Expression of Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Neurotrophin-3 mRNAs in Human Cortical Xenografts

Christian Humpel^{1,2}, Ingrid Strömberg² and Lars Olson²

¹Neurochemistry Unit, Department of Psychiatry, Innsbruck, Austria

²Department of Neuroscience, Karolinska Institute, Stockholm, Sweden

SUMMARY

Trophic factors play an important role in the development of neurons and glia. In order to study the involvement of neurotrophins in human cortical development, human fetal parietal cortical tissue, obtained after early elective abortions, was transplanted to cortical cavities in immunosuppressed rats. Using *in situ* hybridization it was demonstrated that nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 mRNAs are expressed in developing human cortical xenografts. We conclude that neurotrophins may play a role in human cortical development and rat-derived astroglial cells could be involved in establishing reciprocal "permissive sites".

KEY WORDS

trophic factor, neurotrophins, transplant, permissive site, cortex, development

INTRODUCTION

To achieve a better understanding of the involvement of trophic factors in human cortical development, studies of the rodent cortex need to be supplemented with studies of the primate cortex. As an alternative to non-human primates, human-to-rat xenografts provide an interesting possibility /2,5,14,15,25,30,38-41/. The procurement of tissues after early elective routine abortions has

permitted studies of human fetal CNS tissue grafted to immunocompromised rodent hosts and of the expression of different human genes and proteins. Growth factors play an important role in survival and differentiation in the CNS and the guiding/targeting of nerve fiber growth. Nerve growth factor (NGF) and other neurotrophins, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5, are all present in the brain, the highest levels of NGF, BDNF and NT-3 mRNAs being found in the hippocampus /1,6,12,24,31,42/. Neurotrophins are also expressed in other brain areas, such as cerebral cortex, and may have important functional roles in this brain area, as shown *in vitro* and *in vivo* /3,4,10,13,17,20,22,29,34,35/.

Recently, we demonstrated that human fetal cortical tissue fragments survive grafting to cavities of the rat brain /16/. These tissue fragments became integrated with the host brain and formed reciprocal nerve fiber connections /16/. The ingrowth or outgrowth of nerve fibers seemed to occur at specific sites, so called "permissive sites", probably guided by trophic interactions /16/. The human grafts significantly expressed angiotensinogen mRNA and protein, indicating a role for this neuropeptide precursor in cortical development /18/. In a recent study we demonstrated BDNF mRNA in developing rat cortex, grafted *in oculo* to the anterior eye chamber /17/. The aim of the present study was to observe whether the neurotrophin mRNAs (NGF, BDNF, NT-3) are expressed in developing human cortical xenografts. We showed that all three neurotrophins were found in these grafts and rat-derived astroglial cells could be involved in establishing reciprocal connections between graft and host.

Reprint address:
Christian Humpel, Ph.D.
Neurochemistry Unit
Department of Psychiatry
Anichstr. 35
A-6020 Innsbruck, Austria

MATERIALS AND METHODS

Grafting procedure

Grafting was performed as described in detail previously [16,18]. Human fetal parietal cortical fragments were recovered after first trimester (8.5-9.5 weeks) abortions. Women admitted for elective abortions, who had no prior knowledge of this study, were informed of the aims of the study and gave their consent. Anonymity was strictly maintained. The abortions were performed according to hospital routine, with low-pressure vacuum aspiration and with approval by the Regional Ethical Committee of the Karolinska Institute. A cavity in the cortex (bregma: -1 to -2 laterally) of halothane anesthetized female Sprague Dawley rats (B&K Laboratories AB, Sweden, 200-220g, n=5) was made by gentle suction with a modified glass pipette connected to an aspiration pump. The cavity was filled with gelfoam (Spongostan) soaked in Hanks' balanced salt solution (HBSS; Gibco). After 1-2 weeks, the gelfoam was removed under anesthesia to expose the cavity. Human cortical tissue pieces (1-2 mm³) were placed at the bottom of the cavity and the cavity closed with gelfoam soaked in HBSS. For immunosuppression all rats received daily injections of cyclosporine A (10 mg/kg; Sandoz) containing vibramycin (2 mg/kg; Pfizer), starting the day before grafting. The total number of grafts studied was five.

In situ hybridization

At different times after grafting (16.5-34 weeks), animals were sacrificed, their brains removed, immediately frozen on dry ice, sectioned (14 µm) with a cryostat (Leitz) and thawed onto slides (ProbeOnTM slides, Fisher Biotech, USA). *In situ* hybridization was performed as described previously [19,44-46]. Oligonucleotides were labeled at the 3' end with [α -³⁵S]dATP using terminal deoxyribonucleotidyl transferase (NEN, DuPont, Sweden) and purified on nensorb columns (NEN). Sections were hybridized at 42°C overnight in a humidified chamber with 0.1 ml per slide of the hybridization solution [50% formamide, 4xSSC, 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, 0.02%

bovine serum albumin, 10% dextrane sulfate, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl (N-lauroyl sarcosine), 0.02 M Na₃PO₄ (pH 7.0), 50 mM dithiotreitol] containing 1x10⁷ CPM/ml probe. Sections were subsequently rinsed, washed four times (15 min each) at 54°C in 1xSSC, cooled to room temperature, dehydrated through 70%, 90% and 99% ethanol and air dried. Sections were dipped in Kodak NTB-2 photo emulsion (diluted 1:1 in water), exposed for 6 (truncated *trkB*) and 15 weeks (neurotrophins) at -20°C, developed, fixed, and lightly counterstained with cresyl violet. Sections were analyzed using light and dark-field microscopy (Nikon Microphot) and photographed (Kodak Tmax-400). Slides were evaluated under the dark field microscope and the signal estimated using a semi-quantitative scale from 0-4.

The following antisense oligonucleotides were used:

NGF, bp 464-513 (5'-CCT GGG GTC CAC AGT AAT GTT GCG GGT CTG CCC CGC CAC GCG TGC AGC TAT) /43/; BDNF, bp 251-298 (5'-CTC CAG AGT CCC ATG GGT CCG CAC ACC TGG GTA GGC CAA GCT GCC TTG) /33/; NT-3, bp 379-429 (CTC CAA GGG GGT GCT GTC GCT CAG CAG GAC CCG CGG TGA ATT GTA GCG TCT) /26/; rat specific truncated *trkB*, bp 723-767 (5'-ACA TGG GGC AGG CAA GAG AAG CCC TCC AGA AGC CCA AGA CCA GCA) /27/.

RESULTS

All neurotrophin oligonucleotides showed cross reaction with rat and gave the expected hybridization signal in host rat hippocampus (Fig. 1, upper panel). NGF mRNA was strongly expressed in neurons in and around the host granule and pyramidal cell layer of hippocampus, BDNF mRNA was found in neurons in the granule and pyramidal cell layer of the hippocampal formation and NT-3 mRNA was seen in neurons of the granule cell layer of dentate gyrus and in CA2 and medial CA1 areas.

All three neurotrophins were expressed in the developing human cortical xenografts (Fig. 1, middle and lower panel). Clear scattered areas of NGF positive cells were detectable in the human transplants. The NGF mRNA signal was lowest in the younger grafts (16.5-20 weeks), reached a

Fig. 1: Expression of neurotrophin mRNAs in dentate gyrus of the rat host hippocampus and in human cortical xenografts. The oligonucleotides cross react with rat and reveal NGF mRNA expression in neurons (arrows) inside the host granule cell layer of the dentate gyrus and in the hilus (left panel); BDNF mRNA expression in neurons in the granule (gr) cell layer of the hippocampal formation (middle panel), and NT-3 mRNA expression in neurons of the granule cell layer of dentate gyrus (right panel). In the human cortical xenografts clusters of cells were found expressing either NGF, BDNF or NT-3 mRNAs. The pictures show examples of transplants taken 25 (NGF and NT-3) or 16.5 (BDNF) weeks postgrafting. Bright field microscopy reveals specific localization of the silver grains over cresyl violet counterstained cells (lowest panel). Bar (from left to right) = 90, 140, 30 μ m (NGF); 20, 140, 20 μ m (BDNF); 70, 140, 25 μ m (NT-3).

maximum at week 25, and decreased again in the older grafts (32-34 weeks) (Table 1). A strong patchy expression of BDNF mRNA was seen in the human cortical grafts. The signal for BDNF mRNA was highest in the youngest graft (16.5 weeks), slightly decreased thereafter, reaching a moderate expression in the older grafts (Table 1). NT-3

mRNA was found in the grafts in a patchy pattern. The signal for NT-3 mRNA peaked at week 25 (Table 1).

In situ hybridization for rat truncated *trkB* receptor mRNA showed a strong signal over the host cortex (Fig. 2a,b). The grafts partly revealed higher background but did not show a specific

TABLE 1

Quantitative expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) mRNAs in five different human cortical grafts, as evaluated by *in situ* hybridization

graft #	fetal age [weeks]	grafts [weeks]	mRNA expression in the grafts*		
			NGF	BDNF	NT-3
1	9.5	16.5	+(+)	++++	+
2	9	20	++	+(+)	++
3	9.5	25	+++(+)	++	+++(+)
4	8.5	32	+(+)	+(+)	+
5	8.5	34	(+)	+++	+(+)

* The intensity of labeling was semiquantitatively graded: + low, ++ medium, +++ strong, and ++++ very strong.

Fig. 2: High levels of expression of rat truncated *trkB* receptor mRNA are found in the rat host cortex (ctx), while the human transplants (tp) have a somewhat higher background but are negative (a, b). Somewhat stronger signals are found close to the graft-host interface (a, b). The box in Fig. a represents the area depicted at higher magnification in Fig. b. In a few areas a strong signal is found representing “permissive sites” (large arrows, c, d). Bright field microscopy reveals specific localization (small arrows) of silver grains over cresyl violet counterstained cells (d). Bar = 240 μ m (a), 100 μ m (b), 150 μ m (c), 50 μ m (d).

signal (Fig. 2a-d). Often the hybridization signal was increased at the graft-host interface (Fig. 2b). In a few cases a strong signal was found in rat-derived cells, possibly in areas where nerve fibers entered the transplant (Fig. 2c,d).

DISCUSSION

This study is an extension of our previous studies in which human-to-rat cortical xenografts /16,18/ and rat cortex *in oculo* grafts /17/ were characterized. Briefly, the majority of the grafts survived, became integrated with the rat host cortex and formed reciprocal nerve fiber connections. Specific "permissive sites" seem to be involved in this nerve fiber growth from graft to host and from host to graft. The present study demonstrates that NGF, BDNF and NT-3 mRNAs were expressed in the developing human cortical xenografts.

NGF, BDNF and NT-3 cross reacted with rat and gave the expected pattern of expression in the rat hippocampus /9,12,31/. The probe for truncated *trkB* receptor gave strong signals all over the rat host brain, especially in rat cortex /19/. The positive cells in the grafts expressing the neurotrophins and their receptors were identified using bright field microscopy, showing the silver grains to be located over cresyl violet counterstained cells.

Several studies have indicated a role for neurotrophins in cortical development in the rat. Using *in situ* hybridization, BDNF mRNA was found in several cortical areas (frontal, parieto-temporal, cingulate and piriform) in the developing rat from 2 weeks of age and thereafter /10/ and in prefrontal cortex of fetal and adult monkeys /20/. Exogenous NGF affected the development of grafted cortex cerebri /8/ and BDNF, and much less potently NT-3, regulated neuropeptide expression in cultured cerebral cortical neurons /29/. Castrén *et al.* /3/ demonstrated that physiological stimuli (visual input changes) can regulate BDNF and *trkB* mRNAs in the visual cortex. Neurotrophins also seem to play an important role in glutamate-mediated excitotoxicity in cortical neurons /22,33, 34/. Recently, we reported /17/ that BDNF mRNA as well as *trkB* receptor mRNAs were expressed in rat cortex cerebri grafted to the anterior chamber of the eye. BDNF mRNA expression was increased

compared to adult cortices in single grafts and downregulated to near control levels by a compensating second graft, indicating trophic interactions /17/. The present study extends these reports, and describes that all three analyzed neurotrophins, NGF, BDNF and NT-3, are expressed in the human cortical xenografts, likely playing important roles during development. Indeed, a recent report showed that BDNF is required for activity-dependent survival of cortical neurons /13/. Since it was not easy to obtain several grafts of the same age in this study, the different age data need to be carefully interpreted. It might be possible that the different levels of mRNA expression do not represent normal developmentally regulated mRNA expression, but are merely a reflection of intra-transplantation or intra-donor variability.

Earlier studies of similar grafts suggested that most cells in our human grafts were immature /18/. Recently, we reported that the mRNA for a neuropeptide precursor, human angiotensinogen, was expressed mainly in immature protoplasmatic astrocytes in human cortical grafts /18/. As we were not able to use non-radioactive *in situ* hybridization combined with glial immunomarkers, we cannot determine whether the neurotrophins are expressed in immature neurons and/or glial cells as well. However, since the localization of neurotrophins in glial cells has not been demonstrated by *in situ* hybridization *in vivo*, we suggest that the neurotrophin mRNAs are expressed in immature neurons in the human cortical grafts. This is in agreement with Miranda *et al.* /28/, who reported that NGF and BDNF and more rarely NT-3 mRNAs were expressed in the developing rat cerebral cortex.

The neurotrophins bind with high affinity to receptors encoded by members of the *trk* family of proto-oncogenes /6,7,32/. The *trk*-related gene *trkB* encodes a high-affinity signal transducing component, having high affinity for both BDNF and NT-3 /36,37/. The full-length *trkB* contains a tyrosine kinase domain, while truncated *trkB* lacks an intracellular signal-transducing domain /21,27/. In the CNS, the full-length *trkB* mRNA is predominantly expressed in neurons, while expression of truncated *trkB* is predominantly found in glial cells /11,21,44/. Miranda *et al.* /28/ recently demon-

strated that the *trkB* receptor mRNA is expressed in the developing rat cerebral cortex, and is co-expressed with different neurotrophins. Kumar *et al.* /23/ reported that *trkB* is present in astrocytes and upregulated by NGF, suggesting a role in normal glial function. In our study, truncated *trkB* mRNA expression was often very strong in areas at the graft-host interface to the cortical grafts indicating that rat astroglial processes are involved. We have also observed a similar phenomenon in intraocular rat cortical transplants /17/. The present data indicate that glial processes and the high affinity *trkB* receptor might be involved in neuronal and glial communication at the graft-host border.

"Permissive sites" seem to be responsible for establishing reciprocal nerve fiber connections. Several cases of such "permissive sites" were found in our human cortical grafts and nerve fibers labeled with human-specific neurofilament antibody entered such sites (for details see /16,18/). We report now that these "permissive sites" appear to contain rat-derived cells expressing the truncated *trkB* receptor mRNA. These cells seem to form an entry for the ingrowth (or outgrowth) of nerve fibers, starting from a specific point at the host-graft interface. We suggest that these cells are rat astrocytes, because truncated *trkB* is predominantly found in glial cells and neurons, while the expression of full length *trkB* is restricted to neurons. At the "permissive sites", it might be possible that astroglial processes are necessary for guiding/targeting nerve fiber growth and that neurotrophins could play a role in this process.

This study shows that mRNAs for NGF, BDNF and NT-3 are expressed in developing human cortical grafts. We conclude that neurotrophins may play a role during development and nerve fiber growth in the human cortex, and rat-derived astroglia could be involved in establishing reciprocal connections.

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