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NMDA Antagonist Peptide Supplementation Enhances Pain Alleviation by Adrenal Medullary Transplants

Farinaz NasiriNezhad[1] and Jacqueline Sagen

The Miami Project to Cure Paralysis, University of Miami School of Medicine, Miami, FL

Spinal transplantation of adrenal medullary chromaffin cells has been shown to decrease pain responses in several animal models. Improved potency may be possible by engineering cells to produce greater levels of naturally derived analgesics. As an initial screen for potential candidates, adrenal medullary transplants were evaluated in combination with exogenously administered neuropeptides in rodent pain models. Histogranin is a 15-amino acid peptide that exhibits NMDA receptor antagonist activity. The stable derivative [Ser[1]]histogranin (SHG) can attenuate pain symptoms in some animal models. The formalin model for neurogenic inflammatory pain and the chronic constriction injury (CCI) model for neuropathic pain were used to evaluate the combined effects of chromaffin cell transplantation and intrathecal (IT) SHG injections. Animals were implanted with either adrenal medullary or control striated muscle tissue in the spinal subarachnoid space. For evaluation of formalin responses, animals were pretreated with SHG (0.5, 1.0, 3.0 μg) followed by an intraplantar injection of formalin, and flinching responses were quantified. Pretreatment with SHG had no significant effect on flinching behavior in control animals at lower doses, with incomplete attenuation only at the highest dose. In contrast, 0.5 μg SHG significantly reduced flinching responses in animals with adrenal medullary transplants, and 1.0 μg nearly completely eliminated flinching in these animals in the tonic phase. For evaluation of effects on neuropathic pain, animals received transplants 1 week following CCI, and were tested for thermal and mechanical hyperalgesia and cold allodynia before and following SHG treatment. The addition of low doses of SHG nearly completely eliminated neuropathic pain symptoms in adrenal medullary transplanted animals, while in control transplanted animals only thermal hyperalgesia was attenuated, at the highest dose of SHG. These results suggest that SHG can augment adrenal medullary transplants, and the combination may result in improved effectiveness and range in the treatment of chronic pain syndromes.

Key words: Histogranin; Spinal cord; Neuropathic pain; Inflammatory pain; Formalin test

INTRODUCTION

The transplantation of cells into the CNS can have therapeutic benefit by providing a sustained and renewable local source of naturally derived pharmacologically active agents. The use of this approach in the management of pain has been explored in several laboratories. Most of this work has focused on the transplantation of adrenal medullary chromaffin cells in the spinal subarachnoid space, as these cells produce numerous agents with analgesic or antinociceptive activity, including opioid peptides, catecholamines, and endogenous NMDA antagonists (17,29,39,41). Adrenal medullary tissue or isolated chromaffin cell transplants have shown efficacy in various preclinical pain models including the formalin test (3,24,33,35,36,38,42), chronic inflammation (30,40), neuropathic pain models (5,6,10,11), central pain models (2,7,44,45), and wind-up (14). However, analgesic effects of chromaffin cell transplants are not always observed, as described in recent reports using acute pain models and the formalin test (18,19). Variations in transplantation or testing procedures between laboratories may account for some of these differences. A recently completed open label clinical trial utilizing adrenal medullary allografts in terminal cancer patients suggested that opioid requirements for pain control could be decreased or stabilized in a majority of patients in contrast to the usual dose escalation observed during disease progression (15). However, a controlled clinical trial using encapsulated bovine chromaffin cells for terminal cancer pain reportedly did not reach significant clinical efficacy (Astra AB, Sweden; unpublished findings), possibly due

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[1]Current address: Department of Physiology. Basic Science Center, Medical School, Iran University of Medical Sciences, Tehran, Iran.
Address correspondence to Jacqueline Sagen, Ph.D., Miami Project to Cure Paralysis, University of Miami School of Medicine, 1095 NW. 14th Terrace (R-48), Miami, FL 33136. Tel: (305) 243-5618; Fax: (305) 243-3923; E-mail: jsagen@miami.edu

to the relatively low numbers of cells (hence achieving subtherapeutic doses of released analgesic agents) that can be accommodated by the capsules.

Thus, it is likely that adrenal medullary chromaffin cells, while possessing a promising analgesic therapeutic cocktail and the cellular machinery for sustainable local delivery of these agents, may produce levels too low for management of severe chronic pain, particularly in the clinical setting given the practical limitations of cell transplantation. The goal of ongoing studies in our laboratory is to increase the analgesic efficacy of transplanted cells by supplementation with additional or increased levels of therapeutic agents. For this purpose, naturally derived neuropeptides have been selected, as these may be ultimately genetically engineered to produce high potency analgesic chromaffin cells for transplantation. As an initial screen for potential peptide candidates, adrenal medullary transplants are evaluated in combination with exogenously administered neuropeptides in rodent pain models. A promising candidate is the 15-amino acid peptide, histogranin, as it possesses NMDA antagonist-like activity (17,31), a class of agents that have been shown to reduce hyperalgesia and allodynia, attenuate opioid tolerance, and enhance opioid analgesia (20,21,25–28). In addition, chromaffin cells possess the cellular machinery to process histogranin, as the natural form is produced in the adrenal medulla (16). The stable analog, [Ser^{1}]histogranin (SHG) was selected, as it has the potential to produce more sustained NMDA receptor antagonism for chronic pain therapy, and should be synthesizable by engineered mammalian cells. Previous findings in our laboratory have shown that intrathecally injected SHG can attenuate, although not completely block, neuropathic, inflammatory, and NMDA-mediated hyperalgesia (9,12,32,35). The goal of the present studies was to determine whether SHG would be a promising candidate for producing enhanced potency chromaffin cells by screening this agent in combination with adrenal medullary transplants for attenuation of neurogenic inflammatory and neuropathic pain. Preliminary results were presented at the American Society for Neural Transplantation and Repair meeting (22,23).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River) weighing 200–250 g at the initiation of the studies were used. All animal procedures followed NIH guidelines and were approved by the University of Miami Animal Care and Use Committee.

Experimental Design

Animals were assessed for either neurogenic inflammatory pain using the formalin test or peripheral neuropathic pain using the chronic constriction injury (CCI) model (1). For evaluation of effects of SHG supplementation with adrenal medullary transplants in inflammatory pain, animals with intrathecal catheters and either adrenal medullary or control striated muscle transplants in the subarachnoid space (see below for surgical procedures) received intrathecal injections of SHG (0.5, 1.0, or 3.0 μg) 15 min prior to intraplantar formalin injection and observation of pain-related behaviors (see below for behavioral testing procedures). For effects on neuropathic pain, a battery of tests for allodynia and hyperalgesia (see below) was given prior to CCI induction, 1 week following CCI surgery, and every other day beginning 1 week following transplantation through study completion. At each test point following transplantation, baseline responses and responses 15 min following intrathecal injection of SHG or saline were determined. Doses of SHG (0.5, 1.0, or 3.0 μg) or saline vehicle were injected intrathecally in 10 μl volumes followed by 10 μl saline flush. Doses were rotated such that each animal received all doses during the course of the study. There were 9–14 animals in all transplant/pharmacologic treatment groups. SHG was custom synthesized by Research & Diagnostic Antibodies (Berkeley, CA).

Surgical Procedures

To induce unilateral peripheral neuropathic pain, the common sciatic nerve of anesthetized animals was exposed on one side at the midthigh level and four 4-0 chromic gut ligatures spaced about 1 mm apart were loosely tied around the sciatic nerve proximal to the trifurcation, constricting the nerve to a barely discernable degree, such that circulation through the epineural vasculature was not interrupted. Following surgery, the musculature was sutured in layers, and the skin closed with wound clips.

All animals were implanted with an intrathecal catheter prior to initiation of behavioral testing to administer doses of SHG directly into the CSF. For animals with CCI, catheter placement was done 1 week following induction of neuropathic pain. Intrathecal (IT) catheters (8.0 cm of PE-10 tubing) were introduced via a slit in the atlanto-occipital membrane and carefully threaded caudally in the intrathecal space, such that the tips ended at the level of the thoraco-lumbar spinal cord.

At the time of catheter implantation, animals were also transplanted with either adrenal medullary or control striated muscle tissue in the spinal subarachnoid space. Both adrenal medullary tissue and striated muscle tissue were obtained from adult male Sprague-Dawley donors. Adrenal medullary tissue was dissected from two adrenal glands in ice-cold HBSS and cut into small pieces (<0.5 mm^{3}) as described in previous studies (3,11,30,33). Transplant control animal received equal volume of stri-

ated muscle tissue from donor animals. For implantation, a laminectomy was performed to expose lumbar segments L1–L2 and tissue was implanted in the subarachnoid space via a slit in the dura and arachnoid membrane under the aid of a dissecting microscope. Following transplantation procedures the back musculature was sutured with 3-0 silk and the skin closed with wound clips. After all surgical procedures, animals were returned to their cages and food and water were available ad libitum.

Behavioral Evaluation

Animals were evaluated for formalin pain responses 1 week following adrenal medullary transplantation and intrathecal catheter placement. To accomplish this, 50 μl of 5% formalin was injected in the plantar surface of one hind paw. The number of flinches and licking behaviors that occurred during the first minute of each 5-min interval up to 1 h was counted as described in previous studies (3,33,35,42). For evaluation of pain responses using the CCI model, a battery of sensory tests was performed in sequence. Responses to noxious heat (thermal hyperalgesia test) were evaluated using a paw withdrawal test (13). Responses to noxious mechanical pressure (mechanical hyperalgesia) was assessed using a pressure withdrawal test (Randall-Selitto apparatus), and responses to innocuous cold (cold allodynia) were determined using the acetone test. To assess response to a noxious thermal stimulus, rats are placed beneath an inverted clear plastic cage on an elevated glass floor and allowed to acclimate for 5 min. A radiant heat source beneath the glass was aimed at the plantar hind paw and activated a timer. Withdrawal latencies were the length of time between the activation of the heat source and the hind paw withdrawal from the glass (normal baseline ~10 s). To avoid tissue damage in the absence of a withdrawal, the cutoff was set at 20 s. Three trials were given at least 30 s apart, the average values used for statistical analysis. To assess sensitivity to a noxious mechanical stimulus, the rats were wrapped in a towel and an increasing force (48 g/s) was applied to the plantar surface of the hind paw until the rat reacted with a withdrawal response (normal baseline ~10 in arbitrary scale units). The apparatus automatically terminated at 1000 g (25 in scale units) in the absence of a response. To evaluate cold allodynia, the animals were placed beneath an inverted plastic cage on an elevated mesh floor and a drop of acetone was applied to the plantar surface of the foot using polyethylene tubing connected to a syringe. The acetone bubble was gently touched to the heel and quickly spread over the proximal half of the plantar surface of the foot. Acetone was applied five times with at least 3-min intervals between applications. The frequency of foot withdrawal was expressed as a percent (normal intact rats usually do not respond to this stimulus; hence receive a score of 0): No. of trials accompanied by foot withdrawals/No. of total trials × 100.

Data Evaluation

All behavioral studies were done by an observer blinded to the experimental treatment. Data are expressed as means ± SEM. Statistical comparisons between transplant/pharmacologic treatment groups were done using two-way ANOVA (repeated measures) and the Newman-Keuls test for multiple post hoc comparisons (37). For confirmation of adrenal graft viability, a sampling of transplanted animals were perfused intracardially, their spinal cords dissected, and graft tissue stained for tyrosine hydroxylase immunocytochemistry (DiaSorin, diluted 1:1000).

RESULTS

Effects of SHG Supplementation on Antinociceptive Effects Transplants in Inflammatory Pain

Figure 1 shows the effects of pretreatment with 0.5 μg (top panel), 1.0 μg (middle panel) or 3.0 μg (lower panel) SHG on the formalin responses in animals with adrenal medullary or control transplants. Typical biphasic formalin flinching responses were observed in most cases: phase 1, thought to be indicative of acute pain, occurred during the first minute postformalin, and phase 2, thought to be indicative of tonic, more prolonged pain, occurred approximately between 20 and 50 min postformalin, separated by a quiescent interphase between phase 1 and phase 2. Following treatment with the lowest dose of SHG, animals with adrenal medullary transplants showed significantly reduced flinching compared with animals with control striated muscle transplants [overall $F(1, 12) = 22.64$, $p < 0.001$]. The attenuated flinching in adrenal transplanted animals was observed only in phase 2 at this dose, notably at 30–40 min after formalin ($p < 0.05$); no significant differences were noted for phase 1 responses ($p > 0.05$). In contrast, 1.0 μg SHG injected intrathecally produced significantly reduced flinching in both phases of the formalin response in animals with adrenal medullary compared with control transplants [overall $F(1, 12) = 54.61$, $p < 0.001$; $p < 0.05$ for phase 1 and 2]. Phase 1 responses were lower in adrenal medullary transplanted than control transplanted animals following 1.0 μg SHG pretreatment, but these responses were only partially attenuated and not completely eliminated. In contrast, phase 2 flinching was markedly attenuated and nearly completely eliminated for the duration of the response period (20–50 min) in these animals. This dose of SHG was more potent in reducing formalin responses than the lower dose in adrenal medullary grafted animals ($p < 0.05$). In contrast, 1.0 μg SHG did not reduce responses compared with 0.5 μg

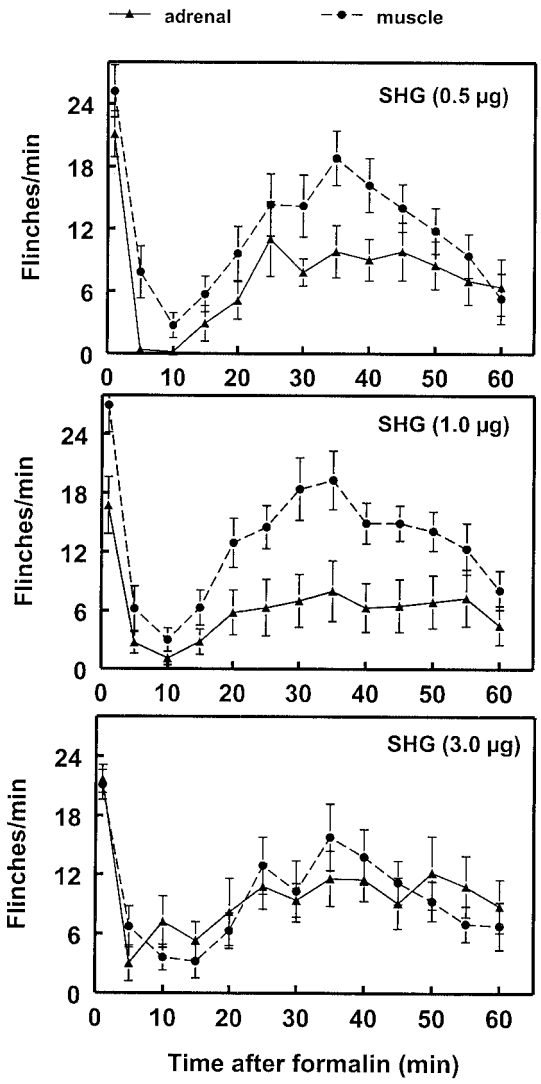

Figure 1. Effects of [Ser[1]]histogranin (SHG) supplementation and adrenal medullary or striated muscle transplants in the spinal subarachnoid space on formalin-evoked flinching responses. SHG was given intrathecally at 0.5 µg (top panel), 1.0 µg (middle panel), or 3.0 µg 15 min prior to intraplantar injection of formalin (5%). Each point represents the mean ± SEM number of hind paw flinches observed during the first minute of each 5-min interval following the formalin in animals with adrenal medullary (triangles) or control (circles) transplants. n = 11–15 animals per transplant group.

SHG in animals with control transplants ($p > 0.05$). The highest SHG dose tested also produced lowered flinching in animals with adrenal medullary transplants, but this was not significantly different from the lower doses of SHG ($p > 0.05$). However, in contrast to the lower SHG doses, 3.0 µg SHG produced moderate attenuation of flinching responses in control transplanted animals ($p < 0.05$). Similarly, SHG in this dose range and other NMDA antagonists have been found to reduce phase 2 flinching in previous reports (4,8,34,43). At this highest SHG dose studied, formalin flinching was reduced to an equivalent extent in both adrenal medullary and control transplanted animals ($p > 0.05$).

Effects of SHG Supplementation on Antinociceptive Effects Transplants in Neuropathic Pain

Figures 2–4 show the effects of SHG pretreatment on neuropathic pain responses in animals with adrenal medullary (top panels) and striated muscle control (lower panels) transplants. Responses to noxious heat are shown in Figure 2. At 1 week following CCI, animals showed reduced hind paw withdrawal latencies (thermal hyperalgesia) compared with presurgical baseline responses in all animals ($p < 0.05$). Following adrenal medullary transplantation, thermal hyperalgesia was attenuated, although not completely reversed (predrug treatment solid bars in Fig. 2 top panel; $p < 0.05$ compared with both CCI and baseline responses). In contrast, thermal hyperalgesia was not altered by control striated muscle transplants (predrug treatment solid bars in Fig. 2 bottom panel; $p > 0.05$ compared with CCI; $p < 0.05$ compared with baseline responses). The combined effects of adrenal medullary transplants supplemented with SHG markedly reduced, and nearly eliminated, the thermal hyperalgesia produced by the peripheral nerve constriction injury compared with effects in the control transplant group [overall $F(1, 22) = 10.06$, $p < 0.001$]. Following the intrathecal pretreatment with SHG, thermal hyperalgesia was reduced and thermal withdrawal responses nearly restored to pre-CCI baseline levels in animals with adrenal medullary transplants ($p > 0.05$ compared with both baseline and saline pretreatment). All SHG doses tested produced this result and further reduced hyperalgesia compared with the effects of the adrenal medullary transplants alone ($p < 0.05$). SHG also attenuated thermal hyperalgesia in control transplanted animals, but only at the highest dose tested ($P < 0.05$ compared with CCI at 3.0 µg SHG). Lower doses of SHG did not alter responses to noxious thermal stimuli in control transplanted animals ($p > 0.05$).

CCI also produced mechanical hyperalgesia observed by 1 week following (Fig. 3; $p < 0.05$ compared with baseline). As observed in previous studies, effects of adrenal medullary transplants on noxious mechanical responses were markedly weaker than those on noxious thermal responses (Fig. 3, top panel). However, supplementation with SHG significantly attenuated mechanical hyperalgesia in these animals ($p < 0.05$ compared with both post-CCI and saline pretreatment responses), nearly reversing responses to noxious mechanical stimuli to pre-CCI levels in these animals ($p > 0.05$ compared with baseline). SHG pretreatment in muscle transplanted animals showed a trend towards attenuating mechanical hy-

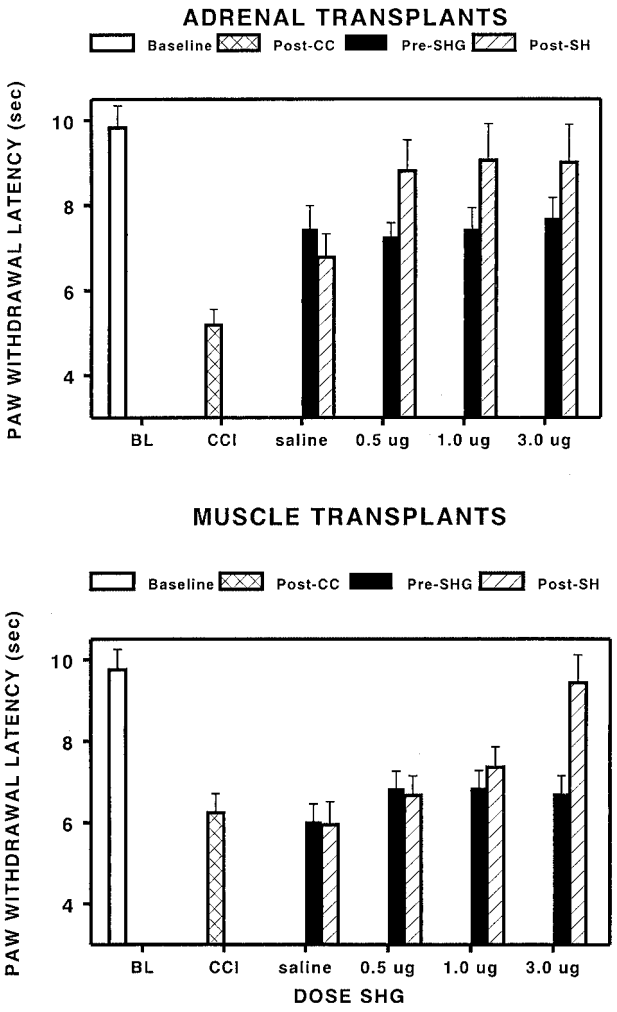

Figure 2. Effect of [Ser[1]]histogranin supplementation and adrenal medullary or striated muscle transplants in the spinal subarachnoid space on complete chronic constriction injury (CCI)-induced thermal hyperalgesia. Baseline latencies (BL, open bars) of rats were evaluated prior to unilateral CCI, after CCI (cross-hatched bars), and before (Pre-SHG; solid bars) and after (Post-SHG, diagonal bars) intrathecal treatment with saline vehicle or SHG (0.5, 1.0, or 3.0 μg). Mean latencies ± SEM in adrenal medullary (top panel, $n = 14$) or striated muscle (bottom panel, $n = 10$) transplanted animals are shown.

peralgesia in muscle transplanted animals, particularly at 3.0 μg, but this did not reach statistical significance ($p > 0.05$ compared with post-CCI and with saline pretreatment). As observed for thermal hyperalgesia, the combined effects of adrenal medullary grafts with SHG supplementation nearly eliminated the mechanical hyperalgesia generated by peripheral nerve injury in these animals, in contrast to striated muscle transplants [overall $F(1, 22) = 9.31$, $p < 0.001$].

Figure 4 shows responses to innocuous cold (acetone application) in response to SHG pretreatment in adrenal medullary (top panel) and control striated muscle (bottom panel) transplants. Prior to CCI, lifting withdrawal responses to hind paw plantar acetone application were nearly zero (baseline). Following CCI, cold allodynia was apparent as approximately 50–60% hind paw lifting in response to acetone. Adrenal medullary transplants showed a nonsignificant tendency toward reduced allodynia. However, following intrathecal pretreatment with SHG, cold allodynia was markedly attenuated in animals with adrenal medullary transplants ($p < 0.05$ compared with both post-CCI and saline pretreatment). In contrast,

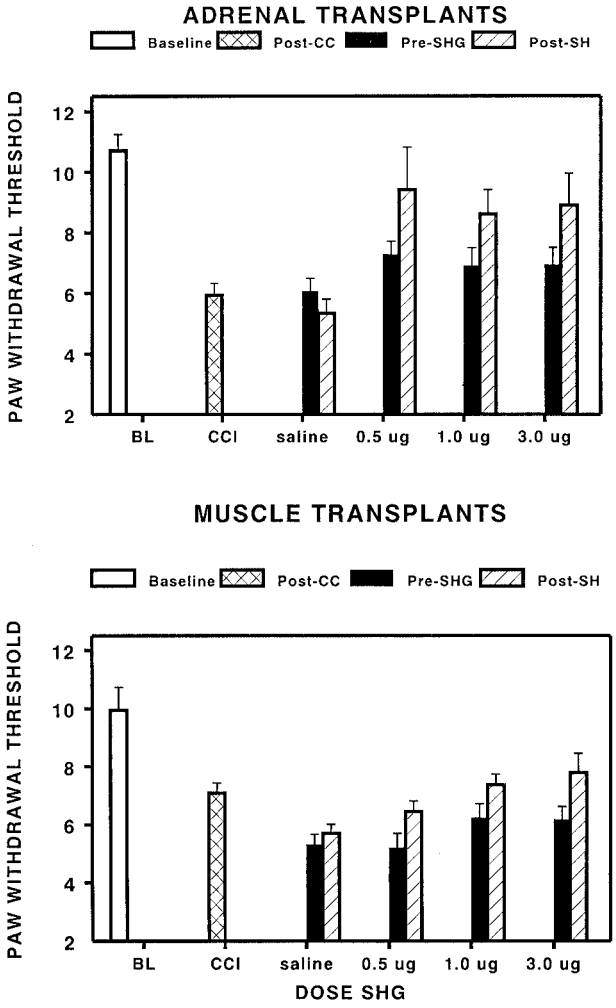

Figure 3. Effect of [Ser[1]]histogranin supplementation and adrenal medullary or striated muscle transplants in the spinal subarachnoid space on complete chronic constriction injury (CCI)-induced mechanical hyperalgesia. Baseline latencies (BL, open bars) of rats were evaluated prior to unilateral CCI, after CCI (cross-hatched bars), and before (Pre-SHG; solid bars) and after (Post-SHG, diagonal bars) intrathecal treatment with saline vehicle or SHG (0.5, 1.0, or 3.0 μg). Mean thresholds ± SEM in adrenal medullary (top panel, $n = 14$) or striated muscle (bottom panel, $n = 10$) transplanted animals are shown.

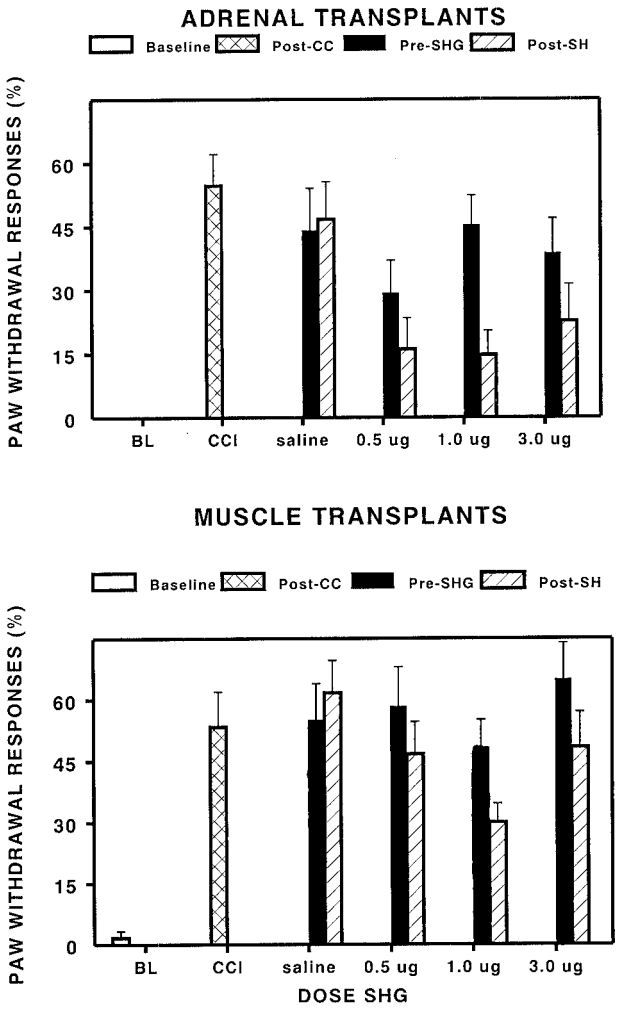

Figure 4. Effect of [Ser^1]histogranin supplementation and adrenal medullary or striated muscle transplants in the spinal subarachnoid space on complete chronic constriction injury (CCI)-induced cold allodynia. Baseline latencies (BL, open bars) of rats were evaluated prior to unilateral CCI, after CCI (cross-hatched bars), and before (Pre-SHG; solid bars) and after (Post-SHG, diagonal bars) intrathecal treatment with saline vehicle or SHG (0.5, 1.0, or 3.0 μg). Mean percent withdrawals (of five trials) ± SEM in adrenal medullary (top panel, $n = 14$) or striated muscle (bottom panel, $n = 10$) transplanted animals are shown.

SHG pretreatment did not significantly alter responses to acetone in animals with control transplants except at the 1.0 μg dose ($p > 0.01$ compared with CCI). The combination of adrenal medullary grafts and SHG supplementation was significantly more effective than combinations with striated muscle grafts in reducing cold allodynia [overall $F(1, 22) = 8.88$, $p < 0.001$].

Figure 5 shows an example of an adrenal medullary graft in the spinal subarachnoid space. Chromaffin cells were stained with tyrosine hydroxylase for identification. The morphology of this example is similar to that observed in previous studies in our laboratory (11,29,33, 35,40).

DISCUSSION

The aim of these studies was to evaluate whether supplementation with a naturally derived analgesic agent could be useful in enhancing the antinociceptive effects of adrenal medullary transplants. This approach is an initial screen for the long-term goal of engineering novel and more potent cell lines producing additional or higher levels of analgesic agents for chronic pain management. Results of the studies indicate that supplementation with SHG, a stable analog of NMDA antagonist peptide histogranin, can augment reduction of neuropathic and inflammatory pain in animals with adrenal medullary transplants in the spinal subarachnoid space.

A histogranin analog was selected, because this naturally occurring peptide has been shown to possess NMDA antagonist activity, a potentially useful pharmacologic supplement in chronic pain treatment, as NMDA antagonists can augment opioid analagesia, reduce tolerance development to opioids, and reduce the abnormal hyperalgesic components of neuropathic and inflammatory pain without altering normal pain sensitivity (20, 21,25–28). Previous findings in our laboratory have demonstrated that SHG can attenuate some symptoms of both neuropathic and inflammatory pain (9,32,34). An additional advantage of this peptide family is that histogranin is produced naturally in chromaffin cells; thus, the cellular machinery for proper processing and secretion should be available if SHG is selected for generation of more potent chromaffin cell lines.

The present studies confirmed the antihyperalgesic effects of SHG, as intrathecal SHG attenuated CCI-induced thermal hyperalgesia and formalin flinching in control transplanted animals as higher dose ranges were reached. In adrenal medullary transplanted animals, intrathecal SHG was significantly more potent in reducing both CCI-induced thermal hyperalgesia and formalin flinching, as effects were observed in the lower dose ranges, and nearly complete pain suppression could be achieved. In addition, attenuation of both CCI-induced mechanical hyperalgesia and cold allodynia, not achieved in the SHG dose ranges tested in the control transplanted group, was observed in animals with adrenal medullary transplants. Thus, the combination of adrenal medullary transplants supplemented with SHG provided a more complete suppression of the constellation of abnormal sensory symptoms in neuropathic and inflammatory pain.

The formalin model is a model for neurogenic inflammation that consists of two phases. The initial phase is thought to reflect acute pain responses due to the direct activation of nociceptors, while the second phase is

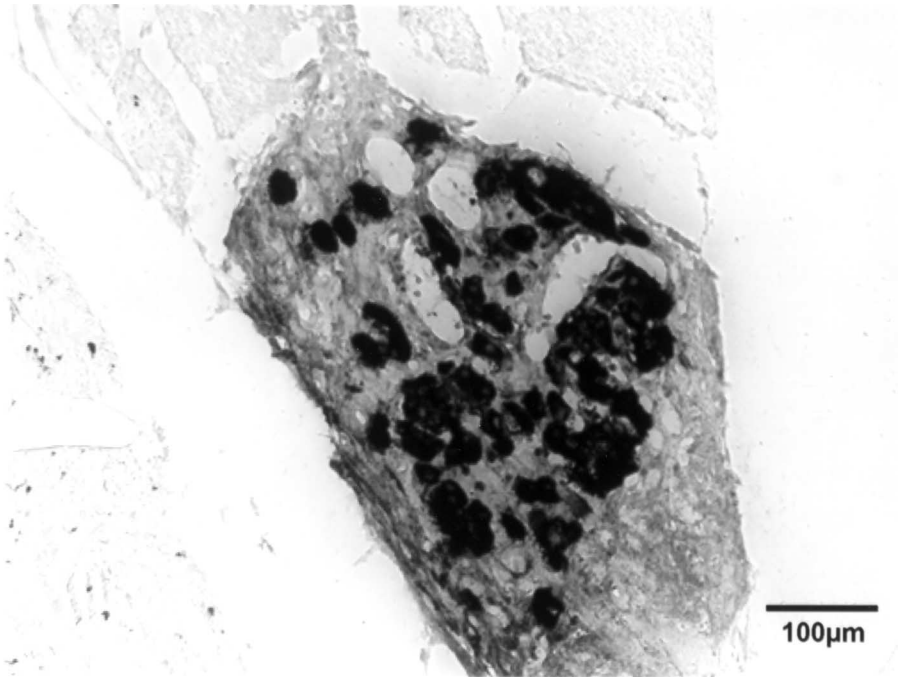

Figure 5. Appearance of adrenal medullary transplants in the spinal subarachnoid space. Healthy clusters of chromaffin cells are shown stained for tyrosine hydroxylase. Cells and clusters are interspersed with connective and vascular tissue.

thought to reflect tonic pain, which may involve central neuroplastic mechanisms developing during the quiescent interphase, modeling a progression to chronic pain. The second phase is thought to be initiated in part by activation of spinal NMDA receptors (4,8,43). Previous findings in our laboratory have demonstrated that intrathecal injection of SHG suppresses the second, but not the first, phase of the formalin test, and that adrenal medullary transplants also more potently and consistently suppress phase 2 in this model (33,34). Thus, it is not surprising that the combination nearly suppressed phase 2 flinching responses, while having only modest effects on phase 1. These findings suggest that this approach may be particularly useful in the treatment of the nonacute stages of chronic inflammatory pain.

Both adrenal medullary transplants and NMDA antagonists have also been shown to produce differing effects on thermal and mechanical hyperalgesia and allodynia, depending on the models and tests employed. In the present study, thermal hyperalgesia following peripheral nerve injury was nearly completely reversed by the combination of low doses of intrathecal SHG and adrenal medullary transplants, and reversed by higher doses of SHG in control transplanted animals. In contrast, the addition of SHG suppressed mechanical hyperalgesia in adrenal medullary transplanted animals, while producing only marginal nonsignificant effects in control transplanted animals in this dose range. Similarly, cold allodynia was markedly, although incompletely, suppressed by the combination of SHG and adrenal medullary transplants, while SHG in this dose range was only marginally effective in control transplanted animals. These findings suggest that neuropathic pain symptoms amenable to adrenal medullary transplants can be further enhanced by the addition of SHG, while symptoms only weakly affected by these transplants can be significantly improved by this addition.

It is interesting to note that pain responses were often incompletely suppressed, even at the highest doses of SHG employed, and that the highest doses were not substantially more potent than lower doses in animals with adrenal medullary transplants. A U-shaped dose–response effect of SHG has been noted in previous antinociceptive studies, with intermediate doses being the most potent, and suggested that SHG may act in a partial agonist fashion at the NMDA receptor (34). Regardless of mechanism, these findings indicate that maximum achievable pain suppression effects of this combination have been reached in this selected dose range, and that some aspects of chronic neuropathic and inflammatory pain are likely to be partially attenuated, but not completely suppressed.

In summary, the results of this study demonstrate that pain alleviation by adrenal medullary transplants can be

further enhanced by supplementation with stable peptide NMDA antagonist SHG. This combination can expand the range of chronic pain symptoms responsive to transplantation therapy as well as strengthen effects on amenable symptoms. A strategy to generate cell lines utilizing this combination may result in improved effectiveness and range in the treatment of chronic pain syndromes.

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