Transplantation of the rat suprachiasmatic nucleus. Functional studies and ex vivo adenoviral vector-mediated gene transfer
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Citation for published version (APA):

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Effect of adenoviral vector-mediated expression of neurotrophin-3 in suprachiasmatic nucleus transplants on restoration of circadian rhythmicity; preliminary results

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Transplants of fetal suprachiasmatic nucleus (SCN) tissue were transduced with an adenoviral vector encoding for neurotrophin-3 (NT-3) and subsequently transplanted in the third ventricle of SCN-lesioned arhythmic Wistar rats. Previously, it was shown that the expression of NT-3 in these transplants resulted in an increase in the number of vasopressinergic (VPergic) and vasoactive polypeptidergic (VIPergic) SCN neurons and a tendency towards an increase in efferent growth of the VPergic SCN neurons into the host brain. In the present study it was investigated whether the adenoviral vector-mediated expression of NT-3 in the transplants resulted in an increase in the percentage of SCN-lesioned arhythmic rats that show restoration of circadian rhythmicity after transplantation. A circadian rhythm in locomotion, drinking, eating and wheel-running activity was restored in 2 out of 7 rats (28%) with a mock-infected control SCN graft, and in 3 out of 5 rats (60%) with a AdNT-3-transduced SCN graft. These preliminary results suggest that expression of NT-3 in fetal SCN transplants promotes the recovery of circadian rhythmicity. Since the number of rats per group is very small, more experiments are necessary to reproduce and substantiate this effect.

Introduction

Transplants of fetal suprachiasmatic nucleus (SCN) can restore circadian rhythmicity of some behavioral parameters of SCN-lesioned rat and hamster [1-5,8-10,12]. Although SCN transplantation studies have been carried out since 1984 [7,11], the mechanism and the pathways involved in rhythm restoration are still not fully understood. Elucidation of
these issues might contribute to the understanding of the circadian pacemaker. A peculiar finding in SCN transplantation studies is the difference in percentages of rhythm recovery in SCN-transplanted hamster and rat (80-100% vs 40-50%). Recovery of wheel-running rhythms in hamster is established relatively independent of the location of the transplanted SCN [1,9] and in the absence of neuritic contact of the SCN neurons with SCN target areas in the host brain [13]. By contrast, in rat, recovery of drinking and locomotion rhythms is only observed when the transplant is located in the third ventricle, i.e. at the SCN lesion site [3,8]. Moreover, a relation with reinnervation of SCN target areas is suggested: all rats that recover their circadian drinking rhythm show outgrowth of vasopressinergic (VP) and vasoactive intestinal polypeptidergic (VIP) fibers into the host brain, and the pattern of outgrowth resembles the innervation pattern of the in situ SCN [8]. However, the density and the extent of the outgrowth is very limited. It is conceivable therefore that an increase of graft-to-host efferents could improve functional repair of circadian rhythmicity in SCN-lesioned rat.

In an attempt to improve functional SCN transplantation by stimulating neuronal survival and neurite outgrowth of the SCN transplant, SCN grafts have been transduced with an adenoviral vector encoding for neurotrophin-3 (AdNT-3) and were subsequently implanted in SCN-lesioned Wistar or Brattleboro rats. The long-term expression of NT-3 resulted in a higher number of VPergic and VIPergic SCN neurons in the transplants, with some indications of increased SCN fiber growth (Chapter 8). In the present study, the performance of AdNT-3-transduced SCN transplants was investigated with respect to restoration of circadian locomotion, wheel-running, drinking and eating rhythms in SCN-lesioned arrhythmic Wistar rats. Elaborate microscopical evaluations as described in Chapter 8, were presently combined with rhythm recordings in order to see whether restoration of rhythmicity could be related to specific features of the SCN transplant.

Materials and methods

Male and female Wistar rats (Harlan, Zeist, The Netherlands) were housed under standard conditions with food and tap water available ad libitum. Female rats served as dam for E17 SCN donor fetuses (E0 is the day after overnight mating). Male rats were SCN-lesioned when weighing 280-350 g [8], and were thereafter housed individually in cabinets with light- and sound-tight ventilated rhythm-recording cages (40 x 40 x 38 cm) under continuous dim red light (DD, 0.2 lux). Recordings of locomotion, drinking, eating and wheel-running rhythms were carried out for 6 or 12 weeks after lesioning to test for arrhythmicity. The bottom of the cage consisted of a metal grid on which a small perspex animal cage with sawdust was placed. Locomotion was detected by two overlapping infrared beams in the ceiling of the cage, wheel-running by movements of a wheel of 34.5 cm in diameter (10 pulses per complete rotation) (Tecniplast, Buguggiate, Italy), drinking by registration of drinking nipple contacts via an electric circuit, and eating by interruptions of an infra-red beam in front of the food bin. The lighting condition in the cage was detected by a light sensor. The rhythm-recording cages were made in our institute (design and
production by H. Overdijk, A. Kamstra, N. Bos, M. Mirmiran and C. Pool) and on-line connected with a computer system collecting automatically the frequency data and lighting condition of 6 min periods (software design D. van der Werf). Cages were cleaned about every 8 days on an irregular basis to avoid entrainment to handling. Circadian locomotion, wheel-running, drinking and eating rhythmicity was analyzed by visual inspection of double-plotted actograms and by Chi-square periodogram analysis [15] of the 6 min period frequency data (Tau, Jonathan Schull, Mini-Mitter Co., Inc. Sunriver, OR, USA). Arhythmicity of SCNX rats was tested on the data of a 3-week period between week 3 and 12 post-lesion. Rats which lack a circadian rhythm (period length between 20 and 26 h) according to Chi-square analysis were defined arhythmic and were used as recipients for SCN transplants.

The construction of the recombinant adenoviral vector encoding for NT-3 (AdNT-3), the ex vivo transduction protocol, and the transplantation surgery of the fetal SCN tissue were described previously [6,8,17]. One group of arhythmic rats received mock-transduced control transplants (n=7; 18 h incubation in culture medium) and a second group received AdNT-3-transduced transplants (n=5; 18 h incubation with 5x10⁶ pfu/ml AdNT-3 in culture medium). After transplantation, rhythm recordings were continued for 10 or 30 weeks for the control rats and 25 weeks for the rats receiving an AdNT-3-transduced transplant. Analysis of restoration of rhythmicity was performed on the data of a 3-week period between week 8 to 12 post-transplantation as described above. The rats were designated rhythmic when 2 or more parameters displayed circadian rhythmicity. The period length of the circadian rhythms was calculated from the slope of an eye-fitted line through the activity onsets of the entire post-transplantation period.

Rats were sacrificed by perfusion fixation and their brains were processed for immunocytochemistry for CPP (C-terminal proopressophysin as a marker for VPergic cells) and VIP as described in detail before (Chapter 8). The transplants were scored for the number and location of the SCN cell clusters (frontal, homotopic or caudal from the lesioned host SCN), and for the estimated number of CPP- and VIP-positive cells and fibers in the transplant and those crossing the graft-host interface (on a scale from - to ++; see Chapter 8). Chi-square analysis at p<0.05 was performed on the scores for immunocytochemical parameters and scores for behavioral rhythms to see if any of the features of the transplanted SCN could be correlated with the restoration of circadian rhythmicity.

Results

Restoration of circadian rhythmicity
Restoration of circadian rhythmicity was observed in 2 out of 7 arhythmic SCNX rats that received a mock-transduced SCN graft (Table 1). The circadian rhythm in the locomotion, eating and running-wheel activity of rat #23 evolved between week 2 and 3 after transplantation with a period length of 24.1 h (Figure 1). Rhythm recordings of rat #29 revealed a weak circadian rhythm in locomotion and eating activity but not in the drinking activity, which evolved only at 11 weeks after transplantation with a period length of 24.2
Figure 1. Double plotted actograms of two control rats housed under DD conditions throughout the registration period. The activity actogram of rat #9 reveals arhythmicity prior and after transplantation (solid triangle), whereas activity, eating and wheel running actograms of rat #23 reveal a restoration of rhythmicity within 20 days after transplantation with a periodicity slightly longer than 24 h (24.1 h).
Figure 2. Double plotted actograms of activity, drinking, eating and wheel running of rat #1404 in DD conditions, which was implanted with a AdNT-3-transduced transplant. The actograms reveal a simultaneous restoration of a circadian rhythm (24.0/24.1 h) in all four behavioral parameters within 50 days after transplantation (solid triangle).
h. For comparison with the outcome of rat #23, Figure 1 also shows the activity actogram of control rat #9 that failed to restore circadian rhythmicity.

In the group of rats that received AdNT-3-transduced transplants, 3 out of 5 rats restored circadian rhythmicity in all 4 parameter studied (#1401, #1404, #1405), while 1 rat (#1402) revealed a circadian rhythmicity only in wheel-running activity (Table 1) and thus was considered arrhythmic. Circadian rhythmicity in the behavioral parameters of the 3 rhythm-restored rats showed up around week 4 (#1401, #1405) and week 7 (#1404) after transplantation with period lengths of 24.2 h (#1401), 24.0 to 24.1 (#1404) and 23.7 to 23.8 h (#1405). Figure 2 shows the activity, wheel-running, eating and drinking actograms of rat #1404 as an example.

Morphology of the transplants
Viable transplants containing one or more SCN cell clusters were located in the third ventricle in all rats. The number and location of the SCN cell clusters, as well as the scores for the CPP and VIP staining are given for each of the individual rats in Table 1. None of the features of the transplant, or the presence of residues of the lesioned endogene SCN, could be correlated with the restoration of circadian rhythmicity. The estimated numbers of CPP- and VIP-positive SCN cells in the transplants were high in some, but not in all rats that displayed restoration of circadian rhythmicity. For instance, the scores for CPP- and VIP-positive cells of rhythm-recovered rat #1401 were low, whereas the scores were high for rat #19 which remained completely arrhythmic (Table 1). The presence of SCN fibers crossing the graft-host interface could also not predict rhythm restoration. All rats showed graft-to-host CPP-positive fiber outgrowth, whereas only some rats showed VIP-positive fiber outgrowth, which was again not related to the occurrence of rhythm restoration (Table 1). The location of the SCN cell clusters within the transplant relative to the site of the ablated host SCN had no influence either on restoration of circadian rhythmicity. Rhythm restoration was observed in rats in which the SCN clusters were located frontal/homotopic from the site of the lesioned endogene SCN (#23 and #29), but also in rats in which the SCN cell clusters were located homotopic/caudal (#1405) or only caudal (#1401, #1404) from the lesioned endogene SCN (Table 1).

Discussion
The present results show that the procedure for ex vivo adenoviral transduction of SCN transplants does not impair the capacity of the transplant to restore circadian rhythmicity of arrhythmic SCNX rats. Transduction with a vector encoding for NT-3 resulted in a high percentage of SCNX arrhythmic rats that restore circadian rhythmicity of locomotion, wheel-running, drinking and eating activities (60%) as compared to control transplants (28%). The two experimental groups were however derived from two separate experiments, so that direct statistical analysis to compare the recovery rates could not be performed. Since moreover the number of animals were low, the positive result on functional restoration of circadian rhythmicity is regarded as a preliminary result.
Table 1. Survey of the rhythm registrations (post-transplantation period length) and immunocytochemistry of the SCNX Wistar rats implanted with a mock-transduced or AdNT-3-transduced SCN transplant.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>post-T period length (h)</th>
<th>clusters (f/h/c)</th>
<th>CPP cells</th>
<th>fibers</th>
<th>outgrowth</th>
<th>VIP cells</th>
<th>fibers</th>
<th>outgrowth</th>
<th>endogene SCN</th>
</tr>
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<tbody>
<tr>
<td>mock 9</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>n.a.</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>23</td>
<td>24.1 n.a.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-/+</td>
<td>++</td>
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<td></td>
<td>25</td>
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<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>24.2 n.a.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-/+</td>
<td>++</td>
</tr>
<tr>
<td>AdNT-3 1401</td>
<td>24.2</td>
<td>24.2</td>
<td>24.2</td>
<td>24.2</td>
<td>0/0/1</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>++</td>
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<tr>
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<td>24.3</td>
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<td>0/2/1</td>
<td>++</td>
<td>+</td>
<td>++/+++</td>
<td>++</td>
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<tr>
<td>1404</td>
<td>23.7</td>
<td>23.7</td>
<td>23.8</td>
<td>0.3/2</td>
<td>0/1/1</td>
<td>++</td>
<td>+</td>
<td>++/+++</td>
<td>++</td>
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<tr>
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<td>23.7</td>
<td>23.7</td>
<td>23.8</td>
<td>0.3/2</td>
<td>0/1/1</td>
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<td>++/+++</td>
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<td>++/+++</td>
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</tbody>
</table>

#, rat number; AC, activity; DE, drink event; EE, eat event; RW, running wheel; clusters, number of SCN cell clusters located frontal (f), homotopic (h), or caudal (c) from the lesioned endogene SCN; n.a., not assayable. For explanation of scores see M&M.

In the present study, for the first time, four different behavioral parameters were recorded simultaneously after transplantation of the fetal SCN in SCNX rodents: locomotion, drinking, eating and wheel-running activity. The pattern of activity on these four parameters was similar. When visible, rhythm restoration was usually observed in all of these actograms, except when the restored rhythm was weak, as for rats #29 and #1402. Previous SCN transplantation studies in rat have either used drinking activity alone or drinking activity in combination with eating and wheel-running activity [3]. The latter study reports also coinciding restoration of circadian rhythmicity for the different parameters. Apparently, the rhythms of these four behavioral parameters in are tightly coupled, indicating that they are driven by the implanted SCN.

A previous study (Chapter 8) has shown that the expression of NT-3 in SCN transplants increased the estimated number of CPP- and VIP-positive cells and fibers in the transplant. In the present study, the extensive immunocytochemical evaluation of the transplants was combined with rhythm recordings to evaluate whether this aspect in the transplants brought about by NT-3, could be related to the restoration of circadian rhythmicity. However, in the present group of rats such a relation was not observed. The presence of SCN neurons in the transplant is certainly a prerequisite for rhythm restoration, but a higher number of surviving SCN neurons does not relate to the occurrence of rhythm restoration. It was previously reported that rhythm restoration is always accompanied by graft-to-host efferent growth, seen as crossing of the graft-host border by VPergic and VIPergic fibers SCN fibers, although also non-recovery rats often reveal efferent growth [8]. Moreover, a relationship between VIPergic fiber outgrowth into the host thalamus and recovery of circadian wheel-running activity of hamsters was reported [16]. Adenoviral vector-mediated expression of NT-3 did not increase the number of fibers crossing the graft-host interface but still appeared to have a positive effect on the number of efferent fibers in the
host brain when studied in the Brattleboro rat (Chapter 8). Presently, the density of SCN fibers crossing the graft-host interface appeared to have no relation with the occurrence of rhythm restoration. A positive effect of NT-3 on the efferent growth from the transplanted SCN to denervated host target areas as observed in the Brattleboro rats, could not be investigated in the currently used Wistar rats because of the presence of endogenous CPPergic and VIPergic fibers of non-SCN sources. It can thus not be excluded that the putative positive effect of NT-3 on the percentage of rhythm recovery is mediated through an increase in the efferent growth of VPergic and VIPergic SCN neurons into the Wistar host brain. In previous studies reinstatement of circadian rhythm could never be related to the presence of the few, usually ventrally located, SCN neurons that escaped the lesion [3,8]. In the present study the presence of a residue of the SCN in SCNX animals did not give any clue for rhythm restoration either (see Table 1). The present AdNT-3 group, however, includes relatively more animals with some residual SCN neurons. An action of transplant-derived NT-3 as neurite growth-promoting factor or as regeneration factor on these host cells, mediating in this way the restoration of circadian rhythmicity, could as yet not be excluded. This confounding factor should thus be excluded in future studies.

Since none of the parameters of the VP and VIP staining could be related to the occurrence of rhythm recovery, the presence in the transplant of SCN neurons and/or their efferent fiber growth other than the VPergic and VIPergic neurons might be more crucial for rhythm restoration. Silver et al. [14] have characterized a group of calbindin-positive cells in the core of the hamster SCN, which might play a crucial role in rhythm generation. Elimination of the calbindin-positive cells resulted in arrhythmic hamsters, even when VPergic and VIPergic neurons remained present, and the presence of calbindin-positive cells in SCN transplants correlated fully with restoration of circadian wheel-running rhythms of SCN-lesioned hamsters (personal communication). Calbindin-positive cells are however not present in the rat SCN (own observation), but yet another SCN cell type with the same functional significance may exist. Similar to the survival-enhancing effect on the VPergic and VIPergic cells in the transplants, one might speculate that adenoviral vector-mediated expression of NT-3 has influenced this cell population and via this way improved rhythm restoration.

The preliminary results presented here suggest that expression of NT-3 in fetal SCN transplants increases the occurrence of rhythm restoration in SCN-lesioned adult Wistar rats, but a firm conclusion can not be drawn since a direct comparison with the control group could not be made. The recovery rate in the control group resembled recovery rates previously reported in SCN-transplanted rats, whereas the recovery rate in the NT-3 treatment group was notably higher. A future study, including preferably both a AdLacZ control group and an untreated control group, should be set up to further investigate the effect of adenoviral vector-mediated NT-3 expression on restoration of circadian rhythmicity. Inclusion of a large number of animals would moreover allow the identification of a possible effect of NT-3 on characteristics of the restored rhythms such as amplitude, period length or time span to rhythm recovery.
References


Since some of the members of the VT and VIP staff were not able to attend the
opening ceremony, the installation of the equipment was delayed. The equipment
was finally installed on the 15th of the month. The equipment was then tested
and the results were satisfactory. However, the installation of the equipment
was not completed on time.

The equipment was designed to perform the following functions:

1. Data collection
2. Data analysis
3. Data storage

The equipment was installed in the room labeled "VT-1" and was connected to
the power supply. The equipment was then tested and found to be functional.

Since the installation was delayed, the equipment was not available for the
event. The equipment was eventually used during the event, but only after the
event was over.

The equipment was designed to withstand harsh conditions and was
expected to have a long lifespan. However, the installation was delayed,
resulting in the equipment being unavailable for the event.

Chapter 10