J Physiol 588.14 (2010) pp 2621–2631

The Journal of Physiology

Pre- and postsynaptic modulation of monosynaptic reflex by $GABA_A$ receptors on turtle spinal cord

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There is growing evidence that activation of high affinity extrasynaptic $GABA_A$ receptors in the brain, cerebellum and spinal cord substantia gelatinosa results in a tonic inhibition controlling postsynaptic excitability. The aim of the present study was to determine if $GABA_A$ receptors mediating tonic inhibition participate in the modulation of monosynaptic reflex (MSR) in the vertebrate spinal cord. Using an *in vitro* turtle lumbar spinal cord preparation, we show that conditioning stimulation of a dorsal root depressed the test monosynaptic reflex (MSR) at long condition–test intervals. This long duration inhibition is similar to the one seen in mammalian spinal cord and it is dependent on $GABA_A$ as it was completely blocked by 20 μM picrotoxin (PTX) or bicuculline (BIC) or 1 μM gabazine, simultaneously depressing the dorsal root potential (DRP) without MSR facilitation. Interestingly 100 μM picrotoxin or BIC potentiated the MSR, depressed the DRP, and produced a long lasting motoneurone after-discharge. Furosemide, a selective antagonist of extrasynaptic $GABA_A$ receptors, affects receptor subtypes with $\alpha_{4/6}$ subunits, and in a similar way to higher concentrations of PTX or BIC, also potentiated the MSR but did not affect the DRP, suggesting the presence of $\alpha_{4/6}$ $GABA_A$ receptors at motoneurones. Our results suggest that (1) the turtle spinal cord has a $GABA_A$ mediated long duration inhibition similar to presynaptic inhibition observed in mammals, (2) $GABA_A$ receptors located at the motoneurones and primary afferents might produce tonic inhibition of monosynaptic reflex, and (3) $GABA_A$ receptors modulate motoneurone excitability reducing the probability of spurious and inappropriate activation.

(Received 22 February 2010; accepted after revision 24 May 2010; first published online 2 June 2010)

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Abbreviations BIC, bicuculline; DRP, dorsal root potential; MSR, monosynaptic reflex; PTX, picrotoxin; VRP, ventral root potential.

Introduction

GABA, the major inhibitory neurotransmitter in the central nervous system, exerts its action by activating the widely distributed $GABA_A$ and metabotropic $GABA_B$ receptors. Unlike $GABA_B$ receptors the ionotropic synaptic $GABA_A$ receptors are formed by a pentamer of subunits that form ligand-gated chloride channels, and play an important role in signalling with high temporal and spatial precision as well as producing synchronization and oscillatory behaviour (Farrant & Nusser, 2005). Recently in neurons of hippocampus and cerebellum a new type of $GABA_A$ receptor has been described. Residing at extrasynaptic or perisynaptic sites, these receptors are activated persistently by the GABA spillover, producing a form of signalling termed tonic inhibition (Farrant & Nusser, 2005; Walker & Semyanov, 2008). Importantly, all tonic extrasynaptic $GABA_A$ receptor-mediated conductances are blocked by high concentrations of bicuculline, picrotoxin or SR-95531 (Semyanov *et al.* 2004; Farrant & Nusser, 2005); for example, in hippocampal interneurones picrotoxin produces a maximal change in holding current at 100 μM (Semyanov *et al.* 2004). However, a submicromolar concentration of SR-95531 selectively blocks only phasic currents mediated by the classical $\alpha_1\beta_2\gamma_2$ synaptic $GABA_A$ receptor (Semyanov *et al.* 2004). Few $GABA_A$ receptor antagonists show clear subunit selectivity, but the diuretic furosemide has ~100-fold selectivity for α_6 over α_1 subunit-containing receptors (Korpi *et al.* 1995),

DOI: 10.1113/jphysiol.2010.188979

and has been used to determine the role of synaptic and extrasynaptic α_6-containing receptors in cerebellar granule cells.

In the spinal cord of adult mice the presence of extrasynaptic $GABA_A$ receptors in substantia gelatinosa neurones has been described recently (Takahashi *et al.* 2006; Ataka & Gu, 2006). However, until now electrophysiological evidence regarding the presence of these receptors at either motoneurones or primary Ia afferents has not been reported. In mammalian spinal cord, there is evidence to show that the monosynaptic reflex (MSR) is modulated by presynaptic inhibition of primary afferents mediated by synaptic activation of $GABA_A$ receptors at axo-axonic synapses with GABAergic interneurons (Schmidt, 1971). Presynaptic inhibition has been associated with primary afferent depolarization (PAD); this signal is propagated to the dorsal root afferents and is recorded as the dorsal root potential (DRP) (Eccles *et al.* 1962; Schmidt, 1971; Rudomin & Schmidt, 1999). In the bullfrog spinal cord a $GABA_A$ agonist, muscimol, depresses the MSR by inducing pre- and postsynaptic inhibition (Peng & Frank, 1989). Interestingly, the muscimol effect was reversely blocked by bicuculline (100 μM), although at that time it was not known that two classes of $GABA_A$ receptors existed. Our previous studies also have shown that muscimol decreased by 60% the motoneurone input resistance and the time constant, whereas bicuculline increased the amplitude of EPSPs in motoneurones by 20% (Delgado-Lezama *et al.* 2004), confirming the presence of $GABA_A$ receptors on turtle lumbar cord motoneurones. In the lamprey $GABA_A$ receptors play an important role in burst frequency regulation during fictive locomotion, increasingly significantly the frequency in the presence of picrotoxin (100 μM) or gabazine (100 μM) (Schmitt *et al.* 2004).

Morphological evidence from immunohistochemical and *in situ* hybridization also supports the presence of distinct $GABA_A$ receptors in afferent terminals and motoneurones composed of α2, α3, α5 and γ2 subunits (Persohn *et al.* 1991, 1992; Wisden *et al.* 1991; Ma *et al.* 1993; Alvarez *et al.* 1996; Bohlhalter *et al.* 1996; Yang *et al.* 1997). In supraspinal neurons extrasynaptic $GABA_A$ receptors include the α5-subunit (Farrant & Nusser, 2005; Walker & Semyanov, 2008). However, the role of these receptors in MSR modulation in the spinal cord is unknown.

In this study, we were able to answer this question pharmacologically with different concentrations of antagonists like picrotoxin, bicuculline and gabazine as well as furosemide, a selective extrasynaptic $GABA_A$ antagonist. Moreover we also observed for the first time that a $GABA_A$ mediated long duration inhibition of the MSR exists in the adult turtle, suggesting that there is indeed presynaptic inhibition of primary afferents and that it resembles the one observed in adult mammals (Eccles *et al.* 1963). We also demonstrated that $GABA_A$ receptor antagonists at concentrations known to preferentially block synaptic receptors (i.e. picrotoxin 20 μM, bicuculline 20 μM and gabazine 1 μM) abolished presynaptic inhibition without facilitation of MSR and partially depressed the DRP. However, a higher concentration of bicuculline or picrotoxin (100 μM) facilitated the MSR producing at the same time an additional depression of the DRP. Importantly, the MSR was followed by a prolonged after-discharge. Likewise, furosemide facilitated the MSR without affecting the DRP, suggesting the presence of $GABA_A$ receptors with $\alpha_{4/6}$ subunits in motoneurones.

Overall, these results suggest that (1) the turtle has a mammalian-like presynaptic inhibitory system in the spinal cord, and (2) $GABA_A$ receptors, probably of the extrasynaptic type, located on motoneurones and/or primary afferents produce tonic inhibition of the MSR.

Methods

All experimental procedures followed the guidelines set out in *The Journal of Physiology* (Drummond, 2009) and were carried out with the approval of the Cinvestav Experimental Ethics Committee and in accordance with the current Mexican Norm for Care and Use of Animals for Scientific Purposes. The animals were provided by the National Mexican Turtle Centre located in Mazunte, Oaxaca (Mexico) with the authorization (DGVS-03821/0907) by the Federal Mexican Government Ministry of Environment and Natural Resources (Secretaría de Medio Ambiente y Recursos Naturales, Semarnat).

Preparation

Fifty adult turtles (*Kinosternon leucostomun* and *Pseudemis scripta,* 15–20 cm carapace length) were anaesthetized with pentobarbitone (100 mg kg^{-1}, I.P.). The plastron was opened and the blood removed by intraventricular perfusion with Ringer solution (~10°C) of the following composition (mM): 120 NaCl, 5 KCl, 15 $NaHCO_3$, 3 $CaCl_2$, 2 $MgCl_2$ and 20 glucose, saturated with 2% CO_2–98% O_2 to give pH 7.6. The laminectomy was made to isolate lumbar spinal enlargement containing segments from D7 to D10 in continuity with the ventral and dorsal roots. For the recordings, the preparation was placed in a recording chamber and perfused with Ringer solution (20–22°C). At the end of the dissection the animals were killed by decapitation.

Recordings

Two dorsal (DRD8 and DRD9) and one ventral root (VRD9) were mounted in suction electrodes (Fig. 1*A*). The

test MSR recorded from VRD9 was evoked by stimulation of the ipsilateral dorsal root (DRD9). Long duration inhibition was induced by applying a conditioning stimulus to DRD8 before the test MSR was evoked; in some experiments the sensory evoked dorsal root potential (DRP) was recorded from DRD9. The recording suction electrodes were connected to a differential AC amplifier (Grass Instruments, Quincy, MA, USA) with a bandwidth of 0.1 Hz to 1 kHz. The dorsal roots were stimulated with a rectangular current pulse (0.5 ms). The threshold was defined as the minimum stimulus intensity that elicits a measurable ventral root potential (VRP). Unless otherwise stated the recordings shown were the average of four to eight stimuli applied every 30 s.

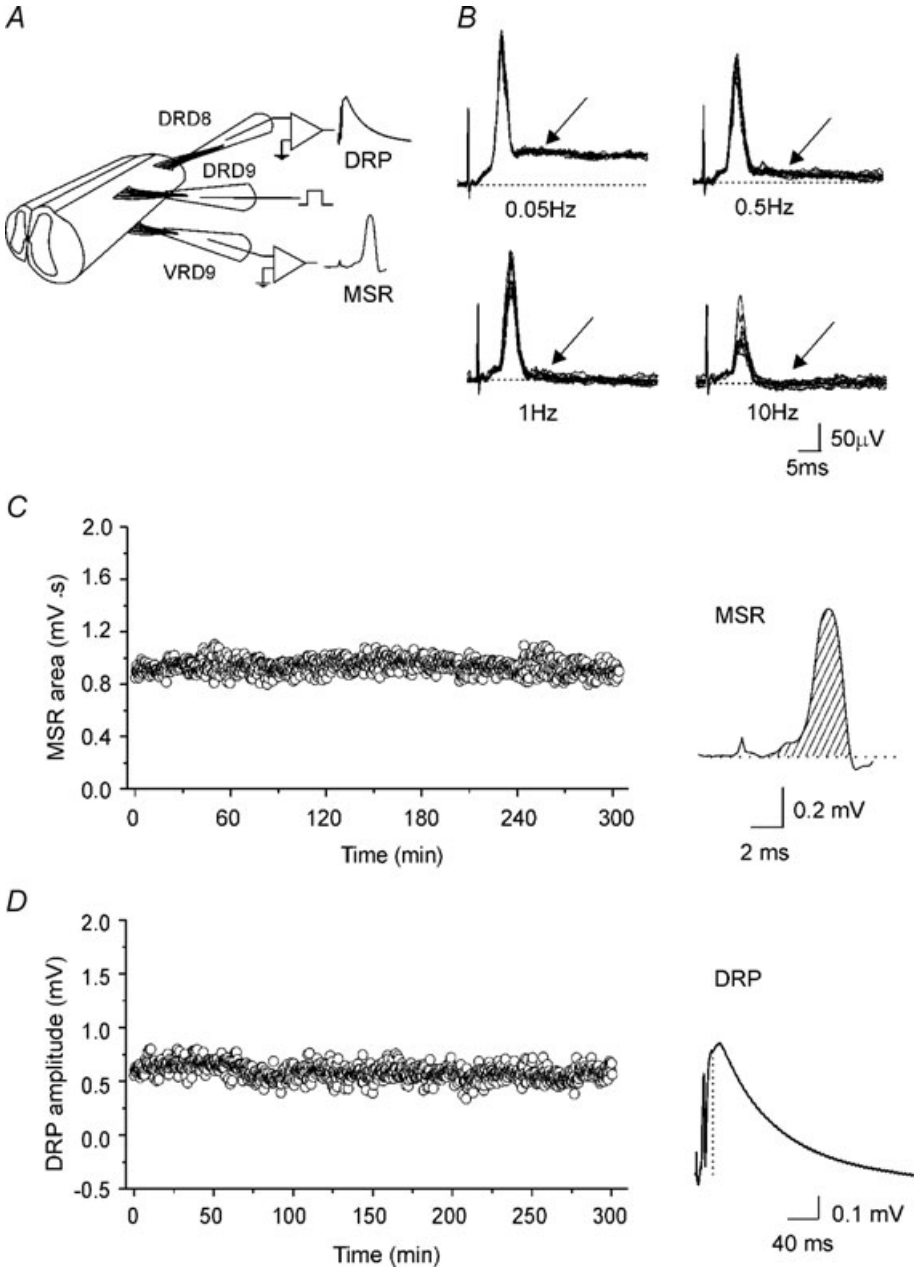

Figure 1. Simultaneous recordings of the monosynaptic reflex (MSR) and the dorsal root potential (DRP) in the turtle spinal cord

A, scheme of two spinal cord segments in continuity with the dorsal and ventral roots attached to suction electrodes for stimulation and recording of the DRP and VRP. *B*, VRP evoked at four different frequency stimulations (2*T*) applied to the ipsilateral dorsal root. The earliest component of VRP is the MSR because it followed one to one frequency stimulation at 10 Hz without jittering and failure. The slower VRP component failed to follow one to one at 1 Hz of dorsal root stimulation. Striped area under the MSR curve (*C*) and the DRP amplitude (*D*) recorded simultaneously every 30 s plotted *versus* time. Traces, average of the MSR and DRP recordings of 10 sweeps.

Drugs and analysis

Bicuculline (20–100 μM), picrotoxin (20–100 μM), gabazine (1 μM) and furosemide (100–200 μM) were applied to the bath solution to block $GABA_A$ receptors. All the drugs were purchased from Sigma. The action of $GABA_A$ antagonists was quantified by measuring the changes of the area under the MSR curve (Fig. 1*C*) and DRP amplitude (Fig. 1*D*). Values are presented as the mean ± S.E.M. The statistical difference between means was determined by Student's *t* test. Means were considered statistically different when $P < 0.05$.

Results

MSR characterization

Ventral root potential was evoked by stimulation (2.5 times threshold) of the homonymous dorsal root (D9); the earliest component presented a peak latency of 7.41 ± 0.10 ms ($n = 40$). These results are in agreement with previous studies showing similar latencies for monosynaptic EPSPs in turtle motoneurones (Yamashita, 1986), and as a result we considered this component to be the monosynaptic reflex (MSR). Likewise, the MSR also followed one to one high frequency stimulation (>10 Hz) without jittering (Fig. 1*B*). At higher stimulation frequencies ≥1 Hz, slow VRP components were depressed, but attenuated MSRs remained (Fig. 1*B*).

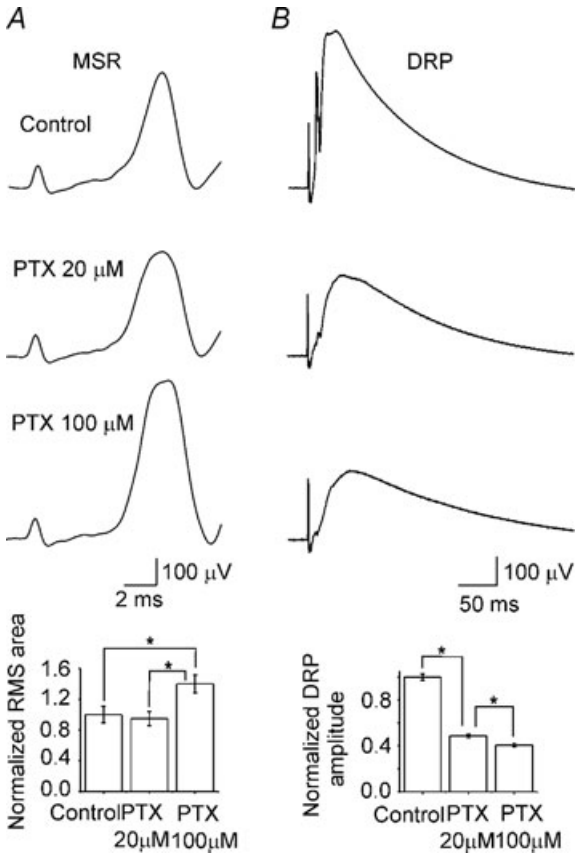

Figure 2. Action of picrotoxin on the MSR and DRP
A and *B*, top to bottom, representative MSR and DRP evoked by dorsal root stimulation (2*T*) and simultaneously recorded in control Ringer solution and in the presence of picrotoxin (PTX) at 20 and 100 μM. Bar graphs, normalized MSR area and DRP amplitude in control Ringer solution and in the presence of PTX. Vertical lines indicate S.E.M. Asterisks indicate statistical difference with respect to the control for the MSR and with respect to the control and between PTX at 20 and 100 μM for the DRP ($n = 11$; $P < 0.05$).

Viability of the preparation and effect of $GABA_A$ antagonists on the MSR and DRP

In order to know if $GABA_A$ receptor antagonists at different concentrations produce any reliable action on the MSR and the DRP we first tested the stability of the preparation by recording both signals every 30 s during 4 h in normal Ringer solution. Figure 1*C* and *D* shows plots of the MSR area and DRP amplitude, respectively. During this time both variables did not change ($P > 0.05$). Therefore, we proceeded to evaluate the action of the drugs.

Modulation of MSR and motoneurone excitability by $GABA_A$ postsynaptic receptors has received little attention (Curtis *et al.* 1971; Peng & Frank, 1989; Vinay & Clarac, 1999; Delgado-Lezama *et al.* 2004). The reported data suggest the presence of $GABA_A$ receptors in motoneurones, which probably are tonically activated by GABA and might modulate the MSR. To assess this possibility, the MSR was recorded in the presence of picrotoxin (20 μM) or bicuculline (20 μM), concentrations reported to block mostly synaptic $GABA_A$ receptors (Semyanov *et al.* 2004; Farrant & Nusser, 2005). Figure 2*A* and *B* shows that the MSR area was not significantly affected ($P > 0.05$), while the DRP was depressed to 46.4 ± 1.5% ($n = 11$) by picrotoxin. Similar actions were observed in the presence of bicuculline (20 μM) (data not shown). However, previous data from our lab reported that motoneurone EPSPs evoked by stimulation of the dorsolateral funiculus increased in amplitude in the presence of bicuculline (40 μM) (Delgado-Lezama *et al.* 2004). Therefore, we decided to investigate if the MSR could be modulated by the extrasynaptic $GABA_A$ receptors reported to be sensitive to high concentrations of picrotoxin or bicuculline (100 μM) (Semyanov *et al.* 2004; Farrant & Nusser, 2005; Walker & Semyanov, 2008). Unexpectedly, 100 μM picrotoxin facilitated the MSR by about 20 ± 2.4% ($n = 11$) with respect to the control (Fig. 2*A*; $P < 0.05$), while the sensory evoked DRP presented an additional depression to 38 ± 2.7% (Fig. 2*B*; $n = 11$; $P < 0.05$). Similar changes were recorded with bicuculline 100 μM ($n = 9$; data not shown). Interestingly, gabazine at 1 μM, a competitive antagonist which has affinity for synaptic

$GABA_A$ receptors, also depressed the DRP by $44 \pm 2.5\%$ without facilitating the MSR ($P > 0.05$; $n = 8$). These results suggest the presence of synaptic $GABA_A$ receptors in the terminals of primary afferents producing PAD due to the effect of low concentrations of picrotoxin (20 μM), bicuculline (20 μM) and gabazine (1 μM) in the DRP. On the other hand the MSR remained unchanged with low concentrations of picrotoxin (20 μM), bicuculline (20 μM) and gabazine (1 μM), suggesting that $GABA_A$ receptors located on motoneurones are not sensitive to low concentrations of antagonist, excluding the possibility of these being synaptic.

Long duration inhibition in the turtle spinal cord

It is well documented that sensory flow information in mammals is modulated by presynaptic inhibition of primary afferents. Sensory evoked presynaptic inhibition is mediated by activation of $GABA_A$ receptors at axo-axonic synapses producing PAD (Schmidt, 1971; Rudomin & Schmidt, 1999). Previous studies in mammals have shown that presynaptic inhibition lasts at least 300 ms (Eccles *et al.* 1962). Therefore we attempted to study sensory evoked presynaptic inhibition by applying conditioned stimulation of the MSR at different time intervals (30–300 ms). We observed that a long latency inhibition mediated by $GABA_A$ receptors regulated transmission from primary afferents. A test MSR evoked by one stimulus to DRD9 was conditioned by applying one stimulus to DRD8 at interstimulus intervals of 30, 60, 100 and 300 ms (scheme, Fig. 3*A*). The conditioning–test MSR in normal Ringer solution was depressed at all interstimulus intervals tested with a maximal depression of about 92% at 30 ms (Fig. 3*B* and *C*). However, in the presence of picrotoxin (20 μM), a conditioning stimulation did not depressed the MSR in the range of interstimulus intervals assessed (Fig. 3*B* and *C*). Interestingly, the sensory evoked DRP recorded on DRD9 and evoked by stimulation of DRD8 presented almost the same duration as the long latency inhibition of the MSR and was depressed about 40% (Fig. 3*B*). A similar result was observed in another three preparations. Blockade of presynaptic inhibition and depression of DRP were also observed in the presence of bicuculline (20 μM; $n = 4$) and gabazine (1 μM; $n = 4$). The fact that gabazine at this concentration is considered to block mainly synaptic $GABA_A$ receptors (Semyanov *et al.* 2004; Farrant & Nusser, 2005) strongly suggests that a long latency inhibition in the turtle, like in mammals, might be produced by activation of synaptic $GABA_A$ receptors located at primary afferent terminals, which consequently might mediate the fraction of DRP depressed by the blockers. As suggested by Eccles *et al.* (1963), the DRP sensitive to picrotoxin, and in our results also sensitive to bicuculline (20 μM) or gabazine, could correspond to the PAD associated with presynaptic inhibition of primary afferents. Consequently, we might conclude that sensory evoked presynaptic inhibition of low threshold afferents in the turtle spinal cord might be mediated by GABA release from GABAergic interneurones establishing axo-axonic contacts with primary afferents, as has been shown in mammals and amphibians (Rudomin & Schmidt, 1999; Peng & Frank, 1989; Vesselkin *et al.* 2001). This inhibition is not tonically active as occurs in the case of presynaptic inhibition of high threshold cutaneous afferents in the cat spinal cord (Rudomin *et al.* 2007).

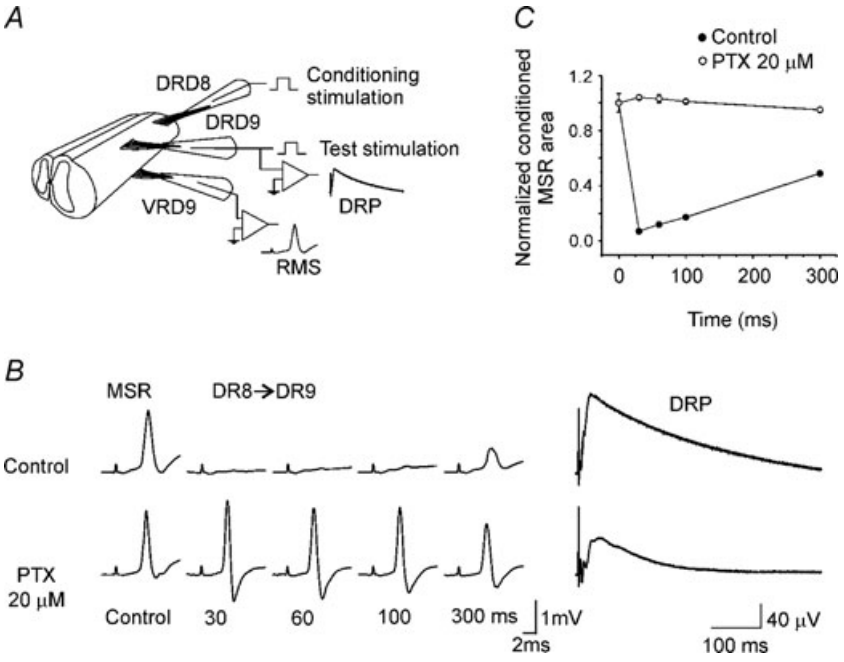

Figure 3. Long duration inhibition of the MSR
A, scheme showing the protocol to induce long duration inhibition of the MSR. MSR recorded from D9 ventral root (VRD9) was evoked by stimulation of the ipsilateral dorsal root (DRD9). Conditioning stimulation was applied through D8 dorsal root (DRD8). *B*, MSR control and conditioned (DR8→DR9) recorded in control Ringer solution (top traces) and in presence of picrotoxin (20 μM; lower traces) at interstimulus intervals indicated bellow. To the right, DRP recorded from DRD9 in control Ringer solution and in the presence of picrotoxin. *C*, plot of normalized MSR area *versus* interstimulus interval in control Ringer solution (filled circles) and in presence of picrotoxin (open circles). Vertical bars indicate S.E.M.

Effect of high concentration of picrotoxin on the long duration inhibition of the MSR

Having shown that long duration inhibition was abolished by $GABA_A$ receptor antagonists at low concentrations, which preferentially blocks synaptic $GABA_A$ receptors at supraspinal nuclei (Farrant & Nusser, 2005; Walker & Semyanov, 2008), we decided to assess the action of picrotoxin at 100 μM on the MSR conditioned by stimulation of an adjacent dorsal root. Figure 4 shows the time course of the conditioned MSR area recorded at interstimulus intervals of 30, 60, 100, 150 and 300 ms. As previously shown, picrotoxin (20 μM) abolished the long duration inhibition without any significant facilitation of the MSR ($P > 0.05$). However higher concentrations of picrotoxin (100 μM) facilitated the conditioned MSR by $20 \pm 1\%$ ($n = 4$). The same result was observed in the presence of bicuculline (100 μM; $n = 4$; not shown). This result suggests the existence of two types of $GABA_A$ receptors on primary afferents and motoneurones. One type, sensitive to low concentration of the antagonists, is probably located on primary afferents, where they are activated synaptically and mediate presynaptic inhibition. It appears that motoneurones do not have this type because the MSR was not facilitated. A second type, sensitive to high concentration of the antagonists, might be located in motoneurones and also in primary afferents.

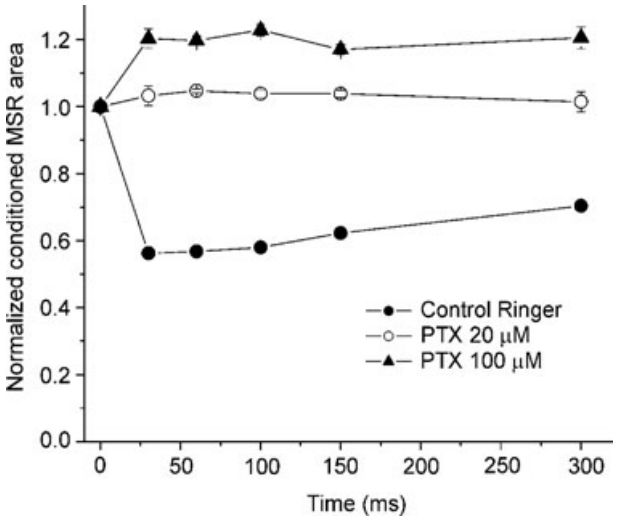

Figure 4. Faciliation of the conditioned MSR by higher concentrations of picrotoxin
Plot shows the normalized area of MSR evoked by stimulation of the DRD9 conditioned by stimulation of an adjacent dorsal root (DRD8) at interstimulus intervals of 30, 60, 100, 150 and 300 ms in control Ringer solution (filled circles) and in the presence of picrotoxin at 20 (open circles) and 100 μM (filled triangles). Vertical bars indicate the S.E.M.

Facilitation of MSR by furosemide sensitive $GABA_A$ receptors

Immunohistochemical and *in situ* hybridization studies have revealed the presence of diverse $GABA_A$ receptors in dorsal horn layers and dorsal root ganglia as well as in motoneurones (Persohn *et al.* 1991; Wisden *et al.* 1991; Ma *et al.* 1993; Alvarez *et al.* 1996; Bohlhalter *et al.* 1996). However, the physiological roles and pharmacological implications of this receptor diversity have not yet been determined (Rekling *et al.* 2000). Therefore we decided to assess the action of furosemide, an antagonist of α_4/α_6 subunit-containing $GABA_A$ receptors at concentration that do not affect the Cl^- transporter (Hochman & Schwartzkroin, 2000), on the MSR and the DRP. In order to block synaptic $GABA_A$ receptors involved in the long duration inhibition of the MSR, we first applied picrotoxin (20 μM) ($n = 4$) or gabazine (1 μM) ($n = 4$). As previously shown, gabazine (1 μM) did not facilitate the MSR ($P > 0.05$) and depressed the DRP amplitude $44 \pm 2.58\%$ ($P < 0.05$; $n = 4$, Fig. 5). Then, we added furosemide (100 and 200 μM). Interestingly the MSR was facilitated by 11 ± 2.6 and $20 \pm 2\%$ respectively, as observed in the presence of picrotoxin and bicuculline (100 μM); however the DRP was not affected ($n = 4$; $P > 0.5$; Fig. 5*B* and *C*). Additionally, similar to the action of picrotoxin during the long duration inhibition, furosemide also facilitated the conditioned MSR (not shown). Therefore, these results suggest that $\alpha_{4/6}$ subunit-containing $GABA_A$ receptors might be located at motoneurones and regulate their excitability by shunting the motoneurone membrane.

Long-lasting after-discharge in motoneurones induced by picrotoxin, bicuculline and furosemide

MSR evoked by one stimulus (2*T*) applied to the ipsilateral dorsal root was followed by some polysynaptic reflexes

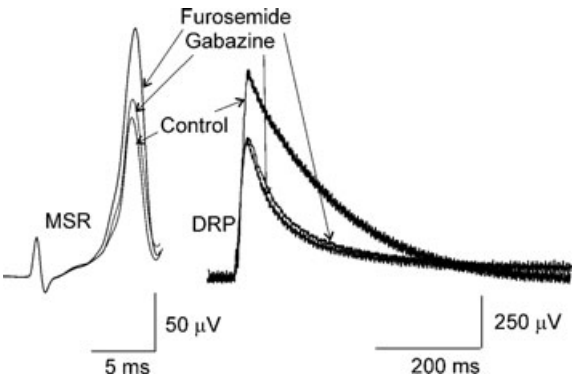

Figure 5. Furosemide effect on the MSR and DRP
The MSR and DRP recorded in control Ringer solution, in the presence of gabazine 1 μM, and plus furosemide 200 μM.

not lasting more than 10 ms (Fig. 6*A*). Nonetheless, in the presence of picrotoxin (20 μM) or bicuculline (20 μM; not shown) as well as gabazine (1 μM; not shown) the MSR was not affected, but polysynaptic reflexes were facilitated and a 40 ms latency long-lasting post-discharge of 2 s duration was evident (Fig. 6*B*). When the antagonist concentration of picrotoxin or bicuculline was increased to 100 and 200 μM, respectively, the post-discharge was facilitated and the latency was shortened (20 ms) (third and fourth traces from the top in Fig. 6*A*; $n = 16$).

Increase of the excitability of motoneurones might be due to two factors, first a large decrease of presynaptic inhibition leading to an increase of excitatory synaptic inputs to the motoneurone, and second, blockade of postsynaptic inhibitory control due to extrasynaptic or synaptic $GABA_A$ receptors that would drive the membrane potential close to the firing threshold through an increase in membrane resistance (Delgado-Lezama *et al.* 2004). This has been observed in hippocampus and cerebellar granule cells where activation of extrasynaptic $GABA_A$ receptors has a profound effect on neuronal excitability (Semyanov *et al.* 2004), as well as a gain modulation control in cells with high variability of synaptic input (Mitchell & Silver, 2003).

Discussion

The modulation of the MSR by $GABA_A$ receptors is mediated by two mechanisms: presynaptic inhibition of Ia afferent terminals and postsynaptic inhibition of motoneurones (Kellerth & Szumski, 1966; Schmidt, 1971; Peng & Frank, 1989; Jonas *et al.* 1998; Rudomin & Schmidt, 1999; Kullmann *et al.* 2005). Despite the morphological and pharmacological evidence available on $GABA_A$ receptors on the motoneurones, the role of these receptors in the postsynaptic modulation of the MSR has been scarcely studied. Although presynaptic inhibition has gained much attention in the past decades, and is one of the most studied mechanisms in the spinal cord, it seems that both presynaptic and postsynaptic sites play a complementary role in maintaining the stability of reflex activity (Solodkin *et al.* 1984). Our results show that $GABA_A$ receptors, located probably at extrasynaptic sites and activated by ambient GABA, along with synaptic

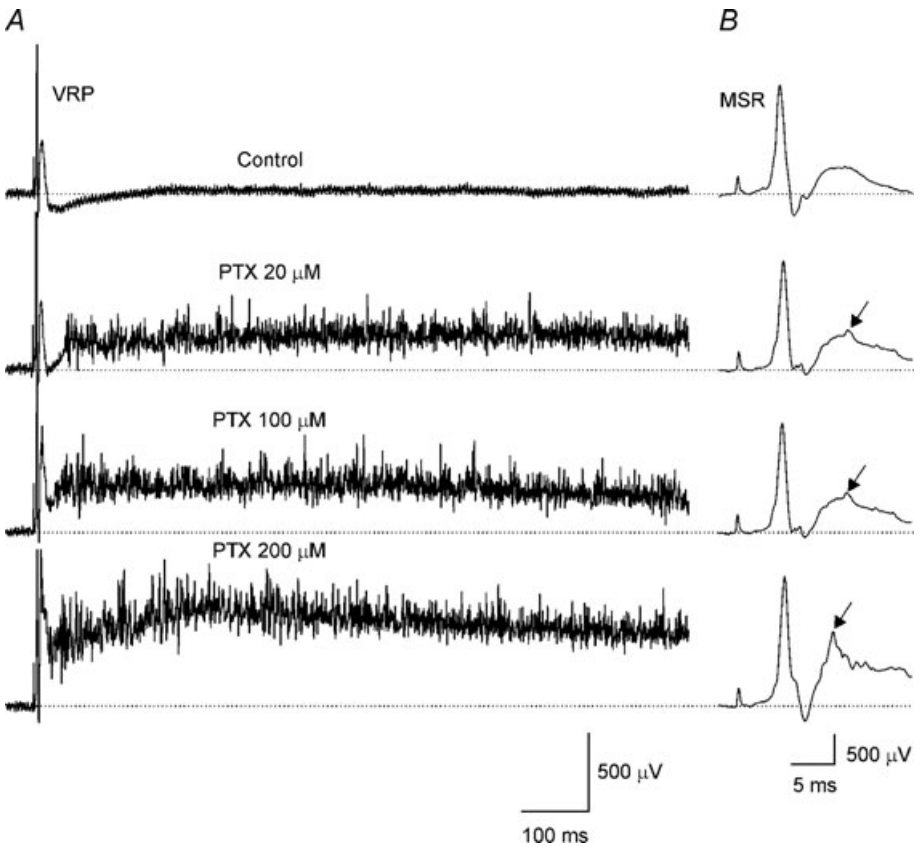

Figure 6. Motoneurone after-discharge in the presence of picrotoxin
A, VRP evoked by ipsilateral dorsal root stimulation (2*T*) recorded, from top to bottom, in control Ringer and in the presence of picrotoxin (PTX) at 20, 100 and 200 μM. *B*, the MSR from *A* at a different time and voltage scale. Arrows indicate polysynaptic reflex.

$GABA_A$ receptors, might have a relevant role in controlling primary afferents and motoneurone excitability.

Monosynaptic reflex in the turtle spinal cord

The early component of the VRP, evoked by dorsal root stimulation, was considered as a MSR because it followed one to one dorsal root stimulation without failure and presented a time to peak similar to the value reported for turtle motoneurone EPSP (Yamashita, 1986). Interestingly, the polysynaptic reflex in control Ringer solution and in the presence of $GABA_A$ antagonists failed to follow frequency stimulation higher than 1 Hz while the MSR was able to followed one to one frequencies higher than 10 Hz. Furthermore, stable recordings of the MSR and DRP were observed for even 300 min, which gives confidence in the action of the drugs.

MSR and DRP modulation by synaptic $GABA_A$ receptors

Our results show that blockade of $GABA_A$ receptors with bicuculline and picrotoxin at 20 μM or gabazine at 1 μM did not facilitate the MSR but depressed the DRP. It is worth mentioning that gabazine at 1 μM, which selectively blocks only synaptic $GABA_A$ receptors at supraspinal nuclei neurons (Farrant & Nusser, 2005; Walker & Semyanov, 2008), like picrotoxin and bicuculline at 20 μM, also depressed the DRP, suggesting that a fraction of the DRP probably is mediated by activation of synaptic $GABA_A$ receptors located at primary afferent terminals. Therefore, like in mammals (Schmidt, 1971; Rudomin & Schmidt, 1999), an important part of the DRP could be produced by synaptic $GABA_A$ receptors located on primary afferents and activated by GABA released at axo-axonic synapses with interneurones. This is supported by previous immunohistochemical studies carried out in mammals and amphibians showing that focal accumulation of β subunit-containing $GABA_A$ receptors on primary afferents is opposed by glutamic acid decarboxylase (GAD)-immunoreactive terminals (Alvarez *et al.* 1996; Vesselkin *et al.* 2001), although this is a point that needs to be addressed further in the turtle in future experiments.

MSR and DRP modulation by extrasynaptic $GABA_A$ receptors

Interestingly, when picrotoxin or bicuculline concentration was increased to 100 μM, the MSR was facilitated while the DRP resistant to picrotoxin, bicuculline (20 μM) or gabazine (1 μM) presented an additional depression. It is well known that in many neurons from supraspinal nuclei these concentrations block preferentially $GABA_A$ receptors that mediate inhibitory tonic current and possess an important role controlling excitatory synaptic integration (Semyanov *et al.* 2004; Chadderton *et al.* 2004; Farrant & Nusser, 2005; Walker & Semyanov, 2008). Therefore, MSR facilitation and DRP depression might have been produced by blockade of tonic $GABA_A$ receptors located at primary afferents and/or motoneurones. This conclusion is partly supported by the action of furosemide, which selectively antagonizes α_4/α_6 subunit-containing $GABA_A$ receptors (Korpi *et al.* 1995; Hamann *et al.* 2002), at a concentration that does not block the Na^+–K^+–$2Cl^-$ cotransporter (Hochman & Schwartzkroin, 2000) and facilitates the MSR without affecting the DRP. This result is in agreement with the finding that $\alpha_{4/6}$ subunit mRNA is not expressed in dorsal root ganglia (Ma *et al.* 1993; Maddox *et al.* 2004). In addition, in adult cerebellar granule cells α_6 subunit-containing $GABA_A$ receptors were found to mediate a tonic current that represents 58% of the total current blocked by bicuculline (40 μM) and picrotoxin (100 μM) (Semyanov *et al.* 2004; Farrant & Nusser, 2005; Walker & Semyanov, 2008). Consequently, lack of effect of furosemide on the DRP strongly indicates that MSR facilitation might be produced by blockade of $\alpha_4/_6$ subunit-containing $GABA_A$ receptors located in motoneurones.

Presynaptic inhibition mediated by synaptic $GABA_A$ receptors

Like in mammals, long latency inhibition of the conditioned MSR presented a time course of 30–300 ms and was blocked by picrotoxin, bicuculline or gabazine (Eccles *et al.* 1963; Schmidt, 1971; Deshpande & Warnick, 1988; Rudomin & Schmidt, 1999). Likewise, the sensory evoked DRP recorded from one dorsal root presented a similar time course to the long duration inhibition of the MSR. Moreover, as occurs in cat and neonate rat, the DRP was not completely blocked by 20 μM bicuculline or picrotoxin (Schmidt, 1971; Kremer & Lev-Tov, 1998; Rudomin & Schmidt, 1999). We therefore assume that the long latency inhibition observed in this study corresponds to sensory evoked presynaptic inhibition of the MSR. Interestingly, removal of presynaptic inhibition and depression of DRP were also produced by gabazine at 1 μM concentration, which has been shown to block only synaptic $GABA_A$ receptors mediating fast IPSCs in hippocampal and cerebellar neurons (Semyanov *et al.* 2004; Farrant & Nusser, 2005; Walker & Semyanov, 2008). This result indicates that the DRP sensitive to $GABA_A$ receptor antagonists at low concentrations probably was mediated by activation of synaptic $GABA_A$ receptors at axo-axonic synapses on primary afferent terminals and might correspond to the PAD associated with presynaptic inhibition as was proposed in mammals initially by Eccles *et al.* (1963),

and others (Schmidt, 1971; Rudomin & Schmidt, 1999). In conclusion, our results demonstrate that presynaptic inhibition of turtle primary afferents presents similar properties to that found in cat (Schmidt, 1971; Rudomin & Schmidt, 1999) and neonate rat (Deshpande & Warnick, 1998), and therefore it might also be produced by GABA release from axo-axonic synapses (Rudomin & Schmidt, 1999).

Motoneurone after-discharge in presence of $GABA_A$ receptor antagonist

Control VRP evoked by an afferent volley was composed of an early synchronous MSR sometimes accompanied by a polysynaptic reflex, as has been recorded in mammals (Deshpande & Warnick, 1998; Jiang & Heckman, 2006). However, in the presence of $GABA_A$ receptor antagonists, the MSR was followed by a long latency post-discharge lasting up to 20 s; this activity of the motoneurones has been reported to occur in the presence of picrotoxin (100 μM) and strychnine (5 μM) in the *in vitro* spinal cord preparation from adult mice (Jiang & Heckman, 2006). We speculate that long-lasting activity of motoneurones might be produced by synaptic activation of a plateau potential mediated by L-type Ca^{2+} channels of last order excitatory interneurones involved in the pathway of primary afferents and motoneurones. Interestingly, similar long-lasting after-discharges have also been evoked in dorsal horn neurons by stimulation of the dorsal root after blockade by bicuculline of $GABA_A$ receptors, which facilitated the plateau potential expression mediated by L-type Ca^{2+} channels (Russo *et al.* 1988). In motoneurones the plateau potential also mediated by L-type Ca^{2+} channels is down-regulated by activation of $GABA_A$ receptors (Alaburda *et al.* 2005). However, we cannot rule out the possibility of a $GABA_A$ tonic conductance in the motoneurone that simply allows a decrease in the membrane resistance reducing the excitability produced by a constant synaptic inflow of segmental and descending pathways. Regardless of the origin of the motoneurone after-discharge, it is clear that $GABA_A$ receptors play an important role in controlling motoneurone excitability, which is crucial to muscle activity.

Functional implications

In this work, we show that $GABA_A$ receptors sensitive to a high concentration of bicuculline, picrotoxin and furosemide are preventing over-excitation of motoneurones and probably interneurones involved in motor activity. Synaptic strength in the spinal cord is a mechanism to control information flow on motoneurones and is regulated accurately by different neurotransmitters and modulators in order to allow motoneurones to respond suitably to the ambient requirements. $GABA_A$ receptors could play an important role as modulators of synaptic strength, as has been shown in nociception. Decreasing with bicuculline the inhibitory control by GABAergic neurones leads to a hyperactivity of dorsal horn neurones projecting to supraspinal nuclei (Woolf & Doubell, 1994; Russo *et al.* 1998). At the motoneurone level, tonic activation of $GABA_A$ receptors can be a mechanism to control information processing by reducing its membrane time constant, thereby narrowing the time window to integrate excitatory synaptic inputs, working as a filter to maintain accuracy of response of motoneurones. Furthermore, the tonic inhibitory conductance mediated by $GABA_A$ receptors might be a low-cost metabolic mechanism to control excitability of motoneurones and interneurones involved in networking spinal activity. Additionally, the proposal of a likely tonic inhibitory state at the motoneurones responsible for modulating the reflex activity during locomotion in cat spinal cord has been proposed before (Gosgnach *et al.* 2000), and there have been experiments on humans which point towards the motoneurone as a possible site for $GABA_A$ reflex activity modulation (Guissard *et al.* 2001).

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Author contributions

W.B.: design of the experimental protocol; collection, analysis and interpretation of data; drafting the manuscript; J.E.L.A.: collection, analysis and interpretation of data. J.A.: design of the experimental protocol; collection, analysis and interpretation of data. R.D.L.: conception and design of the experimental protocol; collection, analysis and interpretation of data; drafting the manuscript; final approval of the manuscript. The work was carried out in the Department of Physiology, Biophysics and Neuroscience of the Centre for Research and Advanced Studies of the National Polytechnic Institute (Cinvestav-IPN), Mexico City.

Acknowledgements

Master fellowships from Conacyt to B.W. and L.A.J.E. are gratefully acknowledged. We thank R. Leyva and R. Panfilo for technical assistance with animals and the National Mexican Turtle Centre for providing the turtles. We also thank Dr Pablo Rudomín and Dr Ricardo Felix for comments and suggestions to improve the paper and Dr David McCrea for revising the manuscript. This work was supported by grant no. 50864-Q from the National Council of Science and Technology (Conacyt, Mexico).