The Role of Doublecortin-like in the Suprachiasmatic Nucleus

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ABSTRACT
The hypothalamic Suprachiasmatic Nucleus (SCN) is known as the central biological clock that regulates circadian rhythmicity in mammals. Through vasopressin (AVP) processing in its shell, the SCN signals to other brain areas and peripheral organs in the body. A novel protein, Doublecortin-like (DCL) was identified to have overlapping expression with that of AVP. Mice with SCN-specific DCL-knockdown show abnormal activity adaption after a change in light-phase, and immunohistochemical AVP staining indicate a distortion of AVP in the SCN of DCL-knockdown mice. Together, we conclude that DCL may be a novel regulator of AVP signalling in the SCN.

Keywords
Suprachiasmatic Nucleus, Vasopressin, Doublecortin-like, Circadian Rhythms

INTRODUCTION
As most living organisms, humans have a circadian cycle guided by an intrinsic biological clock [1]. In mammals, this biological clock is located in the suprachiasmatic nucleus (SCN), within the hypothalamus in the brain. The SCN is composed of two areas working together: the shell and the core [2]. Information from the retina is processed by the core through the neurotransmitter Vasoactive Intestinal Peptide (VIP). Subsequently, GABAergic signaling transmits information from the core to the shell. The shell is the external feature of the SCN and regulates the excretion of vasopressin (AVP) [3] [4]. AVP is both an external and an internal mediator of the SCN. The external functions of AVP include the activation of distant brain areas and the control of the expression other hormones. The internal function is for intracellular communication within the SCN.

The biological clock works on a molecular level: within the SCN the changing balance of gene-expression forms a rhythm in each individual neuron. In normal circumstances, this is a 24-hour rhythm, but a change in environment can cause a shift in rhythm. This shift causes the biological clock to adapt. Humans experience this as a jetlag. Many previous studies show phase-shifts in mice where the day-night rhythm is changed [5]. Other factors also influence the circadian rhythm, including locomotor activity, food uptake and direct release of hormones like cortisol and serotonin.

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The analysis of biological rhythms in the SCN is very important for understanding the role of the SCN. Animal models are observed for neuronal and gene activity and this is linked to locomotor activity and changes in phenotype.
The ability to adapt to changes in rhythm is due to neuroplasticity of the SCN. This report focusses on a specific neuroplasticity protein, doublecortin-like (DCL), which is a member of the doublecortin-family. Recent studies by Saaltink et al. show the importance of this family within the SCN [6].
Doublecortin (DCX), which is similar in length and shares a high number of amino acids with DCL, is a protein associated with the formation of microtubules. It is expressed by neuronal precursor cells and immature neurons [7]. DCX is often used as a marker for neurogenesis, using labeled antibodies against DCX. Doublecortin is a Microtubule Associate Protein (MAP). Doublecortin-like kinase 1 (DCLK1), a member of the DCX family, has a splice variant highly homologue to DCX [8]. The resulting protein is therefore called DCL [9] [10]. Its known function lies in microtubule stabilization within mitotic spindles. However, the role of DCL in the SCN is mostly unknown and under investigation.
Mouse models are commonly used in circadian research. Although mice are nocturnal animals, the activity of the SCN is reversed, and therefore equal to that of diurnal animals [11]. DCL knockdown mice have been developed by the LUMC neurophysiology lab, in which the knockdown can be triggered through doxycycline (dox) ingestion. Saaltink et al. developed a reverse tetracycline (doxycycline)-controlled transactivator (rtTA) regulated model [12]. Using these mice, they have shown that the knockdown of DCL resulted in an inhibition of neuronal proliferation and hence, an inhibition of neurogenesis. Therefore, it can be expected that DCL knockdown mice will have lowered neuroplasticity and will react to environmental changes in a less profound fashion.
The aim of this study is to discover the role of DCL in the SCN using DCL-knockdown mice. The primary hypothesis is that the DCL knockdown mice have an altered reaction to photoperiodic changes, due to alteration in the expression of vasopressin.

METHODS
Mouse model
For these experiments, the DCL-knockdown mice as bred by Saaltink et al. were used. Experiments were approved by the Animal Experiments Ethical Committee of Leiden University. The following setup was used:
4 groups of mice were treated: one DCL-knockdown group and one wildtype group fed with a doxycycline diet and a transgenic- and wildtype-group on normal diet as controls. The mice were exposed to different sets of photoperiods, as shown in figure 1. Two relevant shifts were performed: a shift from 12:12 light:dark (LD) to 8:16 LD and a shift to total darkness (DD), also called freerunning. The

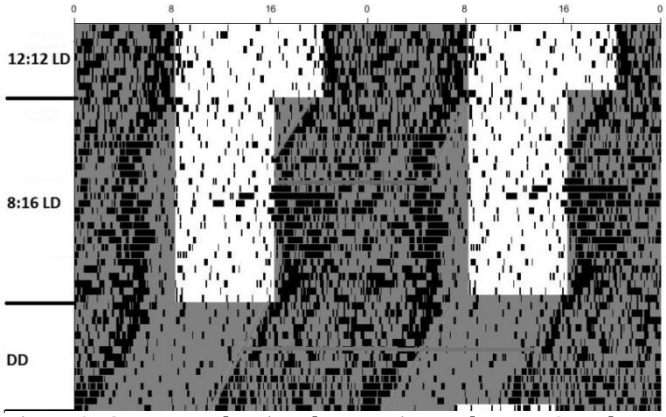

Figure 2: Actogram showing the experimental setup. Gray bars indicate darkness, white bars indicate light in the cage. Black bars indicate activity of the mouse. Each horizontal line consists of 48 hours.

locomotor activity of the mice was recorded [13] using Passive Infrared Registration (PIR) sensors and these recordings were available for analysis by the software program 'Clocklab', in the form of actograms (figure 1).

Clocklab

The Actimetrics developed application Clocklab is routinely used to analyze actograms. Using this program a straight line through the onsets and offsets was drawn. In this way, the tau of each individual mouse was calculated. By definition, the tau is the duration of a single cycle, from the onset of the active period of day n until the onset of the active period of day n+1. In normal rhythm, this would be 24 hours, as it is light controlled, but during a phase shift, tau is an indication of neuroplasticity.

SPSS analysis

In SPSS the data from all the mice were divided into four groups: transgenic mice with chow (transgenic chow group); transgenic mice with doxycycline (transgenic doxycycline group); wildtype mice with chow (wildtype chow group); and wildtype mice with doxycycline (wildtype doxycycline group). Using one-way ANOVA analysis differences between the groups were analyzed, giving an F-value. The F-value is a ratio between two mean squares, equal groups are therefore expected to have an F-value of around 1 and a higher F-value shows a bigger difference between the groups. To find intergroup differences, a Post-hoc Tukey HSD test was performed. Furthermore, using independent t-tests differences between both diets and mouse-lines were examined. Given p values are 2-tailed and $p<0,05$ was considered statistically significant. Note that all SPSS analysis was performed while the experimenter was blind with respect to the background of the animals, in order to overcome bias.

Immunohistochemical staining

The brains sere extracted and stored in 30% sacharose in 0,1M TBS with soda-azide. Subsequently, the brains were sectioned in 30 µm thick slices in a cryostat at -23ºC. After cutting, the slices were stored in an anti-freeze solution containing 40% 0,1 M TBS, 30% ethylene glycol and 30% glycerol. The slices were frozen for staining at -20ºC.

Immunohistochemistry was performed to detect expression of DCL, AVP and VIP in the SCN. For DCL a novel primary antibody was generated by injecting a rabbit with 18-amino acid long synthetic peptide (QRDLYRPLSSDDLDSVG-C), which is the 6th and 7th exon of DCLK1 and resembles the C-terminus of DCL. The obtained polyclonal antibody that was formed by the rabbit was used and has been described in detail [6]. The primary antibodies for AVP and VIP are also polyclonal rabbit antibodies: rabbit-anti-AVP (AB1565, EMD millipore) and rabbit-anti-VIP (T-4246, Peninsula Laboratories International, Inc.). As a secondary antibody, Alexa fluor 594 goat-anti-rabbit (A11037) was used to visualise three primary antibodies.

Microscopy

Using a Leica DM5500 epifluorescence microscope and Leica SP8 confocal laser scanning microscope (Leica Microsystems, Rijswijk, the Netherlands) fluorescence was observed. Using the DAPI stain and the Mouse Brain Atlas as orientation, the SCN was localized. Photos were taken of the results using an attached Leica DFC450 camera. Image Pro Plus was used to cells and to quantify corresponding fluorescent intensities. ImageJ calculates the intensity of each pixel, which corresponds to the local fluorescence and thus the presence of the target protein. For all three antibodies the area of the full SCN was isolated and a pixel-intensity count was performed. A background area was extracted. For the AVP-antibody an intensity count was performed on cellular level. Per slide, 15 full cell bodies were selected and the mean intensity of these cellbodies were calculated. This measurement is semi-quantitative, as exact localisation of every SCN neuron may vary from section to section and also the immunohistochemical procedure may vary from sample to sample. However, the resulting data gives a solid indication of the quantity in which the protein is present.

RESULTS

During analysis of the data, no significant difference was found between the three control groups in any of the experiments. For this reason the DCL-knockdown mice are compared to the wildtype mice on doxycycline in all figures. Note that the analysis has been performed for all groups.

Phase shift from 12:12 LD to 8:16 LD

To analyse possible statistically significant differences in circadian function between the different mouse groups during the 8:16 LD period, the data is analysed using a one-way ANOVA. For the onset *tau*, a significant difference between DCL-knockdown mice and controls is found, with values of $F(3, 31) = 42,841$, $p < 0,0001$. This significant difference is shown in figure 2b.

Phase shift to DD

A similar ANOVA analysis is performed to find significant differences between the mouse groups after the switch to full darkness (DD, freerunning). The mean difference between groups is compared for the onset tau, and a significant difference with values of $F(3, 29)=6,435$,

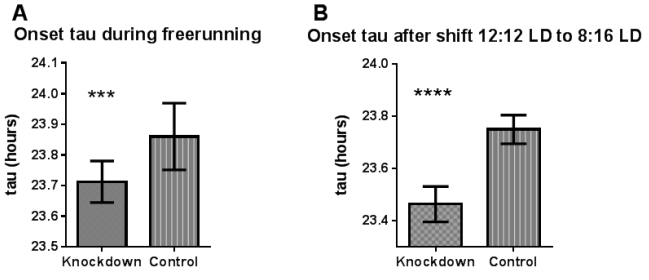

Figure 1: A: mean onset tau during the first 10 days of the 8:16 LD photoperiod. p<0,0001. B: mean onset tau during the last 10 days of DD (freerunning) photoperiod. p=0,002.

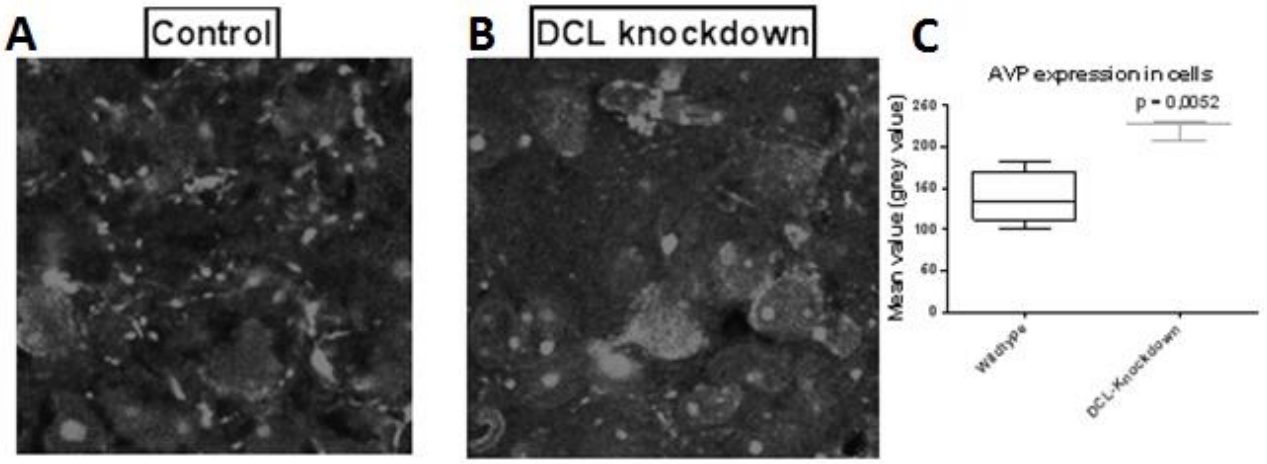

Figure 3: Immunohistochemical AVP staining and fluorescence analysis of the SCN. A: Illustrative example of a control mouse, magn. 40x. Fluorescence shows distributed inside the cell and spreading outside the cell. E: Illustrative example of DCL knockdown mouse, magn. 40x. Fluorescence shows concentrated to inside the cells mainly, with less spreading outside the cells. F: Significant differences found between DCL knockdown mice and control mice showing that there is more DCL inside the SCN cells of the knockdown mice than in the SCN cells of the control mice (p=0,0052).

p=0,002 is found. This significant difference is shown in figure 2a.

Furthermore, looking at the first five days of freerunning, the change in the active phase, per day is found by subtracting the onset from the offset. If the result is a positive number, it means that the active period becomes longer by this number each day. The change of active period per day was analyzed per group. First of all, a normal distribution is found in all groups. The mean difference between groups is compared and a significant difference between knockdown mice and controls is found, with a value of $F(3,29)=19,502$ $p<0,001$. (data not shown)

Immunohistochemistry

Independent t-test of intensity measurements of photos of DCL in the SCN shows a difference of 59,37 (±4,980)% between the DCL knockdown mice and the control groups. (data not shown)

The fluorescent intensity of AVP staining in the SCN showed no significant difference within the whole SCN (data not shown). However, a difference in histology was observed. AVP was more concentrated in the cells of the knockdown mice, but not outside the cells. This shows that the distribution of AVP is altered between the knockdown mice and the control mice. To test this, fluorescence was analyzed for the individual cells and a significant increase in fluorescence within the cells was found between the knockdown mice and the control mice (figure 3). The figures show that there is a buildup of AVP within the cells of the DCL knockdown, but no spreading around the cells.

No significant difference was found comparing VIP expression in SCN of knockdown mice and controls. (data not shown).

DISCUSSION

The results show that DCL-knockdown mice have an altered response to light changes and a different localization of AVP expression in the SCN. This is the first study finding that a structural protein (DCL) is of crucial importance for the circadian functioning of the SCN.

Phase shift measurements

The results from both the switch to 8:16 LD and the freerunning period show a much lower tau in the DCL-knockdown mice. During freerunning, all three groups of control mice stay close to the normal rhythm, with tau of around 23,9 hours, while the knockdown group has a tau of 23,7 hours, which was significantly different from the control groups. These data indicate that the DCL-knockdown mice are less successfully able to stay in rhythm. Rhythm patterns also suggest a gradual loss of rhythm, during the first 5 days of freerunning (data not shown). Similar findings were reported in AVP-receptor knockout mice [14]. These findings agree with our hypothesis that the knockdown mice have a fastened reaction to environmental change due to change in expression of AVP.

Immunohistochemical staining of the SCN

Analysis of the immunohistochemical staining of DCL in the SCN indicates that induced DCL-knockdown was effective. The transgenic mice show a 60 percent knockdown of DCL. This percentage can be regulated through changes in diet, adding more doxycycline to the food will result in lower DCL expression.

Furthermore, the immunohistochemical staining of AVP in the SCN shows significantly heightened AVP expression inside the SCN cellbodies of the knockdown mice, but low expression in axons and varicosities. Overall expression of AVP does not seem to change in the SCN. This suggests a key role of DCL in the transport of AVP from the cellbody to the protrusions. This observation is supported by research by Yoshiaki et al. who found similar actograms from their AVP-receptor knockout mice [14] and the similar anatomical locations of DCL and AVP.

AVP does not seem to co-localize with DCL and high magnification of DCL-AVP double fluorescence staining suggests the expression in other cells than AVP (shown by a fellow student, data not shown). DCL-positive cells surround AVP cells in a way similarly seen in glial cells [15]. For this reason, we believe that the DCL-positive cells are actually astrocytes. The mechanism in which the astrocytes control the transport of AVP from the cellbody to the varicosities is unknown, but we can suggest two possible mechanism. The first mechanism is through the expression of ATP. ATP is released by astrocytes in a circadian rhythm [16] along the cellbodies of the AVP-cells. ATP could induce a pathway controlling AVP release to the axons. DCL would regulate the transport of ATP in the astrocyte, in this model. A second possible mechanism would be mechanical. The astrocytes surrounding the AVP-cell could mechanically push the cell to transport

AVP along the axon in a similar way as GnRH release is physically controlled by tanycytes [17]. In this model, DCL acts as a structural protein. In both models, the change in AVP expression after DCL-knockdown is explained.

Finally, immunohistochemical VIP staining has shown no differences between the knockdown mice and the controls, indicating that DCL does not influence expression and subcellular localization of this neurotransmitter. Our VIP findings are in line with our hypothesis, because the expression of VIP is in the core of the SCN rather than the shell, where DCL is expressed. However, AVP staining shows a network of AVP-positive axons through the core of the SCN where VIP-neurons are located. Change in expression of AVP could result in a changed regulation of VIP. Though we find no change in fluorescence of the VIP cells, the rhythm of VIP cells might be changed in DCL-knockdown mice.

CONCLUSION

The downregulation of DCL in mice shows a change in activity pattern similar to AVP or VIP knockdown mice. Furthermore, the immunohistochemical fluorescent staining of the SCN shows an increase of AVP within the cellbody and a decrease of AVP in axons and varicosities while there is no change in VIP. Increased AVP localization on the one hand and decreased localization in axons and varicosities on the other hand suggests that DCL influences transport of AVP from the cell body to the protrusions. As earlier studies show no co-localization between DCL and AVP, DCL is not actively bound to AVP during this transport. Future research may focus at the transport of AVP in more detail, in order to find the exact function of DCL. Also, the type of cell in which DCL is active should be identified, to see whether it are indeed astrocytes. Another interesting study would be to look at whether there is circadian rhythmicity in the concentration of DCL in the SCN. As so many proteins and compounds have a rhythm, especially in the SCN, the changes in level of DCL might be a good indication of the role and mechanism of the protein in the SCN. In order to examine the mechanisms in which AVP transport could be regulate, I recommend looking at ATP expression of DCL-positive cells, as well as the role ATP plays on AVP-cells.

This study has given new insights in the functioning of DCL and a better understanding of the mechanisms of the biological clock. It also shows DCL as a possible clinical target for AVP regulation and therefore as a pharmaceutical target for medication against jetlag and sleep-cycle related diseases, as earlier described by Yoshiaki et al.. For the time being, this study sheds the first light on DCL in the SCN, but many more steps should be taken to fully understand the role protein.

ROLE OF THE STUDENT

Boris Polm was a bachelor student during this project, under supervision of Dr. Erno Vreugdenhil. The student received the actogram data, as well as the prepared brains by from the supervisor. Sectioning, preparation of the slices, staining and microscopic work were performed by the student. All data presented was attained by Boris Polm, and the opinions enclosed in the discussion were formed by the student, as well as discussed within the research group.

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