GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR IS ESSENTIAL FOR NEURONAL SURVIVAL IN THE LOCUS COERULEUS–HIPPOCAMPAL NORADRENERGIC PATHWAY


*Department of Physiology and Neuroscience and the Center on Aging, Medical University of South Carolina, 26 Bee Street, Charleston, SC 29425, USA
bDepartment of Neurology, Medical University of South Carolina, Charleston, SC 29425, USA
cNational Institute on Drug Abuse, Bethesda, MD 20892, USA
dUmeå University, Umeå, Sweden

Abstract—It has been shown that the noradrenergic (NE) locus coeruleus (LC)–hippocampal pathway plays an important role in learning and memory processing, and that the development of this transmitter pathway is influenced by neurotrophic factors. Although some of these factors have been discovered, the regulatory mechanisms for this developmental event have not been fully elucidated. Gial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor influencing LC-NE neurons. We have utilized a GDNF knockout animal model to explore its function on the LC-NE transmitter system during development, particularly with respect to target innervation. By transplanting various combinations of brainstem (including LC) and hippocampal tissues from wildtype or GDNF knockout fetuses into the brains of adult wildtype mice, we demonstrate that normal postnatal development of brainstem LC-NE neurons is disrupted as a result of the GDNF null mutation. Tyrosine hydroxylase immunohistochemistry revealed that brainstem grafts had markedly reduced number and size of LC neurons in transplants from knockout fetuses. NE fiber innervation into the hippocampal co-transplant from an adjacent brainstem graft was also influenced by the presence of GDNF, with a significantly more robust innervation observed in transplants from wildtype fetuses. The most successful LC/hippocampal co-grafts were generated from fetuses expressing the wildtype GDNF background, whereas the most severely affected transplants were derived from double transplants from null-mutated fetuses. Our data suggest that development of the NE LC–hippocampal pathway is dependent on the presence of GDNF, most likely through a target-derived neurotrophic factor. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: noradrenergic neurons, locus coeruleus, glial cell line-derived neurotrophic factor, hippocampal formation, neuroplasticity, transplantation.

The noradrenergic (NE) innervation of the hippocampal formation has been the subject of numerous anatomical, pharmacological, and physiological studies, due not only to its postulated role in memory and learning but also to the extensive knowledge about its function and anatomy (Cassel et al., 1997; Collier et al., 1988; Gasser and Dravid, 1987; Gerhardt et al., 1991; Madison and Davis, 1983; see also Gibbs and Summers, 2002). Moreover, deficiencies in the NE hippocampal innervation have been found in aged animals, as well as in aged humans and patients with Alzheimer’s and Parkinson’s disease (Chan-Palay and Asan, 1989; Cummings et al., 1998; Petronis, 1999; Miguez et al., 1999; Lee et al., 2001; Ishida et al., 2001). It has been suggested that the loss of NE transmitter function is directly related to memory loss observed during aging and neurodegenerative disease, since replacement of these neurons with locus coeruleus (LC)-containing brainstem transplants could reverse a specific memory loss in aged rats (see e.g. Collier et al., 1987). Furthermore, activation of the brainstem LC neurons and subsequent activation of different adrenoceptor subtypes in the forebrain have been shown to be directly involved in learning (possibly via its role in attention and arousal) and also in memory consolidation (for review, see Gibbs and Summers, 2002). Hence, this important transmitter system paradoxes in many important physiological processes, and yet the regulation of NE innervation of the hippocampus during development is only partially known.

An area of significant focus during the last couple of decades has been the biological impact of target-derived neurotrophic factors on neuronal survival and fiber outgrowth, due to these factors’ importance both during healthy development and disease. Specifically, neurotrophin 3 (NT-3; see Friedman et al., 1993; Reiriz et al., 2002), brain-derived neurotrophic factor (Friedman et al., 1993; Sklar-Tavron and Nestler, 1995), Neurturin and glial cell line-derived neurotrophic factor (GDNF; see Arenas et al., 1995; Granholm et al., 1997a; Holm et al., 2002) have all been shown to affect LC-NE neurons during development. Early in development, the generation of LC neurons is reflected by initial expression of the transcription factors Phox2a and Phox2b in dorsal rhombomere 1, followed by expression of the enzymes dopamine β hydroxylase (DBH) and tyrosine hydroxylase (TH; see Vogel-Hopker...
and Rohrer, 2002). The bone morphogenetic protein 5 (BMP-5) is expressed in dorsal neuroepithelium, and BMP inhibition in stage 10 chick embryos results in lack of LC-NE neurons. The BMPs have also been shown to affect LC neurons later during development, at least in tissue culture experiments (see Reiriz et al., 2002). Conversely, it has been shown that the LC-NE innervation may regulate expression rates of neurotrophic factors in the target region, such as the hippocampus (Hutter et al., 1996). GDNF was first cloned and sequenced in the early 1990s as a region, such as the hippocampus (Hutter et al., 1996). GDNF was subsequently reported to exert its effects by binding to a multicomponent receptor that consists of the Ret receptor tyrosine kinase and a glycosylphosphatidylinositol-linked receptor family (Lin et al., 1993). GDNF was subsequently reported to exert its effects by binding to a multicomponent receptor that consists of the Ret receptor tyrosine kinase and a glycosylphosphatidylinositol-linked receptor family named GFRα1–4 (Treonar et al., 1996; for review, see Saarma and Sariola, 1999), and LC-NE neurons were found to express both GFRα1 and 2 during development (Holm et al., 2002). Recently, more widespread functions of this protein have been revealed. GDNF is expressed in neuronal and glial elements during critical periods of cortical, striatal, and hippocampal development and innervation (Nosrat et al., 1996; Ikeda et al., 1999), which suggests a specific function for GDNF during target innervation and synaptogenesis.

The early development of GDNF knockout (−/−) mice, compared with wildtype (WT) littermates has been examined previously (see Pichel et al., 1996; Sanchez et al., 1996; Moore et al., 1996). Abnormalities were detected in both peripheral and central NE neurons (see Granholm et al., 1997a), while the mesencephalic dopamine neurons remained intact (Pichel et al., 1996; Moore et al., 1996; Sanchez et al., 1996; Granholm et al., 1997a), suggesting that NE neurons are more dependent on GDNF during early development than central dopamine neurons. However, these earlier studies were limited to fetal development, since all GDNF−/− animals die at birth. It has been demonstrated that neurotrophic factors are important not only during development but also for maintenance of neuronal plasticity in adult animals, especially after insults. GDNF-transfected fibroblasts can rescue adult LC-NE neurons from 6-hydroxydopamine-induced neurotoxicity (Arenas et al., 1995). GDNF thus appears to be effective in rescuing both dopaminergic and NE neurons from injury in the adult animal as well (Bowenkamp et al., 1995; Gash et al., 1996; see also Granholm and Hoffner, 2000). However no studies, to our knowledge, have focused on the ability of GDNF or related growth factors to affect innervation of target regions, such as the hippocampus, during development. In order to study the continued maturation of LC-NE neurons, one would need to transplant these cells from GDNF knockout fetuses into adult hosts. We have, therefore, utilized the well characterized intracranial transplantation model (Strömborg et al., 1985; Granholm et al., 1997b) in order to examine the fate of these neurons beyond postnatal day 1, when GDNF knockout animals succumb to renal agenesis (see Pichel et al., 1996). It has been demonstrated previously that there is no TH-positive innervation derived from the host brain in the grafted fetal tissues when placed in the lateral ventricle of an adult host (see Björklund and Strömborg, 1997). Therefore, placement of the transplanted tissues into the lateral ventricle insures that innervation seen in either of the tissues is derived from the other fetal co-graft. We have previously demonstrated that fetal midbrain dopaminergic neurons from GDNF −/− fetuses develop poorly when grafted into the adult WT mouse (see Granholm et al., 2000). However, we have not yet examined the fate of either dopaminergic or NE innervation properties from GDNF −/− neurons innervating a fetal target area in intracranial double transplants.

The aim of the present study was to examine whether a GDNF null mutation affected LC-NE neurons in brainstem grafts and their innervation of co-grafted fetal hippocampal tissue when both are transplanted into the hippocampus or lateral ventricle of adult WT mice. The outcome of this study should shed light on the biological effects of GDNF on NE innervation of the hippocampal formation as well as determine whether intrinsic (within the neurons themselves) neuronal or extrinsic (within the target area of innervation) factors determine the viability of a discrete neuronal population in the brain at the time of innervation of the cortical target area.

**EXPERIMENTAL PROCEDURES**

**Animals**

A nonfunctional allele of the GDNF gene was generated by replacing part of the third exon that encodes GDNF protein with a cassette expressing the selectable marker neomycin phosphotransferase, as described previously (see Pichel et al., 1996; Granholm et al., 1997a). After introducing this construct into embryonic stem cells, six clones were identified with the predicted mutant allele. Four clones produced chimeric mice that transmitted the mutation to their progeny. Heterozygous offspring were viable and fertile, whereas mice homozygous for the mutant GDNF allele (GDNF−/−) died within 24 h of birth, due to lack of renal development. We have established a colony of these mice and housing and breeding of the mice adheres to local IACUC approved protocols and NIH guidelines for animal use and care. All necessary precautions were made to reduce the number of animals in the study, as well as the potential suffering of animals.

**Dissection and transplantation**

Fetal donors of embryonic day 16–17 (E16-17) were obtained from heterozygous dams, mated to heterozygous males. Fetal tissue containing brainstem LC and hippocampus was dissected and transplanted into the lateral ventricle at the level of the hippocampal formation of adult WT mice according to our previous reports (for details see Strömborg et al., 1985; Björklund and Strömborg, 1997; Granholm et al., 1997b). A schematic presentation of the experimental design is shown in Fig. 1. Pregnant heterozygous mice were anesthetized with an overdose of halothane and decapitated, whereafter the entire uterus was removed and placed on ice. The crown-rump length was used for determination of embryonic stage. A tail sample was dissected from each fetus and the kidneys were examined by blunt dissection from the dorsal spine. As previously shown, all GDNF−/− fetuses lacked kidneys. Heterozygous fetuses frequently had only one kidney or renal malformations. Hence, it was possible to design different combinations of tissues prior to transplantation (e.g. knockout...
Brainstem and hippocampal tissue is dissected from embryonic day 16-17 knockout and wildtype fetuses

Using stereotaxic techniques, tissue is placed in lateral ventricle close to the hippocampal formation of adult wildtype siblings

Fig. 1. Schematic drawing of the experimental design. Fetuses from a heterozygous dam were examined for gross kidney morphology and a limb and tail were saved for genotyping. Brainstem tissue containing the LC and hippocampus were dissected from WT or knockout fetuses, and implanted into the ventricle lateral to the hippocampal formation of adult WT controls in differing combinations of double transplants. Following transplantation, the tail samples were genotyped to confirm the initial determination of WT vs. −/− status (lack of kidneys is obvious in −/− fetuses, while heterozygous fetuses usually have only one intact kidney or other abnormalities).

(-/-) LC with WT hippocampus; WT/WT; double −/− etc. The tail sample was kept on ice until all dissections were done, and thereafter DNA was extracted for genotyping (see below). One by one millimeter tissue blocks were dissected from the LC-region of the brainstem and the CA1 of the hippocampus. The tissue blocks were kept in tissue culture medium (Dulbecco’s normal medium) in room temperature until transplantation (within 1/2 h from dissection). Thereafter, tissue blocks were placed in a spinal needle with an insert using suction, and transplanted into the lateral ventricle. Transplants were placed at the following coordinates: 1 mm posterior to Bregma, 3 mm lateral, and 2.5 mm deep. Various combinations of fetal LC or hippocampus from WT and GDNF −/− fetuses at E16-17 were grafted bilaterally to the lateral ventricle next to the hippocampus in adult WT mice, so that each recipient contained one type of graft on one side and a second type of transplant contralaterally. The groups were divided as follows due to tissue availability at the time of grafting: WT/WT (n=8), WT/KO (n=7), KO/WT (n=4), KO/KO (n=8). Of the eight KO/KO grafts, three were treated with GDNF (100 µg/ml) in the incubation bath for 30 min at room temperature prior to transplantation. This control experiment was performed to determine the specificity of the GDNF null mutation. All transplant recipients were killed at 3 weeks post-grafting by euthanasia (halothane) and brains dissected and postfixed according to the protocol detailed below.

PCR genotyping

Genomic DNA was prepared from a 1 cm tail cutting of fetal mice and graft recipients. The tail was homogenated in 0.2 M NaCl, 5 mM EDTA, 100 mM Tris, pH 8.5, 0.2% SDS and 400 µg/ml proteinase K overnight at 55 °C. The suspension was then centrifuged to remove debris, and the DNA in the supernatant was precipitated with one volume isopropanol, pelletted, washed once with 70% ethanol, and resuspended in 20 µl sterile water. The DNA was assayed for the presence of the WT or knockout allele in two separate PCR reactions using WT or knockout specific primers. PCR was performed in a total reaction volume of 50 µl which contained 2 µl of genomic DNA, 2 µM of each primer, 5 mM MgCl2, 200 µM each, dATP, dGTP, dCTP, and dTTP, and one unit Taq polymerase. The genomic DNA was amplified for a total of 35 cycles and the products analyzed for the presence of the WT or −/− allele on a 2% agarose gel. Amplification of the WT allele gave a band of 344 bp, while the mutant allele gave a band of 255 bp (see Granholm et al., 1997a).

Immunocytochemistry

Transplants were studied at 3 weeks postgrafting. Recipient mice were killed and processed for TH immunohistochemistry of graft and host brain tissue. Mice with intracranial grafts were deeply anesthetized with chloral hydrate (600 mg/kg i.p., Sigma, The Woodlands, TX, USA) and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde and 2% picric acid in 0.1 M phosphate buffered saline (PBS). Brains were removed and post-fixed for 24 h, then transferred to 30% sucrose in 0.1 M PBS for a minimum of 16 h. Sections were processed for free-floating immunohistochemistry using antibodies against TH (1:1000 in PBS; Peel-Freeze Inc., Roger, AZ, USA). Immunohistochemistry was performed according to our standard protocol (Granholm et al., 1997a,b). Controls included sections where the primary antibody was omitted, as well as a pre-incubation of the primary antibody with the appropriate antigen. Forty micron sections were cut on a cryostat (Microm, Zeiss, Thornwood, NY, USA), washed, and incubated for 48 h with TH antibodies. The sections were washed and incubated in Tris-buffered saline, incubated with the secondary antibody reacted with the ABC solution (Vectorstain; Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (Sigma) (DAB; 0.0300 g/100 ml imidazole-acetate buffer solution; Fisher Scientific, Fairlawn, NJ, USA). Sections were mounted on glass slides and coverslipped. In the first experiment (12 animals with bilateral transplants), sections were processed on slides using the immu-
nofluorescence method of detection (Gerhardt et al., 1991). This method was used for all animals processed for image analysis of TH staining density since immunofluorescence provides a linear detection of antigen presence in the samples. For photographic purposes, sections from the second group of animals were processed free-floating using the DAB method (see above). All sections were viewed using a Nikon Eclipse 600 light microscope. In order to control for inter-group staining variability, all steps of the immunohistochemical staining, including the DAB reaction, were performed in the same solutions and times for all groups using tissue wells with plastic mesh bottoms on an orbital shaker table. Cresyl Violet staining was performed on every sixth section throughout the transplants to verify graft placement and examine the general morphology of the grafts.

**Densitometry of TH innervation**

Image analysis of innervation staining density was performed using the NIH Image analysis program, on every sixth section throughout the transplants. The image analysis measurements were performed blindly by two independent investigators, blinded to outcome of the study. Image is written using Think Pascal from Symantec Corporation. Image can be used to measure area, average gray value, as well as path lengths and angles of cellular components. Density calibration is performed against an optical density calibration curve that takes into account and subtracts the background from each section that is measured. The gray scale value is within the range of 0–256, where 0 represents white. The first, most rostral, section of the transplant was selected randomly for each animal, and thereafter, every sixth section was stained for TH immunohistochemistry as described above throughout the entire transplant. The entire surface of the hippocampal and the brainstem graft was outlined on five to eight sections from each graft (dependent on the size of the transplants), and density was evaluated separately for the two co-grafts. An additional level of confidence was supplied by the fact that transplants from different groups were placed on opposite sides of the same WT host brain, allowing evaluation of staining density to be performed for two different groups and treatments on the same section of host brain. Statistical analyses between groups were performed using two-tailed Student’s t-test.

**RESULTS**

**Routine morphology**

Evaluation of serial sections with Cresyl Violet of all samples confirmed that transplanted grafts were successfully incorporated into their respective adult host (Fig. 2). Routine histology did not show observable alterations in overall morphology of either brainstem or hippocampal grafts, regardless of whether the donor tissue came from –/− or WT fetuses. In addition, no size differences were observed between either hippocampal or brainstem grafts from the two groups, suggesting that overall neuronal and glial survival was not compromised by the GDNF mutation. As can be seen in Fig. 2, both –/− and WT fetal graft tissue showed a well-developed and organotypic morphology in both brainstem and hippocampal tissue. The hippocampal tissue contained a densely packed layer of large neurons,
Table 1. Summary of microscopy results for knockout and wildtype grafts

<table>
<thead>
<tr>
<th>Summary of TH immunohistochemistry</th>
<th>STaining observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC –/–/Hip WT</td>
<td>Few cell bodies observed in the LC transplant. Hip grafts contained sparse to moderate innervating fibers.</td>
</tr>
<tr>
<td>LC –/–/Hip –/–</td>
<td>Very few (one to two) cell bodies in LC. Significantly reduced or non-existent fiber staining in Hip graft.</td>
</tr>
<tr>
<td>LC WT/ Hip –/–</td>
<td>Small to moderate number of cell bodies present in LC grafts. Very few innervating fibers observed in Hip grafts.</td>
</tr>
<tr>
<td>LC WT/ Hip WT</td>
<td>Numerous distinct cell bodies in LC graft. Robust fiber density observed in Hip transplant.</td>
</tr>
</tbody>
</table>

*LC: locus coeruleus-containing brainstem grafts; Hipp: hippocampal grafts.

reminiscent of the pyramidal cell layer in situ, surrounded by more sparse layers reminiscent of the molecular layer and stratum oriens. Brainstem grafts were less organized, but contained large cell groups that were densely packed in transplants from both WT and –/– fetuses, reminiscent of the organization in this brain region in situ. Hence, there was no obvious general abnormality of gross morphological appearance in either brain area with the GDNF deletion, compared with WT control grafts. The presence of the two transplants in the ventricle did not appear to affect the animals adversely. Due to the small size of the transplanted tissues (1×1 mm), they did not appear to occlude the ventricles and no hydrocephalus was observed in the host brains following transplantation. As shown previously (see Björklund and Strömberg, 1997), transplants into the ventricle of rodents can survive for many months without interference with host brain function and/or morphology.

**TH immunoreactivity in WT transplants**

All brains were subjected to TH immunohistochemistry and the results for all transplant combinations are summarized in Table 1 (see above). All transplanted brains contained remnants of fetal tissue grafts and an obvious needle track that was sometimes filled with transplanted tissue and grafted neurons. Grafts of WT brainstem tissue containing the LC together with WT hippocampus (WT/WT) contained moderate to large amounts of TH-positive neurons and fibers, respectively (Fig. 3A and B). Clusters of large TH-immunoreactive neurons were present in the brainstem graft, sometimes in an organization reminiscent of the LC in situ (Fig. 3B). Large bundles of TH-positive neurites were seen leaving the brainstem graft and growing toward and into the hippocampal portion of the graft (Fig. 3A). In addition, distinct, varicose TH-positive fibers could be seen surrounding neuronal profiles in the hippocampal pyramidal cell layer, suggesting reconstitution of the LC-hippocampal pathway in the WT/WT transplants.

**Effects of GDNF null mutation on TH immunostaining in grafts**

TH immunohistochemistry revealed that the GDNF status of transplanted tissues significantly affected both LC and hippocampal grafts, as evidenced by the staining intensity for TH in transplants from knockout fetuses. Virtually all double GDNF knockout transplants (double –/–) were almost completely devoid of TH immunostaining, both in the brainstem LC and the hippocampal portion (Fig. 3E). Knockout LC grafts contained few or no distinguishable TH-positive cell bodies (Fig. 3F). Finally, very few TH-positive fibers were observed in the hippocampal portion of a double –/– graft. In order to determine the specificity of the GDNF deletion in knockout fetuses, we pre-incubated double GDNF –/– transplants (n=3) with GDNF (100 µg/ml) for 30 min in room temperature prior to transplantation. This concentration is 1.5–2 orders of magnitude above the ED50 for the GFRα-1 receptor, insuring that the plateau of the dose-response curve would be reached (Treanor et al., 1996). Interestingly, these three transplants contained numerous LC neurons and TH immunoreactive neurites, resembling the pattern seen in WT/WT grafts (see Fig. 4C). The innervation density of transplanted tissue was best illustrated by either dark-field or fluorescence photography. Fig. 4 demonstrates darkfield photography of a WT/WT double graft (A), a double –/– graft (B), and a double –/– graft treated with GDNF at the time of grafting (C). Note the almost complete absence of TH immunoreactive neurites in the double –/– graft, suggesting that this combination gave rise to the least target innervation of all groups. On the other hand, a few LC-NE neurons could be seen in the brainstem portion of this graft (bottom of 4B), demonstrating that LC neurons can develop, at least to some extent, in the complete absence of GDNF, although they appear unable to innervate the target area appropriately.

The variable combinations of –/– and WT grafts at the same transplantation site (LC –/–/Hippo WT and LC WT/ Hippo –/–) demonstrated that the LC neurons were most influenced by a GDNF null mutation in the target tissue, the hippocampal graft. Modest innervation of transplants resulted from grafts consisting of LC –/– and WT hippocampus together (Fig. 3D), while the combination of LC WT and hippocampus –/–, on the other hand, elicited only sparse, if any, TH immunoreactive fibers (Fig. 3C). Hence, it appeared that the genotype of the target tissue, rather than the neuronal source population itself, was most important for the development of an organotypic NE innervation in these double graft combinations.

**Image analysis**

Image analysis of TH staining density was performed on a subset of the transplants using fluorescence microscopy. WT/WT (n=6) and double GDNF –/– (n=5) grafts were examined using fluorescence immunohistochemistry and an image analysis densitometry system (NIH Image). As can be seen in Fig. 5, there was a significant decrease in –/– double grafts in terms of TH staining density in the brainstem portion, compared with WT/WT grafts (B,
The mean values for the two groups were 21.9±3.2 (S.E.M.) for the WT/WT group, and 4.69±2.4 for the double −/− group. The density in the hippocampal portion of the double grafts was significantly different between the groups as well (A, P<0.05, Student’s t-test, two-tailed). The mean values in the hippocampal formation were 27.5±8.7 for the WT/WT group, and 6.03±2.2 for the double GDNF −/− group (see Fig. 5A). These data confirm interpretations made by two independent observers on blind-coded slides (see above) and suggest that the presence of GDNF is important for development of an appropriate NE innervation from the brainstem to the hippocampal formation.

DISCUSSION

Our study demonstrates that GDNF appears to be critical for establishment of a viable NE system, not only during early development, as evidenced by our previous findings in newborn GDNF knockout animals (Granholm et al., 1997a), but also for continued innervation into an appropriate target, such as the hippocampus. Using a knockout mouse model, we demonstrate here that the lack of GDNF markedly affected the NE innervation from brainstem-LC grafts to adjacent hippocampal grafts. Immunohistochemical analyses revealed that TH innervation was greatly diminished when the hippocampal graft was derived from GDNF −/− fetuses, whereas the GDNF status of brainstem grafts was somewhat less important. The most strikingly abnormal combination of transplants was the double knockout group (GDNF −/− LC with a GDNF −/− hippocampus), wherein TH immunoreactive cell bodies or neurites were virtually absent in both grafts.

It is well known that LC-NE neurons can respond to injury by increasing synthesis of transmitter and initiating compen-
satory sprouting. Several investigations have found that a partial lesion of the LC-NE neurons gives rise to an upregulation in TH expression in LC cell bodies as well as compensatory sprouting into both the cerebellum and non-dенervated targets, such as the hippocampus (Fritschy and Grzanna, 1992; Kostrzewa et al., 1988). A similar compensatory upregulation of NE systems has been found locally in the hippocampal formation following surgical or chemical axotomy, or after selective removal of other hippocampal inputs, such as the cholinergic system (Gage et al., 1983; Gasser and Dravid, 1987; Madison and Davis, 1983). Thus, these neurons have well-documented regenerative properties, and will continue to elaborate new collateral sprouts over extended time periods following surgical or neurotoxin-induced injury (Wolfman et al., 1994). The significant loss of LC-NE innervation into hippocampal grafts from knockout fetuses described herein is even more striking in view of this regenerative ability; apparently the lack of one neurotrophic factor has severe and long-term consequences for the LC neurons that cannot be overcome by regenerative sprouting later in life. However, it should be noted that these experiments were only continued until 3 weeks post-grafting. Long-term experiments will reveal whether these altered innervation patterns remain throughout the lifespan of the grafted animal.

The LC sends efferent projections to the hippocampus via the dorsal tegmental bundle (Loughlin and Fallon, 1985). Neurons from the dorsal area of the LC provide the majority of the NE innervation to the hippocampus. The areas of the hippocampus that contain the most dense NE innervation are the hilus of the dentate gyrus and the mossy fiber layer of CA3. The NE terminals innervate the dendrites and somata of granule cells in the dentate gyrus and the pyramidal cells of the hippocampus. It is reasonable, therefore, to suggest that NE innervation from the LC may influence the physiology of hippocampal neurons that express neurotrophins. These neurotrophins, in turn, may then serve as target-derived neurotrophic factors for the LC. This ‘target’ interaction hypothesis is further substantiated by the fact that the LC WT/Hippocampus −/− combination transplants were as poorly innervated as the double GDNF −/− combinations, while the LC −/−/Hippocampus WT combinations were not. This hypothesis must, of course, be confirmed by labeling studies, using biotinylated or radioactive GDNF injections into the hippocampal formation, to be traced back to LC-NE neurons in the brainstem. Previous work has demonstrated that GDNF is retrogradely transported within the dopaminergic neurons from the striatum to the substantia nigra in mice (Tomac et al., 1995), and also that the LC-NE neurons express C-Ret and GFR-α receptors throughout the lifespan in the rat (Trupp et al., 1997), but it has not been demonstrated whether the same relationship exists also for the LC–hippocampal NE pathway.

In the present study, we have examined the maturation of LC neurons from GDNF −/− and WT fetal mice, grafted into ventricle of adult WT animals. This approach allowed us to evaluate the effects of lack of a trophic factor in a discrete neuronal population and its target, the hippocampus, and how this relates to normal neuronal development and target innervation. These studies provide a method in which the continued development and maturation of neuronal pathways from trophic factor knockout strains may be studied despite the lack of postnatal viability of the knock-

**Fig. 4.** Darkfield microscopy of TH immunostained sections of a WT/WT graft (A), a double GDNF −/− graft (B), and a double GDNF −/− graft preincubated with GDNF prior to grafting (C). Note the difference in TH immunoreactive neurite density between the three different grafts. GDNF preincubation gave rise to an increase in density of staining both in the LC portion of the graft and the hippocampal portion (C). All of these grafts were embedded in the lateral ventricle. A few TH immunoreactive cell bodies can be seen in the brainstem section (LC) of the double −/− graft in the bottom of (B), but they did not appear to innervate the −/− hippocampal co-graft (H) above it. Border between the grafts is delineated by a line in each figure. Scale bar shown in (A) = 150 μm in A–C.
was performed using two-tailed Student's t-test, with significance level set at P<0.05.

**Fig. 5.** Image analysis of TH immunostaining in WT/WT and double −/− transplants. There was a significant decrease in TH staining in the double GDNF −/− brainstem grafts, compared with WT grafts (B, ** P<0.01), and a significant difference between these two groups in hippocampal innervation as well (A, * P<0.05). The density of TH staining was expressed as relative density, on a scale of 0–256, where 0 represents white and 256 represents black. The statistical evaluation was performed using two-tailed Student's t-test, with significance level set at P<0.05.

---

out mice. Previously, it has not been possible to study these processes, other than by using tissue culture, since these neurotrophic factor knockout mice typically die shortly after birth. We have recently documented the GDNF dependence of dopaminergic midbrain neurons during development, by demonstrating that ventral mesencephalic dopaminergic neurons fail to develop when transplanted from a GDNF −/− fetus (see Granholm et al., 2000). The findings presented here strongly suggest that GDNF may be critical for the long-term survival of the pontine LC-NE neurons as well. Interestingly, routine histology did not indicate that the LC neurons disappeared or are underdeveloped in −/− grafts, but only that they did not express TH; Cresyl Violet staining revealed a large group of magnocellular neurons in the brainstem portion of the −/− double grafts (Fig. 2C and D), which showed no TH immunolabeling. Hence, the neurons may develop but TH expression might be suppressed. This question could be addressed in future studies, using GDNF injections into an already mature GDNF −/− double transplant, and was partially answered in the present study by the fact that GDNF exposure in −/− tissues prior to grafting gave rise to a compensatory upregulation of TH expression in the grafts (see Fig. 4C). Histological evaluation of ventral mesencephalic tissue in grafts from GDNF −/− animals in a previous study (Granholm et al., 2000) did not suggest a similar scenario for dopaminergic neurons, since these transplants were clearly underdeveloped, compared with WT transplants in the same hosts. This difference between the two brain regions in terms of response to GDNF deserves further study. Recent studies by Holm and collaborators (2002) have demonstrated that both GDNF and its family member Neurturin specifically promote the development of neurites from LC-NE neurons. Their finding in tissue culture of brainstem neurons can partially explain our present results, since the most obvious finding reported here was the lack of LC-NE neurites in hippocampal transplants from GDNF knockout animals.

Even though it has been demonstrated that developing LC-NE neurons express GDNF receptors (see Holm et al., 2002), the regulatory mechanism for its biological action on LC-NE neurons is poorly understood. A recent study has clarified some of these mechanisms. Reiriz et al. (2002) found that both NT-3 and GDNF, in the presence of serum, enhanced the production of LC-NE TH immunoreactive neurites from a brainstem-derived tissue culture. However, in serum-free conditions, neither of the neurotrophic factors had effects on survival of these neurons. Addition of BMP-2 to the tissue culture gave rise to a significant functional enhancement of the biological effects of GDNF, NT-3 as well as basic fibroblast growth factor. The greatest effect on LC-NE survival was found with co-administration of forskolin, BMP-2 and GDNF. Thus, their results showed that LC neurons require multiple factors for their survival and development, and also suggest that activation of the neurons by BMP-2 and cAMP plays a decisive role in conferring LC-NE neuron responsiveness to neurotrophic factors. It would therefore be interesting in future experiments to determine if supplementation of the GDNF null mutation transplants with forskolin, BMP-2, and/or other neurotrophic factors would overcome the severe developmental disturbance demonstrated here. Other investigations have revealed that neurotransmitter expression and release may also partake in the neurotrophic factor-regulated development of the LC-NE transmitter pathway (Dreyfus, 1998), further suggesting a complicated interaction between multiple factors at play. Since the fetal knockout tissues were placed in an adult environment, in the ventricle of adult recipient mice, it should be noted that normal expression rates of other factors would not be available to the developing tissues to the same extent as if they were developing in the intact fetus (many factors decrease significantly in their expression in the adult CNS and may of course not be available in the ventricle). Thus, compensatory mechanisms that may be in place in the intact developing brain would be
less effective here, rendering, perhaps, a more severe effect of the growth factor mutation than otherwise expected.

Another possible pathway by which GDNF may influence the development of LC-NE neurons is via the norepinephrine transporter (NET). Neurotrophic factors, such as FGF-2 and NT-3 have been shown to upregulate the expression of NET mRNA in NE neurons during development (Sieber-Blum and Ren, 2000), and NET, in turn, regulates the differentiation of LC neurons by promoting expression of TH and DBH. Even though direct effects of GDNF on the dopamine transporter have been found (see e.g. Hebert and Gerhardt, 1997), it is not known, to our knowledge, whether GDNF also regulates expression of NET mRNA. Since NET may participate in differentiation of the LC-NE neurons, and obviously later in neurotransmission, it would be important to evaluate the effects of the GDNF null mutation on the NET system as well in future studies. Due to the poor availability of GDNF knockout mice at this time, this examination was not within the scope of the present study.

In conclusion, the present study provides evidence for an influence of GDNF upon development and innervation of LC-NE neurons into the hippocampal formation. Tissues from GDNF−/− fetuses failed to develop normal NE neurons, and there was a marked lack of innervation into the adjacent, double-grafted hippocampal formation. Future studies will focus upon the functional consequence of this GDNF dependency, as well as explore interactions with other neurotrophic factors.

Acknowledgements—Thanks are due to Mr. Alfred Moore and Mr. Matt Nelson for excellent technical assistance. This work was supported by USPHS grant AG15239.

REFERENCES


Ren, 2000), and NET, in turn, regulates the differentiation of LC-NE neurons by promoting expression of TH and DBH. Even though direct effects of GDNF on the dopamine transporter have been found (see e.g. Hebert and Gerhardt, 1997), it is not known, to our knowledge, whether GDNF also regulates expression of NET mRNA. Since NET may participate in differentiation of the LC-NE neurons, and obviously later in neurotransmission, it would be important to evaluate the effects of the GDNF null mutation on the NET system as well in future studies. Due to the poor availability of GDNF knockout mice at this time, this examination was not within the scope of the present study.

In conclusion, the present study provides evidence for an influence of GDNF upon development and innervation of LC-NE neurons into the hippocampal formation. Tissues from GDNF−/− fetuses failed to develop normal NE neurons, and there was a marked lack of innervation into the adjacent, double-grafted hippocampal formation. Future studies will focus upon the functional consequence of this GDNF dependency, as well as explore interactions with other neurotrophic factors.

Acknowledgements—Thanks are due to Mr. Alfred Moore and Mr. Matt Nelson for excellent technical assistance. This work was supported by USPHS grant AG15239.

(Accepted 6 November 2003)