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# Chapter 6

## General discussion

## Table of contents

## 1 Introduction

The main objective of this thesis was to study the role of the DCLK1 splice variant DCL in the brain, in particular in adult hippocampal neurogenesis. To map DCL in the adult brain, a DCL-specific antibody was designed and experiments were performed to validate a new genetic mouse model that is based on knockdown of DCL by doxycycline-induced sh-RNA molecules, called the DCL-KD mouse. First we reviewed the existing literature about developmental and adult neurogenesis in chapter 1. In the same chapter we reviewed the role of doublecortin and doublecortin-like 1 in this process and discussed several animal models to study adult neurogenesis. In chapter 2 we developed a DCL specific antibody and mapped the expression of DCL in the adult brain. As expected, we identified DCL in neurogenic areas like the SVZ and SGZ of the dentate gyrus where it co localized with DCX. DCL protein was also found in several non-neurogenic areas which raised novel questions about the function of DCL in these areas. In chapter 3, the DCL-KD mouse model was validated by showing specific DCL knockdown after doxycycline administration. Furthermore, the effect of DCL knockdown was studied on adult hippocampal neurogenesis using stereological techniques. Evidence was found for a role of DCL in the cell cycle exit of proliferating NPCs. In addition, we studied the effect of DCL knockdown on hippocampus-dependent behaviour. Additional evidence for the effect of DCL knockdown on hippocampus-dependent learning using a contextual fear conditioning paradigm is discussed in chapter 4. We describe the possible function of DCL in the hypothalamic tanycytes in chapter 5. In the latter chapter, we studied the question if DCL is involved in the Hypothalamic-Pituitary-Thyroid-axis (HPT-axis) and if DCL knockdown can affect thyroid hormone signalling via deiodinase type 2 activity in hypothalamic tanycytes.

## 2 DCL expression in the adult brain



Doublecortin-like kinase 1 (DCLK1) is, In contrast to doublecortin (DCX), a multiple splice-variants encoding gene containing at least four different splice variants (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). Each of the splice variants has its own functional and expression pattern (Burgess and Reiner, 2002;Engels et al., 2004;Vreugdenhil et al., 2001). Up to now, differentiation between DCLK1 splice variants by immunohistochemical methods was difficult since available antibodies did not differentiate between DCLK1 splice variants (Femia et al., 2013;Kikuchi et al., 2010;Kruidering et al., 2001). There are several other biochemical methods to separate DCLK proteins based upon their length or sequence, but in an immunohistochemical staining no distinction can be visualized under the microscope with a-specific DCLK antibodies. Based on studies in embryonic and postnatal brain tissue it is known that the DCLK splice-variant doublecortin-like (DCL) is expressed in radial glia cells and immature neurons during embryonic and early

postnatal neurogenesis (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). Furthermore, DCL is also expressed in adult hippocampal tissue (Vreugdenhil et al., 2001) but the exact cellular location in this heterogeneous brain region is unknown. As DCL shares a number of biophysical properties with DCX and is also highly homologous to DCX (Vreugdenhil et al., 2007), a well known marker of adult neurogenesis, we hypothesized that DCL, like DCX, might be expressed in the population of migrating immature neurons in the hippocampal dentate gyrus. However, other DCLK1 splice variants like DCLK-long and DCLK-short are also expressed in the hippocampal dentate gyrus (DG) (Vreugdenhil et al., 2001) and may cross-react with antibodies targeting different DCLK1 splice-variants and are not suitable to study DCL. Therefore, we developed a novel antibody targeting specifically DCL and no other DCLK1 splice variants. This antibody appears to be highly specific for DCL and does not stain other DCLK1 splice variants or the highly homologous DCX (Saaltink et al., 2012).

*2.1 DCL expression in neurogenic area's like hippocampus and olfactory bulb.*

As expected, we found DCL expression in the subgranular zone of the DG, the subventricular zone (SVZ), the rostral migratory stream (RMS) and the olfactory bulb (OB)(chapter 2). In all these neurogenic areas, DCL co localizes with DCX confirming several studies showing a functional interaction between both DCX and DCLK1 (Deuel et al., 2006;Koizumi et al., 2006;Tanaka et al., 2006;Tuy et al., 2008). In the embryonic brain (Boekhoorn et al., 2008), DCL is slightly earlier expressed compared to DCX. Within the adult brain such a temporal difference in expression seems to be absent although more detailed studies are needed to support this statement. This overlap in expression suggests a similar role as DCX for DCL in adult neurogenesis (Brown et al., 2003b;Couillard-Despres et al., 2005;Couillard-Despres et al., 2006;Rao and Shetty, 2004). Also during migration via the RMS and within the OB, DCL and DCX are strongly co localised. Only in the periglomerular cells (PGC's) in the granule cell layer (GL) of the OB some single positive DCX cells are found, suggesting a different temporal expression pattern of DCL compared to DCX as found earlier (Boekhoorn et al., 2008).
Images of DCL and DCX double staining show another remarkable fact. Whereas a DCX staining results in a rather empty cell nucleus and densely stained cytosol including dendritic branches, DCL staining exhibits a more speckled pattern without a densely filled cytosol (see Chapter 2, Fig. 5). This suggests different sub-cellular functions for DCL and DCX. Techniques with higher resolutions than confocal laser scanning microscopy are needed to visualize the exact location of DCL in the cell.

*2.2 DCL expression outside the neurogenic areas.*

Beside the neurogenic DG, SVZ, RMS and OB, DCL expression was found in several other brain regions like the suprachiasmatic nucleus (SCN), islands of Calleja (ICj) and hypotha-

lamic tanycytes (chapter 2). The majority of studies show DCL and DCLK to be involved in neurogenesis and neuronal development. During embryonic development, DCL is widely expressed in mitotic neuronal progenitor cells, radial glial cells (RGs) and radial processes and functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). Furthermore, DCL is involved in spine maturation (Shin et al., 2013). These findings raise the question why DCL is expressed in the SCN, in the ICj and in tanycytes, brain areas and cell populations in which neurogenesis has not been established.

As reported in chapter 2, DCL expression in the ICj shows strong resemblance with expression in the OB. DCL is mainly expressed in neuropil or extra-nuclear cytosol. Both OB and ICj consist of granule cells and are part of the olfactory system since ICj are located within the olfactory tubercle. Like the OB, the ICj are formed during brain development via cells born in the SVZ. Whereas the OB is supplied via the RMS, are the ICj supplied with granule cells via the ventral migratory mass (VMM) (De Marchis et al., 2004). The RMS continues with streaming neurons from the SVZ to the OB, whereas the VMM ceases in the adolescent mouse brain. However, 11 days after BrdU injection Shapiro and colleagues found BrdU/NeuN positive cells in the ICj of mice 2 months of age, suggesting some neurogenesis is still present in the adult ICj (Shapiro et al., 2009). Since DCL is expressed in the majority of ICj granule cells, its function in this area might be broader than neurogenesis alone and might reflect a form of plasticity or intracellular transport along the neuronal fibres (see Chapter 1).
DCL expression in the SCN might also be related to plasticity and fast transport along microtubules. The SCN exhibits daily structural rearrangements within the SCN (Girardet et al., 2010a;Meijer et al., 2010), which may demand high levels of neuronal plasticity. DCL might be involved in SCN plasticity, as has been reported for polysialic acid (PSA) and neural cell adhesion molecule (NCAM) (Bonfanti et al., 1992;Glass et al., 2003;Prosser et al., 2003;Shen et al., 1997). PSA/NCAM is thought to play a key role in the daily structural rearrangement of the SCN (Bonfanti et al., 1992;Girardet et al., 2010a;Glass et al., 2003;Prosser et al., 2003) and is thus crucial for the circadian organization of behaviour (Fedorkova et al., 2002;Prosser et al., 2003;Shen et al., 1997). In line with DCL expression in the SCN, PSA/NCAM is also a well-known marker for neurogenesis in the adult brain and is often co expressed with DCX (Bonfanti, 2006;Nacher et al., 2001). Up to now, no neurogenesis in the SCN has been reported and therefore, a functional role of DCL in SCN plasticity is most obvious. However, besides neurogenesis, DCL is involved in fast microtubule-guided retrograde transport of signalling molecules in neuroblasts and neuroblastoma cells (Fitzsimons et al., 2008) and thus might also play such a role in the SCN. Possible implications of DCL as intracellular transport factor will be discussed further below (see paragraph 6.8).
An increasing number of studies show (condition-dependent) neurogenesis in the hypothalamus (Haan et al., 2013;Lee et al., 2012;Xu et al., 2005). Although hypothalamic tanycytes

do have DCL expression, these DCL positive tanycytes do probably not reflect continuous hypothalamic neurogenesis like in the SVZ and DG. The hypothalamus is a highly dynamic region, which plays a crucial role in neuro-endocrine signalling between body and brain (Rodriguez et al., 2010). Several processes are regulated by seasonal time cues, which induce physiological adaptation (Hazlerigg and Loudon, 2008). Like in the SCN, such dynamic processes need cellular rearrangements in which DCL might play a role. DCL might also play a similar role in tanycytes as it does in neuronal progenitor cells (NPC's) were it is involved in sub cellular transport of the glucocorticoid receptor (GR) (Fitzsimons et al., 2008). Like DCX (Friocourt et al., 2001), DCL is equipped with a serine-proline rich C-terminus which functions as an anchor point for interaction with other proteins.

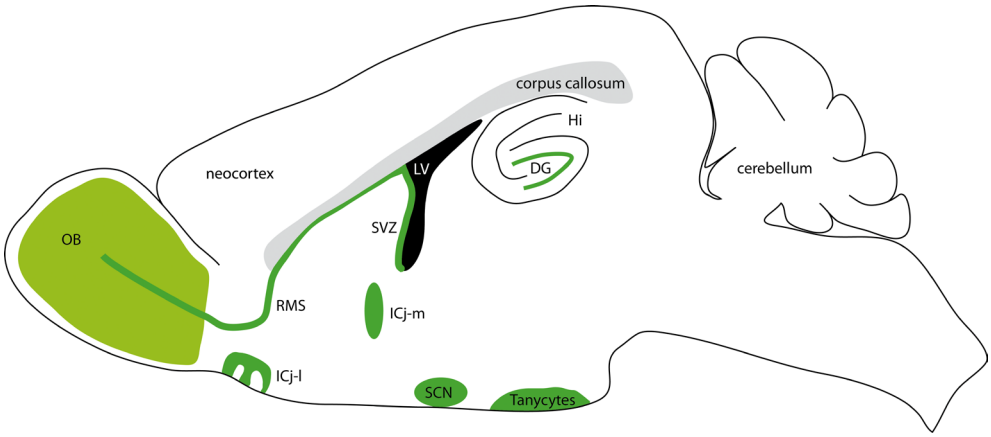



**Figure 1:** Schematic overview of all DCL expression sites (green) in the adult mouse brain. Within the hippocampus (Hi) DCL is expressed in the dentate gyrus (DG). DCL is highly expressed along the walls of the lateral ventricles (LV). From this subventricular zone (SVZ), DCL positive immature neurons migrate along the rostral migratory stream (RMS) towards the olfactory bulb (OB) were DCL is also expressed in the neuropil. Other sites of DCL expression are the major Islands of Calleja (ICj-m), the lateral Islands of Calleja (ICj-l), suprachiasmatic nucleus (SCN) and the hypothalamic tanycytes.

In summary, DCL expression in non-neurogenic brain areas does probably not reflect neurogenesis, but might be an indication of cellular plasticity. The ICj show great resemblance with the OB and therefore, DCL might play a similar role in both these areas. The hypothalamic SCN and tanycytes are areas with functional neuronal plasticity and dendritic rearrangements in which DCL might play a role. Further studies on DCL have to reveal its function within these non-neurogenic brain areas.

## 3 A transgenic mouse model to study DCL in the brain: application of in vivo RNA-interference

The main objective of this thesis is to investigate the role of DCLK1 splice variant DCL in adult neurogenesis. The technical focus in this study is the validation of an in vivo model in which DCL is targeted by RNA interference technology. We hypothesized that DCL is functionally involved in adult neurogenesis. Therefore, by knocking down DCL, we expect to disrupt the integration of neurons into neuronal networks, which will affect the behavioural output. Since DCL plays a crucial role in embryonic corticogenesis (Boekhoorn et al., 2008;Vreugdenhil et al., 2007) and its analogue DCX may compensate for the loss of DCL during development, a conditional model which can be activated on demand in adult life is required. As described in chapter 3, we designed a transgenic mouse model harbouring an short hairpin DNA construct in its germ line of which the expression is blocked by a TET-repressor (TETr). Expression of the shRNA targeting DCL can induced by doxycycline (Seibler et al., 2007).

Firstly, we have investigated induction of the DCL-shRNA expression by doxycycline administration. We found a strong up regulation of shRNA expression after doxycycline (dox) induction. Although this was a 10-fold up regulation in the hippocampus and 25-fold up regulation in the olfactory bulb, we also found some shRNA expression in non-induced transgenic animals. Theoretically, TETr should block the expression of the DCL targeting shRNA. However, incomplete blockade may result in shRNA expression (leakage) and subsequent reduction of DCL levels which might be compensated by DCX. Indeed, our initial experiments indicate reduced DCL expression in the adult brain of non-induced transgenic mice. Therefore, we inspected embryonic and early postnatal tissue to explore possible premature DCL knockdown. However, despite little hairpin leakage, the developing brain was unaffected and no significant reduction of DCL protein was found at this stage of development. Our data indicate normal brain development in which DCL performs like in a wildtype animal without DCX compensation. This finding is important since DCL knockdown at an adult stage might be ineffective when DCX compensates for DCL during brain development (Deuel et al., 2006;Koizumi et al., 2006;Tanaka et al., 2006;Tuy et al., 2008). In contrast to other studies with shRNA mice (Acehan et al., 2011;He et al., 2010;Out et al., 2011), we report the presence of leakage. However, we only observe a clear phenotype after doxycycline administration, indicating that leakage did not affect brain development and brain function.

A major advantage of the DCL shRNA mouse model is the specificity of the hairpin. Since DCL is one of the 10 splice variant of the DCLK1 gene (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001), specific knockdown of DCL, but not of other DCLK splice variants, is a necessary step to study DCL function in the adult brain. Indeed, Western Blot analysis of induced wildtype and transgenic brain tissue shows a highly specific

knockdown of DCL whereas DCLK-short and DCLK-long are unaffected. Especially the distinction between DCL and DCLK-long is important since DCL shares most of its functional protein domains with DCLK-long. Furthermore, DCL and DCLK-short appeared to precisely regulate postsynaptic functions, because these variants have distinct roles in postsynaptic density (PSD) protein accumulation and spine morphogenesis (Shin et al., 2013). Therefore, our mouse model seems also an excellent tool to study the role of DCL in spine morphogenesis.

## 4 Effect of DCL knockdown on neurogenesis in the adult brain.

The first studies with BrdU revealed an unexpected increase of proliferation after DCL knockdown. The DCX domains in the DCX and DCLK1 genes are thought to function as anti-catastrophe factor by stabilizing microtubules (Moores et al., 2004;Moores et al., 2006). Previous observations in our lab showed a function for DCL in mitotic spindle formation and thus a role in cell mitosis (Vreugdenhil et al., 2007). Furthermore, combination treatment of microtubule disrupting agents such as vinca alkaloids and siRNA targeting DCL and DCLK1-long induced apoptosis in neuroblastoma cells in vitro (Verissimo et al., 2010a). However, these studies used siRNA targeting both DCL and DCLK-long. Since the study in chapter 3 used specific constructs targeting DCL only, it seems likely that only DCLK-long might be involved in cell proliferation. However, both DCLK-long and DCL contain microtubules binding domains, which stabilize microtubules. Which role each of the two DCLK1 splice variants plays in proliferation is still unknown. Knocking down both DCLK-long and DCL leads to apoptosis and microtubules destabilization both in vitro and in vivo (Shu et al., 2006;Verissimo et al., 2010a;Vreugdenhil et al., 2007). Specific knockdown of DCL in vivo leads to increased proliferation. Although DCL is expressed in mitotic spindle formations within the embryonic brain, we have no indications that DCL is also expressed in stem cells in the adult brain (unpublished data). Therefore, DCL knockdown might have an indirect effect on proliferation. One explanation might be a feedback mechanism within the hypothalamus. When the flow of immature neurons reaching the network is reduced due to DCL knockdown, the stem cells might be triggered to increase their production of new immature neurons. However, the mechanism of such a feedback system is unknown.



Another possibility is the involvement of DCL in transporting signalling proteins affecting the fate of neuronal progenitor cells such as the glucocorticoid receptor (GR; (Fitzsimons et al., 2008). Stress is a well known inhibitor of neurogenesis (Lucassen et al., 2010a;Schoenfeld and Gould, 2012) with strong effects on both proliferation and differentiation/maturation. Stress is known to increase circulating levels of glucocorticoids (cortisol in humans and corticosterone in rodents) which bind to and activate GRs. The increased corticosterone concentration drives in the dentate gyrus progenitor cells the cell nuclear localization of GR that in turn can modulate gene transcription and suppress cell proliferation (Veenema et al., 2007).

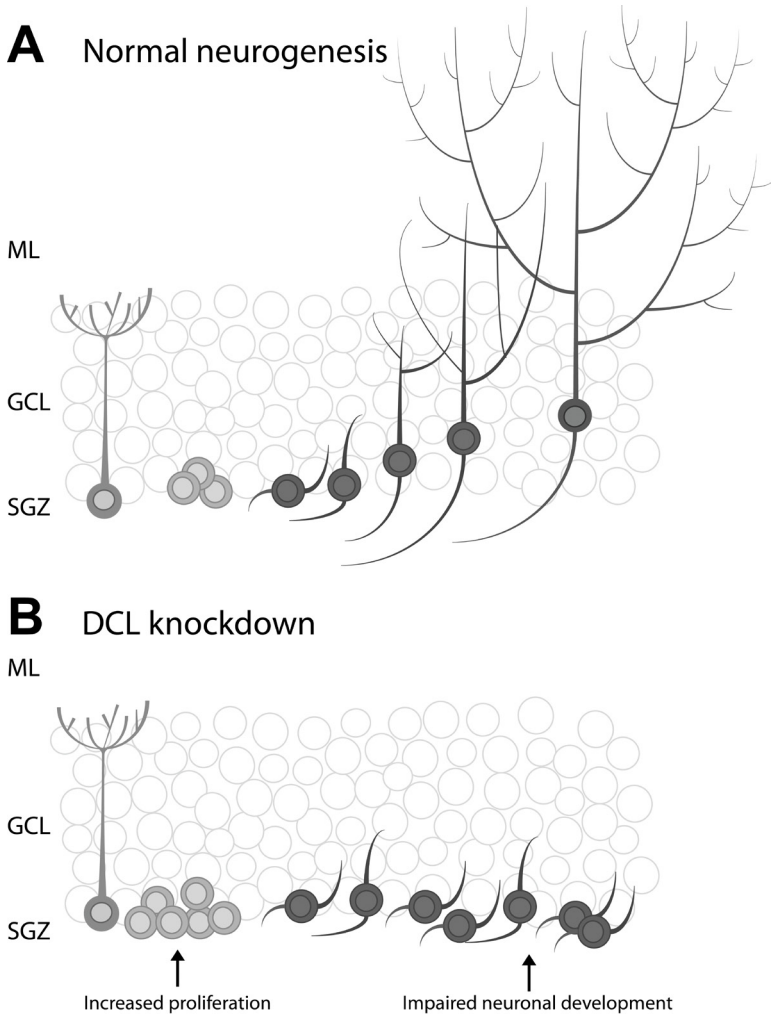



**Figure 2:** Effect of DCL knockdown on hippocampal adult neurogenesis. A: In the subgranular zone (SGZ) reside stem cells which generate neural progenitor cells (NPC's; pink). These new born cells develop into immature neurons (green) which migrate into the granule cell layer (GCL). The immature neurons make contact with the existing network in the molecular layer (ML) and develop into mature neurons (blue). B: After DCL knockdown, proliferation is increased, but immature neurons do not migrate and develop into mature neurons.

Therefore, the increased proliferation of these progenitor cells after DCL knockdown might reflect lower GR transport to the nucleus. Also here, we have no data that actually show DCL expression in neurogenic stem cells.

The first glimpse of a striking DCX phenotype in our DCL-KD mice became visible when hippocampal sections were inspected under the fluorescent microscope (Chapter 3). Nearly

all DCX positive cells reside in the subgranular zone (SGZ) of the DG and show hardly any elongate dendrites towards the granular cell layer (GCL) and molecular layer (ML) suggesting an arrest in neuronal development. Cells seem to stop their development at the point where they have to migrate into the GC and develop elongated dendrites towards the ML. This arrest suggests that DCL might play a key role in dendritic outgrow. Since both DCL and DCLK-short are thought to regulate postsynaptic density (PSD) accumulation and spine morphogenesis (Shin et al., 2013), knockdown of DCL might affect these processes. This seems to be the case since DCL knockdown results in reduced dendritic development in the population of DCX positive cells. DCLK-short is one of the genes which is highly up-regulated after NGF-induced differentiation of PC12 cells (Dijkmans et al., 2008). After NGF induction, PC12 cells develop neurites and differentiate into neurons. DCLK-short has been shown to repress CREB-mediated transcription (Ohmae et al., 2006;Silverman et al., 1999), which is an important transcription factor in NGF-induced processes like PC12 differentiation (Finkbeiner et al., 1997). However, whether DCLK-short is expressed in NPC's is unknown. The question remains whether we look at a cell cycle arrest, or impaired dendritic outgrow. Since type-1 DCX positive cells in DCL-KD mice show small to medium processes (as describe by (Plumpe et al., 2006)) it is likely that immature neurons do not receive the right signals to develop further into mature granule cells. When they fail to integrate into the network, the cells might go into apoptosis and disappear.

DCL is highly homologues to DCX (Vreugdenhil et al., 2007) and both DCLK1 and DCX are thought to compensate each other during development in knockout conditions (Deuel et al., 2006;Koizumi et al., 2006;Tanaka et al., 2006;Tuy et al., 2008). Evidently, this compensation does not occur when DCL knockdown is applied during adulthood. It is quite remarkable that micro (mi)RNA-mediated retroviral knockdown of DCX does not alter adult neurogenesis in either adult neurogenic niche (Merz and Lie, 2013). Although DCX is a well known marker for adult neurogenesis (Brown et al., 2003b;Couillard-Despres et al., 2005;Couillard-Despres et al., 2006;Rao and Shetty, 2004) its function in adult neurogenesis is unknown. Apparently, DCX is dispensable for adult neurogenesis. This study is in contrast with findings in the developing embryo in which RNAi mediated knockdown of DCX had an effect on developmental neurogenesis (Koizumi et al., 2006). The explanation for these opposite findings might be technical. A DCX-KD mouse like the DCL-KD mouse would provide useful information regarding the function of DCX during adult neurogenesis.

In conclusion, DCL knockdown results in aberrant neuronal development and maturation within the hippocampal neurogenic niche. In line with the expected function of DCL, immature neurons do not migrate into the granule cell layer and do hardly develop dendrites reaching the hippocampal network in molecular layer after DCL knockdown (Fig. 2B). Unexpected is the increased proliferation seen in vivo in hippocampal dentate gyrus where

DCL knockdown in vitro in PC12 cells leads to a reduction of cell proliferation. A feedback mechanism involving DCL in glucocorticoid signalling might explain this unexpected finding in vivo in the hippocampus.

## 5 The effect of DCL knockdown on hippocampus-dependent memory tasks.

In the past decades hippocampal adult neurogenesis gained considerable attention in the fields of psychiatric and degenerative brain disorders. Neurogenesis is affected by severe stressors and might form a potential cure for brain degeneration. The hippocampus is involved in higher cognitive brain functions like learning and memory formation. Especially spatial and contextual memory formation is thought to be hippocampus dependent (Marin-Burgin and Schinder, 2012). Adult neurogenesis might play a key role in these hippocampal functions (Kee et al., 2007;Kempermann et al., 1998a;Raber et al., 2004). However, the hippocampus is a complex and heterogeneous brain area in which neurogenesis is just a part of the story. One of the first techniques to target adult neurogenesis was the use of x-ray radiation showing effective blockade of adult neurogenesis (Santarelli et al., 2003). Using this technique, Saxe et al. (Saxe et al., 2006) showed an effect of reduced neurogenesis on a hippocampus-dependent behavioural task measuring contextual fear memory. Using a genetic manipulation strategy, Immayoshi and colleagues (Imayoshi et al., 2008) observed that neurogenesis inhibition affects hippocampus-dependent spatial memory formation using a circular hole board paradigm. A range of studies followed with varying results. We applied similar tests to our mouse model (chapter 3 and 4) and did not find an effect of impaired adult neurogenesis on spatial and contextual memory formation.
In a circular hole board paradigm, DCL-KD animals are capable of learning to find the exit hole like wildtype littermates. Also contextual fear memory formation is unaffected by DCL knockdown. However, some subtle behavioural differences are found. For example, DCL-KD animals have a significant longer escape latency compared to wildtype littermates. In the fear conditioning paradigm, DCL-KD animals show hardly any tail rattling behaviour which suggests reduced levels of basal anxiety. Thus, DCL knockdown affects behavioural output but it seems that the behavioural paradigms, used in our studies, are not suitable tests. However, the most important finding is the normal spatial and contextual fear memory performance despite the impairment of adult neurogenesis.

There are several explanations for our findings. In the first place, we make use of a specific knockdown strategy. Although we see a clear phenotype with respect to adult neurogenesis, neurogenesis is not completely blocked. Proliferation is even increased and we still measured some BrdU/NeuN double positive cells. Neurogenesis blockade by X-ray radiation is less specific and potentially affects the whole hippocampus including the non-neurogenic cell population. In support of the outcome of our approach several other techniques targeting

adult neurogenesis specifically did not result in a change of hippocampus-dependent spatial memory formation (Jaholkowski et al., 2009;Jedynak et al., 2012;Martinez-Canabal et al., 2013;Urbach et al., 2013). These studies raised the question whether adult neurogenesis is involved in hippocampus-dependent learning and memory formation (Frankland, 2013).

Recently, adult hippocampal neurogenesis have been implicated in pattern separation (Aimone et al., 2011;Sahay et al., 2011b;Aimone et al., 2011;Sahay et al., 2011b;Tronel et al., 2012). Pattern separation is the capacity to distinct between two highly similar input patterns (Treves et al., 2008). The process of pattern separation is suggested to be associated with post traumatic stress disorders and with panic disorders (Kheirbek et al., 2012). However, the studies described in this thesis are not suitable for studying pattern separation performance. Therefore, the effect of DCL knockdown on subtle context recognition remains to be speculative.

Another factor of interest is neurogenesis in the olfactory bulb. Although we did not study the OB in detail, the aberrant morphology of DCX positive cells seen in the hippocampus, seems to be absent in the SVZ, RMS and OB. Adult neurogenesis in both OB and hippocampus might play a similar role in spatial and contextual discrimination (Konefal et al., 2013) but seems to be regulated independently (Belnoue et al., 2013). Impairment of neurogenesis in both brain areas might increase the effect on spatial and context discrimination. Due to our limited observations on the SVZ, RMS and OB, we can only speculate about the effect of DCL knockdown on adult neurogenesis in the olfactory system.

It remains the question why the DCL-KD mice take more time to enter their home cage despite the fact they know how to find the exit hole. Unlike rats, mice have some problems with navigating in the Barnes maze (Koopmans et al., 2003). Therefore mice are habituated to the tunnels 1 week before the free exploration trial (FET). Mice with reduced DCL expression might not benefit from this training. Also during the training sessions, DCL-KD mice perform better in the second training of the day suggesting a limited habituation memory. This might be an effect of reduced pattern recognition; the animals fail to recognize the tunnel as safe passageway in a novel context (circular hole board). Why DCL-KD animals fail to remember the tunnel training, but succeed in spatial memory formation remains to be elucidated.

One may also reason the other way around. The behavioural parameters affected by DCL knockdown, i.e. prolonged escape latency and tail rattling, point towards reduced anxiety. Since cue fear memory is thought to be amygdala dependent, the strong freezing response of DCL-KD animals during the first cue presented at the memory retrieval trials is rather difficult to explain from the perspective of impaired neurogenesis. However, the interplay

between amygdala and hippocampus might be affected. In chapter 4 we discussed the fear conditioning paradigm in more detail. A series of multiple cue/shock pairings might be too strong. A fear conditioning paradigm with a delayed or immediate single context-shock pairing might provide a more precise answer on hippocampus related fear memory formation with involvement of the amygdala.

The significant differences in tail rattling and increased freezing behaviour in the first cue suggest an effect of DCL knockdown on trait anxiety. Trait anxiety is defined as an enduring level of anxiety, independent from environmental stimuli whereas state anxiety is temporary anxiety induced by environmental stimuli (Lister, 1990). Both forms of anxiety seem to have genetic components which are differentially rooted in the genetic background of laboratory mouse strains (Avgustinovich et al., 2000). DCL knockdown might not affect state anxiety, which explains the normal fear conditioning response, but might reduce trait anxiety, explaining the reduction in tail rattling behaviour and increased escape latency. Though high levels of trait anxiety are correlated to reduced adult neurogenesis (Earnheart et al., 2007;Sah et al., 2012), we find opposite effects in our DCL-KD animals. Although the numbers of BrdU/NeuN double positive cells are strongly reduced in DCL-KD animals, their proliferation is significantly increased (see chapter 3). Since stress is deleterious for proliferation, increased proliferation in our DCL-KD animals might reflect reduced trait anxiety. DCL is involved in sub cellular transport of the GR (Fitzsimons et al., 2008) which might explain the mechanism underlying this increased proliferation. Exposing DCL-KD animals to more anxiety related behavioural paradigms like novel object exploration in combination with familiar and unfamiliar context, might provide answers to the questions raised here.

In conclusion, DCL-induced impaired neurogenesis does not affect hippocampus-dependent spatial memory and contextual fear memory. DCL-KD animals need equal amount of time to find the exit as their litter mate controls, but their latency to escape the board and enter their home cage is increased. Furthermore, they show a stronger freezing response to the first cue in CFC where after they behave like wildtype littermates except for tail rattling behaviour during fear. These results are highly significant. These findings suggest a role for DCL in anxiety in which DCL facilitates the stress-induced corticosterone signal. Therefore, DCL-KD animals seem a valuable model for further research aimed to address the role of neurogenesis in the processing of fearful information by measuring more subtle aspects of context discrimination and pattern separation.



## 6 DCL in the hypothalamus

Mapping studies with the novel DCL antibody revealed unexpected expression sites within the brain. One of these sites are hypothalamic tanycytes. Tanycytes reside at a crossroad

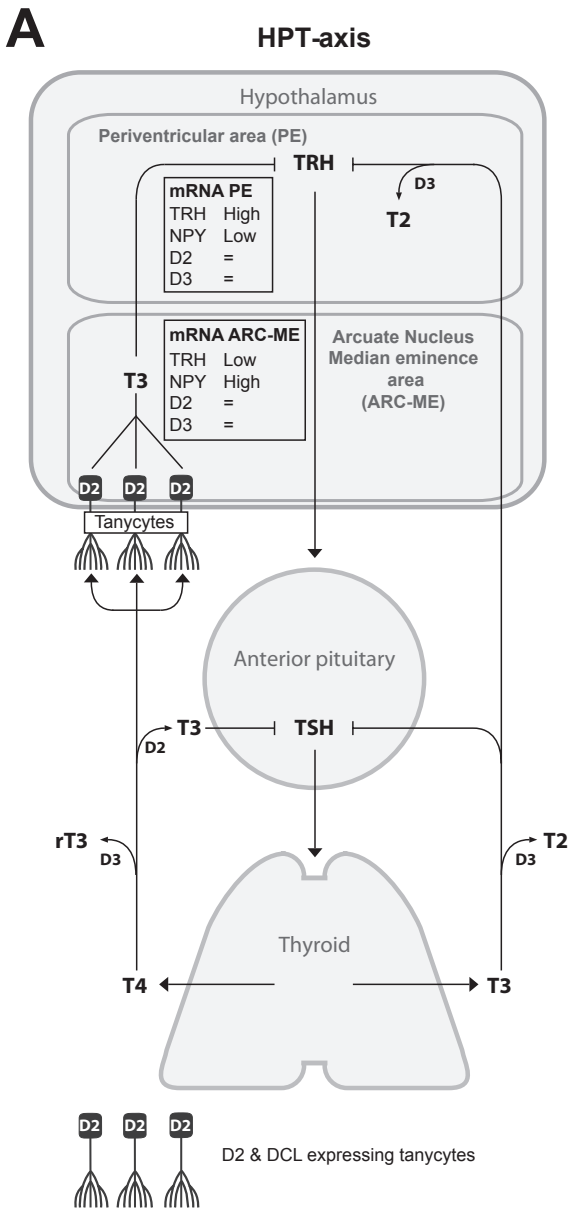
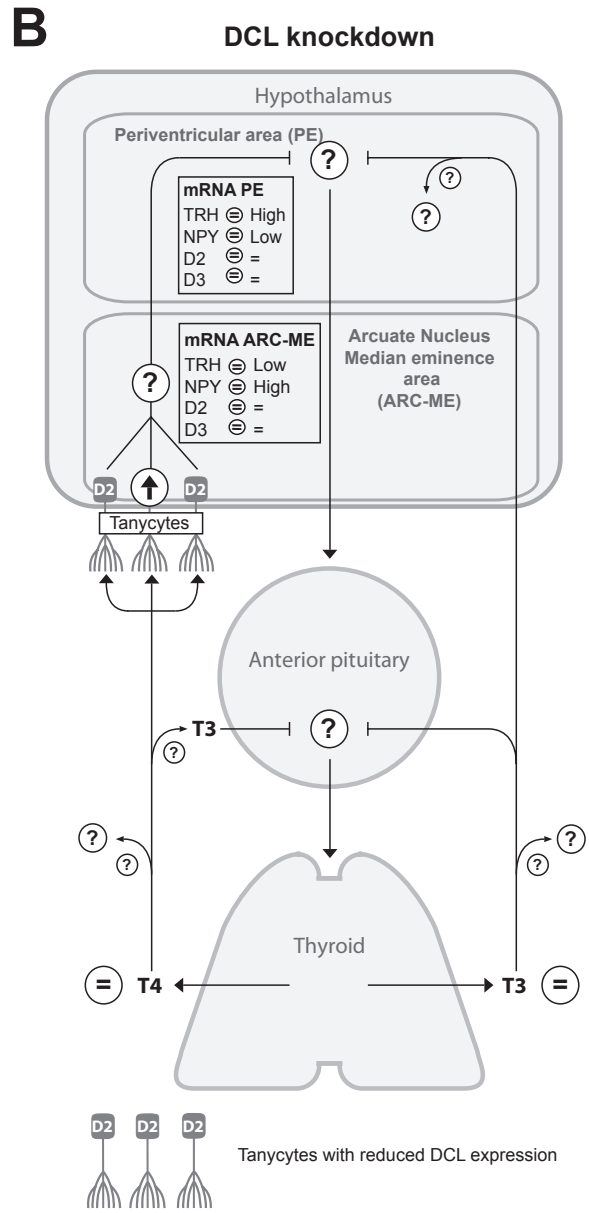



**Figure 3:** Schematic overview of HPT-axis and the effect of DCL knockdown. **A:** Normal condition in which Thyrotropin-releasing hormone (TRH) is released from the periventricular area (PE) of the hypothalamus. TRH stimulates the anterior pituitary to release Thyroid-stimulating hormone (TSH). TSH stimulates the thyroid to release thyroid hormone in 2 variants; inactive T4 and active T3. Deiodinase enzymes D2 and D3 convert respectively T3 into inactive T2 and T4 into active T3. In the Arcuate Nucleus and Median eminence area (ARC-ME) reside DCL positive tanycytes which also contain high levels of D2.




**B:** After DCL knockdown, D2 activity whithin the ARC-ME region is increased, but has no effect on peripheral levels T3 and T4. However, from many parameters it is still unknown what happens after DCL knockdown. Brain areas and organs involved in the HPT-axis are written in grey.

between cerebrospinal fluid (CSF), brain and peripheral bloodstream which may be considered as a peri- or circumventricular organ (CVO). Beside the blood-brain barrier (BBB), CVO's form an alternative route between the peripheral blood stream and the CSF (Rodriguez et al., 2005;Rodriguez et al., 2010).

Tanycytes play an important role as gatekeeper (Rodriguez et al., 2005) and are equipped with transport machinery to transfer substances from blood to CSF and vice versa (Rodriguez et al., 2010). Tanycytes are also the major source of deiodinase type 2 (D2) expression in the adult brain (Guadano-Ferraz et al., 1997;Kalsbeek et al., 2005;Tu et al., 1997). The enzyme D2 converts inactive thyroid hormone T4 into active T3. Within the hypothalamus, thyroid hormone signalling is involved in the regulation of energy balance and lipid metabolism (Bernal, 2002;Murphy and Ebling, 2011). In chapter 5, we studied the effect DCL knockdown on thyroid hormone signalling. Thyroid hormone levels in the peripheral bloodstream were not affected by DCL knockdown. Similarly, mRNA expression levels of several HPT-axis related genes within the hypothalamus are not changed. Interestingly, D2 activity within the hypothalamus was significantly increased. Up to now, we cannot state whether DCL knockdown affects D2 activity directly or indirectly. A possible mechanism is DCL-mediated post-translational modification of D2 proteins, which is independent from transcriptional regulation of the D2 gene. One post-translational modification mechanism that regulate D2 activity is ubiquitination via an ATP-dependent process (Gereben et al., 2000;Steinsapir et al., 2000). As DCL knockdown leads to reduction of ATP production (Verissimo et al., 2013), increased D2 activity might be the result of a blockade of its ubiquitination-mediated degradation in DCL knockdown mice.



Another possibility is the involvement of DCL in hypothalamic neurogenesis. Tanycytes are thought to represent a population of stem cells which can give birth to new neurons (Haan et al., 2013;Kokoeva et al., 2005;Kokoeva et al., 2007;Lee et al., 2012;Perez-Martin et al., 2010;Xu et al., 2005; for reviews see Bolborea and Dale, 2013;Cheng, 2013;Lee and Blackshaw, 2012). Like the SVZ and SGZ, the wall of the third ventricle in the hypothalamus might form a third neurogenic niche although the rate of neurogenesis is low and is context dependent (food availability). Based on morphological characteristics, the third ventricle can be divided into three zones (I, II & III) by (Perez-Martin et al., 2010). The ventral part of the wall is mainly inhabited by multiciliated cubic ependyma cells together with subependymal astrocytes. These cells also form the upper part of zone II in which also tanycytes reside. Zone III consists entirely of hypothalamic tanycytes. Only subependymal astrocytes and tanycytes are thought to be part of the neurogenic niche (Perez-Martin et al., 2010). Since DCL is mainly expressed in zone III near the median eminence, is limited expressed in zone II and is absent in zone I, its expression might be restricted to tanycytes (chapter 2). Moreover, blockade of neurogenesis by X-ray irradiation in the hypothalamus results in a

reduction of weight gain and in increase of energy expenditure and total activity (Lee et al., 2012). In chapter 5, we also report a reduction in weight gain which might be due to DCL knockdown-induced aberrant hypothalamic neurogenesis. Therefore it might be interesting to address the relationship between DCL and neurogenesis in the hypothalamus in future studies. Whether DCL-KD animals show more activity remains also to be determined though DCL-KD animals show increased proliferation which is also induced in animals exposed to exercise (van Praag et al., 1999a;van Praag et al., 1999b).

In conclusion, DCL knockdown in hypothalamic tanycytes results in increased activity of hypothalamic D2 without affecting peripheral thyroid hormone levels and hypothalamic mRNA expression of several HPT-axis related genes (Fig. 3). A possible mechanism is DCL mediated post-translational modification of D2 proteins, which might regulate D2 activity by ubiquitination via an ATP-dependent process. Furthermore, it may be interesting to study the possible relationship between DCL and hypothalamic neurogenesis in future studies.

## 7 The use of a conditional siRNA expressing mouse model to study mental disorders.

In this paragraph I will discuss the sh-RNA DCL mouse as a model for mental disorders. Furthermore, what are the advantages and disadvantages of the use of siRNA technology?

### *7.1 siRNA technology in disease models*

There are several genetic modified mouse models with inducible techniques affecting adult neurogenesis; for an overview see (Dhaliwal and Lagace, 2011;Imayoshi et al., 2011). The majority of these models used inducible site-specific recombinases like Cre which can recognize LoxP sites and cuts out the DNA of interest. In the case of adult neurogenesis targeting models, Cre recombinases are often under control of neuronal stem cell specific promoters, like nestin and GFAP. It is deleterious for adult neurogenesis to flox several stem cell marker genes like Cdk5 (Lagace et al., 2008), Notch1 (Ables et al., 2010), NeuroD1 (Gao et al., 2009) and Sox2 (Favaro et al., 2009). In all studies, the gene of interest is cut out the DNA at a specific time point after brain development. In this respect, these techniques also circumvent knockout possible compensation effects during embryogenesis by homologous genes, e.g. DCX and DCL. Compared to these studies, our DCL-KD animals show comparable effects of DCL knockdown on adult neurogenesis. The population of immature neurons is decreased as well as the number of BrdU/NeuN positive cells. Remarkably, proliferation is increased in DCL-KD animals, though this finding is likely due to DCL knockdown and not the use of siRNA technology.

The strength of the conditional siRNA technique is the specificity: individual splice-variants are targeted by the shRNA, leaving the expression of other splice-variants unaltered. In our model, shRNA targets very precisely DCL without affecting DCLK-long and DCLK-short. The conditional aspect of shRNA expression is of great importance in particularly our DCLK1 study. Embryonic neocortical development DCX (Corbo et al., 2002) and DCLK1 knockout mice is normal, strongly suggesting compensational effects of both DCX and DCLK1 in these transgenic animals (Deuel et al., 2006;Pramparo et al., 2010;Tanaka et al., 2006;Tuy et al., 2008). Conditional knockdown of DCL in adult life circumvents the inactivation of DCLK1 function by DCX compensation. Although some leakage is found in adult animals, studies on embryo's and early postnatal pups in chapter 3 revealed no significant effect of leakage on DCL protein expression suggesting normal development of non-induced DCL-KD animals (data not shown).

siRNA-mediated DCL knockdown is systemic, i.e. after induction by doxycycline, DCL-shRNA is expressed in every cell of the animal. Beside the neurogenic hippocampus and forebrain (SVZ, RMS & OB) DCL is expressed in several other brain areas like ICj, SCN and hypothalamic tanycytes (chapter 2). One technique to specifically hit adult neurogenesis in the dentate gyrus is the use of stereotactic injections of viral vectors with siRNA constructs. Two studies applied this technique on genes closely related to DCL, DCX (Merz and Lie, 2013) and GR (Fitzsimons et al., 2013). In the case of GR, NPC's were targeted using lentiviral vectors with GFP and siRNA targeting the GR. The viral vectors were applied locally into the dentate gyrus and GR knockdown resulted in hyper active neurogenesis with more elaborate dendritic arborisation and increased migration (Fitzsimons et al., 2013). The cellular phenotype resulted in a reduction of contextual fear memory. In contrast, a study (Merz and Lie, 2013) in which DCX was targeted in the dentate gyrus using retroviral vectors did not affect adult neurogenesis. Although DCX knockdown in NPCs appeared successful, new-born cells developed normally suggesting that DCX is dispensable for adult neurogenesis. Retro- or lentiviral vector mediated gene transfer seems a good technique to target neurons within the hippocampus, but stereotactical brain surgery might be associated with substantial mechanical damage and discomfort. Transgenic mice, engineered with shRNA expressing constructs in their germ line, like DCL-KD mice, do not suffer from these side effects but offer similar opportunities to target genes involved in adult neurogenesis.



*7.2 Impaired neurogenesis as phenotype*

The initial aim of this project was to explore the possibilities to develop a model for Major Depressive Disorder (MDD) since neurogenesis is inhibited in acute and chronically stressed animals. Also the effect of many antidepressant drugs and electroconvulsive therapy seemed to be dependent on adult neurogenesis (Duman, 2004). Furthermore, adult neurogenesis

was thought to underlie the hippocampal volume decrease in depressed patients (Sheline et al., 1996). Although there is a strong correlation between neurogenesis and MDD, there are several arguments to support the idea that MMD and hippocampal volume decrease are independent from adult neurogenesis (Czeh and Lucassen, 2007;Sapolsky, 2001;Sapolsky, 2004). Also, cognitive impairment, as observed in MDD patients, is not correctly modelled in mice with inhibited adult neurogenesis (Frankland, 2013). This raised the question to what extend adult neurogenesis is involved in hippocampus functioning.

The selection of our behavioural studies was based on the idea that adult neurogenesis plays an important role in hippocampal related cognition like spatial memory and contextual fear conditioning. However, despite the significant impairment of adult neurogenesis, DCL-KD mice did not show impaired spatial and contextual fear memory formation. Therefore, DCL-KD mice are likely not a suitable model to study cognitive and emotional features of MDD. Nevertheless, adult neurogenesis is significantly affected and therefore the shRNA DCL-KD model is suitable to study the role of adult neurogenesis in other subtle hippocampal functions like context discrimination and possibly pattern recognition (Clelland et al., 2009;Sahay et al., 2011b). These functions are thought to underlie diseases like anxiety disorders, panic disorders and post traumatic stress disorder (PTSD; Kheirbek et al., 2012).

## 8 Perspectives

The studies reported in this thesis provided answers about the role of DCL in hippocampal adult neurogenesis. DCL is expressed in the neurogenic niche in the olfactory forebrain and the hippocampus. DCL knockdown has a deleterious effect on the population of DCX positive immature neurons and the number of BrdU/NeuN double positive neurons. Furthermore, DCL is also expressed in areas of which neurogenic capacity is less well studied. The function within these brain areas remains to be determined.



These findings raise several novel questions and create possibilities for further research. In the first place, the fate of neurons in a DCL knockdown environment is not precisely determined. Stereotactic injections with viral vectors containing green fluorescent protein (GFP) can label and trace new-born neurons (van Hooijdonk et al., 2009). GFP labelling of hippocampal tissue in DCL-KD animals might shed light on the fate of immature neurons in DCL-KD animals. How many of new-born neurons integrate into the network? Can new-born neurons develop into mature neurons with healthy dendrites or is there stagnation in the developmental process?

Another point of interest is the increased proliferation after DCL knockdown. Such a profile is seen in animals with increased exercise or after removal of the adrenals. When adre-

nalectomy (ADX) is applied, the level of circulating glucocorticoids is reduced and proliferation is boosted (Gould et al., 1992;Cameron and Gould, 1994). Increased glucocorticoid signalling by injection of corticosterone or after exposure to a stressor reduces proliferation (Cameron et al., 1998;Wong and Herbert, 2004). How does DCL affect proliferation? In vitro, in PC12 cells DCL knockdown inhibits proliferation of the neuroblastoma cells and also in vivo, neuroblastoma tumor growth is slowed down (Verissimo et al., 2013). A similar effect was expected regarding proliferation within the hippocampus, but we found the opposite. Since proliferation is strongly suppressed by glucocorticoid signalling and DCL is involved in intracellular GR transport (Fitzsimons et al., 2008), DCL knockdown might promote proliferation indirectly via abolished glucocorticoid signalling. Hence, this hypothesis can be tested in an experiment correlating different concentrations of circulating glucocorticoid with its cell nuclear localization in the proliferated cells using the DCL knockdown mouse model.
In hypothalamic tanycytes, DCL knockdown affects D2 activity, but other parameters within the hypothalamic-pituitary-thyroid hormone axis are unaffected. This mild outcome raises the question whether DCL might be involved in other hypothalamic functions as well. The first function to be affected by DCL knockdown may be hypothalamic neurogenesis. Since reduced neurogenesis in the hypothalamus was reported to affect bodyweight, energy expenditure and total activity (Lee et al., 2012), measuring daily activity of DCL-KD animals might provide clues about hypothalamic neurogenesis. Increased daily activity might explain the increase in proliferation. However, intracerebroventricular (icv) infusion of BrdU into the third ventricle is necessary to study hypothalamic neurogenesis properly.

Another interesting hypothalamic site of DCL expression is the suprachiasmatic nucleus (SCN). Within the SCN, DCL is mainly expressed in the area in which also arginine-vasopressin (AVP) is expressed. The SCN is the only source from which AVP is rhythmically released into the extracellular space (Schwartz and Reppert, 1985). In voles, the loss of rhythmic AVP protein expression is correlated to behavioural arrhythmicity in constant darkness. When voles are subject to constant darkness, their behavioural activity is organized by their internal clock. Some of the voles loose this rhythm and become arrhythmic. In these arrhythmic animals, AVP is still expressed, but no longer released resulting in accumulation of AVP protein in SCN neurons (Jansen et al., 2000;Jansen et al., 2007). Preliminary data suggest a similar pattern in DCL-KD animals. After DCL knockdown, the number of AVP positive cells seems to be increased. Since DCL is involved in intracellular transport along microtubules, DCL knockdown might affect AVP transport and hamper subsequent release. Whether this has an effect on normal daily rhythms remains to be determined although it is more likely that DCL knockdown affects adaptation towards transitions in novel environmental cues or “Zeitgebers” that mimic seasonal changes in day length. Also, the hypothalamic tanycytes are involved in seasonal changes (Bratincsak et al., 2007;Hazlerigg and Loudon, 2008) which makes our DCL-KD animals an interesting model to study hypothalamic function in daily

and seasonal regulation of behaviour. However, several parameters need to be studied in DCL-KD animals first, like the circadian rhythm of AVP protein expression, constant registration of daily activity and adaptation towards novel light/dark regimen.

Finally, the DCL-KD model is suitable to study ageing and cognitive impairment. There is a steep decline in the number of new-born neurons during the lifespan, which coincides with a decline in cognitive, sensory and motor functions (Andrews-Hanna et al., 2007;Hof and Morrison, 2004). Although there are still outstanding questions about the role of adult neurogenesis in olfaction and contextual recognition (Lazarini and Lledo, 2010), the olfactory tubercle is subject to changes over time. The DCL expressing Islands of Calleja (ICj) are part of the olfactory tubercle. During lifetime, the number of granule cells inhabiting the ICj decreases and the volume and location of the ICj change (Adjei et al., 2013). The function of DCL in the neuropil and neurogenesis in both OB and ICj remains to be determined. One of the intriguing questions is: does DCL knockdown affect ICj plasticity over time and thereby olfactory function? Altogether, DCL-KD mice may be used for a number of studies related to brain plasticity in the field of cognition, stress, energy metabolism and olfaction.

## 9 General conclusions

The results in this thesis showed for the first time DCL-specific expression in the adult mouse brain. Besides the expected regions with the capacity to generate new neurons (hippocampus and olfactory forebrain), DCL expression was found in three novel brain areas namely hypothalamic tanycytes, suprachiasmatic nucleus (SCN) and Islands of Calleja (ICj). A state of the art conditional shRNA expressing mouse model was used to target DCL mRNA. The analysis of these DCL knockdown animals using qPCR and Western blot revealed strong reduction of DCL protein expression. Subsequent stereological analysis using BrdU and several stem cell and neuronal markers revealed increased progenitor proliferation, but impaired neurogenesis in the hippocampus. This impaired neurogenesis was associated, however, with an apparent normal spatial and contextual fear memory formation in circular hole board and in a contextual fear conditioning paradigm. Therefore, DCL-regulated adult neurogenesis seems not crucial for hippocampus-dependent learning. However, more subtle functions like pattern separation and context distinction might be regulated by DCL. DCL knockdown also increased D2 activity within the hypothalamus. Further studies are needed to reveal the role of DCL in hypothalamus function. Altogether, the DCL-KD mouse seems a good working model to study adult neurogenesis and the role of DCL in this process. Furthermore, this model offers novel opportunities to study several hypothalamic processes like energy metabolism and the circadian rhythm.