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Reduced benzodiazepine tolerance, but increased flumazenil-precipitated withdrawal in AMPA-receptor GluR-A subunit-deficient mice

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ABSTRACT

Pharmacotherapy with benzodiazepines is compromised by rapid sedative tolerance and diverse withdrawal symptoms. To assess the role of AMPA-type glutamate receptor GluR-A subunits in neuroadaptation to subchronic benzodiazepine treatment, GluR-A subunit-deficient mice were rendered tolerant by a high-dose seven-day flurazepam treatment (40 mg/kg, s.c., twice a day for 4 days, 60 mg/kg twice a day for 3 days). The acute effects to flurazepam were not changed in the GluR−/− mice compared with their littermate control mice. GluR-A−/− mice developed less tolerance than their controls as demonstrated in behavioral tests for muscle relaxation and sensory functions. Actually, the knockout mice exhibited slower recovery than their littermates from impaired gait and pelvic position after an acute 40 mg/kg dose of flurazepam. The apparent elimination of flurazepam was similarly increased in the knockout and control mice as assessed by blood and brain concentrations 2 h after acute and chronic treatments, but the active metabolite desalkylflurazepam cumulated similarly in both mouse lines. Withdrawal symptoms, precipitated by flumazenil (20 mg/kg, s.c.) 48 h after discontinuation of the flurazepam treatment, were enhanced in the GluR-A−/− mice. The results stress the importance of the AMPA-receptor system in neuroadaptation to acute and chronic effects of benzodiazepines.

1. Introduction

Benzodiazepine (BZ) treatment is widely used for quick and efficient anxiolytic and sedative responses. However, when the use is extended to longer periods, adverse effects emerge. These include tolerance to the initial drug effect, dependence as revealed by withdrawal symptoms after treatment discontinuation, and abuse problems (O'Brien, 2005; Rosenbaum, 2005).

γ-Aminobutyric acid type A ($GABA_A$) receptors mediate the main effects of BZs (Korpi et al., 2002). BZs bind to the $GABA_A$ receptor and modulate their properties allosterically, which suggests that receptor adaptation could provide possible mechanisms for the development of tolerance and withdrawal symptoms. Many properties of the $GABA_A$ receptor complex have been observed to change during long-term BZ treatment. These include changes in the affinity and number of binding sites of BZs, altered receptor subunit composition, uncoupling of the BZ binding-site from the receptor channel function, and changes in the affinity and number of binding sites of GABA [for review, see Bateson, 2002; Hutchinson et al., 1996; Wafford, 2005]. Furthermore, $GABA_A$ receptor subunit-specificity has been suggested to have a role in the development of tolerance to BZs by downregulation of α5 subunit-containing receptors in the hippocampal dentate gyrus (Li et al., 2000; van Rijnsoever et al., 2004). Most of these studies have employed low to moderate doses of BZs, to which tolerance is quickly developed.

Other neurotransmitter systems have also been implicated as mechanisms underlying tolerance and dependence to BZs. Especially interesting is the main fast excitatory neurotransmission system in the brain using glutamate as the transmitter. It has been suggested that the BZ-induced increase in inhibitory transmission is counteracted by the glutamatergic system through modified glutamate metabolism and/or modified receptor mechanisms (Allison and Pratt, 2003; Izzo et al., 2001). When mice are chronically co-treated with BZs and an antagonist of *N*-methyl-D-aspartate (NMDA) receptors, tolerance develops only partially (Steppuhn and Turski, 1993). Development of tolerance to BZs increases mRNA of NMDA receptor NR1 and NR2B subunits, which can be reversed by NMDA receptor antagonist MK-801 (Almirón et al., 2004). Furthermore, withdrawal symptoms are reduced by the administration of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists in a time-dependent manner: the former is effective when given at the time of withdrawal and the latter when given just prior to the appearance of the symptoms (Steppuhn and Turski, 1993). An increase in the number and activity of AMPA receptors in BZ tolerance is evidenced by increased binding of AMPA-receptor ligand [^{3}H]Ro 48-8587 and by increased electrophysiological responses (Allison et al., 2005; Van Sickle and Tietz, 2002).

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Importantly, the GluR-A (GluR-1) subunit of AMPA-type glutamate receptors has been implicated in neuroadaptational mechanisms, such as long-term potentiation (LTP), learning and memory and drug addiction (Bannerman et al., 2006; Carlezon and Nestler, 2002). During hippocampal synaptic plasticity, GluR-A subunit-containing receptors are targeted to the cell membrane, a pivotal event in LTP (Hayashi et al., 2000; Shi et al., 2001). Pharmacological study of the role of GluR-A subunit-containing AMPA receptors in the development of BZ tolerance is hampered by the lack of a selective ligand. Fortunately, a knockout mouse line has been generated in which the hippocampal LTP is abolished but the synaptic transmission remains largely unmodified (GluR-A−/− mice; Zamanillo et al., 1999). This defect in LTP is rescued by genetic re-expression of the GluR-A subunit (Mack et al., 2001). In the GluR-A−/− mice, chronic morphine administration leads to reduced tolerance and withdrawal symptoms, indicating an interaction between sedative opioid and excitatory glutamate neurotransmitter systems and highlighting the role of AMPA receptor mechanisms in the effects of addictive drugs (Vekovischeva et al., 2001).

Here we studied the effects of BZ flurazepam (FZ) at high doses in acute and chronic settings of tolerance and dependence in GluR-A−/− mice (Zamanillo et al., 1999). We used measurements in which the phenomenon of "learning while intoxicated" should play only a minor role in order to find out the significance of GluR-A subunit-containing AMPA receptors in the adaptation of the brain to the effects of high BZ levels.

2. Methods

2.1. Subjects

GluR-A−/− mice were produced by deletion of the *Gria1* gene as described previously (Zamanillo et al., 1999). The mice were back-crossed to C57BL/6NHsd (Harlan BV., Horst, Netherlands) line seven times. In the experiments, male and female GluR-A−/− mice and their wild-type littermate controls were from heterozygous breeding, and all experiments were carried out at the age of 2.5–4 months. The animals were genotyped using tail-tip biopsy samples at the age of 30 days. The tails were digested overnight with proteinase-K (0.5 mg/ml; Finnzymes Oy, Espoo, Finland) at 55 °C and centrifuged at 15 300 *g* for 10 min followed by PCR on the supernatant as described previously (Zamanillo et al., 1999). The experimental animals were kept 2–4 per cage (37×21×15 cm, Tecniplast, Buguggiate, Italy) containing aspen chips and they had free access to standard rodent food pellets (Harlan) and tap water. The temperature of the animal room was kept at 21–23 °C and relative humidity at 50–60% with the lights on from 7 a.m. to 7 p.m. The experimental procedures were approved by the Experimental Animal Committee of the University of Helsinki and by the Southern Finland Provincial Government.

2.2. Behavioral testing procedures

The drug effects were evaluated by behavioral and functional tests selected from the primary SHIRPA mouse observational assessment protocol based on Irwin's earlier categorization of mouse behavior (Irwin, 1968) and modified as described in Vekovischeva et al. (2004). Briefly, the mice were visually observed in an open arena, on a horizontal wire and on a walking beam. The open arena consisted of an acrylic glass cage (55×33×18 cm), the floor of which was covered with a white plastic sheet divided into 15 squares of equal size. First, transfer arousal was scored by dropping the animal on the center of the arena and observing the immediate reaction of the animal. This was done by putting the animal in an acrylic glass cylinder (15×11 cm) with a metal sheet underneath it, and letting the animal fall from the cylinder by removing the sheet at approximately 25 cm above the arena. Locomotor activity (crossed squares), pelvic elevation, tail elevation and gait were assessed for 30 s immediately after transfer arousal evaluation. Touch escape was determined by touching the animal with a finger from above. The startle reflex was observed by generating a 90-dB click sound 30 cm above the animal using a click-box (MRC Institute of Hearing Research, Nottingham, UK). The grasping reflex of the hindlegs was assessed by holding the animal by its tail above a horizontal metal wire (at the height of 30 cm), allowing it to grip the wire with its forelegs, lowering the animal to the horizontal, and then releasing the animal to test its ability to grasp the wire with its hindlegs. Motor coordination was assessed with a wooden walking beam (length 80 cm, diameter 1.2 cm, height 60 cm) by monitoring the animal's ability to walk on the beam towards a safe laboratory bench. The righting reflex was evaluated by holding the animal by its tail and flipping it to the air in a backward somersault and then observing the landing position. The contact righting reflex was assessed by placing the animal in an acrylic glass tube (20×4 cm). The tube was rolled over to turn the animal upside down and the latency for the animal to right itself was measured. The body temperature was measured by a mouse rectal probe thermometer (BAT-12, Physitemp, Clifton, NJ). The tests were scored as described in the SHIRPA testing protocol, except for the tail elevation which was scored 0–3: 0=dragging tail, 1=horizontally extended, 2=elevated/Straub tail, 3=extensive elevation/Straub tail. The scoring of the measured behaviors is presented in Table 1. The behavioral testing was carried out genotype-blind, i.e., the experimenter was not aware of the mouse genotype while performing the experiments.

Withdrawal symptoms were precipitated by an injection of flumazenil and observed by placing the animal in an acrylic glass cylinder (diameter 20 cm, height 40 cm). Behavioral parameters (jumping, digging, stretching, "wet-dog"—like shakes, forepaw

Table 1
Scoring of the behavioral measurements used in experimental procedures

Behavior	Scoring
Transfer arousal	0=Coma
	1=Prolonged freeze, then slight movement
	2=Extended freeze, then moderate movement
	3=Brief freeze (a few s), then active movement
	4=Momentary freeze, then swift movement
	5=No freeze, immediate movement
	6=Extremely excited ("manic")
Locomotor activity	Number of crossed squares in 30 s
Pelvic elevation	0=Markedly flattened
	1=Barely touches
	2=Normal (3 mm elevation)
	3=Elevated (more than 3 mm elevation)
Tail elevation	0=Dragging
	1=Horizontally extended
	2= Elevated / Straub tail
	3=Extensive elevation / Straub tail
Gait	0=Normal
	1=Fluid but abnormal
	2=Limited movement only
	3=Incapacity
Touch escape	0=No response
	1=Mild (escape response to firm stroke)
	2=Moderate (rapid response to light stroke)
	3=Vigorous (escape response to approach)
Startle reflex	0=None
	1=Preyer reflex (backwards flick of pinnae)
	2=Jump less than 1 cm
	3=Jump more than 1 cm
Wire test	0=Active grip with hindlegs
	1=Difficulty to grasp with hindlegs
	2=Unable to grasp with hindlegs
	3=Unable to lift hindlegs, falls within seconds
	4=Falls immediately
Righting reflex	0=No impairment
	1=Lands on side
	2=Lands on back
	3=Fails to right when placed on back
Contact righting reflex	0=Absent
	1=Present

tremor, paw treading, head twitching, writhing, circulating behavior, and pelvic elevation) were monitored for 15 min for each animal from videotapes. One score point was given for each parameter and the total score was summed up for each mouse. Both FZ-treated and saline-treated mice received flumazenil injections. The withdrawal scores of FZ-treated mice were compared with those of saline-treated mice.

2.3. Drugs and injections

Flurazepam hydrochloride (FZ; Sigma, St. Louis, MO) was dissolved in 0.9% saline. Flumazenil (Sigma) was dissolved in a small volume of Tween 20 (Fluka Chemie GmbH, Buchs, Switzerland), sonicated, and filled to correct volume with saline. Control animals were injected with saline. Both drugs and saline were injected subcutaneously in a 10 ml/kg volume.

2.4. Experimental procedures

2.4.1. Effects of and recovery from an acute dose of flurazepam

FZ was given as a single 40 mg/kg injection and the mice were followed for a 12-h period. Behavioral testing was performed immediately before the drug injection, at 30 min after the injection and thereafter at one-hour intervals.

With another group of mice, the time elapsed in recovering behavior after a single dose of FZ (40 mg/kg) was determined. The criterion for recovery was met when the SHIRPA protocol parameters of gait, pelvic elevation and horizontal beam walking performance had returned to their corresponding baseline values. Body temperature was determined at the time of recovery, and blood and brain tissues dissected for determination of drug concentrations.

2.4.2. Subchronic administration of flurazepam and withdrawal symptoms

Mice received FZ injections twice a day at 12-h intervals for seven days. The dose per injection was 40 mg/kg for the first four days and 60 mg/kg for the next three days, giving total daily doses of 80 and 120 mg/kg, respectively. Development of tolerance was assessed by behavioral testing on the 1st, 4th, 5th and 7th test day of drug treatment, 30 min after the FZ or saline injection using the complete SHIRPA test. A group of treated animals was tested for withdrawal symptoms 48 h after the last FZ injection. Withdrawal was precipitated by injecting both flurazepam and saline-treated mice with 20 mg/kg flumazenil, and the animals were immediately scored for withdrawal signs for 15 min.

2.5. LC-MS analysis for flurazepam and desalkylflurazepam content in blood and brain

For the determination of FZ and desalkylflurazepam (DES) concentrations, blood plasma and brain samples were collected at four sampling time points: 1) 2 h after a single injection of FZ (60 mg/kg), 2) when the mice had recovered from the single 40 mg/kg dose of FZ, 3) 2 h and 4) 48 h after the last 60 mg/kg injection at the end of the seven-day chronic FZ treatment. The DES concentration is at its highest 2 h after FZ injection (Miller et al., 1988a). The mice were decapitated. Trunk blood was collected into tubes containing EDTA and centrifuged. The brains were quickly dissected and frozen on dry ice. The samples were stored at −80 °C until assayed. Before analysis, the brains were homogenized mechanically (Ultra-Turrax, Janke & Kunkel GmbH, Staufen, Germany) in 20 mM Tris–HCl buffer. FZ and DES concentrations were determined by the use of a PE SCIEX API 2000 liquid chromatography-tandem mass spectrometry system (Sciex division of MDS, Toronto, Canada) (Darius and Banditt, 2000; Miller et al., 1988a). The ion transitions monitored were mass-to-charge ratio (m/z) 388 to m/z 315 for FZ, m/z 289 to m/z 140 for DES, and m/z 314 to m/z 268 for the internal standard, flunitrazepam. The limit of quantification for FZ was 0.1 ng/ml (in our samples the limits

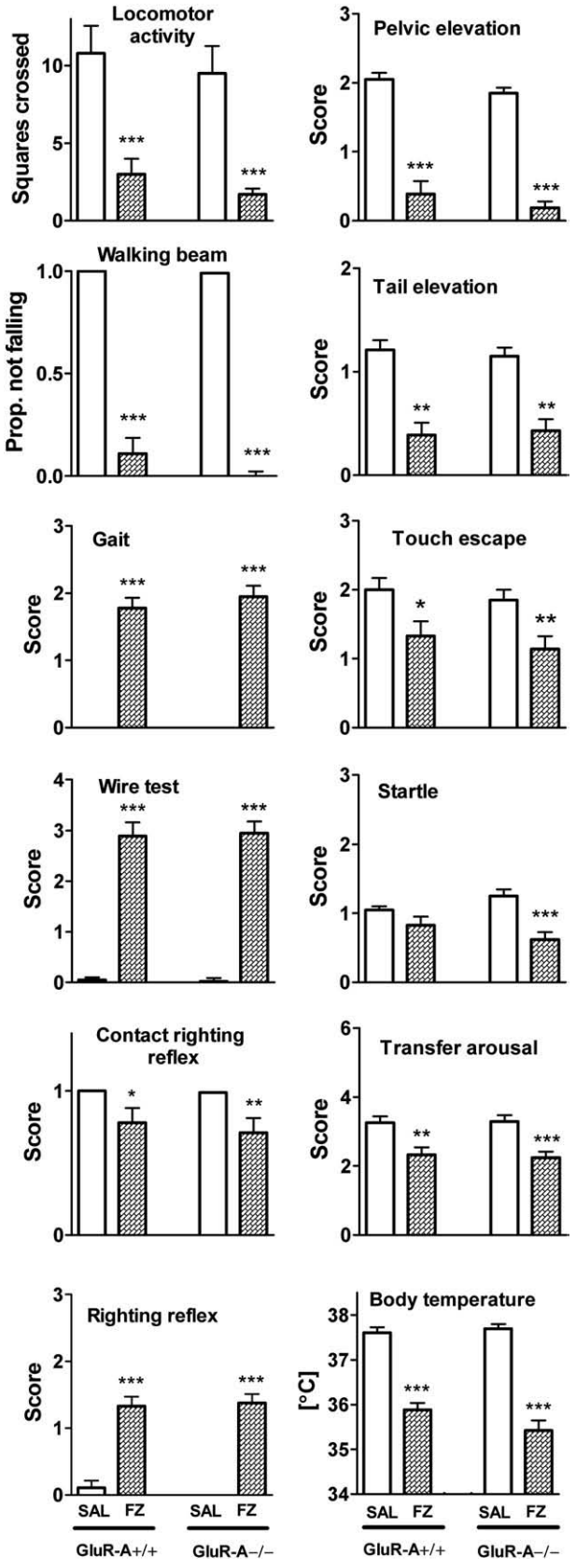


Fig. 1. Acute responses of GluR-A−/− and littermate GluR-A+/+ mice to flurazepam (40 mg/kg s.c.) in various behavioral tests. The behavior was scored 30 min after flurazepam (FZ) or saline (SAL) injections. The effects were not different between the lines in any of the tests, except that the body temperature was 0.4 °C lower in GluR-A−/− mice. FZ affected significantly all behaviors (except the startle scores in the wild-types). Bars are means ± SEM, $n = 18–21$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ for the significance of the difference within line between the flurazepam and saline groups (unpaired t-test).

corresponded to 0.26 nmol/L and 2.6 nmol/kg for plasma and brain tissue equivalents, respectively) and for DES 5 ng/ml (17 nmol/L and 173 nmol/kg for plasma and brain tissue equivalents, respectively).

2.6. Statistics

The data from the acute FZ experiments were analyzed with three-way ANOVA (genotype×gender×drug), and the data on the recovery of behavioral function with two-way repeated-measures ANOVA (genotype×gender, with time as the within-subjects factor). The data from the subchronic FZ experiment were analyzed with three-way repeated-measures ANOVA (genotype×gender×drug, with day as the within-subjects factor), and those from the withdrawal symptoms with three-way ANOVA (genotype×gender×drug). The drug concentration data were analyzed with three-way ANOVA (genotype×gender×sampling time). The SPSS statistical package (version 12.0.1, SPSS Inc., IL, USA) was used in the analyses. When significant differences were indicated by ANOVA, a post-analysis was carried out with unpaired *t*-test, least significant difference post-test, or Wilcoxon matched-pair test. The significance was set at $p<0.05$. To make the results easily readable, the *F*-values and *p*-values are expressed only for the least significant SHIRPA parameter in the acute and subchronic treatment experiments.

3. Results

3.1. Acute effects of flurazepam

The GluR-A−/− mice were compared to their wild-type GluR-A+/+ littermates in regard to sensitivity to FZ (40 mg/kg) by assessing various behavioral parameters according to the SHIRPA protocol 30 min after the injection of the drug or saline. None of the measured parameters showed a difference between the genotypes ($p>0.05$), except for the body temperature that was slightly lower in the knockouts (Fig. 1). The drug effect was clearly detected in all parameters ($F_{1,69}>9.95$, $p<0.01$). There were no significant genotype×drug interactions, nor any gender effects. All parameters reflecting motor functions, reflexes and activity, i.e., locomotor activity, motor coordination on the walking beam, gait, horizontal wire test, righting reflex and contact righting reflex were similarly reduced/impaired in both lines after FZ as compared with saline controls ($p<0.05$, unpaired *t*-test). In addition, muscle relaxation, as assessed by loss of pelvic and tail elevation, was increased in both mouse lines ($p<0.01$). Sensorimotor function, such as touch escape, was significantly affected in both lines ($p<0.05$), but the startle reflex was slightly attenuated only in the GluR-A−/− mice ($p<0.001$). Transfer arousal ($p<0.01$) and body temperature ($p<0.001$) were reduced by FZ in both lines. The measured parameters thus showed strong acute behavioral effects of FZ both in the wild-type and GluR-A−/− mice without any consistent genotype difference.

3.2. Recovery from an acute dose of flurazepam

Next, we wanted to test whether behavioral recovery from the effects of a single FZ injection (40 mg/kg) to naïve animals differed between the GluR-A−/− mice and their wild-type littermates. Because of the protracted behavioral effect of FZ, which is also due to its pharmacologically active metabolite DES (Bradley and Nicholson, 1984), we observed the behavior for 12 h. The acute sedative and motor-impairing effects of FZ were identical in GluR-A−/− and wild-type mice before they started to recover. Also, the baseline values in all tests determined prior to the FZ injection were identical between the mouse lines. The recovery as measured by gait ($F_{1,20}=27.6$, $p<0.001$) and pelvic elevation ($F_{1,20}=26.3$, $p<0.001$) (Fig. 2) was compromised in the GluR-A−/− mice as compared with the GluR-A+/+ littermates. In another set of animals, it also took longer for the GluR-A−/− mice to recover from the FZ injection than for their wild-type littermates ($F_{1,18}=52.1$, $p<0.001$) (Fig. 3). The body temperature at full recovery

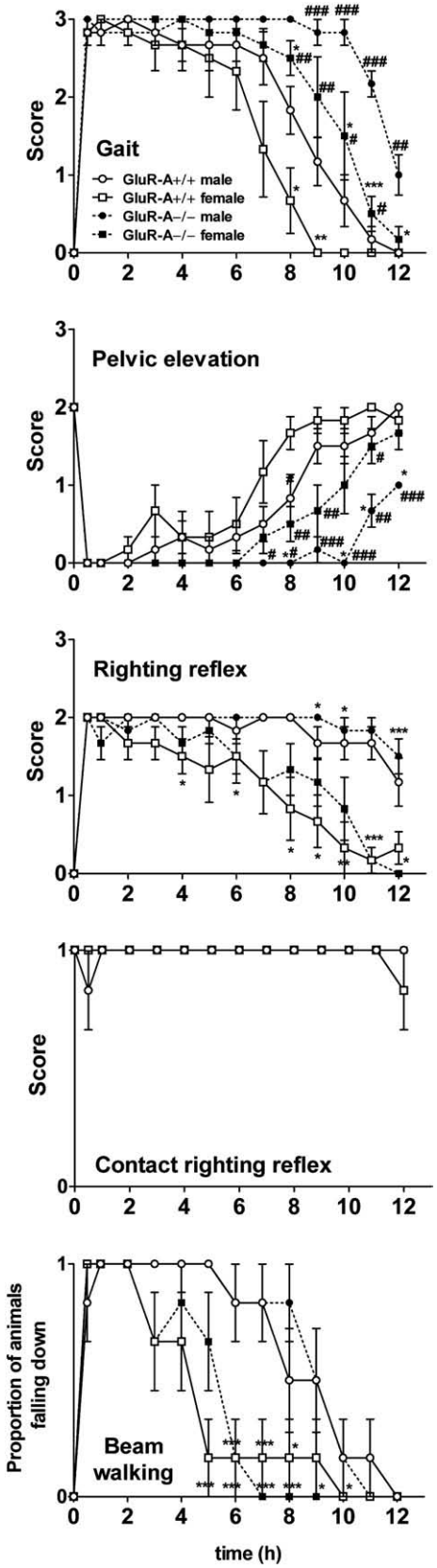


Fig. 2. Acute effects of flurazepam in the GluR-A−/− and littermate control mice in selected tests of motor function and reflexes after 40 mg/kg s.c. The behavioral parameters of GluR-A−/− and GluR-A+/+ mice were determined at baseline and 30 min after the injection, and thereafter at 1 h intervals until 12 h. Data are means±SEM, $n=10–12$ for each genotype. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ between genders; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ between the lines (unpaired *t*-test).

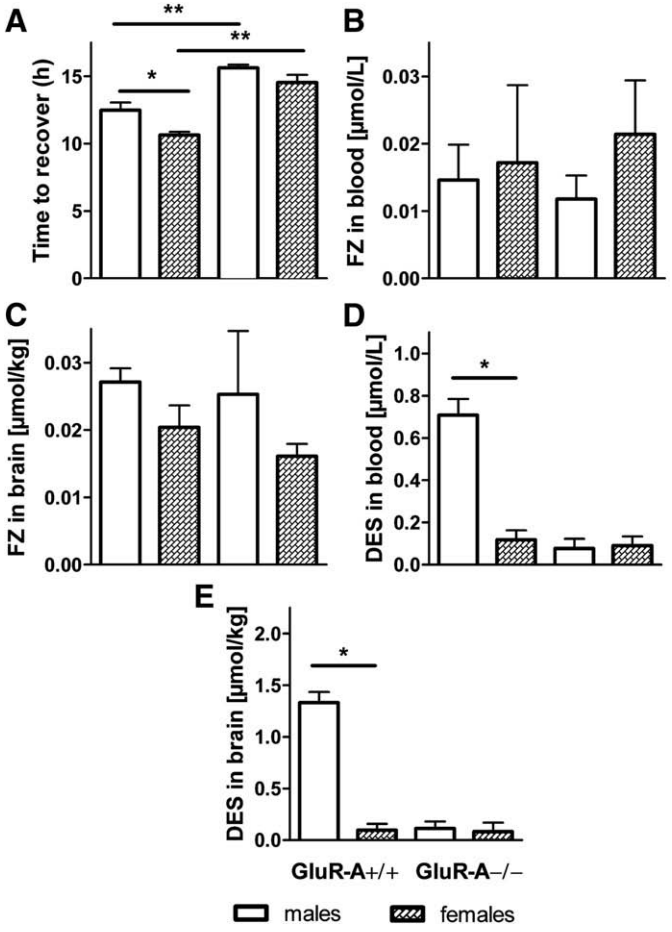


Fig. 3. Recovery of function after an acute dose of flurazepam 40 mg/kg s.c. A. Recovery time in hours after the FZ injection to GluR-A−/− and GluR-A+/+ mice. The criterion for recovery was that the scores of the SHIRPA parameters of gait, pelvic elevation, and horizontal beam walking performance returned to their baseline values. B–E. FZ and DES concentrations in blood and brain tissues. The data are means±SEM, $n=4$–5. $^{*}p<0.05$, $^{**}p<0.01$ (unpaired t-test).

did not differ between the genotypes or genders (data not shown). Gait, pelvic elevation, righting reflex and walking beam performance all showed a gender difference ($F_{1,20}=8.89$, $p<0.01$; $F_{1,20}=7.08$, $p<0.05$; $F_{1,20}=36.1$, $p<0.001$ and $F_{1,20}=20.8$, $p<0.001$, respectively), the female mice ($n=6$ for both GluR-A−/− and GluR-A+/+) recovering from the FZ dose quicker than the males ($n=6$ for both GluR-A−/− and GluR-A+/+).

Blood and brain concentrations of FZ and DES determined 2 h after the FZ injection showed similar levels in both GluR-A+/+ and GluR-A−/− mouse lines (Table 2). A lower level of DES in females suggests a slower rate of formation, a faster metabolism, or a larger volume of distribution of DES in females than males. At the time of behavioral recovery, the FZ concentration was similar in both genotypes as illustrated in Fig. 3B and C ($F_{1,18}=0.007$, $p>0.05$; $F_{1,18}=0.469$, $p>0.05$, for FZ in blood and brain, respectively) and genders ($F_{1,18}=0.560$, $p>0.05$; $F_{1,18}=3.12$, $p>0.05$, for FZ in blood and brain, respectively). The DES levels in blood and brain tissue, shown in Fig. 3D and E, were higher in GluR-A+/+ males than in GluR-A+/+ females and GluR-A−/− males and females (genotype effect in ANOVA: $F_{1,18}=34.33$, $p<0.001$; $F_{1,18}=55.65$, $p<0.001$, for DES in blood and brain, respectively; gender effect: $F_{1,18}=26.08$, $p<0.001$; $F_{1,18}=59.03$, $p<0.001$, for DES in blood and brain, respectively; genotype×gender interaction: $F_{1,18}=28.86$, $p<0.001$; $F_{1,18}=53.51$, $p<0.001$, for DES in blood and brain, respectively). However, the higher levels of DES concentration in GluR-A+/+ males did not result in a longer recovery time (Fig. 3A); this finding was not observed in any other drug condition.

3.3. Development of tolerance to subchronic flurazepam

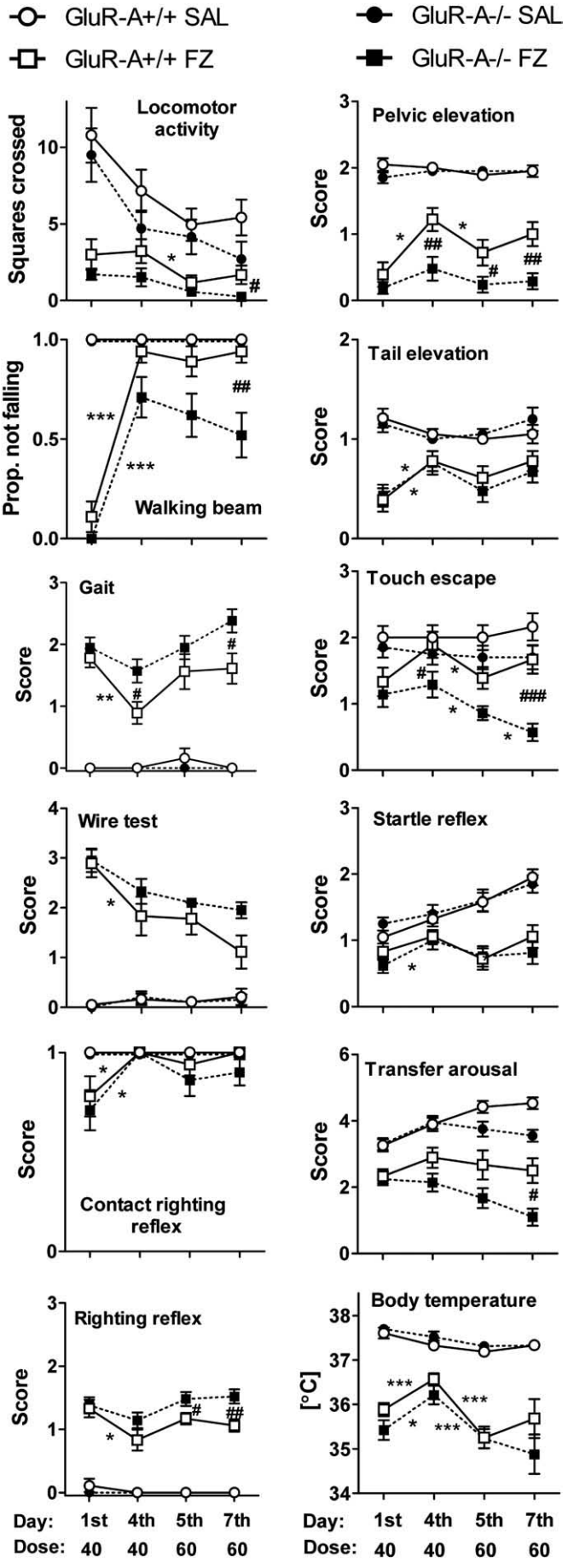
During repeated high-dose treatment with FZ, the performance of the mice was impaired and full tolerance developed only in some tests (Fig. 4). The maximal behavioral effect of FZ was reached in all tests 30 min after drug administration, and this time point was used to test the development of tolerance during subchronic FZ treatment (see Fig. 2). In all scores, there were significant drug effects and drug×day interactions (drug: $F_{1,69}>19.0$, $p<0.001$; drug×day interaction: $F_{3,207}>2.68$, $p<0.05$). In some tests, such as locomotor activity, walking beam, righting reflex, pelvic elevation, touch escape, and transfer arousal, the mouse genotype effect was significant ($F_{1,69}>5.36$, $p<0.01$), whereas in gait, tail elevation, wire test, startle, contact righting reflex, and body temperature, no genotype effect was found ($F_{1,69}<3.64$, $p>0.05$). The gender factor was significant in three tests (locomotion, $F_{1,69}=9.08$, $p<0.01$, wire test, $F_{1,69}=4.90$, $p<0.05$ and body temperature, $F_{1,69}=8.84$, $p<0.01$), and gender×day interaction was significant in locomotion ($F_{3,207}=4.326$, $p<0.01$) and body temperature ($F_{3,207}=7.661$, $p<0.001$) tests. The significant gender effect in locomotion was largely due to the higher activity in females than males after the first FZ injection ($p<0.01$).

To corroborate the finding that the GluR-A−/− mice and their wild-type littermates differed in tolerance development, we ran the Wilcoxon matched-pair test and compared the lines in regard to the behavioral scores obtained on different testing days (Fig. 4). Between the first and fourth day, the wild-types ($p<0.05$) improved their scores significantly

Table 2
Flurazepam (FZ) and desalkylflurazepam (DES) concentrations in blood and brain of acutely and subchronically flurazepam-treated GluR-A−/− knockout mice and GluR-A+/+ wild-type mice

Sample	WT males	WT females	KO males	KO females
2 h after an acute 60 mg/kg injection of FZ				
FZ				
Blood (µmol/L)	2.38±0.68	2.57±0.69	4.51±2.15	4.00±1.59
Brain (µmol/kg)	3.98±0.72	2.23±0.48	3.52±0.81	2.78±0.97
Brain/blood ratio	2.25±0.70	1.29±0.36	2.05±0.90	1.52±1.00
DES				
Blood (µmol/L)	4.03±0.30	1.68±0.28$^{\#\#\#}$	3.70±0.56	1.26±0.15$^{\#\#}$
Brain (µmol/kg)	5.65±0.41	2.24±0.29$^{\#\#\#}$	5.64±0.89	1.96±0.22$^{\#\#}$
Brain/blood ratio	1.41±0.07	1.37±0.07	1.52±0.05	1.56±0.05
DES/FZ ratio				
Blood	1.77±0.41	0.64±0.14$^{\#}$	2.66±1.66	0.50±0.23
Brain	0.97±0.19	1.35±0.22	0.91±0.22	1.94±0.61
2 h after the last 60 mg/kg dose of subchronic FZ				
FZ				
Blood (µmol/L)	0.31±0.09**	0.43±0.16*	0.44±0.26*	0.29±0.05**
Brain (µmol/kg)	1.03±0.24**	1.02±0.34	0.75±0.15**	0.87±0.25*
Brain/blood ratio	4.34±1.17	3.62±1.30	5.37±1.59	3.40±0.12
DES				
Blood (µmol/L)	9.03±0.45***	4.36±0.40$^{\#\#\#***}$	8.60±0.87**	4.23±0.62$^{\#\#*}$
Brain (µmol/kg)	14.8±0.78***	6.45±0.73$^{\#\#\#***}$	14.3±1.21**	6.08±0.69$^{\#\#\#**}$
Brain/blood ratio	1.64±0.06	1.47±0.07	1.69±0.11	1.52±0.10
DES/FZ ratio				
Blood	31.99±6.62**	13.13±3.84$^{\#**}$	48.14±14.71*	13.32±2.53$^{\#**}$
Brain	14.97±3.19**	11.40±4.13*	15.93±2.23**	10.13±2.68

Data are expressed as mean±SEM ($n=4$–9). Three-way ANOVA (genotype, gender, drug) gave significant effects of drug for: blood flurazepam (FZ), $F_{2,68}=33.4$, $p<0.001$; brain FZ, $F_{2,68}=54.2$, $p<0.001$; blood desalkylflurazepam (DES), $F_{2,68}=196.5$, $p<0.001$; brain DES, $F_{2,68}=258.5$, $p<0.001$; blood DES to FZ ratio, $F_{2,68}=31.9$, $p<0.001$; brain DES to FZ ratio, $F_{2,68}=41.6$, $p<0.001$; and of gender for: blood DES, $F_{1,68}=65.4$, $p<0.001$; brain DES, $F_{1,68}=98.4$, $p<0.001$ and DES to FZ ratio in blood, $F_{2,68}=8.50$, $p<0.01$. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ between acute and subchronic treatments within genotype and gender, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ between males and females within genotype (unpaired t-test). No significant differences were observed in brain to blood concentration ratios. Forty-eight hours after the subchronic FZ treatment, the concentrations of both FZ and DES were below the limit of quantification (data not shown).



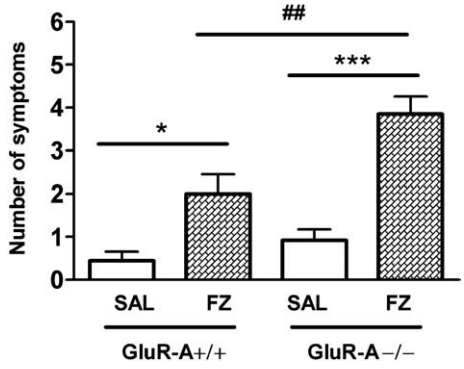


Fig. 5. Withdrawal symptoms precipitated with flumazenil (20 mg/kg s.c.) in GluR-A−/− and GluR-A+/+ mice 48 h after the cessation of subchronic flurazepam treatment. Evaluation of symptoms included jumping, digging, stretching, "wet-dog"—like shakes, forepaw tremor, paw treading, head twitching, writhing, circling behavior, and pelvic elevation; each giving one score point. Each animal was scored immediately after flumazenil injection for 15 min. Data are means ± SEM, $n = 10–13$. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ between FZ and saline groups within line (unpaired t-test). $^{\#\#}p<0.01$ between lines, within FZ-treated groups (unpaired t-test).

in 7 out of 12 tests, while the GluR-A−/− mice improved only in four tests. Between the fifth and seventh day, after the elevated FZ dose, the scores for the GluR-A−/− mice deteriorated further and there was a significant genotype difference in 7 out of 12 scores ($p<0.05$). However, at this high challenge dose, not even the wild-type mice developed statistically significant tolerance, although their performance tended to improve towards the behavioral values obtained after saline injection. The data suggest that the GluR-A−/− mice have a reduced capacity to develop tolerance to the effects of FZ. The decrease in body temperature showed tolerance in both females and males between the first and fourth day of FZ administration ($p<0.01$ and $p<0.05$, for females and males, respectively), but between the fifth and seventh day, the tolerance development was significant only in females ($p<0.01$).

After a challenge dose, FZ concentrations in blood and brain tissue were significantly reduced after subchronic FZ treatment as shown in Table 2 (ANOVA: blood FZ, $F_{2,68}=33.4$, $p<0.001$; brain FZ, $F_{2,68}=54.2$, $p<0.001$). In contrast, DES concentrations in blood and brain were increased after chronic treatment (blood DES, $F_{2,68}=196.5$, $p<0.001$; brain DES, $F_{2,68}=258.5$, $p<0.001$), similarly in GluR-A+/+ and GluR-A−/− mice. The ratio of DES to FZ was greatly (ca. 15-fold) increased both in blood and brain (DES to FZ ratio in blood, $F_{2,68}=31.9$, $p<0.001$ and brain, $F_{2,68}=41.6$, $p<0.001$) (Table 2). No overall genotype effect or genotype × sampling time interaction was found ($p>0.05$). Furthermore, the brain to blood concentration ratios for neither FZ nor DES were changed by the chronic FZ treatment ($F_{2,68}=3.59$, $p>0.05$ and $F_{2,68}=2.84$, $p>0.05$ for FZ and DES, respectively). However, females of both genotypes had significantly reduced DES levels in their blood and brain tissue ($F_{1,68}=65.4$, $p<0.001$ and $F_{1,68}=98.4$, $p<0.001$ for blood and brain, respectively), which was also reflected in a reduced DES to FZ ratio in blood ($F_{2,68}=8.50$, $p<0.01$).

3.4. Withdrawal symptoms after subchronic flurazepam

Flumazenil administration (20 mg/kg) 48 h after the last dose of subchronic FZ treatment provoked withdrawal symptoms in both GluR-A−/− and wild-type mice ($F_{1,38}=16.0$, $p<0.001$; Fig. 5). There were more symptoms in the GluR-A−/− mice than in their littermates

Fig. 4. Performance of GluR-A−/− and littermate control mice in various behaviors during subchronic flurazepam treatment. Scoring of the behaviors was carried out 30 min after the first daily dose of FZ (40 mg/kg s.c. on the 1st and 4th day, and 60 mg/kg on 5th and 7th day) or saline. Data points are means ± SEM, $n = 18–21$. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ within line between test days (Wilcoxon matched-pair test). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ between lines, within FZ-treated groups (unpaired t-test).

($F_{1,38}=6.45$, $p<0.05$). The number of withdrawal symptoms was similar in both genders ($F_{1,38}=0.60$, $p>0.05$). After the chronic treatment, at the 48-h sampling time point, the levels of blood and brain FZ and DES were below the limit of quantification (data not shown).

4. Discussion

Behavioral assessment of the effects of acute and subchronic treatment with FZ showed that the GluR-A subunit-deficient mouse line has a reduced capacity to develop tolerance to this drug. This result indicates that glutamatergic system has an important role in the neuroadaptation process opposing the increased neuronal inhibitory tone caused by BZs. However, precipitated withdrawal symptoms after the treatment were stronger in the GluR-A−/− mice, pointing to further differences in the neuronal/receptor adaptations between the knockout and wild-type mice. As expected, we observed no pharmacokinetic differences between the mouse lines. A strong induction of FZ metabolism was detected in both lines after subchronic treatment, however.

The deletion of the GluR-A subunit did not completely prevent the development of tolerance, indicating that also other mechanisms contributed to the BZ tolerance in the GluR-A−/− mice. Majority of the literature concerning various animal species finds the pharmacodynamic changes to be the most important factor in BZ tolerance (Loscher and Schwark, 1985; Miller et al., 1988b; Scherkl et al., 1985). However, also the metabolic induction leading to faster elimination of BZs might influence the development of tolerance to BZs, (File, 1982). FZ concentrations in blood and brain tissue were greatly reduced, i.e. to about 1/10 in blood and to about 1/2–1/4 in brain, after subchronic treatment in comparison to the concentrations after a challenge injection with the same dose. Importantly, the concentrations of DES were increased about three-fold. Subchronic FZ treatment greatly increased the DES to FZ concentration ratio in both blood and brain suggesting an induction of FZ metabolism. The elimination half-life of DES in the mouse is approximately 6 h (Miller et al., 1988a), which can lead to some cumulation of DES during the dosing of FZ at 12-h intervals. Cumulation of DES is supported by our tolerance data: only a slight further development of tolerance was observed in the latter phase of the subchronic treatment, i.e. between the fifth and seventh day, when the higher FZ dose was administered. Some further impairment was observed in the performance of the GluR-A−/− mice, e.g., in the walking beam test. The concentration of DES was lower in the females of both genotypes, indicating a gender-specificity in FZ metabolism or distribution in mice. Regardless of the gender-difference in FZ pharmacokinetics, the males and females differed in tolerance development only with respect to the body temperature reducing effect of FZ. Since the brain concentrations of DES at 2 h after FZ administration were more than 1000-fold higher than the K_i values for [^{3}H]flunitrazepam binding to rat cortical membranes [0.9 vs. 13 nM for DES and FZ, respectively (Miller et al., 1988a)], the receptor sites were apparently saturated. Functional activity is, however, often detected *in vitro* only with high nanomolar or micromolar concentrations (Yu et al., 1988). In summary, the receptor sensitivity difference between DES and FZ and the difference in their brain concentrations measured in the present high-dose experiment suggest that DES was the main active compound in that study. As there were no differences in the FZ or DES concentrations between the mouse lines, pharmacokinetic differences do not explain the lesser development of tolerance in the GluR-A−/− mice.

The role of the GluR-A subunit in long-term BZ treatment has previously been implicated by gene expression and protein level studies (Izzo et al., 2001; Song et al., 2007). Furthermore, BZ dependence enhances AMPA-mediated synaptic transmission in the hippocampal CA1 region (Van Sickle and Tietz, 2002), and AMPA receptor antagonists have been shown to be able to alleviate withdrawal symptoms when administered before the onset of the symptoms (Steppuhn and Turski, 1993). Our data seem to be at variance with these studies because the GluR-A−/− mice had reduced tolerance to FZ but exhibited more pronounced withdrawal symptoms. Therefore, unlike our earlier findings showing that subchronic morphine induces less tolerance and less withdrawal symptoms in the GluR-A−/− mice than in their littermates (Vekovischeva et al., 2001), the results on FZ administration suggest a divergence of the adaptation mechanisms between the development of tolerance and dependence. It is thus likely that in the absence of an upregulatable GluR-A subunit, which is an essential factor in many kinds of neuronal plasticity events (Bannerman et al., 2006; Kauer, 2004), other adaptation mechanisms, such as other glutamate and GABA mechanisms and/or e.g. the corticotropin-releasing factor mechanisms (Skelton et al., 2007), must be recruited.

Acute effects of FZ were not altered in GluR-A−/− mice, thus providing a solid basis for comparing the effects of repeated drug administration. In addition, GluR-A subunit deficiency did not affect the basal behavior of saline-treated animals, despite the GluR-A−/− mice having been described as hyperactive (Zamanillo et al., 1999; Vekovischeva et al., 2001). Hyperactivity could not be detected in the present tests, which did not include any exploration tasks. The lack of GluR-A subunit-dependent neuroadaptation became evident also during the recovery from an acute dose of FZ, as the GluR-A−/− mice needed a longer time to regain their baseline performance. In fact, it is known that an AMPA receptor turnover, by trafficking of AMPA receptors to synapses, occurs quickly by mechanisms in which the GluR-A subunit has an established role (Malenka, 2003). Thus, glutamatergic compensation during the action of sedative drugs, such as benzodiazepine agonists (this study) and opioids (Vekovischeva et al., 2001), is deficient in GluR-A−/− mice. Such compensation is not, however, needed for adaptation to ethanol, because ethanol effects, tolerance and dependence are unchanged in GluR-A−/− mice (Cowen et al., 2003). Although ethanol inhibits the glutamate receptor function (Lovinger et al., 1989; Möykkynen et al., 2003), it acts via multiple neurotransmitter targets (Weiss and Porrino, 2002) and may thus induce many other adaptational mechanisms to counteract its behavioral effects.

Excitatory glutamate and inhibitory GABA systems are known to regulate and scale each other's functional activity during the development *in vivo* and in *in vitro* cultured neurons (Martikainen et al., 2004; Möykkynen et al., 2007). It thus seems possible that in the absence of GluR-A subunit-containing AMPA receptors, the normal reciprocal interaction is deficient when the brain is challenged by high doses of BZs.

In the present study, flumazenil precipitated withdrawal symptoms after subchronic FZ treatment of mice. The present data allow one to estimate that, although below the limit of exact quantification, low nanomolar concentrations of FZ and DES may still have been present in brain tissue at 48 h after the last FZ dose. Flumazenil, acting as a selective antagonist of the allosteric BZ-site of $GABA_A$ receptors (Hunkeler et al., 1981), apparently displaced the low drug concentrations and induced the withdrawal symptoms by reducing the activity of the $GABA_A$ receptors. Although the residual FZ or DES levels at withdrawal precipitation remain unknown, significantly more withdrawal symptoms were observed in the GluR-A−/− mice than in their littermate controls. Taking into account the reduced tolerance of the knockout mice, one can suggest that a higher remaining inhibitory activity after the seven-day subchronic FZ treatment was prevailing in the knockout brains, perhaps due to the lack of down-scaling of the GABA system. This condition might then result in a more pronounced withdrawal in response to the blockade of the BZ sites. Therefore, tolerance to and withdrawal symptoms from subchronic BZ treatment may have a common neuronal substrate. Previous results using the same mouse model show that both tolerance to and withdrawal from subchronic morphine are decreased (Vekovischeva et al., 2001). Thus, the data derived from the GluR-A subunit-deficient mouse line suggest common mechanisms for opioid and BZ tolerance, probably involving GluR-A subunit-containing AMPA receptors, but different from the ones involved in the withdrawal from these compounds.

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References

Allison C, Pratt JA. Neuroadaptive processes in GABAergic and glutamatergic systems in benzodiazepine dependence. Pharmacol Ther 2003;98:171–95.

Allison C, Pratt JA, Ripley TL, Stephens DN. α-Amino-3-hydroxy-5-methylisoxazole-4-propionate receptor autoradiography in mouse brain after single and repeated withdrawal from diazepam. Eur J Neurosci 2005;21:1045–56.

Almirón RS, Pérez MF, Ramírez OA. MK-801 prevents the increased NMDA-NR1 and NR2B subunits mRNA expression observed in the hippocampus of rats tolerant to diazepam. Brain Res 2004;1008:54–60.

Bannerman DM, Rawlins JN, Good MA. The drugs don't work-or do they? Pharmacological and transgenic studies of the contribution of NMDA and GluR-A-containing AMPA receptors to hippocampal-dependent memory. Psychopharmacology (Berl) 2006;188:552–66.

Bateson AN. Basic pharmacologic mechanisms involved in benzodiazepine tolerance and withdrawal. Curr Pharm Des 2002;8:5-21.

Bradley CM, Nicholson AN. Flurazepam hydrochloride and its principal metabolites: behavioural studies in the monkey (*Macaca mulatta*). Psychopharmacology (Berl) 1984;82:395–9.

Carlezon Jr WA, Nestler EJ. Elevated levels of GluR1 in the midbrain: a trigger for sensitization to drugs of abuse? Trends Neurosci 2002;25:610–5.

Cowen MS, Schroff KC, Gass P, Sprengel R, Spanagel R. Neurobehavioral effects of alcohol in AMPA receptor subunit (GluR1) deficient mice. Neuropharmacology 2003;45:325–33.

Darius J, Banditt P. Validated method for the therapeutic drug monitoring of flunitrazepam in human serum using liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry with an ion trap detector. J Chromatogr B Biomed Sci Appl 2000;738:437–41.

File SE. Recovery from lorazepam tolerance and the effects of a benzodiazepine antagonist (RO 15–1788) on the development of tolerance. Psychopharmacology (Berl) 1982;77:284–8.

Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R. Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 2000;287:2262–7.

Hunkeler W, Mohler H, Pieri L, Polc P, Bonetti EP, Cumin R, et al. Selective antagonists of benzodiazepines. Nature 1981;290:514–6.

Hutchinson MA, Smith PF, Darlington CL. The behavioural and neuronal effects of the chronic administration of benzodiazepine anxiolytic and hypnotic drugs. Prog Neurobiol 1996;49:73–97.

Irwin S. Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia 1968;13:222–57.

Izzo E, Auta J, Impagnatiello F, Pesold C, Guidotti A, Costa E. Glutamic acid decarboxylase and glutamate receptor changes during tolerance and dependence to benzodiazepines. Proc Natl Acad Sci U S A 2001;98:3483–8.

Kauer JA. Learning mechanisms in addiction: synaptic plasticity in the ventral tegmental area as a result of exposure to drugs of abuse. Annu Rev Physiol 2004;66:447–75.

Korpi ER, Gründer G, Lüddens H. Drug interactions at $GABA_A$ receptors. Prog Neurobiol 2002;67:113–59.

Li M, Szabo A, Rosenberg HC. Down-regulation of benzodiazepine binding to α5 subunit-containing γ-aminobutyric $acid_A$ receptors in tolerant rat brain indicates particular involvement of the hippocampal CA1 region. J Pharmacol Exp Ther 2000;295:689–96.

Loscher W, Schwark WS. Development of tolerance to the anticonvulsant effect of diazepam in amygdala-kindled rats. Exp Neurol 1985;90:373–84.

Lovinger DM, White G, Weight FF. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science 1989;243:1721–4.

Mack V, Burnashev N, Kaiser KM, Rozov A, Jensen V, Hvalby O, et al. Conditional restoration of hippocampal synaptic potentiation in GluR-A-deficient mice. Science 2001;292:2501–4.

Malenka RC. Synaptic plasticity and AMPA receptor trafficking. Ann N Y Acad Sci 2003;1003:1-11.

Martikainen IK, Lauk K, Möykkynen T, Holopainen IE, Korpi ER, Uusi-Oukari M. Kainate down-regulates a subset of $GABA_A$ receptor subunits expressed in cultured mouse cerebellar granule cells. Cerebellum 2004;3:27–38.

Miller LG, Greenblatt DJ, Abernethy DR, Friedman H, Luu MD, Paul SM, et al. Kinetics, brain uptake, and receptor binding characteristics of flurazepam and its metabolites. Psychopharmacology (Berl) 1988a;94:386–91.

Miller LG, Greenblatt DJ, Barnhill JG, Shader RI. Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric $acid_A$ receptor function. J Pharmacol Exp Ther 1988b;246:170–6.

Möykkynen T, Korpi ER, Lovinger DM. Ethanol inhibits α-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function in central nervous system neurons by stabilizing desensitization. J Pharmacol Exp Ther 2003;306:546–55.

Möykkynen TP, Sinkkonen ST, Korpi ER. Compensation by reduced L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor responses in a mouse model with reduced γ-aminobutyric acid type A receptor-mediated synaptic inhibition. J Neurosci Res 2007;85:668–72.

O'Brien C. Benzodiazepine use, abuse, and dependence. J Clin Psychiatry 2005;66(Suppl 2):28–33.

Rosenbaum JF. Attitudes toward benzodiazepines over the years. J Clin Psychiatry 2005;66(Suppl 2):4–8.

Scherkl R, Scheuler W, Frey HH. Anticonvulsant effect of clonazepam in the dog: development of tolerance and physical dependence. Arch Int Pharmacodyn Ther 1985;278:249–60.

Shi S, Hayashi Y, Esteban JA, Malinow R. Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell 2001;105:331–43.

Skelton KH, Gutman DA, Thrivikraman KV, Nemeroff CB, Owens MJ. The CRF_1 receptor antagonist R121919 attenuates the neuroendocrine and behavioral effects of precipitated lorazepam withdrawal. Psychopharmacology (Berl) 2007;192:385–96.

Song J, Shen G, Greenfield Jr LJ, Tietz EI. Benzodiazepine withdrawal-induced glutamatergic plasticity involves up-regulation of GluR1-containing α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in hippocampal CA1 neurons. J Pharmacol Exp Ther 2007;322:569–81.

Steppuhn KG, Turski L. Diazepam dependence prevented by glutamate antagonists. Proc Natl Acad Sci U S A 1993;90:6889–93.

van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, et al. Requirement of α5-$GABA_A$ receptors for the development of tolerance to the sedative action of diazepam in mice. J Neurosci 2004;24:6785–90.

Van Sickle BJ, Tietz EI. Selective enhancement of AMPA receptor-mediated function in hippocampal CA1 neurons from chronic benzodiazepine-treated rats. Neuropharmacology 2002;43:11–27.

Vekovischeva OY, Zamanillo D, Echenko O, Seppala T, Uusi-Oukari M, Honkanen A, et al. Morphine-induced dependence and sensitization are altered in mice deficient in AMPA-type glutamate receptor-A subunits. J Neurosci 2001;21:4451–9.

Vekovischeva OY, Aitta-Aho T, Echenko O, Kankaanpaa A, Seppala T, Honkanen A, et al. Reduced aggression in AMPA-type glutamate receptor GluR-A subunit-deficient mice. Genes Brain Behav 2004;3:253–65.

Wafford KA. $GABA_A$ receptor subtypes: any clues to the mechanism of benzodiazepine dependence? Curr Opin Pharmacol 2005;5:47–52.

Weiss F, Porrino LJ. Behavioral neurobiology of alcohol addiction: recent advances and challenges. J Neurosci 2002;22:3332–7.

Yu O, Chiu TH, Rosenberg HC. Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration. J Pharmacol Exp Ther 1988;246:107–13.

Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, et al. Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science 1999;284:1805–11.