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Peptide amphiphile delivery of sonic hedgehog protein promotes neurite formation in penile projecting neurons

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Abstract

Erectile dysfunction (ED) critically impacts quality of life in prostatectomy, diabetic and aging patients. The underlying mechanism involves cavernous nerve (CN) damage, resulting in ED in 80% of prostatectomy patients. Peptide amphiphile (PA) nanofiber hydrogel delivery of sonic hedgehog (SHH) protein to the injured CN, improves erectile function by 60% at 6 weeks after injury, by an unknown mechanism. We hypothesize that SHH is a regulator of neurite formation. SHH treatment promoted extensive neurite formation in uninjured and crushed CNs, and SHH inhibition decreased neurites N80%. Most abundant neurites were observed with continuous SHH PA treatment of crushed CNs. Once induced with SHH, neurites continued to grow. SHH rescued neurite formation when not given immediately. SHH is a critical regulator of neurite formation in peripheral neurons under uninjured and regenerative conditions, and SHH PA treatment at the time of injury/prostatectomy provides an exploitable avenue for intervention to prevent ED.

Summary

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Significance statement: As a step towards clinical translation, we examined how peptide amphiphile nanofiber hydrogels delivering SHH protein to the CN at the time of crush injury (rat prostatectomy model) promote regeneration and improve erectile function. A key mechanism is SHH regulation of neurite formation in penile projecting neurons, which is critical for re-innervation and clinical translation. Development of peptide amphiphile SHH delivery has significant potential for high impact on future treatment of prostatectomy patients, and there is potential for intervention in the regenerative process even after immediate injury, if SHH treatment was not given immediately.

Conflict of interest: There is no conflict of interest for the authors of this work.

Summary sentence: Sonic hedgehog treatment promotes sprouting of neurons that innervate the penis, under normal and regenerative conditions.

Keywords

Neurite formation; Peripheral nerve regeneration; Schwann cells; Cavernous nerve injury; Pelvic ganglia; Sonic hedgehog; Peptide amphiphile nanofiber hydrogel

Peripheral neuropathy is common in diabetic and aging men and in prostate cancer patients, who undergo prostatectomy surgery to treat prostate cancer, resulting in loss of erectile function in up to 85% of patients.^{1,2} The cavernous nerve (CN), which provides innervation to the penis, is subject to crush, tension and resection injuries due to manipulation of the pelvic plexus to remove the prostate. State of the art treatments, including PDE5i, are ineffective in 61%–69% of erectile dysfunction (ED) patients with peripheral neuropathy,^{3,4} so development of novel treatments, which target the regenerative process, are sorely needed to improve clinical outcome. In previous studies we identified that sonic hedgehog (SHH) protein delivered by peptide amphiphile (PA) nanofiber hydrogel to the injured CN, improves erectile function by 60% at 6 weeks after injury in a rat prostatectomy model.⁵ The mechanism of how SHH treatment promotes peripheral nerve regeneration is unclear, but is critically important for translation to prostatectomy patients to prevent ED. In this study we examine the key regulatory role of the SHH pathway in promoting CN regeneration, by examining its function in Wallerian degeneration and neurite formation.

Peripheral nerve regeneration is a complex process, initiated with injury to the CN (Figure 1, A). Separation of proximal and distal ends of the injury site occurs within 30 min⁶ and degeneration of the distal segment begins within 24–36 h (Figure 1, B).⁷ The axonal skeleton and membrane break apart, activating Schwann cells to demyelinate, if myelinated, 8–10 proliferate,^{11,12} phagocytize the debris, and release cytokines that recruit macrophages to finish the clean up process.^{13,14} Neurites initiate from the proximal end of damaged axons within 4 days, and grow towards bands of Büngner, which are formed of aligned Schwann cells.^{15,16} Neurites are attracted by growth factors produced by Schwann cells.

We've shown in previous studies that SHH protein is abundant in Schwann cells (co-localize with S100, a Schwann cell marker), which migrate to the injury site when the CN is damaged.¹⁷ SHH is critical to maintain normal CN architecture, is neuroprotective and plays a significant role in CN regeneration.⁵ With SHH inhibition, demyelination of CN fibers and degeneration of non-myelinated fibers occur.⁵ CN injury decreases SHH protein in major pelvic ganglia (MPG) neurons that innervate the penis,^{18,19} and reintroduction of SHH protein by extended release PA hydrogels improves erectile function (60%), improves neural morphology, and suppresses penile apoptosis which occurs with loss of innervation.⁵ Part of the regenerative mechanism in response to SHH treatment involves maintaining neural–glial interactions.¹⁹ We've shown in previous studies by *in situ* hybridization, that *Shh* mRNA is synthesized in PG neurons, while immunohistochemical analysis identified SHH protein in both neurons and associated glial cells.¹⁸ Similarly the SHH receptor Patched protein was abundant in both neurons and glia, while Smoothened, the regulator of SHH pathway activity, was abundant in satellite glial cells, indicating the importance of the SHH pathway

for neuronal–glial interaction and regulation of the neuronal microenvironment. In other organs, components of the SHH pathway are expressed in human macrophages,²⁰ and Shh may act as a macrophage chemoattractant during initiation of gastritis in the stomach.²¹ These studies identify a role for the SHH pathway in several crucial steps of Wallerian degeneration that take place primarily at the distal segment of the crush injury. Since local conditions influence how neurites emerge from parent axons,²² and SHH is critical to maintain neural–glial interactions,¹⁹ we hypothesize that SHH is a key regulator of regenerative processes taking place at the proximal injury site, including neuronal sprouting/neurite formation. A role for SHH in neurite formation of MPG/CN neurons has not previously been investigated, and is critical to our understanding of how to facilitate peripheral nerve regeneration.

As a step towards clinical translation, we examine the mechanism of how PA SHH protein delivery to the CN at the time of crush injury enhances regeneration and improves erectile function. We show that SHH is critical for neurite formation in regenerating neurons. Development of PA SHH delivery has significant potential for high impact on future treatment of prostatectomy patients, and we show that there is potential for intervention in the regenerative process even after immediate injury, if SHH treatment was not given immediately. Understanding the mechanism of how SHH promotes CN regeneration *via* nanoparticle-based delivery, will allow us to fine tune the delivery for optimal regeneration.

Methods

Animals

Sprague–Dawley rats (n = 66) postnatal day 115–120 (P115–P120) 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.

CN crush surgery

MPG/CN were exposed and microforceps (size 0.02×0.06 mm) were used to crush the CN bilaterally for 30 s. This method of CN crush has commonly been used in the literature^{23,24} and the extent and reproducibility of crush injury were previously verified in our laboratory.⁵ CN crushed rats were sacrificed immediately (n = 3), at 2 (n = 3), 4 (n = 7) and 9 (n = 9) days after injury and were grown in organ culture.

Organ culture

The caudal portion of the MPG, which innervates the penis, and attached CN (n = 64), were identified and isolated. The pelvic, hypogastric and ancillary nerves, along with the adjacent regions of the MPG that innervate the bladder, rectum and prostate, were not included in the dissection. MPG/CN were embedded in sterile 6 well culture plates containing 150 μ l of reduced growth factor Matrigel (Corning Life Sciences 356,231). The type of Matrigel used for the study is critical since some high growth factor forms of Matrigel commonly used can

influence neurite formation. Affi-Gel beads (100–200 mesh, Bio Rad), incubated overnight at 4 °C with 1× PBS (n = 13), DMSO (n = 3), SHH protein (25 µl of a 1 µg/µl solution, R&D Systems, n = 12), 5E1 SHH inhibitor (Hybridoma Bank, 286 µg/ml, n = 6) or cyclopamine (10 µM, n = 6), were embedded in 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% CO₂) at 37 °C for three to five days.

Additional rats underwent bilateral CN crush, and MPG/CN tissues (n = 12) were isolated after three days, and were embedded in Matrigel as described above. Affi-Gel beads were used to deliver either 1× PBS (n = 4), or SHH protein (1 µg/µl, R&D Systems, n = 8). Neurite formation was examined after three days in culture.

Quantification of neurites

Quantification of neurites was performed 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 (representative photos are presented in the figures of this manuscript) were magnified on computer screen until growth cones were clearly visible, and all 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.

Quantification of neurite length was performed by measuring the length of neurites for each tissue grown in organ culture by a blinded observer at 160× magnification. Photos of the MPG/CN were magnified on computer screen until growth cones were clearly visible, and neurite length was measured at seven representative points for each tissue. Neurite length is reported as the average µ length per tissue.

Quantification of perimeter length over which neurites formed in each tissue was performed by a blinded observer at 160× magnification. Photos of the PMG/CN were magnified on computer screen until growth cones were visible, and the distance in cm along the tissue perimeter was measured in which sprouts formed for each tissue. Average perimeter length over which neurites formed was reported in cm.

Bilateral CN crush surgery with SHH or MSA protein treatment by PA

(C16)-V₂A₂E₂-(NH₂) PA was prepared as previously described.⁵ 20 mM CaCl₂ was added to a glass slide and 8 µl of 20 mM PA plus either 2.27 µg SHH or mouse serum albumin (MSA, control) proteins was pipetted onto the slide to form the PA. Bilateral CN crush was performed and PA containing the protein intercalated within the hydrogel, was transferred with forceps on top of the crushed CNs. The release rate of SHH protein from the PA was previously determined to be 90% by 75 h.⁵ MPG/CN were isolated 4 days after CN injury and were grown in organ culture for an additional 4 days with SHH or MSA PA. Groups were: Continuous SHH treatment (n = 6), continuous MSA (control) treatment (n = 5), SHH given initially and then withheld (n = 7), and SHH withheld initially for 4 days and then given *in vitro* (n = 5).

Immunohistochemical analysis (IHC)

Immunofluorescence, a form of immunohistochemical analysis that utilizes fluorescent secondary antibodies, was performed on frozen MPG/CN sections (14 μ m) from rats treated with PBS (control, $n = 5$), or SHH protein ($n = 5$) as described previously.⁴ Crushed CNs ($n = 3$) were also examined. Sections were incubated overnight at 4 °C with primary antibodies against neuronal nitric oxide synthase (nNOS, Transduction Laboratories), growth associated protein 43 (GAP43, Chemicon), or SHH (Santa Cruz, N-19 antibody). Secondary antibodies were 1/150 goat anti-mouse 594 or chicken anti-goat 488 (Molecular Probes). Sections were mounted using DPX Mounting media (Electron Microscopy Sciences) and fluorescence was visualized using a Leica DM2500 microscope.

Statistics

Where comparisons of three or more groups were made, ANOVA with a Scheffe's or Dunnett's post hoc test was performed using SPSS program. Where two groups were compared, a Levine test (SPSS) was first performed to ensure equal variance between groups, and then a t-test. The results were reported $\pm$ the standard error of the mean. Where there was unequal variance between groups, a Mann-Whitney U Test was performed and the results reported as the median with interquartile range. Results were considered significantly different if $P \leq 0.05$.

Results

SHH protein was abundant in crushed CN

SHH protein localization was examined by IHC analysis of CNs that underwent crush injury, and were sacrificed after 4 days ($n = 3$). SHH protein was abundant in cells on either side of the crush site (Figure 1, C). We previously confirmed SHH co-localization with S100 in Schwann cells of the CN that underwent tie placement, simulating a crush injury.¹⁸

Neurite formation in uninjured MPG/CN

Organ culture was performed on MPG/CN taken from uninjured Sprague Dawley rats and tissues were treated with PBS (control). A highly magnified view of a small portion of control MPG shows growing neurites with clearly visible growth cones at the tips and elongating fibers (Figure 2, A, arrows indicate visible growth cones). In order to confirm that neurites that are forming are from only the caudal portion of the MPG that provides innervation to the penis, nNOS IHC was performed. It has previously been well documented that nNOS is abundant in penile projecting neurons.²⁵ Since antibodies do not migrate well in intact tissue, such as our organ culture MPG/CN, we sectioned the tissue prior to IHC analysis. Many of the neurites break off with sectioning, however we were able to identify nNOS in elongating neurites of the CN and in growth cones of the MPG (Figure 2, B), indicating that they originate from penile projecting neurons. In order to confirm that nNOS protein was localized in growing neurites, we performed GAP43 (growth cones marker) staining on serial sections. GAP43 was abundant in elongating neurites of the CN and in growth cones of the MPG (Figure 2, C).

SHH protein promotes neurite formation in uninjured MPG/CN neurons

Organ culture was performed on MPG/CN taken from uninjured Sprague Dawley rats and tissues were treated with PBS (control) or SHH protein. Control MPG/CN showed limited sprouting from neurons in the caudal portion of the PG (region which innervates the penis) and CN (Figure 3, A). Arrows indicate growing neurites. SHH protein treatment caused abundant and extensive sprouting over a wide area (Figure 3, A). Neurites were quantified by counting the number of growth cones visible in highly enlarged photographs of entire MPG/CN tissues (Figure 3, A insert). This made the growth cones easily visible for counting. The number of sprouts increased 2.1-fold (114%, P value = 0.043), with SHH treatment (Figure 3, A and C). The average length of sprouts was also quantified in μ . SHH protein treatment increased the length of sprouts 1.8-fold (82%, PBS = 178.8 ± 47.2 , SHH = 326.1 ± 33.5 , P value = 0.02).

SHH treatment enhanced neurite formation in crushed CNs

Organ culture was performed on MPG/CN taken from Sprague Dawley rats that underwent bilateral CN crush and were isolated after three days and grown in organ culture with PBS or SHH protein delivered *via* Affi-Gel beads. Controls showed abundant neurite formation with CN injury (Figure 3, B). SHH protein treatment caused more abundant and extensive neurite formation. The number of neurites increased 1.5-fold (49%, P value = 0.013) with SHH treatment (Figure 3, D). Neurites were 1.4-fold longer (40%, red arrows) with SHH treatment (Median PBS = 216.3, interquartile range = 57.7, Median SHH = 303.6, interquartile range = 240.7, P = 0.048, Figure 3, B) and neurites appeared over a 1.8-fold (76%) wider area with SHH treatment (PBS = 3.74 ± 0.23 , SHH = 6.57 ± 0.57 , P = 0.003).

SHH inhibition suppressed neurite formation

Uninjured MPG/CN were grown in organ culture for three days with Affi-Gel beads delivering SHH inhibitors (cyclopamine or 5e1) or control treatments (DMSO, vehicle for cyclopamine, or PBS, Figure 4, A and B). Cyclopamine targets the smoothened part of the SHH receptor,^{26,27} while the 5e1 inhibitor prevents binding of SHH to Patched.²⁸ Both SHH inhibitors caused a reduction of neurites near the bead vehicles (Figure 4, A and B), with neurite formation reduced by 5.6-fold (82%, P value = 0.001) with cyclopamine, and 7.3-fold (86%, P value = 0.00003) with 5e1 antibody (Figure 4, C).

Neurite formation after CN injury

MPG/CN underwent CN crush and were isolated 0, 2, 4 and 9 days after injury and grown in organ culture for 4 days. Neurites were observed at all time points after injury, with most abundant neurite formation after 2 days (Figure 5). While less abundant, neurite formation was possible at later time points, with similar neurite sprouting potential observed at 4 and 9 days (Figure 5).

SHH treatment by PA promotes abundant neurite formation

Sprague Dawley rats underwent CN crush and were treated with SHH or MSA (control) protein *in vivo* for four days by PA. Isolated MPG/CN were grown in organ culture an additional four days with SHH or MSA protein delivered by PA. Little neurite formation was

observed with MSA treatment (Figure 6, A), while SHH treatment (Figure 6, B) induced intense neurite formation in a wide area, with neurites increasing by 4-fold (298%, P value = 0.0001). SHH protein was able to rescue neurite sprouting potential, with an increase of 2.8-fold (181%, P value = 0.0001) in the number of neurites, after being withheld initially (Figure 6, C). When SHH was given immediately, and then withheld, neurites increased 2.4-fold (141%, P value = 0.0001), and continued to grow once initiated (Figure 6, D).

Location of SHH delivery influences neurite formation

When SHH was delivered by PA to the MPG (Figure 7, A), neurites were abundant along the MPG and CN and neurites appeared longer in length, particularly along the severed end of the CN (Figure 7, B) in comparison to CN delivery (Figure 7, B).

Discussion

We show that SHH is an important regulator of neurite formation (axonal sprouting) of peripheral neurons, under uninjured homeostatic conditions and under regenerative conditions observed after prostatectomy surgery. Similar CN injury also occurs with aging, and with peripheral neuropathy in diabetic patients, and SHH may have parallel effects on neurite formation in these conditions. The number of neurites was increased with SHH treatment, as well as the length of neurites and the area over which sprouts form. Sprouting of neurons that innervate the penis was confirmed with identification of nNOS and GAP43 in growth cones and regenerating axons. The regulatory role of SHH in neurite formation, was confirmed with targeted inhibition of the SHH receptors, patched and smoothened,^{26–28} which severely reduced the number of neurites (>80%). The most robust neurite formation was observed with continuous SHH protein delivery by PA to the crushed CN, which impacted a wide area of MPG/CN, increasing neurites 298%, and sprouts appeared longer. Once neurites were induced with SHH treatment, they continued to grow when SHH was withdrawn. When SHH was withheld initially, and not given until four days after injury, neurite sprouting was rescued, with initiation of abundant neurites, though less robust than when SHH was given continuously. These studies identify SHH as an essential regulator of neurite formation in peripheral neurons under normal and regenerative conditions, and have important clinical implications for translation. SHH protein abundance decreases in the MPG/CN with CN injury,¹⁸ likely contributing to the poor regenerative response of the CN in prostatectomy and diabetic ED patients and animal models. Our results indicate that treatment of the MPG/CN with SHH protein at the time of CN injury/prostatectomy, would be beneficial to enhance neurite formation and regenerate the CN. This would have the added benefit of maintaining downstream morphology of the penis, which is irreversibly altered in response to loss of innervation.^{18,29} While SHH intervention has the most impact when given immediately with crush injury, it can be useful even at a later time to promote regeneration.

Neurite formation in uninjured MPG/CN was not abundant under the low growth factor conditions chosen for this study. This was intentional since growth factors commonly found in Matrigel, such as VEGF, may influence neurite formation, and compromise experimental conditions. Neurite formation was evaluated from 0 to 9 days after CN crush. Neurites were

minimal the day of crush injury, and was most abundant two days after injury, suggesting that early responding factors produced during this window of time are critical. At four and nine days after injury, neurites were consistently low, however SHH treatment could induce robust neurite formation at four days after CN injury (Figure 6, A and B). Our previous studies show that the active form of SHH protein decreases in the MPG/CN as early as one day after injury, and similar SHH protein levels were observed at 4 and 7 days.¹⁸ These studies suggest a window of time when optimal neurite formation can be induced with exogenous signals. These temporal relationships have therapeutic implications should SHH supplementation transition from bench to the bedside.

Location of SHH delivery may contribute to differences in neurite sprouting potential. SHH protein delivered *via* PA to the crush site, has previously been shown to undergo retrograde transport to MPG neurons that innervate the penis, where SHH maintains signaling between MPG neurons and glia, and prevents neuronal apoptosis.¹⁸ In this study when SHH was applied to the MPG, rather than the CN, neurites formed in a similar manner to CN delivery, however neurites were longer, particularly at the resected portion of the CN, where there was extensive branching. This suggests that SHH delivery to the MPG activates normal neurite sprouting mechanisms more readily so that neurites form more quickly, are longer, and more extensively branched. When thinking about translation to prostatectomy patients, both the CN and MPG are responsive to SHH induction of neurite sprouting, and a treatment program which includes both as SHH targets, would be beneficial to promote regeneration and preserve erectile function.

Conclusions

SHH is an important regulator of neurite formation in peripheral neurons, under uninjured and regenerative conditions observed after prostatectomy surgery, with aging, and with peripheral neuropathy in diabetic patients. Treatment of the MPG/CN with SHH protein at the time of CN injury, would be beneficial to enhance neurite formation and regenerate the CN. This would have the added benefit of maintaining down stream morphology of the penis, which is otherwise irreversibly altered in response to loss of innervation. While SHH intervention has the most impact when given immediately with crush injury, it can be useful even at a later time to promote regeneration.

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Abbreviations:

CN	Cavernous nerve
DMSO	Dimethyl sulfoxide

ED	Erectile dysfunction
GAP43	Growth Associate Protein 43
MPG	Major pelvic ganglia
MSA	Mouse serum albumin
nNOS	Neuronal nitric oxide synthase
PA	Peptide amphiphile
PBS	Phosphate buffered saline
PDE5i	Phosphodiesterase-5 inhibitor
RPMI medium	Roswell Park Memorial Institute
SHH	Sonic hedgehog
VEGF	Vascular endothelial growth factor

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Figure 1.

(A) Diagram of the MG and CN with crush injury. (B) Diagram of Wallerian degeneration in the CN in response to crush injury. The axonal skeleton and membrane break apart. Schwann cells proliferate, phagocytize the debris, and release cytokines that recruit macrophages. The proximal end of damaged axons attempts repair, which involves initial swelling and some retrograde degeneration, clearing of debris and axonal sprouting-neurite formation. Neurites grow towards bands of Büngner, which are formed of aligned Schwann cells. Neurites are attracted by growth factors produced by Schwann cells. (C) Immunohistochemical analysis for SHH protein in the CN four days after crush injury shows abundant SHH protein on either side of the crush site. Arrows indicate SHH protein. 200× magnification. MPG = major pelvic ganglia. CN = cavernous nerve.

Figure 2.

(A) Magnified view of neurites that grow from MPG and CN tissue in organ culture shows both elongating fibers and growth cones. Arrows indicate growth cones. Red line indicates enlarged region. (B) IHC analysis was performed for nNOS, a marker of penile projecting neurons, in order to confirm that dissection of the MPG only included the caudal portion of the MPG that provides innervation to the penis. Since antibodies do not migrate well within intact tissue, we sectioned the MPG/CN tissue prior to staining. nNOS was abundant in elongating neurites in the CN and in sprouting neurons of the MPG. Arrows indicate neurites and growth cones. (C) IHC for GAP43, a growth cone marker, was performed to confirm that what we are visualizing is neurite formation. Arrows indicate staining. Affi-Gel bead delivery vehicles, which were used to deliver PBS control, are high-lighted with asterisks, and non-specifically stain with fluorescent secondary. 100–200× magnification.

Figure 3.

Normal/uninjured MPG and CN tissue grown in organ culture for three days with 1× PBS (control) or SHH protein. (A) Limited sprouting of neurites appears in uninjured MPG/CN treated with PBS, in several discrete regions of the MPG. With SHH treatment, neurite formation increased 114% (C), in a wide area of the MPG and CN, and neurites were 82% longer. Arrows indicate neurites. MPG = major pelvic ganglia. CN = cavernous nerve. 60–160× magnification. (B) The CN was crushed, and after three days, the MPG and CN were isolated and grown in organ culture for three days with 1× PBS (control) or SHH protein. Abundant neurites appeared in the crushed MPG/CN treated with PBS. With SHH treatment, neurites increased 49% (D), were 40% longer, and appeared over a 76% wider area of the MPG and CN (red arrows). White arrows indicate neurites. Red lines represent enlarged regions. 80× magnification.

Figure 4. Normal adult MPG and CN tissues were grown in organ culture for three days with Affi-Gel beads delivering either the SHH inhibitor cyclopamine and DMSO control (**A, C**), or 5e1 SHH inhibitor and PBS control (**B, C**). Neurite formation was inhibited (particularly in the region near the Affi-Gel beads) by 82% with cyclopamine, and 86% with 5e1 SHH inhibitor. Arrows indicate neurites. Red lines represent enlarged regions. 50× magnification.

Figure 5.

MPG and CN tissue from Sprague Dawley rats that underwent bilateral CN crush, and MPG/CN tissues were harvested at time of crush, and after 2, 4 and 9 days, and were grown in organ culture for 4 days. Neurite formation was observed at all time points after CN crush, with most abundant neurites observed at 2 days after CN crush. While less abundant, neurite formation was possible at later time points after CN injury, with a similar neurite sprouting potential observed after 4 and 9 days. Arrows indicate neurites. 50–80× magnification.

Figure 6.

Adult Sprague Dawley rats underwent bilateral CN crush and were treated with either SHH protein or MSA (control) protein *via* peptide amphiphile (PA) nanofiber hydrogels, which delivered protein for 4 days *in vivo* and then in *in vitro* organ culture after isolation of the MPG/CN. (A) Very little neurites were observed after CN injury with continuous MSA treatment. (B) Continuous SHH treatment induced intense neurite formation in a wide area along the MPG and CN. The number of neurites increased by 298% with SHH treatment. (C) SHH protein treatment was able to rescue neurite sprouting potential, with an increase of 181% in the number of neurites even when not given until four days after crush injury. (D) When SHH protein was applied initially for four days *in vivo*, and then withheld in organ culture, neurites increased 141% in comparison to MSA controls, and continued to grow at a lower level once initiated, in comparison to continuous SHH treated MPG/CN. Arrows indicate neurites and the PA vehicle. Red lines represent enlarged regions. 50–80× magnification.

Figure 7. When SHH protein was delivered by PA to the MPG (A), neurites were abundant along the MPG and CN and neurites appeared longer in length, particularly along the severed end of the CN (B) in comparison to CN delivery (B). MPG = major pelvic ganglia. Line indicates length of sprouts. 50–160× magnification.