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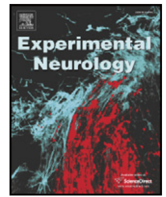

Locus coeruleus promotes survival of dopamine neurons in ventral mesencephalon. An *in oculo* grafting study

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ABSTRACT

Parkinson's disease is a neurodegenerative disorder where dopamine neurons in the substantia nigra of ventral mesencephalon undergo degeneration. In addition to the loss of dopamine neurons, noradrenaline neurons in the locus coeruleus degenerate, actually to a higher extent than the dopamine neurons. The interaction between these two nuclei is yet not fully known, hence this study was undertaken to investigate the role of locus coeruleus during development of dopamine neurons utilizing the intraocular grafting model. Fetal ventral mesencephalon and locus coeruleus were implanted either as single grafts or co-grafts, placed in direct contact or at a distance. The results revealed that the direct attachment of locus coeruleus to ventral mesencephalon enhanced graft volume and number of tyrosine hydroxylase (TH)-positive neurons in ventral mesencephalic grafts. Cell counts of subpopulations of TH-positive neurons also immunoreactive for aldehyde dehydrogenase 1-A1 (ALDH1) or calbindin, revealed improved survival of ALDH1/TH-positive neurons. However, the number of calbindin/TH-positive neurons was not affected. High density of dopamine-β-hydroxylase (DBH)-positive innervation in the ventral mesencephalon placed adjacent to locus coeruleus was correlated to the improved survival. Ventral mesencephalic tissue, implanted at a distance to locus coeruleus, did not demonstrate improved survival, although DBH-positive nerve fibers were detected. In conclusion, the direct contact of locus coeruleus resulting in dense noradrenergic innervation of ventral mesencephalon is beneficial for the survival of ventral mesencephalic grafts. Thus, when trying to rescue dopamine neurons in Parkinson's disease, improving the noradrenergic input to the substantia nigra might be worth considering.

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Introduction

Parkinson's disease is a progressive neurodegenerative disorder where dopamine neurons in the substantia nigra of the ventral mesencephalon (VM) undergo degeneration and consequently, loss of dopamine nerve fibers occurs in the dorsal striatum. The symptoms of the disease appear when at least 50% of the dopamine neurons are lost in the VM, and the dopamine levels are reduced by 80% in the dorsal striatum (Agid, 1991; Kish et al., 1988). However, an extensive cell loss also occurs in the locus coeruleus (LC), where the noradrenaline neurons undergo degeneration (German et al., 1992). This noradrenergic loss in patients suffering from Parkinson's disease was first documented in 1960 (Ehringer and Hornykiewicz, 1960), and has thereafter been described several times (Gesi et al., 2000). Interestingly, in other progressive neurodegenerative disorders such as Alzheimer's disease, loss of noradrenergic neurons in the LC has been documented in addition to the characterized loss of septal cholinergic neurons (German et al., 1992; Mann, 1983). In fact, the decline in noradrenergic neurons in the LC is more pronounced than the loss of dopamine and cholinergic neurons in Parkinson's and Alzheimer's diseases, respectively (Chan-Palay, 1991; German et al., 1992; Mann, 1983; Zarow et al., 2003).

The consequence of degeneration in the LC in neurodegenerative disorders is still unclear. However, noradrenaline *per se* reduces oxidative stress and loss of noradrenergic nerve fibers down-regulates anti-inflammatory agents in the brain (Heneka et al., 2003). Moreover, dopamine neurons in the substantia nigra become more sensitive to various insults in animals with depleted noradrenergic neurons in the LC (Heneka et al., 2003; Mavridis et al., 1991; Srinivasan and Schmidt, 2004), and noradrenergic hyperinnervation of the brain protects the nigrostriatal dopamine neurons from neurotoxins (Kilbourn et al., 1998). Furthermore, it has been demonstrated that lesion of the LC reduces the metabolism of dopamine in the striatum to such an extent that the dopamine D2 receptors become supersensitive (Harro et al., 2003). Although the LC projections are widespread over the brain, including targets such as the substantia nigra and ventral tegmental area (Collingridge et al., 1979; Jones and Moore, 1977), interaction between the LC and substantia nigra is not well established.

Studies of interactions between different brain areas can be performed utilizing isolated tissues of interest in a co-culture system. For long-term studies the *in oculo* grafting model is a useful tool instead of cultures, since the grafts may survive as long as the rat host (Eriksdotter-Nilsson et al., 1988). The LC has been extensively studied

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in intraocular co-grafts with hippocampus, and as triple-grafts together with hippocampus and septum (Srivastava et al., 1997; Willis et al., 2005). The *in oculo* transplants develop an intact blood–brain barrier a couple of weeks postgrafting. Thus, it is possible to create small brain microcircuits under *in vivo* conditions (Granholm et al., 1996). This grafting technique has also been used to monitor effects of neurotrophic factors on survival of dopamine neurons in VM grafts (Björklund et al., 1997; Giacobini et al., 1993; Strömberg et al., 1993).

As already mentioned, LC has been implicated to protect dopamine neurons from various insults in adult animals but more attention is needed to elucidate the role of LC during development of the VM. Therefore, the survival of VM dopamine neurons in the presence or absence of noradrenergic nerve fibers was investigated using the intraocular grafting model.

Materials and methods

Animals

Female Sprague–Dawley rats (150 g) were used as recipients and pregnant females of the same strain served as donors (Scanbur B&K, Sollentuna, Sweden). All animals were housed on a 12-hour light/dark cycle under constant temperature, given free access to water and food pellets. The experiments have been performed in accordance with international standards on animal welfare and were approved by the local ethics committee.

Dissection procedure

Pregnant rats were anesthetized with isofluran (Baxter Medical AB, Sweden) using Univentor 400 anesthesia unit (AgnThos, Sweden) and neck dislocated. Embryonic day 13–14 (crown–rump length 10–13 mm) fetuses were dissected in Dulbecco's modified Eagle medium (DMEM; Gibco, Sweden). Both ventral mesencephalon (VM) and locus coeruleus (LC) were bilaterally dissected from each fetus under a dissection microscope. The mesencephalic flexure was isolated and divided in the midline into two pieces, and the pontine flexure was used as orientation for dissection of the two LC tissue pieces. One unilateral tissue piece of either VM or LC was used per eye when grafted as single transplant, or combined for co-grafts.

Intraocular grafting

Prior to the intraocular grafting, the recipient rats (n=41) were bilaterally sympathectomized under deep isofluran anesthesia to remove the endogenous noradrenergic innervation of the eye. The sympathectomy was performed a minimum of two weeks before grafting to achieve normalized levels of neurotrophic factors after the denervation (Ebendal et al., 1983; Ebendal et al., 1985). Rats with intact superior cervical ganglion (n=6) were also used in this study. Before grafting, atropin was given to each eye of the rats to dilate the pupil in order to prevent injury of the iris when opening the cornea. During anesthesia, the rats received the intraocular transplants through a slit in the cornea made by a razor blade, and the tissue pieces were implanted into the anterior chamber of the eye using a Pasteur pipette with a modified tip. One unilateral tissue piece from VM and LC was transplanted either as single VM (n=19), single LC (n=8), or VM+LC co-grafts (n=12) placed adjacent to each other. Furthermore, VM and LC were also grafted into the same eye and placed opposite to each other (n=10). Eyes, where only one of two tissue pieces in the double grafts had survived, were excluded. Additionally, single VM transplants were implanted either to intact (n=12) or to sympathectomized eyes (n=12). Graft sizes were measured at the time of transplantation, 4, and 8 weeks after transplantation using an operation microscope equipped with a scale mounted in one of the oculars. In co-grafts, the placement of the two tissue pieces was documented at grafting, and then monitored throughout the study.

Immunohistochemistry

Eight weeks postgrafting, the animals were given 0.5 ml pentobarbitalnatrium prior to intracardial infusion of 50 ml Tyrode solution (Ca^{2+} free), followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The intraocular grafts were dissected and postfixed for 1 h in the same fixative, and then rinsed in 10% sucrose in 0.1 M phosphate buffer. The grafts were stored in sucrose solution at 4 °C until processed for indirect immunohistochemistry. The transplants were rapidly frozen in gaseous CO_2, and cryostat sections (14 μm) were collected and thawed onto gelatine-coated glass slides. The primary antibodies used in this study were tyrosine hydroxylase (TH; mouse anti-rat, diluted 1:1500, Immunostar Inc., Hudson, WI, USA; rabbit anti-rat, diluted 1:300, Pel Freez, Rogers, AK, USA), dopamine beta hydroxylase (DBH; mouse anti-bovine, 1:150, Chemicon-Millipore, Stockholm, Sweden), aldehyde dehydrogenase 1A1 (ALDH1; rabbit anti-mouse, 1:150, Abcam, Cambridge, UK), and calbindin$_{D28k}$ (calbindin; mouse anti-chicken, 1:2000, Swant, Bellizona, Switzerland). Sections cut from ventral mesencephalon from the brains of the host animals were processed for ALDH1/TH double staining to evaluate the localization of the two antibodies. The primary antibodies were applied for 48 h at 4 °C. Blocking unspecific binding was performed using 5% goat serum (Sigma-Aldrich, Stockholm, Sweden) in 0.1 M phosphate buffered saline (PBS) for 15 min at room temperature. Incubations in secondary Alexa Fluor® 594 conjugate antibodies (goat anti-mouse, 1:500; goat anti-rabbit, 1:500) or Alexa Fluor® 488 conjugate antibodies (goat anti-mouse, 1:200; goat anti-rabbit, 1:500; Molecular Probes Inc., USA) were performed for 1 h at room temperature. Finally, the sections were mounted in 90% glycerol in 0.1 M PBS. All incubations were performed in a humidified chamber and double labeling was performed in sequence with one antibody at a time. Rinsing the sections in 0.1 M PBS preceded incubations of antibodies. The primary antibodies TH and DBH, and all secondary antibodies were diluted in 0.1 M PBS containing 0.3% Triton X-100, whereas the primary antibodies against ALDH1 and calbindin were diluted in 0.1 M PBS containing 2% goat serum or 10% fetal calf serum (Sigma-Aldrich, Sweden), respectively. When Triton-X-100 was excluded in incubation with primary antibodies, the sections were preincubated in 0.3% Triton X-100 in 0.1 M PBS containing 10% serum for 1 h at room temperature and rapidly rinsed in 0.1 M PBS before primary antibodies were applied.

Image analysis and statistics

The size of the transplants was estimated by measuring the longest diameter and the diameter perpendicular to it. The anterior eye allows the transplants to grow to a thickness of approximately 1 mm, and therefore the volume of the grafts was calculated by multiplying the two linear measurements. This estimation of graft volume has proven valid by comparisons with actual graft weight (Björklund et al., 1980).

Cell counts of TH-positive neurons were performed on every fifth section throughout the grafts to estimate the number of surviving neurons in both VM and LC transplants. The total number of TH-positive neurons within each graft was estimated using the formula of Abercrombie (Abercrombie, 1946). When VM and LC were transplanted together, double labeling using TH/DBH was performed to characterize the grafts. Thus, TH/DBH-positive neurons were counted in the LC, and TH-positive/DBH-negative neurons were counted in the VM. The borderline between the two transplants became obvious when switching to the DAPI-filter. Furthermore, ALDH1/TH-positive neurons, as well as calbindin/TH-positive

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