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Neuropharmacology ■■ (2005) ■■■–■■■

NEURO PHARMACOLOGY

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Antiabsence effects of carbenoxolone in two genetic animal models of absence epilepsy (WAG/Rij rats and *lh*/*lh* mice)

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Received 31 December 2004; received in revised form 8 April 2005; accepted 12 April 2005

Abstract

Carbenoxolone (CBX), the succinyl ester of glycyrrhetinic acid, is an inhibitor of gap junctional intercellular communication. We have tested its possible effects upon two genetic animal models of epilepsy (WAG/Rij rats and lethargic (*lh*/*lh*) mice). Systemic administration of CBX was unable to significantly affect the occurrence of absence seizures in WAG/Rij rats. In particular, intravenous (5–40 mg/kg) or intraperitoneal (i.p.; 10–80 mg/kg) administration of CBX was unable to significantly modify the number and duration of spike-wave discharges (SWDs) in WAG/Rij rats, whereas the bilateral microinjection (0.05, 0.1, 0.5 and 1 μg/0.5 μl) of CBX into nucleus reticularis thalami (NRT) and nucleus ventralis posterolateralis (VPL) thalami produced a decrease in the duration and the number of SWDs. Bilateral microinjection of CBX into nucleus ventroposteromedial (VPM) thalami did not produce any significant decrease in the number and duration of SWDs. On the contrary, i.p. (5–40 mg/kg) or intracerebroventricular (0.5, 1, 2 and 4 μg/2 μl) administration of CBX in *lh*/*lh* mice induced a marked decrease in the number and duration of SWDs in a dose-dependent manner. At the doses used no movement disorders, or other behavioural changes, were recorded in both WAG/Rij rats and *lh*/*lh* mice. No effects were observed in both animal models following systemic or focal administration of glycyrrhizin into the same brain areas where CBX was shown to be effective.

Keywords: Epilepsy; Carbenoxolone; WAG/Rij rat; Lethargic mouse; Gap junction; Connexin; Absence seizures

1. Introduction

The mechanisms of action of established and new antiepileptic drugs are complex and include a variety of targets. In recent years, much attention has been focused on the possible modulation of inhibitory amino acids transmission (e.g. γ-aminobutyric acid (GABA)) or excitatory amino acids, such as glutamate and aspartate. However, a substantial fraction of clinical cases are still refractory to current therapies (Loescher, 2002), therefore, identification of novel pharmacological targets and analysis of the mechanism of action of drugs acting on them is still a priority in epilepsy research. It has been proposed that neuronal gap junctions can represent

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doi:10.1016/j.neuropharm.2005.04.012

a potential novel target for anticonvulsant therapy (Carlen et al., 2000; Perez-Velazquez and Carlen, 2000; Traub et al., 2001b, 2002; Bennett and Zukin, 2004; Nemani and Binder, 2005).

Gap junctions are the ultrastructural substrate of electrical synapses (Bennet, 1997), and connexins are the proteins that form gap junction channels (Willecke et al., 2002). A series of reports has demonstrated that inhibitory GABAergic interneurones in the cerebral cortex, hippocampus, thalamus, striatum and cerebellum are extensively interconnected by electrical synapses (for a review see Galarreta and Hestrin, 2001). Moreover, studies on the cellular localization of Cx36, the principal neuronal connexin in the adult brain (Condorelli et al., 2000, 2002, 2003; Belluardo et al., 2000; Rash et al., 2000), have shown its expression in GABAergic interneurones in several brain regions, including some thalamic nuclei (Belluardo et al., 2000), and analysis of Cx36 knock-out mice have confirmed its role in interneuronal coupling (Deans et al., 2001; Hormuzdi et al., 2001; Landisman et al., 2002). Experimental and theoretical evidence suggests that direct electrotonic communication between neurones via gap junctions, in combination with synaptic and ionic mechanisms, might contribute to the generation or maintenance of seizures (Carlen et al., 2000; Perez-Velazquez and Carlen, 2000; Traub et al., 2001a, 2002). Early experimental evidence is based on the anticonvulsant effects of gap junction blockers in in vitro seizure models (0-Ca^{2+}: Perez Velazquez et al., 1994; high K^+-low Ca^{2+}: Margineau and Klitgaard, 2001; 0-Mg^{2+}: Kohling et al., 2001; Nyikos et al., 2003; 4-aminopyridine: Ross et al., 2000; Traub et al., 2001b; bicuculline: Li et al., 2001; Samoilova et al., 2003; $GABA_B$ antagonists: Uusisaari et al., 2002; repetitive tetanization of Schaffer collaterals: Jahromi et al., 2002). An involvement of electrical coupling was also shown in vivo for the local administration of 4-aminopyridine in the rat neocortex (Szente et al., 2002; Gajda et al., 2003) and synchronization of cat thalamic reticular neurones (Fuentealba et al., 2004).

Gap junctions are known to play a significant role in high-frequency network oscillations (Draguhn et al., 1998; Traub et al., 2001a; Maier et al., 2002) and the onset and maintenance of epileptiform activity in a number of brain areas (e.g. adult guinea pig piriform cortex: de Curtis et al., 1998; rat hippocampus: Traub et al., 2002; Uusisaari et al., 2002; rat dentate gyrus: Schweitzer et al., 2000; rat amygdala: Elisevich et al., 1998). More recently, Liu and Jones (2003) have described the distribution of Cx36 in mouse thalamus and a previous report demonstrated that gap junctions might play a crucial role in thalamic reticular neurones (Landisman et al., 2002).

In human epilepsies, gap junctions have been linked to complex partial (Elisevich et al., 1997), intractable (Lee et al., 1995), mesial temporal lobe (Fonseca et al., 2002) and other seizure types (Carlen et al., 2000; Li et al., 2001; Naus et al., 1991; Perez-Velazquez and Carlen, 2000; Traub et al., 2001a, 2002). These findings have led to gap junction blockers being proposed as potential anticonvulsants.

Carbenoxolone is a commonly used gap junction blocker; it is the succinyl ester of glycyrrhetinic acid, which is an aglycone saponin derived from the liquorice root (Davidson et al., 1986; Davidson and Baumgarten, 1998). The exact mechanism of gap junction blockade by carbenoxolone is not currently known (Davidson and Baumgarten, 1998; Goldberg et al., 1996; Guan et al., 1996; Rouach et al., 2003).

Recently, we have shown that carbenoxolone exerts anticonvulsant effects against audiogenic seizures in genetically epilepsy prone rats (GEPRs) and DBA/2 mice and possesses an additive activity when administered in combination with some classical anticonvulsants such as diazepam, felbamate, gabapentin, phenobarbital and valproate (Gareri et al., 2004a,b).

In the present study, we evaluated the efficacy of systemic (intraperitoneal and intravenous) and focal administration of carbenoxolone in two different genetically prone animal models of epilepsy: the WAG/Rij rat and the lethargic (*lh*/*lh*) mouse models.

WAG/Rij rats present absence seizures and are included among GAERS (genetic absence epilepsy rats from Strasbourg) (Seidenbecher and Pape, 2001). It has been demonstrated that abnormal discharges on EEG are generalized and that the cortico-thalamic network is primarily involved (Inoue et al., 1993; Snead, 1995; Wang and Snead, 2001; Coenen and van Luijtelaar, 2003). Therefore, WAG/Rij rats are an interesting model of gene-linked absence epilepsy (Crunelli and Leresche, 1991; Midzianovskaia et al., 2001; Coenen and van Luijtelaar, 2003). Another useful model for absence epilepsy is represented by the *lh*/*lh* mice (Loscher and Schmidt, 1988; Hosford et al., 1992, 1995; Hosford and Wang, 1997): in this case epilepsy is overt without any sensory stimulation. *lh*/*lh* mice present ataxia, 5–8 Hz spike-wave discharges on cortical EEG, together with concurrent behavioural episodes such as vibrissae spasmus and increase in breath frequency. Behavioural and pharmacological studies have widely validated this genetic model (Hosford et al., 1995; Hosford and Wang, 1997).

2. Materials and methods

2.1. Animals

In this study, we used two genetic animal models of absence epilepsy: male WAG/Rij rats and lethargic (*lh*/*lh*) mice. Male WAG/Rij rats (200–260 g), 6–8 months

of age, were acquired from Harlan Italy (Correzzana, Milano). Male Wistar rats (Harlan Italy, Correzzana, Milano) 6–8 months old were used as control for WAG/Rij rats. Rats were housed three or four per cage (350×530 mm long×180 mm high) under stable conditions of humidity (60±5%) and temperature (21±2 °C) and allowed free access to food and water until the time of experiments. Lethargic (*lh*/*lh*) mice (B6EiC3Sn a/A-Cacnb4^{lh}) lacking the β_4 subunit of voltage-activated Ca^{2+} channels were originally obtained from Prof. B.S. Meldrum (University of London, UK) and inbred in the vivarium facilities of the Faculty of Pharmacy, University of Catanzaro (Italy) under the same conditions as WAG/Rij rats. Procedures involving animals and their care were conducted in conformity with the international and national law and policies (European Communities Council Directive of 24th November 1986, 86/609EEC).

2.2. Experimental design

The animals were placed individually into experimental cages and allowed to habituate to the environment for approximately 1 h; locomotor activity was contemporarily assessed. Control animals were always tested on the same day as the respective experimental groups. Carbenoxolone or glycyrrhizin (the main bioactive component of liquorice) were administered to WAG/Rij rats and to *lh*/*lh* mice; the administration was performed intraperitoneally (i.p.), intravenously (i.v.), or stereotaxically into some predetermined brain areas. Control animals received equal volumes of vehicle (DMSO+saline (1:9) or phosphate buffer solution) at the respective times before the test.

2.2.1. WAG/Rij rats

Increasing doses of carbenoxolone (CBX) or glycyrrhizin were administered i.p. (10–80 mg/kg) or i.v. (5–40 mg/kg). For i.v. administration, animals were anaesthetized with chloral hydrate (400 mg/kg i.p.; Carlo Erba, Milan, Italy) and drugs were injected into one jugular vein at doses of 5, 10, 20 and 40 mg/kg. A time course study was also performed up to 6 h.

Another group of rats was implanted with guide cannulae and received a bilateral microinjection of CBX or glycyrrhizin into thalamic sites at doses of 0.05, 0.1, 0.5 and 1 μg/0.5 μl. Bilateral guide cannulae were stereotaxically implanted into the nucleus reticularis thalami ($A=2.8$; $L=\pm3.4$; $H=5.8$ from bregma), into the nucleus ventralis posteromedialis thalami ($A=3.3$; $L=\pm2.6$; $H=6$ from bregma), or into the nucleus ventralis posterolateralis thalami ($A=2.3$; $L=\pm2.8$; $H=6$ from bregma), according to the coordinates of the atlas of Paxinos and Watson (1986). These nuclei have been demonstrated to be involved in SWDs pathogenesis (Inoue et al., 1993; Steriade et al., 1993; Snead, 1995; Coenen and van Luijtelaar, 2003). The intrathalamic administration was performed in order to point out whether or not microinjection in these brain sites might significantly influence number and duration of EEG features. To this aim, rats were also concomitantly implanted with cortical electrodes. Electrodes were implanted on cerebral cortex surface: two into frontal region (coordinates AP, 11; L, ±2.5), two in parietal region (coordinates AP, 7; L, ±2.5), one ground electrode into the frontal region and another referring electrode into the occipital region. After surgery, animals were allowed at least 1 week for recovery after implantation.

For the intracerebral administration of drugs, animals were gently hand-restrained and drug infusions were made bilaterally using injector cannulae connected by a polyethylene tube to a 1 μl Hamilton syringe. Drugs were infused in a volume of 0.5 μl at a rate of 0.2 μl/min, the cannulae kept in situ for one further minute. Animals were used only once, and at the end of the experiments, injection sites were verified by both macroscopic and histological examination. Each dose and group of experiments required at least 6 animals.

EEG was recorded in a frequency band between 1 and 30 Hz 1 h before and up to 5 or more hours after drug or vehicle administration. The quantification of absence of seizures was based on the number and the duration(s) of electroencephalogram spike-wave discharges (SWDs), as previously described (De Sarro et al., 2000; Russo et al., 2004). Exploratory spontaneous behaviour was contemporarily and independently registered by two independent researchers for all EEG duration according to Coenen and van Luijtelaar (1989).

2.2.2. Lethargic mice

lh/*lh* mice were chronically implanted with five electrodes and a guide cannula for i.c.v. administration under fluothane anaesthesia (according to the atlas of Paxinos and Franklin, 2001). At least 1 week after surgery, each mouse underwent five daily electroencephalogram (EEG) recordings. During each 5-h recording session, mice received i.c.v. either vehicle (0.01 M sodium phosphate buffer-DMSO) or drug (carbenoxolone or glycyrrhizin 0.5, 1, 2 or 4 μg/2 μl) per cannula at 60 min after each baseline recording or intraperitoneally (i.p.) either vehicle (DMSO+saline 1:9) or drug (carbenoxolone or glycyrrhizin 5, 10, 20 or 40 mg/kg, at least 6 mice per dose). During each recording, the behavioural changes after drug treatment in comparison to vehicle were noted. The identification of absence seizures was based on the duration(s) of electroencephalogram spike-wave discharges (SWDs) or poly-spikes, as previously described by Hosford et al. (1992) (i.e. amplitude not less than 60 μV and frequency range of 5–6 Hz; seizures must have a duration no shorter than 0.6 s). The quantification of absence seizures was based

on the duration of electroencephalogram SWDs, as previously described (De Sarro et al., 2000; Russo et al., 2004).

2.3. Expression of Cxs

In situ hybridization and immunohistochemical procedures were used mainly to examine, respectively as mRNAs and proteins, the expression levels of Cx30, Cx36, Cx43, and Cx45 in brain regions where carbenoxolone was injected: the nucleus reticularis thalami, nucleus ventralis posterolateralis thalami and nucleus ventralis posteromedialis thalami. The expression of these Cxs in WAG/Rij rats was compared with that of male Wistar rats 6 months old. Rats were killed by decapitation under deep anaesthesia, brains were rapidly frozen and serial coronal cryostat sections of 14 μm were prepared and processed for the in situ hybridization and immunohistochemical analysis.

2.3.1. In situ hybridization

To obtain anti-sense and sense cRNA probes for connexin mRNA in situ hybridization study a specific sequence for each Cx examined was used as reported in previous works (Condorelli et al., 2002, 2003). Tissue sections were processed for the in situ hybridization as previously described in Condorelli et al. (2003). Following fixation in 4% paraformaldehyde for 15 min, slides were rinsed twice in PBS and once in distilled water. Tissue was deproteinated in 0.2 M HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M ethanolamine for 20 min and dehydrated with increasing concentrations of ethanol. Slides were incubated for 16 h in a humidified chamber at 52 °C with 8×10^5 cpm of probe in 70 μl of hybridization cocktail (50% formamide, 20 mM Tris–HCl (pH 7.6), 1 mM EDTA pH 8.0, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 μg/ml yeast tRNA, 0.1 μg/ml poly-A-RNA, 1× Denhardt's solution and 10% dextran sulphate). Slides were washed twice in 1× SSC at 62 °C for 15 min, and then in formamide: SSC (1:1) at 62 °C for 30 min. After an additional washing in 1× SSC at 62 °C, single-stranded RNA was digested by RNase treatment (10 μg/ml) for 30 min at 37 °C in 0.5 M NaCl, 20 mM Tris–HCl pH 7.5, 2 mM EDTA. Slides were washed twice with 1× SSC at 62 °C for 30 min before dehydration in ethanol and air drying. For tissue localization of Cx mRNAs hybridized sections were exposed for 3 weeks to beta-Max Hyperfilm (Amersham) and subsequently coated with NTB-2 photoemulsion diluted 1:1 in water (Eastman-Kodak Co., Rochester, NY), stored in desiccated light-tight boxes at 4 °C for 4 weeks. Slides were developed with D19 (Eastman-Kodak Co.), fixed with Al-4 (Agfa Gevaert, Kista, Sweden) and counterstained with Cresyl Violet. Control of the hybridization specificity of the cRNA riboprobes was performed using sense ^{35}S-labelled riboprobes. In order to evaluate the mRNA levels of Cx30, Cx36, Cx43 and Cx45 a semiquantitative analysis was performed by measuring the optical density value of the area of interest (Rt, VPL and VPM) in the film autoradiograms on a personal computer using the PC version of the NIH IMAGE program (http://rsb.info.nih.gov./nih.image).

2.3.2. Immunohistochemistry

Cryostat sections of 14 μm thickness were fixed for 3 min in cold (−20 °C) acetone, air-dried for 15 min, rinsed with PBS containing 0.5% BSA and incubated for 2 h at room temperature (RT) with the following antibodies: (a) rabbit polyclonal Cx30 antibody diluted 1:50 (Zymed laboratory Inc., CA, USA); (b) rabbit polyclonal Cx36 antibody (Zymed laboratory Inc., CA, USA) diluted 1:200; (c) mouse monoclonal Cx43 antibody diluted 1:50 (Chemicon Int. Temecula CA, USA); (d) rabbit polyclonal Cx45 diluted 1:200 (Chemicon Int. Temecula CA, USA). After two washings with PBS, the sections were incubated at RT for 1 h with appropriate rhodamine-tagged secondary antibodies, diluted 1:200. After two washing in PBS, sections were cover-slipped and examined under a fluorescence microscope (Leica, DMRBE).

The evaluation of Cx protein levels was obtained by counting the puncta in the area of interest by mean of the analysis of particles module of the NIH IMAGE software.

2.4. Effects on motor movements

WAG/Rij rats were trained just before systemic antiepileptic testing, to do coordinate motor movements continuously for 5 min on a rotarod 6 cm in diameter, 4.5 rpm (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as the inability of the animals to remain on the rotarod for a test period of 5 min, according to Dunham and Mija (1957). The locomotor performance of WAG/Rij rats was usually assessed at 60 min after i.p. drug administration. Behavioural changes and their onset and duration were recorded after drug injection until the time of the rotarod test. In particular, two independent observers followed gross behavioural changes consisting of locomotor activity, ataxia, squatting posture and possible piloerection. These behavioural changes were noted but not statistically analysed.

2.5. Statistical analysis

The behavioural response was recorded for each animal. Statistical analysis of EEG changes in WAG/Rij and in lethargic (*lh*/*lh*) mice was performed through one-way variance analysis (ANOVA) (both for dose and time) followed by multiple comparison by Bonferroni when it was possible. All tests used were two-sided and

$P<0.05$ was considered significant. TD_{50} values (±95% confidence limits) for each compound were estimated using the method of Litchfield and Wilcoxon (1949).

2.6. Drugs

Carbenoxolone, the succinyl ester of 18-β-glycyrrhetinic acid (disodium salt, MW=614.7, water soluble), and glycyrrhizin, the glycoside derivative of 18-β-glycyrrhetinic acid (MW=840), were purchased from Sigma (St. Louis, MO, USA) and dissolved in DMSO+saline (1:9) for i.p. or i.v. administration and in DMSO+phosphate buffer (1:9) for intracerebral microinjection.

3. Results

3.1. WAG/Rij rats

The effects of carbenoxolone on absence seizures in WAG/Rij rat have been studied following both i.p. and i.v. injection. Furthermore, in order to test the efficacy of focal administration of CBX into known anatomical substrates of absence seizures, stereotaxic microinjections into the nucleus reticularis thalami, ventralis posterolateralis thalami and ventralis posteromedialis thalami were also performed.

3.1.1. Effects of intraperitoneal (i.p.) and intravenous (i.v.) administration of carbenoxolone or glycyrrhizin in WAG/Rij rats

Intraperitoneal administration of carbenoxolone (10, 20 and 40 mg/kg) was unable to produce significant changes in the number and duration of SWDs in WAG/Rij rats.

Even the highest dose tested (80 mg/kg i.p.), superior to that administered in previous work in GEPRs (Gareri et al., 2004b), was unable to produce significant changes in the number and duration of SWDs, although a mild non-significant decrease in the number and duration of SWDs was registered (Fig. 1A,B). Similarly, intravenous administration of carbenoxolone at the doses of 5, 10, 20 and 40 mg/kg was unable to significantly affect the number and the duration of SWDs (data not shown). The weak antiabsence effects of carbenoxolone reached a peak between the 2nd (i.v.) or the 3rd (i.p.) hour after injection and disappeared after 6 h.

Glycyrrhizin, a natural analogue of carbenoxolone which is inactive as a gap junction blocker (Davidson et al., 1986), did not affect the number and duration of absence seizures (up to 80 mg/kg by i.p. administration or 40 mg/kg by i.v. injection).

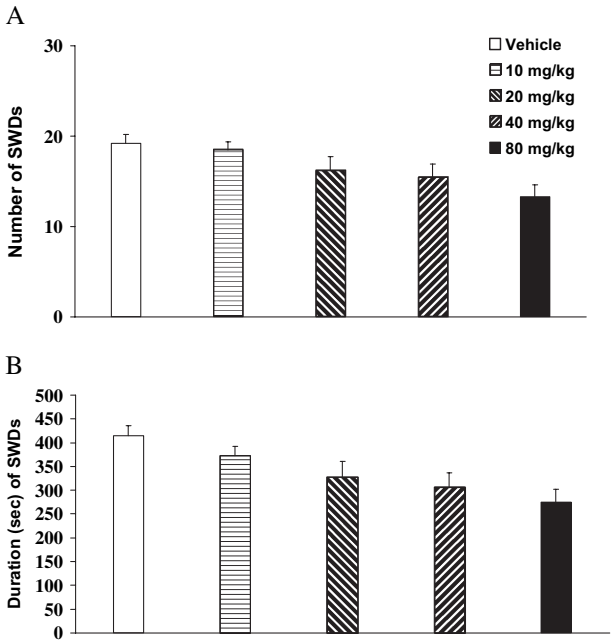

Fig. 1. Effects of various doses of carbenoxolone (10, 20, 40 and 80 mg/kg) administered i.p. 60 min before EEG recording, on the number (A) and duration (B) of spike-wave discharges in WAG/Rij rats. Data are expressed as mean±SEM.

3.1.2. Effects of bilateral microinjection of carbenoxolone or glycyrrhizin into nucleus reticularis thalami

Bilateral microinjection of carbenoxolone (0.5 and 1 μg/0.5 μl) into nucleus reticularis thalami (NRT) produced a significant decrease in the number and duration of SWDs (Fig. 2A,B). Maximal response was recorded 90–150 min after drug microinjection. Lower doses (0.05 μg and 0.1 μg/0.5 μl) did not significantly affect the number and duration of SWDs.

Bilateral microinjection of glycyrrhizin (0.05, 0.10, 0.50 and 1 μg/0.5 μl) into the NRT did not produce a reduction in the number or the duration of SWDs in the dose range examined (data not shown).

3.1.3. Effects of bilateral microinjections of carbenoxolone or glycyrrhizin into ventroposterolateral thalamic nuclei

Bilateral microinjection of carbenoxolone (0.5 and 1 μg/0.5 μl) into ventroposterolateral thalamic nuclei (VPL) produced a significant dose-dependent reduction in the duration and the number of SWDs (Fig. 3A,B). Maximal response was recorded 90 min after drug administration. Lower doses (0.05 μg and 0.1 μg/0.5 μl) did not significantly influence the number and duration of spike-waves epileptic discharges.

Bilateral microinjection of glycyrrhizin into VPL did not produce a reduction in the number or the duration of SWDs (data not shown).

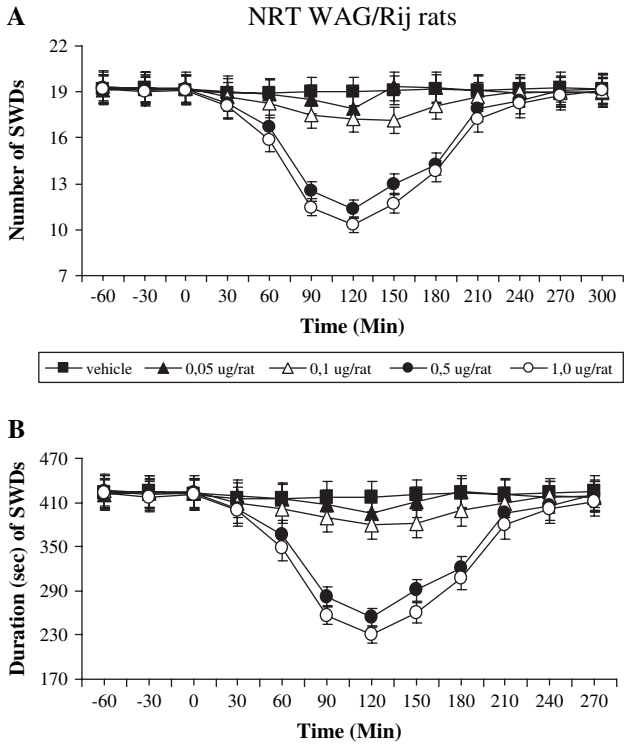

Fig. 2. Effects of the focal administration in the NRT of various doses of CBX on the number (A) and duration (B) of SWDs in WAG/Rij rats.

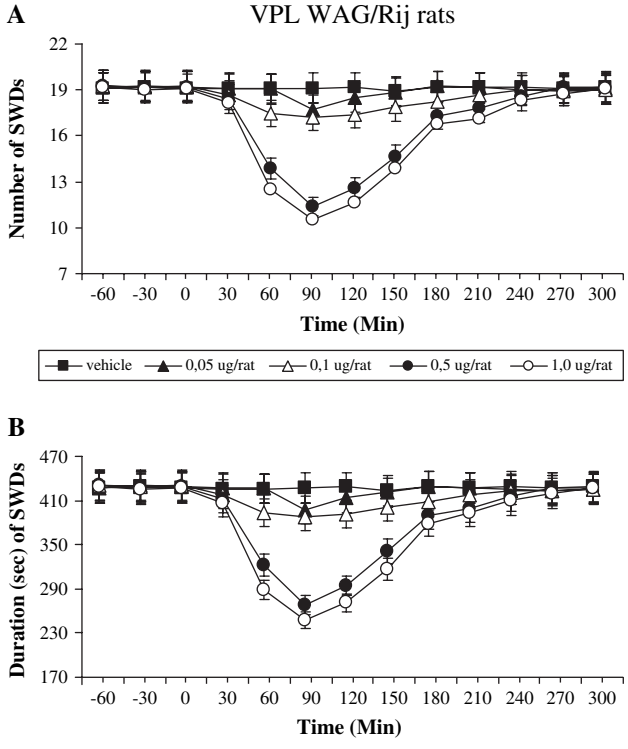

Fig. 3. Effects of the focal administration in the VPL of various doses of CBX on the number (A) and duration (B) of SWDs in WAG/Rij rats.

3.1.4. *Effects of bilateral microinjections of carbenoxolone or glycyrrhizin into nucleus ventralis posteromedialis thalami*

Bilateral microinjection of carbenoxolone or glycyrrhizin (0.05, 0.1, 0.50 and 1 μg/0.5 μl) into nucleus ventralis posteromedialis thalami (VPM) did not produce any significant decrease in the duration and the number of SWDs (Fig. 4A,B).

3.2. *Comparative analysis of connexin expression in the WAG/Rij and Wistar rat brain*

Among Cxs found in the brain, the main connexins expressed in the rat thalamus include Cx30, Cx36, Cx43 and Cx45. The present analysis was undertaken to test the hypothesis that a permanent alteration of one or more of these Cxs in the WAG/Rij rats, as compared to Wistar rats, could be one of the factors responsible for the increased susceptibility towards absence epilepsy in the WAG/Rij rats. Therefore we examined the expression of these Cxs in some thalamic nuclei (reticularis thalami, ventroposterolateral and ventroposteromedial thalamic nuclei) that have been involved in SWDs pathogenesis (Inoue et al., 1993; Steriade et al., 1993; Snead, 1995; Coenen and van Luijtelaar, 2003) and where carbenoxolone has been stereotaxically injected.

In the thalamus Cx36, the principal neuronal connexins, was detected at high levels in the nucleus reticularis thalami (Belluardo et al., 2000), and a comparative

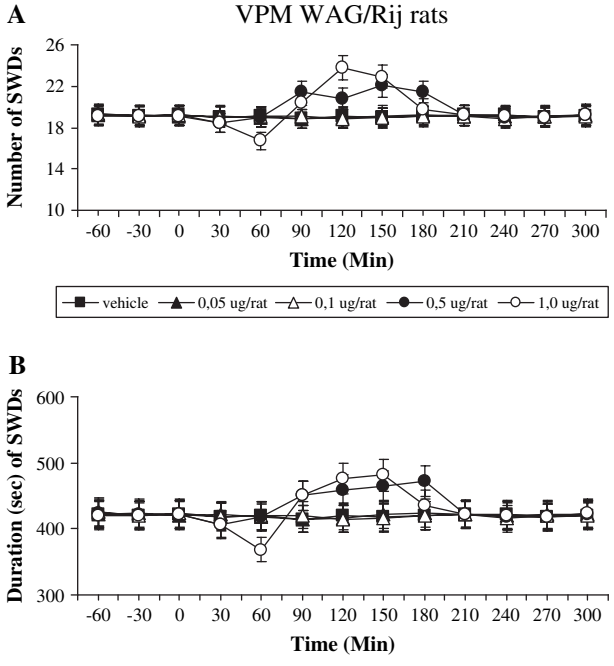

Fig. 4. Effects of the focal administration in the VPM of various doses of CBX on the number (A) and duration (B) of SWDs in WAG/Rij rats.

analysis between Wistar and WAG/Rij rats did not reveal differences at both mRNA and protein level. The neuronal and glial Cx45 (Condorelli et al., 2003) was expressed in all the thalamic nuclei but its expression was unchanged in the WAG/Rij rats as compared to control Wistar rats (Fig. 5). Cx30 and Cx43, two astroglial connexins (Condorelli et al., 2002, 2003), were expressed in all the thalamic nuclei examined. A slight reduction of Cx30 levels was detected in WAG/Rij rats as compared to Wistar rats (Fig. 6).

Taken together the results of both in situ hybridization and protein level analysis revealed no significant modification in Cx expression between WAG/Rij and Wistar rats in the thalamic nuclei examined.

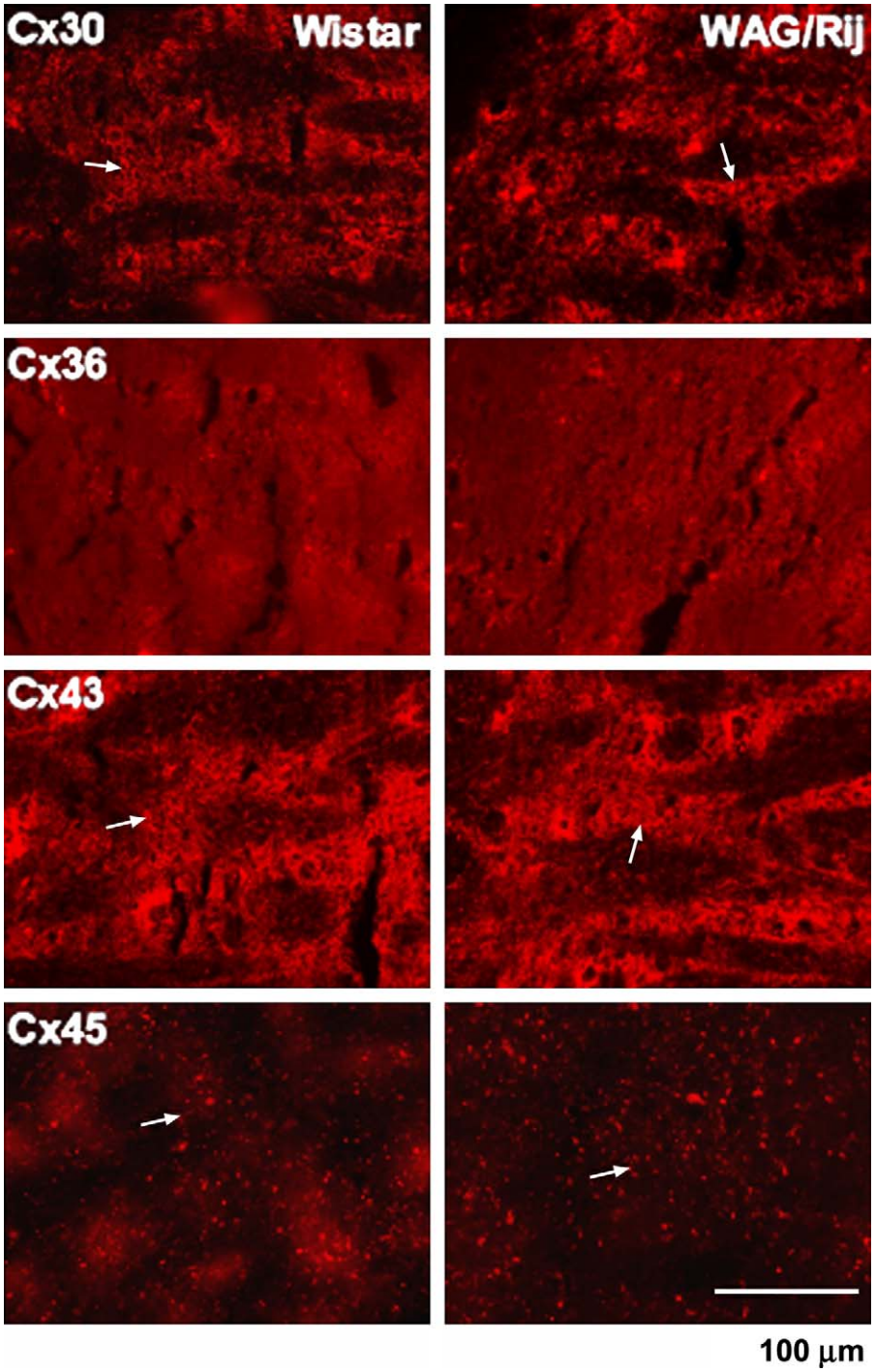

Fig. 5. Immunolabelling of Cx30, Cx36, in the nucleus reticularis thalami (NRT) and Cx43 and Cx45 in the nucleus ventralis posterolateralis (VPL) of rat control (Wistar) and WAG/Rij. Note the immunolabelling levels of different connexins examined in the wag/RiJ are similar to the control. Arrows indicate immunofluorescent puncta. Scale bar: 100 μm.

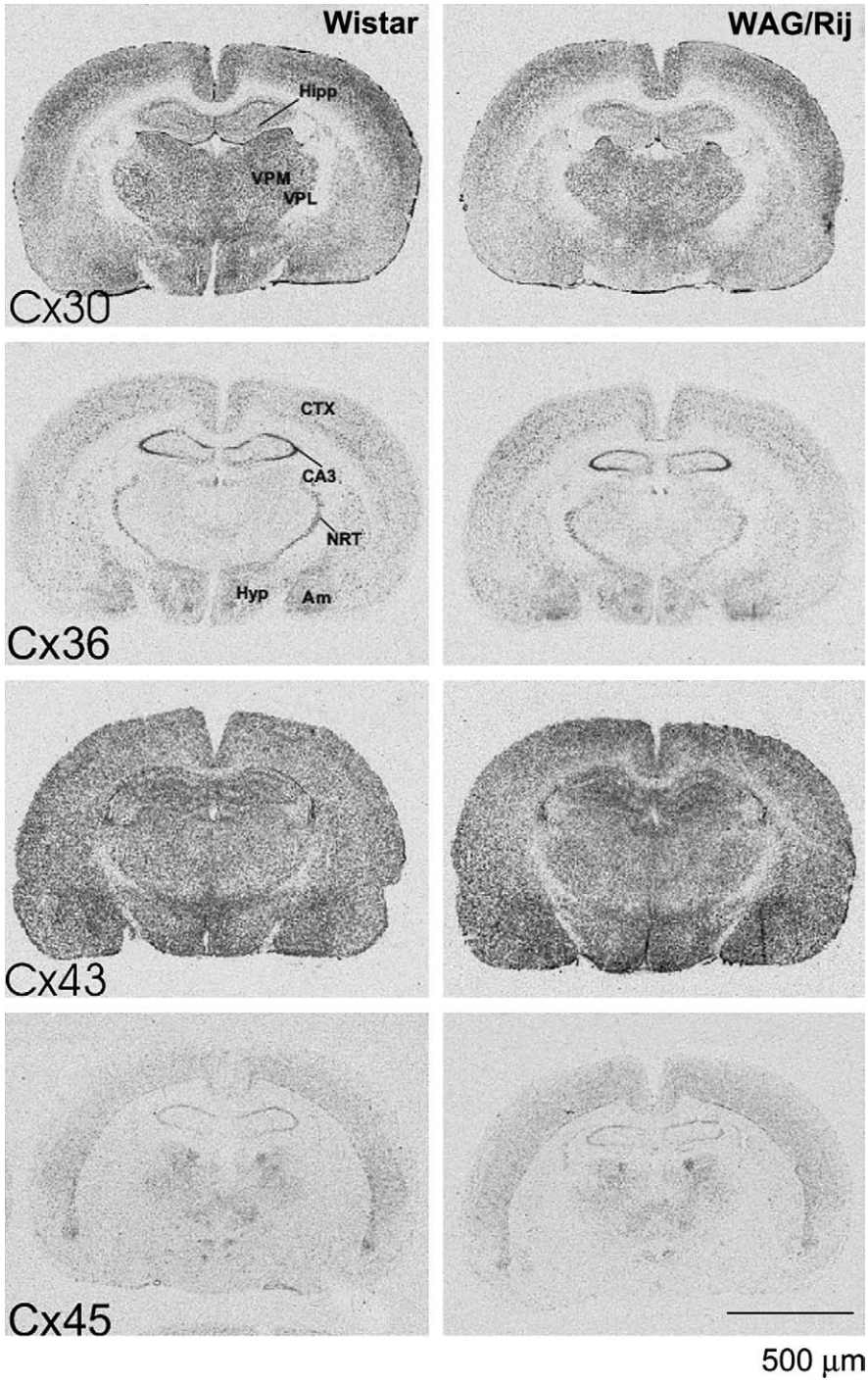

Fig. 6. Autoradiograms showing Cx30, Cx36, Cx43 and Cx45 mRNA expression in the brain of control (Wistar) and WAG/Rij rats. Representative views of coronal brain sections made at the dorsal hippocampal level. Am, amygdala; CTX, cerebral cortex; Hipp, hippocampus; Hyp, hypothalamus; NRT, nucleus reticularis thalami; VPL, nucleus ventralis posterolateralis thalamus; CA1, pyramidal layer of hippocampus. Scale bar, 500 μm.

3.3. Lethargic mice (lh/lh)

3.3.1. Effects of intraperitoneal (i.p.) administration of carbenoxolone or glycyrrhizin to lethargic mice

Intraperitoneal administration of CBX (10, 20 and 40 mg/kg) induced a significant dose-dependent reduction in the number and duration of epileptic discharges with a maximum effect observed around 120 min after CBX administration (Fig. 6A,B); the lowest dose (5 mg/kg) did not cause any significant changes of the typical spike-wave complexes of lethargic mouse EEG (Fig. 7A,B). At the effective anti-epileptic doses (10, 20 or 40 mg/kg)

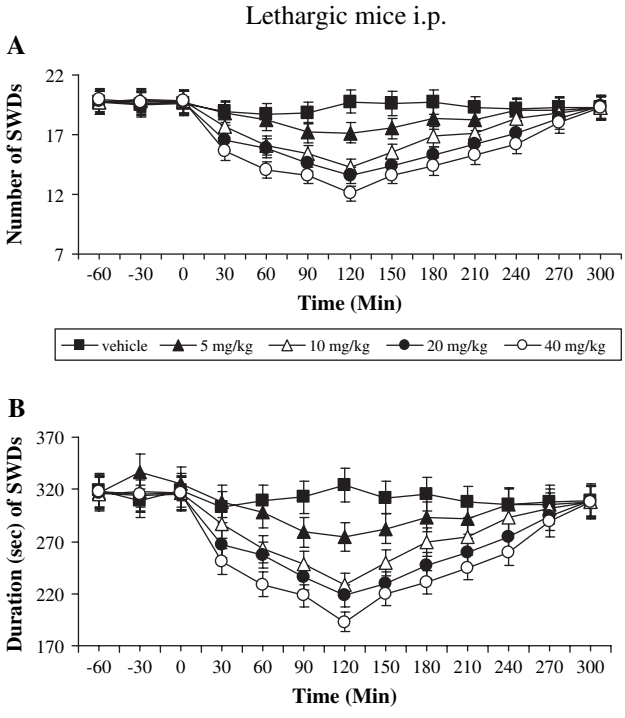

Fig. 7. Effects of the intraperitoneal administration of CBX on the number (A) and duration (B) of SWDs in *lh*/*lh* mice.

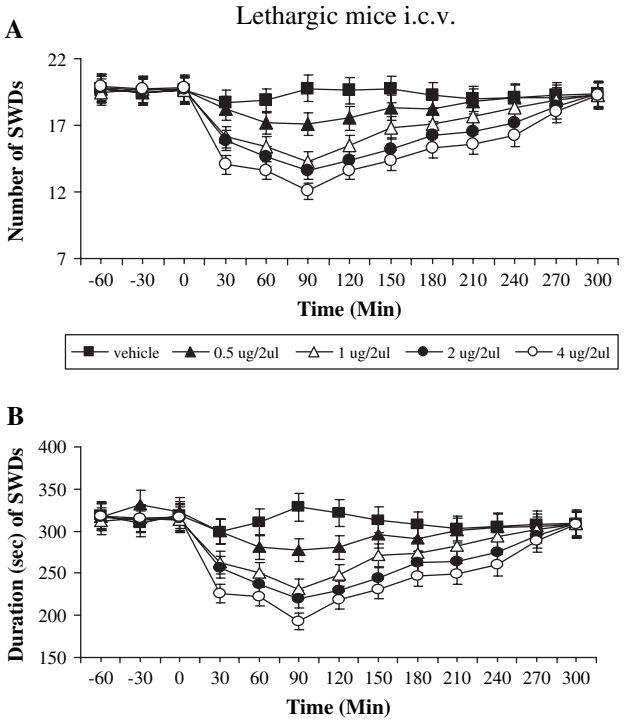

Fig. 8. Effects of the intracerebroventricular administration of CBX on the number (A) and duration (B) of SWDs in *lh*/*lh* mice.

no movement disorders or other behavioural changes were recorded.

Glycyrrhizin, given i.p. at 10, 20, 40 and 80 mg/kg was unable to produce any significant changes in the number and duration of the typical spike-waves of lethargic mice (data not shown).

3.3.2. Effects of intracerebroventricular microinjection of carbenoxolone or glycyrrhizin to lethargic mice

Intracerebroventricular injection of CBX (0.5, 1, 2 or 4 μg/2 μl) determined a dose-dependent and significant reduction in the number and duration of SWDs and did not affect motor coordination (Fig. 8A,B). Intracerebroventricular injection of glycyrrhizin (0.5, 1, 2 or 4 μg/2 μl) did not determine significant changes in baseline EEG recording (data not shown).

3.4. Locomotor activity

3.4.1. Influence of carbenoxolone or glycyrrhizin on locomotor activity of WAG/Rij rats

Carbenoxolone and glycyrrhizin, at the doses administered for evaluating antiabsence effects (up to 80 mg/kg), did not affect motor coordination in the rotarod test. However, doses of carbenoxolone higher than those used in the present study produced a transient ataxia in WAG/Rij rats. In particular, after the i.p. administration of a high dose of carbenoxolone (300 mg/kg), an impairment of locomotor performance was observed in WAG/Rij rats from 0.5 to 6 h (data not shown).

4. Discussion

4.1. Carbenoxolone anti-absence effects in lethargic mice and WAG/Rij rats

We have recently demonstrated that carbenoxolone possesses anticonvulsant effects, in vivo, in audiogenic seizure-susceptible DBA/2 mice and in genetically epilepsy-prone rats (Gareri et al., 2004a,b). Data reported here show that other genetic models of epilepsy are sensitive to the antiepileptic effects of carbenoxolone.

Lethargic mouse is a useful model for the study of absences in man (Hosford et al., 1992, 1995, 1999; Hosford and Wang, 1997). This model has shown a high predictable value in predicting the anti-absence clinical efficacy of several antiepileptic drugs (Hosford et al., 1992, 1995, 1999; Russo et al., 2004). Therefore, the present demonstration of a dose-dependent decrease in the occurrence of absence epilepsy following i.p. and i.c.v. administration of carbenoxolone in lethargic mice may have important implications for human therapy.

WAG/Rij rats present absence seizures and are included among GAERS (genetic absence epilepsy rats from Strasbourg) (Midzianovskaia et al., 2001;

Seidenbecher and Pape, 2001). Electrophysiologic studies indicated that abnormal discharges on EEG are generalized and the hippocampus is not primarily involved; thalamic nuclei, together with reticular thalamic nucleus apparently act as pacemaker for these abnormal discharges.

In WAG/Rij rats, systemic administration of carbenoxolone did not exert any significant antiabsence effects, whereas its focal administration into the reticular or into the ventroposterolateral thalamic nuclei caused a decrease in the number and the duration of spike-waves discharges. The results obtained following intracerebral stereotaxic injections suggest that some anatomical substrates of absence seizures are also targets for carbenoxolone action. The NRT is known as a critical structure in absence seizures and bilateral lesion of this area abolishes absence seizures permanently (Avanzini et al., 1993). Focal microinjection of $GABA_A$ agonists and $GABA_B$ antagonists and lesion experiments have also implicated the thalamic nuclei in the modulation of absences (Crunelli and Leresche, 1991). Accordingly, focal bilateral administration of carbenoxolone into the NRT or VPL clearly demonstrated that a direct action at these brain sites exerts a potent antiabsence effect. Indeed, typical brain connexin, such as Cx43, Cx30 and Cx45 are expressed in all thalamic nuclei examined, whereas the neuronal Cx36 is expressed only in the nucleus reticularis thalami (present work; Belluardo et al., 2000; Condorelli et al., 2003), where it plays an important role in the regulation of neural firing patterns (Landisman et al., 2002; Fuentealba et al., 2004). Moreover, increased expression of brain connexins (such as Cx43 and Cx30) have been observed in experimental epilepsy models in vitro and in vivo and in human epileptic brain tissue (Li et al., 2001; Condorelli et al., 2002, 2003; Szente et al., 2002; Gajda et al., 2003; Naus et al., 1991; Sohl et al., 2000; Aronica et al., 2001; Fonseca et al., 2002). We could not detect any significant difference in the expression of these connexins (Cx43, Cx30, Cx45, Cx36) in the brain of WAG/Rij in comparison to control Wistar rats, thus excluding that an abnormal expression of the main astroglial and neuronal connexins in the thalamic nuclei might contribute to the absence seizure susceptibility phenotype in these animals. Moreover, we have no direct proof that connexins are the real target of carbenoxolone action. Indeed, members of a novel family of gap junction proteins, called pannexins, are expressed at high levels in the rodent brain and channels formed by pannexins are sensitive to carbenoxolone (Bruzzone et al., 2003).

Rouach et al. (2003) suggested that the carbenoxolone blockade of spontaneous neuronal network activity in hippocampal or cortical neuronal cultures is not mediated by an action on gap junctions, but may instead be mediated by direct effects on neurones. Although previous reports have not described significant effects of carbenoxolone on intrinsic neuronal properties (Draguhn et al., 1998; Kohling et al., 2001; Schmitz et al., 2001; Yang and Michelson, 2001), recent evidence suggests a more complex action of this compound on neuronal excitability (Rouach et al., 2003). Carbenoxolone is known to have other pharmacological actions, such as inhibition of 11-β-hydroxysteroid dehydrogenase and mineralocorticoid agonist effects (Jellinck et al., 1993). However, these effects do not seem to be linked to the anticonvulsant action. Ross et al. (2000) reported that the mineralocorticoid antagonist spironolactone was unable to block the ability of carbenoxolone to depress spontaneous epileptiform activity in hippocampal brain slices.

4.2. Effective doses of carbenoxolone

In the present work and in a previous work on audiogenic seizures in DBA/2 mice and GEPRs (Gareri et al., 2004a,b) we found that the anticonvulsant effects of systemic administration of carbenoxolone were significant at doses of 5–40 mg/kg. It is interesting that systemic administration of carbenoxolone at similar doses (7–35 mg/kg) has been reported to affect specific CNS functions in rats (blockade of apomorphine-induced striatal stereotypes in rats; Moore and Grace, 2002). In contrast, the ED_{50} of i.p. carbenoxolone was 280 mg/kg in the pentylenetetrazole epileptic model and doses as high as 400 mg/kg were necessary to observe significant effects in the maximal electroshock model (Hosseinzadeh and Nassiri Asl, 2003). However, at high doses, sedative and muscle relaxant effects and a decline of motor coordination were also observed, suggesting that more non-specific effects on CNS function were taking place (Hosseinzadeh and Nassiri Asl, 2003). In the present study, we did not observe any effect of carbenoxolone on motor coordination at doses that already exert a maximal anticonvulsant effect on absence seizures in lethargic mice, while we confirmed the ataxia-inducing effect of higher doses. Therefore, our results suggest that the anticonvulsant action of carbenoxolone is more evident or specific for audiogenic seizures (Gareri et al., 2004a,b) and absence epilepsy (present work) in comparison to other forms of generalized epilepsy.

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