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Spinal subarachnoid adrenal medullary transplants reduce hind paw swelling and peripheral nerve transport following formalin injection in rats

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Abstract

Previous studies have demonstrated that adrenal medullary chromaffin cells transplanted into the spinal subarachnoid space significantly reduced pain-related behavior following hind paw plantar formalin injection in rats. The data suggests a centrally mediated antinociceptive mechanism. The spinal transplants may have effects on sciatic nerve function as well. To address this, the current study examined the effects of spinal adrenal transplants on hind paw edema and the anterograde transport of substance P (SP) that occur following formalin injection. Robust formalin-evoked edema, as well as hind paw flinching, was observed in striated muscle control-transplanted rats, which were not observed in adrenal-transplanted rats. To visualize transport of SP, the sciatic nerve was ligated ipsilateral to formalin injection and the nerve was processed 48 h later for immunocytochemistry. A significant formalin-induced accumulation of SP immunoreactivity (IR) was observed proximal to the ligation in control-transplanted rats. In contrast, there was significantly less SP IR observed from nerve of adrenal-transplanted rats, suggesting a diminution of anterograde axoplasmic transport by adrenal transplants. The change in SP IR may have been due to an alteration of transport due to formalin injection, thus, transport was visualized by the accumulation of growth-associated protein 43 (GAP43) at the ligation site. Formalin injection did not significantly increase proximal accumulation of GAP43 IR, indicating that formalin does not increase anterograde transport. Surprisingly, however, adrenal transplants significantly diminished GAP43 IR accumulation compared to control-transplanted rats. These data demonstrate that spinal adrenal transplants can attenuate the formalin-evoked response by modulating primary afferent responses.

Keywords

Axonal transport; Chromaffin cell; Dorsal root reflex; Growth-associated protein 43; Nociceptor; Spinal cord; Substance P

1. Introduction

The formalin test has been widely used in rats and mice to rapidly evaluate the efficacy of potential antinociceptive agents as well as to study the pathophysiology of acute and persistent pain (Coderre et al., 1990; Wheeler-Aceto and Cowan, 1991). It is generally believed that the pain-related behaviors of the first, acute phase are mediated mainly by enhanced primary afferent nociceptor activation and that the behaviors of the second, chronic phase result mainly through sensitization of spinal dorsal horn nociceptive neurons, along with changes in primary

afferent and dorsal horn neurochemistry (Coderre et al., 1993; Tjolsen et al., 1992). One change in the neurochemistry that occurs in the ipsilateral small diameter dorsal root ganglia (DRG) neurons and dorsal horn is an increase in the neuropeptide substance P (SP), previously implicated in dorsal horn nociceptive transmission (Kantner et al., 1986; Noguchi et al., 1988; Rokugo et al., 2002).

Substance P appears to mediate the progressive tissue damage in the painful arthritic state (Bileviciute et al., 1993; Decaris et al., 1999; Lotz et al., 1987). The release of SP from primary afferent terminals in the skin has been implicated in the symptoms of tissue inflammation, characterized by vasodilation and edema as well as hypersensitivity (Richardson and Vasko, 2002). Substance P stimulates adjacent primary afferent terminals, vasculature and immune cells, increasing the release of other neuroactive and inflammatory substances from terminals and locally maintaining the hypersensitive state; depletion of SP from nociceptors with capsaicin greatly attenuates this response (Inoue and Nakata, 2006; Levine et al., 1985; Willis, 1999). Neurogenic inflammation is also maintained by antidromic activation of afferent nociceptors and spinal cord dorsal horn circuits via the dorsal root reflex (Donnerer et al., 1992; Sluka et al., 1994; Willis, 1999). Knee joint edema as well as hind paw hypersensitivity is markedly reduced following section of the dorsal roots of the sciatic nerve and spinal infusion of a non-*N*-methyl-D-aspartate receptor antagonist (Sluka et al., 1994; Willis, 1999). Thus, a spinal dorsal horn circuit modulates symptoms of peripheral inflammation and a key component is the peripheral transport of SP.

Adrenal medullary chromaffin cells contain and release numerous antinociceptive substances, including catecholamines and opioid peptides, and growth factors (Czech and Sagen, 1995). Transplanted into the lumbar spinal subarachnoid space, these cells have been shown to reduce pain-related behaviors in a number of rat pain models, including the formalin test (Hama and Sagen, 1994a; Sagen et al., 1986; Siegan and Sagen, 1997; Sol et al., 2004; Vaquero et al., 1991; Wang and Sagen, 1995). The opioid receptor antagonist naloxone and α -adrenoreceptor antagonist phentolamine attenuate the antinociceptive effects of the transplant, indicating that the primary action of these cells is to attenuate the activity of dorsal horn nociceptive neurons (Hentall et al., 2001). However, there is the possibility that these transplants can also exert an antinociceptive “peripheral effect” by inhibiting the dorsal root reflex.

The goal of the current study was to determine whether spinal adrenal medullary transplants can reduce the effects of hind paw formalin injection, including edema and distal transport of SP. To visually assess possible changes in axoplasmic transport following formalin injection, the phosphoprotein growth-associated protein 43 (GAP43) was immunostained along with SP. Although typically found in axonal growth cones during regeneration, GAP43 is also transported in the axoplasm of normal adult peripheral nerve (Li et al., 1995).

2. Results

The results of treatments from each group are summarized in Table 1.

2.1. Formalin test

In sham-transplanted rats, a biphasic response to hind paw 5% formalin injection was observed (Fig. 1a). Rats with control transplants showed a similar response to formalin. Although rats with an adrenal transplant showed similar numbers of total behaviors in phase 1 compared to rats with a sham and control transplants, total phase 2 pain-related behaviors were significantly attenuated in these rats (Fig. 1b; $p < 0.05$ vs. sham and control transplants).

2.2. Hind paw swelling

Transplantation with either control or adrenal tissue did not affect hind paw diameter (data not shown). Prior to formalin injection, the circumference of the left hind paw and the contralateral hind paw was 18.0 ± 1.4 mm (mean $\pm$ S.E.M.) and the difference between the hind paws was 0.2 ± 0.2 mm. In rats with either a sham or a control transplant, a robust increase in ipsilateral hind paw circumference was observed 1 h following formalin injection (Fig. 2). In contrast, the hind paw swelling was significantly attenuated in rats with an adrenal transplant ($p < 0.05$). A noticeable ipsilateral swelling was clearly present in adrenal-transplanted rats, however ($p < 0.05$ vs. pre-formalin injection).

2.3. Substance P immunoreactivity

Forty-eight hours after ligation of the sciatic nerve in sham-transplanted rats that received a hind paw formalin injection, there was a significant accumulation of SP immunoreactivity (IR) proximal to the ligation but no accumulation distal to the ligature (Fig. 3a,b). The SP IR appeared to be mainly within axons and not in Schwann cells or in immune cells. A similar accumulation of SP IR proximal to the ligation was observed in control-transplanted rats (Fig. 3d). In rats that did not receive a formalin injection, there is a slight but measurable accumulation of SP IR proximal to the ligation (Fig. 3c).

The area of SP IR in the proximal ligation area was significantly increased in both sham- and control-transplanted rats that were injected with formalin (Fig. 5; $p < 0.05$ vs. ligation only rats). In contrast to sham- and control-transplanted rats, a low accumulation of SP IR was observed in formalin-injected adrenal-transplanted rats (Fig. 3e), which was not significantly different from that of rats that were not injected with formalin (Fig. 5; $p < 0.05$ vs. sham- and control-transplanted rats).

2.4. Growth-associated protein 43 immunoreactivity

Forty-eight hours after ligation and formalin injection of control-transplanted rats, there was a significant accumulation of GAP43 IR proximal to the ligation with a slight area of IR distal to the ligature (Fig. 4a,b, Fig. 5). The GAP43 IR was mainly found in axons and not in Schwann cells. An increase in GAP43 IR was also observed in sham-transplanted formalin-injected rats (Fig. 4c, Fig. 5). In rats that did not receive a formalin injection, the accumulation of GAP43 IR proximal to the ligation did not appear to differ from that of either control- or sham-transplanted rats (Fig. 4d, 5; $p < 0.05$). Compared to sham- and control-transplanted rats, a smaller accumulation of GAP43 IR was observed in adrenal-transplanted rats (Fig. 4e, 5; $p < 0.05$ vs. sham- and control-transplanted rats).

3. Discussion

The current data demonstrated that lumbar spinal adrenal medullary transplants reduce pain-related behavior and symptoms of peripheral inflammation, possibly through a suppression of the anterograde transport of neuroactive substances down the primary afferent following tissue injury, which leads to a reduction of the symptoms of peripheral inflammation, including edema and pain. Adrenal transplants significantly reduced formalin-induced hind paw edema and accumulation of the pro-inflammatory neuropeptide SP in the sciatic nerve. In contrast, control transplants did not affect any of these parameters. Prior formalin injection did not appear to further increase GAP43 IR, indicating that formalin injection does not significantly affect axoplasmic transport. Interestingly, GAP43 was significantly attenuated in adrenal-transplanted rats, which, in combination of the reduction in SP, suggests decreased axoplasmic transport. The results indicate that spinal adrenal transplants inhibit a mechanism originating from the dorsal horn, such as the dorsal root reflex.

Previous studies in rat models of acute and chronic pain have demonstrated the antinociceptive efficacy of spinally transplanted adrenal medullary chromaffin cells (Hama and Sagen, 1994a; Sagen et al., 1986; Siegan and Sagen, 1997; Sol et al., 2004; Wang and Sagen, 1995). Adrenergic and opioid agonists are antinociceptive when intrathecally injected either alone or in combination (Kuraishi et al., 1985; Przesmycki et al., 1997; Sherman et al., 1988). Likewise, chromaffin cells release catecholamines and opioid peptides and the antinociceptive effects are blocked with either phentolamine or naloxone (Czech and Sagen, 1995; Siegan and Sagen, 1997; Vaquero et al., 1991). There are other less well-characterized substances that are synthesized and released by chromaffin cells that may have antinociceptive effects as well (Lemaire et al., 1995; Wang et al., 1995). Direct effects of adrenal transplants on afferent-induced dorsal horn neuron sensitization are supported by electrophysiological studies. Wide dynamic range neurons in control-transplanted rats displayed significant after-discharges following repetitive c-fiber stimulation, whereas neurons in adrenal-transplanted rats did not (Hentall et al., 2001).

It is possible that there may be more removed effects of adrenal transplants mediated through the dorsal horn. Primary afferent nociceptors conduct signals from the periphery to the dorsal horn, but signals can also travel distally. Nociceptors, then, can serve an efferent function, antidromically releasing SP and other substances into the site of inflammation (Willis, 1999). Infusion of a non-*N*-methyl-D-aspartate receptor antagonist within the spinal dorsal horn attenuated inflammatory edema of the rat knee joint as well as hind paw hypersensitivity (Sluka et al., 1994). Opioids and adrenergic agonists have been shown to decrease peripherally directed release of SP from nociceptors (Willis, 1999). In the current study, it is likely that opioid peptides and catecholamines released from the adrenal transplant decreased hind paw edema and transport of SP.

In the current study, to demonstrate alterations in axoplasmic transport of SP in the sciatic nerve, a short term ligation was employed to enhance SP IR for visual quantitative analysis (Brimijoin et al., 1980; Dahlstrom et al., 1974). It is possible that the ligation itself induced an increase in the rate of anterograde SP transport, but other studies have not reported an enhancement of the rate of neuropeptide transport (one to two days) following either a transection or crush injury (Tonra et al., 1998). In the current study, tight ligation without previous hind paw formalin injection leads to a small but measurable level of SP proximal to the ligation. (In none of the treatment groups was SP IR noted distally to the ligation, thus confirming anterograde-only transport of SP.) However, formalin injection significantly increased SP IR in sham- and control-transplanted rats. A previous study demonstrated that retarding microtubule function with colchicine treatment of the central terminal significantly suppressed formalin-induced accumulation of SP IR in the dorsal horn (Kantner et al., 1986). Thus, the current data demonstrate that formalin increases axoplasmic transport of SP to the periphery.

In addition to the increased transport of SP, it is possible that SP synthesis was also increased following formalin injection. An increase of about 30% in SP IR in the DRG was obtained after brief (about 5 min) formalin stimulation and a significant increase in SP mRNA occurred beginning 3 h after formalin injection (Noguchi et al., 1988; Rokugo et al., 2002). Noguchi et al. (1988) further mentioned that 24 h following formalin injection, there was no parallel increase in SP IR despite a sustained increase in mRNA. Thus, most of the SP IR observed in the current study was likely to be SP that was already present in the DRG.

Short term ligation of the sciatic nerve also resulted in an increased accumulation of GAP43 proximal to the ligature. The increase is likely due to increased anterograde axoplasmic flow but it is possible that novel GAP43 may have also accumulated at the ligation site in response to axonal damage due to ligation (Leslie et al., 1995; Li et al., 1995; Wiese et al., 1992). The

initial increase of GAP43 in DRG neurons in those studies appears to be mainly in small diameter DRG neurons, primarily nociceptive neurons. The current data suggest that a similar (if not the same) population of axons were IR for both SP and GAP43. Thus, anterograde transport within nociceptive primary afferents was increased following formalin.

Both SP and GAP43 IR were significantly attenuated in rats with a spinal adrenal transplant. In conjunction with a reduction of hind paw edema in these rats, the probable mechanism underlying these observations is the reduction of the dorsal root reflex. As mentioned earlier, inhibition of the dorsal root reflex and the associated antidromic release of SP by dorsal horn infusion of various substances significantly reduces peripheral edema as well as inflammation-induced nociception (Sluka et al., 1994; Willis, 1999). Alternatively, if synthesis of GAP43 was, in fact, increased (along with elevated axoplasmic transport), a possible means by which adrenal transplants could reduce GAP43 synthesis is via the central release of nerve growth factor-like substances to attenuate this synthesis (Hirata et al., 2002). Thus, in addition to immediate effects on dorsal horn neurons, the transplants may also involve a long-term trophic effect.

The current study suggests an involvement of a dorsal root reflex-type mechanism in attenuating inflammatory nociception by spinal adrenal medullary transplants, although the exact substances released from the transplants responsible for reducing this reflex have yet to be identified. By virtue of proximity, several effects of adrenal transplants on dorsal horn neurons have been previously noted (Hama and Sagen, 1994b; Ibuki et al., 1997; Siegan et al., 1996). The current data demonstrates an additional “peripherally” directed mechanism, whereby anterograde transport of SP and the associated edema is reduced. A treatment for chronic pain should be able to address both the peripheral and the central mechanisms that maintain the chronic pain state as both of these mechanisms appear to work in concert in clinical pain.

4. Experimental procedures

4.1. Animals

Male Sprague Dawley ($n=24$; Taconic, Germantown, NY; 275–325 g at the time of the experiments) rats were used as recipients and male Sprague Dawley retired breeders were used as tissue donors. Rats were housed in a 12 light–dark room and allowed free access to food and water before and after surgery. The procedures were reviewed and approved by the University of Miami Animal Care and Use Committee and followed the guidelines of the *Guide for the Care and Use of Laboratory Animals*. Behavioral testing and image analysis were performed in a blinded manner.

4.2. Transplantation surgery

Donor rats were rendered unconscious by carbon dioxide overdose. The adrenal glands were removed and the medullae were dissected into small pieces ($<1 \text{ mm}^3$) in ice-cold Hanks' Balanced Salt solution (Sigma-Aldrich, St. Louis, MO). Medullary tissues from two glands were transplanted into each recipient ($n=6$). For control transplants, striated muscle tissue was obtained from the abdominal wall of donor rats and was also dissected into small pieces ($n=6$).

The recipient was anesthetized with pentobarbital (50mg/kg, intraperitoneal). The back was shaved and the skin cleaned with chlorhexidine. As previously described, a laminectomy was performed using aseptic technique on spinal vertebrae L1–L2 (Siegan and Sagen, 1997). The dura was exposed and pierced and the tissue pieces were slid into the subarachnoid space and pushed under the dura to come to rest at about the spinal levels L3–L4. An additional control sham-transplanted group ($n=6$) received a laminectomy but no transplant. Following the

transplantation procedure, the muscles were sutured closed and the skin closed with wound clips. Rats were coded and the tester was unaware of the transplant that each rat received. Subsequent tissue processing was also done in a blinded manner.

4.3. Formalin test and assessment of hind paw edema

Two weeks following transplantation surgery, rats were subcutaneously injected with 25 μ l of 5% formalin into the left plantar hind paw. Immediately following injection, rats were placed in clear plexi-glas containers and the numbers of hind paw licks and flinches were counted over a 2 min period every 10 min up to 50 min following formalin injection.

Following the last testing time point, the circumference of the formalin-injected hind paw and the contralateral right paw was measured with a flexible tape measure and the difference between the injected and uninjected hind paws was calculated.

4.4. Sciatic nerve ligation

To visualize axoplasmic transport of substances within the sciatic nerve, the nerve was tightly ligated (Dahlstrom et al., 1974). Two hours after formalin injection, rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Using aseptic technique, the ipsilateral sciatic nerve at mid-thigh level was exposed and the sciatic nerve was tightly ligated with 5-0 silk suture. The muscle was sutured shut and the skin closed with cyanoacrylate. Rats were perfused for immunocytochemistry two days after sciatic nerve ligation surgery.

To evaluate the effect of ligation alone on axoplasmic transport, some rats ($n=6$) were not injected with formalin.

4.5. SP and GAP43 immunocytochemistry

Two days after formalin testing and nerve ligation, rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and rapidly transcardially perfused with 200 ml cold saline followed by 450–650 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). The sciatic nerves were harvested and cryoprotected overnight in 30% sucrose in PBS at 4 °C. Without removing the ligature, longitudinal sections (20 μ l thickness) were cut on a cryostat and thaw-mounted onto gelatin-coated slides.

Nerve sections were incubated with blocking solution (4% normal goat serum in PBS with 0.4% Triton X-100) for 1 h at room temperature. Slides were then incubated overnight at 4 °C with primary antibody (either rabbit anti-SP, 1:1000; ImmunoStar, Hudson, WI or rabbit anti-GAP43, 1:2000; Chemicon, Temecula, CA) diluted in blocking solution. Following incubation in primary antibody, slides were rinsed with PBS and then incubated with biotinylated goat anti-rabbit secondary antibody (1:200; Vector Labs, Burlingame, CA). Finally, slides were incubated with avidin: biotinylated enzyme complex (ABC) in PBS (1:500) for 1 h at room temperature then visualized with 0.02% 3',3'-diaminobenzidine, 0.25% nickel ammonium sulfate, 0.002% H₂O₂ in PBS as the chromogen. The reaction was stopped after 5 min of incubation by rinsing in PBS and then slides were dehydrated in ascending alcohols and xylenes and cover-slipped.

The sections were coded, thus the evaluator was blinded to treatment conditions. Immunoreactivity was quantified using a Zeiss Axiophot research microscope furnished with a fully motorized 3D LEP stage, Optronix cooled video camera and Stereo Investigator software (Micro Brightfield, Inc., VT). Under a 10x objective, the immunoreactive area adjacent to the ligation, extending 200 μ m from the ligation towards the “core” of the nerve, a total area of about 80,000 μ m², was measured from six random sections from each rat. The coding was broken following the completion of the imaging experiment.

4.6. Statistical analysis

Comparison between the treatment groups was performed with a one-way ANOVA with a Dunnett's test for post-hoc comparisons. Statistical significance was taken at $p < 0.05$.

Abbreviations

DRG, dorsal root ganglia; GAP43, growth-associated protein 43; IR, immunoreactivity; NGF, nerve growth factor; SP, substance P.

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Fig. 1.

Pain-related behaviors over time in the formalin test in rats. Following injection of 5% formalin into the plantar hind paw, the total numbers of hind paw licking and flinching over 2 min were counted every 10 min. (a) Pain-related behaviors over time. (b) Total number of behaviors in phase 1 (0–9 min), interphase (10–19 min) and phase 2 (20–52min). $n=6$ /group. Data are expressed as mean $\pm$ S.E.M. * $p<0.05$ vs. sham- and control-transplanted groups.

Fig. 2.

Hind paw swelling following formalin injection. Hind paw diameter was measured in rats prior to injection of formalin. One hour after formalin injection, the circumference of the widest portion of both hind paws was measured (mm). The change (Δ) in paw diameter= injected hind paw-contralateral hind paw. $n=6$ /group. Data are expressed as mean $\pm$ S.E.M. * $p<0.05$ vs. sham and control transplants.

Fig. 3. Substance P immunoreactivity in representative longitudinal sections of the rat sciatic nerve. Forty-eight hours after tight ligation of the sciatic nerve, rats were perfused. (a), (b) Taken from a sham-transplanted formalin-injected rat. In the nerve distal to the ligation (L) (a), there is little SP IR, whereas abundant SP IR (↓) can be observed proximal to the ligation (b). (c) In a rat that did not receive hind paw formalin injection prior to nerve ligation, the nerve proximal to ligation had very little SP IR. (d) In a formalin-injected rat with a control transplant, robust SP IR was observed proximally to the ligation. (e) In a formalin-injected with an adrenal transplant, there was reduced observable SP IR proximal to the ligation. Scale bar=200 μm.

Fig. 4.

Growth-associated protein 43 immunoreactivity in representative longitudinal sections of the rat sciatic nerve. Forty-eight hours after tight ligation of the sciatic nerve, rats were perfused. (a) A low power view of sciatic nerve taken from a sham-transplanted rat without a previous formalin injection. In the nerve distal (left, $\uparrow$) to the ligation (L), there is little GAP43 IR, whereas abundant GAP43 IR can be observed proximal (right, $\downarrow$) to the ligation. (b) From a formalin-injected control-transplanted rat, the nerve proximal to ligation had significant GAP43 IR. (c) Robust GAP43 IR was observed proximal to ligation, from a formalin-injected sham-transplanted rat. (d) GAP43 IR was found in nerve proximal to the ligation, from a sham-

transplanted rat without prior formalin injection. (e) In a formalin-injected rat with an adrenal transplant, there was a reduced area of GAP43 IR proximal to the ligation. Scale bar=200 μ m.

Fig. 5.

Presence of SP and GAP43 IR in sciatic nerve. Two hours after formalin injection, the sciatic nerve ipsilateral to hind paw formalin injection was tightly ligated in the sham-, muscle- and adrenal-transplanted groups. In an additional group, no formalin (-) was injected into the paw but the sciatic nerve was ligated. All rats were perfused 48 h after ligation and the area (μm^2) of SP and GAP43 IR were measured near the ligation area. $n=6/\text{group}$. Data are expressed as mean $\pm$ S.E.M. * $p<0.05$ vs. ligation only group. # $p<0.05$ vs. sham and control transplants.

Table 1

Summary of the treatment groups

Group	Formalin	Ligated	Transplant	Change in	
				SP	GAP43
1	N	Y	None	↑ ^a	↑ ^a
2	Y	Y	None ("sham")	↑↑	↑
3	Y	Y	Control tissue	↑↑	↑
4	Y	Y	Adrenal tissue	↓↓	↓↓

n=6/group.Y, yes; N, no; ↑, increase; ↓, decrease; ↑↑, significant increase vs. group 1 ($p<0.05$); ↓↓, significant decrease vs. group 2 and 3 ($p<0.05$).^a compared to contralateral unoperated sciatic nerve.