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REVIEW

Tolerance to allopregnanolone with focus on the GABA-A receptor

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Many studies have suggested a relationship between stress, sex steroids, and negative mental and mood changes in humans. The progesterone metabolite allopregnanolone is a potent endogenous ligand of the γ-amino butyric acid –A (GABA-A) receptor, and the most discussed neuroactive steroid. Variations in the levels of neuroactive steroids that influence the activity of the GABA-A receptor cause a vulnerability to mental and emotional pathology. There are physiological conditions in which allopregnanolone production increases acutely (e.g. stress) or chronically (e.g. menstrual cycle, pregnancy), thus exposing the GABA-A receptor to high and continuous allopregnanolone concentrations. In such conditions, tolerance to allopregnanolone may develop. We have shown that both acute and chronic tolerances can develop to the effects of allopregnanolone. Following the development of acute allopregnanolone tolerance, there is a decrease in the abundance of the GABA-A receptor α4 subunit and the expression of the α4 subunit mRNA in the ventral-posteriomedial nucleus of the thalamus. Little is known about the mechanism behind allopregnanolone tolerance and its effects on assembly of the GABA-A receptor composition. The exact mechanism of the allopregnanolone tolerance phenomena remains unclear. The purpose of this review is to summarize certain aspects of current knowledge concerning allopregnanolone tolerance and changes in the GABA-A receptors.

Abbreviations

CNS, central nervous system; EEG, electroencephalography; GABA-A, γ-amino butyric acid –A; HRT, hormone replacement therapy; NMDA, N-methyl-D-aspartic acid; PMDD, premenstrual dysphoric disorder; PMS, premenstrual syndrome; SS, burst suppression of 1 s or more in the EEG (silent second); THDOC, tetrahydro-deoxycorticosterone; VPM, ventral-posteriomedial nucleus

Introduction

The development of tolerance to foreign compounds with effects in the central nervous system (CNS) is a common phenomenon. Whether tolerance can occur with endogenous substances and in specific disorders is less well known. The purpose of this review is to examine the possibility that chronic production of steroids active on the γ-amino butyric acid –A (GABA-A) receptor may induce tolerance under certain conditions. In particular, inhibitory compounds working on the GABA-A receptor are known to induce tolerance. Well-known examples are benzodiazepines, barbiturates and alcohol, all of which enhance the inhibitory effect of GABA on the chloride ion flux through the GABA-A receptor (Allan *et al.*, 1992; Pesold *et al.*, 1997; Wallace *et al.*, 2007). Endogenous GABA-A receptor agonists exist in the 3α-hydroxy metabolites of progesterone, testosterone and deoxycorticosterone, known as allopregnanolone, pregnanolone, androstanediol and tetrahydro-deoxycorticosterone (THDOC). These compounds are steroids and act as agonists at the GABA-A receptor, and hence are known as GABA steroids (Mellon and Griffin, 2002; Backstrom *et al.*, 2003). The first evidence for the development of tolerance to chronic pregnanolone exposure in cell cultures was provided by Friedman and colleagues (Friedman *et al.*, 1993). Another study showed that chronic

allopregnanolone treatment in cell cultures reduced the receptor responses and down-regulation of the receptor content, suggesting a classical tolerance development (Yu *et al.*, 1996a).

γ-amino butyric acid steroids are produced in high quantities giving substantial serum concentrations in specific situations at which tolerance to endogenous allopregnanolone exposure can occur in humans. In women, large amounts of the GABA steroid allopregnanolone are produced during the luteal phase of the menstrual cycle, during pregnancy and during hormone replacement therapy, which includes progesterone (Luisi *et al.*, 2000; Bicikova *et al.*, 2002; Ottander *et al.*, 2005; Andreen *et al.*, 2006; Nyberg *et al.*, 2007). In male and female humans and animals, high concentrations of both allopregnanolone and THDOC are produced during acute stress (Purdy *et al.*, 1991; Droogleever Fortuyn *et al.*, 2004). However, during chronic stress the concentrations decrease somewhat, at least in animals (Serra *et al.*, 2000). This is similar to the situation for cortisol, where the blood concentration decreases under chronic stress (Yehuda, 2001). Due to metabolism allopregnanolone serum concentrations in women are highly correlated to progesterone levels during the luteal phase and during pregnancy (Luisi *et al.*, 2000; Nyberg *et al.*, 2007). THDOC and allopregnanolone are during stress correlated to cortisol and to each other, and also stimulated by corticotrophin-releasing hormone and adrenocorticotropic hormone (Meczekalski *et al.*, 2000; Droogleever Fortuyn *et al.*, 2004; Reddy, 2006; Girdler and Klatzkin, 2007)

The question arises whether endogenously produced GABA-A receptor active compounds produced over a long period, giving high serum concentrations, can induce tolerance. A second question is whether, and in what areas of the brain, there are changes in the GABA-A receptor due to tolerance development. A third question is whether the induced GABA-A receptor tolerance is involved in CNS-related disorders, and how the signs of tolerance in pathological situations and disorders may be recognized. One sign of endogenously induced tolerance could be that the sensitivity to other GABA-A receptor active compounds is decreased due to cross-tolerance between GABA steroids and other GABA-A receptor active compounds. A second sign could be withdrawal symptoms at a state where the endogenous production of the GABA-A receptor agonists is rapidly ended. This would reveal compensatory changes responsible for the tolerance and might indicate a down-regulated GABA-A receptor function. In women, a rapid decrease in GABA steroid production occurs for example during menstruation at the end of the luteal phase, and during the postpartum period when steroid production rapidly declines. In both men and women, high production of GABA steroids occurs during stress, and a withdrawal situation may thus occur when the person relaxes after a period of increased stress.

Definitions of tolerance

The effect of a certain substance can change through repetitive or constant input of that substance. It is common knowledge that regular users of sedative drugs and alcohol generally become able to tolerate larger amounts of these substances after repeated exposures. In other words, the same dosage does not have the same clinical effect and the dose must be increased in order for the initial effect to be reached. Thus this acquired tolerance is a reduced effect of a defined dose or a need to increase the dose to reach a defined effect and can be subdivided in the following types: pharmacodynamic, pharmacokinetic and learned tolerance with the two subgroups behavioural or conditioned tolerance (O'Brien, 2006). In this subdivision acute tolerance is also a separate entity but the possible relation to chronic tolerance was discussed already in the review by Kalant and coworkers (Kalant *et al.*, 1971).

Pharmacodynamic tolerance (functional tolerance) can be regarded as a defence against the actions of the used drug. The primary components involve a decrease in sensitivity in the target tissue when this tissue is influenced by an active concentration of the drug (tolerance at the receptor site). However, the decreased function can also partly be compensated by increased activity in other brain systems as illustrated by the activity in the muscarinic component of the cholinergic system after long-term exposure to barbiturate, ethanol and benzodiazepines (Nordberg and Wahlstrom, 1992). A related compensatory brain mechanism is the learned behavioural or conditioned tolerance. As shown already by Goldberg (1943) this type of tolerance is similar to a pharmacodynamic tolerance and tends to reduce the drug effects with extended exposure. However, from the psychological point of view, learned tolerance is interesting, as it shows that compensatory mechanisms can be remembered and reduces pharmacological effects. However, also the learned effects are due to the pharmacological effects of the drug. Thus also in learned tolerances, the organism learns to decrease the behavioural effect of a certain dose of the drug (Siegel, 1989). We know that tolerance need not solely be due to the mechanisms that are directly induced by the drug, but could also be due to secondary compensatory mechanisms that are indirectly influenced by the primary drug effect. An example is the spatial learning in the Morris maze where different treatments with allopregnanolone before the initial test did not influence the inhibitory effects of allopregnanolone on learning during the initial session but showed a clear decreasing inhibitory effect with increased learning at the following daily sessions (Turkmen *et al.*, 2006). Learned tolerance probably induced some complex changes responsible for these secondary effects. In conditioned tolerance Pavllovian conditioning could be involved (Siegel, 1989) but at present, no general agreement exists on the mechanisms underlying behavioural tolerance (Leblanc *et al.*, 1976; Demellweek and Goudie, 1983; Tiffany *et al.*, 1992).

Pharmacokinetic tolerance (dispositional or metabolic tolerance) comprises changes in drug absorption, distribution, excretion and metabolism by which the body increases its ability to get rid of the drug; for example, an increase in the level of enzymes in the body that breaks down the drug can lead to a reduction in the concentration and duration of drug contact in the target tissues. The term usually describes a phenomenon of increased drug clearance (Kalant *et al.*, 1971). In fact, as all effects of a drug have a pharmacological basis, it cannot be restricted to one kind of pharmacological effect (dynamic or kinetic). It must be pointed out that the different mechanisms of tolerance discussed above are not mutually exclusive; pharmacodynamic and pharmacokinetic

changes can for instance be found at the same time (Wahlstrom and Bolander, 1985; Kalant, 1993; Wahlstrom, 1998).

It is known that sensitivity to a substance can decrease after a single administration (acute tolerance) or after multiple exposures to the substance (chronic tolerance) (Kalant *et al.*, 1971). The term 'rapid tolerance' has been suggested by some investigators to describe a decrease in sensitivity to a drug hours after the duration of a single exposure to that drug (Kalant, 1993). A distinction is also made between tolerance to a specific drug, to related drugs (selective or homologous cross-tolerance) and to drugs from unrelated drug classes (heterologous cross-tolerance) (Kalant *et al.*, 1971; Greenblatt and Shader, 1978).

The GABA-A receptor and allopregnanolone

The GABA-A receptors system is the major inhibitory component of the CNS (Majewska *et al.*, 1986; Rupprecht, 2003), but within this single system there is a number of complications involved in the GABAergic inhibition such as: (i) an excitation component depending on age, cell type, compartment or functional state of the network; (ii) a phasic or tonic activity depending on the position of the receptor in relation to the synapse; and (iii) a strong variation depending on the receptor subunit composition (Birke and Draguhn, 2010). These isoforms consist of transmembrane heteropentameric GABA-receptor structures, which are composed of five subunits that belong to different subunit classes including α1 to α6, β1 to β4, γ1 to γ3, δ, ε, π, θ and ρ1 to ρ3 (Barnard *et al.*, 1998; Mehta and Ticku, 1999; Sieghart and Sperk, 2002). The different components are responsible for a large heterogeneity of GABA : A receptors, which also is mirrored by the number of different drugs that beside the physiological agonists are claimed to act through it. These drugs include anxiolytics, anticonvulsants, barbiturate, general anaesthetics, ethanol and several neurostereroids (Mehta and Ticku, 1999). Subunit composition appears to determine the pharmacological profile of the different GABA-A receptors where the anaesthetic effect has been the subject of extensive investigations searching for a basic pharmacological mechanism using a number of different *in vivo* and *in vitro* experimental techniques (Antkowiak, 1999; Belelli *et al.*, 1999; Krasowski and Harrison, 1999).

Of the anaesthetic drugs active on the GABA-A receptors, the neurosteroids are the only ones that can be synthesized both in the brain and outside in glands such as adrenals gonads and placenta (Paul and Purdy, 1992; Mellon and Griffin, 2002; Ottander *et al.*, 2005). They can from that point of view be regarded as endogenous anaesthetics (Harrison *et al.*, 1987), which are acting directly on some physiological inhibitory neural systems. During recent years allopregnanolone (5α-pregnane-3α-ol-20-one), which is a metabolite of progesterone, has been studied with increasing interest in this group of substances (Belelli and Lambert, 2005). *In vitro* testing with different techniques are often difficult to compare but indicate that allopregnanolone is active on the GABA-receptor system in the nM range (Harrison *et al.*, 1987; Belelli and Lambert, 2005) while effects of different standard anaesthetics are found in the μM to the mM range with propofol (around 1 μM) as more potent than pentobarbital (around 50 μM) (Antkowiak, 1999; Belelli *et al.*, 1999). These relations between effect and concentrations *in vitro* can be compared with *in vivo* data on dose – effect relations recoded with a threshold testing technique for deep anaesthesia in male rats (Table 1). All the substances in Table 1 where tested by intravenous (i.v.) infusion to reach a defined electroencephalography (EEG)-criterion (a burst suppression lasting 1 s or longer, a silent second SS) using an optimal infusion rate (Korkmaz and Wahlstrom, 1997). The optimal infusion rate for each substance was determined in separate sessions as the minimum dose needed to reach the criterion when tested with different infusion rates (the results of the these original experiments are given in the references found in table 2 in Zhu *et al.*, 2001). It is clear from Table 1 that pregnanolone and allopregnanolone were the most potent anaesthetics infused with the fastest optimal rates. In these experiments with the neurosteroids infused at different rates the rats were killed at the EEG-criterion for deep anaesthesia and the concentrations of the neurosterioids were analysed in different parts of the brain (Zhu *et al.*, 2001). In most parts the concentrations increased with the increased infusion rate, but in hippocampus and a sample consisting of midbrain and medulla oblongata the concentrations were stable and uninfluenced by the infusion rate. The stable neurosteroid concentration in above brain areas at the reached criterion indicates that they are the site of action. That is the sensitivity of the threshold was stable and reached independent of infusion rate, when the anaesthesia criterion was reached.

A further analyse of Table 1 shows that methohexital infused with a much slower optimal rate reached the criterion at a dose level only slightly larger than allopregnanolone and pregnanolone. It is also evident that the ratio between propofol and pentobarbital founded on the *in vitro* data presented above (50 times more potent) has been reduced to only three times in these *in vivo* studies. Table 1 also shows that optical isomers of the barbiturate hexobarbital differed much more than the corresponding isomers of narcobarbital. In both cases, the dose of the racemate needed to reach the criterion, lay between that of the two isomers.

In both humans and animals, allopregnanolone levels in brain and plasma can change during various physiological and pathological conditions, for example, during the menstrual cycle, at stress and pregnancy (Bixo *et al.*, 1997; Droogleever Fortuyn *et al.*, 2004; Parizek *et al.*, 2005). The brain concentration of allopregnanolone is significantly higher than the plasma level in both cycling and pregnant female rats, and both brain and plasma levels temporally follow those of progesterone (Corpechot *et al.*, 1993). In humans, allopregnanolone accumulates in the brain and increases in both the brain and plasma during luteal phase of the menstrual cycle (Bixo *et al.*, 1997). Allopregnanolone is rapidly redistributed from the brain, and the first compartment half-life is very short (Purdy *et al.*, 1990; Johansson *et al.*, 2002; Timby *et al.*, 2006). However, long-term clearance is slow, probably due to large accumulation in fat tissue (Zhu *et al.*, 2004; Turkmen *et al.*, 2008). The brain and plasma concentrations of allopregnanolone are regulated by different mechanisms (Paul and Purdy, 1992). It has also been indicated that there is a significant regional and interspecies diversity in the progesterone metabolic pathways and

Table 1

Relation between potency of different anaesthetic drugs and allopregnanolone tested at identical conditions

Substance (Sub)	Dose (mmol·g^{-1})	Optimal rate (mmol·g^{-1}·min^{-1})	Relative dose Sub/Allo
Allopregnanolone	44	13	–
Pregnanolone	33	11	0.75
Flurazepam	186	39	4.2
Propofol	75	56	1.7
Barbiturates dissolved and calculated as sodium salts			
Hexobarbital rac	243	58	5.5
Hexobarbital (+)-S	178	58	4.1
Hexobarbital (–)-R	572	58	13.0
Narcobarbital rac	223	46	5.1
Narcobarbital (+)	188	46	4.3
Narcobarbital (–)	259	46	5.9
Pentobarbital rac	225	20	5.1
Thiopental	145	38	3.3
Methohexital	49	35	1.1

All basic doses are those that can induce a burst suppression of 1 s or more in the EEG at induction of anaesthesia tested with the optimal rate (mg·kg^{-1}·min^{-1}) earlier defined for each of the substance as the dose-rate, which gives a minimum dose to reach the criterion. Rac = racemate (+) and (–) indicate optical isomers. Data calculated from table 2 in Zhu *et al.* (2001) where the references to the different studies involved are given.

allopregnanolone accumulation in the brain. In rat brain slices incubation of progesterone leads to accumulation of 5α-dihydroprogesterone and allopregnanolone, whereas incubation of mouse brain slices leads to the accumulation of 5α- dihydroprogesterone and 20α- dihydroprogesterone and that of monkey brain slices leads to the accumulation of only 20α- dihydroprogesterone (Korneyev *et al.*, 1993). Many of the physiological functions of allopregnanolone in the human brain remain to be determined, but this neurosteroid plays an important role in the regulation of the GABAergic system.

However, allopregnanolone and the GABA-A receptors mostly studied within higher centres of the mammalian brain. It has been shown that the rat spinal cord actively produces allopregnanolone (Venard *et al.*, 2008). Progesterone metabolites allopregnanolone and THDOC regulate neural plasticity and selectively increase the expression levels of GABA-A receptor subunits α2, α3, α4 and δ in the lumbosacral spinal cord (Peng *et al.*, 2009).

The interaction of allopregnanolone with the GABA-A receptor is still not completely understood. Allopregnanolone is an allosteroic modulator, which at nM concentrations potentiates the GABA effect at the GABA-A receptors. At higher concentrations (μM) allopregnanolone may in neurons also directly open the GABA-A receptor chloride channel (Belelli and Lambert, 2005). The modulating action of allopregnanolone is assumed to be mediated by binding to the GABA-A receptor (Majewska *et al.*, 1986; Paul and Purdy, 1992; Rupprecht, 2003). Recently, studies have reported the presence of two distinct allopregnanolone binding sites on the GABA-A receptor (Hosie *et al.*, 2006) that are highly conserved in all α subtypes of the GABA-A receptor (Hosie *et al.*, 2009). These domains mediate the potentiating and direct activation effects of neurosteroids by binding to the α-subunit transmembrane domains and interfacial residues between α and β subunits respectively. The authors suggest that significant receptor activation by neurosteroids relies on occupancy of these domains (Hosie *et al.*, 2006). It has been suggested that the effects of neurosteroid at physiological concentrations (low nM) may be influenced by the subunit composition of the GABA-A receptor (Lambert *et al.*, 2001). Subunit composition appears to determine the pharmacological profile of the receptor. However, allopregnanolone has been shown to act at a wide variety of GABA-A receptor subtypes with almost similar potency and efficacy with no recorded GABA-A subunit specificity, possibly because of a broad spectrum of action in the CNS (Puia *et al.*, 1990; Mitchell *et al.*, 2008). The presence of specific subunits can, however, alter the nerve cell responses to drugs; for instance, benzodiazepine has no pharmacologically effect on GABA-A receptors without γ subunit, regardless of the α subunit present. Likewise, changes in α4 or δ subunit expression may underlie the altered sensitivity to allopregnanolone (Zhu *et al.*, 1996; Smith, 2002).

Many of the behavioural and physiological effects of allopregnanolone are similar to those of benzodiazepines and other positive modulators of the GABA-A receptor, such as barbiturates and ethanol (Paul and Purdy, 1992; Follesa *et al.*, 2006), but in contrast to ethanol and benzodiazepines, fewer studies have focused on the tolerance that might result from exposure to allopregnanolone.

Allopregnanolone tolerance in clinical conditions

Evidence suggests that allopregnanolone exert clinically significant effects on the function of the CNS. The effects of allopregnanolone on the CNS via the GABA-A receptor can be divided into three categories: (i) direct effects on the receptor function; (ii) induction of tolerance; and (iii) withdrawal (Backstrom *et al.*, 2003). There are examples of conditions and symptoms that may be due to tolerance development to GABA steroids.

PMDD/PMS

One interaction between reproductive hormones and CNS is in premenstrual dysphoric disorder (PMDD) or premenstrual syndrome (PMS). The aetiology of PMDD is not yet clearly known, but evidence from studies indicates that it involves both ovarian steroids and an altered GABAergic function (Wang *et al.*, 1996; Monteleone *et al.*, 2000; Sundstrom Poromaa *et al.*, 2003). Although several studies have tried to find differences in serum concentrations between PMDD/PMS patients and controls, the results suggest that differences in steroid concentrations are not the reason why certain women experience PMDD/PMS and others do not. Instead, the results show that PMDD patients differ in terms of their sensitivity towards sex steroids, especially those affecting GABA function compared with controls.

The major findings in PMDD/PMS patients indicate that tolerance against GABA-A receptor modulators develops during the luteal phase of the menstrual cycle. PMDD/PMS patients are less sensitive to GABA-A receptor modulators such as benzodiazepines, ethanol, and GABA steroids (pregnanolone) during the luteal phase when they are exposed to allopregnanolone, but not during the follicular phase when the GABA steroids are absent (Sundstrom *et al.*, 1997; 1998; Nyberg *et al.*, 2004). The results indicate that patients with PMDD have a reduced GABA-A receptor sensitivity to these substances in the CNS, while this was not the case in women without PMDD(Sundstrom *et al.*, 1997; 1998; Nyberg *et al.*, 2004). Women with PMDD also show changes in startle response and pre-pulse inhibition during the luteal phase compared with the follicular phase, a phenomenon not seen in controls (Gulinello *et al.*, 2001; Epperson *et al.*, 2007; Kask *et al.*, 2008). fMRI studies show different follicular to luteal phase responses in PMDD patients compared with controls (Protopopescu *et al.*, 2008). In addition, patients with severe PMDD/PMS symptoms were less sensitive to the administered pregnanolone or benzodiazepines compared with PMS patients with more moderate symptoms. In an animal model of PMS/PMDD, the allopregnanolone effect on mood behaviour occurred in parallel with an up-regulation of the hippocampal α4 subunit of the GABA-A receptor and decreased benzodiazepine sensitivity (Gulinello *et al.*, 2001). This is in line with the decreased benzodiazepine sensitivity in women with PMDD (Sundstrom *et al.*, 1997).

In addition, severe negative mood effects of oral contraceptives are mainly found in women with PMS/PMDD, and not controls without PMS (Cullberg, 1972). Reintroduction of estradiol or progesterone in women with medically inhibited ovarian hormone production resulted in recurrence of symptoms in women with PMS/PMDD, but not in controls (Schmidt *et al.*, 1998; Segebladh *et al.*, 2009). Postmenopausal women with a history of PMS/PMDD responded with more negative symptoms on progestagens compared with women without such history (Bjorn *et al.*, 2000).

Catamenial epilepsy

In catamenial condition, some women with epilepsy show an exacerbation in seizure frequency during menstruation (Laidlaw, 1956; Reddy, 2004; Penovich and Helmers, 2008). This has been interpreted as a withdrawal symptom suggesting that there is an antiepileptic factor present during the luteal phase that is rapidly removed at the time of menstruation, provoking withdrawal symptoms with increased seizure frequency. A withdrawal syndrome is a response to a tolerance developed before the end of the drug exposure, again indicating that a tolerance can develop in certain individuals during the luteal phase of the menstrual cycle. That allopregnanolone and its isomer pregnanolone have antiepileptic effects has been shown in several studies (Landgren *et al.*, 1987; Backstrom *et al.*, 1990). The changes in hormone secretion during the menstrual cycle and the abrupt decrease in progesterone and allopregnanolone secretion during the premenstrual period play a central role in the pathogenesis of the catamenial seizure pattern (Backstrom, 1976; Moran and Smith, 1998). The menstrual seizure exacerbations could be explained on the basis of withdrawal of the antiseizure effects of allopregnanolone. Tuveri and colleagues showed that women with catamenial epilepsy have a lower serum concentration of THDOC and dehydroepiandrosterone sulphate/cortisol ratio than control group. This finding probably does not related to fluctuations in gonadal hormone secretion, as similar decreases were detected in every phase of the menstrual cycle. The serum concentrations of progesterone, pregnenolone and allopregnanolone were not different between groups (Tuveri *et al.*, 2008).

Sedation during pregnancy

Pregnant women experience marked alterations in both psychology and physiology. Memory impairment, cognitive changes and mood changes during pregnancy and in the postpartum period have been documented (Zonana and Gorman, 2005). Allopregnanolone increases substantially during pregnancy (Paul and Purdy, 1992; Hill *et al.*, 2007). During the first part of a pregnancy marked sleepiness is observed, which is substantially decreased later in pregnancy, even though progesterone and allopregnanolone levels are increasing and sedation occur in much lower concentrations of allopregnanolone in non-pregnant women (Timby *et al.*, 2006). This finding indicates that tolerance to allopregnanolone develops during pregnancy. In animals, GABA-A receptor subunit composition changes in parallel with the increase in allopregnanolone concentrations during pregnancy (Concas *et al.*, 1998). In isolated cortical neurons, chronic allopregnanolone treatment results in reduced receptor responses to this neurosteroid (Yu *et al.*, 1996a).

Postpartum blues and depression

If GABA-A receptor down-regulation occurs during pregnancy, the rapid postpartum fall of progesterone metabolites

will increase excitability (withdrawal effect), due to the down-regulated receptors. This could be an explanation for postpartum neurological and mood disorders. An increased seizure frequency is noticed during labour and delivery among women with epilepsy (Tomson, 1997), and changes in mood such as 'third day blues', postpartum depression and postpartum psychosis (Maguire and Mody, 2008; Nemeroff, 2008; Pearlstein *et al.*, 2009) also indicate that hormone-related phenomena such as PMDD are related to the occurrence of postpartum mood disorders (Stowe and Nemeroff, 1995; Bloch *et al.*, 2006). These results support the hypothesis that the aetiology for postpartum mood disorders may be related to differential neurosteroid sensitivity.

Negative mood symptoms on HRT in postmenopausal women

Negative mood changes are also clinically well-known side effects of combined hormone replacement therapy (HRT; estrogen + progesterone), and constitute a major problem concerning compliance. In postmenopausal women taking continuous combined estrogen/progestogen HRT, negative side effects arise shortly after the introduction of the treatment, but the severity of the symptoms decreases after 3–6 months, suggesting that an adaptation (tolerance) to the treatment has occurred (Robinson, 2001; Odmark *et al.*, 2004; Wihlback *et al.*, 2005). Women on estrogen-only HRT do not develop negative mood changes (Klaiber *et al.*, 1997). All progestagens (hormones with similar effects to progesterone) seem to be able to induce negative mood changes (Bjorn *et al.*, 2000; Andreen *et al.*, 2006). In sequential treatment, negative mood symptoms begin shortly after adding progesterone to the treatment and continue to rise in severity until the progesterone treatment has ended (Bjorn *et al.*, 2000; Andreen *et al.*, 2006).

Changes during acute and chronic stress

The brain and plasma concentrations of neurosteroids are partly regulated by independent mechanisms, given that the brain like the gonads and adrenals, is a steroidogenic organ that is able to produce neurosteroids (Mellon and Griffin, 2002). Serum concentrations of neuroactive steroids might therefore inaccurately reflect the brain levels of these compounds.

Allopregnanolone and THDOC are released during stress as stress activates the hypothalamus-pituitary-adrenal axis that releases corticosteroids, including THDOC and allopregnanolone (Purdy *et al.*, 1991; Serra *et al.*, 2000; Reddy, 2003). Metabolites of glucocorticoids have also been shown to enhance the GABA-A receptor effect of allopregnanolone (Stromberg *et al.*, 2005). The neurosteroid allopregnanolone has previously been shown to interfere with noradrenergic and corticosteroid-mediated regulation of corticotrophin-releasing hormone release and gene transcription (Patchev *et al.*, 1996). Those observations indicate that allopregnanolone might affect the neuroendocrine response to acute stress by influencing the hypothalamus-pituitary-adrenal axis (Patchev *et al.*, 1996; 1997). A repetitive stress condition in animals has been shown to result in alterations in function and sensitivity of the GABA-A receptor (Deutsch *et al.*, 1994; Serra *et al.*, 2000; Dong *et al.*, 2001; Guidotti *et al.*, 2001; Biggio *et al.*, 2007), pointing to the possibility that chronic stress condition is related to the development of tolerance against GABA-A receptor active compounds from the adrenal glands. Allopregnanolone and THDOC have emerged as responsible substances in the mechanism of the above mentioned clinical conditions (Deutsch *et al.*, 1994; Guidotti *et al.*, 2001; Reddy, 2003). Chronic exposure to stress and high levels of corticosterone results to changes in the mRNA levels of GABA-A receptor subunits and decreases GABA-A receptor function (Orchinik *et al.*, 1995; Verkuyl *et al.*, 2004; Serra *et al.*, 2008). Chronic stress could alter neuroactive steroid synthesis (Guidotti *et al.*, 2001), and synthesis that occurs following acute stress might be reversed during chronic stress. Studies have shown that in socially isolated animals, the brain and plasma concentrations of allopregnanolone and THDOC are decreased (Serra *et al.*, 2000; Matsumoto *et al.*, 2003). The mechanisms that underlie the persistent decrease in the abundance of neuroactive steroids, reduced sensitivity and function of the GABA-A receptors by chronic stress remain unclear.

Animal studies on tolerance induced by allopregnanolone and other GABA steroids

In vivo experiments in animals reveal tolerance to and withdrawal from progesterone/allopregnanolone for several but not all tested functions. Long-term progesterone (allopregnanolone) treatment gives insensitivity to benzodiazepine in the hippocampus CA1 followed by a withdrawal after ending the progesterone treatment (Costa *et al.*, 1995). The changes in the hippocampus seem to be related to up-regulation of the α4 subunit (Smith *et al.*, 1998a,b). Allopregnanolone has been shown to induce tolerance against ethanol-induced hypothermia. An investigation of the cross-tolerance between allopregnanolone and ethanol for initial hypothermic effects in mice showed differences in the impact of genetic factors in the development of tolerance to these two substances (Palmer *et al.*, 2002). Another study revealed a cross-tolerance in the neurophysiological effects of allopregnanolone in rats with a history of chronic ethanol exposure (Slawecki *et al.*, 2005). In mice, the anticonvulsant effect of allopregnanolone is also decreased in alcohol withdrawal tested with induction of pentylenetetrazol seizures and this effect was only seen in genotypes with intense ethanol withdrawal (Finn *et al.*, 2000; Beckley *et al.*, 2008). Tolerance developed to the anticonvulsant activity of allopregnanolone on picrotoxin-induced seizures after repeated intracerebroventricular administration of allopregnanolone at the effective doses ED(85) once or twice daily for 5 days (Czlonkowska *et al.*, 2001).

Studies from our laboratory show that in rats both acute and chronic tolerance to allopregnanolone effects can develop (Zhu *et al.*, 2004; Turkmen *et al.*, 2006; 2008). Theoretically, the observed tolerance occurred because the neuronal systems had become less sensitive to allopregnanolone after GABA-A receptor subunit changes. The induction of acute tolerance to allopregnanolone was accomplished by maintaining a deep anaesthesia at the level of burst suppression of 1 s [silent second level, SS, measured by electroencephalography (EEG)-threshold method] for longer periods

(maximum 90 min) (Korkmaz and Wahlstrom, 1997). After induction of acute tolerance, possible changes in GABA-A receptor mRNA and proteins were analysed with *in situ* hybridization and immunohistochemical methods. When the anaesthesia was increased to 60 or 90 min (Zhu *et al.*, 2004; Turkmen *et al.*, 2006), an acute tolerance was recorded both as an increase in the need to administer allopregnanolone to maintain the anaesthesia and as an increased concentration of allopregnanolone in serum and in hippocampus, midbrain + thalamus + hypothalamus and medulla oblongata. No changes were recorded in cortex, cerebellum and striatum. Bulbus olfactorius and amygdala were also analysed but showed no concentration change (Birzniece *et al.*, 2006). Thus when combined with determination of substance concentrations in different brain regions, this *in vivo* method can be used to study the brain areas that are of importance for tolerance development. An increased concentration was also recorded in fat but not in muscle after longer anaesthesia indicating fat as a depot of administered allopregnanolone. The induced tolerance to allopregnanolone still remained when tested after 24 h, but was not present 48 h after induction by an anaesthesia lasting 90 min (Zhu *et al.*, 2004; Birzniece *et al.*, 2006; Turkmen *et al.*, 2008)

Chronic treatment with minaxolone, a potent ligand for the neurosteroid binding site of the GABA-A receptor, 100 $mg \cdot kg^{-1}$, orally, once daily for 7 days resulted in a loss of sedative response to an acute dose of minaxolone, indicating development of tolerance (Marshall *et al.*, 1997). Ganaxolone (3α-hydroxy-3β-methyl-5α-pregnane-20-one), an orally active synthetic analogue of allopregnanolone and an allosteric modulator of GABA-A receptor, induced a cross-tolerance with diazepam after 7 days treatment but not after 3 days treatment, and no tolerance developed to ganaxolone itself (Reddy and Rogawski, 2000).

However, divergent results have also been obtained; allopregnanolone in a dose of 15 $mg \cdot kg^{-1}$ given once per day intraperitoneally (i.p.) for 5 days did not induce any tolerance when sleep induction was studied (Damianisch *et al.*, 2001). In addition, a chronic treatment with the GABA steroid pregnanolone did not induce tolerance to the anticonvulsive effect when given as three daily injections of 15 mg i.p. for 14 days (Kokate *et al.*, 1998). It is worth noting that allopregnanolone was given i.p. in both of these studies, and allopregnanolone like other anaesthetic steroids given by this route is to a large extent resorbed by the intestine and subject to first passage metabolism when passing the liver (Selye and Stone, 1944). This may be one factor explaining the divergent results. Other factors could be that different functions are differently sensitive to the development of tolerance against allopregnanolone.

In vivo development of allopregnanolone tolerance and interfering factors

The introduction of a substance (e.g. allopregnanolone) into a biological system and the distribution of substance molecules to the receptor sites are far more complicated than adding a compound into a test tube. The initial dosage of the drug, the route of administration, the rate of absorption, the rate of elimination and many other factors enter into this complex process.

Studies have shown that single or repeated exposure to allopregnanolone results in the development of tolerance. Factors of importance for development of allopregnanolone tolerance are the exposure dosage and the timescale of its development. Tolerance to the anaesthetic effects of allopregnanolone (high dosage) develops more rapidly than tolerance to the anticonvulsant and memory impairment effects (lower dosage) (Czlonkowska *et al.*, 2001; Zhu *et al.*, 2004; Turkmen *et al.*, 2006).

We have investigated whether chronic allopregnanolone treatment generates development of tolerance to the spatial learning impairment produced by allopregnanolone administration immediately before the Morris water maze test session (Turkmen *et al.*, 2006). In the study four different allopregnanolone pretreatment regimes was used: (i) 3 days of once-daily i.v. injections of allopregnanolone in β-cyclodextrin; (ii) 3 days of two times per day i.v. injections; and (iii) 7 days of twice-daily i.p. injections of allopregnanolone in sesame oil all with corresponding placebo injections. This study revealed that chronic allopregnanolone treatment once per day in 3 days did not reduce allopregnanolone's effect on learning. Pretreatment two times per day in 3 days partly reduced and pretreatment two times per day during 7 days clearly induced tolerance against the negative allopregnanolone effects on spatial learning in the Morris water maze test. The more frequent and longer pretreatment gave a stronger and more clear-cut tolerance. The differences in performance may be due to difference in treatment duration and to the fact that an insufficient steroid concentration reached the brain over 24 h in rats injected just once a day. However, it is remarkable that there was no effect of any of the pretreatments on the effect of allopregnanolone on the initial performance during the first Morris maze session after the pretreatments. We have previously shown that allopregnanolone concentrations in brain tissue after an i.v. injection decrease to half within 12 min (Johansson *et al.*, 2002), resulting in a marked fluctuation of receptor occupancy by the drug. It is known that the interval between drug exposure and the concentration of the drug needed (the treatment interval) are important factors influencing the strength of tolerance development (Kalant *et al.*, 1971), and allopregnanolone has a very short half-life after single injections.

Sesame oil was used as a vehicle for i.p. injections (Turkmen *et al.*, 2006). This produced a slow and continuous absorption of the steroid, probably creating more optimal conditions for tolerance development as the duration of drug exposure was prolonged (Chien, 1981). When allopregnanolone is injected i.p., the neurosteroid is taken up into the portal circulation, and metabolism of the drug is started in the liver before the drug reaches the brain, thus a larger amount of steroid is required to induce an effect. Therefore, the i.p. dosage of allopregnanolone was high, 20 $mg \cdot kg^{-1}$, while in the experiment with 3 days of i.v. pretreatment, the dosage was kept constant at 2 $mg \cdot kg^{-1}$ during pretreatment and Morris water maze testing. These results highlight the fact that many factors, often involving pharmacokinetic components, can influence the development of chronic allopregnanolone tolerance including longer pretreatment or a change in allopregnanolone delivery.

We also know that allopregnanolone has a wide spectrum of effects, including negative effects on spatial learning, anxiolysis and an anaesthetic effect (Bitran *et al.*, 1991; Johansson *et al.*, 2002; Zhu *et al.*, 2004). Our studies suggest that the speed of tolerance development to allopregnanolone effects is related to the amount of drug administered during induction of tolerance. Tolerance to the anaesthetic effect of allopregnanolone was obtained with a total dose of 80 mg·kg^{-1} (intravenous injection) over 90 min (Zhu *et al.*, 2004), while with i.p. injections of allopregnanolone 20 mg·kg^{-1} twice a day it took 7 days to induce chronic allopregnanolone tolerance to negative effects in the Morris water maze test (Birzniece *et al.*, 2006; Turkmen *et al.*, 2006).

Tolerance to substances active at GABAergic system

Chronic treatment with positive allosteric modulators that act at different sites of the GABA-A receptor results in changes in drug sensitivity and expression of receptor subunit mRNAs (Impagnatiello *et al.*, 1996; Ito *et al.*, 1996a; Smith *et al.*, 1998b; Loh and Ball, 2000; Birzniece *et al.*, 2006). The role of GABA-A receptors in the development of tolerance against the positive modulator substances has earlier mostly been investigated using benzodiazepines, ethanol and barbiturates (Roca *et al.*, 1990). However, it has also been shown that a chronic exposure to GABA itself induces a reduction in GABA-A receptor sensitivity, and thus tolerance (Mhatre and Ticku, 1994).

Benzodiazepines are potentially addictive drugs; psychological and physical dependence can develop within a few weeks or months of regular or repeated use (Miller *et al.*, 1988). Furthermore, benzodiazepines decrease in effectiveness after a certain time of regular use. Much of their efficacy is lost due to tolerance development. When tolerance develops, 'withdrawal' symptoms can appear even though the user continues to take the drug. This is the cluster of symptoms that reflects the rebound hyper-excitability following chronic exposure to benzodiazepines and neuronal adaptations. Withdrawal can imply the development of dependence as well as tolerance. Thus, the symptoms suffered by many long-term users are a mixture of adverse effects of the drugs and 'withdrawal' effects due to tolerance (Bateson, 2002; Wafford, 2005). Symptoms of benzodiazepine withdrawal that appear after tolerance development are time-limited (1–2 weeks), but the duration varies according to the drug and to individual properties (Laurijssens and Greenblatt, 1996).

Acute tolerance was initially noticed to appear after ethanol exposure (Mellanby, 1919). The same type of tolerance development has also been reported for many GABA modulator substances, such as barbiturates (Brodie *et al.*, 1951; Aston, 1966) and benzodiazepines (Wong *et al.*, 1986; Ihmsen *et al.*, 2004) in both humans and animals, and the neurosteroid allopregnanolone in rats (Zhu *et al.*, 2004; Birzniece *et al.*, 2006). Repeated ethanol intake leads to the development of tolerance that changes the behavioural response to ethanol. Tolerance results in a reduction in the anxiolytic, motor discoordinating and sedative/hypnotic effects of ethanol (Le *et al.*, 1987).

Barbiturates are CNS depressants that were earlier used as sedatives, hypnotics, anaesthetics and anticonvulsants. However, prolonged use produces tolerance and physical dependence (Wahlstrom, 1979). Barbiturates act on GABA-A receptors through three distinct mechanisms, resulting in positive allosteric modulation, direct activation and inhibition. These effects are observed at different concentrations and are differentially affected by the subunit composition of GABA-A receptors (Yu and Ho, 1990; Ito *et al.*, 1996a,b; Drafts and Fisher, 2006). However, possible changes in GABA-A receptor subunit composition during tolerance to barbiturates have not yet been studied.

It is well established that there are alterations in GABAergic neurotransmission following chronic exposure to the above mentioned substances. These alterations contribute to the symptoms of tolerance, dependence and withdrawal (Klein and Harris, 1996; Sanna *et al.*, 2003). However, the nature and mechanism of these changes are not clear (Allison and Pratt, 2003; Kan *et al.*, 2004; O'Brien, 2005). It has been suggested that benzodiazepine treatment of cortical neurons in cultures results in GABA-A receptor down-regulation (Holt *et al.*, 1996; 1997). Although the molecular mechanisms responsible for the changes in GABA-A receptor function induced by exposure to positive modulators remain unclear, post-translational modifications, receptor trafficking, effects on receptor density and changed subunit expression have been proposed (Tehrani and Barnes, 1991; Biggio *et al.*, 2003). The GABA-A receptor down-regulation is proposed to be in the following order: (i) desensitization (tachyphylaxis) characterized by decreased receptor currents during a continuous GABA exposure; (ii) sequestration (endocytosis) of subunit polypeptides and uncoupling of allosteric interactions between the GABA and benzodiazepine binding sites; (iii) subunit polypeptide degradation; and (iv) repression of subunit gene expression(Tehrani and Barnes, 1991; Barnes, 2000). Consequently, the end point of these changes can be observed as changes in the GABA-A receptor subunit mRNA expression and finally benzodiazepine tolerance.

On the other hand, it has been shown changes in the GABA-A receptor subunit composition at tolerance to these substances.

Benzodiazepine treatment to determine tolerance development and changes in the GABA-A receptor subunit expression revealed that tolerance developed after 7 days flunitrazepam treatment in rats (Tietz *et al.*, 1999). In the same study, quantitative *in situ* hybridization showed that the α1 and β3 subunit mRNAs were decreased and β2 subunit mRNA increased in the hippocampus of tolerant animals (Tietz *et al.*, 1999). Another study revealed that 7 days diazepam treatment increased α4, β1 and γ3 subunit mRNA levels, which were sustained at 14 days treatment along with increases in α3 and α5 and a decrease in γ2 subunit mRNA (Holt *et al.*, 1996). In a third study, rats were treated with diazepam continuously (s.c.) for several weeks (Wu *et al.*, 1994). This treatment decreased the α1 subunit mRNA level in the hippocampus but not in the cortex or cerebellum. The α5 subunit mRNA level was decreased in the cerebral cortex and hippocampus, and γ2 subunit mRNA level decreased only in the cortex (Wu *et al.*, 1994). However, even if the acute sedative effect of diazepam is related to GABA-A receptors including the α1 subunit (McKernan *et al.*, 2000), tolerance to the sedative action of diazepam is suggested to be associated with a decreased level of the α5 subunit in the

hippocampal dentate gyrus (van Rijnsoever *et al.*, 2004). These studies revealed that chronic activation of the GABA-A receptors by diazepam is sufficient to induce tolerance, and those changes in subunit mRNAs leading to changes in the amounts of specific subunits could possibly account for the mechanism of tolerance development. However, tolerance develops to some, but not all, behavioural actions of benzodiazepines. Tolerance to the anxiolytic action of benzodiazepine can develop, but little or no tolerance develops to the anterograde amnesia after chronic treatment in either humans or animals (Ghoneim *et al.*, 1981; Hughes *et al.*, 1984). In rats, the Morris water maze test has been used to show that tolerance can develop to the amnesic effect of diazepam on spatial learning (McNamara and Skelton, 1997). The same study revealed that the amnesic effect of diazepam is not secondary to sedation, and the amnesic and sedative effects are independent factors

The development of ethanol tolerance and dependence is associated with alterations in many receptor systems (Nordberg and Wahlstrom, 1992; Hoffman *et al.*, 2000; Chastain, 2006). The properties of the GABA-A receptors are influenced, as well as alterations have been recorded in the expression of distinct GABA-A receptor subunit mRNA and peptides in various brain regions. The levels of GABA-A receptor α1, α2 and α3 subunit mRNA are reduced in the cerebral cortex, while the α4, β1, β2, β3, γ2 and γ2 subunit peptide or mRNA levels are increased in the cerebral cortex following chronic ethanol exposure (Mhatre and Ticku, 1992; Devaud *et al.*, 1997; Mahmoudi *et al.*, 1997; Matthews *et al.*, 1998). Selective alterations have been seen in cortical GABA-A receptor subunit mRNA levels of rats undergoing withdrawal from ethanol. In that study (Devaud *et al.*, 1996), α1 and α4 subunit mRNA levels showed only slight alterations during withdrawal, whereas previous observations (Devaud *et al.*, 1997) had shown a significant decrease in α1 and a significant increase in α4 mRNA levels in ethanol dependent (not withdrawing) animals. β2, β1 and γ1 mRNA levels increased significantly during ethanol withdrawal (Devaud *et al.*, 1996; 1997).

Studies using barbiturates have shown that the time needed to develop tolerance varies. One day of pentobarbital exposure decreased the duration of loss of righting reflex, but tolerance to the hypothermic effects of thiopental and barbital progressed after 7 days (Saunders *et al.*, 1990). Increased sensitivity to pentylenetetrazol-induced seizures was first observed after 3 days of pentobarbital exposure (Lin and Sutherland, 1977; Saunders *et al.*, 1990). The same study proposed that the acute effects of barbiturates on the GABA-A receptor complex are reversible. Chronic pentobarbital treatment induces expression of α6 subunit mRNA in the brain of tolerant rats, while α1 and γ2 subunit mRNAs are unchanged. This is in contrast to withdrawal condition that triggers a surge in levels of α1 and γ2 subunit mRNAs (Ito *et al.*, 1996a).

Chronic allopregnanolone effects on GABA-A receptor function and expression

Long-term exposure of cultured cortical neurons to allopregnanolone has been shown to produce down-regulation of the GABA and [35S]t-butylbicyclophosphorothionate binding sites and uncoupling of the GABA-A receptor complex (Yu and Ticku, 1995). This *in vitro* study also showed that chronic neurosteroid treatment decreases the efficacy of benzodiazepine ligands and neurosteroids at the GABA-A receptor complex in cortical neurons. This was associated with decreased efficacy of GABA-induced Cl(–) influx as well as decreased efficacies of other positive modulators to potentiate the GABA-induced currents and effects on single cell GABA-A response (Yu *et al.*, 1996a). Chronic allopregnanolone treatment also decreased the β2, β3, α2 and α3 subunit mRNA levels, but did not alter the γ2s subunit mRNA level in cortical neurons (Yu *et al.*, 1996b). Another *in vitro* cellular experiment revealed that chronic neurosteroid treatment uncouples interactions between neurosteroids and benzodiazepine recognition sites (Friedman *et al.*, 1993). In pregnant rats, the brain concentration of the progesterone is increased between days 10 and 15, whereas allopregnanolone and THDOC peaked around day 19 (Corpechot *et al.*, 1993; Concas *et al.*, 1998; Sassoe-Pognetto *et al.*, 2007). The response to muscimol was decreased on days 15 and 19 of pregnancy and increased 2 days after delivery, indicating a tolerance followed by a withdrawal. The expression of γ2L mRNA in the cerebral cortex and hippocampus decreased during pregnancy and returned to control values 2 days after delivery.

Administration of Finasteride, a 5α-reductase blocker, to pregnant rats prevented the decreases in both the stimulatory effect of muscimol on 36Cl- uptake and the decrease of γ2L mRNA observed during pregnancy. These results indicate that the plasticity of GABA-A receptors during pregnancy and after delivery is a function of 5α-reduced neuroactive steroids produced during pregnancy (Concas *et al.*, 1998). The effects of long-term progesterone/allopregnanolone treatment appear to be mediated through modulation of GABA-A receptor signalling mechanisms that control the expression of specific receptor subunit genes. The outcome of change in signalling appears to differ among neurons derived from different regions of the brain. Neuroactive steroids such as allopregnanolone might have differential actions on GABA-A receptor plasticity in distinct neuronal cell populations, which could account for some of the physiological effects induced by these compounds (Follesa *et al.*, 2001). Short-term exposure to allopregnanolone and the discontinuation of neurosteroid after long-term exposure (sudden decrease in the neurosteroid level) result in increased expression of hippocampal GABA-A receptor α4 subunit mRNA (Gulinello *et al.*, 2001). The increased expression of the α4 subunit was correlated with increased anxiety and reduced sensitivity of the GABA-A receptors to classical benzodiazepine agonists and zolpidem as well as with a distinct pattern of regulation by flumazenil and other positive and negative modulators (Gulinello *et al.*, 2001).

Possible mechanisms for acute and chronic allopregnanolone tolerance

The mechanistic relationship between the different forms of tolerance remains unclear. There are two main hypotheses about the relation between acute and chronic tolerance. The

first hypothesis assumes a mechanistic relationship between acute and chronic tolerance, in which chronic tolerance is a reflection of the more rapid development or greater magnitude of acute tolerance (Kalant *et al.*, 1971). The second hypothesis proposes that acute and chronic tolerances are not related, and may have different underlying mechanisms (Tabakoff and Kiianmaa, 1982; Tabakoff *et al.*, 1982). Acute and chronic tolerance could be compared with the changes that take place during short-term and long-term memory development respectively. It has been suggested that short-term memory involves a post-translational modification of proteins, while long-term memory requires synthesis of new proteins (Kauderer and Kandel, 2000).

We have conducted a number of studies to evaluate the pharmacodynamic and pharmacokinetic properties of allopregnanolone at induction of anaesthesia and acute tolerance in rats, showing a rapid loss of allopregnanolone from the blood (Zhu *et al.*, 2001; 2004; Johansson *et al.*, 2002; Birzniece *et al.*, 2006; Turkmen *et al.*, 2008). As mentioned above, male rats were anaesthetized with i.v. allopregnanolone to a well-defined criterion, the deep anaesthesia level of a burst suppression in the EEG of 1 s or more (a silent second, SS) (Korkmaz and Wahlstrom, 1997). When anaesthesia was maintained for 60 min at the SS level the allopregnanolone concentrations in serum remained stable and similar to the concentrations recorded in the control groups analysed directly after a single induction of the EEG threshold (Zhu *et al.*, 2004). However, around 60 min of anaesthesia the dosages of allopregnanolone needed to maintain the anaesthesia criterion at the SS level started to increase and the concentrations in some parts of the brain consisting of hippocampus, medulla oblongata, pons, midbrain, thalamus and hypothalamus had also started to increase. After 90 min the concentration of allopregnanolone had increased significantly in serum and a further increase was seen in the parts of the brain given above. A significant increase in allopregnanolone concentration in fat was also observed compared with the control group. This increase was related to the duration of anaesthesia. It is evident that there is active redistribution of allopregnanolone to the fat as the duration of anaesthesia (allopregnanolone infusion) is increased. After 90 min of anaesthesia, allopregnanolone concentrations were clearly increased in hippocampus, medulla oblongata, pons, midbrain, thalamus and hypothalamus regions of the brain, but it must be pointed out that no increase was seen in the other areas investigated (cortex, striatum and cerebellum). We interpret this to mean that in the regions where the brain allopregnanolone concentration increased in parallel with the increase in maintenance dosage and serum level, the receptor sensitivity had decreased and therefore an increase in dosage was needed to obtain a brain region concentration to keep the anaesthesia at the SS level. As the receptor sensitivity had decreased a higher tissue concentration was needed to maintain the effect. Thus, this cannot be solely a pharmacokinetic effect. In that case the brain concentration would have decreased or stayed constant in parallel with the maintained effect. This indicates that of the brain regions investigated, the hippocampus, medulla oblongata pons, midbrain, thalamus and hypothalamus seem to be the most heavily involved in the induction of acute allopregnanolone tolerance for anaesthesia (Birzniece *et al.*, 2006). The increased concentration of allopregnanolone in fat show that redistribution to the fat is an important component in the elimination of allopregnanolone from the brain. Thus the increase in certain brain regions may be due to increased binding at neurosteroid binding sites on the GABA-A receptor, where changes in receptor structure and/or number could determine binding capacity. Furthermore, it is possible to state that during acute tolerance to the anaesthetic effect of allopregnanolone, the tolerance type is pharmacodynamic (Zhu *et al.*, 2001; 2004; Birzniece *et al.*, 2006; Turkmen *et al.*, 2008).

Furthermore, there was a statistically significant decrease in the GABA-A receptor α4 subunit mRNA and protein level in the thalamic ventral-posteriomedial nucleus (VPM) of the tolerant rats that had been anaesthetized for 90 min when compared with the control group (Birzniece *et al.*, 2006). The detected reduction in GABA-A receptor α4 subunit level in the VPM of the thalamus might be the primary component causing the need for an increase in dose of allopregnanolone to maintain anaesthesia. A reduced number of α4 subunits could mean a decreased number of functional GABA-A receptors or a change in efficacy of the receptor by a changed subunit composition. With regard to the number of GABA-A receptors, we did not measure the amount of receptors present in different areas, but each GABA-A receptor includes two α subunits and we found no compensatory up-regulation of other α subunits analysed in the thalamus. Therefore, there is no evidence for a change in subunit composition where the α4 subunits would have been replaced with other α subunits. The GABA system is inhibitory, and a decreased number of functional GABA-A receptors will cause less inhibitory activity. Because we keep rats at the deep anaesthesia level during the use of the EEG threshold method, we have to increase the dose of neurosteroid to maintain anaesthesia in situations with lower GABA system inhibitory activity. Therefore, the decrease of the GABA-A receptor α4 mRNA level in the thalamus could be considered the most important finding favouring changed subunit expression as a mechanism for development of acute anaesthesia tolerance to allopregnanolone. Another finding in favour of this interpretation is the negative correlation seen between the individual increases in the dose of allopregnanolone needed to maintain the anaesthesia during the last period of anaesthesia (65–85 min) and the mRNA expression of the GABA-A receptor α4 subunit in the VPM of thalamus. This indicates that the stronger the tolerance that has developed in the individual animal, the lower the GABA-A receptor α4 subunit in the VPM of the thalamus. For the GABA-A receptor subunits α1, α2, α5, β2 and δ, no difference in mRNA expressions between groups was found in the studied regions of the brain (Birzniece *et al.*, 2006; Turkmen *et al.*, 2008).

In line with our results, there are data indicating that a 4 day application of allopregnanolone to developing neuronal cells reduces the GABA-A receptor α4 subunit in a concentration-dependent manner (Grobin and Morrow, 2000). However, in pregnant rats, when there are high allopregnanolone levels for a long period, no change in α4 subunit expression can be found in the cortex or hippocampus. On the seventh day postpartum (withdrawal from allopregnanolone), an increase in this subunit was detected in the hippocampus, while the thalamus was not investigated (Concas *et al.*, 1999).

Alternate mechanisms to our results, besides change in receptor composition, may be involved in organizing the GABA-A receptors function in later periods of the acute allopregnanolone exposure. In fact, allopregnanolone induced GABA-A receptor regulation has not been considered only in terms of alterations in the subunit composition of the receptor. Changes in receptor function can occur independently of alterations in number of receptors and subunit types (Brussaard and Koksma, 2002; 2003). In the GABA-A receptor structure, both the N and C terminals of the receptor subunits extend outside the cell membrane, and the intracellular M3-M4 loop is the most important domain involved in regulating receptor function (Olsen and Tobin, 1990; Chen and Olsen, 2007). A rapid way of changing the receptor function is phosphorylation, a temporary chemical modification of the protein by enzymes inside the cells. The enzymes involved (phosphokinases) might be important in allopregnanolone tolerance. Although the molecular mechanism is not yet understood, it is clear that the interaction of positive modulator substances (e.g. allopregnanolone) with the GABA-A receptor can be regulated by the relative activity of kinases and phosphatases (Brandon *et al.*, 2002; Brussaard and Koksma, 2002; Kittler *et al.*, 2005).

The hypothesis that both tolerance and drug withdrawal syndrome appear after adaptive regulation of drug receptors in the CNS was introduced many years ago by Hihmmelsbach (Himmelsbach, 1941). Post-translational mechanisms are now believed to be at least as important as changes in the receptors themselves (Pandey, 1998; Brussaard and Koksma, 2003). By this mechanism, receptor function can be altered without any change in the overall numbers of the receptor protein, involving a temporary chemical changes of the GABA-A receptor protein by enzymes that cause a rapid change in receptor function (Brussaard and Koksma, 2003; Tasker, 2004). Theoretically, the receptor protein phosphorylation is sufficiently rapid to be the mechanism for acute effects of allopregnanolone, and it has been suggested that allopregnanolone may affect the function of some receptors by altering their state of phosphorylation (Fancsik *et al.*, 2000; Koksma *et al.*, 2003; Tasker, 2004). This mechanism may also participate in a rapid homeostatic mechanism that may underlie phenomena such as acute or rapid tolerance. In conditions of acute or rapid tolerance, adaptations that are rapidly established (within minutes or hours) are also rapidly removed. This is certainly the case for receptor phosphorylation, as dephosphorylases can remove the phosphate group within minutes and thus restore receptor function to normal (Gyenes *et al.*, 1994; Brussaard and Koksma, 2002; Jovanovic *et al.*, 2004), but it is doubtful if such rapid mechanisms are involved in the acute tolerance induced by the threshold method as a reduced change was still present 24 h after induction (Turkmen *et al.*, 2008).

Another regulatory mechanism that can change the function of the GABA-A receptors is internalization (Barnes, 2000). The continued presence of a GABA-A receptor agonist drug such as benzodiazepine leads the nerve cell to internalize certain receptor subunit proteins over a period of hours (Tehrani and Barnes, 1991). Once internalized, these receptors are unavailable to the drug, thus decreasing the drug effect. This mechanism may cause some form of rapid tolerance, as internalization usually takes several hours. If allopregnanolone can induce rapid tolerance is still an open question.

The body responds to a single exposure or the continued presence of a drug with a series of adjustments that tend to overcome the drug effects. In the case of the endogenous substance allopregnanolone, compensatory changes can occur in the GABA-A receptor, making the GABA-A receptor less responsive, so that the inhibitory actions of allopregnanolone are decreased. At the same time there are changes in secondary systems affected by allopregnanolone, such as the cholinergic, dopaminergic, and glutamate (NMDA) systems, so that the activity of excitatory neurotransmitters tends to be enhanced (Nordberg and Wahlstrom, 1992; Dazzi *et al.*, 1996; Sanna *et al.*, 1998; Johansson and Le Greves, 2005). Evaluation of these changes is beyond the scope of this review.

In conclusion, we have shown that both acute and chronic tolerances can develop to the effects of allopregnanolone. The development of acute allopregnanolone tolerance in its anaesthetic effect leads to a decrease in the abundance of the GABA-A receptor α4 subunit and the expression of the α4 subunit mRNA in the VPM nucleus of the thalamus. Tolerance to the different effects of allopregnanolone may vary between individuals, probably as a result of differences in intrinsic genetic, neurological, and chemical properties and depend on CNS levels and exposure duration. In light of these studies, one might conclude that different systems and different brain areas are involved in various effects of allopregnanolone and might have different sensitivity to develop tolerance.

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Conflict of interest

None.

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