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Research Report

Hindbrain raphe stimulation boosts cyclic adenosine monophosphate and signaling proteins in the injured spinal cord

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ABSTRACT

Early recovery from incomplete spinal cord contusion is improved by prolonged stimulation of the hindbrain's serotonergic nucleus raphe magnus (NRM). Here we examine whether increases in cyclic adenosine monophosphate (cAMP), an intracellular signaling molecule with several known restorative actions on damaged neural tissue, could play a role. Subsequent changes in cAMP-dependent phosphorylation of protein kinase A (PKA) and PKA-dependent phosphorylation of the transcription factor "cAMP response element-binding protein" (CREB) are also analyzed. Rats with moderate weight-drop injury at segment T8 received 2 h of NRM stimulation beginning three days after injury, followed immediately by separate extraction of cervical, thoracic and lumbar spinal cord for immunochemical assay. Controls lacked injury, stimulation or both. Injury reduced cAMP levels to under half of normal in all three spinal regions. NRM stimulation completely restored these levels, while producing no significant change in non-injured rats. Pretreatment with the 5-HT7 receptor antagonist pimozide (1 mg/kg, intraperitoneal) lowered cAMP in non-injured rats to injury amounts, which were unchanged by NRM stimulation. The phosphorylated fraction of PKA (pPKA) and CREB (pCREB) was reduced significantly in all three regions after SCI and restored by NRM stimulation, except for pCREB in lumbar segments. In conclusion, SCI produces spreading deficits in cAMP, pPKA and pCREB that are reversible by Gs protein-coupled 5-HT receptors responding to raphe-spinal activity, although these signaling molecules are not reactive to NRM stimulation in normal tissue. These findings can partly explain the benefits of NRM stimulation after SCI.

1. Introduction

Although spinal cord injury (SCI) in adult mammals typically leads to permanent impairment of motor, sensory and autonomic functioning, some degree of improvement usually occurs in the first weeks (Muramatsu et al., 2009; Onifer et al., 2011). We have previously proposed that recovery from mechanical trauma in either the spinal cord or brain is controlled by the serotonergic raphe nuclei of the brainstem. The system of raphe nuclei as a whole sends axonal

Abbreviations: cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; ELISA, enzyme-linked immunosorbent assay; NRM, nucleus raphe magnus; PKA, protein kinase A; pPKA, phosphorylated PKA; pCREB, phosphorylated CREB; SCI, spinal cord injury; TBI, traumatic brain injury; TRH, thyrotropin-release hormone

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projections to all likely sites of damage. Our model holds that raphe neurons respond to typical sensory and chemical sequelae of neurotrauma by widespread axonal release of restorative substances (principally serotonin and certain neuropeptides). Experimentally, we have shown that prolonged stimulation of the hindbrain's nucleus raphe magnus (NRM) in rats can boost this natural repair system after SCI (Hentall and Burns, 2009; Hentall and Gonzalez, 2012), and that stimulation of the midbrain dorsal or median raphe is similarly beneficial after traumatic brain injury (Carballosa Gonzalez et al., 2013).

The NRM's highly branched fibers terminate in essentially all spinal segments and laminae (Allen and Cechetto, 1994; Rajaofetra et al., 1989; Sur et al., 1996). They respond to the sensory correlates of severe collision, such as pain and low blood pressure (Fields et al., 1991; Gao and Mason, 2001), and to chemical products of tissue injury, such as cytokines, purines and eicosanoids (Heinricher et al., 2004; Nason and Mason, 2006; Selden et al., 2007; Wei et al., 2008). As well as releasing serotonin, which has various trophic and neuroprotective actions (Trakhtenberg and Goldberg, 2012), many NRM-spinal terminal co-release thyrotropin-release hormone (TRH) (Hokfelt et al., 2000; Kachidian et al., 1991), which has also proved to have positive effects on recovery after SCI (Faden et al., 1989). Electrical stimulation of the NRM enduringly improves the recovery of both sensory and motor performance after incomplete thoracic SCI in rats, while increasing the numbers of serotonergic terminals within gray matter and myelinated fibers in white matter around the lesion epicenter (Hentall and Burns, 2009; Hentall and Gonzalez, 2012). Multiple mechanisms probably contribute to these improvements, given the multifarious cells and processes affected by mechanical trauma. Conversely, from the perspective of evolutionary fitness, any molecular or cellular mechanism with a major beneficial effect on repair is likely to be mobilized.

We tested this reasoning with respect to the important intracellular signaling cascade initiated by cyclic adenosine monophosphate (cAMP). Increased cAMP is known to facilitate recovery from SCI (Nikulina et al., 2004; Pearse et al., 2004; Qiu et al., 2002), probably by acting in several distinct ways. It subdues the inhibitory actions of the proteins produced by myelin breakdown on axonal growth, it modulates glia-propagated inflammation and it leads to upregulated expression of pro-inflammatory cytokines such as TNFα (Christiansen et al., 2011; Gerlo et al., 2011; Ghosh et al., 2012; Zhang et al., 2002). Some of these beneficial effects occur through cAMP-dependent phosphorylation of protein kinase A (PKA) and subsequent activation of the cyclic AMP response element-binding protein (CREB), which governs the transcription of an array of regeneration-associated genes (Hannila and Filbin, 2008; Moore and Goldberg, 2011).

We first sought to establish whether NRM stimulation can normalize the spinal cord concentration of cAMP, which has been reported to fall in the zone of a thoracic contusion (Pearse et al., 2004). We furthermore assayed both total and phosphorylated PKA and CREB. All three molecular species were measured separately in cervical, thoracic and lumbar spinal cords of rats with three-day thoracic (T8) contusion injury, some of which had received 2 h of NRM stimulation just prior to tissue extraction, as well as in non-injured controls. We also analyzed the role of the 5-HT7 receptor, by pretreatment with the antagonist drug pimozide. This is the predominant serotonin receptor subtype in the spinal cord with a stimulatory effect on adenylate cyclase. Our results generally support the proposition that an increase in serotonergic NRM activity enhances restoration of the injured spinal cord, and also, somewhat more surprisingly, show a generalized spinal response to local injury.

2. Results

2.1. *Effect of NRM stimulation on cAMP after T8 SCI or pimozide pretreatment*

Mean cAMP concentration was measured in the spinal cords of rats with or without 3-day old injuries, some receiving NRM stimulation and some receiving an inactive implant. After SCI, cAMP values in cervical, thoracic and lumbar segments were found to be considerably lower than in corresponding non-injured tissue (Fig. 1). Post hoc comparisons against non-injured, non-stimulated controls showed significance at $p<0.01$ (Fig. 1) in the 4 rats from each group that provided an initial batch of cAMP measurements, so an additional batch was not analyzed. Stimulation of the serotonergic NRM for 2 h restored cAMP levels in all three regions to roughly normal. However, this stimulation caused little change in cAMP levels in non-injured rats (Fig. 1).

Pimozide, a 5-HT7 antagonist, was administered 1 h prior to the start of stimulation to uninjured rats; as before, there were 4 rats in the two groups (with or without stimulation). Pimozide produced a clear reduction in cAMP in all segments. Specifically, in lumbar and cervical segments, cAMP levels following pimozide pretreatment were approximately those measured in rats with untreated SCI (Fig. 1), with somewhat less of a fall in thoracic segments. Stimulation of the NRM following administration of pimozide had no effect, so that cAMP levels remained significantly lower than in non-injured controls. Both non-stimulated and stimulated groups after pimozide treatment were significantly different from non-injured, non-stimulated controls in post hoc comparison (Fig. 1). Preliminary observations that this dose of pimozide given immediately after moderate contusion at C5 caused fatal inanition within a few days ($n=3$) while survival of controls was adequate (>90%) led us to omit testing

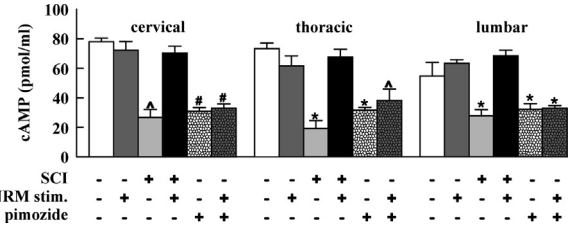

Fig. 1 – Intracellular cAMP levels 3 days after moderate thoracic (T8) contusion or in non-injured controls, showing effects of NRM stimulation, pimozide pretreatment or both. In each of the 18 groups (3 regions ×6 treatments), there were 4 rats. Marks indicate comparison with normal controls (no SCI, no stimulation) in pairwise Bonferroni contrasts for each region: #$p<0.05$, ^$p<0.01$, *$p<0.001$.