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CONGENITAL ABSENCE OF CORTICOSPINAL TRACT DOES NOT SEVERELY AFFECT PLASTIC CHANGES OF THE DEVELOPING POSTNATAL SPINAL CORD

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Abstract—The arrival and refinement of corticospinal afferents are likely to influence the maturation of the spinal cord and sensory-motor networks. To understand this better, we studied the revision of monosynaptic muscle afferents, the expression of activity-related genes, neurotrophins and their receptors in the cervical spinal cord from postnatal day (P) 0 to 21. We compared control and *Celsr3*|*Emx1* mice, in which corticospinal axons never develop. The corticospinal tract (CST), labeled by anti-protein kinase C gamma (PKCγ) antibody in the dorsal funiculus, increased gradually in the control, but was never visible in the mutant. Using anti-parvalbumin and choline acetyltransferase double immunostaining, close contacts between proprioceptive afferent fibers and spinal motor neurons appeared at P0 and were gradually eliminated thereafter, with no difference between control and mutant mice. In both genotypes, the number of parvalbumin-positive interneurons increased similarly from P7 to P21, and a comparable upregulation of c-Jun protein was seen at P7. Contrary to control samples, in which ciliary neurotrophic factor (CNTF) protein levels increased from P0 to P7 and gradually decreased after P14, CNTF concentrations were time-invariant in mutant samples. The dynamic profile of neurotrophin-3 (NT3) expression was also moderately affected in mutant mice. In control spinal cord, NT3 was increased at P7 and decreased at P14, but remained more stable in mutant samples. In contrast, expression profiles of brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase (Trk) B, TrkC, p75 neurotrophin receptor (p75^{NTR}) and glial cell-line-derived neurotrophic factor (GDNF) were similar in both genotypes. In conclusion, with the possible exception of CNTF and NT3 expression, most events that accompany maturation of the spinal cord appear largely independent of corticospinal inputs. **© 2015 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: corticospinal tract, axonal elimination, neurotrophins, c-Jun, neural activity, *Celsr3*.

INTRODUCTION

In rodents, corticospinal axons reach the lower cervical spinal cord around birth, and the refinement of locomotor spinal networks is complete by the end of the third postnatal week, with a series of plastic changes occurring during that interval (Joosten et al., 1992; Oudega et al., 1994). Corticospinal axons ramify exuberantly in the gray matter of the lower cervical spinal cord from P4 to P10, after which redundant branches are eliminated from some areas, such as the lateral part of ventral and dorsal horn (Curfs et al., 1994). Muscle afferents initially make inappropriate, redundant connections to motor neurons, which are gradually eliminated (Seebach and Ziskind-Conhaim, 1994; Gibson and Clowry, 1999), and this leads to a decrease in the strength of the monosynaptic stretch reflex (Kudo and Yamada, 1987; Ozaki and Snider, 1997). The intrinsic circuitry is reorganized, and the distribution of spinal interneurons is refined in parallel to maturation of corticospinal inputs (Chakrabarty et al., 2009; Russ et al., 2013). Parvalbumin and c-Jun are markers of neuronal activity and their expression changes in parallel to the arrival of corticospinal inputs, and is modified by lesions of the early, but not mature motor cortex (Clowry et al., 1997, 2004; Gibson et al., 2000). In mice with an isolated cortex, the spinal motor network does not mature fully (Han et al., 2013).These findings suggest that corticospinal inputs influence spinal cord maturation, and raise questions about responsible mechanisms, particularly the role of growth factors.

Neurotrophins are secreted proteins involved in multiple events during neural development, such as proliferation, survival, differentiation and growth. They bind to receptors of the tropomyosin-related kinase (Trk) receptor family and to the common receptor p75^{NTR} (Hennigan et al., 2007; Skaper, 2008). Trk receptors display ligand-binding specificity: TrkA binds preferentially to nerve growth factor (NGF), TrkB to brain-derived neurotrophic factor (BDNF), and TrkC to neurotrophin-3 (NT3) (Davies et al., 1995; Ryden and Ibanez, 1996;

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Abbreviations: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CST, corticospinal tract; EDTA, ethylenediaminetetraacetic acid; GDNF, glial cell-line-derived neurotrophic factor; NT3, neurotrophin-3; p75^{NTR}, p75 neurotrophin receptor; PKCγ, protein kinase C gamma; SG, substantia gelatinosa; Trk, tropomyosin-related kinase.

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Bothwell, 2014). Virtually all corticospinal motor neurons express mRNA coding for TrkB, TrkC and p75 neurotrophin receptors ($p75^{NTR}$), but not TrkA (Giehl and Tetzlaff, 1996; Giehl, 2001). TrkB and TrkC are expressed as two isoforms: full-length receptors with a tyrosine kinase domain, and truncated isoforms lacking the kinase domain (Klein et al., 1990; Middlemas et al., 1991; Bartkowska et al., 2013). In general, neurotrophic factors promote neuronal survival via Trk receptors, but can also induce apoptosis through $p75^{NTR}$ in the absence of Trk receptors (Kaplan and Miller, 1997; Bamji et al., 1998). Endogenous BDNF and NT3 can promote the survival of axotomized corticospinal neurons in rats (Giehl et al., 2001), and upregulation of TrkB expression in corticospinal motor neurons may regulate fiber sprouting after corticospinal tract (CST) injury (Hollis et al., 2009; Fouad et al., 2010), while knockdown of BDNF or TrkB has opposite effects (Ueno et al., 2012).

Two other neurotrophins, ciliary neurotrophic factor (CNTF) and glial cell-line-derived neurotrophic factor (GDNF), are also important for peripheral and central nervous system development and function. CNTF is produced by astrocytes and microglia following brain injury and supports the survival of a variety of neuronal populations, as well as remyelination (Moore et al., 2011; Tanaka et al., 2013). In the spinal cord, GDNF is secreted by motor neurons and oligodendrocytes, and is implicated in plasticity (Nakamura and Bregman, 2001; McCullough et al., 2013). GDNF is also produced from other sources such as limb mesenchyme. Defective GDNF production alters the location of developing spinal motor neurons that innervate the limbs and perturbs steering of motor axons to target muscles (Haase et al., 2002; Kramer et al., 2006).

We previously showed that the changes in expression of neurotrophic factors and receptors after brachial plexus avulsion and root re-implantation are associated with differences in motor neuron survival rates between normal and *Celsr3|Emx1* mice with congenital absence of CST (Ding et al., 2014). Here, we used the same mouse model to assess the role of corticospinal fibers on spinal cord maturation, by studying the remodeling of monosynaptic connections, the expression of activity-related genes, and that of neurotrophins BDNF, NT3, CNTF and GDNF, and receptors TrkB, TrkC and $p75^{NTR}$.

EXPERIMENTAL PROCEDURES

Animals

Animal procedures followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Laboratory Animal Ethics Committee at Jinan University (Permit Number: 20111008001). We used *Celsr3*$^{f/-}$*;Emx1-cre* mutant mice (*Celsr3|Emx1* for short) and *Celsr3*$^{f/+}$*;Emx1-cre* as controls. In those mutant mice *Celsr3* is conditionally inactivated in cortical excitatory neurons upon expression of Cre under the *Emx1* promoter (Gorski et al., 2002), and the CST does not develop (Zhou et al., 2008; Tissir and Goffinet, 2013). The genotyping of animals was described before (Ding et al., 2014). Male or female animals were used indiscriminately, and the day of birth was taken as P0. The numbers of animals in each experimental set are mentioned in Table 1.

Immunostaining

Under deep anesthesia with 10% chloral hydrate, mice (P0, P7, P14 and P21) were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Spinal C5-T1 segments were collected and post-fixed in the same fixative overnight, and then immersed in 30% sucrose until they sank. Thirty-μm-thick transverse sections were prepared with a sliding microtome (Leica). After washing in 0.1 M phosphate-buffered saline three times, the sections were blocked in 10% goat serum plus 3% bovine serum albumin for 2 h, and incubated with primary antibodies overnight at 4 °C. Signal was detected with Alexafluor 546- or 488-labeled fluorescent secondary antibodies (1:1000, Invitrogen, Carlsbad, CA, USA). Primary antibodies were: rabbit anti-protein kinase C gamma (PKCγ) (1:400, ab109539, Abcam, Cambridge, MA, USA), rabbit anti-BDNF (1:1000, ab6201, Abcam), goat anti-TrkB (1:1000, AF1494, R&D, Minneapolis, MN, USA), rabbit anti-NT3 (1:1000, ab65804, Abcam), rabbit anti-CNTF (1:1000, ab46172, Abcam), mouse anti-parvalbumin (1:1000, MAB1572, Millipore, Billerica, MA, USA) and goat anti-choline acetyltransferase (1:500, AB144p, Millipore).

Western blot

Mice were sacrificed under deep anesthesia and tissue samples from C5-T1 segments were quickly collected and dissociated in lysis buffer (containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride) on ice for 30 min, during which they were homogenized by sonication for 20–60 s (Sonics & Materials, INC., USA). Lysates were frozen/thawed four times and cleared by centrifugation at 14,800 rpm for 15 min at 4 °C. Supernatants were pooled and protein concentrations were measured with a BCA kit (Biotechnology CO., LTD, China). For Western blot, the supernatants were mixed with loading buffer (50 mM Tris–HCl, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol and 2 mg/ml bromophenol blue) in a 1:1 ratio, and boiled for 5 min. Samples containing 30-μg total protein were analyzed by polyacrylamide gel electrophoresis (8% gels for c-Jun, $p75^{NTR}$, TrkB and TrkC, and 10% gels for BDNF, NT3, CNTF and GDNF), and transferred to polyvinylidene fluoride membranes (Millipore) by electroblotting (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk and 0.1% Tween20 (pH 7.4) for 2 h, and incubated with the following antibodies: rabbit anti-BDNF (1:2000, ab6201, Abcam), goat anti-TrkB (1:1000, AF1494, R&D), rabbit anti-NT3 (1:2000, ab65804, Abcam), rabbit anti-TrkC (1:1000, 3376, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-$p75^{NTR}$ (1:2000, G3231, Promega, Wisconsin, USA), rabbit anti-CNTF (1:2000, ab46172, Abcam), rabbit anti-GDNF (1:1000, ab18956,

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