

Analgesia induced by isolated bovine chromaffin cells implanted in rat spinal cord

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Communicated by Dominick P. Purpura, June 9, 1986

ABSTRACT Chromaffin cells synthesize and secrete several neuroactive substances, including catecholamines and opioid peptides, that, when injected into the spinal cord, induce analgesia. Moreover, the release of these substances from the cells can be stimulated by nicotine. Since chromaffin cells from one species have been shown to survive when transplanted to the central nervous system of another species, these cells are ideal candidates for transplantation to alter pain sensitivity. Bovine chromaffin cells were implanted into the subarachnoid space of the lumbar spinal region in adult rats. Pain sensitivity and response to nicotine stimulation was determined at various intervals following cell implantation. Low doses of nicotine were able to induce potent analgesia in implanted animals as early as one day following their introduction into the host spinal cord. This response could be elicited at least through the 4 months the animals were tested. The induction of analgesia by nicotine in implanted animals was dose related. This analgesia was blocked by the opiate antagonist naloxone and partially attenuated by the adrenergic antagonist phentolamine. These results suggest that the analgesia is due to the stimulated release of opioid peptides and catecholamines from the implanted bovine chromaffin cells and may provide a new therapeutic approach for the relief of pain.

In the last several years, the possibility of successfully transplanting neural tissue to the central nervous system has renewed interest in the potential for neural implants to restore functional deficits (cf. refs. 1-3). A particularly promising source of tissue for transplantation is the adrenal medulla, since it contains cells of neural crest origin that synthesize and secrete neuroactive substances while circumventing the ethical problems involved with using fetal neuronal tissue. Adrenal chromaffin cells have been shown to survive for long periods of time when transplanted into the central nervous system and to have the ability to restore motor deficits in animals with lesions (1-5). Adrenal medullary homografts have been transplanted to the striatum of human Parkinson patients (6).

These results have opened up the exciting possibility for repair of damaged neuronal circuitry. In our laboratory, we are interested in the modulation of pain sensitivity. Since pain is not necessarily the result of damaged neuronal tissue, it is essential to establish the function of neural transplants in intact, nonlesioned animals.

Adrenal medullary chromaffin cells are ideal candidates for these transplantation studies since they contain and release several neuroactive substances implicated in the modulation of pain sensitivity in the central nervous system, including norepinephrine, epinephrine, methionine- and leucine-enkephalin (7-10), neurotensin (11), vasoactive intestinal polypeptide (12), and other neuropeptides (13). These studies suggest that some catecholamines and neuropeptides may be

co-stored and co-released by chromaffin cells (14). Another advantage of using these cells for transplantation studies is that the rate of release of neuroactive substances from chromaffin cells can be readily modified by common pharmacological agents such as nicotine.

The superficial dorsal horn of the spinal cord, where primary afferent fibers carrying nociceptive information terminate (15, 16), contains enkephalinergic interneurons and high densities of opiate receptors (17-20). Moreover, the intrathecal injection of morphine into the spinal subarachnoid space elicits potent analgesia (21). In addition to the influence of opioid peptides on spinal cord pain transmission pathways, catecholamines also appear to play an important role in pain modulation. Histochemical studies reveal a dense concentration of noradrenergic fibers in the superficial laminae of the spinal cord (22, 23). The intrathecal administration of norepinephrine or noradrenergic agonists produces analgesia (24), while the blockade of noradrenergic receptors in the spinal cord by antagonists (25, 26) or the depletion of spinal cord norepinephrine by neurotoxins (27, 28) produces increased sensitivity to noxious stimuli. Opioid peptides and catecholamines may act synergistically to produce their effects, with the maximum effect dependent on the coactivation of both systems in the spinal cord (29).

Thus, the transplantation of adrenal medullary tissue into the spinal cord may provide the ideal combination of neuroactive substances for the relief of pain. Moreover, the degree of release of these substances could be modulated by nicotine. We have reported that analgesia could be induced by the transplantation of rat adrenal medullary tissue into the rat spinal cord (30). One of the disadvantages of transplanting solid tissue pieces of adrenal medulla is that the graft contains a heterogeneous cell population. The adrenal medulla contains several cell types besides chromaffin cells, including endothelial cells, fibroblasts, and ganglionic cells. To circumvent these variables, a homogeneous preparation of chromaffin cells can be used. Furthermore, since the survivability of the graft depends on its ability to obtain nutrients from the host environment, it is likely that cell suspensions will be better able to survive the initial phases. Finally, due to the relative immunological protection of the central nervous system (31-34), it is possible to implant chromaffin cells from other species (5) that produce greater amounts of opioid peptides. The purpose of the present study was to determine the potential for implants of bovine chromaffin cells in the spinal cord to produce alterations in pain sensitivity and to examine the characteristics of this response. A preliminary account of these experiments has been reported (35).

MATERIALS AND METHODS

In all of the following studies, male Sprague-Dawley-derived rats weighing 300-350 g served as hosts. Pain sensitivity was measured in these animals using three standard analgesio-metric tests sequentially: the tail-flick test (36), the paw-pinch test, and the hot-plate test (37). To elicit the tail-flick

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response, a focused beam of high intensity light is applied to the dorsal surface of the rat's tail. The time interval between the onset of the stimulus and the tail-flick response is measured at three regions of the tail, the average of which is defined as the "tail-flick latency." To prevent tissue damage in the absence of a response, the stimulus is terminated at 14 sec, and the tail-flick latency is assigned a value of 14. The paw-pinch response is elicited by a commercially available apparatus (Ugo-Basile) that applies pressure at a constant rate of 64 g/sec. The force is applied to the ventral surface of both hind paws sequentially until the animal reacts by a withdrawal response. The hot-plate response is determined by placing the rat on a 55°C copper plate enclosed in a plexiglass cylinder. The interval between placement on the hot plate and the response of either licking the hind paws or jumping off the plate is defined as the "hot-plate latency." In the absence of a response, the animal is removed after 40 sec and assigned a hot-plate latency of 40. Thus, thermal and mechanical pain stimuli were employed, as well as reflexive and integrated pain responses.

In all of the following studies, animals were initially screened for baseline pain sensitivities and pain sensitivities following a low dose of nicotine (free base, 0.1 mg/kg, s.c.). The cells were obtained from the adrenal glands of steers or cows as described by Pollard *et al.* (38). The resultant preparation from 7–9 glands contains $0.5\text{--}1.0 \times 10^9$ chromaffin cells and is essentially free of other cell types. Suspensions of primary cultures of bovine chromaffin cells were shipped in air-tight containers of culture media the day after preparation. For implantation, cells were concentrated by centrifugation and resuspended in small volumes of Hank's buffer containing 2.5S nerve growth factor (0.1 $\mu\text{g}/\text{ml}$) and kept on ice until they were placed in the rat spinal cords. The cells were injected via an intrathecal catheter according to a modification of the technique of Yaksh and Rudy (39). Under ether anesthesia, a small incision was made in the dura overlying the atlanto-occipital junction, a catheter made of polyethylene (PE 10) tubing was threaded through the incision into the subarachnoid space and down the spinal cord to the level of the lumbar enlargement. Cell suspensions were injected through the catheter in 15- μl volumes over 20–30 sec, followed by a 10- μl flush with Hank's buffer. Each animal received approximately 100,000 cells (counted in a hemocytometer). Cell viability was determined at the end of the surgical procedures by trypan blue exclusion to be 80–90%. Control animals received an equal volume of either heat-killed cells or Hank's buffer containing nerve growth factor.

Animals that exhibited motor abnormalities following surgical procedures were discarded from the study. The remaining animals were returned to their cages and allowed free access to food and water. They were tested for pain sensitivities according to the protocols described in the following sections.

Experiment 1. Initially, animals were tested 6–8 weeks following cell implantation, since this had been determined to be sufficient for establishment of behavioral responses (40). Pain sensitivity was assessed for implanted and control animals by the three analgesiometric tests. The animals then received an injection of nicotine (0.1 mg/kg, s.c.) and were tested again 2, 10, 20, and 30 min later.

Experiment 2. To determine the potential for long-term changes in pain sensitivity, another group of animals with identical implants was tested for pain responsiveness before and after nicotine at several time intervals following the implantation procedures. Rats were tested at 1 day, 1 week, 2 weeks, 4 weeks, 8 weeks, and 16 weeks after receiving cell or control implants.

Experiment 3. To determine the sensitivity of chromaffin cell implants to nicotine, another group of implanted animals

received several doses of nicotine at weekly intervals on a rotating dose schedule, such that one-third of the animals received each of the doses on each test day. The doses of nicotine used were 0.05 mg/kg, 0.1 mg/kg, and 0.2 mg/kg.

Experiment 4. Since the analgesia induced by stimulation of chromaffin cells may be due to the release of neuroactive substances from chromaffin cell granules, it was of interest to determine the contribution of catecholamines and opioid peptides to this response. Rats with spinal cord bovine chromaffin cell implants received an injection of opiate antagonist naloxone (2 mg/kg, s.c.), α -adrenergic antagonist phentolamine (10 mg/kg, s.c.), or saline vehicle 5–10 min before the nicotine injection. These antagonist doses were chosen since they do not produce any alterations in pain sensitivity.

Statistical analysis was done using two-way analysis of variance and the Newman-Keuls test for multiple post hoc comparisons (41).

RESULTS

Prior to the implantation of chromaffin cells, nicotine (0.1 mg/kg) did not produce any alterations in pain sensitivity as assessed by the tail-flick, paw-pinch, or hot-plate tests. In contrast, the injection of nicotine induced potent analgesia in animals with spinal cord chromaffin cell implants ($P < 0.01$ for all three tests). This is shown in Fig. 1. The peak increase in pain threshold was at 2 min following the nicotine injection. Both tail-flick latency and paw-pinch threshold remained elevated for 20 min, tending toward baseline levels by 30 min, while hot-plate latencies returned to baseline by 20 min. The injection of nicotine had no significant effect on pain sensitivity in animals with control implants. Since there was no difference between control animals with heat-killed cell implants or buffer, the data from both of these groups was pooled.

The ability of nicotine to induce analgesia in implanted animals was tested at several intervals over a 16-week period. Results are summarized in Fig. 2. Since this dose of nicotine did not significantly alter pain sensitivity at any time in control animals ($P > 0.05$), the data for these animals are omitted for clarity. Analgesia induced by nicotine stimulation could be observed as early as 1 day following cell injection. However, at this time, the difference between the pre- and post-nicotine response latencies were smaller than at other time points, particularly for the tail-flick and paw-pinch tests. An explanation for this is that the baseline pain sensitivities (pre-nicotine) were higher at 1 day following cell implantation than at other times during the study. Compared to the pre-implantation pain sensitivities, tail-flick latency was elevated from 3.2 ± 0.4 sec to 5.4 ± 0.6 sec and paw-pinch threshold from 10.5 ± 0.5 to 13.1 ± 0.7 . These differences were statistically significant ($P < 0.05$).

The ability to induce analgesia with nicotine in transplanted animals was well maintained at least through 4 months. The differences between the pre- and post-nicotine pain sensitivities were statistically significant at all the tested time points for all three tests ($P < 0.01$). However, there appeared to be a slight decrement in response toward the end of the study, although this was not significant.

The sensitivity of the implanted chromaffin cells to low doses of nicotine was determined by using several doses of nicotine. Results are illustrated in Fig. 3. The lowest dose of nicotine, 0.05 mg/kg, produced a small, but statistically significant elevation in tail-flick latency in animals with spinal cord bovine chromaffin cell implants ($P < 0.05$). This dose also appeared to produce an increase in paw-pinch threshold and hot-plate latency, but these were not statistically significant. At the highest dose of nicotine (0.2 mg/kg), the elevations in all three tests were nearly maximal (91%

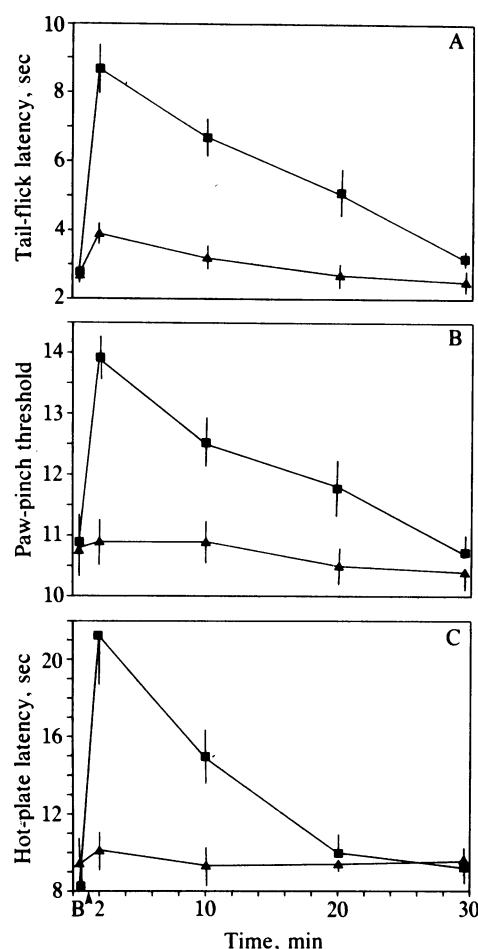


FIG. 1. Effect of spinal cord bovine chromaffin cell implants on pain sensitivity. The ordinate is the threshold for response to noxious stimuli as measured by the tail-flick test (A), paw-pinch test (B), and hot-plate test (C). Each point represents the mean \pm SEM. The abscissa is the time course of responses in min to noxious stimuli following nicotine stimulation. Time 0 indicates the preinjection values. The arrowhead indicates the point at which nicotine (0.1 mg/kg, s.c.) was injected. Symbols: squares, animals with bovine chromaffin cell implants in the spinal cord ($n = 14$); triangles, animals with control implants in the spinal cord ($n = 10$).

maximum tail-flick latency and 92% maximum paw-pinch threshold). However, at this dose, there was also a small but significant elevation in the pain threshold of control animals.

To determine the contribution of catecholamines and opioid peptides to the analgesia induced by nicotine in implanted animals, a group of animals with spinal cord bovine chromaffin cell implants were pretreated with either opiate antagonist, naloxone, adrenergic antagonist phentolamine, or saline vehicle. These preinjections did not alter pain sensitivity as determined 10 min after the injection (not shown). The injection of nicotine (0.1 mg/kg) in saline-pretreated animals resulted in the usual induction of analgesia (Fig. 4). In contrast, this analgesia was severely attenuated in animals pretreated with naloxone as assessed by all three analgesiomeric tests ($P < 0.01$). Phentolamine pretreatment completely blocked the elevation in hot plate latency ($P < 0.01$) and appeared to partially attenuate the elevation in tail-flick latency and paw-pinch threshold, but these were not statistically significant.

DISCUSSION

Results of this study indicate that it is possible to alter pain sensitivity by implanting chromaffin cells into the subarach-

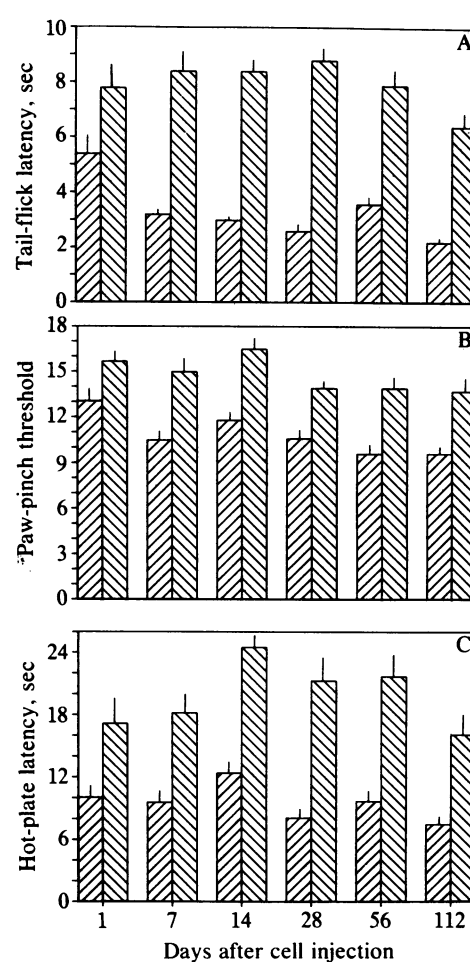


FIG. 2. Long-term changes in pain responsiveness in animals with spinal cord bovine chromaffin cell implants. The ordinate is the threshold for response to noxious stimuli as determined by the tail-flick test (A), the paw-pinch test (B), and the hot-plate test (C). The bars represent the mean \pm SEM for each measurement ($n = 15$ animals). The ordinate is the time after chromaffin cell implantation. Each set of bars represents the response latencies before (right-hatched bars) and 2 min after (left-hatched bars) nicotine injections (0.1 mg/kg, s.c.) in implanted animals. Since nicotine had no effect on pain sensitivity in animals with control implants at any of these times, these values are omitted for clarity.

noid space of the spinal cord. The ability of low doses of nicotine to induce analgesia in implanted animals suggests that the chromaffin cells survive and retain their functional ability to respond to nicotinic stimulation by releasing neuroactive substances. This analgesia can be induced by chromaffin cells as early as 1 day following transplantation. However, the pain threshold of these animals this soon after the implantation is slightly elevated compared to animals with control implants. The most likely explanation for this result is that the cells are initially releasing large amounts of their granular contents into the subarachnoid space following the trauma of manipulation during implantation. Since this elevated pain threshold is not observed at longer time periods following the implantation, these results suggest that either there is minimal basal (i.e., nonstimulated) release of neuroactive substances from the chromaffin cells or that spinal cord receptors become tolerant to the low level of catecholamines and neuropeptides spontaneously released by chromaffin cells.

The changes brought about by the implanted cells appear to be maintained for at least 4 months since nicotine could still induce analgesia at this time. This suggests that neural tissue

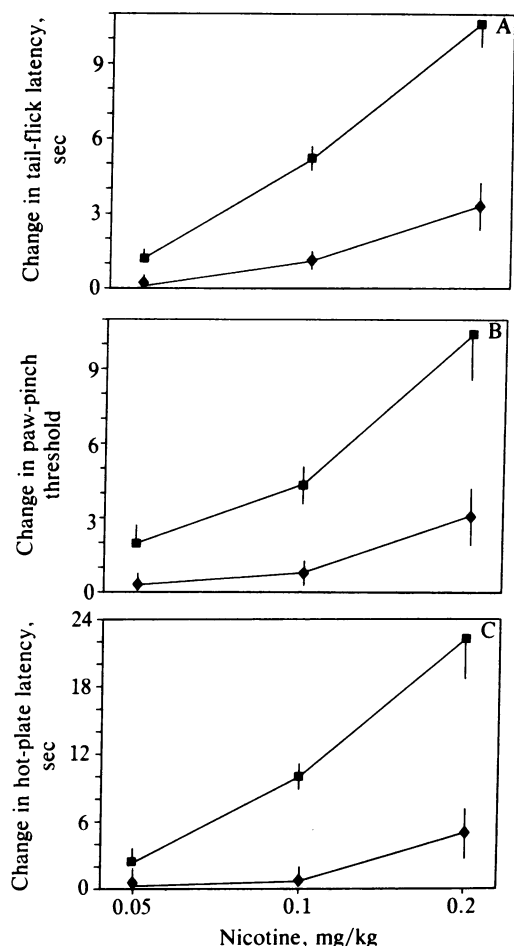


FIG. 3. Dose-response relationships for the effect of nicotine on pain sensitivity in animals with spinal cord bovine chromaffin cell implants. Ordinate is changes in nociceptive threshold for tail-flick test (A), paw-pinch test (B), and hot-plate test (C). Values were obtained by subtracting the prenicotine response latencies from the latencies determined 2 min following nicotine injections. Each point represents the mean \pm SEM. Abscissa is nicotine doses (mg/kg) plotted on a logarithmic scale. Symbols: squares, animals with bovine chromaffin cell implants in the spinal cord ($n = 9$); diamonds, animals with control implants in the spinal cord ($n = 8$).

transplanted across species may provide a long-term therapeutic approach to neurological disorders. However, there did appear to be a slight decrement in the responsiveness to nicotine toward the latter part of the study, suggesting that some of the implanted cells may ultimately die. One possible explanation for this may be an immunological rejection, since plasma cells can be found infiltrating the transplants (40). In support of this possibility, preliminary results show that transplanted animals receiving the immunosuppressant cyclosporin appear to be more responsive to lower doses of nicotine.

The response to nicotine stimulation is dose related, suggesting that the implants are responding to higher doses with an increased release of chromaffin granule content. At the highest dose of nicotine, the increases in pain threshold were nearly maximal for the tests employed. However, there was also a slight increase in the pain threshold of control animals. This is not surprising, since nicotine in higher doses (0.5–2.0 mg/kg) has been shown to produce antinociception (42, 43). However, it does complicate the interpretation of the effect of transplants on pain sensitivity, since the relative contribution of nicotine itself to the analgesia must be considered. Therefore, the intermediate dose of 0.1 mg/kg

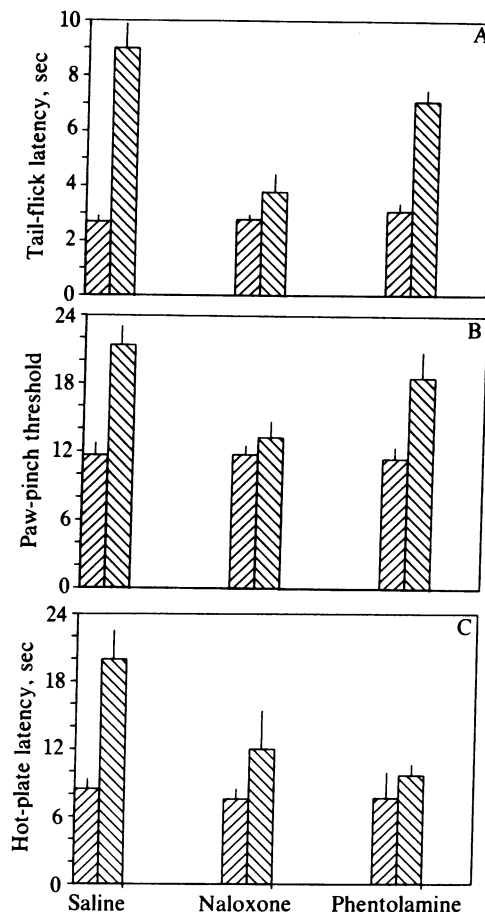


FIG. 4. Effect of antagonists on the analgesia induced by nicotine in animals with spinal cord bovine chromaffin cell implants. The ordinate is the threshold for response to noxious stimuli as determined by the tail-flick test (A), the paw-pinch test (B), and the hot-plate test (C). Each bar represents the mean \pm SEM ($n = 7$). The bar on the left in each set is the pain threshold measured 5 min following the injection of saline, naloxone (2 mg/kg, s.c.), or phentolamine (10 mg/kg, s.c.). The preinjection values are not shown, since the antagonists did not alter these response latencies. The bar on the right in each set is the response to nicotine (0.1 mg/kg, s.c.), injected 5 min after the pretreatment with antagonists.

was determined to be optimal for these studies, since it does not alter pain sensitivity in control animals.

The ability of naloxone to block the analgesia normally induced by nicotine in the implanted animals supports the notion that this analgesia is the result of the release of opioid peptides from the transplanted chromaffin cells. This result is supported by studies that showed that the analgesia induced by nicotine in rats with rat adrenal medullary solid tissue transplants could be completely reversed by naloxone (30). The partial attenuation by phentolamine suggests that catecholamine release may also be involved. The contribution of other neuropeptides in the chromaffin cells has not been determined. It is possible that the corelease of two or more pharmacologically active agents (such as norepinephrine and enkephalin) from implanted chromaffin cells would act synergistically to produce their effects. The synergistic action of opiates and catecholamines in the intrathecal induction of analgesia has been suggested (29).

The results of these studies demonstrate that it is possible to induce analgesia by implanting chromaffin cells into the spinal cord. This is potentially clinically useful, since it would provide a local and readily available source of opioid peptides and catecholamines for the relief of pain. One aspect that requires further study is the question of tolerance, with

respect to nicotine's ability to stimulate release of neuroactive substances from chromaffin cell granules and with respect to spinal cord sensitivity to repeated release of opioid peptides and catecholamines from chromaffin cells. Interestingly, it has been demonstrated that while there is significant tolerance to analgesic potency with repeated morphine injections intrathecally, the concurrent injection of lower doses of morphine and noradrenergic agonist produces no decrement in analgesic potency (21). Since chromaffin cells have been shown to co-release catecholamines and opioid peptides (14), they may provide an ideal combination for avoiding the development of tolerance. Furthermore, Eiden *et al.* (7) have shown that continued exposure of chromaffin cells to nicotine results in methionine-enkephalin mRNA induction and increased methionine-enkephalin synthesis, an effect that may offset a possible tolerance effect to repeated injections of nicotine. Preliminary work in our laboratory suggests that analgesia can be elicited by nicotine injections at daily intervals in animals with chromaffin cell implants.

In summary, results of this study indicate that adrenal chromaffin cells transplanted into the subarachnoid space of the spinal cord can reliably produce analgesia when stimulated by nicotine. This is most likely due to the stimulated release of neuroactive substances from the chromaffin cell granules. Thus, these cells can provide a local supply of opioid peptides and catecholamines, readily available on demand. This technique may provide a new therapeutic approach for the relief of intractable pain.

We are indebted to Dr. Herbert K. Proudfit for his generous support and advice and for lending us his analgesiometric equipment. This work was supported, in part, by Award HHS 1-F32 NS07630 from the National Research Service and by Research Grant GM 37326 from the Department of Health and Human Services.

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