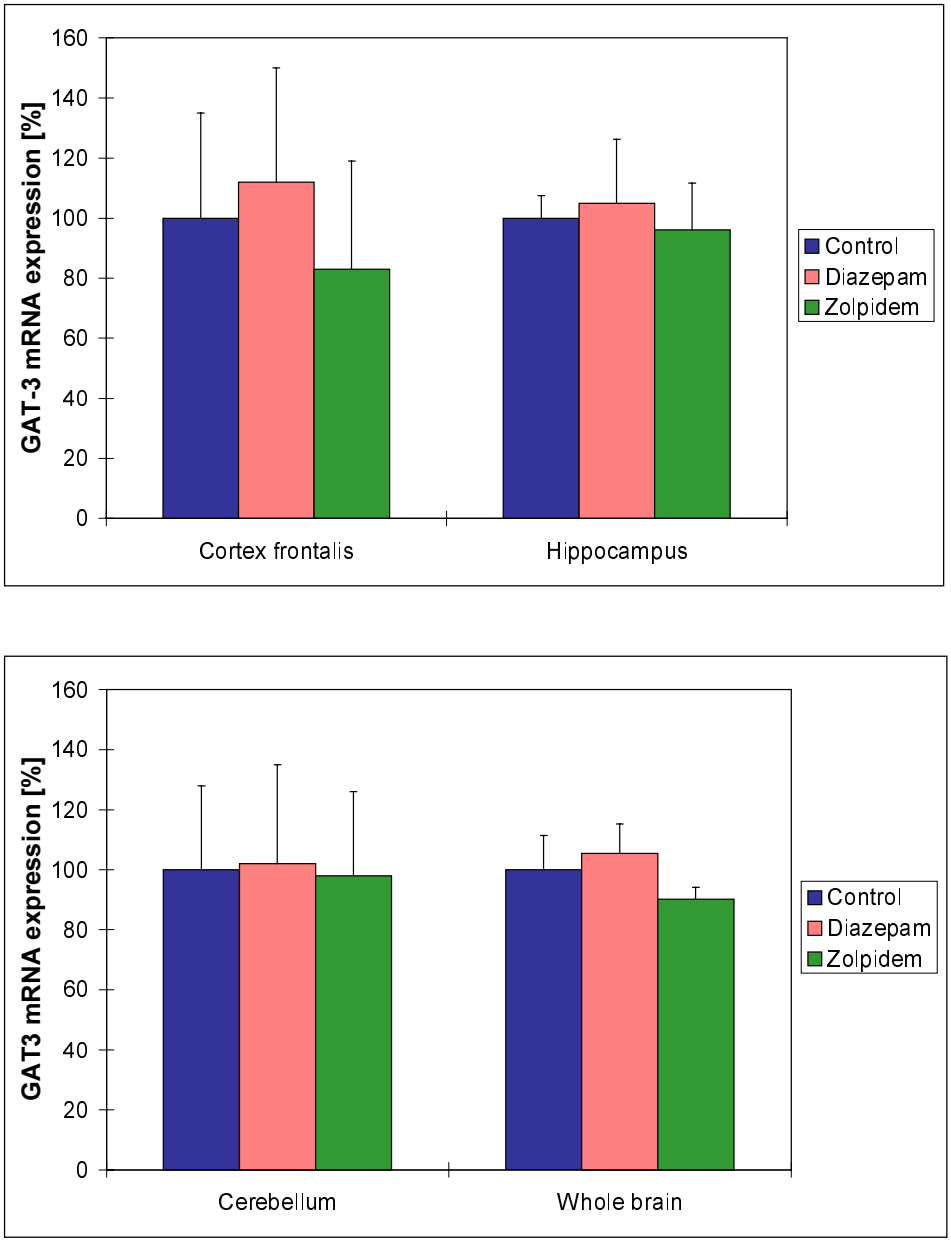

Figure 3.21

GAT-3 mRNA expression after chronic treatment with either diazepam or zolpidem: The GAT-3 mRNA was analysed by competitive RT-PCR. The mRNA levels of GAT-3 were measured in cortex frontalis, hippocampus, cerebellum and whole brain samples from control, diazepam- and zolpidem-treated rats. Each sample was examined in triplicate measurements. The mean values and the standard deviations from three animals are plotted.

3.2.3.3.2 Differential Display analyses

Another interesting question was whether the chronic administration of diazepam or zolpidem influences the expression of other genes than GABA transporters. To test this, whole brain samples were analysed by the means of differential display method (2.20). Three total RNA samples were analysed from each treatment group. Under the established conditions, the obtained band pattern was constant for tested sample in repeated experiments and samples from one treatment group gave the same band pattern (figure 3.22). In the assay, 36 primer combinations were tested (2.20.2). The tested range of primer combinations revealed no differential expression of any gene after 24 days of treatment with either diazepam or zolpidem.

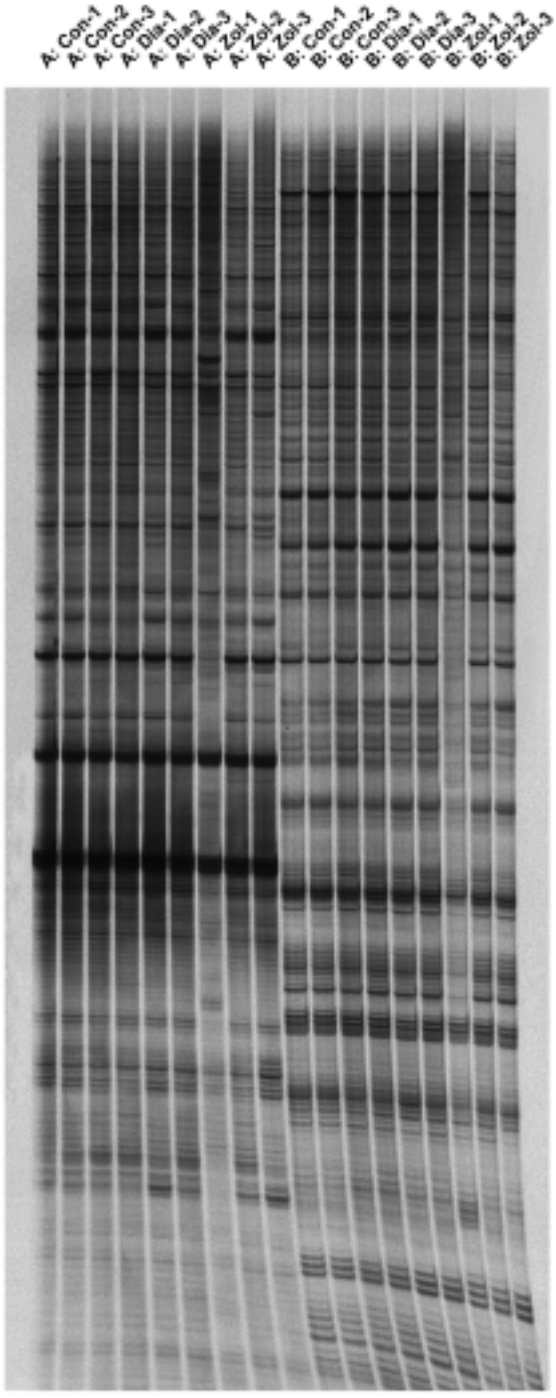

Figure 3.22

Differential display: An example gel of differential display autoradiogram. In the experiment, samples from control (**Con**), diazepam-treated (**Dia**) and zolpidem-treated (**Zol**) animals were compared. Total RNA isolated from whole brains was analysed with different primer combination. Shown is an example of D3/U2 (**A:**) and D4/U2 (**B:**) primer combinations. The numbers 1 - 3 represent samples from different animals of the same treatment group.

4 Discussion

The number of genes in the human genome is estimated at 50-100 thousand. However, only a fraction of these genes are expressed in any given cell. Moreover, the level of gene expression in cells may vary with time, physiological conditions and disease states. This differential gene expression is generally reflected by the different number of mRNA species expressed in a given cell at any time point, and changes in relative mRNA levels may have important implications in the development of the pathological processes. Therefore, discovery of differentially expressed genes is essential for the understanding of the molecular mechanisms involved in normal and pathological states, as well as providing new insights for discovery of new molecular targets for pharmacological manipulation and drug development. Hence, a number of techniques have been developed to identify genes that are differentially expressed in disease states. For example, Northern blot hybridisation, RNase protection assay, quantitative RT-PCR has been successfully utilised to identify discordantly expressed known genes. Other techniques, such as differential hybridisation and substractive library screening, have been used successfully for the discovery of differentially expressed genes with known or unknown sequences. Although these two techniques have proved to be useful, they are technically difficult and labour intensive, and require large amounts of mRNA. In addition, a number of PCR-based methods to uncover differentially expressed genes have been developed and used for novel gene discovery, these include mRNA differential display (Liang and Pardee, 1992) and arbitrary primed PCR (Sokolov and Prockop, 1994).

In the present work the ribonuclease protection assay, competitive RT-PCR and mRNA differential display were used, whereas the first two were applied for quantification of specific mRNAs and the last one was used for the qualitative detection of the differential mRNA expression. The accuracy of these measurements was largely influenced by the quality of sample RNA, which is characterised by factors like: the integrity of RNA, the accuracy of photometric

sample RNA quantification and the absence of DNA contamination in the total RNA preparation.

4.1 RNA preparation

The integrity of the sample RNA was important for the preciseness of employed methods. Because of this, a great attention was paid to isolation and purification of RNAs. In order to minimise the activity of RNases, RNAs were isolated into a solution of strong RNase inhibitor guanidium-thiocyanate (Chomczynski and Sacchi, 1987) and all necessary solutions and instruments were chilled on ice prior to use. Moreover, a phenol/chloroform extraction step was added at the end of the RNA isolation procedure to remove RNases.

For the preparation of total RNA, several protocols have been published for different sources (Boom et al., 1990; Mulder et al., 1994; Piatak et al., 1993). In addition, several RNA isolation kits are commercially available.

The next factor influencing the measurements was the photometric determination of RNA concentration. The accurate concentration determination of different sample RNAs is fundamental to the applied assays. Therefore, it is important to use samples of RNA with the highest possible purity. In order to control for purity of RNAs, absorbance at 240, 260 and 280 nm were measured and ratios calculated of the absorbance at 260 nm to those at 240 and 280 nm (R260/240 and R260/280, respectively). To minimise the variability of detected values it is necessary to use RNAs with similar OD ratios. To reach this, if possible, all RNA samples to be compared were prepared simultaneously. For the experiments, RNAs were used with ratios (OD_{260}/OD_{280})=1.8 to 2.0 and ratios (OD_{260}/OD_{240})= 1.6 - 1.8. Using both absorbance ratios increases the validity of RNA purity characterisation by spectrophotometry (Glasel, 1995). Generally, photometric RNA quantification might be the source of error most difficult to eliminate (Wilfinger et al., 1997).

Another important factor influencing the purity of the RNA is contamination with DNA. Even a small amount of DNA in the sample influences the reliability of the RNA detection and quantification in all three methods. It is important especially in

those assays that involve PCR (competitive RT-PCR, mRNA differential display) since each DNA fragment, which is homologous to the target RNA can be co-amplified and so misrepresent the results of the experiment.

In the present work, the isolated total RNAs were treated with RNase-free DNase I in order to remove the DNA contamination. This essential reaction was carried out in the presence of $MnCl_2$, since $MnCl_2$ has been reported to increase DNase I activity to a larger extent than the conventionally used $MgCl_2$ (Melgar and Goldthwait, 1968). Moreover, the mechanism of DNA cleavage by DNase I largely depends on the metal cofactor used in the reaction. In the presence of magnesium, DNase I cleaves each DNA strand independently, and the cleavage sites are distributed in a statistically random manner (Sambrook et al., 1989). In the presence of manganese, DNase I cleaves both DNA strands at approximately the same site, creating fragments of DNA that are blunt-ended or have protruding termini of one or two nucleotides. Bauer and colleagues have shown that DNase I treatment of RNA samples is more efficient in the presence of manganese than magnesium (Bauer et al., 1997). The benefit of the manganese in DNase I cleavage is probably based on both altered cleavage mechanism and enhanced activity of the enzyme, creating shorter fragments with a reduced likelihood for reassociation.

The DNA contamination causes in competitive RT-PCR and RPA shifts in the determined amount of the target mRNA. In mRNA differential display, presence of DNA results in amplification of PCR products that can be misinterpreted as products of regulated transcripts. To minimise the number of false positive results in differential display, it is recommended to analyse more samples from the same treatment group simultaneously. In competitive RT-PCR, the problem with DNA contamination can be avoided by using primers, which recognise sequences in two different exons. The amplification from the contaminating DNA either is inefficient, because of the large size of the intron between the two recognised exons or it gives rise to a product which, because of the intron, is larger than the target and can be easily distinguished from it. It is also possible to use one junction primer, which recognises sequence that contains parts from two exons and so allows only selective amplification of spliced transcripts. However, there is not always the possibility of this choice for the target sequence design since the

gene of interest is intronless or for the target gene only the cDNA sequence is known and there is no information available about the intron/exon boundaries. Because of this, a lot of attention was paid to purification of RNA and keeping it free from DNA contamination.
Also, the RNA probes (RPA) and RNA competitors (RT-PCR) had to be free from DNA contamination. Because of this, they were purified, in addition to DNase treatment, by separation in polyacrylamide gels.

4.2 RNase Protection Assay (RPA)

RPA was used for quantification of GAT-1 mRNA in kindled animals. The established assay was able to detect mRNA for GAT-1, however, 5-10 µg of total RNA were necessary for each assay. Consumption of RNA in RPA was similar to that of a Northern blot. Because of this, the competitive RT-PCR was used in all other experiments, in which only small amounts of total RNA were available, for detection of GABA transporters in various rat brain regions. Unlike the sensitivity, the fidelity of the RPA was comparable to that of competitive RT-PCR with respect to their intra- and inter-assay variability.
RPA method employs so called "in solution hybridisation" since the hybridisation step is carried out in reaction tube after direct addition of the labelled probe to the sample total RNA. Using solution hybridisation methods, low abundance mRNAs can be detected more readily and quantified more accurate than using hybridisation protocols that rely on RNA bound to a solid support, for example, Northern blots and dot blots (Lee and Costlow, 1987). Another advantage of RPA, compared to Northern blot, is that the target mRNA does not have to be completely intact, since the probes used in nuclease protection assays usually span only a subregion of several hundred base-pairs of the target transcript. Breaks that occur outside the hybridising region have no effect on nuclease protection results, since the probe rather than the target is size-selected. However, this means that the probe must be uniform in length. Therefore, unlike for Northern blot, the gel purification of probes is frequently necessary for nuclease protection

assays. The RPA is more sensitive than Northern blot, however its sensitivity is also limited. Some transcripts could not be detected with RPA and were detected only with RT-PCR (Lee et al., 1996).

The RPA, together with the S1 protection assay, ranks among group of nuclease protection assays. RPA has been utilised to study various aspects of RNA and DNA structure. It has been used for detection and quantification of specific RNA species in complex mixture of total cellular RNA. Because of the high resolution of the polyacrylamide gel system used to analyse the protected probe, nuclease protection assays are also well suited for mapping positions of external and internal junctions, e.g. transcription initiation (Schimada et al, 1987) and termination sites (Yang and Melera, 1992) and intron/exon boundaries (Berk and Sharp, 1977; Calzone et al., 1987). Both S1 nuclease and RNase are able to cleave hybridised probes at looped out single stranded regions resulting from deletions in the target RNA; in addition, RNase A is capable of cleavage at base-pair mismatches that result from point mutations (Myers et al., 1985). Because of these features, RPA can be also used to discriminate between closely related targets, for example, members of multigene families.

A number of innovations have made nuclease protection assays faster and more convenient. Commercial kits are available that contain pre-optimised reagents (Ambion, Promega, Boehringer Mannheim). Some of these products have streamlined the procedure by facilitating the direct analysis of mRNA in cell or tissue lysates, without prior purification of the RNA fraction (Ambion Direct Protect, USB Lysate RNase protection kit) or by increasing the hybridisation rate between probe and target by several orders of magnitude (Ambion HybSpeed). Procedures have also been described for nonisotopic RNase protection assays (Turnbow and Garner, 1993; Wundrack and Dooley, 1992).

4.3 Competitive RT-PCR

The RT-PCR is one of the most sensitive methods available for detection of specific transcripts. It has been used for detection and/or quantification of either very rare transcripts (Lee et al., 1996; Garcia de Viedma et al., 1999), transcripts from very small tissue samples, e.g. biopsies (Slade et al., 1999; Bulun et al., 1996; Mularoni et al., 1996) or even in a single cell (Comer et al., 2000). There are a number of different protocols available for quantification by means of competitive RT-PCR. Different approaches vary in a number of factors involved in the assay. This includes the kind of the competitor, the sequence and the size of the competitor, the selection of enzymes used in the reverse transcription reaction and in the PCR.

4.3.1 The kind of the competitor

In the submitted work, RNA competitors were employed as internal standard for quantification of GAT-1 and GAT-3 mRNAs in the rat brain.

Both, DNA and RNA molecules can be used as competitors. The RNA competitors are the most similar to the target of the assay - mRNA. They are added at the beginning of the assay and they mimic and compete with mRNA in all steps of the assay. On the other hand, the use of RNA competitors is more labour and time consuming than DNA competitors and strict precautions have to be made in order to avoid any contamination with DNA or RNases. The use of DNA rather than RNA as competitor has been advocated to avoid or minimise the hassles of generating and handling unstable RNA fragments. Both, double stranded (Gilliland et al., 1990) and antisense single stranded (deKant et al., 1994) DNA fragments have been used as competitor. However, with these variants, there is no control of the efficiency of the reverse transcription step. Another disadvantage is that the estimation of cDNA/competitor ratio does not allow the calculation of an “absolute“ number of mRNA molecules in the investigated sample.

4.3.2 Sequence and size of the competitor

In the present study, two kinds of competitors were employed for mRNA quantification by means of RT-PCR. First, the GAT1-mut competitor was used that carried sequence homologous to the amplified GAT-1 cDNA fragment with addition of a small insertion behind the forward primer sequence. Placing the insertion next to the primer sequence should inhibit unwanted heteroduplex formation during the PCR (Piatak et al., 1996a). This strategy was used for GAT-1 quantification in brain of kindled animals. The heteroduplex formation was diminished in comparison to obligatory amplification of similar sequences, e.g. analysis of VNTR polymorphism. Despite of the decrease in the heteroduplex formation, the amplification using the GAT1-mut competitor was not free from heteroduppleces. Because of this, competitors carrying β-actin derived sequences (GAT1-βac and GAT3-βac) were used in all other experiments.

To date, different strategies have been advocated for constructing the DNA template for the generation of competitor RNA. To ensure the fidelity of the assay, target RNA and competitor RNA should be reverse transcribed and amplified with equal efficiencies. To eliminate potential differences in the reaction efficiencies the target and the RNA competitor should be amplified with the same set of primers and their sequences should be as similar as possible. Next to sequence differences, significant size difference between target RNA and competitor RNA may also cause inequalities in amplification efficiency. The longer the competitor, the less efficient is its amplification (McCulloch et al., 1995).

Different protocols for the construction of internal standards have been published. To ensure the equal amplification efficiency, templates identical to the target sequence except of small insertion or deletion have been used most often as internal control (Gilliland et al., 1990; Grassi et al., 1994; Mularoni et al., 1996). On the other hand, the use of almost identical sequences can also negatively influence the quantification. The disadvantage of this approach is the possibility of heteroduplex formation. Heteroduppleces are relatively stable formations and are not dissolved during the obligatory used native gel electrophoresis. Heteroduplex formation influences the fidelity of the obtained data. Heteroduppleces occur preferentially at higher amplification product concentrations when the reaction approached the plateau phase (McCulloch et al., 1995) and the relative amount of

heteroduplexes increases with cycle number (Schneeberger et al., 1995). To avoid this problem, one can quantify the amount of the product in the early phase of PCR before the heteroduplexes begin to form. However, this requires sensitive detection systems such as radionucleotide labelling, which somehow defeats the purpose of performing competitive RT-PCR. One of the reasons this approach is so widely used is that, if both amplicons amplify with the same efficiency, the PCR can be extended in the later, nonexponential phase of the reaction and thus detected by simple systems such as gel electrophoresis analysis and ethidium bromide detection. It has been also reported, that electrophoresis conditions may be modified in order to minimise heteroduplex formation (Boer and Ramamoorthy, 1997). Furthermore, placing the mutation (insertion or deletion) in the vicinity of one priming site can be preferable to placing it near to the centre of the competitor sequence. The mismatch at the end of the sequence destabilises heteroduplex formation (Piatak et al., 1996a).

To avoid the problem with heteroduplex formation, a completely unrelated sequence, native or engineered, may be used for construction of the competitor (Siebert and Larrick, 1993; Wang et al., 1989). The chosen sequence has to be flanked with the same primer sequences as the target fragment and has to be similar in size and CG content in order to be amplified with the same efficiency as the target. This strategy was used in the present work using GAT1-βac and GAT3-βac competitors.

Another way to circumvent heteroduplex formation is to employ a sequence identical to the target sequence except of some point mutations that create or destroy endonuclease recognition site within the sequence (PATTY assay). Target and competitor are identical in size and almost identical in sequence, therefore, they are amplified with the same efficiencies. The products can be then easily distinguished by gel electrophoresis following specific digestion (Becker-Andrè and Hahlbrock, 1989; McCulloch et al., 1995). The disadvantage of the method is an additional enzymatic reaction (restriction) necessary for the product resolution, which increases the labour and financial costs of the assay.

4.3.3 Integrity and amount of competitor in the assay

The RNA competitor is used in minute concentrations and the RNA solutions are susceptible to degradation. Because of this, degrading of competitor RNA should be suspected when consecutive assays lead to increasing RNA values. RNA samples can be stored in RNase free water at -70 °C. Thaw-freeze cycles should be avoided (Piatak et al., 1996b). For stock solutions and long term storage the RNA can be dissolved in stabilised formamide solution or the RNA can be co-precipitated in defined ratios with carrier RNA (e.g. yeast transfer RNA) in ethanol and stored at -70 °C (Chomczynski, 1992; Ferré et al., 1996).

If no quantitative data are available on the amount of mRNA present in the tissue it is necessary to test a range of different RNA competitor amounts. In the present work, the range from 10^2 to 10^{10} molecules of competitor RNA was tested in a reaction that contained 100 ng of total RNA. Once the titration equivalence point of the reaction was estimated, a second set of reactions could be performed for the actual measurements. It is recommended to use at least five different competitor RNA concentrations. This multipoint titration effectively provides the equivalent of multiple confirmatory reactions for each sample, minimising the potential effects of aberrant reactions on accurate quantification and so decreasing the assay variability. Moreover, the detection of the titration equivalence point becomes more precise with smaller differences between the defined measurement points. The closer to each other the measurement points are the more precise is the quantification, e.g. the measurement with five dilution points covering a range of one order of magnitude is more precise than the measurement using five points covering five orders of magnitude.

4.3.4 Reverse transcription and PCR conditions

The murine moloney leukemia virus reverse transcriptase (MMLV-RT) was chosen over avian myeloblastosis virus reverse transcriptase (AMV-RT) because of its lower RNase H activity. The higher RNase H activity may lead to the cleavage of RNA in the RNA/DNA hybrids near the 3`end of the growing cDNA strand, resulting in uncomplete cDNA synthesis.

However, due to the lower temperature optimum of MMLV-RT as compared to AMV-RT (42°C versus 50°C) the efficiency of cDNA synthesis may be decreased in regions with a high CG content and thus stable secondary structures (Kotewitz et al., 1988). Therefore, a denaturation step (5 min) was included prior to the reaction. Moreover, to prime the cDNA synthesis, not the original antisense PCR primer (GAT3-rv) was used but the 3`-shortened GAT3-RT primer that has a melting temperature close to the temperature optimum of MMLV-RT. Using the short primers, which have melting temperature close to the temperature optimum of the synthesising enzyme, increases the specificity of the reverse transcription (Pfeffer et al., 1995).

In order to improve the specificity of the assay PCR, it is possible to use so- called hot start PCR. In this modification the DNA polymerase has access to the reactants only when the PCR mix has heated up and unspecific priming is prevented. Several variants of this method have been described including the manual addition of polymerase to the hot reaction mix (Mullis, 1991) and the use of wax separating the enzyme and the reaction mix until melting (Chou et al., 1992). Also, a recombinant DNA polymerase has been developed that requires 9-12 min of activation at 95°C (AmpliTaq Gold™, Perkin Elmer). A similar approach is followed in so called touchdown PCR. The strategy of touchdown PCR involves starting the cycling with a very high annealing temperature and then lowering the annealing temperature with successive cycles, so increasing the specificity of the reaction.

Under certain buffer and temperature conditions, RT-PCR can be performed in one tube rather than in two separate steps. In this coupled reaction, reverse transcriptase and Taq DNA polymerase are present in the reaction mix from the beginning of the assay (Aatsinki et al., 1994; Mallet et al., 1995; Wang et al., 1992). There are a number of kits employing this approach e.g. GeneAmp® RNA PCR kit from Perkin Elmer with MMLV-RT+Taq polymerase or Titan™ One tube RT-PCR system from Boehringer Mannheim with AMV-RT +Taq polymerase. Another possibility to perform one tube RT-PCR is to use *Thermus thermophilus* (Tth) DNA polymerase that possesses RNA/DNA reverse transcriptase or

DNA/DNA polymerase activity in presence of Mn^{2+} or Mg^{2+}, respectively. Since this enzyme is thermostable and thermoactive, reverse transcription can be performed at elevated temperatures (Mulder et al., 1994; Myers and Gelfand, 1991) (e.g. GeneAmp® RNA PCR kit from Perkin Elmer with Tth DNA polymerase). The disadvantage of these one-tube methods is that amplification products might be obtained more easily and specifically in two independently optimised reaction steps.

For detection and analysis of amplified PCR products a variety of procedures are available (reviewed in Ferré et al., 1996; Zimmermann and Mannhater, 1996). PCR products are typically separated by gel electrophoresis, as in the present work, by capillary electrophoresis or by HPLC. The detection can be most easily achieved by direct staining with dyes like ethidium bromide. Alternatives include radioactive, fluorescent or antigenic labelling of either nucleotides or primers. It has been reported that the sensitivity of the gel electrophoresis detection can be greatly enhanced (10-100-fold) using the SYBR Green 1 dye instead of the ethidium bromide (Schneeberger et al., 1995). If high background cannot be reduced otherwise, PCR products may be gel-separated, blotted onto membranes and detected after hybridisation with specific probes.

4.4 mRNA Differential Display (DD)

Differential display was applied in the present study to the analysis of the gene expression after long-term diazepam and zolpidem treatment. Thirty-six primer combinations were tested. The analysis did not reveal any differentially expressed transcripts in the tested samples.

Messenger-RNA differential display has been developed as a tool to detect and characterise altered gene expression in eukaryotic cells. It has been developed for gene expression detection in cancer research; however, it has been applied in many other fields of biology and medicine including neuroscience (Fischer et al., 1998).

Compared with the conventional methods for the discovery of genes with altered expression in disease states, such as differential hybridisation and substractive

library screening, the differential display technique has several advantages. The major strengths of DD are its simplicity and ease of use. It should be possible to amplify most cDNAs from a given cell type by using sufficient primer combinations, and to compare those with cDNAs from many other samples. More importantly, all of this is done using a relatively small amount of total RNA from each of the tissues being studied: the amount necessary to amplify all of the mRNAs in a given cell type is less than 5 µg, whereas, using standard methods to construct a representative cDNA library from the same tissue would require 100-500 µg total RNA. The nature of the method allows a large number of comparisons and rapid isolation of fragments of genes of interest. The strengths of the technique include multiple comparisons between several tissues at the same time, versatility in the detection of both up- and down-regulated genes and high sensitivity due to PCR amplification. In addition, the time required for the given study is relatively short when compared to substraction library construction. However, there are some disadvantages in using differential display technique. The major concerns are the high incidence of false positives and the labour intensive nature of this procedure for large-scale screening. In addition, the cDNA fragments isolated by this method are typically small, and frequently located in the 3´-untranslated region. Therefore, in order to identify the differentially expressed gene, one may need to screen a cDNA library to isolate the full-length cDNA clone. Finally, there is a number of questions considering the sensitivity of the method and the preference in detection of differentially expressed genes. The first consideration is whether the method is able to uncover large changes in transcription of genes that are normally relatively abundant. It has been suggested that the method is bias toward cloning of low abundance genes (Guimaraes, 1995). A number of genes, which are normally expressed in low levels and whose transcription is increased by from 50% to several hundred per cent have been identified (Livesey and Hunt, 1996). The second consideration is whether the technique detects only on/off and similar changes in transcription or whether it is able to reveal the changes smaller than 50%. On the other hand, significant improvements and modifications have been made to the method as originally described (Liang and Pardee, 1992) in order to overcome some of the existing problems in this technique. For example, emphasis has been placed

on the importance of DNA-free RNA samples and multiple displays of samples; this reduces the frequency of false positives (Liang et al., 1993), longer primers are used, e.g. 18-20 -mers, in so called enhanced differential display (EDD) (Linskens et al., 1995), this not only increases the reproducibility of DD, but also allows direct sequencing after PCR amplification (Reeves et al., 1995), the potential hazardous nature of ^{35}S as a radiolabel for DD has been noted and either ^{32}P or ^{33}P have been recommended as alternative labels (Trentmann et al., 1995; Tokuyama and Takeda, 1995), use of only three downstream primers with only one anchor base has been introduced (Liang et al., 1994).

In addition, other strategies aimed at discovering novel genes emerged, such as methodology of serial gene expression (SAGE) (Velculescu et al., 1995) and representational difference analysis (RDA) (Lisitsyn et al., 1993). Moreover, the micro-array technique became recently available for high throughput analysis of differentially expressed genes. The application of these techniques will facilitate the discovery of novel therapeutic targets and will help to understand the molecular mechanisms of disease. However, more other steps characterising the functions of the particular gene of interest are necessary for functional study and target validation for the importance of this gene in disease processes.

4.5 Basal mRNA expression profile of GAT-1 and GAT-3

The present work concentrated on the study of CNS specific transporters GAT-1 and GAT-3. One of the aims of the present study was to analyse the distribution pattern of these transporters in the rat brain. The levels of appropriate mRNAs were detected in cortex frontalis, striatum, thalamus, hypothalamus, hippocampus, cortex occipitalis, medulla oblongata and cerebellum by means of competitive RT-PCR. The detected expression of GAT-1 was much higher than that of GAT-3. This is in harmony with the hypothesis that GAT-1 is the major GABA transporter in mammalian brain (Smith et al., 1996). GAT-1 mRNA was detected in all brain regions examined and its expression levels were similar in all regions. These data are consistent with *in situ* hybridisation (Durkin et al., 1995) and immunoblot

studies (Ikegaki et al., 1994, Saito and Tanaka, 1996) on rat brain. The GAT-3 mRNA could be also detected in all brain regions tested. However, GAT-3 mRNA seemed to be more region specific. The strongest GAT-3 expression was detected in striatum, medulla oblongata and thalamus. Very low levels of GAT-3 mRNA were detected in cortex frontalis and cerebellum. This regional distribution fits to expression pattern obtained from *in situ* hybridisation and Western blot analyses (Durkin at al., 1995, Ikegaki et al., 1994, Saito and Tanaka, 1996).

The distribution of GAT-1 mRNA corresponds closely to that of GABAergic neurones (Rattray and Priestley, 1993; Borden, 1996) as determined by the localisation of GABA and its synthesis enzyme GAD. Further, the vast majority of cells positive for GAT-1 mRNA have the morphological appearance of neurones (Borden, 1996). Taken together, these findings suggest that GAT-1 is expressed by GABAergic neurones and are thus consistent with presynaptic mode of action. In contrast, there are certain regions of the brain in which there is an apparent lack of concordance between GAT-1 and GAD localisation (Durkin et al., 1995; Snow et al., 1992; Swan et al., 1994). In these regions, for example cortex, subthalamic nucleus or ventral horn of the spinal cord, the neurones do not synthesise GABA; however, they receive GABAergic innervation, suggesting that GAT-1 in these regions may function postsynaptically. Moreover, GAT-1 mRNA has been observed also in glial cells in the retina (Brecha and Weigmann, 1994), in the cerebellum (Rattray and Priestley, 1993), in addition, GAT-1 immunoreactivity has been observed in glia in the cerebellum, hippocampus and substantia nigra (Radian et al., 1990).

GAT-3 mRNA is present in many brain areas. Examination of its mRNA distribution by in situ hybridisation revealed two different patterns (Durkin et al., 1995). In some brain areas the signal was observed in neurones, however, in other areas of the brain GAT-3 mRNA label was detected in astrocytes. Additional studies support the hypothesis that GAT-3 is present in both neurones and astrocytes (Itouji et al., 1996; Borden, 1996).

Taken together, GAT-1 is predominantly located on presynaptic neurones, however it may be also expressed in postsynaptic neurones and glial cells. GAT-3 is also expressed in both neurones and glia. Expression of GAT-1 and GAT-3 is restricted to CNS and they are expressed in neurones. Because of this,

alternations in expression or activity of GAT-1 and GAT-3 may be involved in the modulation of GABAergic transmission in different pathophysiological states. In the present work, two hypotheses were tested, whether GAT-1 and/or GAT-3 mRNA levels rat brain are altered in kindling model of epilepsy or after long-term treatment with diazepam or zolpidem, drugs modulating GABAergic transmission.

4.6 GAT-1 and GAT-3 in animal model of epilepsy

The epilepsies constitute a family of disorders characterised by spontaneous disturbances in the normal electrical activity of the brain associated with changes in behaviour (seizures). Temporal lobe epilepsy (TLE) is the most common form of this disease (40% of epilepsies in adult). During and colleagues (During et al., 1995) proposed that nonvesicular GABA release mediated by reversal of GABA transporter may be important for suppressing seizure activity. This hypothesis is supported by observation of decreased glutamate-induced release of GABA due to the reversal functioning of GABA transporter and also decreased nipecotic acid binding, a GABA transporter inhibitor, in animal model of TLE.

Whether the observed decrease in binding of nipecotic acid by GABA transporters in kindled animals is due to an altered expression of GABA transporters was tested in the present work. The mRNA levels of GABA transporters GAT-1 and GAT-3, the only transporters specifically expressed in the CNS, were measured in hippocampus and whole brain preparations from kindled animals. Small but not significant decrease in GAT-1 mRNA was detected in kindled animals by competitive RT-PCR in both regions tested. No significant change was detected in mRNA levels of GAT-3 transporter in any region tested. These results are consistent with the findings of Hernandez and co-workers (Hernandez et al., 1995). They measured amino acid transporter levels in the brain of electrically kindled animals. Immunocytochemical examination was used for detection of the cellular distribution of the GABA and glutamate transporters. Only an increase of the glial type of the glutamate transporter (GLT-1) was detected. No significant

change has been observed after kindling in protein levels of other glutamate and in any of the GABA transporters.

These data suggest that the observed diminished activity of GABA transporter in hippocampus of kindled rats is not due to the decreased expression of GAT-1 and GAT-3 genes. GABA transporter activity is also regulated posttranslationally. To date, however, there is no information about the regulation of GAT-3 activity. In the case of GAT-1, the modulation of activity by protein kinase C (PKC) has been reported (Sato et al., 1995) for rat GAT-1 expressed in *Xenopus* oocytes. Modulation of uptake by PKC has been reported for a number of transporters (Sato et al., 1995b; Corey et al., 1994; Casado et al., 1993; Cushman and Wardzala, 1980). Stimulation of PKC exerts its effects by changing the number of functional transporters expressed on the plasma membrane (Qian et al., 1997; Quick et al., 1997; Davis et al., 1998).

In addition to activity of GABA transporters, also changes in GAD and $GABA_A$ receptors in animal models of epilepsy have been observed. Decrease in GABAergic transmission occurs primarily in epileptogenic hippocampus. A recovery can be observed after some weeks (Franck et al., 1988). These findings are supplemented by neurochemical studies showing an initial decrease in glutamate decarboxylase activity in hippocampus of kindled animals (Sperk et al., 1983). This decrease in activity of the key enzyme is probably due to the loss of a subpopulation of GABAergic neurones after the seizures (Lehmann et al., 1998; Sperk et al., 1992; Obenaus et al., 1993). At later intervals, however, increased activity of GAD in hippocampus and cortex (Esclapez and Houser, 1999; Davenport et al., 1990; Marksteiner et al., 1988) and increased expression of the respective mRNAs (Schwarzer and Sperk, 1995; Feldblum et al., 1990) indicate augmented GABAergic activity in surviving neurones. Studies on $GABA_A$ receptors observed an increased $GABA_A$ receptor binding in the hippocampus of electrically and chemically kindled animals (Fritschy et al., 1999; Titualer et al., 1995). Moreover, an increase in number of synaptic $GABA_A$ receptors has been detected in hippocampus of electrically kindled rats (Nusser et al., 1998). Also changes in expression of distinct $GABA_A$ receptor subunits have been reported for rat hippocampus after chemical kindling (Schwarzer et al., 1997; Kamphius et al., 1995 Clark et al., 1994).

These data suggest that in spite of some loss of GABA neurones within hippocampus, surviving GABA neurones may be capable to enhance the synthesis of transmitter and also recover the $GABA_A$ receptor function by increasing the transcription of appropriate genes. These changes in gene expression together with the decrease in the activity of GABA transporters may imply potent compensatory mechanisms against the increased neuronal activity in the chronic epileptic state.

4.7 GAT-1 and GAT-3 after chronic treatment with diazepam or zolpidem

4.7.1 Treatment

In the present study, the oral administration of the drugs was used. The drugs were administered via the drinking water. The advantage of this method is the stress-free application of the drug. With respect to the fast rodent metabolism, the daily dose of diazepam of 10 mg/kg was used which is 36 times the mg/kg anxiolytic dose for diazepam in humans (Hutchinson et al., 1996) and approximately 5 times the acute (injection) anxiolytic dose in rats (Schmitt and Hiemke, 1998). Another advantage of the drug administration via drinking water is the division of the daily dose in several smaller doses. The rats do not drink only once but several times during their active period and so divide their daily water consumption in several parts (Spanagel et al., 1996). This increased number of drinking sessions divides the daily drug dose, which is sedative when administered acutely (Matsubara and Matsushita, 1982), in several smaller doses. This way of administration simulated the oral administration in humans with several application time-points over the day.

The amount of consumed water was controlled every day and the body weight of rats every third day. There was no significant difference between the treatment groups. Moreover, the obtained water consumption and body weight data are in accordance with the data provided by Harlan-Winkelmann Company for the rats of

PVG strain. These data show that there was no deprivation effect caused by the drug treatment.

In many animal studies, injections of drug (intraperitoneal, subcutan) or silastic implants have been used for the treatment. The route of administration is also different from the human clinical use of benzodiazepines. Humans usually take benzodiazepines orally, leading to significant first-pass metabolism by the liver. This means that active metabolites can have a significant impact on the behavioural effects of the drug. Rodents, the most frequently used test animals, metabolise benzodiazepines very rapidly compared to humans (Klotz et al., 1976; Greenblatt et al., 1986; Friedman et al., 1986). Diazepam has a mean elimination half-life of 32.9 hr in humans and 1.1 hr in rats (Klotz et al., 1976). Because of this, the drug doses much higher than in humans have to be used in tests with rodents. One way of overcoming the rapid rodent metabolism is to employ divided daily intraperitoneal injections or oral administration (Smith and Darlington, 1994). Dividing the daily dose results in smaller fluctuations in benzodiazepine plasma levels, and longer periods of $GABA_A$ receptor occupation, than a single bolus dose.

4.7.2 Behavioural analysis

For the analysis of behavioural effects, the open field and elevated plus-maze tests were used. Both methods rank among ethologic test models. The advantage of these tests is the analysis of natural behaviour of animals and therefore, there is no need of learning and training period prior to the test. The disadvantage of these tests is habituation of the animals to the environment of the test arena and because of this, it is possible to test each animal only once in each arena. For the repeated testing of the animals, long time period has to be between two tests (Fernandes and File, 1996; File, 1995).

In both tests used, the basal activity of the animals was analysed and there was no significant difference observed between different treatment groups. In addition to basal activity, exploration behaviour was analysed in the open field and anxiety related behaviour was analysed in the elevated plus-maze. Unlike basal activity,

there were changes observed in exploration behaviour. Both zolpidem-treated groups showed significantly increased number of entries into the centre, which is analysed as an increased exploration of the arena. Diazepam treated animals did not show any change in this parameter. Furthermore, acutely and chronically diazepam-treated animals spent more time with moving than control group. However, despite of the increased movement time, diazepam-treated animals moved for comparable distance as controls. This increase in the time spent for movement which does not results in the increase of the distance can be explained by the increase of number of small movements (e.g. rears or sniffing) which do not result in extension of the path length. These movements also rang among exploration activities. Hence, the increase of "time moving" without prolongation of the path length is caused by increase in exploration activities.
In opposite to diazepam treatment, the zolpidem-treated animals spent less time with moving than controls, however, without altering the total "distance moved". Zolpidem-treated animals showed increased number of entries into the centre zone of the test arena. The centre zone is more lit than the border parts. The exploration of this zone by rats is characterised by careful entering and exploring the centre followed by fast leaving this zone back to the "safe" darker corner and border areas. Therefore, reaching the comparable values of "distance moved" by zolpidem-treated animals despite of the decrease in the time spent for moving compared to the controls can be explained by transient increase in speed of the movement.

In anxiety related parameters, no significant change compared to controls was detected after zolpidem treatment. From diazepam-treated animals, only acutely treated animals showed a decrease in anxiety-like behaviour. The chronically diazepam-treated animals did not show any change in the analysed parameters. This difference in effects suggests development of tolerance after chronic diazepam treatment. This hypothesis was confirmed by testing of double treated animals. In this group, the acute administration of diazepam was not able to decrease the anxiety-related parameters as in acutely treated animals. Therefore, the loss of anxiolytic effect of acute diazepam treatment following the chronic

diazepam treatment showed that tolerance developed to diazepam effects during the long-term administration.

Taken together, in both tests, the chronic treatment with neither diazepam nor zolpidem did affect the basal activity of animals. The obtained data show increase in the exploration activity. In each treatment group, there was always only one parameter changed significantly compared to the control. However, these data are in concordance with study, which showed differential influence of exploration activities of rats by diazepam and zolpidem (Schmitt and Hiemke, 1998). In the anxiety related parameters, zolpidem treatment did not cause changes neither after acute nor after chronic administration. The chronically diazepam-treated groups revealed no difference from controls in contrast to the acutely diazepam-treated group. The missing of the anxiolysis in chronically diazepam-treated animals confirms the development of tolerance to anxiolytic effect of diazepam during the chronic treatment.

Drug tolerance may lead to dose escalation and increased risk of physiological dependence. There are several studies analysing the tolerance-producing effects of zolpidem. The pre-clinical studies conducted with rodents suggested that tolerance does not develop to behavioural effects of zolpidem (Cox et a., 1988; Perrault et al., 1992; Sanger and Zivkovic, 1987; Sanger and Zivkovic, 1992). The results of a study conducted with non-human primates, baboons, suggested that repeated administrations of zolpidem produce tolerance. Several clinical trials that assessed the efficacy of zolpidem failed to find tolerance to its effects following repeated administration (Fairweather et al., 1992;Poirrier et al., 1994; Roger et al., 1993; Scharf et al., 1994; Schlich et al, 1991; Shaw et al., 1992). Only one study with humans suggested that tolerance develops to the effects of zolpidem (Ware et al., 1997).

There is a number of studies on animals about effects of repeated administration of benzodiazepines. There is a large body of evidence which documents the development of tolerance to muscle relaxant, locomotor activity and anticonvulsant effects of benzodiazepines within 2-3 weeks of continuous administration (reviewed in Hutchinson et al., 1996). Most of the studies agreed that tolerance to muscle relaxant effects develops relatively quickly (within 5-10

days) at a wide range of doses. Oral administration of diazepam in wide range of doses (2.5-20 mg/kg) caused tolerance in rat within 7 days (Matsubara and Matsushita, 1982). Benzodiazepines have sedative effects on locomotor activity. However, at lower doses with some benzodiazepines the acute administration causes increase in activity of the animals (Matsubara and Matsushita, 1982). The majority of studies testing the activity of animals have observed development of tolerance to effects influencing the activity caused by benzodiazepines following 2-14 days of treatment. Diazepam administrated acutely in a dose of 2.5 mg/kg increased the activity of the rats (Matsubara and Matsushita, 1982). The same dose administrated orally for 14 days did not develop tolerance to this effect (Matsubara and Matsushita, 1982). However, intraperitoneal injections of 2-5 mg/kg of diazepam caused within 3-14 days development of tolerance to its effect in increasing of exploration activity (File and Fernandes, 1994; Harro et al., 1990). Diazepam administered in a dose of 10 -20 mg/kg decreased the activity of the animals. Repeated oral treatment with 20 mg/kg of diazepam caused development of tolerance to the sedative effect of diazepam already after 2 days (Matsubara and Matsushita, 1982). The studies about development of tolerance to anxiolytic effects of benzodiazepines obtained different results. While a number of studies demonstrated that tolerance developed (Vellucci and File, 1979; Treit 1985; File et al., 1987; Davis and Gallager, 1988; Ishihara et al., 1993; Luscombe et al., 1994), there are also studies that failed to obtain the evidence of tolerance (Sansone, 1979; Harro et al., 1990; Hijzen et al., 1990). Diazepam administered to rats intraperitoneally in dose of 5 mg/kg did not cause tolerance after 5-21 days of treatment (Davis and Gallager, 1988; Harro et al., 1990). However, diazepam in dose of 20 mg/kg caused tolerance to anxiolytic effects after already 2 days (Davis and Gallager, 1988). Moreover, diazepam in dose of 5 mg/kg administered continuously via silastic capsules caused tolerance to its effects after 5 days in opposite of the same dose administered by intraperitoneal injections (Davis and Gallager, 1988). The discrepancy between these findings can be partly explained by the type of benzodiazepine employed, the dose used, and by route and duration of administration. The factors increasing the likelihood of anxiolytic tolerance are longer half-life of the drug, higher dose and continuous administration. These factors are consistent with the suggestion that $GABA_A$

receptor occupation for longer periods may be important for the development of tolerance (Davis and Gallager, 1988).

4.7.3. GAT-1 and GAT-3 mRNA expression

Prolonged administration of benzodiazepines in vivo is known to evoke tolerance and dependence, implying the existence of endogenous mechanisms regulating the interaction of such modulators with the $GABA_A$ receptors. The molecular mechanisms underlying the development of tolerance to BZ are not understood. In the present work, one aim was to test the hypothesis, whether changes in GABA transporter mRNA expression are accompanying the long-term BZ administration. The mRNA levels of GAT-1 and GAT-3 were detected after chronic diazepam or zolpidem treatment. Diazepam is a classical benzodiazepine and is known to cause tolerance when administered chronically, as it also was shown in the present work. Zolpidem is an imidazopyridine, which binds to the BZ binding site on $GABA_A$ receptors and is reported to have a little tolerance liability. It differs from diazepam also in its pharmacology, by binding with high affinity only to BZ binding site of α_1 subunit containing $GABA_A$ receptors. There are only two studies suggesting the tolerance potential of zolpidem. These two substances were compared with respect to their effects on GABA transporter mRNA production. The mRNA levels were analysed in cortex frontalis, hippocampus, cerebellum and whole brain samples from animals chronically treated with zolpidem and animals chronically treated with and also tolerant to diazepam (3.2.3.2.4). Neither chronic zolpidem nor chronic diazepam treatment caused significant changes in the mRNA expression of any GABA transporter in any region tested. Therefore, it is seemed unlikely that changes in expression of GABA transporters are involved in adaptation of the GABAergic system to the drug treatment and in development of tolerance to it.

However, the GAT-1 mRNA analysis showed similar expression pattern in all tested regions (Figure 3.20) with no change in GAT-1 mRNA level after zolpidem administration and increase in GAT-1 mRNA, which was, however, not significant,

after diazepam treatment. One can speculate that this increase in mRNA level may cause increase in protein production, which, furthermore, leads to increased activity of GAT-1. As observed in part of the present work describing kindling experiments, it is obvious that there in a posttranslational mechanism regulating the functionality of GAT-1 by activation of PKC. Increase in production of GAT-1 may be understood as a regulation mechanism working additionally to the posttranslational one. Therefore, small not significant increase in amount of GAT-1 may support the effect of the posttranslational regulation and so result in increased number of functional transporters and so the activity of the GAT-1 in the synaptic cleft. These would be in accordance with hypothesis that benzodiazepines strengthen the effect of GABA released into the synapse and the regulation mechanisms do in opposite of this effect by inhibiting the GABA. One of the possibilities is to increase the GAT-1 activity and so indemnify for faster removal of GABA from the synaptic cleft. Functional analysis of GAT-1 activity after long-term benzodiazepine treatment is necessary in order to test whether GAT-1 is involved in adaptation to and development of tolerance to benzodiazepines.

4.7.4 Differential expression of other genes

Moreover, in the submitted work, differential display technique was employed to analyse the changes in transcription of various genes after chronic diazepam or zolpidem treatment. However, this attempt did not reveal any differentially expressed transcripts. One of the reasons could be that changes in transcription occurred only in distinct brain region or they were in the range of 20% and so in both cases they would be under the detection threshold of the applied method.

To explore the regulation mechanisms controlling the GABAergic system, the effects of chronic exposure to benzodiazepines on $GABA_A$ receptors have been examined. There are several studies showing that long-term BZ administration is followed by decrease in BZ binding (Wu et al., 1994; 1995). However, one study failed to detect decrease of BZ binding after midazolam treatment (Ramsey-Williams et al., 1994). Several studies analysed mRNA

expression for different $GABA_A$ receptor subunits in rodent brain after long-term BZ treatment. Most of these studies agree with each other in a finding that expression of $\gamma 2$ mRNA is downregulated in cortex. Some of these findings include decrease of $\gamma 2$ in hippocampus (Zhao et al., 1994) and some of them excluded it (Primus and Gallager, 1992; Kang and Miller, 1991; Impagnatiello et al., 1996; Wu et al., 1994). The second target of interest was $\alpha 1$ subunit. Part of the studies detected decrease of $\alpha 1$ in cortex and hippocampus (Impagnatiello et al., 1996), some only in hippocampus (Wu et al., 1994), some only in cortex (Kang and Miller, 1991) and some of them detected no change in $\alpha 1$ mRNA levels after long-term BZ treatment (Zhao et al., 1994). To date, there is a general lack of agreement about the regulation of transcription of $\alpha 1$ and other $GABA_A$ receptor subunits. However, these results suggest that GABA regulates the expression of its homologous receptor population by regulation (reduction) in the expression of, at least, $\gamma 2$ receptor subunit. The mechanisms which underlie these effects are unknown, but may involve alternation in transcription rates or mRNA stability. To date, this is the leading theory about development of tolerance to BZ. It takes place by changing the subunit configuration of $GABA_A$ receptors that does not change the total amount of receptor complexes and so does not affect the GABA response. However, this swapping of subunits decreases the affinity of the receptor to BZ and so decreases the effect of BZ. The most obvious candidate for this regulation seemed to be the $\gamma 2$ subunit, which is necessary for the assembly of the BZ binding site on $GABA_A$ receptor complex. Decrease in $\gamma 2$ expression would explain decrease in BZ binding and following decrease in or loss of BZ effect after long-term administration. However, to date, decrease in expression of $\gamma 2$ subunit was readily detected only in the cortex. This local change is expression is probably not sufficient to explain changes in all complex anxiolytic, sedative, muscle-relaxant and anticonvulsant effects of BZ. Because of this, probably also other subunits and/or parts of GABAergic system are involved in regulation of its response and/or adaptation to altered conditions. To explore this, additional studies on this field are necessary.

5. Summary

The submitted work concentrated on the study of mRNA expression of two distinct GABA transporters, GAT-1 and GAT-3, in the rat brain. For the detection and quantification of the chosen mRNAs, appropriate methods had to be established. Two methods, ribonuclease protection assay (RPA) and competitive RT-PCR were emloyed in the present study. Competitive RT-PCR worked out to be 20 times more sensitive as RPA. Unlike the sensitivity, the fidelity of both techniques was comparable with respect to their intra- and inter-assay variability.

The basal mRNA levels of GAT-1 and GAT-3 were measured in various brain regions. Messenger RNAs for both transporters were detected in all tested brain regions. Depending on the region, the observed mRNA level for GAT-1 was 100-300 higher than for GAT-3. The GAT-1 mRNA levels were similar in all tested regions. The distribution of GAT-3 mRNA seemed to be more region specific. The strongest GAT-3 mRNA expression was detected in striatum, medulla oblongata and thalamus. The lowest levels of GAT-3 were in cortex frontalis and cerebellum.

Furthermore, the mRNA expression for GAT-1 and GAT-3 was analysed under altered physiological conditions; in kindling model of epilepsy and also after long-term treatment drugs modulating GABAergic transmission. In kindling model of epilepsy, altered GABA transporter function was hypothesised by During and coworkers (During et al., 1995) after observed decrease in binding of nipecotic acid, a GAT ligand, in hippocampus of kindled animals. In the present work, the mRNA levels were measured in hippocampus and whole brain samples. Neither GAT-1 nor GAT-3 showed altered transcription in any tested region of kindled animals compared to controls. This leads to conclusion that an altered functionality of GABA transporters is involved in epilepsy rather than a change in their expression.

The levels of GAT-1 and GAT-3 mRNAs were also measured in the brain of rats chronically treated with diazepam or zolpidem, $GABA_A$ receptor agonists. Prior to the molecular biology tests, behavioural analysis was carried out with chronically and acutely treated animals. In two tests, open field and elevated plus-maze, the basal activity exploration and anxiety-like behaviour were analysed. Zolpidem treatment increased exploratory activity. There were observed no differencies

between chronically and acutely treated animals. Diazepam increased exploratory activity and decresed anxiety-like behaviour when applied acutely. This effect disappeard after chronic administration of diazepam. The loss of effect suggested a development of tolerance to effects of diazepam following long-term administration. Double treatment, acute injection of diazepam after chronic diazepam treatment, confirmed development of a tolerance to effects of diazepam. Also, the mRNAs for GAT-1 and GAT-3 were analysed in cortex frontalis, hippocampus, cerebellum and whole brain samples of chronically treated animals. The mRNA levels for any of tested GABA transporters did not show significant changes in any of tested region neither after diazepam nor zolpidem treatment. Therefore, changes in GAT-1 and GAT-3 transcription are probably not involved in adaptation of GABAergic system to long-term benzodiazepine administration and so in development of tolerance to benzodiazepines.

6. Abbreviations

5-HT	5-Hydroxytryptamine
AMV	Avian Myeloblastosis Virus
BGT	Betaine GABA Transporter
BSA	Bovine Serum Albumin
BZ	Benzodiazepine
CACA	Cis-4-Aminocrotonic Acid
cAMP	Cyclic Adenoside-Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
DD	Differential Display
DEPC	Diethyl Pyrocarbonate
DI	Diazepam-Insensitive
DMCM	Methy-6,7-Dimetoxy-4-Ethyl-β-Carboline
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside 5`-Triphosphate
DS	Diazepam-Sensitive
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EtBr	Ethidium Bromide
GABA	γ-Aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GAT	GABA Transporter
HPLC	High Pressure Liquid Chromatography
MMLV	Murine Moloney Leukemia Virus
MMLV	Murine Moloney Leukemia Virus
mRNA	Messenger Ribonucleic Acid
nAchR	Nicotinic Acethylcholine Receptor
NTP	Nucleoside 5`-Triphosphate
PA	Polyacrylamide
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PTZ	1,5-Pentamethylentetrazole
QN^+	Hexadecyl-trimethyl Ammonium Bromide
RNA	Ribonucleic Acid
RPA	Ribonuclease Protection Assay
RT-PCR	Reverse Transcription - Polymerase Chain Reaction
SEM	Standard Error of Mean
TACA	Trans-4-Aminocrotonic Acid
TBPS	T-Butylbicyclophosphorothionate
TLE	Temporal Lobe Epilepsy
Tth	*Thermus thermophilus*
VGAT	Vesicular GABA Transporter
VNTR	Variable Number of Tandem Repeats
vs.	versus

7. Literature

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Curriculum Vitae

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