Experimental Neurology 312 (2019) 10–19

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Sonic hedgehog regulation of cavernous nerve regeneration and neurite formation in aged pelvic plexus

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ARTICLE INFO

Keywords:
Neurite
Aging
Cavernous nerve regeneration
Prostatectomy
Sonic hedgehog

ABSTRACT

Introduction: Erectile dysfunction (ED) is a significant health concern that greatly impacts quality of life, and is common in men as they age, impacting 52% of men between the ages of 40 and 70. A significant underlying cause of ED development is injury to the cavernous nerve (CN), a peripheral nerve that innervates the penis. CN injury also occurs in up to 82% of prostatectomy patients. We recently showed that Sonic hedgehog (SHH) protein delivered by peptide amphiphile (PA) nanofiber hydrogel to the CN and penis of a prostatectomy model of CN injury, is neuroprotective, accelerates CN regeneration, improves erectile function ~60%, preserves penile smooth muscle 56% and suppresses collagen deposition 30%. This regenerative potential is substantial in an adult prostatectomy model (P120). However prostatectomy patients are typically older (61.5 ± 9.6 years) and our models should mimic patient conditions more effectively when considering translation. In this study we examine regenerative potential in an aged prostatectomy model (P200–329).

Methods: The caudal portion of the pelvic ganglia (MPG) and CN were dissected from adult (n = 11), and aged (n = 13) Sprague Dawley rats, and were grown in organ culture 3 days. Uninjured and 2 day CN crushed MPG/CN were exposed to Affi-Gel beads containing SHH protein, PBS (control), or 5e1 SHH inhibitor. Neurites were quantified by counting the number of growth cones normalized by tissue perimeter (mm) and immunohistochemistry for SHH, patched1 (PTCH1), smoothened (SMO), GLI1-3, and GAP43 were performed.

Results: SHH treatment increased neurites 3.5-fold, in uninjured adult, and 5.7-fold in aged rats. Two days after CN crush, SHH treatment increased neurites 1.8-fold in adult rats and 2.5-fold in aged rats. SHH inhibition inhibited neurite formation in uninjured MPG/CN but not in 2 day CN crushed MPG/CN. PTCH1 and SMO (SHH receptors), and SHH transcriptional activators/repressors, GLI1-3, were abundant in aged MPG/CN with unaltered localization. ROCK1 was induced with SHH treatment.

Conclusions: Reintroduction of SHH protein in an aged prostatectomy model is even more effective in promoting neurite formation/CN regeneration than in the adult. The first 48 h after CN injury are a critical window when growth factors are released, that impact later neurite formation. These studies are significant because most prostatectomy patients are not young and healthy, as with adult rats, so the aged prostatectomy model will more accurately simulate ED patient response. Understanding how neurite formation changes with age is critical for clinical translation of SHH PA to prostatectomy patients.

1. Introduction

Erectile dysfunction (ED) is a significant health concern that greatly impacts quality of life, and is common in men as they age, impacting 52% of men between the ages of 40 and 70 (Feldman et al., 1994) and 22% of men under 40 (Heruti et al., 2004). Current treatments, including PDE5i, are ineffective in 61–69% of patients with peripheral neuropathy of the cavernous nerve (CN), which provides innervation to

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https://doi.org/10.1016/j.expneurol.2018.11.001
Received 7 August 2018; Received in revised form 27 September 2018; Accepted 1 November 2018
Available online 02 November 2018
0014-4886/ Published by Elsevier Inc.

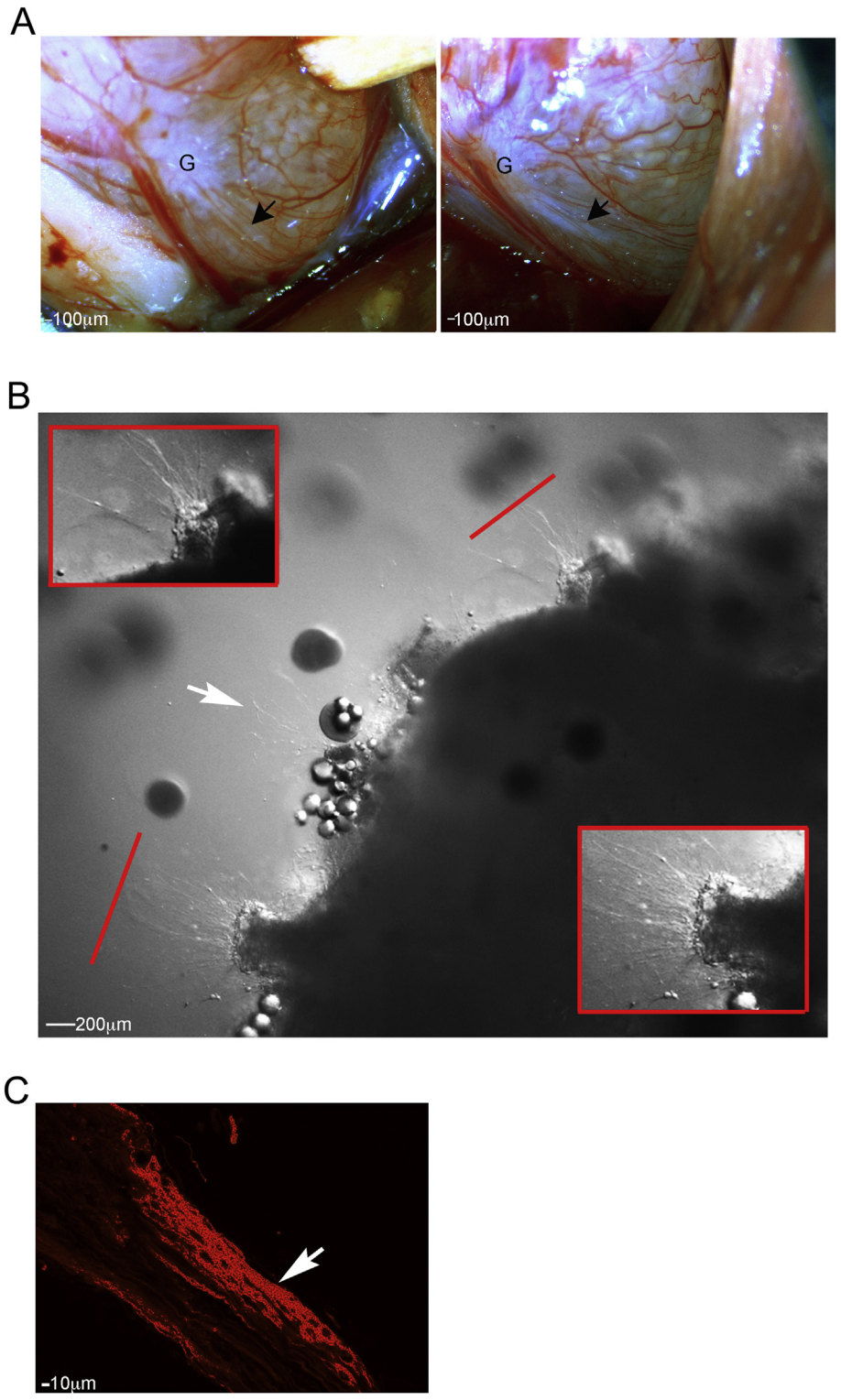


Fig. 1. (A) Photos of the pelvic plexus from adult (P115–120) and aged (P200–329) Sprague Dawley rats. The CN is flat and thin in the adult rat with a small, clearly defined MPG. In the aged rat, the CN is thicker and rounder in appearance, with a larger, less well-defined MPG. The vascular supply does not appear diminished with age. (B) Magnified view of MPG treated with SHH protein, shows growing neurites with clearly visible growth cones at the tips and elongating fibers. (C) Neurites were confirmed with GAP43 (growth cones marker). 100-800× magnification.

the penis (Pace et al., 2010; Perimenis et al., 2002). Injury to the CN and associated major pelvic ganglia (MPG) occurs during radical prostatectomy surgery to treat prostate cancer, in diabetic patients, and in aging patients. When the CN is injured (crush, tension, resection injury), this causes extensive down stream remodeling in the corpora cavernosa of the penis. With loss of innervation smooth muscle undergoes abundant and intensive apoptosis in the first week after injury in rodent models (User et al., 2003; Angeloni et al., 2013a) followed by collagen induction (Choe et al., 2016), making the tissue unable to respond to normal neurotransmitter (nitric oxide) signaling mechanisms, and ED is the end result. Parallel changes in corpora cavernosal remodeling have been documented in prostatectomy and

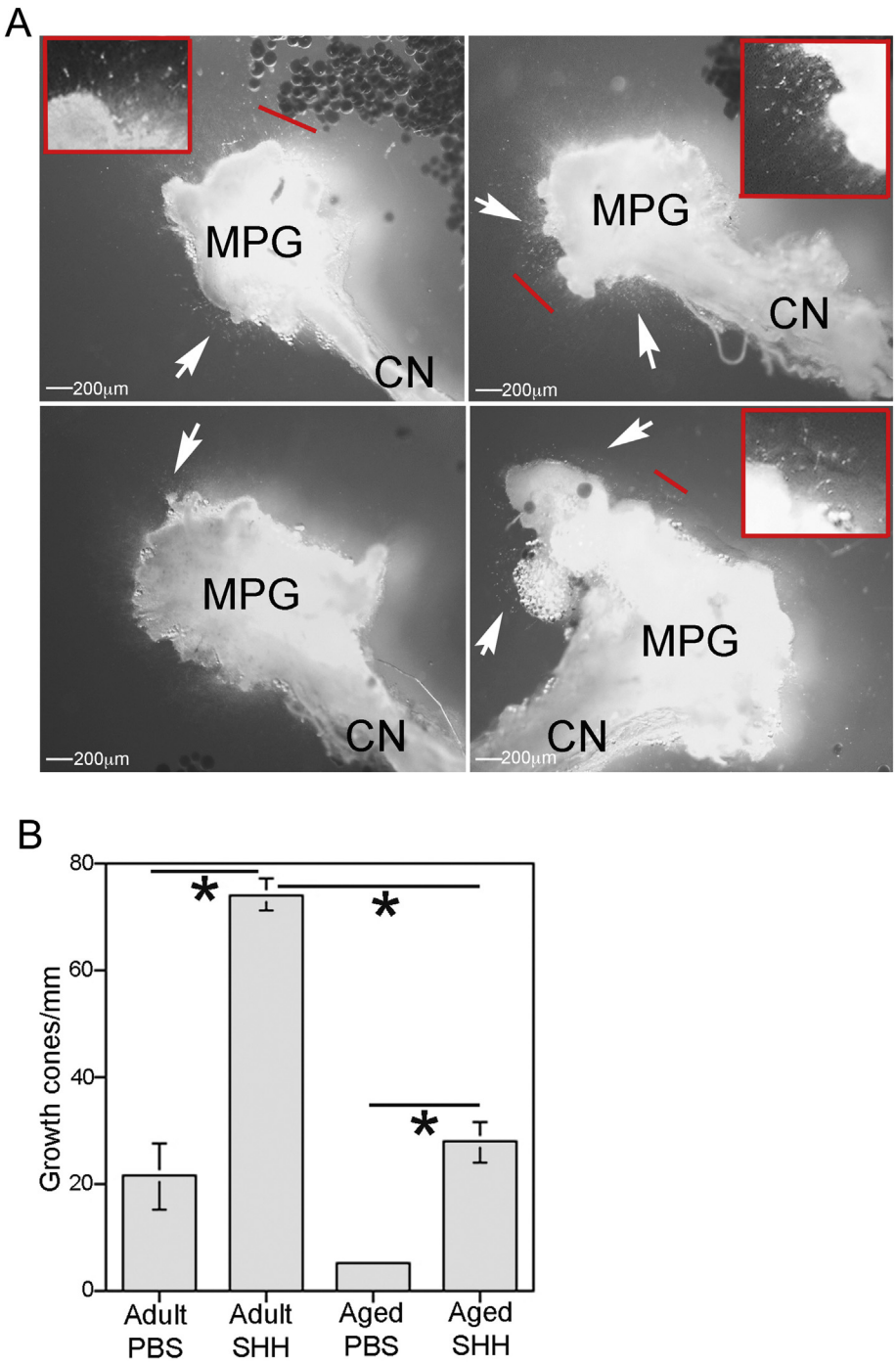
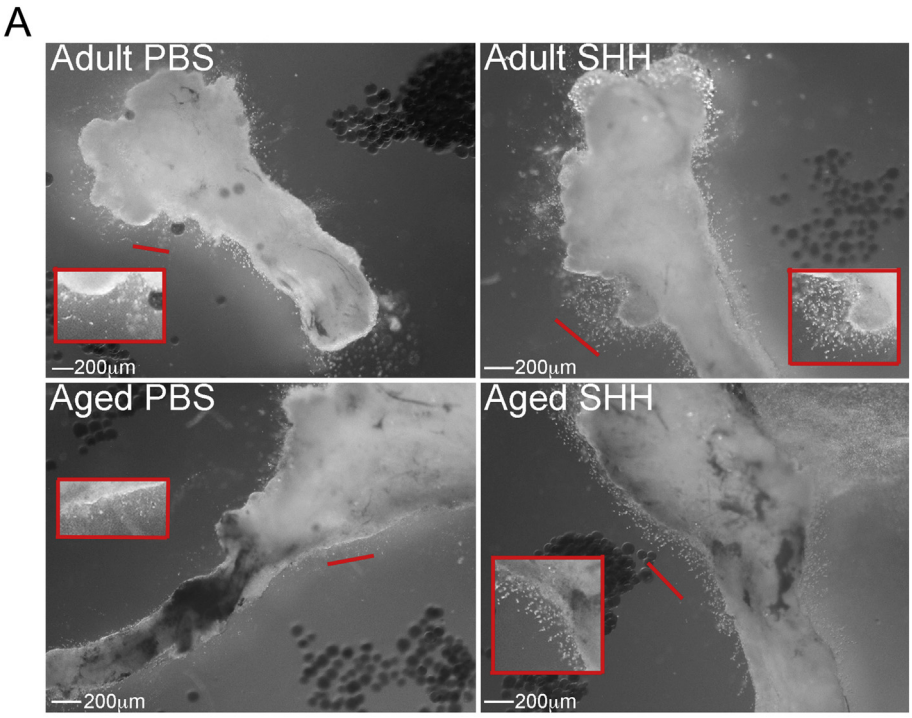


Fig. 2. Uninjured adult and aged MPG were grown in organ culture for 3 days with PBS (control) or SHH protein and neurites were quantified (A and B). The number of neurites/mm increased 3.5 fold with SHH treatment in adult rats (249%, $p = 0.0001$). The number of neurites/mm increased 5.7-fold with SHH treatment in aged rats (468%, $p = 0.013$). There was no difference in neurite formation in adult and aged rats treated with PBS (control, $p = 0.085$). SHH treatment was 2.7-fold less in aged rats in comparison to SHH treated adult rats (63%, $p = 0.001$). 800× magnification.

diabetic patients with ED (Angeloni et al., 2013b), although the remodeling process takes place over a longer time so there is a greater window of opportunity for intervention.

Regeneration of the CN is a primary goal in prostatectomy patients, with up to 85% of patients suffering ED after surgery (Kendirci and Hellstrom, 2004; Emanu et al., 2016). Some factors are known to be decreased in the MPG/CN with CN injury, such as nNOS, neurturin, glial cell line-derived neurotrophic factor alpha2, artemin, neurotrophin-4, and cilliary neurotrophic factor (7 days after CN resection) (Hlaing et al., 2013). Several other factors have been implicated to play a role in neurite outgrowth, a key regulatory step in regeneration of peripheral nerves. BDNF and VEGF promote neurite outgrowth from cultured MPG neurons (Lin et al., 2010), as does SHH (Dobbs et al., 2018). The SHH pathway is essential for embryonic development of many organs and its continuing role in adult tissues is less well defined. SHH is abundantly expressed in normal adult urogenital organs including the penis and MPG/CN tissue that provides innervation (Podlasek et al., 2003). SHH protein is abundantly expressed in MPG neurons and glia (Angeloni et al., 2013a; Choe et al., 2017), and SHH protein is decreased within a day in the MPG and CN of adult rats with



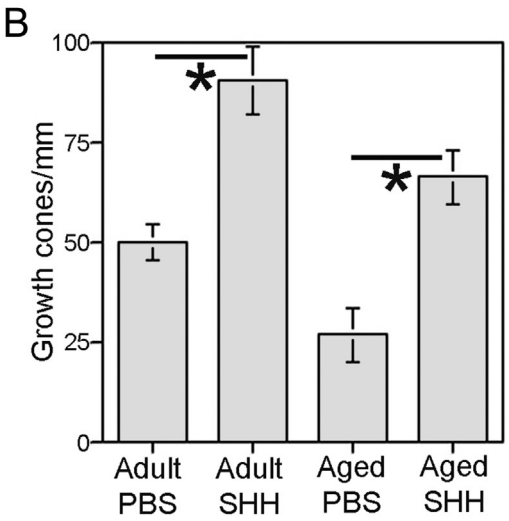


Fig. 3. Adult and aged MPG that underwent CN crush and were isolated after two days were grown in organ culture for 3 days with PBS (control) or SHH protein, and neurites were quantified (A and B). The number of neurites/mm increased 1.8 fold with SHH treatment in adult rats (82%, $p = 0.044$). The number of neurites/mm increased 2.5-fold with SHH treatment in aged rats (150%, $p = 0.030$). There was no difference in neurite formation in adult and aged rats treated with PBS (control, $p = 0.298$) or with SHH protein ($p = 0.197$). Red line indicates enlarged region. 800× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CN crush injury (Angeloni et al., 2013a). SHH treatment of cultured MPG/CN that were either uninjured or CN crushed, caused abundant neurite outgrowth of penile projecting neurons [Dobbs et al., 2018]. This finding is supported by our previous studies in which *in vivo* SHH treatment with peptide amphiphile nanofiber hydrogels after CN injury was neuroprotective, promoted CN regeneration, and improved erectile function ~60% 6 weeks after CN injury (Angeloni et al., 2013a; 2011).

Factors that are upregulated or delivered in the first two days after CN injury have a profound effect on later sprouting potential (Dobbs et al., 2018). Aging also greatly impacts the ability of the MPG neurons to support neurite outgrowth. Neurite growth from cultured MPGs derived from aged rats was not as robust as it was from MPGs from younger rats [L12], and neurite outgrowth in response to BDNF and VEGF was more robust in MPGs derived from young rats (6 months) than from aged rats (24 months) (Lin et al., 2010). GFRα2 and nNOS mRNA expression levels in RT-PCR showed age-related decreases in 1–24 month old rats, and *in situ* hybridization showed that the number of GFRα2 positive neurons in MPG decreased with aging (Hisasue et al., 2006). Morphology changes have also been observed in aged rat MPG including neuronal vacuolar degeneration with preserved nuclei (Golomb et al., 2001). In humans, pathological changes were identified after prostatectomy in pelvic plexus neurons including neuronophagia, neuron cell vacuolization, satellite cells vacuolization, cell pyknosis, and nageotte nodules. A number of these changes were increased with age (Rath-Wolfson et al., 2017). In other organs such as skeletal muscle, SHH pathway signaling is impaired in aged mice with decreased up-regulation of the pathway in response to injury (Piccioni et al., 2014; Renault et al., 2013; Lauth, 2014). We've shown that SHH protein decreases with age in the MPG/CN, with the precursor protein decreasing 37% and the active form 77% (Angeloni et al., 2013a). Since SHH signaling is important to maintain the architecture of the CN (Angeloni et al., 2011), increased age might affect not only cavernous tissue but also the neural plasticity of the CN related to erectile function (Hisasue et al., 2006).

The regenerative potential of SHH delivered by peptide amphiphile nanofiber hydrogel is promising and substantial in MPG/CN of an adult

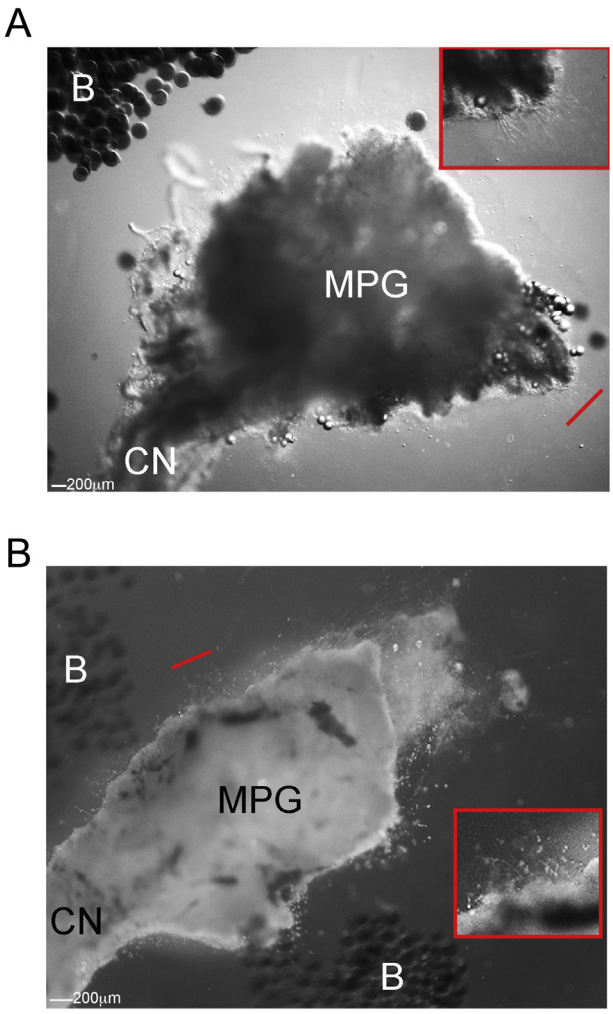


Fig. 4. Uninjured (A) and 2 day CN crushed (B) aged MPG/CN were grown in organ culture for three days with Affi-Gel beads delivering 5E1 SHH inhibitor. SHH inhibition of uninjured MPG/CN had neurite formation in only one small region away from the Affi-Gel beads containing SHH inhibitor (A). In MPG that underwent CN crush and then were cultured after 2 days, SHH inhibition did not decrease neurite formation (B). 800× magnification.

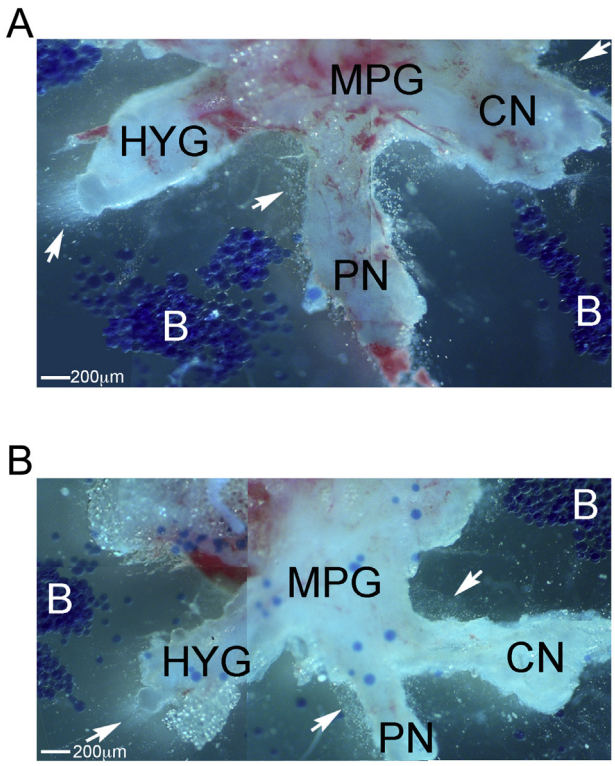


Fig. 5. MPG from aged Sprague Dawley rats that underwent CN crush/MPG tension injury were dissected after two days and grown in organ culture for three days with PBS (A) or SHH protein (B). Aged rat MPG responded to CN injury with neurite formation in the CN, and also in the pelvic nerve and hypogastric nerve, which innervate the bladder (A). Aged pelvic plexus were responsive to SHH treatment (B). 800× magnification.

prostatectomy model (P120). However a P120 rat is comparable to a 20 year-old man, which is unlikely to develop ED. This model is state of the art in the ED field as most investigators study younger rats that have not finished penile development, and yet their findings are being considered equivalent to observations in ED patients. This occurs because of the higher cost and difficulty obtaining older rats, and no other investigators have performed penile postnatal development studies (Podlasek et al., 2003; Hisasue et al., 2006; Bond et al., 2010), so may not be aware of the model limitations. In our tissue bank, the average ED patient age ranges from 52 to 71 years with an average of 61.5 ± 9.6 years. This is consistent with average ED patient ages reported in the literature (Feldman et al., 1994) and is equivalent to 1–2 rat years. It is important and innovative to accurately simulate ED patient conditions in our animal models to obtain clinically relevant information.

Thus we examine SHH pathway signing in an aged prostatectomy model that more accurately mimics ED patient conditions, and hypothesize that if the SHH receptors are intact in aged MPG/CN, that reintroduction of SHH protein to MPG/CN in an aged prostatectomy model would be more effective in promoting CN regeneration (neurite outgrowth) than in the adult. We will examine this in organ culture of uninjured, CN crushed, and SHH inhibited MPG/CN.

2. Materials and methods

2.1. Animals

Adult Sprague-Dawley rats postnatal day 115–120 (P115-P120, n = 11) and aged Sprague Dawley rats (P200-P329, n = 13) were obtained from Charles River (Wilmington, MA). The study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal care protocol was approved by the Office of Animal Care and Institutional Biosafety at the University of Illinois at Chicago and animals were cared for in accordance with institutional approval.

2.2. CN crush surgery

MPG/CN were exposed and microforceps (size 0.02 × 0.06 mm) were used to bilaterally crush the CN 30 s. This is a commonly used method of performing CN crush in rats (Mullerad et al., 2006; Nangle and Keast, 2007) and the extent and reproducibility of crush injury were previously validated in our laboratory (Angeloni et al., 2011), with a visible indent and change in color of the nerve apparent with crush injury. CN crushed rats were sacrificed after 2 days and were grown in organ culture.

2.3. Organ culture

The MPG and attached CN were dissected from adult and aged Sprague Dawley rats. Only the caudal portion of the MPG, which innervates the penis was included in the culture. The pelvic, hypogastric and ancillary nerves, and the regions of the MPG that innervate the bladder, rectum and prostate, were excluded from culture. MPG/CN

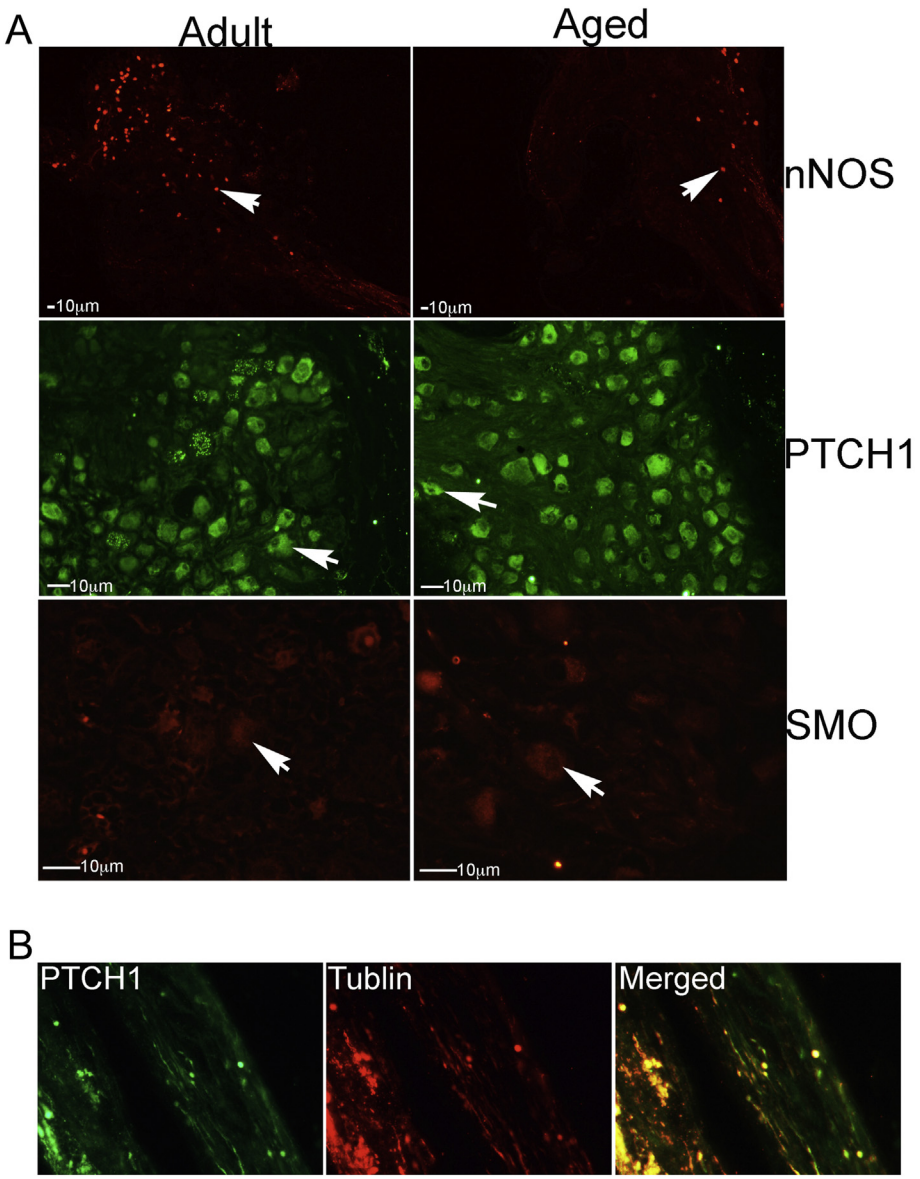


Fig. 6. IHC analysis was performed in uninjured adult and aged rat MPG/CN that was grown in organ culture for 3 days. (A) In aged rats, nNOS was less abundant by visual observation in MPG neurons that innervate the penis. The SHH receptors, PTCH1 and SMO, were identified in adult and aged rat MPG neurons that innervate the penis, with no apparent difference with age. (B) PTCH1, the binding part of the SHHH receptor, was identified in growing neurites, as confirmed with dual staining for β–III tublin. Arrows indicate protein. 100-1000× magnification.

were placed in sterile culture plates containing 150 μl of reduced growth factor Matrigel (Corning Life Sciences 356,231). Reduced growth factor Matrigel was used for this study so that the Martigel did not influence neurite formation. Affi-Gel beads (100–200 mesh, Bio Rad) were incubated overnight at 4 °C with 1XPBS, SHH protein (25 μl of a 1 μg/μl solution, R&D Systems), or 5E1 SHH inhibitor (388 μg/ml, Hybridoma Bank), and were placed on top of the Matrigel near the MPG/CN. Matrigel was gelled at 37 °C for 5 min prior to adding RPMI media (Sigma) and an antibiotic cocktail containing Penicillin-Streptomycin-Glutamine (100×, Thermo Fisher Scientific). Culture plates were placed in an atmosphere controlled incubator (5% CO_2) at 37 °C for three days. Groups were uninjured adult and aged MPG/CN treated with PBS (control, n = 6), SHH protein (n = 6) and 5E1 SHH inhibitor (n = 2), and CN crushed MPG/CN from adult and aged rats treated with PBS (n = 9), SHH protein (n = 6), and 5E1 SHH inhibitor (n = 2). Additional aged rat MPG/CN including the hypogastric and pelvic nerves, underwent CN crush and after 2 days *in vivo*, were grown in organ culture with PBS (n = 2) or SHH protein (n = 2) as outlined above.

2.4. Quantification of neurites

Neurites were quantified by counting the total number of growth cones visible for each tissue grown in organ culture by a blinded observer at 160× magnification, normalized by tissue perimeter length (mm). Photos of the MPG/CN were magnified on the computer screen until growth cones were visible, and growth cones in the entire tissue were counted and the perimeter was measured. Three to five photos for each tissue were counted and averaged. Images were obtained using an Olympus SZ-CTV dissecting microscope and Axiocam R1.2 Carl Zeiss digital camera. Representative photos for each group are presented in the figures.

2.5. Immunohistochemical analysis (IHC)

IHC was performed on frozen MPG/CN which were cut to 12 μm

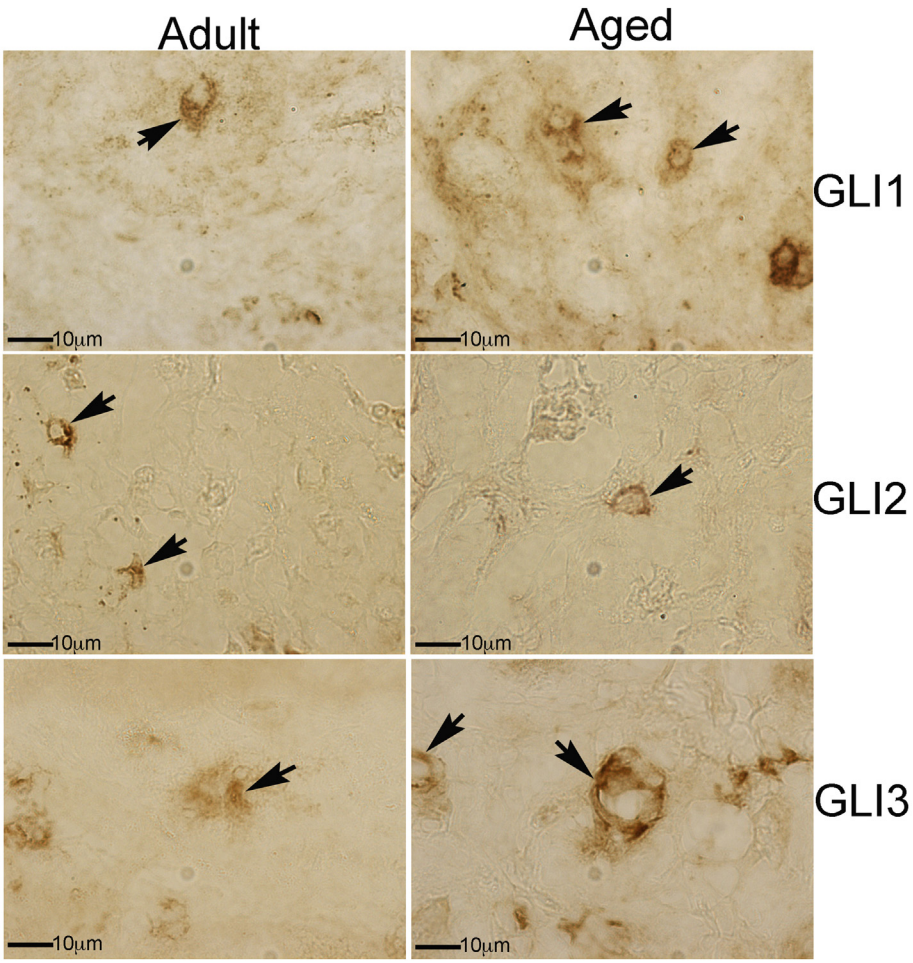


Fig. 7. IHC analysis was performed on uninjured adult and aged rat MPG/CN that was grown in organ culture for 3 days and were assayed for GLI1-3. All three GLI isoforms were identified in glial cells surrounding MPG neurons of adult and aged rats. Arrows indicate GLI1-3 proteins. 1000× magnification.

thickness and were post fixed in acetone for 15 min at 4 °C. OCT was removed with 1× PBS washes prior to blocking with 3% milk in PBS at 4 °C for one hour. Sections were incubated overnight at 4 °C with 1/100 goat polyclonal antibody against patched (PTCH1) and GLI1 (Santa Cruz), rabbit polyclonal antibody against smoothened (SMO, LSBio), GLI2 and GLI3 (Rockland), and mouse monoclonal antibodies against neuronal nitric oxide synthase (nNOS, Transduction Laboratories), β–III tublin (Abcam), growth associated protein 43 (GAP43, Chemicon), Rho-activated serine/threonine kinase (ROCK1, Transduction) and ROCK2 (Santa Cruz). Fluorescent secondary antibodies were chicken anti-goat 488, chicken anti-rabbit 594, and donkey anti-mouse 594 (Molecular Probes, 1/150). HRP secondary antibodies were 1/150 donkey anti-goat (Sigma-Aldrich), and mouse anti-rabbit (Santa Cruz). Control slides in which the primary antibody was omitted, were performed for all secondary antibodies, to ensure artifact staining was not present from the secondary antibodies. Sections were mounted using DPX Mounting media (Electron Microscopy Sciences, Hatfield, PA). Microscopy was performed using a Leica DM2500 microscope.

2.6. Statistical analysis

Statistical analysis was performed by ANOVA with a Scheffe's posthoc test using the SPSS program. The results were reported ± the standard error of the mean. Results were significantly different if $p \leq 0.05$.

3. Results

3.1. Anatomy of the pelvic plexus

Photos were taken of the pelvic plexus from adult (P115–120) and aged (P200–329) Sprague Dawley rats (Fig. 1A). The pelvic plexus was identified. The CN appears flat and thin in the adult rat with a small, clearly defined MPG (Fig. 1A). There is extensive vascular supply accompanying the neural tissue. In the aged rat, the CN is thicker and rounder in appearance (Fig. 1A). The MPG is larger and less well defined. The vascular supply does not appear diminished with age.

3.2. Neurite formation in uninjured MPG/CN

Organ culture was performed on MPG/CN taken from uninjured Sprague Dawley rats and tissues were treated with SHH. A highly magnified view of the MPG shows growing neurites with clearly visible growth cones at the tips and elongating fibers (Fig. 1B, arrows indicate visible growth cones). In order to confirm the presence of neurites, we performed GAP43 (growth cones marker) immunohistochemical analysis. GAP43 was identified in elongating neurites of the CN (Fig. 1C).

3.3. Comparison of neurite formation in uninjured/normal adult and aged rat MPG

Uninjured/normal adult and aged MPG were grown in organ culture for 3 days with PBS (control) or SHH protein and neurites were quantified by counting the number of growth cones/mm of tissue. The

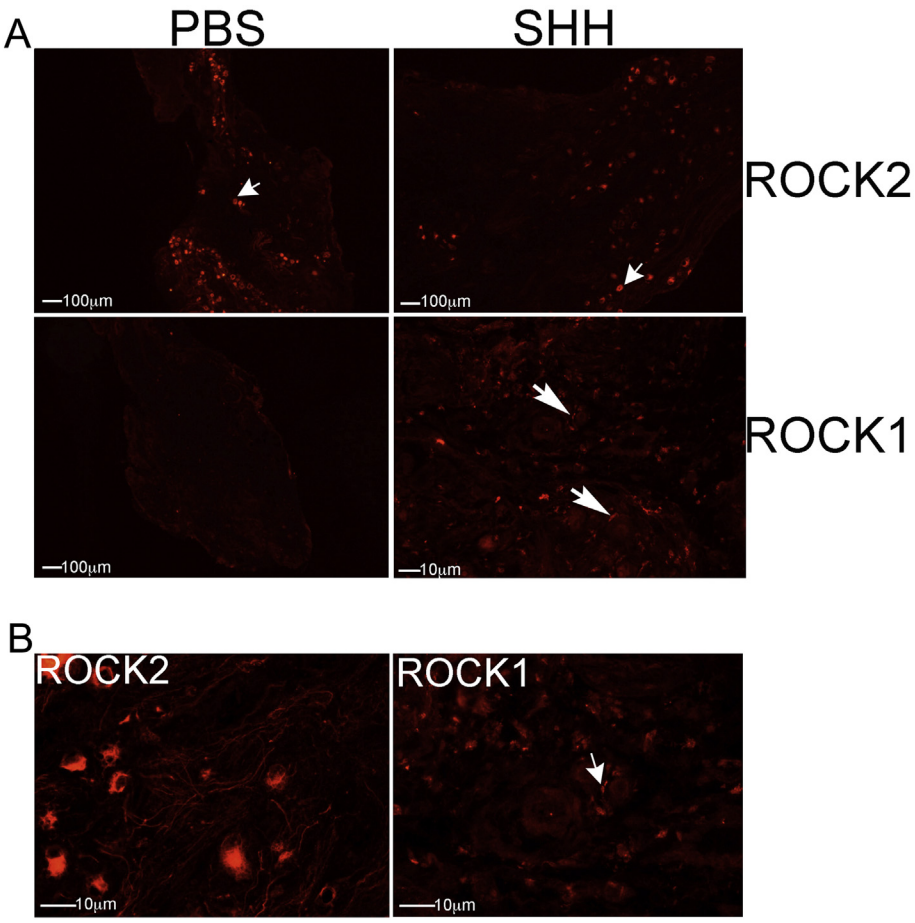


Fig. 8. IHC analysis was performed on uninjured MPG/CN from aged rats that were treated with PBS or SHH protein. ROCK2 was abundant in uninjured MPG/CN treated with PBS and SHH (A). ROCK1 was not identified in uninjured MPG/CN (A), however ROCK1 was induced in response to SHH treatment (A). ROCK2 was localized in MPG neurons, while ROCK1 was present in associated glial cells (B). 50-400× magnification.

number of neurites/mm increased 3.5 fold with SHH treatment in adult rats (249%, p = 0.0001, Fig. 2A and B). The number of neurites/mm increased 5.7-fold with SHH treatment in aged rats (468%, p = 0.013, Fig. 2A and B). There was no difference in neurite formation in adult and aged rats treated with PBS (control, p = 0.085, Fig. 2A and B). SHH treatment was 2.7-fold less in aged rats in comparison to SHH treated adult rats (63%, p = 0.001, Fig. 2A and B).

3.4. Comparison of neurite formation in 2 day CN crushed adult and aged rat MPG

Adult and aged MPG that underwent CN crush were isolated after two days and were grown in organ culture for 3 days with PBS (control) or SHH protein and neurites were quantified. The number of neurites/mm increased 1.8 fold with SHH treatment in adult rats (82%, p = 0.044, Fig. 3A and B). The number of neurites/mm increased 2.5-fold with SHH treatment in aged rats (150%, p = 0.030, Fig. 3A and B). There was no difference in neurite formation in adult and aged rats treated with PBS (control, p = 0.298) or SHH (p = 0.0.197, Fig. 3A and B).

3.5. SHH inhibition suppressed neurite formation

Uninjured aged MPG/CN were grown in organ culture for three days with Affi-Gel beads delivering 5E1 SHH inhibitor or PBS control. SHH inhibition of uninjured MPG/CN had neurite formation in only one small portion away from the Affi-Gel beads containing SHH inhibitor (Fig. 4A). In MPG that underwent CN crush and then were cultured after 2 days, SHH inhibition did not decrease neurite formation (Fig. 4B), indicating that the first 48 h after CN injury are a critical window when growth factors, including SHH, are released, that impact later neurite formation.

3.6. Neurite formation occurs in all nerves of the pelvic plexus

MPG from aged Sprague Dawley rats that underwent CN crush/MPG tension injury were dissected after two days and grown in organ culture for three days with PBS or SHH protein. Aged rat MPG responded to CN injury with neurite formation in the CN, and also in the pelvic nerve and hypogastric nerve, which innervate the bladder (Fig. 5A). Aged pelvic plexus were responsive to SHH treatment (Fig. 5B).

3.7. Immunohistochemical analysis of MPG neurons

IHC analysis was performed on uninjured adult and aged rat MPG that was grown in organ culture for 3 days. In aged rats, nNOS was less abundant by visual observation in MPG neurons that innervate the penis (Fig. 6A). This is in keeping with previous observations in the literature. The SHH receptors, PTCH1 and SMO, are abundant in adult and aged rat MPG neurons that innervate the penis, with no apparent difference with age (Fig. 6A). PTCH1, the binding part of the SHHH receptor, was identified in growing neurites (Fig. 6B). This was confirmed with dual staining for β–III tublin, which is present in elongating neurites (Fig. 6B) (Paden et al., 1995). GLI, the transcriptional activator of the SHH pathway, has three isoforms which were all identified in glial cells surrounding MPG neurons of adult and aged rats (Fig. 7), indicating that the SHH pathway is active in cultured MPG/CN.

G-coupled proteins have been implicated as targets of SHH signaling in other organs. ROCK2 was abundant in uninjured MPG/CN treated with PBS and SHH (Fig. 8A). ROCK1 was not identified in uninjured MPG/CN (Fig. 8A), however ROCK1 was induced in response to SHH treatment (Fig. 8A). ROCK2 was localized in MPG neurons, while

ROCK1 was present in associated glial cells (Fig. 8B).

4. Discussion

Little is known about the role of SHH signaling in the development and adult maintenance of peripheral nerves, such as the CN. MPG neurons more than doubled between birth and adulthood (Yan and Keast, 2008) and the adult number of neurons was achieved in the MPG/CN by P7 for sympathetic neurons, and P21 for parasympathetic neurons. Much more is known about the role of SHH signaling in the central nervous system than the peripheral nerves. During early development, Shh induces the differentiation of dopaminergic neurons (Zhou et al., 2016), and Shh, and its receptors, PTCH1 and SMO, are expressed in postnatal and adult hippocampal neurons (Mitchell et al., 2012). Shh signaling regulates the structure and functional properties of presynaptic terminals of hippocampal neurons (Mitchell et al., 2012). Shh and its receptors were expressed in adult dorsal root ganglia neurons, axons and glia and trended toward higher levels following axotomy injury (Martinez et al., 2015). In our studies of the MPG/CN, we have shown that SHH is abundantly expressed in penile projecting neurons and associated glia of the MPG/CN, SHH inhibition causes axonal degradation of CN fibers, SHH protein is upregulated in Schwann cells that surround the injury site in the CN to facilitate regeneration (Bond et al., 2008), there is decreased precursor and active SHH protein in the MPG/CN with CN injury (Podlasek et al., 2007), and decreased SHH protein with age in the MPG/CN (precursor protein decreasing 37% and the active form 77%) (Angeloni et al., 2011).

While the importance of Hh signaling in embryonic development is undisputed, the requirement for pathway activity in the adult organism is less well defined (Lauth, 2014). Hh signaling is activated in situations of tissue repair, a process considered less effective in older individuals (Lauth, 2014). In other tissues such as skeletal muscle, the SHH pathway is postnatally recapitulated after injury and during regeneration to regulate angiogenesis and myogenesis (Piccioni et al., 2014). Hh signaling and the subsequent improvement in angiogenesis and myogenesis are impaired in aged mice and can be improved by introduction of exogenous Hh ligands (Lauth, 2014). Total Hh activity or its inducibility upon damage was significantly dampened in older animals, contributing to an overall phenotype with less regenerative capability (Lauth, 2014). In organ culture of both uninjured and CN crushed MPG/CN, we observed decreased neurite formation with age (Figs. 2 and 3). CN injury increased neurite formation in both young and aged rat MPG/CN. Surprisingly, we identified that SHH treatment was more effective in promoting neurite formation in both uninjured and CN crushed MPG/CN of aged rats (1.6 and 1.4-fold increased with age) in comparison to adults (Figs. 2 and 3). This is likely because the signaling machinery (PTCH1, SMO and GLI) are intact in aged rats, it is just the overall level of SHH protein that is decreased in MPG/CN (Angeloni et al., 2013a). In ischemic muscle repair in old mice, delay in repair was associated with an impaired upregulation of Gli1 (Renault et al., 2013), and SMO expression was significantly down regulated (Renault et al., 2013). While a detailed quantitative analysis of the MPG/CN is not possible because of the minute tissue size ($< 100\,\mu$), we did not observe a difference in localization or apparent abundance of PTCH1 and SMO on visual observation of sections of the MPG/CN cultures, despite nNOS being clearly less abundant in the aged rat MPG/CN (Fig. 6). The increased effectiveness of SHH treatment in promoting neurite formation in aged MPG/CN suggests that the nanofiber hydrogel delivery of SHH that we have previously developed for adult CN regeneration (Angeloni et al., 2011), will likely be even more effective in our aged prostatectomy model.

We previously showed that SHH, PTCH1 and SMO proteins are abundantly expressed in both MPG neurons and associated glial cells, with SHH mRNA only expressed in neurons by *in situ* hybridization (Angeloni et al., 2013a). Whereas GLI1-3, the transcriptional activators and repressors of the SHH pathway, are exclusively expressed in satellite glial cells surrounding penile projecting neurons of the MPG. This suggests that the glial cells are the target of SHH signaling from the associated neurons. Glial cells play a significant role in maintenance of MPG neurons and during regeneration. Glial cells are active partners in neuronal communication (Gallina et al., 2012). Bidirectional signaling selectively occurs between specific subpopulations of glia, neurons, and synapses (Pace et al., 2010) and is important for neuronal function. Local conditions influence how initial regenerative axon sprouts emerge from parent axons (Podlasek et al., 2007), while CN injury triggers a cascade of events in MPG neurons, including changes in expression of neurotransmitters, neurotrophic factors, cytokine production, and SHH pathway signaling (User et al., 2003; Angeloni et al., 2013a; Kendirci and Hellstrom, 2004). Since glial cells undergo apoptosis prior to neuronal apoptosis following CN injury (Hehemann et al., 2018), they appear to be more sensitive to change in the MPG microenvironment.

The processes that are set into motion after CN injury are complex with a defined time frame for Wallerian degeneration. Separation of proximal and distal ends of the injury site occurs within 30 min (Kerschensteiner et al., 2005) and degeneration of the distal segment begins within 24–36 h (Wang et al., 2015). Neurites initiate from the proximal end of damaged axons within 4 days (Sunderland, 1978; Lundborg et al., 1994). In uninjured MPG/CN cultured with SHH inhibitor, neurite outgrowth was inhibited (Fig. 4). However if the CN was crushed and the tissue left *in vivo* for two days, later SHH inhibition did not affect neurite outgrowth (Fig. 4). This finding suggests that the first 48 h are an important window after CN injury in which growth factors are released that later impact neurite outgrowth. Ideally a therapy to regenerate the CN should be applied immediately in this early window when it has the opportunity to significantly impact later neurite outgrowth. Two days after CN injury, the process of neurite formation has already been set in motion and inhibiting SHH at this time cannot reverse the already primed neurite sprouting process.

ROCK1 and ROCK2 do not always have the same functions, and their activation can be isoform-specific (Hartmann et al., 2015). We show that ROCK2 is normally expressed in MPG/CN, however ROCK1 is not normally expressed in cultured MPG/CN, but was up-regulated in response to SHH protein treatment (Fig. 8). Depending on their subcellular localization, activation, and other environmental factors, ROCK signaling can have different effects on cellular function (Hartmann et al., 2015). We observed different localization for ROCK1 and ROCK2 with ROCK2 present in MPG neurons and ROCK1 identified in associated glial cells. This is consistent with glial cells being the target of SHH produced in MPG neurons since GLI1-3, the transcriptional activators and repressor of the pathway, are expressed in glial cells (Fig. 7). Non-canonical signaling of SHH involving Rho/ROCK has been suggested in other systems, including angiogenesis of endothelial cells (Renault et al., 2010), in astrocytes (He et al., 2013), and in microparticles carrying SHH (Soleti et al., 2009). The function of ROCK1 in this system requires further future investigation.

5. Conclusions

Reintroduction of SHH protein in an aged prostatectomy model is even more effective in promoting neurite formation/CN regeneration than in the adult. The first 48 h after CN injury are a critical window when growth factors are released, that impact later neurite formation. These studies are significant because most prostatectomy patients are not young and healthy, as with adult rats, so the aged prostatectomy model will more accurately simulate erectile dysfunction patient response. Understanding how neurite formation changes with age is critical for clinical translation of SHH to prostatectomy patients.

Summary sentence

Reintroduction of sonic hedgehog protein in an aged prostatectomy model is even more effective in promoting neurite formation/cavernous

nerve regeneration than in the adult. These studies are significant because most prostatectomy patients are not young and healthy, as with adult rats, so the aged prostatectomy model will more accurately simulate erectile dysfunction patient response. Understanding how neurite formation changes with age is critical for clinical translation of sonic hedgehog delivery to prostatectomy patients.

Funding

NIH/NIDDKDK101536.

Declaration of interest

Declarations of interest include iEDISON 0577703-18-0007, -0008, -0009.

Acknowledgments

This project was supported by NIH/NIDDK Award number R01DK101536. Cell culture equipment was provided by the laboratory of Gail Prins, Ph.D.

References

Angeloni, N.L., Bond, C.W., Tang, Y., Harrington, D.A., Zhang, S., Stupp, S.I., McKenna, K.E., Podlasek, C.A., 2011. Regeneration of the cavernous nerve by Sonic hedgehog using aligned peptide amphiphile nanofibers. Biomaterials 32, 1091–1101.

Angeloni, N., Bond, C.W., Harrington, D., Stupp, S., Podlasek, C.A., 2013a. Sonic hedgehog is neuroprotective in the cavernous nerve with rush injury. J. Sex. Med. 10, 1240–1250.

Angeloni, N.L., Bond, C.W., McVary, K.T., Podlasek, C.A., 2013b. Sonic hedgehog protein is decreased and penile morphology is altered in prostatectomy and diabetic patients. PLoS ONE 8, e70985.

Bond, C., Tang, Y., Podlasek, C.A., 2008. Neural influences on sonic hedgehog and apoptosis in the rat penis. Biol. Reprod. 78, 947–956.

Bond, C.W., Angeloni, N.L., Podlasek, C.A., 2010. Analysis of testosterone effects on sonic hedgehog signaling in juvenile, adolescent and adult Sprague dawley rat penis. J. Sex. Med. 7, 1116–1125.

Choe, S., Veliceasa, D., Bond, C.W., Harrington, D.A., Stupp, S.I., McVary, K.T., Podlasek, C.A., 2016. Sonic hedgehog delivery from self-assembled nanofiber hydrogels reduces the fibrotic response in models of erectile dysfunction. Acta Biomater. 32, 89–99.

Choe, S., Bond, C.W., Harrington, D.A., Stupp, S.I., McVary, K.T., Podlasek, C.A., 2017. Peptide amphiphile nanofiber hydrogel delivery of sonic hedgehog protein to the cavernous nerve to promote regeneration and prevent erectile dysfunction. Nanomedicine 13, 95–101.

Dobbs, R., Choe, S., Kalmanek, E., Harrington, D.A., Stupp, S.I., McVary, K.T., Podlasek, C.A., 2018. Peptide amphiphile delivery of sonic hedgehog protein promotes neurite formation in penile projecting neurons. Nanomedicine 14, 2087–2094.

Emanu, J.C., Avildsen, I.K., Nelson, C.J., 2016. Erectile dysfunction after radical prostatectomy: prevalence, medical treatments, and psychosocial interventions. Curr. Opin. Support Palliat Care 10, 102–107.

Feldman, H.A., Goldstein, I., Hatzichristou, D.G., Krane, R.J., McKinlay, J.B., 1994. Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. J. Urol. 151, 54–61.

Gallina, A., Ferrari, M., Suardi, N., Capitanio, U., Abdollah, F., Tutolo, M., Bianchi, M., Sacca, A., Salonia, A., Rigatti, P., Montorsi, F., Briganti, A., 2012. Erectile function outcome after bilateral nerve sparing radical prostatectomy: which patients may be left untreated? J. Sex. Med. 9, 903–908.

Golomb, E., Scolnik, M., Koren, R., Servadio, C., Sandbank, U., Abramovici, A., 2001. Effects of senescence and citral on neuronal vacuolar degeneration in rat pelvic ganglia. Neurotoxicology 22, 73–77.

Hartmann, S., Ridley, A., Lutz, S., 2015. The function of Rho-Associated kinases ROCK1 and ROCK2 in the pathogenesis of cardiovascular disease. Front. Pharmacol. 6, 276.

He, Q.W., Xia, X.P., Chen, S.C., Wang, Y., Huang, M., Huang, Y., Li, J.Y., Li, Y.N., Gao, Y., Mao, L., Mei, Y.W., Hu, B., 2013. Astrocyte-derived sonic hedgehog contributes to angiogenesis in brain microvascular endothelial cells via RhoA/ROCK pathway after oxygen-glucose deprivation. Mol. Neurobiol. 47, 976–987.

Hehemann, M., Choe, S., Kalmanek, E., Harrington, D., Stupp, S.I., McVary, K.T., Podlasek, C.A., 2018. Pelvic and hypogastric nerves are injured in a rat prostatectomymodel, contributing to the development of stress urinary incontinence. Sci. Rep (November 6, in press).

Heruti, R., Shochat, T., Tekes-Manova, D., Ashkenazi, I., 2004. Justo D (2004) Prevalence of erectile dysfunction among young adults: results of a large-scale survey. J. Sex. Med. 1, 284–291.

Hisasue, S., Kato, R., Suetomi, T., Kato, K., Suzuki, K., Kobayashi, K., Itoh, N., Kiyama, H., Tsukamoto, T., 2006. Age-related alteration of neurturin receptor GFRa2 and nNOS in pelvic ganglia. Neurobiol. Aging 27, 124–1530.

Hlaing, S.M., Garcia, L.A., Kovanecz, I., Martinez, R.A., Shah, S., Artaza, J.N., Ferrini, M.G., 2013. Sildenafil promotes neuroprotection of the pelvic ganglia neurons after bilateral cavernosal nerve resection in the rat. BJU Int. 111, 159–170.

Kendirci, M., Hellstrom, W.J., 2004. Current concepts in the management of erectile dysfunction in men with prostate cancer. Clin. Prostate Cancer 3, 87–92.

Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., Misgeld, T., 2005. In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nat. Med. 11, 572–577.

Lauth, M., 2014. Sonic the Hedgehog: a game about aging? Emerging evidence for anti-geriatric effects of Hedgehog signaling. BioEssays 36, 1128.

Lin, G., Shindel, A.W., Fandel, T.M., Bella, A.J., Lin, C.S., Lue, T.F., 2010. Neurotrophic effects of brain-derived neurotrophic factor and vascular endothelial growth factor in major pelvic ganglia of young and aged rats. BJU Int. 105, 114–120.

Lundborg, G., Dahlin, L., Danielsen, N., Zhao, Q., 1994. Trophism, trophism, and specificity in nerve regeneration. J. Reconstr. Microsurg. 10, 345–354.

Martinez, J.A., Kobayashi, M., Krishnan, A., Webber, C., Christie, K., Guo, G., Singh, V., Zochodne, D.W., 2015. Intrinsic facilitation of adult peripheral nerve regeneration by the sonic hedgehog morphogen. Exp. Neurol. 271, 493–505.

Mitchell, N., Petralia, R.S., Currier, D.G., Wang, Y.X., Kim, A., Mattson, M.P., Yal, P.J., 2012. Sonic hedgehog regulates presynaptic terminal size, ultrastructure and function in hippocampal neurons. J. Cell Sci. 125, 4207–4213.

Mullerad, M., Donohue, J.F., Li, P.S., Scardino, P.T., Mulhall, J.P., 2006. Functional sequelae of cavernous nerve injury in the rat: is there model dependency. J. Sex. Med. 3, 77–83.

Nangle, M.R., Keast, J.R., 2007. Reduced efficacy of nitrergic neurotransmission exacerbates erectile dysfunction after penile nerve injury despite axonal regeneration. Exp. Neurol. 207, 30–41.

Pace, G., Del Rosso, A., Vicentini, C., 2010. Penile rehabilitation therapy following radical prostatectomy. Disabil. Rehabil. 32, 1204–1208.

Paden, C.M., Zhou, X., Watt, J.A., Burton, R., Pickett, J., Oblinger, M.M., 1995. Coordinated upregulation of alpha 1- and beta II-tubulin mRNAS during collateral axonal sprouting of central peptidergic neurons. J. Neurosci. Res. 42, 402–412.

Perimenis, P., Markou, S., Gyftopoulos, K., Athanasopoulos, A., Giannitsas, K., Barbalias, G., 2002. Switching from long-term treatment with self-injections to oral sildenafil in diabetic patients with severe erectile dysfunction. Eur. Urol. 41, 387–391.

Piccioni, A., Gaetani, E., Neri, V., Gatto, I., Palladino, M., Silver, M., Smith, R.C., Giarretta, I., Pola, E., Hkatky, L., Pola, R., 2014. Sonic hedgehog therapy in a mouse model of age-associated impairment of skeletal muscle regeneration. J. Genontol. A Biol. Sci. Med. Sci. 69, 245–252.

Podlasek, C.A., Zelner, D.J., Jiang, H.B., Tang, Y., Houston, J., McKenna, K.E., McVary, K.T., 2003. Sonic hedgehog cascade is required for penile postnatal morphogenesis, differentiation, and adult homeostasis. Biol. Reprod. 68, 423–438.

Podlasek, C.A., Meroz, C.L., Tang, Y., McKenna, K.E., McVary, K.T., 2007. Regulation of cavernous nerve injury-induced apoptosis by sonic hedgehog. Biol. Reprod. 76, 19–28.

Rath-Wolfson, L., Shvero, A., Bubis, G., Buzaverov, G., Zeidman, A., Ran, E., Koren, R., 2017. Morphological changes in peri-prostatic sympathetic ganglion cells in aging males. Mol. Clin. Oncol. 6, 713–717.

Renault, M.A., Roncalli, J., Tongers, J., Thorne, T., Klyachko, E., Misener, S., Volpert, O.V., Mehta, S., Burg, A., Luedemann, C., Qin, G., Kishore, R., Losordo, D.W., 2010. Sonic hedgehog induces angiogenesis via Rho kinase-dependent signaling in endothelial cells. J. Mol. Cell. Cardiol. 49, 490–498.

Renault, M.A., Robbesyn, F., Chapouly, C., Yao, Q., Vandierdonck, S., Reynaud, A., Belloc, I., Traiffort, E., Ruat, M., Desgranges, C., Gadeau, A.P., 2013. Hedgehog-dependent regulation of angiogenesis and myogenesis is impaired in aged mice. Arterioscler. Thromb. Vasc. Biol. 33, 2858–2866.

Soleti, R., Benameur, T., Porro, C., Panaro, M.A., Andriansitohaina, R., Martinez, M.C., 2009. Microparticles harboring sonic hedgehog promote angiogenesis through the upregulation of adhesion proteins and proangiogenic factors. Carcinogenesis 30, 580–588.

Sunderland, S., 1978. Nerves and Nerve Injuries. Churchill Livingstone, Edinburgh.

User, H.M., Hairston, J.H., Zelner, D.J., McKenna, K.E., McVary, K.T., 2003. Penile weight and cell subtype specific changes in a post-radical prostatectomy model of erectile dysfunction. J. Urol. 169, 1175–1179.

Wang, L., Sanford, M.T., Xin, Z., Lin, G., Lue, T.F., 2015. Role of Schwann cells in the regeneration of penile and peripheral nerves. Asian J. Androl. 17, 776–782.

Yan, H., Keast, J.R., 2008. Neurturin regulates postnatal differentiation of parasympathetic pelvic ganglion neurons, initial axonal projections and maintenance of terminal fields in male urogenital organs. J. Comp. Neurol. 507, 1169–1183.

Zhou, X., Pace, J., Filichia, E., Davis, B., Hoffer, B., Selman, W., Luo, Y., 2016. Effect of the sonic hedgehog receptor smoothened on the survival and function of dopaminergic neuros. Exp. Neurol. 283, 235–245.