Neonatal Mice Spinal Cord Interneurons Sending Axons in Dorsal Roots

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Abstract

Background: Spinal cord interneurons send their axons in the dorsal root. Their antidromic fire could modulate peripheral receptors. Thus, it could control pain, other sensorial modality, or muscle spindle activity. In this study, we assessed a staining technique to analyze whether interneurons send axons in the neonate mouse's dorsal roots. We conducted experiments in 10 Swiss-Webster mice, which ranged in age from 2 to 13 postnatal days. We dissected the spinal cord and studied it in vitro.

Results: We observed interneurons in the spinal cord dorsal horn sending axons through dorsal roots. A mix of fluorochromes applied in dorsal roots marked these interneurons. They have a different morphology than motoneurons. Primary afferent depolarization in afferent terminals produces antidromic action potentials (dorsal root reflex; DRR). These reflexes appeared by stimulation of adjacent dorsal roots. We found that in the presence of bicuculline, DRR recorded in the L4 dorsal root evoked by L5 dorsal root stimulation was reduced. Simultaneously, the monosynaptic reflex (MR) in the L5 ventral root was not affected; nevertheless, a long-lasting after discharge appeared. The addition of 2-amino-5-phosphonovalric acid (AP5), an antagonist of NMDA receptors, abolished the MR without changing the after discharge. Action potentials persisted in dorsal roots even in low Ca^{2+} concentration.

Conclusions: Thus, firing interneurons could send their axons by dorsal roots. Antidromic potentials may be characteristics of the neonatal mouse, probably disappearing in adulthood.

Introduction

Spinal cord interneurons send their axons in the dorsal root. Their antidromic fire could modulate peripheral receptors. Spontaneous firing and occasional bursting in dorsal roots (DR) occurred after elevating the extracellular potassium concentration in isolated spinal cords of neonatal rats (1). The increase in potassium concentrations is also associated with seizures episodes. They occurred with primary afferent depolarizations and antidromic discharges of nerve impulses in DR fibers (1, 2). Antidromic activity occurred in the dorsal root ganglia in chronically axotomized rats (3). They can block or affect orthodromic impulses colliding with incoming afferent volleys (1, 4). This mechanism would require high firing frequencies in the antidromic discharge (5). Interneurons sending axons via DR in the spinal cord produce antidromic action potential regulating different types of peripheral receptors (6).

Ventral funiculus stimulation also evoked antidromic discharges in dorsal roots in a Petri dish brain stem–spinal cord preparation of neonatal (0-5-day old) rats. These discharges occurred by the underlying afferent terminal depolarization reaching firing threshold (7). Spontaneous interneuron activities play a critical role in the development of neuronal networks. Their discharges were conducted antidromically along the DR preceding those in the ventral root (VR) lumbar motoneurons. The action potential propagates centrally and triggers EPSPs in motoneurons. An indication of axons in dorsal roots coming from spinal cord interneurons is staining neurons with fluorochromes.

Results

Neuron Labeling

We analyzed the fluorescent marker patterns in all spinal cords ($n = 10$) used for this study. The RDA application in the L4 dorsal root produced the red fluorescent staining in afferent fibers. It also stained first-order interneurons by RDA leakage due to lower MW. They were marked close to terminal branches (Fig. 2A). Application of FDA (green) in dorsal root L5 only marked afferent fibers. Some interneurons were marked in red only when they were close to RDA afferent fibers (Fig. 2B, the afferent fiber are indicated by arrows). Application of a mixture of RDA and FDA in DR produced yellow staining. We only considered interneurons sending their axon by the dorsal roots when stained in yellow. We found some interneurons marked in yellow (Fig. 2C-D, indicated by arrows) at the dorsal horn or in the intermediate nucleus.

To determinate the interneuron localization, we marked some spinal cord DR's exclusively with FDA, and most of the afferent fiber ends in the dorsal horn (Fig. 3A). In other cases, we retrogradely marked some interneurons with FDA in L5 and RDA in L4 dorsal root and localized interneurons in the dorsal horn (Fig. 3B). FDA marked interneurons seem to indicate that they project their axons through the dorsal roots. In contrast, the RDA application in L5 and FDA in L4 dorsal root did not stain interneurons located in this region. With an FDA and RDA mixture applied in L4 and FDA mainly in L5 dorsal roots, we found marked yellow interneurons close to the intermediate nuclei region (Fig. 3C). Their morphology is different from motoneurons stained by the mixture of fluorochromes applied in the L5 ventral root (Fig. 4B).

The afferent fibers arriving in the motor nucleus exhibited a bulb-like terminal when we applied the fluorochrome mixture in dorsal roots (Fig. 4A). With FDA, we marked some fibers green (Fig. 4B). We did not see any RDA leakage. The mixture applied in L5 VR stained neurons, revealed the motoneuron morphology. However, no interneurons showed marks in this ventral motor region. In addition, some marked cells resembled neurons traveling in rafts on the spinal cord dorsal surface when the fluorochrome mixture penetrated the dorsal roots (Fig. 4C). Then, they began to penetrate the deep layers of the spinal cord (Fig. 4D).

In Fig. 5A-B, the spinal cord cut exhibits one and two nuclei at different stages. We found only one nucleus with small-sized neurons at P2, and we observed two nuclei at P13. We counted the number of neurons at P2 and P13 and measured the cell size (Fig. 5C-F). The graphs in Figs. 5C and D illustrate the size of all neurons stained in P2 and P13. We performed a linear regression to determine the mean value. In P2, most neurons were less than 2000 μm . In P13, the size and number of neurons in both nuclei increased; even the smallest neurons were more extensive than in P2 (Fig. 5E). The difference in the mean values of the two groups (P2 and P13 < average value) are higher than those occurred by chance; there is a statistically significant difference between the two groups ($P < 0.05$). In P13, the mean value of the size of the neurons in both nuclei was approximately 8000 μm (Fig. 4F).

Discharges in the dorsal and ventral roots

In 10 day old mice ($n = 4$), we stimulated the L5 dorsal root to produce a monosynaptic reflex. It registered in the L5 ventral root and the DRR in the L4 dorsal root. We took control of the monosynaptic reflex, the dorsal, and ventral reflex activity in normal aCSF (Fig. 6A). Recordings were obtained and were similar in all animals ($n = 4$) in these experiments.

Bathing with bicuculline (10–20 μ M) eliminated DRR but not the monosynaptic reflex (Fig. 6B). Interestingly, a long latency reflex occurred after the bicuculline application. Bicuculline has already described inducing locomotion episodes after rhythmic activity recorded in the ventral roots (not illustrated) (Duenas SH & Eidelberg, 1979). Similar activity has been observed in spinal cord motor neurons in the turtle in the presence of bicuculline (9).

AP5 and bicuculline application decreased MR and DRR (Fig. 6C-D); after a few minutes, it eliminated, but not after discharge. We recorded sporadic action potentials in DR (Fig. 6C-D).

After washing out bicuculline and AP5, the normal MR and DRR were reestablished (Fig. 6E). A low Ca + solution was then applied; MR, DRR, and after discharge disappeared; interestingly action potentials were observed in DR (Fig. 6F).

Discussion

In our experiments, spinal interneurons send axons through dorsal roots. We localized most of these interneurons close to the intermediate nucleus. They have several shapes that differ from motoneurons.

In our study, we did not study dendritic arborizations nor their changes with age, as assessed in previous studies. In previous studies, Westerga & Gramsbergen observed a considerable increase in motoneuron soma size in rats, but with different distribution and arborizations patterns in a developing stage, which are longer and more extensive at first in cervical than in the lumbar region (10). This temporal and spatial differences may influence the motor development in a rostrocaudal manner (11). Dendrite bundles appeared relatively late in the Soleus' motoneuron compared to the Tibial anterior; this is related to the fine-tuning of neuronal activity, rather than patterning of motor activity (10). These observations will be studied in neonatal mice.

Developing serotonergic motoneuron innervation is related to the postnatal development of motor function already recognized in the second postnatal week (11). In our study, we found a significant neuronal soma size increase at a similar postnatal age. Marked neurons are not of the same type or from a specific neuron group. That could be related to a different organization of the activation pattern.

We found some cells traveling in the spinal cord dorsal surface. We did not know if these cells are neurons or glia. In a developmental study of kittens, the volume of the lateral cervical nucleus and the

glial cells increased sixfold during 120-day observation, as did both the volumes of myelinated axons (12).

As we noticed cells traveling in rafts in the dorsal horn surface of the spinal cord in the mouse spinal cord, further immunohistological studies could reveal the type of cells and clarify if some of them are progenitor neurons (13–15).

We cannot confirm whether the recorded interneurons produce activity (action potentials) traveling antidromically in dorsal roots. However, we found antidromic activity in dorsal roots, even in bicuculline, AP5, and low calcium. In another study, 2–4 postnatal day mice presented depression curves unexplained by presynaptic activation failure (suppressed by AP5). Low calcium concentration reduced average amplitude and depression, and a higher calcium concentration increased average amplitude and depression. Increasing the bath temperature from 24 to 32 Celsius produced little change in amplitudes, but the depression was noticeably reduced at most frequencies (16). Therefore, these AP could be generated by these interneurons when their axons are sufficiently depolarized.

5HT, DA, and NA produced no change in the compound antidromic potentials evoked by intraspinal microstimulation, indicating that DRP depression is unrelated to direct changes in the excitability of intraspinal afferent fibers (17). Thus, antidromic activity could have an origin other than PAD, and consequently, other functions. Ephaptic interaction in afferent fibers could also produce antidromic firing (18).

Antidromic spike function in dorsal roots could participate in regulating activity in the afferent inflow of information related to inflammation and pain. DRR in afferent fiber raises the hypothesis that mediated antidromic activity contributes to neurogenic inflammation (19). Sectioning the sciatic nerve of neonatal rat's triggers growth of afferent fiber in VR, and stimulation in the L5 spinal cord evoked long latency antidromic potentials in the L5 ventral root. However, in normal rats, such potentials rarely appeared (20). Several experimental conditions, such as axotomy of sensory afferents, produced ectopic antidromic activity in their respective DRG, due to branched sensory afferents fiber (3).

In our experiments, the antidromic activity in DR, even in low calcium concentration, is indicative of axons in dorsal roots. We cannot assert their functional significance or action in the neonatal mouse. It would be essential to find out whether these antidromic potentials in dorsal afferent fibers are favoring some spinal circuit formation which remain in adulthood or are only part of a development process.

Sympathetic preganglionic neurons (PGNs) in the neonatal rat's isolated spinal cord could be synaptically activated either by the dorsal root or spinal pathway stimulation. Dorsal root projections already appeared mature in the neonatal rat, and primary afferents did not appear to project directly to PGNs (21).

Conclusions

In our experiments, spinal interneurons send axons by dorsal roots. Thus, the AP comes to the interneurons sending axons in dorsal roots. Some spikes also occurred in ventral roots. In neonatal mice, spinal cord bipolar neurons could exist, sending axons through ventral and dorsal roots. Thus, AP could be produced by neurons with axons in ventral and dorsal roots. The presence of these interneurons in their maturity and their functional role in neonatal mice should be analyzed.

We used the double labeling technique, which to our knowledge, is the first time that it has been employed to identify interneurons with axons in dorsal roots. The final location of these interneurons in adult mice spinal cords and their function will be investigated to elucidate the functional connections in adulthood.

Materials And Methods

The first purpose in this study was to assess the presence of spinal cord interneurons sending axons in dorsal roots. The second aim was to evaluate whether there are antidromic potentials in the neonatal mouse spinal cord dorsal roots. For studying dorsal root functionality, we also analyzed DRR in the L5 dorsal root. Likewise, we studied MR modulation produced by electrical stimulation on the L5 DR and recorded this reflex in L5 VR. We also added bicuculline, a GABA antagonist drug, and the glutamic antagonist AP5 for analysing neural transmission implied in these reflexes.

Subjects

We did experiments in 10 Swiss-Webster mice isolated spinal cords in vitro preparations at 2 to 13 postnatal days. They were housed one single mouse per cage at room temperature. Experimental protocols and animal care were under the NIH guidelines (USA) and approved by the Institutional Ethics Committee in the Health Science Research Center under Mexican Official Norm (NOM-062-ZOO-1999).

Animals were anesthetized by inhalation with methoxy-flurane. When fully anesthetized, they were decapitated. After ventral laminectomy, we used a tungsten needle to perform a longitudinal hemisection and kept ventral and dorsal roots between the T6 and sacral spinal cord segments. Other researchers followed this procedure in previous studies (22–24). One hemicord was placed in a Sylgard silicone elastomer tube at the bottom of a recording chamber. The hemicord was perfused with oxygenated ACSF flowing at 10–14 ml/min. The bath solutions inflowed aCSF through a servo-controlled heater (TC-324B, Warner instruments) for temperature monitoring. The bath solution recirculated at all times, even during wash out.

Fluorescent labeling

We analyzed the presence of axons and their interneurons with fluorescent markers applied in the L4-L5 dorsal roots (n = 10). For interneuron retrograde labeling, we used fluorescent dextran-amines (Molecular Probes, Eugene, Ore.), including rhodamine dextran amine (RDA, MW 3000) and fluorescein dextran-amine (FDA, MW 10 000).

In most cases, we used RDA and FDA in 50%. By mixing the markers we assured that the interneurons were marked correctly, thereby avoiding an RDA transsynaptic flow leak or insufficient FDA antidromic traveling distally to afferent fiber terminals. Lower RDA molecular weight could produce to leakage, whereas the higher molecular weight could not even travel deep enough.

In some experiments, we labeled DR afferent fibers by applying FDA, RDA, or the mixture of both fluorochromes to the cut L4 or L5 or both DR's for marking the afferent fiber ending in the motor nuclei (Fig. 1B). We also retrogradely labeled motoneurons by applying RDA and FDA to the L4-L5 ventral root ($n = 7$).

We used negative pressure to introduce the roots in the tubes producing a tight seal, avoiding any fluorescent marker leakage. We used the markers diluted in a aCSF ten mmol/L solution, with 0.2% TritonX-100 (Sigma Chemical Co.). We employed fine suction electrodes pulled from polyethylene tubing (PE-190, Clay Adams, Parsippany, NJ.). After 18–24 hours, the spinal cord was fixed by immersion in 4% of PFA in a 0.1% phosphate buffer (7.4 pH) overnight. After ascending sucrose cryoprotecter concentrations, we cut the spinal cords in coronal slices on a freezing microtome. Tissue sections placed on slides, dehydrated in ascending alcohol concentrations, cleared with Xylene and covered with an antifade mounting medium (Vectashield, Vector Laboratories Inc. Burlingame, CA). We examined tissue sections with an inverted Zeiss microscope and a laser scanning confocal imaging System (LSM 510). We analyzed images containing several optical sections in the Z plane and saved them from evaluating the morphology and synaptology of interneurons, motoneurons, and afferent fibers. We reconstructed three-dimensional arrangements with Zeiss LSM 510 software.

Stimulation and recording

We placed the dorsal and ventral roots of segments L4 and L5 into the polyethylene suction electrodes for either stimulation or recordings.

We produced MR and DRR by stimulating the dorsal root filament at the L5 segment in the afferent fibers. To continue, we applied ten pulse trains (0.5 ms pulse duration with 2-min intervals) ranging from 16 Hz to 0.125 Hz. We recorded the MR at the L5 ventral root segment, and the DRR at the L4 dorsal root (Fig. 1A). Ca^{2+} concentration was zero in some experiments. We labeled these experiments as low calcium concentrations.

Data Acquisition

The signals obtained from the recording suction electrodes on DR and VR were amplified with Cyberamp 380 amplifiers (axon instruments: band 10–10 kHz) and digitized at 10 kHz with 16 bits resolution A/D converter (National Instruments NBIO-16) and then stored in the computer. We did data analysis off-line using NIH institute software packages.

Drugs used

Drugs were added to the aCSF bathing solution using gravity-feedline (flow rate: 10–14 ml/min) to a recording chamber. We used the 2-amino-5 phosphonovaleric acid (AP5, 100 μ M) to block the monosynaptic response. Bicuculline (10–20 μ M) methiodide was administered for DRR inhibition. The halved spinal cord was placed in the chamber containing: 1) artificial aCSF: 128 NaCl mM, 4 KCl mM, 2 CaCl mM, 1 MgSO₄ mM, 0.5 NaH₂PO₄ mM, 25 NaHCO₃ mM, 30 mM Glucose; 2) aCSF with low calcium concentration (0.8 mM) and 1 Mg mM; 3) aCSF with bicuculline 2–5 μ M or, and 4) A solution with bicuculline 2 μ M and 2-amine 5-phosphonovaleric acid (AP5). That was perfused continuously by the respective oxygenated aCSF.

Statistical analysis.

In some experiments, we measured the ventral horn neuron soma size. We studied them at 2 and 13 postnatal days (P2 and P13). We carried out a linear regression analysis to establish the average soma size value at the respective age, using the Sigma-Plot software v11. We applied Normality tests (Shapiro-Wilk) to the three groups (P2, P13 < average value, and P13). We performed a t-test to compare the soma size among different groups.

Declarations

Ethical Approval

We carried out experiments in full compliance with ethical standards approved by the NIH guidelines (USA) and approved by the Institutional Ethics Committee, according to the Mexican Official Norm (NOM-062-ZOO-1999).

Consent for publication

I Judith Marcela Duenas Jimenez hereby declare that I participated in the study and in the development of the manuscript titled. I have read the final version and give my consent for the article to be published in BMC Neuroscience.

Availability of data and material

The datasets in this study are available on request to the corresponding author.

Competing interests

I declare that I have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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Author's contribution

All authors contributed to the study design and performed experiments. Sergio Horacio Dueñas Jiménez developed the concept and performed the material preparation, data collection, and analysis. Luis Castillo Hernandez wrote the first draft of the manuscript. All authors commented on previous versions of the paper and approved the final manuscript.

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Figures

A Electrical Stimulation

B Fluorescent labeling

Figure 1

Mouse spinal cord drawing illustrating ventral and dorsal roots in thoracic and hemisected spinal cord lumbar segments. A) The stimulation suction electrode (SSE) did administer at the L5 dorsal segment: the recordings electrodes applied in the L4 dorsal segment for dorsal root reflex (DDR), and monosynaptic reflex in the L5 ventral root (MR-VR). B) For neurons with axon in DR fluorescent dextran amines, and a mixture of both were added in suction electrodes in the dorsal roots L4 or /and L5 for orthograde labeling (OL). For motoneuron retrograde labeling (RL), ventral roots L4 or L5 were filled with fluorochromes.

Figure 2

Images of the spinal cord in the L4-L5 segments with fluorescent markers applied in dorsal roots. A) RDA administered in L4 and FDA in L5. RDA leakage stained some interneurons red (scale bar= 10 μ m). B) Afferent fibers marked by FDA and RDA mixture applications in L5 dorsal root. The arrows indicate a fiber stained red, close to this fiber fluorochrome leakage labeled some red neurons (scale bar= 10 μ m). C-D) Images of the dorsal fibers terminal afferents in the spinal cord, the arrows indicate small size neurons, stained yellow (scale bar= 10 μ m).

Figure 3

Spinal cord images in L4-L5 segments with fluorescent markers. A) Afferent fiber labeled FDA (scale bar, 100 μm). Note a limited number of afferent fibers in the spinal cord ventral horn. B) A single interneuron marked FDA with no RDA leakage marking interneurons. The FDA in the L5 dorsal root and RDA in the L4 dorsal root (scale bar= 10 μm). In A, B, and C, the fluorescent marking made in different spinal cord tissues. C) FDA and RDA mixture applied in L4 dorsal root and FDA in the L5 dorsal root. Several interneurons stained in an orange hue with no fluorochrome cell body leakage (scale bar= 10 μm).

Figure 4

Images of the spinal cord in L4-L5 segments with the mixture of RDA and FDA fluorescent markers. A) Terminal afferent fibers arriving at the ventral spinal cord stained by applying the fluorescent mixture in L5 dorsal root (scale bar, 10 μm). B) The mixture administered through the L5 ventral and dorsal root. It is important to note that the motoneurons and the afferent fiber arriving at the motor nucleus were stained (scale bar, 50 μm). C) Neurons were traveling in rafts on the spinal cord dorsal surface. D) The neurons were penetrating a deep region in the spinal cord.

Figure 5

Nuclei formation in the ventral horn. A) At Postnatal day 2, few neurons were stained with RDA. B) At postnatal day 13, two nuclei correctly identified. C-D) The neurons in both nuclei were also segregated by size. The number of neurons is in the abscissa and the size in micrometers in the ordinate. The line in the graphs indicates linear regression. E) Comparing the size in P2 and P13 neurons under linear regression (average value). Note that in the P2, the size of most of the neurons are under 2000 μm . At P13; there was

a statistically significant increase in the size and number of neurons ($P < 0.05$). F) The mean value of neuron size in both nuclei at P13 increased up to four times.

Figure 6

DRR and MR Recordings. DRR and MR control recording (indicated by arrows, upper and lower traces in A). They were recorded in L4 dorsal and L5 ventral roots, respectively. A: aCSF control, B: aCSF with bicuculline (10-20 μM). C and D illustrating MR and DRR in the presence of bicuculline plus AP5 (100

μM). Bicuculline eliminated DRR, and long-latency reflexes observed in VR. MR depression began 2-4 min after applying AP5. Note that most of the MR were almost fully eliminated, but DR action potentials still appeared. E) Dorsal and ventral root reflexes recovered after drug washout. F: VR and DR recording under low Ca^{2+} environment; the ventral and dorsal reflexes were eliminated, but spiking persisted in both ventral and dorsal roots (indicated by arrows).

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