Transplantation of Hypocretin Neurons into the Pontine Reticular Formation: Preliminary Results

Oscar Arias-Carrión, MA[2]; Eric Murillo-Rodriguez, PhD[1]; Man Xu, MD[1]; Carlos Blanco-Centurion, PhD[1]; Rene Drucker-Colín, MD[2]; Priyattam J. Shiromani, PhD[1]

[1]*West Roxbury VA Medical Center and Harvard Medical School, Massachusetts, USA;* [2]*Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, México*

Study Objectives: The sleep disorder narcolepsy is now considered a neurodegenerative disease because there is a massive loss of neurons containing the neuropeptide, hypocretin, and because narcoleptic patients have very low cerebrospinal fluid levels of hypocretin. Transplants of various cell types have been used to induce recovery in a variety of neurodegenerative animal models. In models such as Parkinson disease, cell survival has been shown to be small but satisfactory. Currently, there are no data indicating whether hypocretin neurons can survive when grafted into host tissue. Here we examined the survival of hypocretin-containing neurons grafted into the pontine reticular formation, a region traditionally regarded to be key for rapid eye movement sleep generation.

Design: In 2 experiments, a suspension of cells from the posterior hypothalamus of 8- to 10-day old rat pups was injected into the pons (midline, at the level of the locus coeruleus) of adult rats. Control rats received cells from the cerebellum, tissue that is devoid of hypocretin neurons. In the first experiment (n = 33), the adult rats were sacrificed 1, 3, 6, 12, 24, or 36 days after transplant, and cryostat-cut coronal sections of the brainstem were examined for presence of hypocretin-immunoreactive neurons. In the second experiment (n = 9), the transplant medium was modified to include agents that stimulate cell growth, and recipient rats were sacrificed 9, 12, and 36 days after receiving the graft.

Settings: Basic neuroscience research laboratory.

Measurements and Results: In the first experiment, clearly defined hypocretin-immunoreactive containing somata and varicosities were visible in pons of rats sacrificed 1 day after grafting of posterior hypothalamic cells but not in rats receiving cerebellum tissue. The hypocretin-immunoreactive somata were not visible in rats sacrificed at 12, 24, or 36 days, indicating that the neurons had died. However, in the second experiment, where enriched transplant medium was used, clearly defined hypocretin-immunoreactive somata with processes and varicosities were present in the graft zone 36 days after implant. These somata were similar in size and appearance to adult rat hypocretin-immunoreactive neurons.

Conclusions: These results indicate that hypocretin neurons obtained from rat pups can be grafted into a host brain, and efforts should be made to increase survival of these neurons.

Key Words: Narcolepsy, hypocretin, grafted, sleep, lateral hypothalamus

Citation: Arias-Carrión O; Murillo-Rodriguez E; Xu M et al. Transplantation of hypocretin neurons into the pontine reticular formation: preliminary results. *SLEEP* 2004;27(8):1465-70.

INTRODUCTION

NARCOLEPSY IS A SLEEP DISORDER CHARACTERIZED BY EXCESSIVE DAYTIME SLEEPINESS, CATAPLEXY, HYPNAGOGIC HALLUCINATIONS, AND SLEEP-ONSET RAPID EYE MOVEMENT (REM) SLEEP PERIODS (FOR REVIEW SEE[1]). Narcolepsy is now identified to be a neurodegenerative disease because there is a massive loss of specific neurons in the brain.[2,3] These neurons contain the neuropeptides hypocretin-1 (HCRT-1) and hypocretin-2 (HCRT-2), which are also known as orexin-A and orexin-B.[4,5] Patients with narcolepsy have a severe reduction in the levels of HCRT in the cerebrospinal fluid,[6] a finding consistent with the HCRT neuronal loss. Rats with lesions of the HCRT neurons also show a decline in cerebrospinal fluid (CSF) HCRT levels.[7] The neurons containing HCRT are located only in the perifornical region of the posterior hypothalamus from where they project to major arousal centers.[8] In narcolepsy, it is not known which HCRT innervation to which target site is responsible for the disease, although extensive research based on lesion, pharmacologic, and electrophysiologic studies suggests that the HCRT innervation to the pontine reticular formation may be key to the phenomenology of the disease.[9,10]

Grafting is a procedure that has been used to reverse disease[11] and is defined as implantation of living neuronal or nonneuronal tissue into a host system. Several studies have demonstrated that grafted tissue survives, integrates within the host brain, and provides functional recovery following brain injury in various animal models.[11-16]

Currently, there is no evidence that a graft of cellular tissue containing HCRT neurons can survive if grafted into a host brain. Therefore, the purpose of this study was to undertake a preliminary evaluation of the survival of grafted HCRT neurons into a region of the brain that is heavily innervated by HCRT axons but where the HCRT somata are normally not present.

Disclosure Statement

This is not an industry supported study. Drs. Arias-Carrion, Murillo-Rodriguez, Xu, Blanco-Centurion, Drucker-Colin, and Shiromani have indicated no financial conflicts of interest.

Submitted for publication April 2004

Accepted for publication August 2004

Address correspondence to: Priyattam J. Shiromani, PhD, Harvard Medical School & VA Medical Center, Bldg 3 Room 2C109, 1400 VFW Parkway, West Roxbury, MA 02132; Tel: (617) 323-7700 X6162;
E-mail: pshiromani@hms.harvard.edu

MATERIAL AND METHODS

Experiment 1

Subjects

Male Sprague-Dawley rats (Charles River, Mass) weighing 200-250 g were housed at a constant temperature (21°C ± 1°C) and under a controlled light-dark cycle (lights on: 7:00 AM-7:00 PM). Food and water were provided ad libitum.

Neonatal Transplantation Procedure

Male Sprague-Dawley pups (8-10 days old) were anesthetized (pentobarbital) and sacrificed by decapitation. A cell suspension of posterior hypothalamic tissue that contained HCRT neurons was prepared from the brains of the rat pups. Control implants consisted of cerebellum tissue (cerebellum vermis at the level of lobule VIII, control rats) where no HCRT neurons are located. Immediately the rats were decapitated, the brain was placed (ventral surface up) in a brain matrix (Ted Pella, Inc, Redding, CA 96049-2477) immersed in cold artificial CSF with a continuous flow of oxygen. A single-edged razor blade was used to make a coronal incision posterior to the optic chiasm. A second razor blade was placed anterior to the pituitary gland. The coronal tissue within the 2 razor blades was gently lifted out of the tissue matrix and placed in a Petri dish covered with ice. A dissecting microscope was used to identify the ventral edge of the fornix and a rectangular section (2.0 mm × 1.0 mm × 2.0 mm) was cut out from both hemispheres using a scalpel. A similar volume of tissue (2.0 mm × 1.0 mm × 2.0 mm) was dissected from the cerebellum (cerebellum vermis at the level of lobule VIII) where no HCRT neurons are located.

The tissue was immersed in ice-cold culture medium, which consisted of DMEM (GIBCO, Invitrogen Corp, Carlsbad, Calif) supplemented with 10% fetal bovine serum (Sigma; A-7030), 10 mM HEPES, 100U/mL Penicillin-Streptomycin (Sigma; P3539) and 250 µL antibiotic-antimycotic (Sigma; A7292). Tissue was mechanically dissociated using a 1-mL Eppendorf pipette until a uniform cell suspension was made. Then, tissue was centrifuged (600 rpm) for less than 2 minutes. The supernatant was discarded, and the pellet was collected into a stainless-steel cannula (tip diameter = 150 µm, 23-gauge) connected to a 10-µL Hamilton syringe. This procedure is described in detail elsewhere.[17] Tissue from 1 donor pup was grafted into 1 host rat. The tissue was grafted into a host rat within 5 minutes after extraction.

Host rats (weighing 200-300g) were placed in the stereotaxic apparatus under deep anesthesia (cocktail of acepromazine [0.75 mg/kg], xylazine [2.5 mg/kg], and ketamine [22 mg/kg] administered intramuscularly). A stainless-steel cannula connected to a 10-µL Hamilton syringe was lowered to the injection site in the pons (coordinates: AP = -9.1mm; L = 0 mm; V = -8.0 mm; Paxinos and Watson[18]). The above DMEM solution (4 µL) containing the cell aggregate of posterior hypothalamus or cerebellum tissue (control group) was injected at a flow rate of 1 µL/minute. All rats were treated in accordance with American Association for Accreditation of Laboratory Animal Care policy on care and use of laboratory animals. All efforts were made to minimize animal suffering and to minimize the number of animals in the experiments. Operations were performed under aseptic conditions.

The host animals were sacrificed 1, 3, 6, 12, 24, and 36 days after the transplants with a lethal dose of pentobarbital (intraperitoneal) and perfused transcardially with 150 mL of saline solution (0.9%) followed by 200 mL of ice-cold 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.2-7.4. The brains were removed and post fixed overnight in the same fixative at 4°C and immersed in 30% sucrose in phosphate buffered solution for 48 hours. Coronal sections of brains (30-µm thickness) were cut on a cryostat and thaw mounted onto gel-coated slides (1:5 serial orders).

To confirm the presence of HCRT neurons in the donors, a group of rat pups (n = 3) were sacrificed with an overdose of pentobarbital, and the brain was fixed with formalin. Coronal brain sections (30-µm thickness) were processed for visualization of HCRT neurons in the perifornical area as described below.

Antibodies

Tissue from all of the rats was processed at the same time in 2 batches to control for staining. Goat anti-HCRT-1 (1:10,000; Santa Cruz Biotechnology, Calif) was used. The tissue sections were processed for HCRT-1 labeling as described previously.[7,19] The slides were coded so that the inspection of the graft zone was done in a blind manner. The code was broken once the sections were examined. Three investigators independently inspected the pontine area where the cells were implanted for presence of HCRT-immunoreactive (HCRT-ir) somata in the brains of host animals, and there was strong agreement between them.

Experiment 2

Subjects

Male Wistar rats weighing 250 to 300 g at the beginning of the experiments were used as recipients. Donor tissue was from Wistar rat pups that were 8 to 10 days old. The animals were housed under a controlled cycle (12-hour light:12-hour dark cycle, light on at 7:00 AM) and controlled room temperature (22°C ± 1°C). Food and water were provided ad libitum. Wistar rats were selected because it is an inbred strain that may promote graft survival relative to the outbred Sprague-Dawley rats where tissue rejection might be worse [20].

Neonatal Transplantation Procedure

The posterior hypothalamus was dissected as in Experiment 1. However, preparation of the cell suspension differed in that tissue fragments were enzymatically dissociated in Spinner's saline solution with 2 mg per mL of collagenase (Worthington, type I. Sigma; M1802) and 15 mg per mL of deoxyribonuclease (Type II) (Sigma; D8764) for 45 minutes at 37°C. Dissociated cells were kept in culture medium (Dulbecco's modified eagle media: DMEM; GIBCO), supplemented with 4.5 µg insulin (Sigma; I5500), 100 U/mL penicillin (Sigma; P3539), 100 mg/mL streptomycin and 2.5 mg/mL fungizone [GIBCO]) until their transplant. Then, tissue was centrifuged (600 rpm) for < 2 minutes.

Host rats were placed in the stereotaxic apparatus under deep anesthesia (cocktail of acepromazine [0.75mg/kg], xylazine [2.5 mg/kg], and ketamine [22mg/kg] administered intramuscularly), and the cells were aspirated into a transparent glass micropipette with a tip diameter (120 µm) attached to a 25-µL Hamilton syringe mounted in a manually driven microinjector (Kopf Instruments, Tujunga, CA 91042).

The stereotaxic coordinates employed were as in Experiment 1. The above DMEM solution (4 µL) containing a cell aggregate of perifornical hypothalamic or cerebellum tissue (control group) was injected at a flow rate of 1 µL per minute. The host animals were sacrificed 9, 12, or 36 days after the transplants with a lethal dose of pentobarbital (intraperitoneal) and perfused transcardially as described in Experiment 1.

In this preliminary study, no attempt was made to count the

number of HCRT-ir neurons that were aspirated into the cannula and initially implanted, as cells can only be used for counting or grafting, but it is not feasible to do both.

RESULTS

In Experiment 1 there was clear evidence of HCRT-ir somata in the graft zone of rats sacrificed 1 day (rats = 4) after receiving tissue from the posterior hypothalamus. These somata were smaller (see Figure 1B) compared to the size of the somata in the donor pups (Figure 1A). On posttransplant day 3 (rats = 9) and day 6 (rats = 10), the HCRT-ir somata that were visible appeared round without processes (Figure 1C). Such somata were not visible on days 12 (rats = 3), 24 (rats = 4), or day 36 (rats = 3). Thus, by day 12, grafted HCRT neurons were not evident.

Experiment 2 was conducted to determine whether modification of the culture media could improve survivability of the grafted neurons. Rats receiving tissue from the perifornical area of the hypothalamus showed HCRT-ir somata on day 9 (rats = 3) that were round without any visible processes (Figure 2A). Some of the somata, although smaller in size, had a morphology that was similar to healthy HCRT neurons in donor pups (see Figure 2B). There was evidence of HCRT cell death because the cell membrane was ruptured and the HCRT-ir was found in small clusters (Figure 2A). By day 12 (rats = 3), we noticed HCRT-ir somata with a swollen nucleus and thin cytoplasm (Figure 2C), suggesting that these HCRT neurons might not be healthy and therefore dying. The presence of any HCRT-ir somata on day 12 is in sharp contrast to Experiment 1 in which no such somata were visible past day 12.

On day 36 (rats = 3), we noticed HCRT-ir somata that had a size and morphology similar to adult HCRT neurons (see Figure 3). These HCRT-ir neurons had clearly established processes in and around the graft zone and were evident in all 3 host rats (Figure 3). HCRT-ir somata with a swollen nucleus or ruptured membrane were not visible on day 36. In all recipient rats, HCRT-ir somata, both round and normal looking, were present within the graft zone.

Rats that received grafts of tissue from the cerebellum showed no HCRT-ir somata in the pons (Figure 1D).

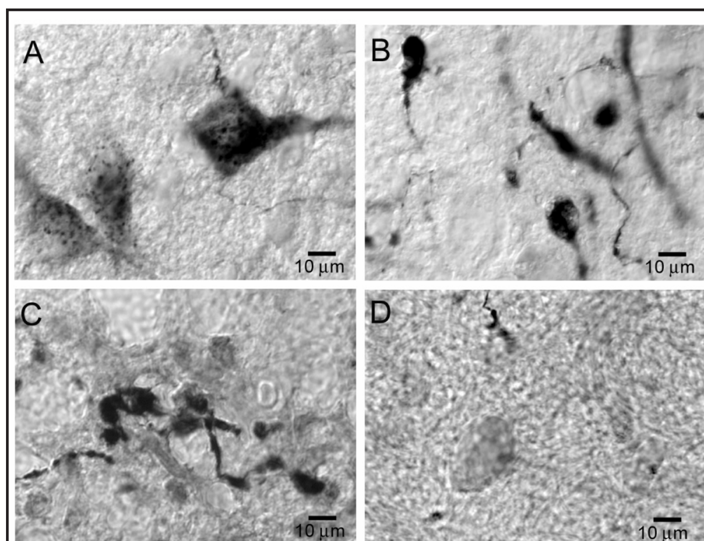

Figure 1—Hypocretin-immunoreactive (HCRT-ir) neurons in Experiment 1. Representative HCRT-ir neurons in rat pups are shown in panel A. A cell suspension containing HCRT-ir neurons was placed in the pons of adult rats and examined 1 day (panel B) or 6 days (panel C) after grafting. HCRT-ir neurons were not evident when cerebellum tissue was grafted into the pons (panel D). The HCRT-ir neurons present 1 day after grafting were round and smaller than in the donor rat pups (panel A versus panel B or C).

DISCUSSION

The results of this preliminary experiment provide evidence that HCRT neurons can be grafted into the pons and survive in an area where such neurons are normally not present. Very few

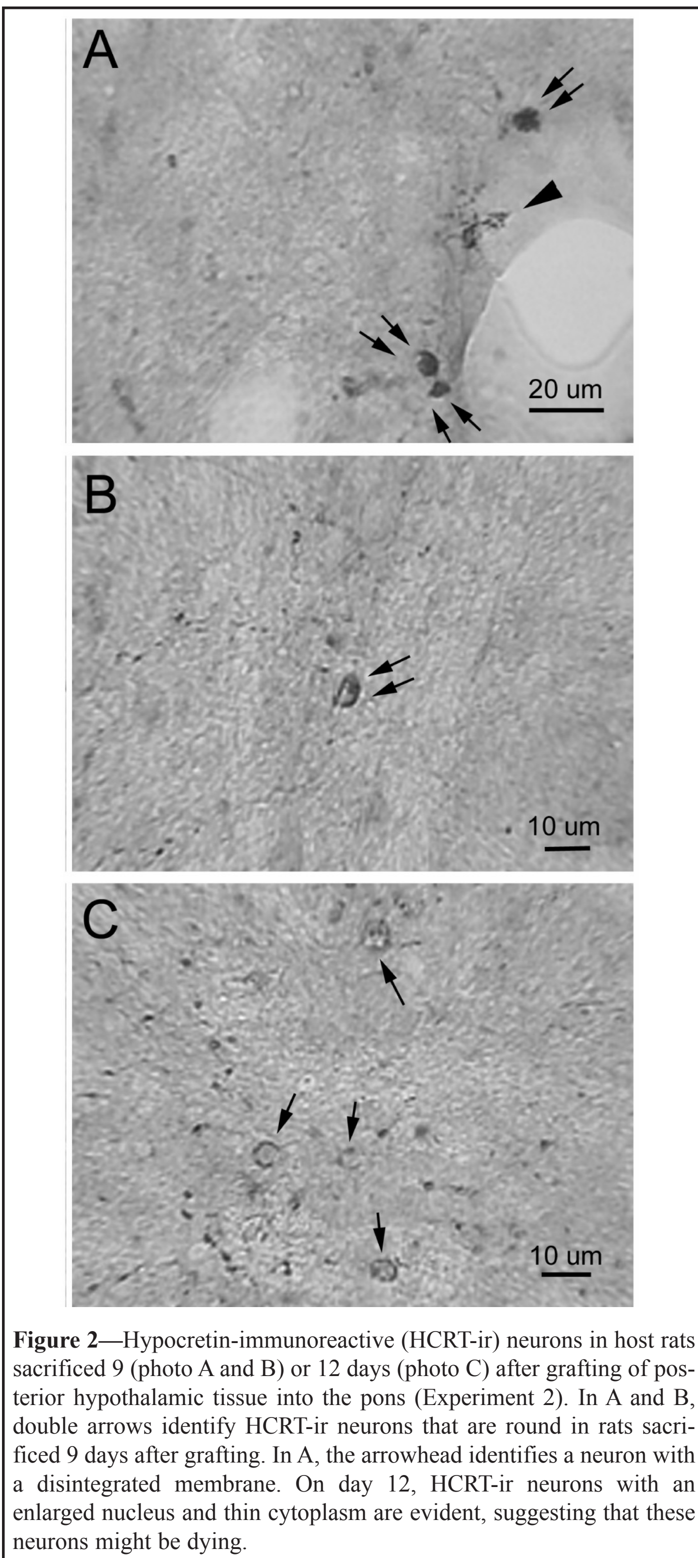

Figure 2—Hypocretin-immunoreactive (HCRT-ir) neurons in host rats sacrificed 9 (photo A and B) or 12 days (photo C) after grafting of posterior hypothalamic tissue into the pons (Experiment 2). In A and B, double arrows identify HCRT-ir neurons that are round in rats sacrificed 9 days after grafting. In A, the arrowhead identifies a neuron with a disintegrated membrane. On day 12, HCRT-ir neurons with an enlarged nucleus and thin cytoplasm are evident, suggesting that these neurons might be dying.

HCRT-ir neurons survived until day 36, but those that did survive had clearly established processes and appeared in size and morphology like normal adult HCRT-ir neurons. A number of experiments have shown that a high percentage (95%-99%) of transplanted cells die after grafting.[21,22] Our findings are consistent with most graft studies of other types. Cell death after transplant occurs via apoptosis or necrosis.[23-25] Apoptotic death is distinguished from necrosis by morphologic and biochemical changes, including preservation of membrane integrity and diminution of cellular volume, both present in apoptosis.[26] Since, as seen in Figure 2, on day 6 the size of the somata of grafted HCRT-ir neurons was dramatically reduced and the membrane was conserved, we suggest that an apoptotic cell death was triggered, probably as a consequence of loss of trophic factors.

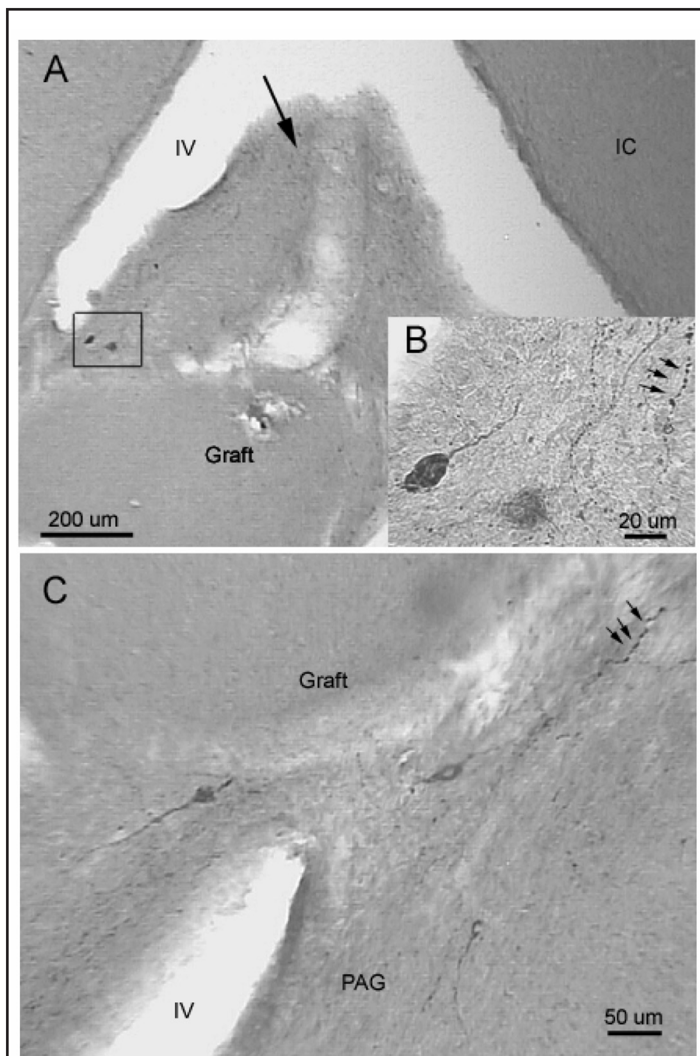

Figure 3—Hypocretin-immunoreactive (HCRT-ir) neurons with processes in adult rats sacrificed 36 days after receiving a graft in the pons of dissociated posterior hypothalamic tissue from 8- to 10-day-old rats pups (Experiment 2). Photos taken from host rats show that some of the grafted tissue accumulated in the fourth ventricle (IV) and HCRT-ir neurons with a morphology and size found in adult rats were evident. Inset B represents a close-up of the section identified by a box in A, and 2 neurons (1 out of the focal plane) with processes are evident. In photo C, 3 HCRT-ir neurons are evident in the graft zone, and 1 is present in the periaqueductal grey (PAG) area. All of the HCRT-ir neurons have clear processes with varicosities (identified by arrows in B and C). The arrow in photo A points to the graft in the ventricle.

The survival of grafted neurons is influenced by many factors. Perhaps, the most important is the age of the donor, since it has been shown that young embryonic tissue survives better than neonatal or adult donor tissue.[27] Here, we used tissue from rat pups that were 8 to 10 days old. We did not take tissue from rat fetuses, since the HCRT neurons have been described to fully develop by postnatal day 10.[28] Future studies may consider using fetal rat tissue so that the undifferentiated neurons may be placed in a site where, with appropriate stimuli, HCRT expression may occur.

Data from transplant studies indicate that the survival of cells and their ability to establish connections also depend on release of diffusible factors such as hormones, neurotransmitters, or neurotrophic factors.[29,30] We assume that the pons is able to secrete factors that entice HCRT axonal growth because normally this region is heavily innervated by HCRT fibers.[8] These factors are still present and promote cell growth in adults, since a few HCRT neurons were evident 3 days after transplant. Addition of neurotrophins or caspase inhibitors in the transplant media and/or inhibition of glia could improve survival.[29,30] It is also known that to prevent rejection, hosts are immunosuppressed through injections of cyclosporin.[31]

HCRT-containing neurons project to the entire brain and spinal cord, providing especially heavy innervation to forebrain and brainstem neuronal populations implicated in wakefulness.[8] In our study, we placed the grafts in the pons, since extensive data indicate that this region is involved in regulating sleep-wakefulness[9] and receives HCRT innervations that modulate sleep.[10] Thus, even though narcolepsy is characterized by loss of HCRT neurons in the lateral hypothalamus,[2,3] it is necessary to place the grafts of HCRT neurons in target regions responsible for the behavior, which has been done with Parkinson disease.[32]

Once the problem of survivability of the HCRT neurons is improved, it will be necessary to determine impact of the transplanted HCRT neurons on sleep-wake behavior. In the present study, we did not examine such behavior because the parameters influencing HCRT survival are still not fully understood. It will be necessary to measure HCRT release, most likely in the CSF. We recently demonstrated that CSF HCRT levels decline with HCRT neuronal loss and that there is no subsequent recovery in HCRT levels once the HCRT neurons are destroyed.[7] This is a particularly useful finding for transplant studies, since we can expect an increase in HCRT levels with HCRT transplants. However, it may be necessary to wait several months before appreciable increases in CSF levels of HCRT and/or a change in sleep behavior is evident in transplanted animals. For instance, some suprachiasmatic nucleus-lesioned animals bearing suprachiasmatic nucleus grafts remain arrhythmic for several months before animals begin to exhibit circadian rhythms of locomotor activity.[15,33-35] This delay in emergence of behavior may reflect the time required for appropriate reestablishment of neuronal connectivity between the donor and the host.

The lateral hypothalamus where the HCRT neurons are located contains a mixed variety of neuronal phenotypes,[36] and the HCRT neurons represent a small proportion relative to other phenotypes such as the melanin concentrating hormone-containing neurons. Clearly, in our method, other phenotypes, including the HCRT neurons, were transplanted. It would be preferable to transplant only HCRT neurons, perhaps using a transgenic mouse

model where the green fluorescent protein identifies HCRT neurons. In these mice, the lateral hypothalamus could be dissected and the green fluorescent protein-HCRT neurons separated under a microscope and implanted into host animals. This method would also provide a way of quantifying the number of HCRT neurons that are being transplanted. The host animals would be mice where the HCRT neurons have been eliminated[37] or mice that lack the HCRT gene,[38] since they represent excellent models for testing the efficacy of transplant or gene therapy. In these models, the symptoms of narcolepsy are present, and targeted transplants could be used to reverse 1 or more of the symptoms. In the mouse model, downstream effector neurons are still responsive to HCRT, since ectopic HCRT expression and HCRT administration reverse the behavior.[39] One has to be cautious, since neuronal transplant and gene therapy have limitations[40,41] and might not be suited to treat a disease such as narcolepsy, which can be managed by pharmacotherapy. However, pharmacotherapy also has its limitations, such as tolerance and side effects, and thus the need to develop alternative strategies.

ACKNOWLEDGMENTS

We thank Dr. Dmitry Gerashchenko for helpful discussions. We also thank Diana Millán-Aldaco and Jill Winston for providing excellent technical assistance and Ms. Teresa Torres-Peralta for typing the manuscript. This work was supported by NIH grants NS30140, AG09975, AG15853, MH55772, Medical Research Service of the Department of Veterans Affairs, CONACyT 25122-M, DGAPA IN-208799 and Fideicomiso UNAM to RDC.

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