Transplantation of the rat suprachiasmatic nucleus. Functional studies and ex vivo adenoviral vector-mediated gene transfer
van Esseveldt, K.E.

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General discussion

Transplantation of the suprachiasmatic nucleus

Though transplantation studies have resulted in a better understanding of the functioning of the suprachiasmatic nucleus (SCN), these studies have also yielded several unexpected observations, thereby generating new questions that may contribute to our further understanding of the functioning of the SCN in vivo. SCN transplantation studies have been carried out in rat and hamster with different results. The most striking difference concerns the success rate of SCN grafts to restore a circadian rhythm in SCN-lesioned arrhythmic host animals. In hamster studies, recovery rates are usually between 80 and 100%, whereas in rat studies the recovery rates are variable, but rarely exceed 60% (Table 1). In addition, the success of the transplantation appears to depend on the location of the transplanted SCN in the rat, but less so in the hamster. In the rat, grafts in the third ventricle appear to be most successful for rhythm restoration [1, 12, 37, 66], while transplants in the ventricular system near the PVT can restore circadian drinking rhythms in a few cases only (Van Esseveldt, unpublished observations; Table 1) and transplants in the lateral ventricle were never able to restore a rhythm [12, 37, 67]. In the hamster, rhythm recovery appears to be less dependent of the location of the graft, and is achieved by grafts in the third ventricle, but also in the lateral ventricle, and even in the foramen of Monro [3, 51, 53]. However, the accuracy of the restored rhythm in the hamster was dependent on the location of the graft [52].

The discrepancy between the results in rat and hamster might partly be explained by methodological differences. In rat studies, drinking activity is usually monitored, while in hamster studies, wheel-running activity is the most often used read-out parameter for circadian rhythmicity. In the intact animal, the SCN is thought to influence the motivational state of the animal through innervation of the paraventricular nucleus of the thalamus (PVT), the paratenial nucleus and the lateral and medial septum, structures which project to the limbic system. Projections of the SCN to the medial preoptic nucleus (MPN), on the other hand, are thought to involve circadian regulation of fluid homeostasis (reviewed in [87]). The notion that these two brain structures may be involved in the two different activities agrees with the finding that restoration of circadian wheel-running rhythmicity in hamster is correlated with an innervation of the host PVT by vasoactive intestinal polypeptidergic (VIPergic) SCN neurons of the graft [82], and that restoration of circadian drinking rhythmicity in the rat is most efficiently established by SCN grafts near the lesion site [12, 37, 67]. However, such a direct relation between the optimal location of the graft and restoration of the different rhythms is disputed by the finding that in the hamster restoration of the circadian wheel-running rhythm is relatively independent of the location of the graft, and by several rat studies showing that locomotor or wheel-running rhythms were restored along with the drinking rhythm [12, Chapter 9 of this thesis]. Such a
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coinciding restoration of different rhythms suggests that the rhythmic signal from the grafted SCN reaches either all target areas in the same period or only the main target area of the SCN, the subparaventricular zone (sPVZ), which in its turn projects to the PVT and MPN.

Apart from the different rhythms that were measured, the other methodological differences in transplantation studies complicate a comparison of the results. Table 1 gives an overview of the transplantation studies and the method variations. The evaluation criteria of the activity pattern before and after transplantation and the criteria for the transplants to be included in the calculations of the various studies are different and sometimes not well documented either. The inclusion or exclusion of animals with grafts lacking an SCN, for instance, influences the recovery percentage. Other variables among transplantation studies include the number and age of the donor SCNs used and the dissection and preparation method (tissue block vs. cell suspension). Comparison of transplantation results obtained by grafts of ages between Embryonic day (E) 10 and Postnatal day (P) 28 showed that maximal recovery (60-90%) is achieved with donor tissue taken between E14 and P6 [47]. Such an extensive range of donor ages has not been investigated in rat transplantation studies. Usually E16/17 tissue is implanted, but implantation of a fetal rat SCN of E20 gave similar results (Van Esseveldt, unpublished observations; Table 1). In most hamster and rat studies, the donor SCN is usually dissected as a block of tissue including the surrounding anterior hypothalamus, which is directly implanted into the host animal. Other studies cut the tissue into a few fragments, minced it to multiple small pieces, isolated the SCN by taking a micropunch to avoid implantation of non-SCN tissue, or dissociated the SCN into separate cells before implantation. In the hamster, the latter two dissection methods results in recovery percentages of 100% and 91%, respectively, when calculated according to the criteria mentioned in Table 1. In the rat, no clear difference was found in the recovery percentage after transplantation of the SCN as a block or minced (44% and 41%, respectively) (Van Esseveldt, unpublished observations; Table 1).

Table 1. Selection of SCN transplantation studies illustrating the different methods that have been used. Variations in the properties of the donor SCN include the species (H, hamster; M, mouse; R, rat), the age (E, embryonic day; P, postnatal day), the preparation method, and the number of donors per host. The transplants are located in the third ventricle (3V), the lateral ventricle (LV), the foramen of Monro (FM), or the thalamus or hypothalamus parenchyma. Rhythmicity of the host (wheel-running, drinking or eating activity) is studied under light-dark (LD) or constant dark (DD) conditions and the period between the SCN lesion and transplantation varies between 2 and 37 weeks. In order to allow a comparison of the transplantation results, for each study the number of SCN lesioned animals that receive an implant (total n), the number of animals which appear to have a viable transplant (T+), the number of animals with an SCN-containing transplant (SCN+), and the number of animals that recovered a circadian rhythm (Rec) are indicated. The recovery percentages are calculated by dividing the number of rhythm recovered animals by the number of animals having an SCN-containing transplant, since the presence of an SCN in the graft is a prerequisite for rhythm recovery. When the number of animals having an SCN-containing graft is not available, the percentage is given between brackets and calculated on the basis of the total number of animals that were implanted [29,67], or on the number of animals with a viable transplant [2,68]. For these studies the results are not well interpretable, especially when information on the presence of an SCN in the transplant is lacking.
Whether the observed differences in the results of rat and hamster SCN transplantation studies reflect a real species difference or are only due to methodological differences should, however, still be investigated in a study in which both species are subjected to the same procedures and evaluation criteria. An additional indication that the rat SCN is indeed less capable of restoring a circadian rhythm may have come from two studies by Sollars and colleagues [81,82], who showed that grafting of an E16 rat SCN is not as effective in restoring the circadian wheel-running rhythm in hamster as grafting of an E13/E14 hamster SCN (50/59% vs 83%). This might, however, be due to an immunological reaction or the immunosuppression therapy in the hamsters that received the rat xenograft. If, in a reverse experiment, a fetal hamster SCN turned out to be more effective in restoring a circadian rhythm in rat than a fetal rat SCN, this would give a stronger indication that the two species indeed differ in their capacity to restore circadian rhythms.

Having summarized the methodological differences between SCN transplantation studies (see also Table 1), it appears that although these differences result in a variation in percentages of rhythm recovery, they are not likely to explain the discrepancy in rhythm recovery between rat and hamster.

**Features of the transplanted SCN and their relation to rhythm restoration**

The exact reason why only 40-50% of SCN grafts restore the circadian drinking rhythm of SCN-lesioned rats is still not clear, but it offers an opportunity to study which features of the grafted SCN underlie rhythm restoration. Several aspects, such as the presence of vasopressin (VP)- and VIPergic SCN neurons in the transplanted SCN, have been studied extensively. Based on VP and VIP immunocytochemistry, the grafted fetal SCN seems to mature well in the environment of the adult host brain [37,89]. Rhythmicity of grafted rat fetal SCN has been demonstrated by *in vitro* recording of neuronal firing rate and measurement of 2-deoxyglucose uptake [4]. Using the latter technique, fetal hamster SCN transplants have been shown to be entrained by the intact host SCN after 14 days after implantation [75]. These techniques can, however, not be applied conveniently to study the rhythm of the grafted SCN in relation to the behavioral rhythm of the host animal.

As a marker for the presence of a circadian rhythm in the grafted SCN, VP levels in the CSF of grafted rats were measured (Chapter 2). VP release by the SCN has frequently been assayed *in vitro* and *in vivo* as a way to monitor the presence of a 24 h rhythm. *In vitro*, the pattern of VP release appears to be similar to the rhythm in electrical activity of the SCN neurons [36]. In fact, the VP rhythm in release has been suggested to be causally related to the activity rhythm of SCN neurons [19]. Besides monitoring rhythmicity of the transplanted SCN, measurement of CSF VP levels offers the possibility to investigate the relation between the VP rhythm of the graft and the overt rhythmicity in individual host animals. The results described in Chapter 2 show that several transplants failed to restore a circadian rhythm in SCN-lesioned arhythmic rat, but did cause a circadian rhythm of the CSF VP levels. Apparently, in these cases, a lack of rhythmicity of the grafted SCN was not the cause of the failure to restore circadian rhythmicity of the host animal. Whether the
other non-functional transplants lack a circadian rhythm could, however, not be established with absolute certainty, since in some cases the number of successive CSF samples that could be obtained may have been too low to detect a rhythm in VP levels with a low amplitude.

The most interesting conclusion drawn from the results described in Chapter 2 is that transplanted SCNs with a circadian rhythm sometimes do not induce a circadian rhythm in the host. This might be explained in two ways. First, the grafted SCN releases VP in a circadian fashion, but is otherwise not functional as a clock. This would imply that the circadian release of VP is not a good marker for to support this possibility. The second possible explanation is that the grafted SCN is functional as a clock, but that the rhythmic signal fails to reach the appropriate targets in the host animal, or that the target areas are unable to receive the signal. Most studies investigating the mechanisms of rhythm restoration have focussed on the properties of the transplanted SCN. It might well be, however, that properties of the host brain, which has been damaged by the lesion, play a role in the capability of the graft to restore a circadian rhythm. Lesions are usually restricted to the site of the SCN and its immediate surroundings and sometimes involve the optic chiasm. SCN target areas adjoining the third ventricle, such as the sPVz, MPN/POA, retrochiasmatic nucleus (RCH), ventromedial nucleus (VMH) or dorsomedial nucleus (DMH), are generally spared by the lesion. A study investigating the extent of a lesion in relation to the ability of SCN transplants to restore a circadian rhythm is certainly needed.

With regard to the pathways along which the transplanted SCN transmits its rhythmic signal to the appropriate target areas in the host brain, it was initially proposed that the appropriate neural connections are essential [12,37,67,82]. However, encapsulated SCN grafts in hamster have shown that a humoral factor may be involved which may be sufficient to mediate a circadian wheel-running rhythm [79]. As discussed in Chapter 2, VP released into the CSF is not likely to be a signal used by the SCN to transmit its rhythm. This does not exclude the CSF as a medium for signal transduction in general, however. When the results of the encapsulated SCN grafts in hamster became available, the idea of signalling by the SCN through the CSF was controversial. Recently, however, non-synaptic signalling routes have been suggested to play a role in the input pathway to the SCN, and within the SCN itself. Following light exposure of the knee joint, blood-borne messengers can apparently entrain the human SCN [20]. This entrainment was proposed to be mediated through soluble gases with neuroactive properties, such as CO and NO. Within the SCN, these molecules are thought to mediate communication between the SCN cells and to be responsible for the synchronization of firing rates of the individual SCN neurons in the absence of synaptic transmission [13,31,71,73,76,88]. The demonstration of circadian transcription of several substances in a fibroblast cell line that could be induced by a serum shock [6] resulted in speculations that in principle each cell contains an autonomous oscillator. These oscillators are then entrained by the SCN, the master pacemaker. For such an entrainment, the blood circulation probably provides the communication medium. In analogy with blood circulation, the circulation of the CSF might provide a transport medium for the signalling molecules of the transplanted SCN to
the target areas within the host brain. This idea may be supported by the finding that in hamster, SCN transplants located upstream from the lesion site can restore a circadian wheel-running rhythm with a more precise timing of the onset of activity than transplants located downstream from the lesion site [52]. However, transplants located downstream and far away from the lesion site are still able to restore a circadian rhythm in SCN-lesioned hamsters, indicating that the CSF is not the only medium transporting circadian information.

In the light of these findings, the results obtained in the rat and described in Chapter 2, showing that SCN transplants with a rhythmic VP release are not able to restore a circadian rhythm of the host, are at least curious. When VP is rhythmically released by an SCN transplant, one would expect that other factors involved in the signalling by the SCN, including the factor responsible for rhythm restoration by encapsulated SCN grafts in SCN-lesioned hamster, are rhythmically released as well. Further research concerning this species difference is needed, but the results of Chapter 2 suggest that the humoral factor responsible for rhythm restoration in the hamster is not involved in rhythm restoration in the rat. This discrepancy might, however, again be ascribed to the different behavioral parameters that are used in hamster studies and in rat studies. The previously discussed difference in host brain areas, which may be responsible for these two different behaviors, might coincide with different signaling pathways, and even with different kinds of signals. Once more, the importance of applying the same transplantation and evaluation criteria to both species becomes apparent.

The VP-deficient Brattleboro rat has frequently been used to study the role of VP in a variety of processes, including its possible role in circadian rhythmicity. Despite the absence of functional VP, several processes in the SCN and in the VPergic SCN cells appear to be normal in the Brattleboro rat. A circadian rhythm can be observed in mutant VP mRNA levels, albeit with a lower amplitude, and in V1a receptor levels [84,94]. SCN cells of Brattleboro mutants expressing the V1a receptor have a circadian rhythm in electrical activity, albeit with a lower amplitude than SCN cells of control heterozygous Brattleboro rats. Moreover, cells with a rhythm in basal activity can be excited by exogenous VP to the same degree as SCN cells of Wistar rats, indicating that the VP receptors are functional [43].

The lower amplitude in the electrical activity rhythm and mutant VP mRNA levels in Brattleboro SCN neurons may account for the lower amplitude of several behavioral rhythms [38]. Other differences in circadian rhythms in the Brattleboro rat as compared to control rats concern a different sleep pattern, with a reduced duration of paradoxical sleep [26], and a change in the period length of free-running rhythms. Homozygous Brattleboro rats have been reported to have a longer period length as compared to heterozygous Brattleboro rats [38].

The observed differences in circadian processes in the adult Brattleboro rat may result from a lack of VP, or be caused by compensatory mechanisms that evolve during maturation of the brain and take over the role of VP. In the adult Brattleboro rat probably both mechanisms are involved, and the separate contribution of each of the two factors to the changes in the Brattleboro brain are difficult to distinguish. With respect to the SCN,
General discussion

the lack of VP might have consequences for internal processes and also for its input and output pathways. Transplantation of a fetal SCN from a Brattleboro rat into an SCN-lesioned Wistar host might make it possible to investigate whether VP is essential for the transplanted SCN to restore circadian rhythmicity. Moreover, it might also reveal which deviations of Brattleboro circadian rhythmicity can be attributed to changed processes within the SCN itself.

The results presented in Chapter 3 of this thesis show that a VP-deficient SCN is able to restore the circadian drinking rhythm of a normal Wistar rat whose SCN has been lesioned. Apparently, VP is not essential as an output signal of the transplanted SCN or for rhythm restoration of the SCN-lesioned arhythmic host rat. The VPergic SCN cells, however, appear to play a crucial role in the generation of overt circadian rhythmicity, since elimination of the VPergic SCN cells in adult rat in vivo results in a loss of circadian rhythmicity [77]. These findings suggest that another neuroactive substance colocalized with VP plays a crucial role in the SCN and its output pathway.

The results in Chapter 3 also show that the altered period length in the adult Brattleboro rat results from a lack of VP in the SCN itself and not elsewhere in the brain. The difference in period length tau between adult homozygous and heterozygous Brattleboro rats can also be observed after transplantation of a fetal SCN derived from these genotypes. Transplantation studies with the tau mutant hamster have confirmed the idea that the period length is genetically determined in the SCN [64]. However, since external manipulations also influence the period length to some extent, in vitro studies on the difference in period length between homozygous and heterozygous Brattleboro rats seem to be necessary to elucidate the background of this difference. A partial SCN lesion has, for instance, been shown to shorten the tau, whereas after a period of LD, the tau gradually lengthens after return to DD. The cause for the difference in tau in the Brattleboro rat is difficult to establish, and may be a developmental or direct effect of a lack of VP in the SCN. In the next paragraph, a hypothetical role for VP in the SCN is proposed, based on its effect on the period length as well as on the other effects described for VP on SCN cells.

A possible role for VP in the mammalian clock

The increasing knowledge of the clock mechanisms in Drosophila and the discovery of many mammalian homologs of Drosophila clock genes make it tempting to speculate about similar clock mechanisms in mammals. The discovery of mammalian homologs for Drosophila clock genes in the SCN suggests that the mammalian SCN may have a similar clock mechanism. As VP is the most well-studied peptide of the SCN and its exact role is still unclear, it might be interesting to see to what extent the current knowledge about VP in the SCN can be incorporated in a Drosophila-like clock mechanism.

When the criteria for a clock gene, mentioned in the introduction of this thesis, are applied to VP, it is clear that VP does not fulfill these criteria since the absence of VP does not lead to arhythmicity (see Chapter 3 and discussion on Brattleboro rat). Moreover, observations in the Brattleboro rat show that, unlike Drosophila PER and TIM, VP has no negative
feedback on its own transcription. Mutations in the *per* or *tim* genes halt the rhythm of mRNA abundance of both genes, whereas mutation in the VP gene in the Brattleboro rat only results in a lower amplitude of the mutant VP mRNA rhythm [84,94]. It should be noted, though, that the Brattleboro mutation is located in the neurophysin part of the precursor, and not in the VP part, and has been studied in the magnocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON). Expression of the mutated VP gene has not been studied in detail in the parvocellular cells of the SCN.

Several characteristics of VP expression in the SCN suggest that it is regulated by the circadian mechanisms of the SCN. Especially the unchanged diurnal rhythm of VP mRNA and VP levels in the SCN under DD conditions show that the VP gene can be considered a 'clock-controlled gene'. VP shows a remarkable expression pattern in the rat SCN, since peak levels of the protein coincide with peak levels of the transcript [19]. The time lag between transcription and translation is thus close to 24 h (Fig. 1), whereas for Drosophila *per* the time lag is between 6 and 8 h [80]. The regulation of expression of both transcripts and protein and the origin of the unusual timing are still not known. As both the level of VP mRNA and that of VP itself depend on many factors, it is difficult to draw conclusions on steady state levels only. However, more data became available concerning processes involved in VP gene expression in the SCN. One of the most interesting findings is a day-night difference in the length of the VP mRNA poly(A) tail [21,65], which appears to be unaffected by extrinsic neuroendocrine signals and environmental lighting conditions, and is thus controlled directly by the clock mechanism [21]. The length of the mRNA poly(A) tail may affect mRNA stability [95] and translational efficiency [62]. The predominantly short poly(A) tail of VP mRNA during the night time might thus explain its low levels.

![Figure 1](image_url)

**Figure 1.** Expression patterns of VP mRNA and protein in the mammalian SCN. Dark bars represent night time. Peak VP levels lag 20 h behind peak mRNA levels. The lower part of the figure shows the period of presence of the short form of VP mRNA poly(A) tail (data derived from [19,65,92]).
through a higher breakdown. However, the diurnal rhythm of VP transcript levels appears to be regulated in the nucleus [22], and can thus not be explained by an effect on its stability in the cytoplasm. It is therefore likely that the circadian rhythm in VP mRNA is a direct result of a rhythmic transcription of the VP gene. Peak levels of VP mRNA at circadian time (CT) 8 coincide with peak levels in electrical activity [44], and glucose utilization [72], suggesting a common regulator within the clock. A day-night difference in translation efficiency due to the changes in the poly(A) tail of the VP mRNA might cause the rhythm in VP levels in the SCN. Transcripts with a long poly(A) tail only begin to accumulate around CT2 [65], which might explain the peak VP level several hours later (CT8) (see Fig. 1). Other processes, such as VP transport, degradation and release at the axon terminals, also influence the VP levels. Release, in turn, is dependent on the electrical activity of the neuron. The coupling between electrical activity and VP release may explain the identical phases of both rhythms.

The widespread presence of the V1a receptor in a subpopulation of VPergic and VIPergic SCN neurons [94] with a circadian rhythm in their electrical activity [43] indicates that VP can also exert its action on the pacemaker cells of the SCN. Activation of the V1a receptor results in phosphoinositol hydrolysis and mobilization of intracellular calcium [45]. This change in calcium may not only result in an increase in excitability of the SCN neurons, but might also affect processes involved in the clock mechanism, such as activation of kinases and phosphatases and of transcription of clock genes. Via these pathways the rhythmic VP output of the SCN may influence processes in the clock, and by its excitatory action enhance the amplitude of rhythmic expression of clock genes, amongst others (Fig. 2).

Other effects of VP on the pacemaker have been proposed based on observations in the VP-deficient Brattleboro rat. The observed deviation in period length in this strain might indicate a direct effect of VP on the clock mechanism. Mutants resulting in an altered period length in Drosophila have been shown to affect levels of clock proteins either directly (per and tim mutants) or indirectly by post-translational processes which influence the stability of the clock proteins (dbt mutants) (see Introduction of this thesis). In mammals, VP might likewise influence the levels of clock proteins, either directly or through the mentioned increase in calcium levels. The increase in calcium levels might for instance affect enzymes involved in the processing of the clock proteins, such as casein kinase, the mammalian homolog of Drosophila DBT which phosphorylates PER, thereby mediating its breakdown. As more putative clock genes are identified in mammals, the effect of VP on the level of clock proteins can be studied, which might shed more light on the role of VP in the molecular mechanisms of the SCN.

Based on deviations in circadian rhythms, VP has been associated with the strength of the circadian rhythm. Several studies have reported a correlation between the number of VPergic neurons in the SCN and the strength and consistency of circadian activity rhythms. In different rat strains and mice selected for differences in nest-building behavior, the animals with the highest number of VPergic neurons in the SCN had a strong unimodal activity pattern, whereas the animals with the lowest numbers of VPergic neurons had a weak multimodal activity pattern [14,15,90]. However, the reverse relation was reported
for common voles, i.e. the animals with the highest number of VPergic neurons had no circadian activity rhythms [34]. It was speculated, though, that this finding was the result of reduced VP release rather than increased VP synthesis [34]. Since the actual VP level VP in SCN cells is the result of a balance between production, transport, degradation and release, the way these data are to be interpreted is not yet clear, and additional information on VP mRNA levels is required. Intraspecies differences in the molecular clock mechanism may underlie the observed difference in VP content of the SCN cells and explain the difference in overt circadian rhythmicity. In this case, the alterations in VP levels are not the cause of the alterations in rhythmicity, but both are the result of alterations in the SCN.

Ex vivo gene transfer

In the present thesis, the optimization and application of adenoviral vectors in the SCN transplantation model are described, primarily aimed at obtaining fetal SCN grafts with an enhanced expression of neurotrophic factors, which might improve transplantation results. The results obtained so far show that the first generation of adenoviral vectors are suitable to introduce a foreign gene in fetal SCN transplants for a long period, i.e. seven months at least. Since adenoviral vectors have been reported to infect cells in all areas of the brain, a more general application of these vectors in neurotransplantation seems feasible and seems to offer many opportunities for the future.

Direct injection of adenoviral vectors in the brain results in a severe immune response and elimination of most transduced cells within a month after the injection [16-18,41]. The presence of viral particles or viral products appears to be the main cause of the immune response rather than the expression of viral genes by the transduced cells. An injection with an adenoviral vector exposed to ultraviolet irradiation to prevent transcription from the viral DNA still resulted in an infiltration of immune cells and MHC I expression [17]. Non-infectious particles, which are always present in adenoviral vector preparations (W. Hermens, personal communication), are not taken up by CNS cells but by antigen-presenting cells. This results in an immune response against the transduced cells.

The results of ex vivo gene transfer to SCN transplants described in Chapters 5 and 6 of this thesis show that the use of adenoviral vectors results in a prolonged expression of the marker gene LacZ. The idea that an immune response is the cause of loss of transgene expression in vivo, probably elicited by the extracellular presence of viral particles in the brain, is supported by our ex vivo experiments. Viral particles that are not taken up by the cells are in our procedure washed away before the donor tissue is implanted into the host.
Figure 3. SCN cell suspension transduced with an adenoviral vector for the reporter gene LacZ (AdLacZ) and immunocyto-chemically stained for the transgene product β-galactosidase after 10 days in vitro. Large number the glial cells and neuronal cells in the culture produce β-galactosidase. (R. Arens et al., unpublished observations).

brain. The few cytotoxic T-lymphocytes observed in the transduced transplants might result from the release of viral antigens after the death of transduced cells in the initial phase after transplantation (see Chapters 5 and 6).

A remarkable finding is the poor infectability of the SCN within the transplant (Chapters 5 and 6). Numerous transduced cells were visible throughout the transplants, except at the sites where SCN cell clusters were present. AdLacZ infection of cell suspension cultures of the fetal SCN showed transduction in a high percentage of the cells [93, Van Esseveldt, unpublished results] (Fig. 3). However, no double stainings were performed to confirm the SCN origin of the cells, but Yamazaki and colleagues report a 100% infection efficiency in the cell suspension culture under certain conditions [93]. The high infection efficiency in SCN cell cultures thus indicates that SCN cells are in principle transducible by adenoviral vectors. Recently a cell surface receptor (coxsackievirus and adenovirus receptor; CAR) was identified which is responsible for internalization of the adenoviral particle [10]. The localization of this receptor in the hypothalamus has not yet been described, but based on the successful transduction of the fetal SCN graft, the presence of this receptor seems obvious. As discussed in Chapter 5 and 6, the reason for the low number of infected cells within the SCN of the transplant may be its compact structure. The viral particles do not actively penetrate the tissue, but for their spread they are probably dependent on the flow of the extracellular fluid. Within the SCN, this flow might be limited by the small extracellular space, which could result in poor penetration of the viral particles.
General discussion

into the area of the SCN. This problem was also encountered when an adult peripheral nerve was \textit{ex vivo} transduced with adenoviral vectors (B. Blits, unpublished results). Injection of the vector in the peripheral nerve instead of immersion, resulted in an efficient transduction, and such a procedure might enhance transduction in the SCN as well.

Using the current procedure, transduction of the majority of the SCN cells throughout this structure will be difficult if the structure is kept intact. Investigation of, for instance, the role of clock genes in SCN neurons by means of \textit{ex vivo} adenoviral vector-mediated gene transfer thus necessitates an adjustment of the currently used transduction method or transplantation method. The hamster might be a more suitable host since it offers the possibility of using an SCN cell suspension for transplantation. Cell suspensions have been shown to be readily infected by adenoviral vectors [93, Van Esseveldt, unpublished results] and to restore circadian rhythmicity in SCN-lesioned hamsters [78]. SCN transplantation in combination with adenoviral vectors may still be useful to improve the functionality of the transplants in rat. Application of neurotrophic factors, which has been shown to be beneficial in other transplantation models, could be achieved by transduction of a number of cells within the transplant, without transduction of the SCN cells themselves. The recently developed adeno-associated viral vector and the lentiviral vector are associated with a greatly reduced immune response and might eventually be used to manipulate gene expression in the \textit{in vivo} SCN by a direct injection in the brain.

Application of neurotrophic factors in the SCN transplantation model

Neurotrophic factors have been applied in several transplantation models and have been shown to improve the survival and neurite growth of transplanted fetal neurons and also functional recovery in several cases (see for instance [33]). Since the results of SCN transplantation in the rat are relatively poor in terms of functionality and neurite outgrowth to the host brain, application of neurotrophic factors to the fetal SCN neurons might be advantageous as well. The neurotrophin nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5) all have more or less their own high affinity receptor, TrkA, TrkB and TrkC. Only a few studies have reported on the presence of neurotrophins and their receptors in the adult SCN [50,55,56], while no information was available about the presence or possible role of neurotrophins in the developing SCN.

To describe in more detail the localization of the Trk receptors in the SCN and surrounding hypothalamus, the localization of TrkC, the high affinity receptor for neurotrophin-3 (NT-3), was studied by in situ hybridization in E15 to P9 and adult rat brain. The results in Chapter 7 show the presence of TrkC mRNA in the prenatal anterior hypothalamus, with at E18 a slightly more intense signal in the SCN as compared to the area around it. In many brain areas the TrkC mRNA levels decrease with development, but levels remain more or less the same in the SCN at all ages investigated. Notably, TrkC seems to be expressed at slightly higher levels in the anteroventral part of the adult SCN as compared to the dorsomedial part. This difference in signal intensity might result from
a difference in cellular composition between these two parts. However, the presence of small and densely packed cells in the dorsomedial SCN might predict the highest signal in this part of the SCN. An opposite but more pronounced pattern was reported for TrkB, the receptor for BDNF, in the adult rat, in which expression is present in the dorsomedial SCN, but not in the ventrolateral SCN [55,56]. Together these data plead for a differential expression of the different Trk receptors in the SCN.

The TrkC receptor has several appearances which probably all have different functions. A distinction can be made between full-length and truncated receptors, the latter lacking the intracellular kinase domain which is activated by ligand-mediated oligomerization of the receptors [58,83,85]. Several functions have been proposed for the truncated receptors, like ligand clearance or transport [48]. The increase in their relative abundance during maturation [32] might, moreover, regulate the sensitivity of the neurons to the neurotrophins. The induction of truncated receptors upon neuronal injury suggests their involvement in ligand presentation and perhaps axonal guidance during axonal growth and regeneration [9]. Support for this suggestion comes from Drosophila, in which Trk receptors have a function in neural cell adhesion [63]. The full-length receptor has been reported to have several isoforms with inserts in the kinase domain [49,83,85], resulting in the activation of different signalling cascades and different effects on cellular responses upon receptor activation [39,49]. The results described in Chapter 7 show the presence of the extracellular part of the trkC receptor in the perinatal brain, and thus make no distinction between truncated receptors or full-length receptors with their different inserts in the intracellular domain. Further studies will have to take into account these differences in receptor isoforms for a more detailed description of the presence of the TrkC receptors in the SCN. A combination with immunocytochemistry for the neuropeptides of SCN cells would allow identification of the SCN cells carrying this receptor.

In vivo studies in the adult CNS have focused on the effect of NT-3 application to lesioned or intact brain areas. The implantation of genetically modified cells expressing NT-3 could prevent the degeneration of noradrenergic neurons of the locus coeruleus in a 6-hydroxydopamine lesion model [5]. Transplantation of NT-3 producing cells in the adult spinal cord results in sprouting of sensory neurons [60,74] and neurons of the corticospinal tract [70]. Although NT-3 stimulates survival of dopamine cells in culture [42], it does not enhance neurite growth and functional efficacy of nigral transplants in a 6-hydroxydopamine Parkinson model [40].

In Chapter 8, the first data are presented on the effect of NT-3 on the developing SCN. SCN transplants transduced with an adeno viral vector encoding for NT-3 revealed an increase in the VIP and CPP content. The increase in immunocytochemical staining for VIP and c-terminal prepressophysin (CPP), the c-terminal part of the VP precursor, was presumed to be caused by an increase in the number of VIP- and VP-positive SCN cells in the transplant, which might have been the result of a survival-enhancing effect of NT-3 on these neurons. A similar effect of NT-3 has been reported for several populations of neurons [35,61,86]. Alternatively, the increase of VP and VIP immunoreactivity might result from an effect of NT-3 on the peptide expression of the SCN cells, as reported for
cholinergic motoneurons and calbindin-positive hippocampus neurons [25, 91]. Theoretically, the increase in immunoreactivity for VP and VIP could also result from an inhibition of transport, degradation or release of the peptides. However, such effects have not been described for NT-3. With the set-up of the experiments of Chapter 8, no distinction can be made between these two possibilities. A more detailed study on the processing of peptides in SCN neurons requires investigation of mRNA levels. However, characterization of the peptidergic content of the TrkC-positive SCN neurons would allow a more direct study of the effect of NT-3 on SCN neurons.

In addition to the differentiating and survival-enhancing effect on several neuron populations, NT-3 has also been reported to stimulate proliferation of neural precursors and oligodendrocyte precursors [8, 24, 46], to influence synaptic functioning [23, 57], and to stimulate neurite outgrowth [59, 60, 70]. The latter effect appears to coincide with an upregulation of the expression of two growth-associated proteins, GAP-43 and α-tubulin [59]. The effect on the SCN transplant size might result from a proliferative effect on the stem cells along the wall of the third ventricle that are present in the E17 tissue dissected for grafting (see [11]). Whether NT-3 stimulates neurite outgrowth in the transplants could not easily be determined, since both VPergic and VIPergic fibers are also present in the host brain endogenously. However, the results in the VP/CPP-deficient Brattleboro hosts, in which the outgrowth of CPP-positive fibers into the host brain could be estimated, suggest an increase of outgrowth of CPP-positive fibers into the host brain (Chapter 8).

Studies on the temporal and spatial expression of TrkC and NT-3 in the in vivo brain have indicated that NT-3 does probably not act as a target-derived growth factor for CNS neurons, but is rather involved in the support of neurite outgrowth and survival of immature neurons during the process of axon growth [54, 69]. During development, NT-3 expression is most prominent in the CNS regions in which proliferation, migration, and differentiation of neurons and their precursors are ongoing (reviewed in [7]). In the view of these observations, the application of NT-3 near the maturing SCN cells in the transplant seems well chosen to stimulate their survival and neurite outgrowth. Little is known, however, of the role of NT-3 in the direction of axon growth. Other neurotrophic factors, such as NGF and BDNF, have been reported to act as target-derived factors and to mediate the formation of correct synapses between the axon and its target neuron [27, 28]. In the SCN transplantation model, this action of BDNF could potentially be used by application of this neurotrophin to areas in the host brain which are to be reinnervated by the transplanted SCN.

The results described in Chapter 9 suggest that SCN transplants transduced with an adenoviral vector encoding for NT-3 are more successful in restoring of circadian rhythmicity than non-infected control transplants. These preliminary results suggest that an increase in the survival and neurite outgrowth of the transplanted SCN neurons may indeed improve the transplantation results. The present thesis shows the first results of adenoviral vector-mediated gene transfer in combination with transplantation of solid pieces of fetal primary CNS tissue. The results described in this thesis point to the exciting new possibility of using adenoviral vectors in studying the SCN. The currently used ex vivo approach in the SCN could, however, also be a model for other transplantation studies using
fetal brain tissue. The ongoing refinement of the viral vectors will soon allow their application in human transplantation studies, thereby increasing the potential of *ex vivo* gene transfer.

**References**

Chapter 10


