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Peripheral targets influence sensory-motor connectivity in the neonatal spinal cord: Sciatic nerve axotomy in *Bax*-deficient mice

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Abstract

In neonatal animals, peripheral nerve axotomy induces cell death in the corresponding dorsal root ganglion neurons and motoneurons, indicating that trophic interactions between these neurons and their targets control neuronal survival at this age. However, axotomy-induced cell death masks the role of peripheral tissues in regulating the central connections between these neurons in neonates. Since we have shown in *Bax*-deficient mice (*Bax*−/−) that transection of the sciatic nerve at postnatal day (P) 0 rarely induced apoptosis in motoneurons, we examined whether peripheral nerve axotomy eliminates synaptic connections between group Ia afferents and motoneurons in *Bax*−/−. After the axotomy, we observed in P7 *Bax*−/− that many axons survived in the fourth lumber (L4) dorsal root and that primary afferent projections to L4 motor pools also remained. Sciatic nerve stimulation evoked synaptic responses in L4 ventral roots in these mice although the amplitudes were considerably smaller and the onset latencies longer compared with the controls. Our results suggest that the monosynaptic connection between group Ia afferents and motoneurons is morphologically and functionally preserved following axotomy. Peripheral tissues may modulate synaptic connectivity but do not contribute to the maintenance of primary afferent projections in the stretch reflex pathway at an immature stage.

Keywords: *Bax*; Axotomy; Spinal cord; Stretch reflex; Primary afferent; Motoneuron; Monosynaptic response

1. Introduction

The simplest of the spinal reflex pathways, namely the stretch reflex pathway, consists of spinal motoneurons and group Ia afferents derived from proprioceptive neurons in the dorsal root ganglion (DRG). Group Ia afferents, which innervate muscle spindles of a given muscle, make monosynaptic connections with motoneurons that innervate the same (homonymous) muscle. In the developing spinal cord, group Ia afferents must recognize and make synapses with their proper partner motoneurons. Development of primary afferent projections to the spinal cord is described for several species of animals so far examined (e.g., rat, Kudo and Yamada, 1987; mouse, Ozaki and Snider, 1997; chick, Lee et al., 1988). In the lumbar spinal cord of rat embryos, several collaterals of afferent axons reach the dorsal part of the dorsal horn by embryonic day (E) 15.5, a small number of afferent collaterals reach the motor pools at E17.5 and afferent volleys evoke monosynaptic excitatory potentials in the ventral root at E18.5 (Kudo and Yamada, 1987).

When group Ia afferents from proprioceptive DRG neurons make monosynaptic connections to spinal motoneurons, a variety of factors derived from their common target (the homonymous muscle) are suggested to regulate the formation of stretch reflex pathways. Transcription factors play a major role in regulating the differentiation and maturation of neurons in the developing nervous system. Motoneurons that innervate a given muscle are grouped in clusters known as motor pools in the spinal ventral horn. Combined expression of transcription factors, such as those of the LIM and ETS families, is

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implicated in establishing a motoneuronal identity in the motor pool during early developmental stages (Landmesser, 2001). These transcription factors are also expressed in both a subset of proprioceptive DRG neurons and a subset of motoneurons that innervate the same target muscles (Lin et al., 1998). If a limb bud is removed in chick embryos before the arrival of peripheral nerves, ETS expression on both spinal motoneurons and proprioceptive DRG neurons is eliminated, indicating that the peripheral target regulates the coordinated gene expression (Lin et al., 1998). Deficiency of ER81, a member of the ETS family, causes defects of monosynaptic connections between group Ia afferents and motoneurons in mice (Arber et al., 2000). These findings suggest that the interaction between neurons and their peripheral target muscle is essential of inducing transcriptional cascades for forming central connections of the stretch reflex pathway. In addition to their role in regulating neuronal differentiation, targets are important to determine the number of indispensable neurons in a neuronal population in developing nervous systems. In prenatal animals, naturally occurring programmed cell death of neurons is observed in the spinal cord and DRG as a result of apoptotic gene expression. The death of neurons is due to a limited supply of neurotrophic factors provided by the target tissue. Even after birth, transection of peripheral nerve axons induces apoptosis, so-called axotomy-induced cell death, in the axotomized motoneurons and DRG neurons, indicating that trophic interactions with their target are still the major factor in regulating neuronal survival during the neonatal period. It is difficult, however, to study when peripheral targets begin to be involved in modulating the central connections of proprioceptive DRG neurons with motoneurons, since axotomy-induced cell death prevents us from investigating the further effect of target muscles in neonatal animals in which basic functions of the stretch reflex pathway have been established (Kudo and Yamada, 1985). Proteins of the Bcl-2 family, including *Bax*, are known to be regulators of apoptosis within the nervous system (Merry et al., 1997). Naturally occurring programmed cell death has been reported to be suppressed in wide areas of the nervous system of *Bax*−/− mice, resulting in a supernumerary but mixed population of normal and atrophic motoneurons (White et al., 1998). In *Bax*−/− neonatal mice, axotomy-induced cell death is also suppressed in motoneurons in the facial nuclei (Deckwerth et al., 1996) as well as in the spinal cord (Kinugasa et al., 2002). Neurotrophin deprivation-induced cell death is also inhibited in DRG neurons from *Bax*−/− mice, in vitro (Lentz et al., 1999) and supernumerary neurons were observed in vivo (White et al., 1998). Thus, in *Bax*−/− neonatal mice, DRG neurons can survive even after peripheral nerve axotomy and, thus, the *Bax*−/− mouse should be a powerful tool for clarifying the effect of peripheral nerve axotomy on development of the stretch reflex pathway just after birth. In the present study, we examined whether peripheral nerve axotomy induces elimination of primary afferent projections and synaptic connections between group Ia afferents and motoneurons in the spinal cord in *Bax*−/− mice.

2. Materials and methods

Bax mice were purchased from the Jackson Laboratory (Knudson et al., 1995; Bar Harbor, Maine). Wild-type (*Bax*+/+) and *Bax*−/− mice were obtained from the overnight mating of *Bax*+/− mice. On P0, mice were anesthetized by hypothermia and the sciatic nerve was unilaterally axotomized at its exit-point from the sciatic notch. In some cases, a portion (more than 1 mm length) of nerve trunk was removed from the proximal sciatic nerve stump. Only completely axotomized neonates were used in the following study.

Mouse genotyping was performed by PCR using a set of three primers: *Bax* exon 5 forward primer (5′-TGATCAGAACCATCATG-3′), *Bax* intron 5 reverse primer (5′-GTTGACCAGAGTGGCGTAGG-3′) and Neo reverse primer (5′-CCGCTTCCATTGCTCAGCGG-3′). Cycling parameters were 5 min at 94 °C for 1 cycle plus 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. PCR products were resolved on 2% agarose gels.

At P7, previously operated mice were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). In order to retrogradely label motoneurons and primary afferents, crystals of a lipophilic fluorescent dyes, DiI and DiD were placed in the fourth lumber ventral root (L4VR) and dorsal root (L4DR), respectively, on both sides of fixed, whole mount preparations. The dyes were allowed to transport for 7 days at 37 °C and the preparations were then cut in 75 μm thick sections for microscopy. 10 μm thick optical sections were stacked and reconstructed by confocal laser scanning microscopy (Leica TCS, 20× objective). Images of the sections were digitally stored and adjusted their contrast and brightness automatically with Adobe Photoshop®. For quantitative analysis of the density of primary afferents, confocal images from both the control and axotomized sides of the L4 ventral horn were acquired under the same conditions in each section. Windows (187 μm × 187 μm, 13,924 pixels) were fit to the corresponding area of the dorsolateral and ventrolateral motor pools. Mean fluorescence intensity (256 gradation) of afferent axons in the selected area was digitally measured with Adobe Photoshop®. The intensity on the axotomized side as a ratio of that on the intact side was calculated in each section. Five animals of each genotype were examined.

For semithin sections, mice were transcardially perfused with 2% glutaraldehyde in 0.1 M PB. The L4DR on both sides were dissected out and 1 μm thick transverse sections were stained with toluidine blue. The sections were digitally photographed using 20 and 100× objectives (Zeiss Axioplan 2). The cross-sectional areas of L4DR and myelinated axons in those roots from five mice of each genotype were measured using NIH Image and the values averaged for *Bax*+/+ and *Bax*−/− mice.

P7-8 mice were deeply anesthetized and the spinal cords with L4DR, L4VR and the sciatic nerve were removed. Isolated spinal cord preparations were superfused with an oxygenated Krebs solution (NaCl 127 mM, KCl 1.9 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.0 mM, $MgSO_4$ 1.0 mM, $NaHCO_3$ 26.0 mM, glucose 16.6 mM, bubbled with 95% O_2 + 5% CO_2, pH 7.4) at room temperature. L4DR or sciatic nerves were placed in glass suction electrodes and stimulated with a single pulse of 0.2 ms duration (interval, 0.033–10 Hz). Ventral root potentials evoked in L4VR (L4VRPs) were recorded with glass suction electrodes (bandwidth, 0.08 Hz–10 kHz). Afferent volleys evoked by stimulation of the sciatic nerve at the proximal stump of axotomy or at the corresponding site on the intact side were recorded from the proximal end of L4DR with glass suction electrodes (bandwidth, 0.08 Hz–10 kHz). Signals were stored with a computer system for off-line analysis.

The examiner was blind as to the axotomized side and genotype of the specimens. Statistical comparisons were made using the Wilcoxon signed rank test with a significance limit of $p < 0.05$. Comparing the cross-sectional areas of myelinated L4DR axons, the Mann–Whitney's test was employed with a significance limit of $p < 0.05$.

3. Results

3.1. The effect of sciatic nerve axotomy on axons in L4DR

In order to examine the effects of sciatic nerve axotomy on primary afferent axons in each genotype, 1 μm thick transverse