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IDENTIFICATION OF INTERNEURONS IN SPINAL CIRCUITS THAT REGULATE MICTURITION

Hypothesis / aims of study

Parasympathetic preganglionic neurons, which lie in the sacral parasympathetic nucleus in the spinal cord, cause bladder contractions and voiding when bladder pressure reaches a level that triggers a micturition reflex. Spinal neurons involved in reflex control can be identified anatomically by the presence of immunoreactivity for Fos, the protein product of the immediate early gene *c-fos*, which occurs in the nuclei of activated neurons. Although the number of Fos-immunoreactive spinal neurons is known to increase substantially after micturition reflexes, the neurochemical phenotype of the activated neurons has not been defined. To test our hypothesis that spinal interneurons are an important part of the neuronal circuitry controlling voiding, we used micturition reflexes to evoke Fos immunoreactivity and then did double immunoperoxidase staining for Fos plus one of three neurochemical markers that identify parasympathetic preganglionic neurons.

Study design, materials and methods

To identify parasympathetic preganglionic neurons, we gave young adult male Sprague-Dawley rats under isoflurane the retrograde tracer cholera toxin B subunit (CTB) by pressure injection into the major pelvic ganglion, which contains post-ganglionic neurons innervating the bladder, lower bowel and reproductive organs.

Fourteen days later, micturition reflexes were evoked in urethane-anesthetized, CTB-injected rats by a 90-minute continuous infusion of saline into the bladder at 120 microlitres/min. Bladder pressure was recorded throughout the infusion. Control rats were treated similarly but no saline was infused. Two hours after the infusion began, we perfused the rats with phosphate-buffered formaldehyde. Their spinal cords were removed and post-fixed for immunocytochemistry.

Segments L5-S2 from an experimental and a control rat were embed together in albumen gelatine, infiltrated with sucrose and cut transversely at on a cryostat into three series of sections. Fos-immunoreactivity was detected by peroxidase immunocytochemistry and a black reaction product. Next, immunocytochemistry with a brown peroxidase reaction product was used to localize one of three neurochemical markers for parasympathetic preganglionic neurons, either (1) choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine, (2) CTB, which had been retrogradely transported by parasympathetic preganglionic axons exposed to the tracer, or (3) nitric oxide synthase, the enzyme that synthesizes nitric oxide, which occurs in the majority of PPN in the male rat. After immunostaining, sections were dried onto slides, dehydrated and coverslipped.

Quantification was done on sections double-stained for Fos plus ChAT. We selected 8 sections from each of 6 experimental and 6 control rats that had been embedded and stained together in pairs. In one dorsal quadrant from each section, we counted neurons with the following immunoreactivities: Fos+ChAT, Fos or ChAT only. We used paired T-test to compare data from experimental and control groups. Counts are presented as mean number of neurons $\pm$ standard error.

Results

Micturition reflexes were very effective at inducing Fos in spinal neurons. In control rats, Fos-immunoreactivity occurred in the nuclei of some neurons in the dorsal and lateral horn. However, many more neurons were Fos-positive in the dorsal and lateral horns of rats in which bladders had been infused with saline. In the infused rats, the neurons activated to produce Fos by micturition reflexes were particularly concentrated in the parasympathetic nucleus and in the region just dorsal to it.

Some of the neurons activated to express Fos by micturition reflexes were parasympathetic preganglionic neurons, marked either by immunoreactivity for ChAT, nitric oxide synthase or retrogradely-transported CTB. However, most of the Fos-positive neurons lacked these markers, indicating that they were non-cholinergic, non-nitrergic and did not project to the major pelvic ganglion.

Since ChAT is the only marker of the three that we used which reliably labels ALL parasympathetic preganglionic neurons, counts were made on sections double-stained for Fos plus ChAT. A total of 227.0 $\pm$ 11.1 neurons in saline-infused rats (n=6) had Fos-immunoreactive nuclei whereas only 27.3 $\pm$ 5.1 neurons showed Fos-immunoreactivity in rats that did not receive saline infusions (n=6). This difference was statistically significant ($p < 0.001$). The numbers of ChAT-immunoreactive neurons did not differ between experimental and control groups (123.0 $\pm$ 8.9 versus 115.0 $\pm$ 3.4, respectively; $p > 0.01$).

The numbers of cholinergic and non-cholinergic neurons immunoreactive for Fos in a 400 micron x 400 micron area that included the parasympathetic nucleus and the dense cluster of Fos-positive neurons immediately dorsal to it were also compared between experimental (n=6) and control (n=6) rats. This region in infused rats (n=6) contained 61.3 $\pm$ 5.9 neurons immunoreactive for Fos + ChAT, 165.7 $\pm$ 12.1 neurons immunoreactive for Fos only and 61.7 $\pm$ 10.0 neurons immunoreactive for ChAT only. The equivalent numbers for uninfused control rats (n=6) were 9.3 $\pm$ 3.9 Fos + ChAT neurons, 18.0 $\pm$ 4.1 Fos only neurons and 115.0 $\pm$ 3.1 ChAT only neurons. The numbers for each of these neurochemically-defined neuronal types were statistically significantly different between experimental and control groups ($p < 0.001$).

Interpretation of results

In the male rat, micturition reflexes triple the number of spinal neurons that express Fos in the dorsal and lateral horn of L6 and S1, where the parasympathetic nucleus is located. The number of parasympathetic preganglionic neurons that express Fos increases by 7-fold. However, a 9-fold increase occurs in the number of Fos-expressing non-cholinergic neurons that lie just dorsal to the parasympathetic preganglionic neurons. There is also an increase in the

number of non-cholinergic neurons expressing Fos outside this restricted lateral region. Since these non-cholinergic, Fos-positive neurons also lack immunoreactivity for nitric oxide synthase and do not innervate the major pelvic ganglion, they are spinal interneurons.
Taken together, these results suggest that interneurons are likely to be very important in processing the information that triggers parasympathetic preganglionic neurons to produce the bladder contractions that result in voiding.

Concluding message
To understand how spinal nerve pathways help to maintain continence, it will be important to define the physiological functions, neurochemistry and connections of the bladder-regulating interneurons identified in this study.

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