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Stathmin is enriched in the developing corticospinal tract

Heidi R. Fuller [a,b,c], Robert Slade [b,c], Nataša Jovanov-Milošević [d], Mirjana Babić [d], Goran Sedmak [d], Goran Šimić [d], Matthew A. Fuszard [e,1], Sally L. Shirran [e,1], Catherine H. Botting [e], Monte A. Gates [b,*]

[a] Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry SY10 7AG, UK
[b] Institute for Science and Technology in Medicine, Keele University, Keele, Staffordshire ST5 5BG, UK
[c] Postgraduate Medicine, Keele University, Staffordshire ST5 5BG, UK
[d] Croatian Institute for Brain Research, Zagreb 10000, Croatia
[e] BSRC Mass Spectrometry and Proteomics Facility, Biomedical Sciences Research Complex, University of St Andrews, North Haugh, St Andrews, Fife KY16 9ST, UK

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ABSTRACT

Understanding the intra- and extracellular proteins involved in the development of the corticospinal tract (CST) may offer insights into how the pathway could be regenerated following traumatic spinal cord injury. Currently, however, little is known about the proteome of the developing corticospinal system. The present study, therefore, has used quantitative proteomics and bioinformatics to detail the protein profile of the rat CST during its formation in the spinal cord. This analysis identified increased expression of 65 proteins during the early ingrowth of corticospinal axons into the spinal cord, and 36 proteins at the period of heightened CST growth. A majority of these proteins were involved in cellular assembly and organization, with annotations being most highly associated with cytoskeletal organization, microtubule dynamics, neurite outgrowth, and the formation, polymerization and quantity of microtubules. In addition, 22 proteins were more highly expressed within the developing CST in comparison to other developing white matter tracts of the spinal cord of age-matched animals. Of these differentially expressed proteins, only one, stathmin 1 (a protein known to be involved in microtubule dynamics), was both highly enriched in the developing CST and relatively sparse in other developing descending and ascending spinal tracts. Immunohistochemical analyses of the developing rat spinal cord and fetal human brain stem confirmed the enriched pattern of stathmin expression along the developing CST, and in vitro growth assays of rat corticospinal neurons showed a reduced length of neurite processes in response to pharmacological perturbation of stathmin activity. Combined, these findings suggest that stathmin activity may modulate axonal growth during development of the corticospinal projection, and reinforces the notion that microtubule dynamics could play an important role in the generation and regeneration of the CST.

1. Introduction

The mammalian corticospinal tract (CST) is the longest efferent axonal projection in the central nervous system (Stanfield, 1992; Sakai and Kaprielian, 2012). Apart from the substantial distance that CST axons must grow, upper motor neurons from layer V of the (mostly primary motor, supplementary motor, premotor and somatosensory, but also widespread regions of the parietal and frontal) cortex extend axons that must navigate a complex route to reach their targets in the anterior horns of the spinal cord. From the cortex, CST axons must first course caudally through the corona radiata and capsula interna to reach the brain stem, where a majority of these axons decussate in the medullary pyramids (Donkelaar et al., 2004). Thick bundles of fasciculated corticospinal axons must then continue a contralateral projection within well defined white matter tracts of the spinal cord until they reach a target segment of the cord, where, after a delay, they mostly synapse with an appropriate interneuron in the ventral gray matter that then synapses with a lower motor neuron.

At the protein level, formation of the CST is achieved via the intracellular responses of growing axons to signals in the extracellular environment. Though the proteins involved in the precise growth of corticospinal axons from the cortex to targets in the spinal cord are not fully understood, it is generally thought that a process of chemoattractive/chemorepulsive signaling (via extracellular proteins) and catastrophe/rescue responses (via intracellular proteins) all play a role. Initially, extracellular expression of semaphorins appear to have both chemoattractive and chemorepulsive properties that facilitate the exiting of corticospinal fibers from the cortex (Bagnard et al., 1998). From here, the expression of netrins may provide

Abbreviations: CNS, central nervous system; CST, corticospinal tract; CRL, crown-rump length; FA, formic acid; FDR, false discovery rate; IPA, ingenuity pathway analysis; iTRAQ, isobaric tag for relative and absolute quantitation; MeCN, acetonitrile; P, postnatal day; PBS, phosphate buffered saline; PFA, paraformaldehyde; RT, room temperature; SCI, spinal cord injury; TBS, tris-buffered saline; TEAB, tetraethylammonium bromide; wg, weeks of gestation.

* Corresponding author at: Keele University, Department of Life Sciences, Huxley Building, Staffordshire ST5 5BG, UK.
E-mail address: m.a.gates@keele.ac.uk (M.A. Gates).

[1] These authors contributed equally.

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an attractive signal in the extracellular environment to facilitate the caudal growth of CST axons into the diencephalon (Richards et al., 1997; Métin et al., 1997). At the medullary pyramids, the absence of inhibitory proteins (or an inhibitory glial barrier), as well as the presence of netrin related proteins, are both thought to allow/facilitate CST axons to cross the midline (Joosten and Gribnau, 1989; Finger et al., 2002). From this point, however, much less is known about how CST neurons navigate their course down the spinal cord itself.

In contrast to human corticospinal neurons (which mostly traverses the lateral CST), the development of a vast majority (~95%) of rat CST axons proceeds caudally through the spinal cord along the ventral wedge of the dorsal columns. Anatomically, the first axons of the rat CST to enter the spinal cord appear in the upper cervical region at the day of birth. These axons subsequently extend into upper thoracic regions by postnatal day (P) 3, and reach the most distal sacral regions by P9 (Donatelle, 1977; Schreyer and Jones, 1982). At the molecular level, the extracellular expression of the protein L1/CAM appears crucial for maintaining the fasciculation of CST fibers as they grow, and to the eventual functioning of the CST. However, L1/CAM is not thought to be involved in stimulating pathfinding of the tract caudally (Dobson et al., 2001). Similarly, the presence of ephrin B3 or B4 ligand-receptor complexes along the spinal cord midline is considered important for maintaining the bilateral segregation of corticospinal axons along the length of the spinal cord (Yokoyama et al., 2001). However, these proteins appear more important for maintaining the laterality of CST axons, rather than actual CST formation (Harel and Strittmatter, 2006).

Little, in fact, is known about the intracellular and extracellular proteins that are important to the growth of corticospinal neurons caudally down the spinal cord. This is unfortunate due to the fact that regeneration of the CST within the spinal cord is of great therapeutic importance. Each year, there are an estimated 6500 cases of spinal cord injury (SCI) in Western Europe, and between 100,000–200,000 incidences worldwide (Lee et al., 2014). One hope is that by identifying key intracellular and extracellular proteins involved in the development of the corticospinal system, the regeneration of the mature CST might be enhanced by manipulating these developmental constituents.

The aim of this study, therefore, was to conduct a comprehensive quantitative proteomics analysis of the rat CST during its formation. By conducting both a spatial and temporal comparison of the developing CST with the more mature CST and other white matter tracts of the developing spinal cord, we show that stathmin 1 — a major intracellular regulator of microtubule dynamics — is highly enriched in the developing CST. Also, we have shown that manipulating stathmin activity in vitro significantly reduces neurite growth from embryonic rat cortical neurons. Such findings suggest that stathmin may have an important role in the growth of corticospinal neurons during development, and that future work clarifying the function of developmentally regulated proteins in the CST may provide insights into how axonal growth along the spinal cord may be facilitated.

2. Materials and methods

All in vivo procedures were approved by the Animal Welfare & Ethical Review Body (AWERB) at Keele University, and were carried out under the licensed authority of the UK Home Office. All adult Sprague Dawley rats were housed in a 12–12 h light–dark environment, and given free access to food and water throughout the study.

2.1. Tissue extraction for mass spectrometry and western blot analysis

Animals were given an overdose of pentobarbitone anesthetic (via i.p. injection) and transcardially perfused with ice-cold sterile 0.9% sodium chloride (saline). The spinal cord was quickly removed and placed in a small Petri dish filled with fresh ice-cold saline. To identify differences in the protein profile of the CST temporally (i.e., to see what proteins are highly expressed in the developing vs more mature CST), tissue was collected from the CST in the cervical spinal cord of rats at P 0, 3, 14 and 28 (Fig. 1A). To identify differences between the protein profile of the developing CST and other developing tracts of the spinal cord, tissue was separately collected from the CST and two other white matter tracts of the spinal cord from P3-aged animals (a period of heightened corticospinal development) (Fig. 1B).

For a temporal analysis, dissected tissue from each animal (8 animals per time point) was homogenized individually in 4 volumes (*w*/*v*) of 6 M urea, 2 M thiourea, 2% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (CHAPS) and 0.5% sodium dodecyl sulfate (SDS) using a pellet pestle (30 strokes with the pestle, left on ice for 10 min, followed by another 30 strokes with the pestle). The extracts were sonicated briefly and left on ice for 10 min, followed by centrifugation at 13,000 *g* for 10 min at 4 °C to pellet any insoluble material. For mass spectrometry analysis, aliquots of extracted proteins from each sample group were pooled and precipitated in 6 volumes of ice-cold acetone overnight at −20 °C. The remaining extracts were stored un-pooled at −80 °C for western blotting. The acetone precipitates were pelleted by centrifugation at 13,000 ×*g* for 10 min at 4 °C and the supernatant was carefully removed and discarded. The pellets were resuspended in 500 mM of

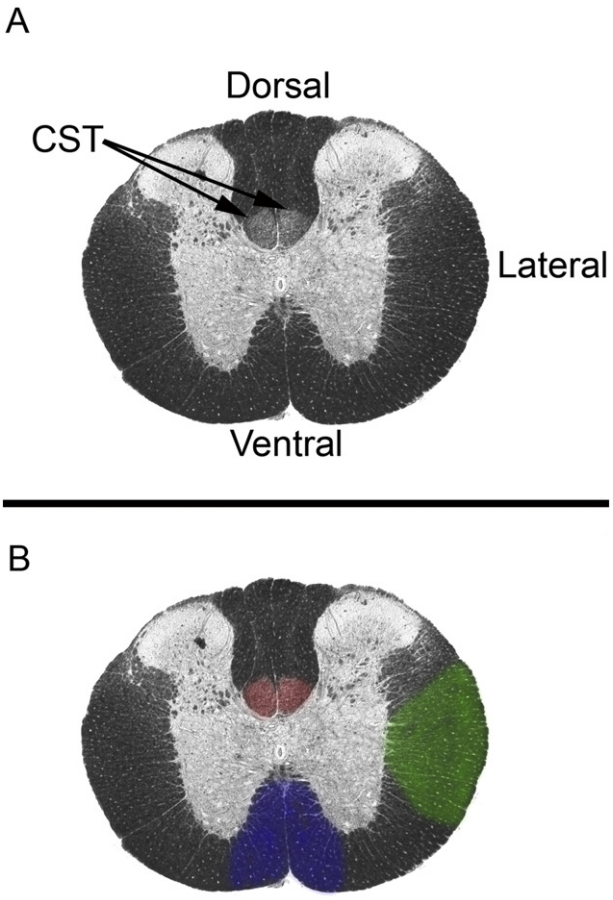

Fig. 1. Diagram illustrating regions of the spinal cord used to identify temporally and spatially specific protein expression in the developing spinal cord. (A) An unstained, bright field image of a cross section through the adult rat spinal cord illustrating the position (arrows) of the corticospinal tract (CST) in rats. (B) To detail protein expression in the CST as it develops, protein was extracted from the CST (red shaded area) at P0, P3, P14 and adult rats and analyzed using iTRAQ. Note that, unlike the human corticospinal projection, the CST fibers in rats predominantly traverse the ventral segment of the dorsal column of the spinal cord. To compare spatial difference in protein expression in developing white matter tracts of the spinal cord, protein was extracted from the CST (red shaded area) of P3 rats, and compared to the proteins expressed in the lateral (green shaded area) and ventral (blue shaded area) white matter tracts of the same animals.