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ORIGINAL INVESTIGATION

C. Bonavita · A. Ferrero · M. Cereseto · M. Velardez · M. Rubio · S. Wikinski

Adaptive changes in the rat hippocampal glutamatergic neurotransmission are observed during long-term treatment with lorazepam

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Abstract *Rationale:* Chronic treatment with benzodiazepines induces tolerance to most of their pharmacological effects. The best-studied neurochemical correlation to this phenomenon involves GABAergic adaptive changes. However, some compensation by excitatory neurotransmission could also be postulated. *Objective:* The aim of this work was to investigate the effect of chronic treatment with benzodiazepines on several parameters of hippocampal glutamatergic neurotransmission. *Methods:* Rats were injected (IP) with a single dose or daily doses (21 days) of 1 mg/kg lorazepam (LZ) or vehicle. Thirty minutes after the last dose, animals were killed and parameters were measured in the dissected hippocampi. We determined one presynaptic parameter, in vitro glutamate release induced by a 60 mM K^+ stimulus. [^{3}H]MK-801 binding to postsynaptic NMDA receptors and the NMDA-stimulated efflux of cGMP were also evaluated. *Results:* While no changes were observed in any of the parameters after a single dose of the drug, we found an increase of 206% in in vitro glutamate release in chronically treated animals [two-way ANOVA: $F(1,16)=6.22$], together with an increment of 103% in the NMDA-stimulated cGMP efflux [two-way ANOVA: $F(1,18)=14.05$]. No changes either in K_D or in B_{max} values for [^{3}H]MK-801 binding to hippocampal membranes were observed. *Conclusions:* Taken together, these changes strongly suggest that a compensatory increase in the glutamatergic response develops in the hippocampus during chronic treatment with LZ. Our findings might indicate a contribution of glutamatergic mechanisms to the tolerance to hippocampal-mediated effects of LZ, such as amnesic and anticonvulsant activities.

Keywords Benzodiazepine tolerance · Hippocampus · cGMP efflux · Glutamate release · Glutamatergic neurotransmission · [^{3}H] MK-801 binding

C. Bonavita · A. Ferrero · M. Cereseto · M. Rubio · S. Wikinski (✉)
Instituto de Investigaciones Farmacológicas (ININFA), CONICET, Junín 956 Piso 5 (CP 1113), Buenos Aires, Argentina
e-mail: swikinsk@ffyb.uba.ar
Tel.: +54-11-49615949
Fax: +54-11-49638593

C. Bonavita · A. Ferrero · M. Cereseto · M. Rubio
Cátedra de Farmacología,
Facultad de Farmacia y Bioquímica, UBA,
Junín 956 Piso 5 (CP 1113), Buenos Aires, Argentina

S. Wikinski
1era Cátedra de Farmacología,
Facultad de Medicina, UBA,
Paraguay 2155 Piso 15 (CP 1113), Buenos Aires, Argentina

M. Velardez
Centro de Investigación en Reproducción,
Facultad de Medicina, UBA,
Paraguay 2155 Piso 3 (CP 1113), Buenos Aires, Argentina

Introduction

Benzodiazepines are frequently used drugs with anxiolytic, hypnotic, anticonvulsant, sedative and amnesic properties. These compounds are mainly prescribed on a chronic basis, but prolonged treatment induces tolerance to most of their pharmacological effects.

The best-known neurobiological mechanisms underlying such tolerance are $GABA_A$ receptor down-regulation (Zeng 1999), benzodiazepine-recognition site down-regulation (Itier 1996) and functional uncoupling of the $GABA_A$-benzodiazepine receptor ionophore complex (Hu 1994). These changes in $GABA_A$ receptor are related to changes in the expression of its α subunits (Li 2000; Tietz 1999a, 1999b) and are in accordance with a reduction in the drug effect.

On the other hand, some sort of excitatory compensation was also thought to play a role in the adaptive changes to chronic treatment with benzodiazepines. Some authors reported an increase in several parameters of glutamatergic activity in diverse areas of the central nervous system of benzodiazepine-withdrawn rats (Mortensen 1995; Tsuda 1997; Suzuki 1999). However, few studies have focused on glutamatergic neurotransmission in the tolerant condition.

The induction of long term potentiation (LTP) in the hippocampus is associated to seizure facilitation and to memory consolidation. The neurochemical sequence for LTP in hippocampus involves presynaptic glutamate release, AMPA, metabotropic and NMDA receptor activation and the participation of at least four postsynaptic pathways involving different protein kinases (see review by Izquierdo 1997). Among them, activation of PKG via the nitric oxide (NO)/cyclic GMP (cGMP) pathway has been described. This pathway is under GABAergic control (Fedele 1997, 1999). GABA-mediated inhibition of LTP seems to be the underlying mechanism for the anticonvulsant and amnesic actions of benzodiazepines. As mentioned above, prolonged treatment with these compounds induces tolerance to these effects. While a diminution in GABA inhibitory signal can explain this tolerance, a compensatory increment in glutamate-mediated excitation cannot be ruled out. In fact, Marin et al (1996) demonstrated a diminution in the threshold for LTP induction in hippocampi from rats treated with diazepam for 4 days.

Therefore, the objectives of this work were to study the effect of the long term administration of benzodiazepines on two parameters of hippocampal glutamatergic neurotransmission, the in vitro glutamate release and the binding of [^{3}H]MK-801 to NMDA receptors, together with one postsynaptic signal coupled to NMDA activation, the in vitro cGMP efflux.

Materials and methods

Animals and drug administration

Male adult Wistar rats, weighing 180–200 g at the beginning of treatment, were housed in groups of four or five in a room with constant temperature and a 12-h light-dark cycle. They received food and water ad libitum. The animals were treated according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington D.C., 1996). All efforts were made to reduce the number and the suffering of the animals employed.

Two treatment schedules were used. Chronic treatment was performed by 21 daily IP injections of lorazepam (LZ) 1 mg/kg or vehicle (55:45, polyethylene glycol: H_2O), while acute treatment consisted of a single dose of LZ or vehicle. Volume of injections was 1 ml/kg. Last doses of chronic treatments or single doses of acute treatments were administered 30 min before the animals were killed by decapitation.

3H-MK-801 binding to hippocampal membranes

Hippocampi were rapidly removed and frozen at –70ºC. Membranes were prepared according to the method described by Weiland et al. (1997). Briefly, tissue was thawed and homogenized (1:20 w/v) in 0.32 M sucrose containing 5 mM EDTA, and the homogenate was then centrifuged for 10 min at 1000 *g*. The supernatant was centrifuged at 30,000 *g* and the pellet resuspended (1:100 w/v) in 5 mM EDTA and incubated for 30 min at 30ºC. Membranes were centrifuged and washed twice by resuspension in 5 mM EDTA and incubation at 30ºC for 30 min. An aliquot was assayed for total proteins using the method of Bradford. The final pellet was frozen at –70ºC overnight. On the day of the experiment, membranes were resuspended in 20 mM HEPES/5 mM EDTA, pH 7, at an approximate protein concentration of 0.5 mg/ml, and saturation binding assays were performed (50 μg of protein per tube) using 1–50 nM [^{3}H]MK-801 (specific activity 22.5 Ci/mmol). All tubes also contained 100 μM glutamate, 20 μM glycine and 20 μM spermidine. Non-specific binding was determined in the presence of 10 μM MK-801. Samples were incubated at 30ºC for 3 h. The reaction was terminated by the addition of 4 ml of ice-cold 5 mM Tris-chloride buffer (pH 7.4) and vacuum filtration through Whatman GF/B glass-fiber filters. The filters were rinsed 3 more times with the same volume of buffer and the radioactivity retained was measured with a liquid scintillation spectrometer. The parameters B_{max} and K_D were estimated with the GraphPad Prism software.

In vitro glutamate release

The experimental procedure was similar to that described by Bonavita et al. (2002). The brains were removed and immediately placed on a Petri dish containing ice-cold Krebs buffer (5 mM KCl, 127 mM NaCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$., 0.8 mM Na_2HPO_4, 25 mM $NaHCO_3$, 11 mM glucose) pregassed with a mixture of O_2 (95%) and CO_2 (5%). Each hippocampus was cut in slices (250 μm) by means of a Sorvall tissue sectioner, and these were placed in a superfusion chamber maintained at 37ºC, perfused at a constant rate of 0.4 ml/min with gassed Krebs buffer. Perfusate samples were collected every 2.5 min. After an initial washout period of 60 min following slicing, to allow tissue stabilization, two samples were collected to assess basal release levels. To elicit glutamate release, the perfusing Krebs buffer was switched for 5 min to one containing 60 mM KCl (NaCl adjusted to 72 mM to maintain osmolarity), and then switched back to the original buffer. Samples were stored at –70ºC and the glutamate content assayed in a 250 μl-aliquote by HPLC with electrochemical detection. Results are expressed as pmol glutamate per mg tissue per ml perfusate.

NMDA-stimulated cyclic GMP efflux

Hippocampal slices (250 μm) were placed in the superfusion chamber as described for the in vitro glutamate release experiments. Perfusate samples were collected every 2.5 min. After an initial 60-min period to allow tissue stabilization, two samples were collected to assess basal efflux levels. To elicit cGMP efflux, tissue was superfused for 5 min with Krebs solution containing 200 nM NMDA and then switched back to Krebs buffer. Samples continued to be collected each 2.5 min during 22.5 min and were frozen at –70ºC until cGMP assay was performed.

Cyclic GMP was quantified by radioimmunoassay with a rabbit cGMP antibody (Chemicon, USA). As basal values were not different between groups, results were expressed as differences between basal and stimulated cGMP efflux, in fmol of cGMP per hippocampus.

Statistical analyses

Differences between basal and stimulated glutamate release or cGMP efflux were expressed as mean±SEM. K_D and B_{max} values from binding experiments were expressed as mean±SEM of absolute values. Comparisons were performed by a 2×2 two-factor ANOVA test (treatment×chronicity) followed by Bonferroni test. The α level was set at 0.05 for all statistical tests.

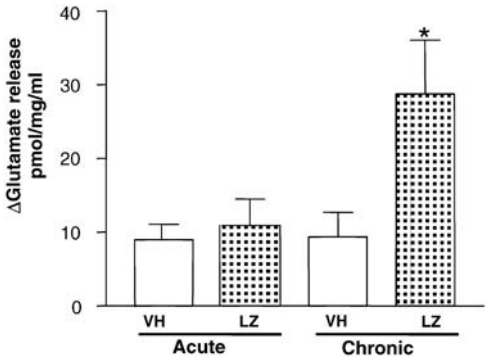

Fig. 1 In vitro release of glutamate was evaluated in hippocampus after a single dose (*Acute*) or the last dose of a 21-day (*Chronic*) treatment with 1 mg/kg of LZ or vehicle (*VH*). Results are shown as the mean±SEM (6–9 animals per group) of the differences between 60 mM KCl-stimulated and basal release. **P*<0.05 versus chronic VH. *F*(1,16)=6.22, two-factor ANOVA followed by Bonferrroni post test

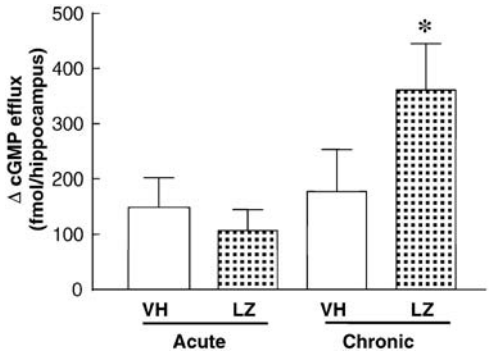

Fig. 2 cGMP efflux in hippocampal slices was evaluated after a single dose (*Acute*) or the last dose of a 21-day (*Chronic*) treatment with 1 mg/kg of LZ or vehicle (*VH*). Results are shown as the mean±SEM (5–8 animals per group) of the differences between 200 nM NMDA-stimulated and basal efflux. **P*<0.001 versus chronic VH. *F*(1,18)=14.05, two-factor ANOVA followed by Bonferroni post test

Results

^{3}H-MK-801 binding to hippocampal membranes

Table 1 shows the binding parameters obtained after a single dose of LZ or 21 days of treatment with the drug. No differences were found either in B_{max} or in K_D values after the acute or the chronic treatments.

In vitro glutamate release

No statistical differences were observed in the basal glutamate release between groups. Mean±SEM values were as follows (pmol/mg/ml): acute vehicle, 5.71±1.4, acute LZ, 7.7±2.7; chronic vehicle, 7.5±1.3 and chronic lorazepam, 6.3±1.5. Therefore, results of K^+-induced glutamate release are expressed as differences between stimulated and basal release (Fig. 1). We observed an increase (206%) in K^+-stimulated glutamate release in chronically LZ treated rats in comparison with chronic vehicle [*F*(1,16)=6.22]. No differences were found either between acute LZ and vehicle or acute and chronic vehicle.

Table 1 Parameters of [^{3}H]MK-801 binding to hippocampal NMDA receptors. Animals were killed by decapitation 30 min after the single dose or the last dose of a 21-day treatment. Hippocampal membranes were prepared and kept at −70ºC until used. On the day of the experiment, saturation curves were prepared with [^{3}H]MK-801 in concentrations ranging from 1 to 50 nM. Binding parameters were estimated by GraphPad Prism software. Results are expressed as mean±SEM of 5–6 animals per group

	K_D (nM)	B_{max} (fmol/mg protein)
Acute treatment	5.3±1.0	2868±635
Controls	6.4±0.9	2014±43
Chronic treatment	4.01±0.56	2506±135
Controls	5.51±0.3	2442±146

NMDA-stimulated cGMP efflux

Neither acute nor chronic treatment with lorazepam induced modifications in basal cGMP efflux. Basal values (mean±SEM) were as follows (fmol/hippocampus): acute vehicle, 179.0±7.5; acute LZ, 212.3±22.0; chronic vehicle, 155.2±32.0 and chronic LZ, 266.0±31.0. Results of NMDA-induced efflux, expressed as mean±SEM of the difference between stimulated and basal values, are shown in Fig. 2. Chronic treatment with LZ induced a significant increment (103%, versus chronic vehicle) in NMDA-stimulated cGMP efflux [*F*(1,18)=14.05], while no changes were observed after acute treatment.

Discussion

We investigated the effect of chronic treatment with LZ on two parameters of the hippocampal glutamatergic neurotransmission, [^{3}H]MK-801 binding and in vitro glutamate release, and on one postsynaptic coupled mechanism, NMDA-stimulated cGMP efflux.

Our experiments demonstrate that long term treatment with LZ increases glutamate release in response to a depolarizing stimulus with K^+ and also an increases in cGMP efflux after an NMDA stimulus, while no differences were found in the affinity or density of NMDA receptors. These changes are not attributable to an acute effect of the drug, since they were seen only after prolonged treatment. Therefore, there appears to be an adaptive modification of hippocampal excitatory neurotransmission elicited by chronic administration of LZ.

Changes in glutamatergic neurotransmission in relation to chronic treatment with benzodiazepines have been widely studied after the withdrawal of these drugs and were associated with abstinence symptoms (e.g. diminution of the convulsive threshold). In contrast, while tolerance was fully investigated with respect to GABA activity, only a few studies focused on excitatory

neurotransmission responses during this condition. We recently reported that tolerance to the sedative effect of these drugs correlates with a decrease in cortical glutamate release and in the affinity of NMDA receptors for [^{3}H]glutamate (Bonavita 2002).

Izzo et al (2001) investigated the expression of hippocampal GluR1 and NR1 subunits of AMPA and NMDA receptors, respectively, during tolerance and after withdrawal of diazepam. In abstinent animals, these authors found no changes in NR1 mRNA production, while GluR1 mRNA was significantly increased. During tolerance, on the other hand, they failed to observe significant changes either in GluR1 or in NR1 mRNA synthesis. The presence of an NR1 subunit is essential to conform a functional NMDA receptor. Only such receptors are identified by the [^{3}H]MK-801 binding experiments that we performed, because MK-801 binds to a site inside the ion channel, which becomes exposed by previous activation with glutamate. Therefore, our binding results are in agreement with the lack of changes in NR1 mRNA production reported by Izzo, as we also found no alterations in NMDA receptor density. However, other changes in NMDA receptor configuration may be induced which, in turn, could modify its response to ligands of the glutamate site (Layer 1993; Nowak 1998).

When we analyzed the in vitro release of glutamate, both basal and after a 60 mM K^+ stimulus, we found that a single dose of 1 mg/kg LZ did not change either basal or stimulated glutamate release. Conversely, after chronic treatment with LZ, we found an increase in the response of the hippocampal tissue to the K^+ stimulus. As mentioned above, the best-known adaptive changes correlating with chronic benzodiazepine treatment are those involving GABAergic neurotransmission. Glutamatergic neurons in hippocampus are inhibited by GABA acting at $GABA_A$ receptors and are stimulated by glutamate acting at NMDA receptors. Therefore, the in vitro glutamate release reflects an equilibrium between inhibitory and excitatory signals. Depolarization with K^+ induces both glutamate and GABA release in the hippocampal slices, but if GABA receptors are downregulated as a consequence of long term treatment with LZ, an increase in glutamate release could be observed. However, the hyperreactivity to the K^+ stimulus that we observed could also be the result of an increase in NMDA receptor response. Our observations on cGMP efflux support this possibility, as an increase in this parameter was observed after stimulation with NMDA which, acting at NMDA receptors, stimulates NOS/soluble guanylyl cyclase pathway (Fedele and Raiteri 1999). We have also observed that an NMDA stimulus induces an increment in glutamate release of about 100% (data not shown). Therefore, an eventual action of the endogenous neurotransmitter on AMPA receptors after an NMDA stimulus cannot be ruled out. Glutamate acting on AMPA receptors has been shown to exert both inhibitory and excitatory actions on cGPM production (Fedele and Raiteri 1999). The inhibitory effect consists of stimulation of GABAergic neurons, resulting in an increased GABA release. However, $GABA_A$ receptors are supposed to be downregulated after chronic treatment with LZ; thus a synergistic stimulatory effect of AMPA and NMDA receptors on cGMP effluxs appears to be likely. Our results seem to be in accordance with those reported by Marin et al. (1996). These authors demonstrated a diminution in the threshold for LTP induction in hippocampi from tolerant rats. As LTP requires the activation of glutamate receptors, the facilitation observed in the tolerant animals could be interpreted as sensitization of hippocampal glutamatergic coupled pathways as a consequence of the prolonged potentiation of GABA inhibition.

Finally, if we compare this effect with our previous findings in cerebral cortex, where we described a functional diminution in glutamatergic parameters (Bonavita 2002), it seems likely that adaptive changes in response to chronic treatment with benzodiazepines are area specific.

Several authors reported that 14 or more daily doses of benzodiazepines, equivalent to that used in the present study, induce tolerance to the amnesic (Ishihara 1993; Longone 1996) and anticonvulsant (Gupta 2000; Amano 2001) effects of the drug. Our experiments strongly suggest that an increased compensatory glutamatergic response develops in hippocampus during chronic treatment with LZ. As glutamatergic hippocampal neurotransmission is essential in seizure induction and memory formation, it is tempting to speculate that these adaptive changes could at least in part, explain the tolerance to the amnesic and the anticonvulsant properties of benzodiazepines observed both in experimental settings and in clinical practice.

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