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Regeneration of sensory but not motor axons following visceral nerve injury

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ABSTRACT

Following peripheral nerve injury, restoration of function may occur via the regeneration of injured axons or compensatory sprouting of spared axons. Injury to visceral nerves that control urogenital organs is a common consequence of pelvic surgery, however their capacity to reinnervate organs is poorly understood. To determine if and how sensory and motor connections to the bladder are re-established, a novel surgical model of visceral nerve injury was performed unilaterally in adult male Wistar rats. Bladder-projecting motor and sensory neurons in pelvic ganglia and lumbosacral dorsal root ganglia, respectively, were identified and characterised by retrograde tracing and immunofluorescence. Application of tracers ipsi- and contralateral to injury distinguished the projection pathways of new connections in the bladder. In naive animals, the majority of sensory and motor neurons project ipsilaterally to the bladder, while ~20 % project contralaterally and ~5 % bilaterally. Injured axons of motor neurons were unable to regenerate by 4 weeks after transection. In contrast, by this time many injured sensory neurons regrew axons to reform a substantial plexus within the detrusor and suburothelial tissues. These regeneration responses were also indicated by upregulation of activating transcription factor-3 (ATF-3), which was sustained in motor neurons but transient in sensory bladder-projecting neurons. Axotomy had little or no effect on the survival of bladder-projecting sensory and motor neurons. We also found evidence that uninjured motor and sensory neurons develop additional projections to the denervated bladder tissue and return connectivity, likely by undergoing compensatory growth. In conclusion, our results show that visceral sensory and motor neurons have a different capacity to regenerate axons following axotomy, however in both components of the circuit uninjured bladder neurons spontaneously grow new axon collaterals to replace the lost terminal field within the organ. For a full functional recovery, understanding the environmental and cellular mechanisms that reduce the ability of pelvic ganglion cells to undergo axonal regeneration is needed.

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Introduction

Peripheral nerve injury studies have extensively investigated the effects of axotomy on somatic nerves that innervate targets such as skin and skeletal muscle (Bloechlinger et al., 2004; Groves et al., 1997; Shin et al., 2014; Tandrup et al., 2000). However, comparatively little is known of the responses and molecular events that occur following injury to visceral nerves that innervate urogenital organs. Damage to visceral nerves is difficult to avoid during pelvic surgery, such as prostatectomy and resection of bowel tumours (Lange et al., 2008; Nishizawa et al., 2011; Wallner et al., 2008). Such damage invariably leads to bladder, bowel and/or sexual dysfunction because autonomic neurons that innervate urogenital organs are located in a network of ganglia (the inferior hypogastric plexus) that are closely apposed to the surgical site. This plexus also forms a conduit for sensory nerves that innervate pelvic organs, such that injury often has widespread effects on neural regulation of urogenital function (Keast, 2006). Understanding the impact of injury on these visceral nerves is essential for developing treatments that are neuroprotective and promote repair.

The anatomy of sensory and autonomic innervation of urogenital organs is well defined in rodents (Keast, 2006; Keast and de Groat, 1989). The major pelvic ganglia (PG), functionally equivalent to the inferior hypogastric plexus in humans, contain a mixture of parasympathetic and sympathetic neurons that provide motor innervation to the bladder and other urogenital organs. Sensory neurons that innervate the bladder have somata in lumbosacral dorsal root ganglia (DRG), and project via the pelvic ganglia to their targets. Therefore, damage to nerve bundles that project from the PG to the bladder invariably injures both motor and sensory axons. The first aim of our study was to establish an experimental model of visceral nerve injury that allows assessment of the response of both sensory and motor neurons to injury. We performed unilateral transection of bladder nerves that contain both sensory and motor axons, while axons that project from neurons

Abbreviations: ATF-3, activating transcription factor-3; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion/ganglia; GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF family receptor alpha 1; IB-4, isolectin B4; NF200, neurofilament 200; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; PG, pelvic ganglion/ganglia; PGP 9.5, protein gene product 9.5; TH, tyrosine hydroxylase.

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in spared ganglia remain uninjured. This model of bladder nerve injury therefore allows the assessment of the innate capacity of injured and spared sensory and motor axons to reinnervate bladder tissues. Recovery of both motor and sensory innervation is essential to restore normal voiding and continence behaviour.

Following peripheral nerve injury, reinnervation may occur via the regeneration of injured axons or compensatory growth of spared axons (Cobianchi et al., 2014; Navarro et al., 2007). Most studies of pelvic visceral nerve injury focus on strategies to understand and improve the response of penis-projecting motor neurons after prostatectomy (Albersen et al., 2012; Canguven and Burnett, 2008). Rodent studies show that the cavernous (penile) nerve, which predominantly contains pro-erectile parasympathetic axons, has a limited capacity to regenerate following transection (Carrier et al., 1995; Kato et al., 2003), although axons regrow slowly after crush (Nangle and Keast, 2007). Following an incomplete (unilateral) injury, reinnervation of cavernosal tissue may also occur by the compensatory growth of spared axons (Carrier et al., 1995; Nangle and Keast, 2007). The growth of collaterals from neurons in the spared pelvic ganglion occurs in parallel with an increase in expression of growth-associated-protein 43 (GAP43), a marker of neuron regeneration and plasticity (Kato et al., 2003). This collateral growth from spared neurons likely contributes to the restoration of function (Nangle and Keast, 2007). Therefore, the second aim of this study was to determine how neural connections are restored to the bladder, whether by the regeneration of injured axons, the compensatory growth of spared axons, or both. This aim was addressed by applying retrograde tracer to the bladder ipsi- and contralateral to injury following unilateral transection of the bladder nerves. A separate analysis of injured and spared neurons that project to the bladder allowed the direct assessment of regenerative and compensatory axonal growth. This was combined with analysis of sensory and motor innervation within tissues on each side of the bladder.

Injured peripheral neurons undergo numerous changes to switch to a regenerative phenotype. This involves upregulation of early response transcription factors and growth-associated genes, protein synthesis and cytoskeletal reassembly to form a growth cone, axonal elongation and the re-establishment of synaptic contact to target tissue (Bradke et al., 2012; Navarro et al., 2007; Scheib and Hoke, 2013). The basic leucine zipper transcription factor, activating transcription factor-3 (ATF-3), has regenerative and anti-apoptotic roles in axotomised neurons (Francis et al., 2004; Seijffers et al., 2006). Viral vector delivery of ATF-3 increases neurite outgrowth of cultured DRG neurons (Seijffers et al., 2006) and prevents kainic acid-induced apoptosis of hippocampal neurons in vivo (Francis et al., 2004). Furthermore, ATF-3 is widely regarded as a marker of neuronal injury as it is rapidly upregulated in all injured DRG neurons following sciatic nerve transection, and downregulated after axons reinnervate their target (Tsujino et al., 2000). However, the expression profile of this regenerative marker in injured bladder sensory and motor neurons is unknown. The early response transcription factor, c-Jun, is also recognized as a functional marker of axonal injury (Herdegen et al., 1997). Recent evidence suggests that c-Jun expression is also associated with injury-independent 'sprouting,' and is upregulated in spared neurons that are undergoing compensatory growth. For example, deafferentation of pelvic ganglion motor neurons stimulates the upregulation of c-Jun and extensive local sprouting of axon collaterals, even though these neurons themselves are not injured (Nangle and Keast, 2009; Uvelius and Kanje, 2010). Furthermore, genetic deletion of c-Jun in CNS neurons reduces the degree of perineuronal sprouting following facial nerve injury (Raivich et al., 2004). Therefore, as the regeneration and compensatory growth of axons is indicated by the expression of ATF-3 and c-Jun, the third aim of this study was to determine the expression of these injury markers within injured and spared motor and sensory neurons innervating the bladder following unilateral transection of bladder sensory and motor nerves.

Death of injured peripheral neurons that supply somatic targets is well described and recognized as a major contributor to poor functional recovery (Scheib and Hoke, 2013; Terenghi et al., 2011). The majority of axotomy-induced apoptosis occurs between 1 day and 2 weeks following injury, with a 10–40 % loss of DRG neurons detected by 2 weeks following sciatic nerve injury (Groves et al., 1997; McKay Hart et al., 2002; Vestergaard et al., 1997). The severity of loss depends on where the injury occurs, and the nature of the injury, in that proximal transection of the sciatic nerve invokes a 3 fold higher loss of DRG neurons compared to the loss seen following a distal injury (Ygge, 1989). Ventral root avulsion causes a severe 70 % loss of somatic motor and preganglionic parasympathetic neurons (Hoang et al., 2006). The impact of injury on survival of pelvic visceral sensory and motor neurons has not, to our knowledge, been directly examined. Preventing the loss of injured neurons is a key therapeutic strategy for improving functional outcome. Therefore the final aim of this study was to determine whether visceral neurons survive axotomy.

Materials and methods

Animals and surgical procedures

All experiments were approved by the Animal Ethics Committee of the University of Melbourne, and complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia). Male Wistar rats (6 weeks; Animal Resource Centre, Perth, Australia) were housed in groups of 3, under a 12 hour light–dark cycle, with *ad libitum* access to water and standard chow.

All surgical procedures were conducted under isoflurane anaesthesia (3 % induction, 2–2.5 maintenance in 1.5–2 % oxygen). For post-operative care, animals were administered Temgesic (buprenorphine 0.05 mg/kg, subcutaneous) and Terramycin (oxytetracycline 10 mg/kg, intramuscular) immediately following surgery, and another dose of Temgesic 8 h later. Pelvic ganglia (PG) in male rats lie on the dorsolateral aspect of the prostate gland. A bundle of nerves exit the ventromedial aspect of the major pelvic ganglion and project to the lower urinary tract and reproductive organs (Keast, 2006). A small cluster of microganglia (accessory ganglia) is embedded along some of these projections and are considered as an extension of the major PG (Keast et al., 1989). Therefore this study refers to these projections as "accessory nerves". In order to study the effects of axotomy on bladder-projecting neurons, unilateral injury of the accessory nerves was performed under conditions described previously (Nangle and Keast, 2009). In brief, the lower abdominal cavity was opened and pelvic organs exposed. Accessory nerves that project ventrally towards the bladder were identified, isolated from underlying prostate tissue with fine forceps and cut with iris scissors at approximately 1 mm from the ganglion. Resection of the accessory nerve was not necessary as upon transection the proximal and distal nerve stumps retracted by approximately 2–3 mm. Transection of the accessory nerves was predicted to axotomise the majority of bladder-projecting neurons residing in lumbosacral DRG and PG. To standardise the injury and ensure that all branches of the accessory nerves were cut, injury was performed and retrograde tracer dye applied immediately to the same side of the bladder ($n = 3$). Ganglia were dissected 1 week following injury and numbers of retrogradely labelled neurons counted. Care was taken to avoid damaging blood vessels, although some bleeding could not be avoided because some micro-vessels are embedded with the accessory nerves. Abdominal muscle and skin were sutured and post-operative care administered.

Retrograde tracing

The neural connectivity of the bladder in naive animals ($n = 4$) was assessed by applying retrograde tracers FluoroGold (FG) and Fast Blue (FB) to opposite sides of the bladder under anaesthesia as described

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