OLGA Yu. VEKOVISCHEVA

The Role of α6 Subunit-containing $GABA_A$ Receptors in Behavioral Effects of Alcohol and Drug Treatments

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Tampere School of
Public Health, Medisiinarinkatu 3, Tampere,
on May 23rd, 2003, at 12 o'clock.

Acta Universitatis Tamperensis 928
University of Tampere
Tampere 2003

ACADEMIC DISSERTATION
University of Tampere, Medical School
University of Turku, Department of Pharmacology and Clinical Pharmacology
Finland

Supervised by
Professor Esa R. Korpi
University of Helsinki
Docent Mikko Uusi-Oukari
University of Turku
Professor Simo Oja
University of Tampere

Reviewed by
Professor Sture Liljequist
Karolinska Institutet
Docent Tomi Taira
University of Helsinki

Distribution

University of Tampere
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 215 6055
Fax +358 3 215 7685
taju@uta.fi
http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 928
ISBN 951-44-5662-9
ISSN 1455-1616

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2003

Electronic dissertation
Acta Electronica Universitatis Tamperensis 251
ISBN 951-44-5663-7
ISSN 1456-954X
http://acta.uta.fi

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-V.

I **Vekovischeva OY**, Haapalinna A, Sarviharju M, Honkanen A and Korpi ER (1999): Cerebellar $GABA_A$ receptors and anxiolytic action of diazepam. Brain Res 837:184-187.

II **Vekovischeva OYu**, Haapalinna A, Näkki R, Sarviharju M, Honkanen A, Heikkilä J and Korpi ER (2000): Enhanced locomotor stimulation by NMDA receptor antagonists in alcohol-sensitive ANT rats. Pharmacol Biochem Behav 67:793-799.

III Korpi ER, Koikkalainen P, **Vekovischeva OY**, Mäkelä R, Kleinz R, Uusi-Oukari M and Wisden W (1999): Cerebellar granule-cell-specific $GABA_A$ receptors attenuate benzodiazepine-induced ataxia: evidence from $\alpha 6$ subunit-deficient mice. Eur J Neurosci 11:233-240.

IV **Vekovischeva OY**, Uusi-Oukari M and Korpi ER (2000): Chronic ethanol treatment and $GABA_A$ receptor $\alpha 6$ subunit gene expression: a study using $\alpha 6$ subunit-deficient mice. Addiction Biol 5:463-467.

V **Vekovischeva O**, Uusi-Oukari M and Korpi ER (2003): Tolerance to diazepam-induced motor impairment: a study with $GABA_A$ receptor $\alpha 6$ subunit knockout mice. Neurochem Res 28:763-770.

ABBREVIATIONS

α6+/+	α6 subunit wild-type mouse line
α6-/-	α6 subunit knockout mouse line
ANT	alcohol-nontolerant rat line (alcohol-sensitive)
AT	alcohol-tolerant rat line (alcohol-insensitive)
BZ	benzodiazepine
CL218 872	3-methyl-6-(3-trifluoromethylphenyl)triazolo[4,3-b]pyridazine
CNS	central nervous system
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
$GABA_B$	γ-aminobutyric acid type B receptor
$GABA_C$	γ-aminobutyric acid type C receptor
GLM	general linear model
L-701,324	7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolinone
LY235959	[3S-(3α4aα, 6β, 8aα)]decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid
MK-801	(5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (dizocilpine)
NMDA	N-methyl-D-aspartate
PCP	phencyclidine
Ro 15-4513	ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate
SAP	stretch attend posture
SR 95531	2'-(3'-carboxy-2',3'-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide
TBPS	*tert*-butylbicyclophosphorothionate
TM	transmembrane region
U85575	12-chloro-5-(5-cyclopropyl-1',2',4'-oxadiazol-3'-yl)-2,3,-dihydrodiimidazo[1,5-a;1,2-c]quinazoline
U92330	5-acetyl-3-(5'-cyclopropyl-1',2',4'-oxadiazole-3'-yl)-7-chloro-4,5-dihydro[1,5-a]quinoxaline

ABSTRACT

The main inhibitory neurotransmitter receptor system, γ-aminobutyric acid type A ($GABA_A$) receptors in the brain, regulates essential processes such as anxiety, memory functions, epileptogenic activity, vigilance, and muscle tension. The receptors comprise heteropentameric subunit assemblies of combinations from 19 mammalian subunits. The receptor forms integral anion channels mostly responsible for hyperpolarisation of postsynaptic cells. As the key features of the receptor subtypes remain unclear, we used a model system to understand the roles of one brain region-restricted subtype, the α6 subunit-containing receptor.

The α6 subunit is exclusively expressed in cerebellar and cochlear nucleus granule cells. The α6 subunit-containing receptors are insensitive to diazepam, a benzodiazepine (BZ) site agonist. The absence of selective ligands to probe these receptors in vivo necessitates use of genetic modifications to establish the significance of the subunit.

Two animal models relevant to subunit α6 were used in the present study: 1) ANT/AT rat lines, 2) an α6 subunit knockout mouse line. Alcohol-tolerant (AT) rats similar to non-selected heterogenous control rats and alcohol-nontolerant (ANT) rats having a point mutation in the α6 subunit were originally developed to study ethanol intoxication. The point mutation renders the normally benzodiazepine (BZ) agonist-insensitive α6 subunit-containing $GABA_A$ receptors BZ agonist-sensitive. In the α6 knockout mouse line the α6 subunit gene is inactivated by disruption at exon 8 and, consequently, the α6 subunit is not produced in α6-/- mice. The use of these rat and mouse models made it possible to study the effects of a pharmacologically critical mutated native subunit and a complete lack of the subunit on different aspects of $GABA_A$ receptor functions.

The lack of the α6 subunit did not affect motor functions in mice but exclusively increased the motor-impairing effect of diazepam. The slightly increased anxiety level and slowed latency to thermal pain were additional phenotypic features of the α6-/- mice. The lack of the α6 subunit was not crucial for the development of tolerance to the motor-impairing effects of ethanol and diazepam. Chronic administration of moderate ethanol doses did not affect the transcriptional control of the α6 subunit gene.

In addition to being involved in enhanced BZ-induced motor impairment, cerebellar mutant $GABA_A$ receptors in ANT rats participate in $GABA_A$ receptor-activation-induced anxiolysis. NMDA antagonists had greater locomotor effects in ANT rats and lesser effects in α6-/- mice, suggesting that pharmacologically the α6 subunit does not regulate sensitivity to NMDA receptor antagonists.

In conclusion, activation of α6 subunit-containing $GABA_A$ receptors should be avoided to limit impairment of motor performance, although this action might contribute to anxiolysis. The presence of the wild-type α6 subunit staves off abnormally strong diazepam sensitivity, although the subunit does not participate in the development of tolerance to chronic ethanol and diazepam administrations.

1. INTRODUCTION

The major part of γ-aminobutyric acidergic (GABAergic) inhibitory neurotransmission is mediated by the ligand-gated ion channels of $GABA_A$ receptors. The $GABA_A$ receptors are composed of numerous subtypes formed by a pentameric subunit assembly around an integral anion channel. The diversity of $GABA_A$ receptor subtypes depends on distinct combinations of 19 mammalian subunits. These have been cloned and are encoded by different genes (Barnard et al. 1992, Mckernan and Whiting 1996, Korpi et al. 2002b). The receptor subtypes (subunit combinations) have their own brain topography and pharmacological properties. Numerous binding sites mediating effects of alcohol, barbiturates, neurosteroids and benzodiazepines (BZ) determine the importance of $GABA_A$ receptors in many behavioral processes (Hevers and Lüddens 1998). These receptors may play a role in the regulation of anxiety (Rickels et al. 2000, Ballenger 2001), the generation of epilepsy (Alldredge and Lowenstein 1999) and sleep disorders (Kales et al. 1979) and the production of alcohol withdrawal (Little 1991, Lewohl et al. 1996), and in pain mechanisms (Trapani et al. 2000). Numerous changes in subunit expression are seen to be induced by chronic drug treatment, suggesting adaptive alterations of the receptors (Mhatre and Ticku 1993, Ito et al. 1996, Pesold et al. 1997, Grobin et al. 1998, Grobin and Morrow 2000), but it remains to investigate how the receptor subtypes are regulated by acute or chronic treatments in order to understand the consequences resulting from tolerance and dependence. At present the key features of the $GABA_A$ receptor subtypes can be reproduced by combinations of three main subunits, α, β and γ2 (Ehya et al. 2003). Autoradiographic studies have revealed the brain regional densities and modulations of ligands binding to $GABA_A$ receptor subtypes. Recombinant expression studies have yielded information on specific high-affinity assemblies between subunits (Klausberger et al. 2001a). At the same time the significance of data obtained *in vitro* must be incremented by *in vivo* observations.

Gene-targeting techniques to create knockout animals allows to assessment of the contributions of genes to behavior. Thus, for example, studies on $GABA_A$ receptor knockout mice have shown the importance of a few subunits such as β3 and γ2, the deficiency of which cannot be compensated for in the developing brain (Gunther et al. 1995, Homanics et al. 1997b). Most other $GABA_A$ receptor knockout models are viable and can be applied in pharmacological and behavioral studies (Korpi et al. 2002a).

The present series concentrated on evolutionarily conserved subunit α6, which is exclusively expressed in cerebellar and cochlear nucleus granule cells (Lüddens et al. 1990, Varecka et al. 1994, Wisden et al. 1996). The cerebellum is a well-described brain structure containing a small number of cell types (Ito 1984, 1990). It is involved in motor action, motor memory storage and, possibly, in some aspects of cognition (Rossi et al. 2001, Molinari et al. 2002, Pirnik and Kiss 2002). The importance of the α6 subunit for alcohol effects (Korpi 1994) gave reason for checking its role in the auspicious and adverse effects of sedative compounds. Genetic animal models were chosen to assess the functional role of α6 subunit-containing $GABA_A$ receptors. One of these models, an alcohol-nontolerant (ANT) rat line, has a point mutation in the $GABA_A$ receptor α6

subunit gene, resulting in an amino acid change which renders their cerebellar α6-containing $GABA_A$ receptors abnormally sensitive to BZ agonists (Korpi et al. 1993). The second animal model is the α6 gene knockout mouse line (α6-/-) (Jones et al. 1997), which was produced by disrupting the α6 subunit gene through homologous recombination (Homanics et al. 1997a, Jones et al. 1997). The combined results of both animal models are important in promoting our understanding of the function and pharmacology of the cerebellar $GABA_A$ receptor subtypes.

2. REVIEW OF THE LITERATURE

2.1. *The inhibitory γ-aminobutyric acid system. General overview*

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. It regulates many physiological functions and emotional and cognitive behaviors through neurosynaptic contacts widespread in the brain (Costa 1982). It is estimated that 20-50% of all central nervous system (CNS) synapses use GABA as their neurotransmitter (Bloom and Iversen 1971). GABA is formed in nerve terminals from L-glutamate via a reaction catalysed by the enzyme glutamic acid decarboxylase (Sze 1979). It is released from nerve terminals by depolarization in a Ca^{2+}-dependent mechanism (Oja et al. 1977). The GABA released is taken up from the synaptic cleft by Na^{+}-dependent GABA transporters into neuronal and glial cells (Soudijn and Van Wijngaarden 2000). GABA is mainly catabolized to regenerate glutamate via mitochondrial GABA transaminase, GABA-T (Oja et al. 1977). It acts on 3 types of receptors which are phylogenetically conserved across different species: $GABA_A$, $GABA_B$ and $GABA_C$ receptors (Friedl et al. 1988).

Like $GABA_A$ receptors, $GABA_C$ receptors are ligand-gated ion channel receptors (Lüddens and Korpi 1995, Sigel 1995, Johnston 1996, Enz and Cutting 1998) and localized in the retina (Qian and Ripps 2001). $GABA_C$ receptors differ in their pharmacological profile compared with $GABA_A$ and $GABA_B$ receptors (Bormann and Feigenspan 1995), as they are insensitive to bicuculline and baclofen, but are responsive to *cis*-4-aminocrotonic acid, a structural analogue of GABA. It has been suggested that the $GABA_C$ receptors should be classified as a subset of $GABA_A$ receptors (Barnard et al. 1998).

The $GABA_B$ receptor does not include an integral ion channel; its subunits have seven hydrophobic transmembrane domains and act via the cooperation of trimeric G proteins through inhibition of adenylate cyclase and regulation of K^{+} and Ca^{2+} channels (Costa 1998, Enz and Cutting 1998). The receptors are bicuculline insensitive and can be activated by the $GABA_B$ agonist baclofen (Bormann and Feigenspan 1995).

The $GABA_A$ receptors incorporate more than ten distinct binding sites (Korpi 1994, Korpi et al. 2002a), which have made this receptor a well-recognized target for drug development.

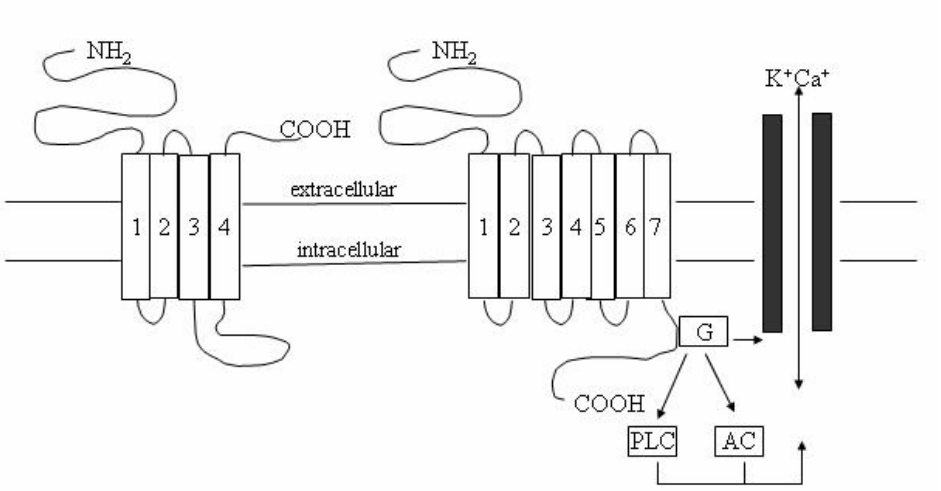

Fig. 2.1 Schematic representation of individual subunits of $GABA_A$ and $GABA_C$ receptors (left) and $GABA_B$ receptors (right). Left: By analogy to the nicotinic acetylcholine receptor, subunits if ligand-gated neurotransmitter receptors consist of four tramsmembrane regions (TM 1-4), a long extracellular N-terminus, a cytoplasmic loop between TM3 and TM4 and a short extracellular C-terminus. The N-terminus contains domains important for ligand binding. The pore of the ion channel is formed by TM2. Right: $GABA_B$ receptors are members of the seven transmembrane protein family (TM 1-7). Binding of an agonist causes activation of a G-protein (G), that in turn can either regulate separate ion channels (permeable for K^+ or Ca^+) directly or use second messenger system such as phospholipase C (PLC) or adenyl cyclase (AC) (Enz et al. 1998).

2.2. $GABA_A$ receptors

2.2.1. Molecular biology of $GABA_A$ receptors

The $GABA_A$ receptors are members of the ligand-gated ion channel superfamily which also includes nicotinic acetylcholine, $GABA_C$, glycine and serotonin 5-HT_3 receptors. $GABA_A$ receptors are the primary mediators of GABA-induced rapid inhibitory neurotransmission (Sieghart 1995) and appear to constitute hypothetical heteropentameric membrane-spanning protein complexes which conduct chloride ions through the neuronal membranes in response to agonist binding.

The $GABA_A$ receptor subtypes are composed of five variable polypeptide subunits, these being members of distinct subunit classes (α, β, γ, δ, ε, π and θ). The known subunits (α1-6, β1-3, γ1-3, δ, ε, π, and θ) have been cloned and their sequence similarity is about 70% within and about 30% between classes (Barnard et al. 1992, Mckernan and Whiting 1996, Korpi et al. 2002b). Alternative splicing of various subunits produces even greater structural diversity (Barnard et al. 1998, Bonnert et al. 1999).

The ligand-gated neurotransmitter receptor subunits consist of a long extracellular N-terminus, four transmembrane regions (TM 1-4), a cytoplasmic loop between TM3 and TM4 and a short extracellular C-terminus. The N-terminus contains domains important for binding of ligands such as BZs (Klausberger et al. 2001a, b, Korpi et al. 2002a). The pore of the ion channel is lined by TM2 regions of the subunits (Unwin 1993, Enz and Cutting 1998) (Fig. 2.1).

The specificity of subunit combinations and interactions between subunits inside the heteropentameric assembly of $GABA_A$ receptors is determined by signaling sequences of the subunits. For example, the residues 58-67 within the α subunit isoforms are important in establishing αβ and αβγ subunits. These residues mediate oligomerization of the α1 and β3 subunits without affecting the assembly between the α and γ subunits (Taylor et al. 2000). Certain domains of α1 (80-100) and γ2 (91-104) subunits are necessary for subunit interaction and assembly and formation of the BZ binding site in the recombinant α1β3γ2 receptor (Klausberger et al. 2000, 2001a). Another region of the γ2 subunit (83-93) might be needed for interaction with the β3 subunits (Klausberger et al. 2001a). While C-terminally truncated N-terminal extracellular domains of α1 and γ2 subunit dimers are enough to form BZ binding sites, [^{3}H]muscimol binding to GABA sites apparently requires transmembrane domains of the α1 subunits together with the C-terminally truncated N-terminal extracellular β3 subunit (Klausberger et al. 2001a, b).

Many proteins interact with $GABA_A$ receptors: gephyrin, a protein widely expressed in the CNS as well as in peripheral tissues (Kneussel et al. 1999), $GABA_A$ receptor-associated protein GABARAP (Wang et al. 1999), and the ubiquitin-like protein Plic-1 (Bedford et al. 2001). All of these are responsible for facilitation of $GABA_A$ receptor cell surface expression and intracellular stabilization of the subunits. These mechanisms are specific for $GABA_A$ receptor subunits and for different receptor subtypes (Moss and Smart 1996).

The subunit composition and stoichiometry of native $GABA_A$ receptors are not completely known, but many studies suggest that the pentameric composition is formed from 2 α, 2 β and 1 γ subunits (Tretter et al. 1997). The γ subunit in some receptor subtypes is apparently substituted by another subunit variant, for example δ. Numerous drugs, including ethanol, BZs and barbiturates are known to interact with the receptors (Homanics et al. 1998a, Loh and Ball 2000), inducing anxiolytic, myorelaxant, anticonvulsant and sedative actions. A part of the compounds can affect receptor function directly (i.e., opening the chloride channel in the absence of GABA) while others act indirectly by binding to allosteric modulatory sites. This latter class of compounds includes BZs, neurosteroids, barbiturates, the anticonvulsant loreclezole, and some anesthetics (Sieghart 1995).

2.2.2. Diazepam as a classical benzodiazepine modulator of $GABA_A$ receptors

BZs have been widely used as anxiolytics, hypnotics, sedatives, anticonvulsants, muscle relaxants and pre-anesthetic sedatives, and to reduce the symptoms of ethanol withdrawal (File 1985, Woods et al. 1992). The main side-effects of BZs are related to their sedative action and include drowsiness, psychomotor incoodination and ataxia (Shader and Greenblatt 1993). Behavioral effects of diazepam such as ataxia (Henauer et al. 1984) and anticonvulsant actions (Paul et al. 1979) have been observed to correlate with occupancy of the BZ binding site of the $GABA_A$ receptor (Korpi et al. 2002a).

In turn, the BZ binding site is allosterically linked to the GABA binding site (Sieghart 1992, Haefely et al. 1993). The binding of GABA to the $GABA_A$ receptor complex elicits a conformational change which causes the anion channel to open, allowing an influx of Cl^- into the mature neuron (Sieghart 1992). When a BZ agonist binds to the $GABA_A$ receptor, the ability of GABA to open the Cl^- channel is enhanced, leading to an increased frequency of Cl^- channel opening and facilitation of neural inhibition (Twyman et al. 1989, Sieghart 1992, Haefely et al. 1993). Thus, BZ ligands may be regarded as allosteric modulators, which alter the gain of the $GABA_A$ receptor channel (Haefely et al. 1993).

Compounds acting through the BZ site can be divided into a number of chemical classes: classical BZs (diazepam, flunitrazepam), imidazobenzodiazepines [ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (Ro 15-4513), flumazenil], β-carbolines [methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), methyl carboline-3-carboxylate], imidazopyridines (zolpidem), pyrazoloquinanolones (2-phenylpyrazolo[4,3-C]quinolin-3(5H)-one), triazolopyridazines [3-methyl-6-(3-trifluoromethylphenyl)-triazolo[4,3-b]pyridazine (CL 218 872)], cyclopyrrolones (zopiclone, suriclone), and imidazoquinoxalines [12-chloro-5-(5-cyclopropyl-1',2',4'-oxadiazol-3'-yl)-2,3,-dihydrodiimidazo[1,5-a;1,2-c]quinazoline (U85575), 5-acetyl-3-(5'-cyclopropyl-1',2',4'-oxadiazole-3'-yl)-7-chloro-4,5-dihydro[1,5-a]quinoxaline (U923330)]. Their efficacies range from full agonists, which bring about a maximum increase (3-5 fold) in the efficacy of GABA, to full inverse agonists which cause a substantial decrease in GABA action. Between these extremes lie partial agonists and partial inverse agonists, which have intermediate effects, and antagonists which do not alter GABA efficacy but competitively block the BZ site (Haefely et al. 1993).

All selective benzodiazepine site ligands currently used are α1-subunit-preferring and have been initially screened for α1β2γ2, the main $GABA_A$ receptor subtype in the brain (Wingrove et al. 2002). This receptor subtype has been thought to mediate anticonvulsant and sedative (Zhang et al. 1998) actions of BZs (Mckernan and Whiting 1996), whereas α2 subunit-containing $GABA_A$ receptors may mediate anxiolysis (Crestani et al. 2001, Mohler et al. 2002).

Some $GABA_A$ receptor subtypes are insensitive to classical BZs like diazepam. The α6 subunit is selectively expressed in the cerebellar granule cell layer (Lüddens et al. 1990). Despite the presence of the γ2 subunit, important for BZ binding sites, the α6β2/3γ2 assemblies are insensitive to benzodiazepine agonists such as diazepam. The diazepam insensitivity of the α6-containing $GABA_A$ receptors results from an arginine residue at position 100 instead of histidine identified in the diazepam-sensitive α1, α2, α3 and α5 subunits

(Wieland et al. 1992). Due to the arginine residue, the α4βγ2 receptors are also diazepam-insensitive (Sur et al. 1999).

Ro 15-4513, an imidazobenzodiazepine, is a benzodiazepine partial inverse agonist (Allan and Harris 1986, Buck and Harris 1990, Buck et al. 1991) which reverses GABA-induced effects and antagonizes the sedative effects of ethanol (Bonetti et al. 1989, Corda et al. 1989). Two classes of Ro 15-4513 binding sites are known: diazepam-sensitive and diazepam-insensitive (Turner et al. 1991). The binding domain for Ro 15-4513 is located within the 1-101 residues of the α6 subunit (Duncalfe and Dunn 1996).

A physiological withdrawal syndrome has been observed in numerous studies following abrupt discontinuation of long-term diazepam treatment (Pevnick et al. 1978, Winokur et al. 1980, Rickels et al. 1983). The development of diazepam tolerance has been shown in its muscle relaxant and ataxic effects (Matsubara and Matsushita 1982), its general locomotor activity-depressing action (De Angelis and File 1979, Harro et al. 1990, File and Fernandes 1994), and anticonvulsant effects (File 1983, Ramsey-Williams et al. 1994) but not in anxiolytic effects (De Angelis and File 1979, Rodgers and Shepherd 1993). Most studies involving animal models have used daily diazepam doses of at least 5 mg/kg (approximately 18 times more than the anxiolytic dose for humans) (Henauer et al. 1984, File 1985, Smith and Darlington 1994) to attain a high diazepam concentration in the animal brain (Friedman et al. 1986). Usually, the route of administration in animal studies is also different from that in human practice (Smith and Darlington 1994), which favours the oral route. Although diazepam is widely used in clinics, the details of its chronic effects must be understood at the behavior and receptor levels, and this can be achieved with the use of animal models (Hutchinson et al. 1996).

2.2.3. Ethanol as a modulator of $GABA_A$ receptor function

Acute and chronic ethanol administrations produce behavioral effects such as anxiolytic, sedative, hypnotic, and motor-impairing closely similar to the effects of BZs and barbiturates (Grobin et al. 1998). Although several neuronal signaling systems are sensitive to the effects of ethanol, $GABA_A$ receptors appear to be one of the main targets (Crews et al. 1996). Using $GABA_A$ receptor modulators it has been suggested that $GABA_A$ receptors are one of the key sites for the behavioral effects of ethanol (Lister and Linnoila 1991).

Ethanol increases $GABA_A$ receptor-mediated Cl^- influx. Persistent ethanol exposure produces both tolerance and dependence. Receptor alterations induced by chronic ethanol treatment concern expression of various $GABA_A$ receptor subunit mRNAs (Mhatre and Ticku 1992) following changes in receptor subunit compositions. Immutable mRNA expressions have only been shown for the α5, γ2L, γ3 and δ subunits in the cerebral cortex (Devaud et al. 1995). In the cerebellum a decrease in the α1subunit mRNA and an increase in α6 subunit mRNA levels have been found (Mhatre and Ticku 1992). It has been suggested that the mechanism might reflect tolerance and/or dependence development (Grobin et al. 1998). The alterations in the assembly of $GABA_A$ receptors correspondingly modify receptor function and binding. Thus, the ability of acute ethanol to potentiate GABA or muscimol-stimulated Cl^- uptake is lost following

chronic ethanol administration in both cerebral cortex and cerebellum (Sanna et al. 1993). An increase in α4 and α6 subunit expression results in the enhancement of binding of the BZ inverse agonist and alcohol antagonist [^{3}H]Ro 15-4513 especially in the cerebral cortex and cerebellum (Mhatre et al. 1988, Mehta and Ticku 1989), accentuating the significance of both brain areas.

A recent study designed to identify trait loci and candidate genes participating in alcohol withdrawal indicated that 20 % of these genes are related to GABAergic neurotransmission. It was noted that the three genes encoding $GABA_A$ receptor subunits α1, α6, and γ2 are located in the same gene cluster on mouse chromosome 11 (Buck et al. 1997), which would imply the participation of the gene cluster in alcohol withdrawal.

2.3. Cerebellum and cerebellar $GABA_A$ receptors

The cerebellum contains more than half of all brain neurons (Ghez and Thach 2000). These neurons are organized in regular manner as repeated units, each of which constitutes a basic circuit module (Delgado-Garcia 2001, Rossi et al. 2001, Otis 2002). At the same time the cerebellum is divided into several distinct regions, receiving projections from different brain areas and spinal cord and sending them to different motor systems (Glickstein 1997, Ozol and Hawkes 1997, Sotelo and Chedotal 1997, Fanardzhian 2001). Three aspects of the cerebellar organization underlie this function. First, the cerebellum is provided with extensive information on the goals, commands and feedback signals associated with the programming and execution of movement. Second, the output projections of the cerebellum are focused on the premotor and motor systems of the cerebral cortex and brain stem, systems which control spinal interneurons and motor neurons directly. Third, synaptic transmission in the circuit modules can be modified, this being crucial for motor adaptation and learning (Dietrichs et al. 1994, Thompson et al. 1997, 1998, Strata and Rossi 1998, Schultz and Dickinson 2000).

Damage to the cerebellum disrupts the spatial accuracy and temporal coordination of movement, impairs balance and reduces muscle tone, and also markedly impairs motor learning and certain cognitive functions (Botez 1993, Daum et al. 1993, Daum and Ackermann 1995, Steinlin et al. 1999, Scott et al. 2001).

The anatomy and physiology of the cerebellum are markedly regular over different cerebellar regions and highly conserved across different species (Paulin 1993, Williams and Holland 1998, Fanardzhian 2000). The cerebellar cortex is a simple three-layered structure consisting of only five types of neurons: the inhibitory stellate, basket, Purkinje and Golgi neurons, and the excitatory granule cells (Hawkes et al. 1993, Dunn et al. 1998a, b, Goldowitz and Hamre 1998, Voogd and Glickstein 1998, Armstrong and Hawkes 2000).

The outermost molecular layer contains the cell bodies of two types of inhibitory interneurons, the stellate and basket cells, dispersed among the excitatory axons of granule cells and the dendrites of inhibitory Purkinje cells, whose cell bodies lie in deeper layers (Meek 1992, Strata and Rossi 1994). The layer displays the pharmacological characteristics expected from the α1β2/3γ2 subunit composition. It shows a type-I BZ pharmacology (Lüddens and Wisden 1991) and

10 times lower sensitivity to GABA than the granule cell layer. Furthermore, furosemide, the first $GABA_A$ receptor subtype-specific antagonist, is not able to antagonize the inhibition of [^{35}S]*tert*-butylbicyclo-phosphorothionate ([^{35}S]TBPS) binding by GABA in this layer (Korpi et al. 1995).

The *Purkinje cell layer*, consisting of a single layer of large, GABAergic Purkinje cell bodies, lies beneath the molecular layer.

The *granule cell layer* lies innermost and contains a vast number (estimated at 10^{11} for the human brain) of granule cells and a few Golgi interneurons. The mossy fibers, the major source of afferent inputs to the cerebellum, terminate in this layer. The bulbous terminals of the mossy fibers contact granule cells and Golgi neurons in the synaptic complexes known as cerebellar glomeruli (Castejon and Castejon 1991). The schematic structure of the glomerulus is presented in Fig. 2.2. (III).

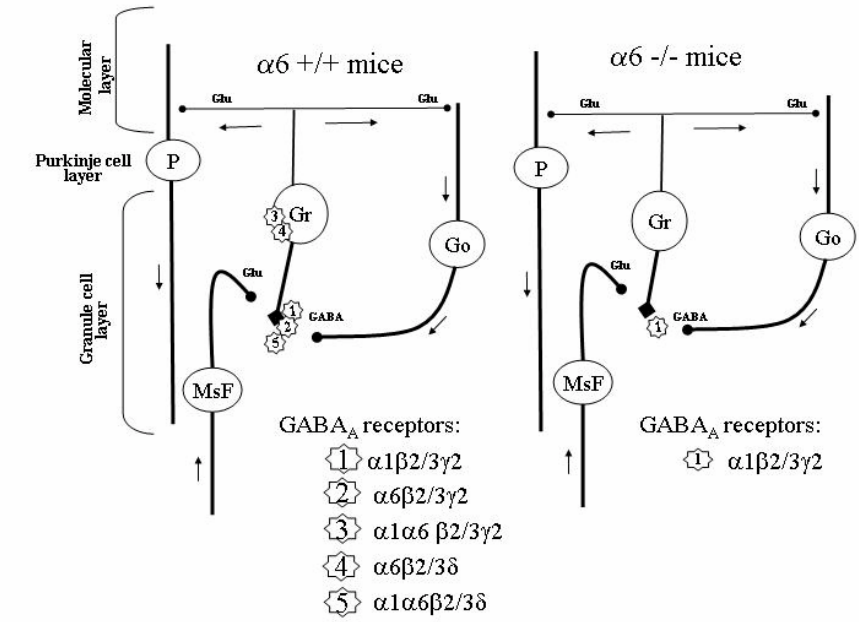

Fig. 2.2. A description of pathways and synapses in the cerebellar cortex associated with glutamatergic (Glu) granule cells. Granule cells (Gr) are activated by excitatory mossy fiber terminals and give rise to parallel fibres, which synapse onto GABAergic Purkinje cells (P) and GABAergic Golgi neurons (Go) in molecular layer. P provide the only efferent pathway from the cerebellar cortex, mainly to deep cerebellar nuclei. Go provide the inhibitory feedback to granule cells. Known $GABA_A$ receptor subunit combinations are given for the wild-type α6+/+ and knockout α6-/- mice for the Golgi-granule cell synapse (Jones et al. 1997), receptors containing the α6 subunit combined with the δ subunits are extrasynaptic (Nusser et al. 1998). Black arrows indicate the diarection of information flow (III).

Glutamatergic granule cell dendrites are activated by mossy fiber terminals. Axons of granule cells form parallel fibers which synapse onto Purkinje cells in the molecular layer, whose axons in turn project into the underlying white matter to deep cerebellar or vestibular nuclei, forming the only different pathway from the cerebellum cortex. GABAergic Golgi neurons in the granule layer provide the inhibitory feedback to granule cells. Abundant expression of $GABA_A$ receptor α1,

α6, β2, β3, γ2 and δ subunits has been detected in mature granule cells (Nusser et al. 1995, Thompson et al. 1996). The restricted expression pattern and the sequence of the α6 subunit have been highly conserved over a long evolutionary period in fishes, birds, rodents and humans, which implies the importance of their functions (Hadingham et al. 1996). The restricted number of $GABA_A$ receptor subtypes in granule cells (α1β2/3γ2, α6β2/3γ2, α1/6β2/3γ2, α6β2/3δ, and α1/6β2/3δ) (Jones et al. 1996, Khan et al. 1996, Jechlinger et al. 1998, Nusser et al. 1998) and the well-known regular structure of the cerebellum make this organ an ideal model for a study of the function of $GABA_A$ receptors (Wisden et al. 1996).

2.4. Animal models to study the role of cerebellar $GABA_A$ receptors

The alcohol-sensitive ANT rat line developed to study the intoxicating effects of ethanol comprises a model of modified cerebellar $GABA_A$ receptors which have a point mutation in the α6 subunit making the normally BZ agonist-insensitive α6 subunit-containing $GABA_A$ receptors BZ agonist-sensitive (Korpi and Seeburg 1993). Alcohol-insensitive AT rats similar in their features to non-selected heterogenous rats were controls for the ANT rats.

The mouse model (α6-/- mice) has a disruption of the α6 subunit gene at exon 8 and, therefore, α6 subunit-containing $GABA_A$ receptors are completely absent in the cerebellum (Homanics et al. 1997a, Jones et al. 1997). Targeted mutations and gene inactivation constitute novel experimental strategies developed to assess the roles of receptor subunits in behavior and in drug effects. The protein of interest is mutated or inactivated to evaluate its contribution to the phenotype of the animal.

Autoradiography of histological sections of adult brains demonstrate less total [^{3}H]Ro 15-4513 binding to the cerebellar granule cell layer in α6-/- mice, with intermediate binding in heterozygotes and highest binding to that in α6+/+ mice (Jones et al. 1997, Mäkelä et al. 1997). A similar trend has been found in another α6 knockout mouse line (Homanics et al. 1997a).

The available experimental data concerning the physiological and behavioral roles of $GABA_A$ receptor subunits are collected in Table 2.1. It is clear that not all subunits can be studied in homozygous null mutants, because some of the subunits are important for brain development and survival. It has however been shown that the lack of α5, γ3, γ2L, α6 and δ subunits does not produce any essential destruction of the behavioral phenotype of the animals (Culiat et al. 1994, Homanics et al. 1997a, Jones et al. 1997, Mihalek et al. 1999). Even the lack of the most important subunits such as α1 and β2 is not associated with a lethal effect or serious behavioral abnormalities (Sur et al. 2001). The findings suggest the presence of functional compensation for their lack by internal resources of the $GABA_A$ receptor system or/and other systems.

2.4.1. Alcohol-sensitive ANT rat line

AT and ANT rat lines have been developed by selective outbreeding based on differential sensitivity to ethanol. The effects of an acute moderate dose (2 g/kg) of ethanol on motor impairment were evaluated in a tilting plane test (Eriksson and Rusi 1981). The behavioral test was designed to assess rapid postural adaptation (Arvola et al. 1958). The similarity of ethanol kinetics and metabolism in both AT and ANT rats (Eriksson and Rusi 1981) indicated that other factors are responsible for their different ethanol sensitivities. At the same time the ANT rats also appeared to be more sensitive to the motor-impairing effects of the barbiturate sodium barbital (Hellevuo et al. 1989), the anestetic propofol (Yildirim et al. 1997), and the NMDA glutamate receptor antagonist dizocilpine (MK-801) (Toropainen et al. 1997). The motor impairment by the BZ agonists diazepam and lorazepam (Hellevuo et al. 1989) as well as by ethanol and barbital is antagonized by picrotoxin. This strongly suggests that $GABA_A$ receptor-mediated mechanisms are involved.

The enhanced BZ sensitivity of motor reflexes in ANT rats is explained by the point mutation in the α6 subunit (arginine at 100 replaced by glutamine), which alters the normally diazepam-insensitive receptors to diazepam-sensitive (Korpi and Seeburg 1993). No other brain regional differences in $GABA_A$ receptor benzodiazepine pharmacology have been detected, nor does brain BZ uptake differ between the ANT and control rats (Uusi-Oukari and Korpi 1992).

This point mutation cannot of itself, however, explain the enhanced ethanol sensitivity of the ANT rats (Korpi and Uusi-Oukari 1992). The presence of NMDA receptors in synapses between GABAergic Golgi neurons and glutamatergic granule cells and their participation in altered motor performance in ANT rats under ethanol (Korpi 1994) suggested a new potential target for study. However, no differences between the lines were found in the binding of the noncompetitive NMDA receptor antagonist [^{3}H]MK-801 in various brain regions (Näkki et al. 1995). Ethanol-induced decreases in cerebellar and hippocampal cGMP levels and NMDA receptor subunit mRNA expression levels were similar in the two rat lines (Toropainen et al. 1997). At the behavioral level, however, MK-801 impaired motor function in the tilting plane test more markedly in ANT than AT rats. The impairment was potentiated by low doses of ethanol in the ANT rats (Toropainen et al. 1997). This would imply that neither dopaminergic nor GABAergic mechanisms can be involved in the motor impairment induced by MK-801 (Toropainen et al. 1997) and a search for glutamatergic differences between the ANT and AT rat lines remains to be undertaken.

If, moreover, the cerebellum is involved in behavioral processes such as anxiety (Bloedel and Bracha 1997), ANT rats might provide a unique model to assess the putative role especially of the cerebellar granule cell $GABA_A$ receptors in behaviors other than motor function.

2.4.2. $GABA_A$ receptor α6 subunit knockout mouse line

Two 129/SvJ x C57BL/6J-derived mouse lines have been independently developed, in which exon 8 of the α6 subunit-coding gene is disrupted. Gene targeting included insertion of either a selectable marker neomycin

phosphotransferase gene (Homanics et al. 1997b) or an *Escherichia coli* enzyme β-galactosidase reporter cassette and neomycin resistance gene (Jones et al. 1997). Reduction of α6 subunit mRNA was demonstrated in one of the mouse lines (Homanics et al. 1997a) while in both knockout lines the α6 subunit protein was missing (Homanics et al. 1997a, Jones et al. 1997).

At the same time, a reduction down to 77% in cerebellar δ subunit protein in the plasma membrane was also detected in α6-/- mice, although the δ mRNA level was normal (Jones et al. 1997, Nusser et al. 1999). The amounts of cerebellar β2, β3 and γ2 subunit proteins were reduced while the amount of α1 subunit protein was not changed. The pharmacological feature of α6 subunit-containing $GABA_A$ receptors, diazepam-insensitive [^{3}H]Ro 15-4513 binding, was absent on the cerebellar granule cell layer of the mutants, as were the high-affinity bindings of [^{3}H]muscimol, a GABA agonist, and [^{3}H]2'-(3'-carboxy-2',3'-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide ([^{3}H]SR 95531), a competitive GABA antagonist (Homanics et al. 1997a, Jones et al. 1997).

Despite the well-proven loss of the α6 subunit, the knockout mice are viable and fertile and have grossly normal brain cytoarchitecture (Homanics et al. 1997a, Jones et al. 1997). These mutant mice showed no substantial baseline differences in movement co-ordination, maintenance of balance or motor learning as compared to their wild-type controls (α6+/+ mice) (Homanics et al. 1997a, Jones et al. 1997).

Ethanol and BZs can be grouped together on the basis of their anxiolytic effect acting through $GABA_A$ receptors (Grobin et al. 1998, Mehta and Ticku 1999, Mihic 1999). Likewise chronic use of ethanol or BZs is attended by the development of tolerance and dependence (Lader 1995, Grobin et al. 1998, Ballenger 2001). Taking into account that the α6 subunit mRNA level is increased in the cerebellum by chronic ethanol treatment (Mhatre and Ticku 1992) α6-/- mice offer a suitable model to study chronic ethanol or BZ effects. However, one of the two α6-/- mouse lines and their wild-type controls did not differ in acute sensitivity to ethanol, ethanol metabolism, acute functional tolerance to ethanol, withdrawal hyperexcitability or protracted tolerance to ethanol (Homanics et al. 1998b). In the present study the other α6-/- mouse line (Jones et al. 1997) was checked for ethanol and diazepam sensitivity and tolerance.

Table 2.1. A summary of $GABA_A$ receptor subunit knockout mouse lines

Gene knockout	Regions of expression		Main effect s	Response to various drugs
α1 subunit	Widely distributed in all brain regions and accounts for 40% of all brain $GABA_A$ receptors			➢ Insensitive to the sedative, amnestic, and anticonvulsant effects of diazepam ➢ Sensitive to the anxiolytic diazepam effect (Holmes 2001)
α5 subunit	Hippocampus		➢ improved performance in water maze (Collinson et al. 2002)	
β3 subunit	Cerebral cortex, hippocampus, hypothalamus, cranial nerve ganglia, spinal cord		➢ 90% of mice homozygous for the mutation die as neonates ➢ 60% have a cleft palate ➢ $GABA_A$ receptor function is severely impaired ➢ Surviving animals are runt, hyperactive, have difficult swimming, walking and frequently fall off platform and rotarod	➢ Normally sensitive to pentobarbital and ethanol ➢ Less sensitive to etomidate and midazolam (Delorey et al. 1998, Homanics et al. 1997b)
γ2 subunit	Majority of $GABA_A$ receptors	Short splice variant	➢ Most KO mice die in early neonatal period ➢ Sensomotor abnormalities: hyperactivity, impaired grasping and righting reflex, abnormal gait	➢ Normally respond to bicuculline, picrotoxin and pentobarbital ➢ Unresponsive to flunitrazepam and diazepam (Homanics et al. 1999)
		Long splice variant	➢ Increased level of anxiety	➢ Insensitivity to pentobarbital and etomidate ➢ No differences in ethanol effects (Homanics et al. 1999)
δ subunit	Cerebellar granule cells, thalamus, hippocampus			➢ Reduced ethanol consumption ➢ Attenuated withdrawal from chronic ethanol exposure ➢ Reduced anticonvulsant effect of ethanol ➢ Insensitive to the anxiolytic effects of neuroactive steroids ➢ Normal hypothermic response to ethanol ➢ Normal anxiolytic response to ethanol (Mihalek et al. 2001)
α6 subunit	Expressed exclusively in cerebellar granule cells Low level of expression in dorsal cochlear nuclei	Homanic's mice	➢ The examination of motor functions was not carried out	➢ Normal hypnotic response to midazolam, ethanol, Ro15-4513, pentobarbital ➢ Normal response to ethanol in terms of acute functional tolerance, protracted tolerance, and withdrawal hyperexcitability (Homanics et al. 1998b)
		Wisden's mice	➢ On motor performance tests (rotarod, horizontal wire, pole descending, staircase, swimming tests) no robust baseline differences were found between α6-/- mice and their littermates (III) ➢ A slightly suppressed free locomotor activity (previously unpublished results) ➢ A slightly increased level of anxiety (previously unpublished results)	➢ Motor performance is more sensitive to impairing effect of acute diazepam but not of acute ethanol (III) ➢ Normal response to chronic ethanol treatment (IV) ➢ Slightly increased response to chronic diazepam treatment (unpublished results) ➢ Insensitive to stimulation by MK-801 (previously unpublished)

3. AIMS OF THE STUDY

There is little information on the neuron-specific role of any $GABA_A$ receptor subtype. The selective expression of the α6 subunit in cerebellar granule cells (and cochlear nuclear granule cells) makes the $GABA_A$ receptors containing this subunit unique. The present study aimed at defining alterations in the behavior of mice with a deficient α6 subunit. We also compared the results in a rat model with a pharmacologically critical mutation in the α6 subunit: the alcohol-sensitive ANT rats. These rats are known to be highly sensitive to motor impairment induced by BZ agonists such as diazepam (see above) (Korpi et al. 1993).

The experiments were planned to answer the following questions:

1. Are the cerebellar granule cell $GABA_A$ receptors involved in anxiety and explorative behavior?
2. Are the glutamate receptor-mediated processes associated with differences in innate ethanol sensitivity in the ANT/AT rat model?
3. Are the motor functions abnormally sensitive to ethanol and diazepam in full knockout of the $GABA_A$ receptor α6 subunit in mice?
4. Does chronic treatment with ethanol or diazepam alter tolerance and/or receptor adaptation in α6-/- mice?
5. Are other behaviors such as pain sensitivity and explorative behavior abnormally diazepam-sensitive in α6-/- mice?

4. MATERIALS AND METHODS

4.1. Animals

The ANT rats have a pharmacologically critical point mutation in the α6 subunit-containing (arginine-100 replaced by glutamine) $GABA_A$ receptors, which alters the normally diazepam-insensitive receptors to diazepam-sensitive (Korpi et al. 1993). The AT rats are ethanol- and BZ-insensitive controls, and their characteristics are similar to those of non-selected heterogenous rats (Korpi 1994). Adult males from F_{50} and F_{54} generations were used. The animals were housed in polypropylene cages (25x42x15 cm) (3-6 rats/cage). A total of 167 ANT and 169 AT rats were used (I, II).

Mice lacking the $GABA_A$ receptor α6 subunit in the cerebellum (III-V) have been produced by homologous recombination with a targeting vector having a truncated α6 subunit gene supplemented with an internal ribosome entry site and lac-Z gene (Jones et al. 1997) in the 129/SvJxC57BL/6J background. Homozygous breeding pairs were transferred to Turku from Cambridge, UK. Ninety- to 210-day old animals of the second generation group housed in polypropylene Macrolon cages (25x42x15 cm) (4-12 mice/cage) were used in the experiments. A total of 127 α6-/- and 76 control mice were used.

Adult animals were used for the experiments. All animals were kept in a 12/12-h light/dark cycle (lights on at 7 a.m.), air-conditioning (20 ± 1°C) and a relative humidity of 50 ± 10% with access to tap water and pellets ad libitum. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Turku.

4.2. Equipment and behavioral tests

The detailed experimental procedures are presented in Table 5.1. and in papers I-V. A summary is given below.

Motor performance testing was carried out by several tests (III-V). Motor capabilities appear important in analyses of the behavioral phenotypes of transgenic and knockout animals (Anagnostopoulos et al. 2001), especially if the alteration of gene expression is observed or expected in the cerebellum. Models of behavior such as anxiety and locomotion are widely used in pre-clinical psychopharmacology for both drug screening and assessment of drug effects.

Rotarod test allows assessment of the ability of mice to learn to stay on an accelerating rotating rod for a fixed time interval. The reduction of this rotarod time by drug treatment in trained animals was the criterion in evaluating levels of drug-induced ataxia (III-V).

Pole test (Ogawa et al. 1985) was used to assess motor coordination, placing the mice on the top of a rough-surfaced iron pole (1 cm in diameter and 55 cm high). The time required for the mouse to turn completely downwards and then to descend to the platform was recorded (III).

To assess the ability of mice to lift their hind paws onto a thin **horizontal wire** after initially hanging from their forepaws, a horizontal wire test was used

(Hellevuo and Korpi 1988) (III). This test also measures motor coordination and myorelaxation.

Swimming behavior was observed for 2 min in warm (25^{o}C) water in a 2-liter beaker. Immobility time was determined (III).

Horizontal locomotion (ambulation) was measured by locomotor boxes (25x42x19 cm) equipped with computer-controlled photocells automatically monitoring the animals' movements (Photobeam Activity System; San Diego Instruments, San Diego, CA) (II, unpublished).

The level of anxiety was evaluated by widely used models (Ferrari et al. 1998, Rodgers et al. 1997) based on the suppression of spontaneous behaviour in rodents by their natural aversion to heights and open spaces such as are used in **plus-maze**, **staircase** and **light-dark** (shuttle box) tests (I, unpublished).

The **elevated plus-maze** made of dark-brown Plexiglas comprised two open (40x10 cm) and two closed (40x10x45 cm) opposing arms extending from a common central platform (10x10 cm). The entire apparatus was elevated to a height of 50 cm above floor level. Placing a mouse on the central platform facing an open arm initiated the 5-min test. Arm entries were defined as entry of all four paws into an arm.

This model of anxiety in animals has been validated for both rats and mice and is sensitive to both anxiolytic and anxiogenic manipulations. The calculation of behavior associated with open-arm behavior (see behavioral parameters in I) was supplemented by a range of specific plus-maze behaviors reflecting aspects of defense and, along with the traditional indices, apparently enhancing the sensitivity of the test (Cole and Rodgers 1994), previously unpublished data). The behavior occurring in closed arms and central platform was indicated as "protected" while that observed in open arms was regarded as "unprotected". The main behavioral categories were chosen according to Rodgers and Dalvi (1997): anxiety, locomotion, risk assessment, exploration. Level of anxiety was evaluated by total arm entries, open arm entries, % open entries, % open time, % closed time, % center time, closed arm returns, % protected head-dipping, % protected stretch attend posture (SAP). Level of locomotion was assessed by total arm entries and closed arm entries. The category of exploration involved total head-dips and total SAP. Risk-assessment behavior was determined through total SAP.

The **staircase test of explorative behavior** consists inplacing a mouse in an enclosed wooden staircase with five steps (Simiand et al. 1984). The time before a move to the first step (latency), the number of steps climbed, rears, grooming and SAP were observed for 3 min.

A two-compartment box divided into a dark and a white area (Costall et al. 1989) (30x30x35 cm) with an open door (12x9 cm) between them was used for light-dark test based on the innate aversion of rodents to brightly illuminated areas (Crawley and Goodwin 1980). The time spent in the white compartment and the numbers of crossings between the compartments were measured for 5 min.

The **tail-flick** and **plantar tests** were designed to assess thermal pain reaction in the α6-/- mice, since synergistic interaction between μ-opioid and $GABA_A$ receptors in the spinal processing of thermally-evoked pain has been suggested (Yanez et al. 1990). Latency of pain reaction was measured in handled animals in a tail-flick and plantar apparatus (Ugo Basile, Comerio, Italy). The tail-flick test means warming the tail by focused beam from a light bulb provoking a tail movement away (cut-off time 10 s). The latency of a specific pain reaction

(shaking or licking) of the hind paw from the focused heating beam (cut off time is 16 s) was assessed by the plantar test. Firstly, different levels of beam intensity (30, 35, 40 for tail-flick and 47, 52, 57 for plantar tests) were tested in drug-naïve animals.

4.3. Drug treatments

The acute effects of ethanol (2.0 and 2.5 g/kg, i.p.; Primalco, Rajamäki, Finland, 12% weight/volume, diluted in 0.9% NaCl) and diazepam [10 and 20 mg/kg, i.p.; Stesolid (Dumex, Copenhagen, Denmark) diluted with Intralipid (Pharmacia, Stockholm, Sweden) to a final concentration of 2 g/l] were assessed in rotarod test in α6-/- mice and their controls (III).

The acute diazepam effect was evaluated in rats (I) and mice (unpublished) by elevated plus-maze and in mice by staircase test (unpublished), since the drug has customarily been used in regulation of anxiety level through $GABA_A$ receptors.

The effect of the noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) class of glutamate receptors MK-801 (Research Biochemicals, Natick, MA), was tested on free locomotion in rats (II) and mice. Other noncompetitive NMDA antagonists such as phencyclidine (PCP), (Research Biochemicals) and ketamine (Research Biochemicals) and the competitive NMDA antagonist [3S-(3α4aα, 6β, 8aα)]decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid (LY 235959, Tocris Cookson, Bristol, UK), and the glycine-site antagonist 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1*H*)-quinolinone (L-701,324, Tocris Cookson) were tested on rats to conduct a more detailed analysis of NMDA receptor functions in AT and ANT rats at the behavioral level. Use of numerous NMDA receptor ligands was intended to elucidate the divergence of molecular and behavioral results obtained by Toropainen and colleagues (1997) (II).

The acute effects of morphine hydrochloride (5 mg/kg, s.c.; European Pharmacopoeia), a classical analgesic acting through μ-opioid receptors, naloxone hydrochloride (1 mg/kg, s.c.; Research Biochemicals), an antagonist of μ-opioid receptors, and diazepam (1 and 3 mg/kg, i.p.; Dumex) were compared by mouse tail-flick test with a beam intensity of 40 (previously unpublished).

Since chronic use of ethanol or diazepam is associated with the development of tolerance and withdrawal symptoms following chronic treatment (Grobin et al. 1998, Mehta and Ticku 1999, Mihic 1999), we used the rotarod test to evaluate the development of tolerance to ethanol (diluted in water) or diazepam in α6-/- mice in the course of 21 days (IV, V). Both drugs were used in moderate doses per os to imitate human conditions. Thus, treatment was started from 2 g/kg ethanol and 5 mg/kg diazepam injections. If tolerance to drug-induced ataxia developed before the treatment schedule ended, the chronic and test doses were slightly increased to obtain a clear effect. In addition to behavioral parameters, body temperature was measured by means of a rectal probe (BAT-12, Physitemp, Clifton, NJ).

4.4. Ligand autoradiography

The effect of chronic diazepam treatment of α6-/- mice and their wild-type controls on $GABA_A$ receptor-associated binding sites was studied on 14-μm horizontal brain slices using quantitative autoradiography (V). [^{3}H]Ro 15-4513 binding to the BZ site in αxβxγ2 receptor subtypes (Lüddens and Wisden 1991) and [^{35}S]*tert*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding to the convulsant site, showing the functional state of $GABA_A$ receptors in the cerebellum (Im and Blakeman 1991) were used according to the method described by Mäkelä and associates (1997).

4.5. Statistical analyses

Statistical analyses were conducted using SAS-STAT software (release 6.11; SAS Institute, Cary, NC). Analysis of the descriptive statistical procedure by SAS-STAT Univariate demonstrated that some data were not distributed normally (Shapiro-Wilk's test). In this case the data were normalized by rank statistical procedure.

Results from simple behavioral experiments were analysed using one-way ANOVA to find differences between mutant and control animals. Thus, the strain factor (knockouts or their wild-type control) was the first step to be analysed for all experiments. Additional factors such as gender or experimental conditions, environment influences or drug treatment were dealt with in two- or three-factorial ANOVA using a combination of the rank and general linear model (GLM) procedures. Repeated–measures design was applied when necessary. Usually, data were ranked and the ranks subsequently subjected to ANOVA (GLM procedure for unbalanced design with unequal group size). Duncan's or Dunnett's tests were conducted for post hoc analyses of between-group comparisons (only when ANOVA revealed significant main effects). Null hypothesis was rejected at the $p<0.05$ level.

5. RESULTS

Previously unpublished results are presented here in detail, while results published in reports I-V are only summarized. Previous studies on ANT/AT rats have been recently reviewed in detail (Pispa et al. 1986, Eriksson 1990, Kiianmaa and Hellevuo 1990, Loh and Ball 2000). All our AT/ANT rat studies were published in full (see I, II).

5.1. Behavioral profile of the ANT and AT rats

5.1.1. Level of anxiety (I)

Acute diazepam treatment was effective in the elevated plus-maze test in ANT rats, reducing their level of anxiety, while AT rats appeared to be nonresponsive to the treatment.

5.1.2. Locomotor effects of NMDA receptor antagonists (II)

The stimulant effect of MK-801 was greater in ANT rats. The differences emerged in a subpopulation among the AT rats which were completely unresponsive to the drug. The insensitivity to MK-801 correlated with the lack of c-fos induction in retrosplenial and cingulated cortices. Similarity of c-fos induction after MK-801 in ANT and AT rats was observed in inferior olivary nucleus only. ANT rats appeared to be more stimulated by ketamine and LY235959. PCP and L-701,324 effects did not differ between the rat lines.

5.2. Behavioral profile of α6-/- mice

The results on α6-/- mice and their wild-type controls are presented in Table 5.1. Most of the statistical results are described in the publications. New unpublished results are presented in full below.

5.2.1. Motor abilities

Inactivation of the α6 subunit did not lead to any serious impairment of motor performance (horizontal wire, pole descending, staircase and swimming test) (III, V). Both α6-/- and α6+/+ mouse lines learned more or less similarly [$F(1,320)=0.02$, ns for line factor] to stay on the accelerating rod during the 8-day training period [$F(1,320)=11.02$, $p<0.01$ for training day factor] (Fig. 5.1). The level of horizontal activity (ambulation) in an unfamiliar environment was decreased in α6-/- mice [$F(1,21)=5.9$, $p=0.0252$] (Fig. 5.2.A). The mice habituated to the test environment, showing a similar level of locomotion irrespective of the genotype (Fig. 5.2.C, D).

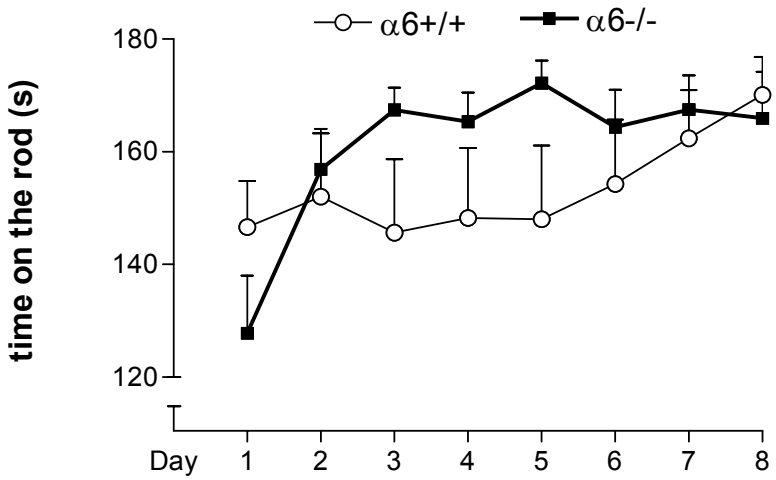

Fig. 5.1. Learning to stay on an accelerating rod. Points depict performance time until falling (mean±SEM, n=7-9) from the rod accelerating from 5 to 15 r.p.m. during 180 s.

The noncompetitive NMDA receptor antagonist MK-801 did not affect ambulation in α6-/- mice, though increasing it in their wild-type controls [$F(1,47)=5.37$, $p=0.0256$ for strain factor, $F(4,47)=7.22$, $p=0.0021$ for drug effect] (Fig. 5.2.C, D). Vertical activity was suppressed similarly in both mouse lines.

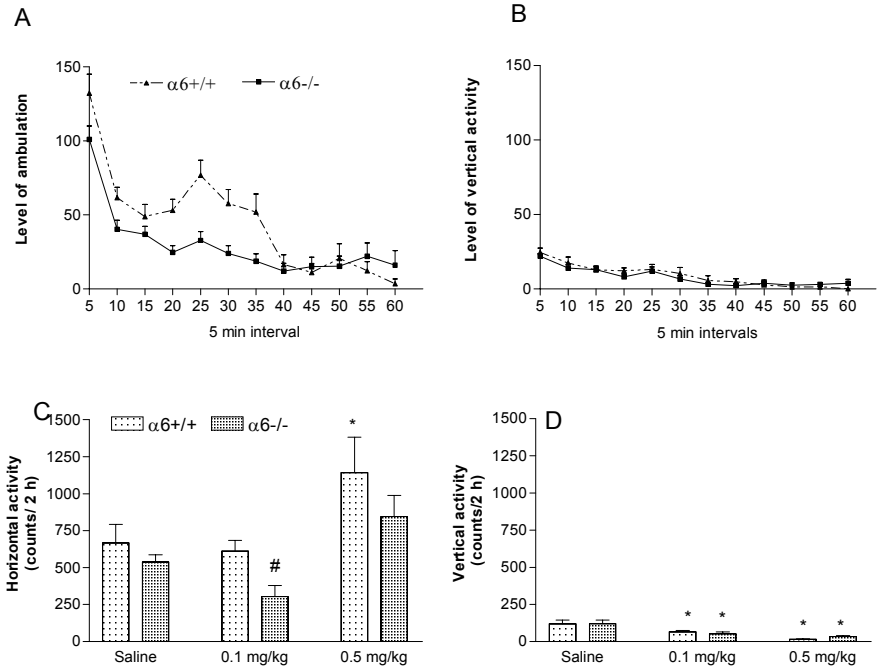

Fig. 5.2. The level of horizontal (A,C) and vertical (B,D) locomotor activity of α6-/- mice and their controls in unfamiliar locomotor cages (A, B) and the effect of MK-801 on the activity (C,D). Points and bars indicate means±SEM. # $p<0.05$ for differences from the corresponding activity of the control line; *$p<0.05$ for differences from saline group within a line; Duncan test.

5.2.2. Ethanol and diazepam effects on motor function and $GABA_A$ receptor binding

The α6-/- mice were clearly more seriously impaired in motor coordination on the rotarod after an acute injection of diazepam, but not after ethanol, as compared to their wild-type controls (III).

Chronic ethanol treatment led to the development of motor tolerance in α6-/- mice on the rotating rod (IV). Since the inactivated α6 gene was so constructed that the *LacZ* gene is expressed under the α6 gene promoter, we sought to establish whether the α6 gene expression was enhanced by ethanol in these mice. However, chronic ethanol failed to affect transcriptional control of the $GABA_A$ receptor α6 gene (IV).

Behavioral tolerance to chronic diazepam treatment developed similarly in both α6+/+ and α6-/- mice. The increase in rectal temperature in diazepam-treated α6-/- mice means that chronically diazepam-treated α6 knockout mice continued to be slightly more responsive to the chosen diazepam treatment regime than their wild-type controls (V). Moreover, vehicle-treated α6-/- mice continued to be more responsive to challenge doses than vehicle-treated wild-type mice.

Chronic diazepam treatment did not affect ligand binding differences in α6+/+ or α6-/- mice. Significant changes were found in [^{35}S]TBPS binding in the presence of GABA and diazepam in vehicle-treated α6-/- mice in comparison with diazepam-treated α6-/- mice in the cerebral cortex (V). Other differences between the mouse lines were associated with the lack of the α6 subunit.

5.2.3. Level of anxiety

The α6-/- mice spent less time on the central square [$F(1,38)=6.5$, $p=0.0152$] and in closed arms [$F(1,38)=5.46$, $p=0.0252$] than the α6+/+ mice (Fig. 5.3.A). Diazepam treatment (0.5 mg/kg or 1.0 mg/kg; i.p.) was ineffective in both α6-/- mice and their controls, as the alterations found were independent of the drug treatment factor. However, strain differences were observed for both saline-treated and diazepam-treated animals [$F(1,112)=25.12$, $p=0.0001$ and $F(1,112)=21.51$, $p=0.0001$ for central and closed arm time respectively].

In the staircase test of exploration (Fig. 5.3.B), the α6-/- mice were less active [$F(1,23)=60.18$, $p=0.0001$], showed lower vertical activity (rears) [$F(1,23)=7.13$, $p=0.0142$], and increased SAP [$F(1,23)=4.8$, $p=0.0398$] as compared to α6+/+ mice. Both α6-/- and α6+/+ mice were insensitive to diazepam injections in this test. The previously observed strain differences remained under diazepam treatment [$F(1,73)=55.88$, $p=0.0001$ for locomotor climbing activity, $F(1,73)=11.78$, $p=0.0010$ for rearing, $F(1,73)=4.17$, $p=0.0450$ for SAP].

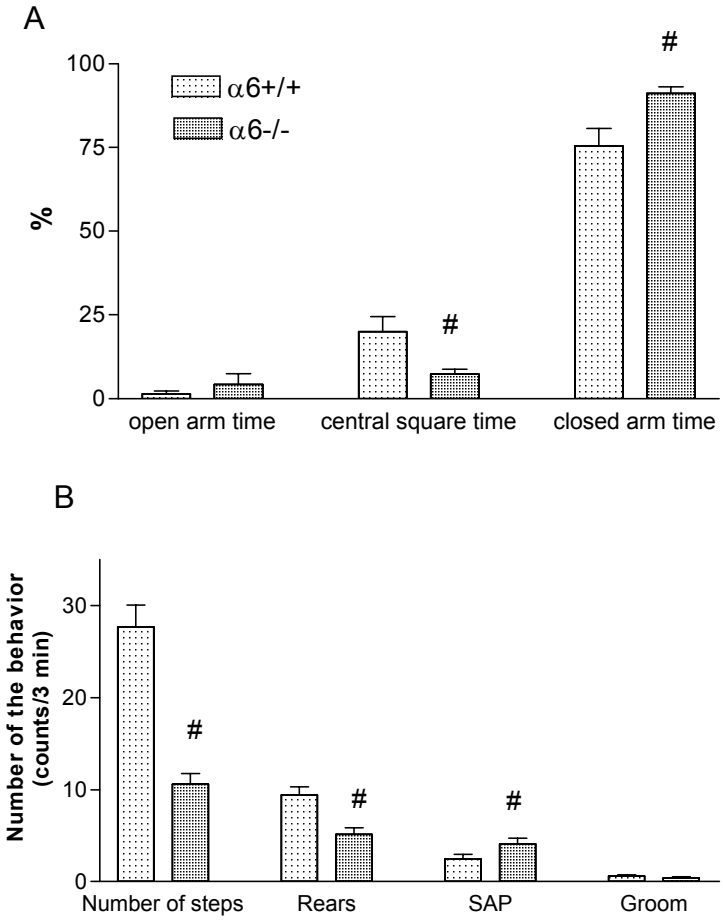

Fig. 5.3. Behavioral profile in plus-maze test (A) and staircase test (B).
Bars indicate means ± SEM.
# $p<0.05$ for differences from the corresponding activity of the control line.

5.2.4. Pain sensitivity

The baseline thermally-evoked pain reaction differed in α6-/- mice and their controls in spinal reflex only (Fig. 5.4.A): an influence of line factor was found for the tail-flick test [$F(1, 94)=31.42$, $p=0.0001$), while in the pain threshold this was not the case. The reaction of both knockout and wild-type mice in the plantar test was thus similar ($F(1,102)=0.01$, ns].

Drug treatment for the tail-flick test (Fig. 5.4.B-F) indicated that 5 mg/kg morphine or 1 mg/kg naloxone were similarly effective on the pain reaction in both α6-/- and α+/+ mice [$F(1,72)=0.13$, ns; $F(1, 64)=2.37$, ns, respectively, for the strain factor], while diazepam treatment (1 and 3 mg/kg) shifted the pain threshold more intensively in α6-/- than α6+/+ mice [$F(1, 127)=7.9$, $p=0.0089$]. Saline-treated α6-/- mice showed longer pain latency than α6+/+ animals at all time points [$F(1,72)=8.5$, $p=0.0101$].

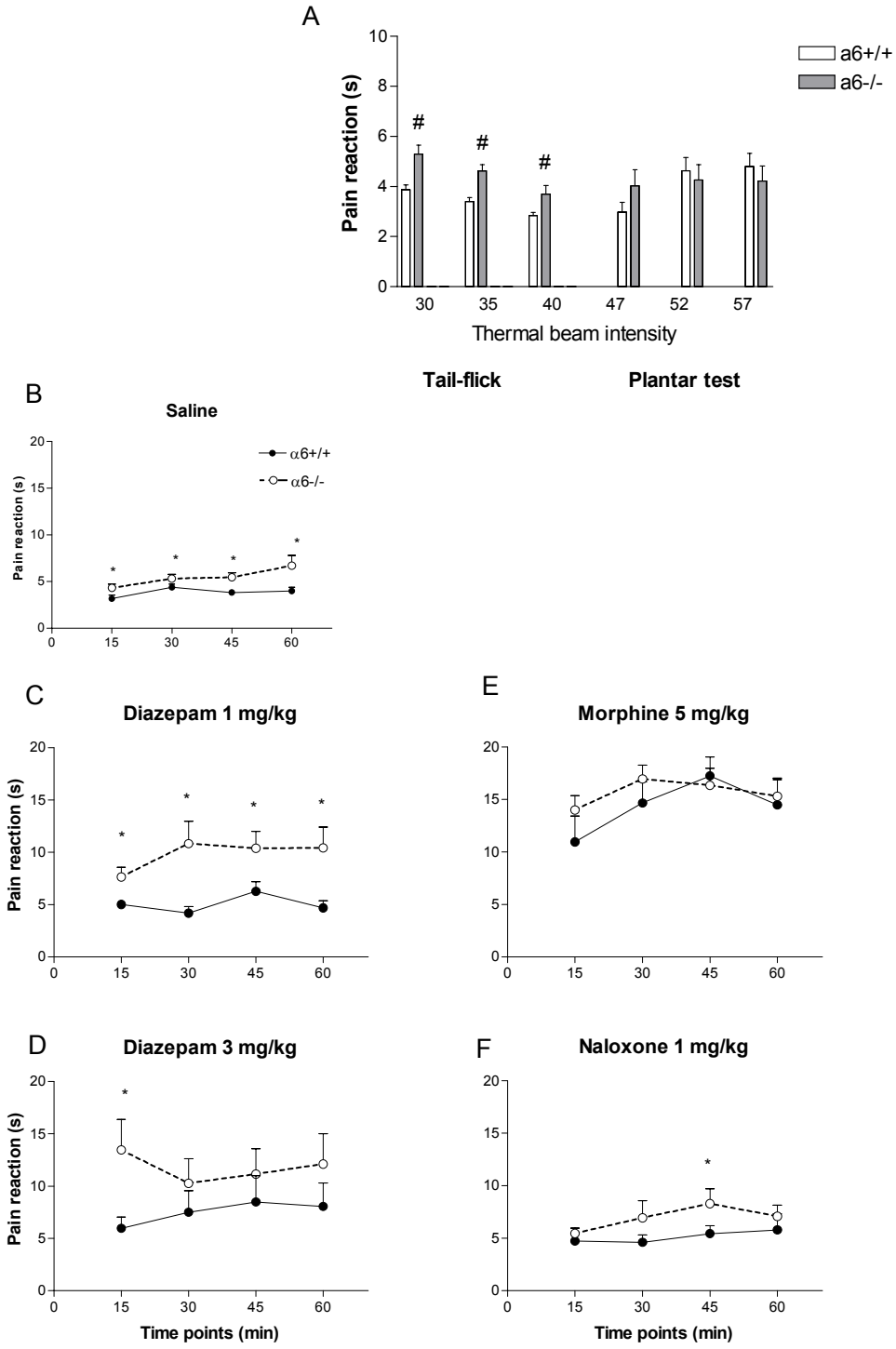

Fig. 5.4. The pain reaction of α6-/- mice and their controls. The initial pain reaction in tail-flick and plantar test (A) and effects of drugs on pain reactivity in tail-flick test (B-F).
Points and bars indicate means ± SEM. # $p<0.05$ for differences from the corresponding activity of the control line; *$p<0.05$ for differences from saline group within a strain; Duncan test.

Table 5.1. The experimental behavioral tests, drug treatments and findings for α6-/- and α6+/+ mice.

Experimental task	Test	Treatment	Summary of results
Motor abilities	Free loco-motion	Drug free	Level of ambulation: α6-/- < α6+/+ (see Results, Fig. 5.2.A, B)
		MK-801 (0.1 & 0.5 mg/kg)	Sensitivity to MK-801: α6-/- < α6+/+ (see Results, Fig. 5.2.C, D)
	Rotarod test	Drug free	Motor performance: α6-/- = α6+/+ (Fig. 5.1).
To determine whether the α6 gene is activated by chronic ethanol in the absence of functional α6 subunit		Chronic ethanol	Chronic administration of moderate ethanol doses leading to motor tolerance does not affect the transcriptional control of the α6 subunit gene (II)
		Chronic diazepam	Motor tolerance upon chronic administration of moderate diazepam doses: α6-/- = α6+/+ Response to challenge diazepam doses: α6-/- > α6+/+ (V)
Level of anxiety	Plus-maze test	Drug-free	Time in closed arms: α6-/- > α6+/+ Time on central square:α6-/- < α6+/+ Level of anxiety: α6-/- ≥ α6+/+ (see Results, Fig. 5.3.A)
		Diazepam (0.5; 1.0 mg/kg)	Diazepam effects: α6-/- = α6+/+ (see Results)
	Staircase test	Drug-free	Number of traversed steps: α6-/- < α6+/+ Number of SAP: α6-/- > α6+/+ (see Results, Fig. 5.4.B). Gender factor significant in latency to move
		Diazepam (0.5; 1.0 mg/kg)	No changes in number of steps by the drug in either mouse line. Number of rears by dose 1 mg/kg: α6-/- < α6+/+ (see results)
Pain sensitivity	Tail-flick; Plantar test	Drug-free	Pain sensitivity by tail-flick: α6-/- < α6+/+ Pain sensitivity by plantar test: α6-/- = α6+/+ (see Results, Fig. 5.4.A)
	Tail-flick	Morphine 5 mg/kg (s.c.) Naloxone 1 mg/kg (s.c.) Diazepam 1.0; 3.0 mg/kg (i.p.)	Morphine analgesia: α6-/- = α6+/+ Naloxone effect on nociception: α6-/- = α6+/+ Diazepam analgesia: α6-/- > α6+/+ (see Results, Fig. 5.4.B-F)

6. DISCUSSION

6.1. Motor performance is not disrupted by the lack of α6-containing receptors

The general methodological approach was designed to check functional changes in the cerebellum in both rat and mouse models involving disruptions in α6 subunit-containing cerebellar $GABA_A$ receptors. The mouse ability to perform distinct motor tasks such as remaining on the accelerating rod, lifting hind paws, descending the subjacent platform, and swimming, was tested in first place. On the whole, motor-related phenotype was not significantly disrupted by the lack of α6-containing receptors under drug-free conditions despite the cerebellum-associated modes of motor function (Bahn et al. 1996), and despite the fact that $GABA_A$ receptor-mediated inhibition of granule cells is critical for normal cerebellar function (Maex and Schutter 1998). The mutant α6-/- mice are viable and fertile, and evince grossly normal brain cytoarchitecture (Homanics et al. 1997a, III).

Granule cells receive a continuous, or 'tonic', synaptic input, which increases their tonic membrane conductance, and so modifies the excitability of granule cells. Tonic inhibition mediated by α6-containing $GABA_A$ receptors is capable of altering the response of granule cells to excitatory synaptic input and modulates granule cell excitability (Brickley et al. 1996). Despite the loss of tonic conductance of $GABA_A$ receptors, granule cells in α6-/- mice have membrane properties similar to those of α6+/+ mice. An unaltered response to depolarizing current injection or excitatory postsynaptic potential properties in α6-deficient mice was explained to be due to compensation. This compensation was found in an increased leak K^+ conductance in α6-/- mice which was similar to the magnitude of tonic conductance in wild-type granule cells and maintained a normal input conductance. This balanced input conductance in α6-/- mice might prevent inappropriate activation of granule cells and compensate motor deficit in adult α6-/- mice (Brickley et al. 2001).

6.2. The lack of α6-containing receptors results in increased sensitivity to diazepam but not to ethanol

If the phenomenon of undisturbed motor skills were explained as due to possible compensatory mechanisms (Brickley et al. 2001), the discovery of increased sensitivity to diazepam but not to ethanol was surprising. Initially, α6 subunit-containing $GABA_A$ receptors appear to be benzodiazepine-insensitive (Korpi et al. 1993), but genetically induced inactivation of the subunit led to strongly impaired learned motor performance in response to acute diazepam in comparison with wild-type controls (III). The BZ site antagonist flumazenil reversed these diazepam effects, thereby displaying the involvement of the remaining α1β2/3γ2 $GABA_A$ receptors at the Golgi neuron-granule cell synapse (Fig. 2.2).

No dramatic effect of moderate chronic ethanol doses on α6 subunit gene transcription was found, although the development of behavioral ethanol tolerance

was clearly observed in α6-/- mice (IV). Because of the absence of wild-type control it is impossible to evaluate the relationship between α6 subunit expression and this developing ethanol tolerance. Studies by another research group have indicated normal ethanol sensitivity in α6 knockout mice as compared with their wild type control (Homanics et al. 1997a,1998b): similar ataxia and sleep time response after an acute injection of ethanol and a similar reduction in both effects after the development of tolerance to repeated ethanol inhalations.

The failure to find increased α6 expression associated with the ethanol tolerance developed means that the elevated expression of α6 subunit mRNA and the protein induced by chronic ethanol treatment (Mhatre and Ticku 1992) cannot be due to direct activation of gene transcription, unless the regulation of this gene differs between the rat (Mhatre and Ticku 1992) and the mouse (IV). Probably, the administration of very high ethanol concentrations could affect the changes in gene expression. On the other hand, the expression of α6 subunit mRNA in ethanol-naïve withdrawal seizure-prone mice is about 60% of that in withdrawal seizure-resistant animals, which would indicate that the amount of the α6 subunit may be important for genetic differences between the mouse lines but not for ethanol tolerance development (Buck et al. 1991).

Chronic diazepam treatment produced a similar development of behavioral tolerance in both α6-/- and α6+/+ mice. However, it should be noted here that α6-/- mice showed increased sensitivity to challenge diazepam doses under the chronic procedure: the wild-type α6+/+ vehicle-treated mice developed tolerance to challenge doses more quickly than α6-/- mice. This means that α6-/- mice need heavier exposure or a longer period of diazepam treatment than their controls to develop the tolerance (V).

Brain-regional binding analysis with [^{3}H]Ro 15-4513 and [^{35}S]TBPS brought out no differences associated with chronic diazepam treatment in either mouse line, although it has been suggested that functional and behavioral tolerance to chronic BZ exposure is associated with an uncoupling of the BZ and GABA binding sites (Ali and Olsen 2001). We found that GABA (0.5 μM) inhibition of [^{35}S]TBPS binding was more markedly potentiated by 1 μM diazepam in the cerebral cortex of diazepam-treated α6-/- mice in comparison with vehicle-received α6-/- mice, suggesting sensitization rather than tolerance (V).

According to other studies, chronic exposure of cortical cultures to flurazepam (Hu and Ticku 1994) as well as withdrawal from chronic treatment with lorazepam and diazepam (Braestrup et al. 1979, Gallager et al. 1984) do not alter [^{3}H]Ro 15-4513 or [^{35}S]TBPS binding. However, the coupling of the GABA sites with the receptor-associated anion channels is reduced after chronic diazepam administration (Gallager et al. 1984), a result also observed *in vitro* in recombinant receptors (Ali and Olsen 2001). These findings can explain why no brain regional ligand binding differences associated with chronic diazepam treatment were found in the α6+/+ mice, although behavioral tolerance was observed (V).

On the other hand, additional effects of chronic diazepam administration on [^{35}S]TBPS binding were found in the case of chronically diazepam-treated α6-/- and α6+/+ mice in the presence of 3 μM GABA in the thalamic area: the binding in diazepam-dependent α6-/- mice appeared less sensitive to 3 μM GABA than their chronically treated α6+/+ controls. Another small mouse line difference in the thalamic and cerebellar granule cell layer areas was observed in the presence of 1

μM DMCM on 3-μM GABA-inhibited [^{35}S]TBPS binding: the DMCM effect was greater in the vehicle-treated α6-/- than vehicle-treated α6+/+ mice.

The findings on receptor binding level indicate that chronic treatment with diazepam is associated with small differences between the mouse lines (III), but we conclude that the development of tolerance to diazepam-induced ataxia is not dependent on the presence of $GABA_A$ receptor α6-subunits in the cerebellum.

6.3. The lack of α6-containing receptors induces changes in other $GABA_A$ receptor subtypes

One reason why the lack of α6 subunit resulted in increased diazepam but not ethanol sensitivity, lies in changes provoked in other receptor subunits (Uusi-Oukari et al. 2000a). The α6 subunit-containing $GABA_A$ receptor subtypes represent approximately 45% of cerebellar receptors, and their absence has led to a reduction in the basal binding of the radioactive ligands, [^{3}H]SR 95531, [^{3}H]Ro 15-4513 and [^{35}S]TBPS, which bind to most $GABA_A$ receptor subtypes in the granule cell layer (Jones et al. 1997, Mäkelä et al. 1997, Nusser et al. 1999). Moreover, the lack of subunit α6 is associated with reduced β2, β3, and γ2 subunit proteins in the cerebellum (Jones et al. 1997) as well as α1 and β2 subunit mRNA and protein in the forebrain (Uusi-Oukari et al. 2000a), this resulting in slightly decreased density of receptors containing these subunits. Only one type of $GABA_A$ receptor with high affinity to diazepam remains in the cerebellum of α6-/- mice: α1β2/3γ2 (Fig. 2.2). This absence of other $GABA_A$ receptor subtypes may explain the enhanced diazepam-induced ataxia seen in α6-/- mice. On the other hand, the precise molecular sites of ethanol, which have many signs, symptoms and mechanisms in common with those of barbiturates and benzodiazepines (Sellers 1988, Buck et al. 1991), are as yet incompletely charted (Franks and Lieb 1997). Thus, the lack of high ethanol affinity and pharmacological specificity (Deitrich et al. 1989) may mean realization of the effects of ethanol through other molecular targets than $GABA_A$ receptors.

The lack of the α6 subunit also causes a marked reduction in the level of the δ subunit protein in spite of normal subunit mRNA levels (Jones et al. 1997). The need of the α6 subunit for the δ subunits to be assembled into native cerebellar granule cell $GABA_A$ receptors, even in the presence of other α subunits, is evidenced in the reduced binding of [^{3}H]muscimol, high-affinity with δ-containing receptors, to GABA sites in cerebellar sections from α6-/- mice (Mäkelä et al. 1997). The binding is also reduced in the cerebellum and in the forebrain of δ-deficient mice (Mihalek et al. 1999), although the behavioral changes are different when the subunit is completely inactivated. Thus, the associated reduction in cerebellar δ-containing receptors in α6-/- mice does not affect the development of ethanol tolerance and withdrawal responses (Homanics et al. 1998b), while δ-/- mice display attenuated ethanol withdrawal and other changes in ethanol-associated behaviors such as reduced ethanol consumption and a reduced anticonvulsant effect of ethanol (Mihalek et al. 2001).

6.4. Hypothetical partnership between distinct types of $GABA_A$ receptors

BZ binding sites can be formed by N-terminal extracellular domains in $GABA_A$ receptor α1 and γ2 subunit dimers, whereas [^{3}H]muscimol binding requires α1 together with β3 subunits (Klausberger et al. 2001a, b). Judging by the analogy of subunit interactions, a similar interconnection could exist between functional assemblies, or distinct $GABA_A$ receptor subtypes (Moss and Smart 1996). The lack of the α6 subunit and, consequently, of the δ subunit might then disrupt this kind of interaction and the remaining α1β2/3γ2 assemblies would be deprived of α6δ subunits, this resulting in an increased diazepam effect. This means that α6 subunit-containing $GABA_A$ receptor subtypes suppress the BZ site sensitivity of α1-containing receptors in the normal cerebellum by preventing (or regulating, or concealing) their *in vitro* binding effects.

Such a conception is supported by the experimental findings. Thus, the explanation for the increased motor-impairment by diazepam but not ethanol becomes more acceptable in that, as noted above, diazepam is much more selective for BZ sites in $GABA_A$ receptors than ethanol for $GABA_A$ receptors. Although many ethanol effects are mediated through $GABA_A$ receptors (Ticku 1990, Korpi 1994, Metten and Crabbe 1999), it equally affects other neurotransmitter systems (Overstreet et al. 1994, Söderpalm et al. 2000, Wilce et al. 2001).

6.5. The lack or point mutation in $GABA_A$ receptor α6 subunit affects NMDA receptor-related behaviors

The failure to explain the enhanced ethanol sensitivity of the ANT rats solely on the basis of the point mutation (Korpi et al. 1992) led to the suggestion that NMDA receptors could be involved in ethanol sensitivity differences between ANT and AT rats. While no differences between the lines were found in the binding of a noncompetitive NMDA receptor antagonist, [^{3}H]MK-801, in various brain regions (Näkki et al. 1995), in the ethanol-induced decrease in cerebellar and hippocampal cGMP levels (Toropainen et al. 1997), or in alteration of NMDA receptor subunit mRNA expression, MK-801 by itself stimulated locomotion more potently in ANT than AT rats. This effect did not involve dopaminergic or GABAergic mechanisms (Toropainen et al. 1997, II).

The finding that NMDA receptor-mediated processes may correlate with innate alcohol sensitivity, as shown for the ANT/AT rat model (II), gave impulse to check the effect of MK-801 on locomotion in α6-/- mice. These mice were insensitive to MK-801, while their wild-type controls normally increased the horizontal activity shown for rodents (Kuribara et al. 1992). This implies that the point mutation in the α6 subunit leads to increased sensitivity to MK-801, while lack of the subunit results in insensitivity to the stimulus effect of MK-801. Interpretation of this result is difficult in that the connections between receptors of different neurotransmitter systems are not fully understood. However, there is compelling evidence in primary cultures of rat cerebellar granule cells to indicate that the regulation of mRNA expression of various $GABA_A$ receptor subunits is controlled by activation of the NMDA receptor complex (Memo et al. 1991). Moreover, an association has been demonstrated between NMDA receptor-

mediated calcium ion conductance and selective change in the $GABA_A$ receptor complex in the cerebral cortex (Deutsch et al. 1995). It might be suggested that the delicate balance between GABAergic and glutamatergic transmission (Deutsch et al. 1995) is two-way. Since no significant changes were found for NMDA receptors in the ANT rats (Toropainen et al. 1997), the subunit can be only indirectly involved in the modulation of NMDA receptor sensitivity.

6.6. The two animal models used to study behavioral effects of α6-containing $GABA_A$ receptors are inherently different

The ANT rats evince increased sensitivity to both ethanol and diazepam, suggesting that the point mutation making α6 subunit-containing $GABA_A$ receptors diazepam-sensitive does not disrupt the $GABA_A$ subtype hypothetical interactions but supplements BZ sites which enhance both ethanol and diazepam effects. The complete lack of the α6 subunit results in increased diazepam- but not ethanol-induced ataxia. Stimulation of locomotor activity by the noncompetitive NMDA receptor antagonist MK-801 results in the opposite effects in rats having a point mutation in the subunit and in α6-deficient mice. It seems that the α6-/- mouse model and the rat model are inherently different and that only the former allows assessment of the role of the α6-containing $GABA_A$ receptors in the cerebellum. It might however be borne in mind that also the forebrain $GABA_A$ receptors were altered in the present α6-/- mice (Uusi-Oukari et al. 2000b).

6.7. The lack of the α6 subunit slightly disturbs the emotional state

The lack of subunit α6 in α6-/- mice led to a slightly increased level of anxiety; these mice showed slightly suppressed locomotion in unfamiliar boxes, spent more time in the closed arms of plus-maze, and traversed fewer steps in the staircase. The reduced locomotor activity disappeared after habituation, suggesting an emotional reason for the initial suppression rather than a motor upset not indicated for the α6-/- mouse behavioral profile (III). The observed behavior could also be a result of changes in forebrain $GABA_A$ receptors following the lack of the α6 subunit in the cerebellum (Uusi-Oukari et al. 2000b).

The effect of diazepam was not significant in the plus-maze test either for the α6-/- or the α6+/+ mice but equalized the difference between them. Diazepam also suppressed the number of rears on the staircase in α6-/- mice (previously unpublished data; see Table 5.1). If vertical activity combines exploratory behavior and anxiety (Blanchard et al. 1993), its decreasing level means that the sensitivity of α6-/- mice to the anxiolytic effect of diazepam is higher than that of the wild type control. Low diazepam doses exclude the suppressive diazepam effect in the staircase test.

Increased activity in open arms in the plus-maze test induced by diazepam was found in the ANT rats (I). This enhanced responsivity to diazepam is consequent upon the α6 subunit mutation, making previously diazepam-insensitive α6 subunit-containing $GABA_A$ receptors diazepam-sensitive. This explains why low doses were effective for ANT but not for AT rats.

The cerebellum might not be an important brain structure for emotional status (Grey and Mcnaughton 2000), but some recent studies indicate that it is involved in processing information with emotional and cognitive value together with the amygdala, dorsal periaqueductal grey matter, dorsomedial hypothalamus and septal nuclei (Bloedel and Bracha 1997, Schmahmann and Pandya 1997, Tesche and Karhu 1997). Our study with the ANT rats also supported the conception of an active role of the cerebellum for emotional status, but this was not confirmed in experiments with the α6-/- mice.

6.8. The lack of the α6 subunit causes slight disturbance in pain reactivity

The lack of the α6 subunit increased pain reactivity in α6-/- mice in analgesic tests, suggesting participation of cerebellar GABAergic mechanisms in the spinal process of thermally-evoked pain. Here the GABAergic pathways could be involved, since the cerebellum is generally known for its lack of opiate receptors (Sivam et al. 1982, Yanez et al. 1990). The differences noted between the mouse lines in the tail-flick but not in the plantar test (Table 5.1) indicate that the lack of the α6 subunit affected pain reactivity on spinal level while superspinal pain mechanisms continued to be similar. The pain reaction in α6-/- mice was slightly drugged and specifically diazepam-sensitive, while classical analgesic morphine and its antagonist naloxone affected the pain reaction similarly in both α6+/+ and α6-/- mice. A low diazepam dose (1 mg/kg) reduced pain sensitivity (increased latency of pain reaction) in α6-/- mice while a moderate diazepam dose induced analgesia in α6-/- mice during the first 15 minutes. Since the motor ability in α6-/- mice was normal (III), their initial increased pain threshold cannot be explained by any motor difficulties. Reduced pain reactivity in α6-/- mice previously subjected to drug treatment indicates that α6-containing receptors could participate in spinal pain mechanisms. The efficacy of such small diazepam doses could be explained by the increased diazepam sensitivity of the remaining α1β2/3γ2 subtype $GABA_A$ receptors in the absence of α6 subunit-containing receptors.

Behavioral study of the role of $GABA_A$ receptor α6 subunit thus indicated that the mutation in the subunit and complete lack of the subunit might exert their effects on behavior through distinct mechanisms and reflect different aspects of receptor mechanisms. The lack of the α6 subunit in cerebellar $GABA_A$ receptors indicated that this subunit is not crucial or is fully compensated by other mechanisms for motor functions and learning motor performance. Its absence leads to very slight disturbances in emotional state and pain reactivity, and in increased diazepam- but not ethanol-induced ataxia. The present results would suggest a hypothesis whereby the functional role of the α6 subunit consists in its interactive linking of $GABA_A$ receptor subtypes in the cerebellum. Since the present α6-/- model suffers from a technical problem arising from the neomycin gene effect on forebrain $GABA_A$ receptor expression (Uusi-Oukari et al. 2000a), future models should be generated to test more selectively the cerebellar granule cell receptor subtypes.

7 CONCLUSIONS

1 The enhanced BZ action on the cerebellar granule neurons seen here in the mutant alcohol-sensitive ANT rats may be associated with an anxiolytic response. The data supported the conception that the cerebellum is capable of mediating emotional status.

2 ANT rats are more sensitive to the noncompetitive NMDA receptor antagonist MK-801- and ketamine-induced stimulation of locomotor activity than the alcohol-insensitive AT rats. The results do not suggest any direct involvement of the ANT rat α6 subunit point mutation in the differences between the rat lines in sensitivity to NMDA receptor antagonists.

3 $GABA_A$ receptor α6-/- mice showed normal performance in motor tasks and motor learning. These mice were more seriously impaired by acute diazepam, but not by ethanol, than their wild-type controls. The enhanced behavioral diazepam sensitivity was reversed by the BZ site antagonist flumazenil, indicating the involvement of the remaining α1β2/3γ2 $GABA_A$ receptors in the granule cells.

4 The α6-/- mice showed ethanol tolerance, indicating that the α6 subunit was not crucial for its development. Chronic administration of moderate ethanol doses did not affect the transcriptional control of the α6 subunit gene. The development of diazepam tolerance to moderate chronic diazepam treatment was slightly slower in the knockout mice, but ligand autoradiography revealed no clear changes in brain $GABA_A$ receptors in the wild-type or knockout mice by diazepam.

5 The lack of subunit α6 led to slightly increased anxiety and slowed latency to pain reaction in α6-/- mice. Curiously, diazepam acted here as an analgesic, but not clearly an anxiolytic. The α6-/- mice were not sensitive to the motor-stimulant effect of noncompetitive NMDA glutamate receptor antagonist MK-801, unlike the ANT rats.

The data obtained here indicate that the knockout mouse and mutant rat models are inherently distinct and allow study of different aspects of cerebellar $GABA_A$ receptor function.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Pharmacology and Clinical Pharmacology in the University of Turku, during the years 1998-2002. The writing of the summary was completed at the Pharmacology Department in the Institute of Biomedicine, University of Helsinki, during the first months of the year 2003.

During the five-year period the study was supported by the International Graduate Programme in Neuroscience, University of Tampere, The Centre for International Mobility (CIMO), the Academy of Finland, TEKES, the Finnish Foundation for Alcohol Studies, and the Sigrid Juselius Foundation.

This period of active work in Finland has been an important experience for my scientific life and I remain indebted to all those who supported and helped me during that time.

I extend my deep appreciation to my first supervisor in Finland, Professor Esa R. Korpi, M.D., Ph.D. I thank him warmly for his patience, tact and moral support to finish the study successfully. His vital energy helped me to develop new scientific skills and to grow up as a researcher.

I wish to express profound thanks to docent Mikko Uusi-Oukari, Ph.D., my second supervisor, co-author and friend, who never refused to help me and who was open to my questions every day.

I am deeply grateful to Professor Simo S. Oja, M.D., Ph.D., my third supervisor, who supported me with much success from Tampere. His advice and diplomacy helped to finally realize the project.

I would also express my special thanks to the official reviewers of my thesis for their responsive reading, valuable comments and constructive criticism: Professor Sture Liljequist, Karolinska Institute, and Docent Tomi Taira, Ph.D., University of Helsinki.

I am also very grateful to Mr. Robert MacGilleon, M.A., for his expert revision of my English language and to Mrs. Sari Luokkala for her skilful secretarial help in preparing this manuscript.

I wish to thank all personnel at the Department of Pharmacology and Clinical Pharmacology with whom I have been working in Turku. The charm of the town with its friendly atmosphere facilitated my work. I am sincerely grateful to Professor Mika Scheinin for allowing me to use the excellent facilities for my studies. I am especially thankful to our secretaries: Ulla-Elina Hurme, Anja Similä, Marjaana Stedt and Hanna Tuominen, who were always concerned about me and helped me to understand many official papers.

I wish to acknowledge a profound dept to all my co-authors, without whom the work could not have been carried out: Antti Haapalinna, Riitta Näkki, Maija Sarviharju, Aapo Honkanen, Jari Heikkilä, Paula Koikkalainen, Riikka Mäkelä, Raymonde Kleinz, and William Wisden. I am also very grateful to Eija Lehtovirta for her valuable help with autoradiography study.

I have appreciated my contacts with people from the GABA group in Turku over the last 5 years for constructive scientific discussions and the interest they showed in my research work.

I am grateful to my new colleagues at the Department of Pharmacology in the University of Helsinki, where I finished writing the thesis, for their friendly attitude towards me from the beginning of my work there.

I am greatly indebted to my first supervisor and teacher Professor Edvin Zvartau, MD, Ph.D., in Saint-Petersburg, who guided my first thesis in Russia and continues to encourage me.

I treasure my colleagues from the laboratory of Behavioral Pharmacology l, Pavlov Medical University in Saint-Petersburg, who are at the same time my close friends and collaborators: Anton Bespalov, Irina Belozertceva, Irina Sukhotina, Elena Verbitskaja, Olga Dravolina, Vladimir Kashkin, Andrei Malishkin, and Ivan Medvedev. I highly value their support and participation in my life which allows to feel more confident a long way from home.

I wish to thank all my friends for never ending our relations overthis long period. My warm thanks to my dear friends, almost my family, Marija Shishova, Vlad Emeljanov, Elena Lyakso and Anastasija Vinogradova. Especially my sincere thanks go to my close-knit group of companions in Turku making my life full of human warmth: Margo and Andrei Glazov, Anna and Nikolai Bogdanov, Slava Koblik, Andrei Tauber and Andrei Berdyugin. I very much appreciate other relationships established in Finland and the numerous nice people I have met.

I wish to give my warmest thanks to my daughter Natalia for her keeping the home fires burning. I am also greatly indebted to Maija Aleksandrovna for her invaluable concern for Natalia and her belief in me.

Helsinki, March, 2003

Olga Vekovischeva

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11. APPENDIX: ORIGINAL PUBLICATIONS I-V