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Chronic GABAergic blockade in the spinal cord in vivo induces motor alterations and neurodegeneration

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ABSTRACT

Inhibitory GABAergic and glycinergic neurotransmission in the spinal cord play a central role in the regulation of neuronal excitability, by maintaining a balance with the glutamate-mediated excitatory transmission. Glutamatergic agonists infusion in the spinal cord induce motor neuron death by excitotoxicity, leading to motor deficits and paralysis, but little is known on the effect of the blockade of inhibitory transmission. In this work we studied the effects of GABAergic and glycinergic blockade, by means of microdialysis perfusion (acute administration) and osmotic minipumps infusion (chronic administration) of GABA and glycine receptors antagonists directly in the lumbar spinal cord. We show that acute glycinergic blockade with strychnine or GABAergic blockade with bicuculline had no significant effects on motor activity and on motor neuron survival. However, chronic bicuculline infusion, but not strychnine, induced ipsilateral gait alterations, phalange flaccidity and significant motor neuron loss, and these effects were prevented by AMPA receptor blockade with CNQX but not by NMDA receptor blockade with MK801. In addition, we demonstrate that the chronic infusion of bicuculline enhanced the excitotoxic effect of AMPA, causing faster bilateral paralysis and increasing motor neuron loss. These findings indicate a relevant role of GABAergic inhibitory circuits in the regulation of motor neuron excitability and suggest that their alterations may be involved in the neurodegeneration processes characteristic of motor neuron diseases such as amyotrophic lateral sclerosis.

1. Introduction

Activity of neuronal circuits is controlled by the action of excitatory or inhibitory neurotransmitters. Dysregulation of this synaptic control has been associated with several neurological and psychiatric diseases that have as a common endpoint hyperexcitability, which may be consequence of a decrease in inhibitory neurotransmission or an increase in excitatory neurotransmission. Overactivation of excitatory neuronal networks can trigger neurodegeneration by excitotoxicity, causing neuronal death and irreversible functional damage according to the affected area. Most experimental studies on excitotoxicity have been focused on excessive glutamatergic transmission, using glutamate receptor agonists, despite the fact that alterations of inhibitory circuits have been shown to be involved in the pathophysiology of several diseases, such as epilepsy, schizophrenia, autism and amyotrophic lateral sclerosis (ALS).

ALS is characterized by the loss of motor neurons (MNs) in cortical areas, brainstem and spinal cord, in both familial and sporadic forms, and the hyperexcitation observed has been associated with alterations of the function of spinal circuitries, controlled by neuronal networks formed by several kinds of cells. The most important components that regulate excitability are the abundant inhibitory interneurons V2b, V0C/G, V0D and V0V, as well as the Renshaw cells, that receive afferents from the MNs and directly inhibit them through the release of GABA and glycine (Ramírez-Jarquín et al., 2014; Schneider and Fyffe, 1992; Todd and Sullivan, 1990). Whereas the glutamate-mediated excitotoxicity has been amply studied, little is known on the effect of the blockade of GABAergic and glycinergic inhibitory transmission in vivo, and this is the purpose of the present work.

For this purpose, we studied the effects of the direct administration of GABAergic and glycinergic antagonists in the lumbar spinal cord, as well as their interaction with glutamate receptor agonists and antagonists, to know whether the alteration of excitatory-inhibitory balance could result in MN hyperexcitation and degeneration. We administered the drugs acutely, by means of

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reverse microdialysis, and chronically using osmotic minipumps. Using these procedures, we have previously shown that acute AMPA perfusion produces MN loss and paralysis in 3–12 h (Corona and Tapia, 2004), whereas its chronic infusion results in progressive MN degeneration and gradual paralysis along several days (Tovar-y-Romo et al., 2007).

2. Material and methods

2.1. Animals

All the experiments were made using adult Wistar male rats (270–300 g), handled in accordance with the Rules for Research and Health Matters (Mexico) and with international standards of research animal welfare (including ARRIVE guidelines), and with approval of the local Animal Care Committee (Approval No. RTI21-14). Animals were housed in a controlled laboratory environment: 12 h light/dark cycle, ad libitum access to regular animal chow and water. All surgical procedures were performed under general anesthesia. All efforts were made to minimize suffering of the animals.

2.2. Drugs

AMPA, CNQX and MK801 were purchased from Tocris Bioscience, and bicuculline methbromide (Bic) and strychnine (Stry) from Sigma Aldrich. For acute treatment (microdialysis) Bic and Stry (1 or 5 mM each) were dissolved in Krebs-Ringer solution containing (in mM) 118 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 4.0 $Na_2H_2PO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose, pH 7.4, in all cases osmolarity was maintained by reducing the NaCl concentration proportionally For chronic treatment, osmotic minipumps (Alzet model 2004, volume ~250 μL, flow rate 6 μL/day) were filled with one of the following solutions: saline or phosphate buffer (PB) for control groups; Bic 5, 10 or 17.5 mM; Stry 20 mM; Bic 10 mM + CNQX 1 mM; Bic 10 mM + MK801 14 mM; AMPA 3.8 mM; and AMPA 3.8 mM + Bic 10 mM. Pumps were incubated for 48 h in filtered saline solution at 37 °C for stabilization. These concentrations were chosen on the basis of previously published results and on preliminary experiments (Corona and Tapia, 2004; Lazo-Gómez and Tapia, 2016; Tovar-y-Romo et al., 2007). In the case of AMPA, we reduced the previously used 7.5 mM concentration to 3.8 mM in order to diminish the severity of the excitotoxic effect.

2.3. Surgical procedures

Surgery for the microdialysis procedure was performed essentially as previously described (Corona and Tapia, 2004). Rats were anesthetized with 5.0% isoflurane in carbogen (95% O_2/5% CO_2 mixture) and placed in a stereotaxic spinal unit; isofluorane concentration was gradually diminished to 1.5–2.0% during the surgery. A median sagittal incision (3.5–4 cm long) was made in the back (skin was shaved, cleaned and disinfected previously) and the underlying fascia and muscle tissue were dissected. The spinous process was removed with a drill, and a ~2 mm diameter hole was drilled in the right lamina of the third lumbar vertebra. The meninges were carefully removed with a metallic hook and a microdialysis probe (CMA7, Carnegie, Sweden) was lowered into the right dorsal horn of the spinal cord. The probe was perfused at a flux rate of 2 μL/min with Krebs medium during 1 h for stabilization, and then perfused for 25 min (50 μL) with one of the media indicated above, using a microinjection pump (CMA/100, Carnegie, Sweden). After the experiment, the microdialysis probe was gently removed and the skin incision was sutured, and after recovered from the anesthesia animals were placed in individual cages and subjected to the motor tests at the times indicated in Results, and finally sacrificed for histological studies.

Surgery for the chronic procedure was performed as previously described (Tovar-y-Romo et al., 2007), slightly modified. The initial tissue dissection was made as described above, and after the spinous process was removed a stainless-steel screw (3.7 mm long, 1 mm diameter) was fixed in the base of the process. A cannula (1 mm long, 50 μm internal diameter and 80 μm external diameter, VitroCom Inc.) was carefully advanced down into the dorsal horn; this probe was attached to the catheter of the osmotic minipumps; union was sealed with cyanoacrylate and the implant was fixed with dental cement. Osmotic minipumps were subcutaneously implanted in the back of the animal. Finally, the skin incision was closed with surgical stainless-steel clips, anesthesia was withdrawn and animals received a single intraperitoneal antibiotic shot. They were kept in individual cages with food and water ad libitum during the period of the motor tests.

2.4. Motor behavior evaluation

Seven days prior to surgery, rats were trained in rotarod (Columbus Instruments, Columbus, OH, USA) and a variation of the paw grip endurance (PGE) task. After surgery animals were evaluated in each test daily until fixation. For rotarod test, rats walked on an accelerating (0.2 rev/min per s) rod, starting from 10 rpm with a cut-off of 120 s, the average time for three attempts was scored. For the PGE, rats were placed on a horizontal placed grid (40 × 25 cm) that was gently turned until reaching a vertical position. Average time of three attempts to climb to the top of the grid or the latency to fall when they were unable to climb was scored with a cut-off of 40 s. In addition, stride pattern of the hind footprints was recording by inking the hindpaws with non-toxic Chinese ink and make the animals walk along a paper 10 × 100 cm runway.

2.5. Histology and immunofluorescence

At the end of each motor test period, rats were perfused and fixed for histological and immunohistological analyses as previously described (Corona and Tapia, 2004; Tovar-y-Romo et al., 2007). Animals were deeply anesthetized with an intraperitoneal injection of pentobarbital and perfused transcardially with 250 mL of ice-cold 0.9% saline, followed by 250 mL of ice-cold 4% paraformaldehyde in 0.1 MPB, pH 7.4. Spinal cord was removed, post-fixed in 4% paraformaldehyde at 4 °C for a week, and successively dehydrated in sucrose gradients (up to 30%). Twenty transverse sections (40 μm thick) of the lumbar region, at the site of the cannula, were obtained in a cryostat. Alternate sections were stained with cresyl violet or immunostained for choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP).

Immunofluorescence for ChAT and GFAP was performed on floating slices which were blocked with 5% of bovine serum albumin in PB 0.1 M with Triton X-100 (0.3%) for 2 h and after exposed to goat polyclonal anti-ChAT (ChAT, 1:200; Chemicon, Temecula, CA, USA) and mouse polyclonal anti-GFAP (1:1000, Sigma Aldrich) as primary antibodies for 48 h at 4 °C. Sections were washed three times for 15 min in PB-Triton and incubated with biotynil-conjugated mouse anti-goat IgG (1:200; Vector, Burlingame, CA, USA) for 1.5 h. After three washes, sections were incubated for 2 h with biotin-conjugated horse anti-goat IgG (1:200, Vector Labs) and after with avidin-Texas Red conjugate (1:200, pH 8.2; Vector) and FITC-conjugated anti-mouse antibody (1:250; Zymed, Carlsbad, CA, USA) as secondary antibodies. Slices were mounted on silane (Sigma Aldrich) treated glass slides and coverslipped with fluorescent mounting medium (DAKO, Carpinteria, CA, USA). Sections were visualized under a confocal Olympus FV10i microscope,