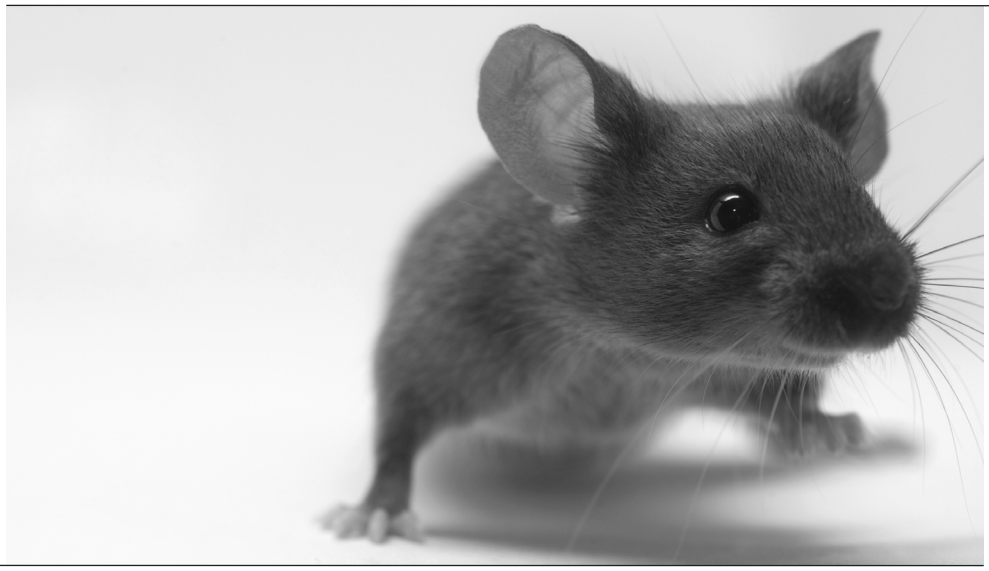
Chapter 4

Blockade of adult neurogenesis by Doublecortin-like knockdown does not affect contextual fear memory formation



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Abstract

Doublecortin-like (DCL) is a microtubule-associated protein that is highly homologous to doublecortin and is crucially involved in embryonic neurogenesis. Previously, we have shown that DCL plays also an important role in adult neurogenesis. As adult neurogenesis has been implicated in anxiety, we have investigated the role of DCL in contextual fear memory formation using transgenic DCL knockdown (KD) mice producing inducible shRNA molecules that specifically target DCL. DCL-KD mice were tested in a contextual fear conditioning (CFC) paradigm. We found that DCL-KD and associated impaired neurogenesis does not abolish hippocampus-dependent contextual fear memory. However, DCL-KD animals show a significant stronger freezing response to the first cue where after they behave like wildtype littermates. In addition, DCL-KD mice exhibit significant reduced tail rattling behaviour during fear. Therefore, DCL-KD animals may form a valuable model to address the role of neurogenesis in the processing of fearful information by measuring more subtle aspects of context discrimination and pattern separation.

Introduction

Doublecortin-like (DCL), a splice-variant of the doublecortin-like kinase (DCLK) gene, encodes a neurogenesis-related microtubule associated protein (MAP) that shares a high amino acid sequence identity with doublecortin (DCX) over its entire length (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). During embryonic development, DCL is widely expressed in mitotic radial glial cells (RGs) and in radial processes. DCL functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). In addition, DCL knockdown by RNA-interference technology leads to significantly reduced cell numbers in the inner proliferative zones and dramatically disrupted mostly radial processes (Vreugdenhil et al., 2007). In the adult brain, DCL is within the DG expressed in the neurogenic niche (Saaltink et al., 2012) and thought to be involved in suppression of spine maturation (Shin et al., 2013). Furthermore, DCL regulates fast retrograde transport of the glucocorticoid receptor (GR), a crucial mediator of the stress response, in neuronal progenitor cells (NPC's) (Fitzsimons et al., 2008). In the adult hippocampus, DCL knockdown resulted in increased proliferation of NPC's but in reduced numbers of post-mitotic NPC's and neuroblasts suggesting a key role for DCL in migration and maturation (chapter 3).

Neurogenesis occurring at adulthood in the subventricular zone (SVZ) of the ventricle walls and the hippocampal dentate gyrus (DG) (Kempermann, 2012;Ming and Song, 2011) is affected by a wide variety of conditions. For instance, neurogenesis is increased by exercise and environmental enrichment (Brown et al., 2003a;Farmer et al., 2004;Kempermann et al., 1997b;Kempermann et al., 1998a;Rhodes et al., 2003;van Praag et al., 1999b;van Praag et al., 1999a) whereas it can be inhibited by severe and chronic stress (Lucassen et al., 2010a;Schoenfeld and Gould, 2012). Since adult neurogenesis takes place in the hippocampus, it is thought that adult neurogenesis function is related to hippocampus dependent memory formation, like spatial and contextual memory. An often-used paradigm to test hippocampal function is contextual fear conditioning (CFC). Fear memory can be acquired during a training program using a cue (light and tone) as conditioned stimulus (CS) and a mild electrical shock as unconditioned stimulus (US). By presenting the CS to animals in the shock compartment (context), fear memory can be tested, which then is subject to extinction several days later. Fear for cue and context is processed in different brain areas. Context-related fear memory depends on the hippocampus and cue-related fear memory is amygdala dependent (Phillips and Ledoux, 1992). CFC appears susceptible to altered neurogenesis (Fitzsimons et al., 2013;Imayoshi et al., 2008;Latchney et al., 2013;Pan et al., 2012;Saxe et al., 2006;Tronel et al., 2012).

In chapter 3 we showed that inhibition of neurogenesis by DCL knockdown did not result in impaired hippocampus dependent spatial memory formation in a circular hole board paradigm. To validate this finding and to distinguish hippocampus dependent context memory formation from amygdala dependent fear memory formation, we tested our DCL-KD animals in the CFC paradigm. Although we observed subtle effects in mice with DCL knockdown-mediated neurogenesis inhibition, contextual fear memory formation is not affected.

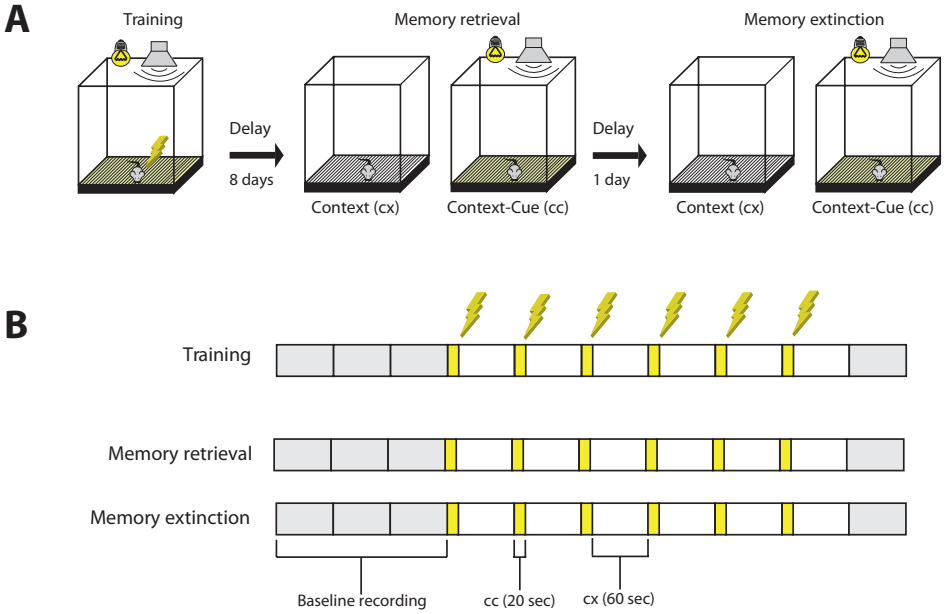

Figure 1: Contextual fear conditioning paradigm. A: Schematic overview of the experiment. During the training, an animal is put in a box with a metal grid. The animal is presented to a cue (tone (70dB) and light (260 lux)) functioning as conditioned stimulus (CS) which is followed by an unconditioned stimulus (US) represented by a shock (0.4 mA) via the grid. After 8 days, the animal is put in the same box to measure memory retrieval. During a context/cue (cc) period only the CS is presented for 20 seconds. 6 of these periods are interspersed with 1 minute of context (cx) without CS. 1 day after memory retrieval, the memory extinction is programmed in the same way. B: Program of the contextual fear conditioning. During the training, 3 minutes of baseline recording (grey) are followed up by a 20 seconds of cc (yellow) with CS together with a mild shock (lightening). The cc is followed by a cx (white) of 1 minute. Such a block is rehearsed 5 times and followed by a single post period of 1 minute (grey). Memory retrieval and extinction are programmed in the same way, however, without an electrical shock (US).

Methods

Animals

Three-month-old DCL KD transgenic (n=15) (Chapter 3) and wildtype (n=17) male mice were obtained from our outbred colony (derived from TaconicArtemis, Cologne, Germany). The animals were kept under a 12:12 light-dark cycle (lights on from 7:00 to 19:00 hours), in a temperature-controlled room (23°C). shRNA targeting DCL, was induced by doxycycline (dox) via dox containing food pellets (Dox Diet Sterile S3888, 200mg/kg, BioServ, New Jersey, USA). Water and food were available ad libitum. After 8 weeks of dox induction the animals were subject to contextual fear conditioning.

Apparatus

The fear conditioning paradigm was performed as described previously (Brinks et al., 2009). In short, a blinded Plexiglas chamber measuring 25x 25x 35 cm high was used as fear conditioning box. The floor consisted of a stainless steel grid connected to a shock generator (0.4 mA). In the wall a speaker was attached at 25 cm height and connected to a tone generator (70dB). A white light source (260 lux) and a camera were placed on top of the chamber (see also Fig. 1).

Procedure

Animals were placed in the chamber for 3 minutes baseline recording followed by 6 light/tone + shock pairings with 60 seconds interval. Light and tone were paired for 20 seconds. An electric shock was applied during the last 2 seconds of the light/tone pairing. 120 seconds after the last shock the animals returned to their home cage. Subsequently, to test memory (day 8) and memory extinction (day 9), this paradigm was repeated without the shock (see also Fig. 1).

Behavioural assessment

Video recorded behaviour was analyzed by an experimenter unaware of the genotype. Freezing behaviour was defined as immobility of the body including the head without any interaction with the environment. Also the number of tail rattles was scored. Behavioural scoring and analysis is performed with ObserverXT version 9.0 (Noldus BV, Wageningen, The Netherlands).

Statistics

Results are expressed as mean ±S.E.M. and unless stated otherwise a Student's t-test was performed using Prism 4.00 (GraphPad Software Inc., San Diego, CA). Behavioural data is tested with a General Linear Model (GLM) with repeated measurements. Tail rattling data is tested with an Univariate Analysis of Variance (UAV). Both tests are performed in SPSS statistical software version 20 (IBM, SPSS Inc. Chicago,IL).

Results

The fear conditioning paradigm consists of three tests. During training, the animals develop a fear for the electrical shock (US), which is associated with the presented cue (CS) and context. Fear memory is measured 8 days later during the memory retrieval test. Due to the lack of the painful US, this fear memory is partly extinguished. Fear memory extinction is measured during the third test at day 9. Analysis of all test series did not reveal significant effects of DCL knockdown on memory formation ($F(1)=0.047$, $p=0.830$), retrieval ($F(1)=0.276$, $p=0.603$) and extinction ($F(1)=1.026$, $p=0.319$). All DCL-KD animals performed similarly as wildtype littermates.

Fear memory training

During the initial training at day 1, the animals developed equal amounts of fear behaviour during both cue and context condition (Fig. 2A). Before each test series baseline behaviour was recorded for 3 minutes. Before training, animals were naïve and showed very low levels of fear behaviour (Fig. 2A). DCL-KD animals were not more or less naïve compared to wildtype littermates ($F(1)=5.770$, $p=0.242$). A GLM for repeated measurements showed a significant increase of freezing behaviour over time during cue and context alternations ($F(3.45)=34.784$, $p<0.001$). DCL knockdown did not affect the learning curve ($F(1)=0.047$, $p=0.830$). However, DCL-KD animals showed a more jagged learning curve with slightly more freezing behaviour in the cue-settings, compared to the subsequent context-setting (see Fig. 2A; ($F(1)=6.221$, $p=0.018$).

Fear memory retrieval

Eight days after training, fear memory was tested during the memory retrieval test (Fig. 2B). Over time animals did not freeze more or less ($F(5)=1.457$, $p=0.208$) but in the cue-setting animals showed more freezing behaviour compared to the context-setting ($F(1)=44.503$, $p<0.001$). DCL knockdown does not affect memory retrieval. However, the first cue elicited a much stronger freezing response in DCL-KD animals compared to wildtype littermates (Fig.

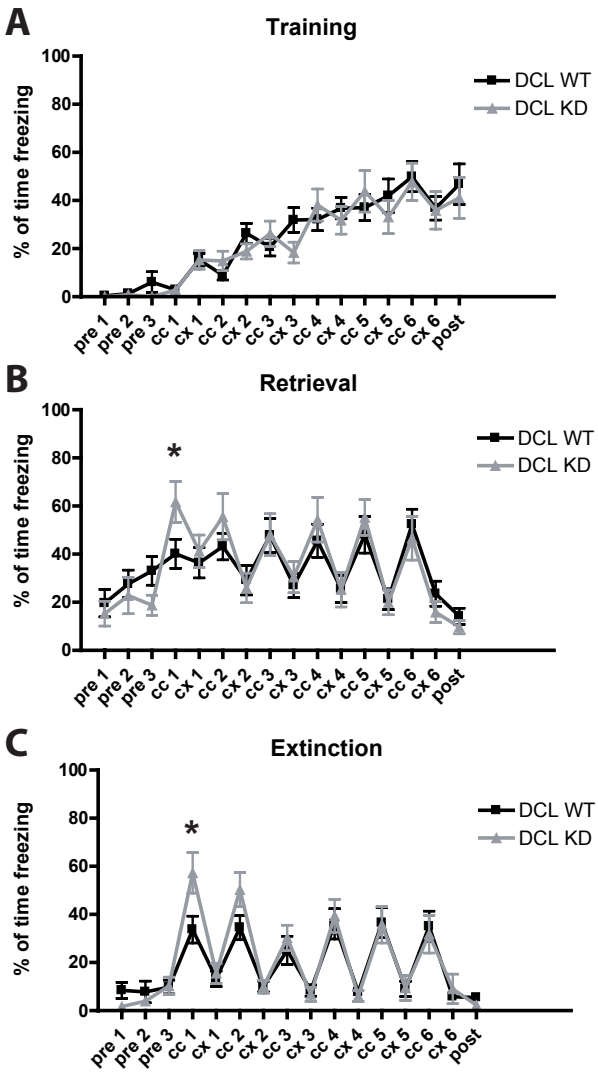

Figure 2: Fear behaviour during contextual fear conditioning. A: Percent of time the animals showed freezing behaviour during the training. DCL knockdown did not affect conditioning to the stimulus (F(1)=0.047, p=0.830). B: Percent of time the animals showed freezing behaviour during memory retrieval. DCL knockdown did not affect fear memory and retrieval (F(1)=0.276, p=0.603) except during the first cue (cc1, marked with an *, t(30)=-2.107, p=0.044). C: Percent of time the animals showed freezing behaviour during memory extinction. DCL knockdown did not affect fear memory extinction (F(1)=1.026, p=0.319) except during the first cue (cc1, marked with an *, t(30)=-2.413, p=0.022). CX: context CC: context-cue.

2B, cc1; t(30)=-2.107, p=0.044). Prior to the first cue, all animals showed an increase in freezing behaviour over the period of 3 minutes (F(2)=5.049, p=0.009), DCL knockdown had no effect on fear behaviour in this period (Fig. 2B; (F(1)=2.413, p=0.132).

Fear memory extinction

At day 9, fear memory was tested again (Fig. 2C). However, due to the previous test without an US, fear memory was slightly extinguished. All animals showed a decrease in freezing

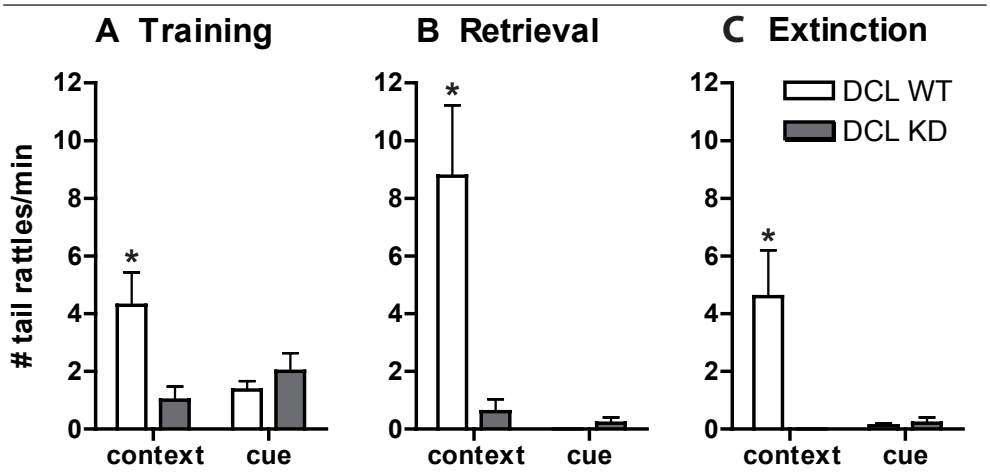

Figure 3: Total number of tail rattles per minute during contextual fear conditioning. In all tests, only in the context situation wildtype animals showed more tail rattling compared to DCL-KD littermates ($p<0.01$). A: During training, an effect for DCL knockdown was found ($F(1)=12.818$, $p=0.001$). B: During memory retrieval, the wildtype animals showed significant more tail rattling during context situation, compared to cue situation ($F(1)=11.024$, $p=0.002$). C: Like memory retrieval, the wildtype animals showed significant more tail rattling during context situation, compared to cue situation in the extinction test ($F(1)=6.439$, $p=0.014$).

behaviour over time in the alternating cue-context settings ($F(2.931)=4.089$, $p=0.010$). Like memory retrieval, animals showed less fear behaviour in the context-setting compared to the cue-setting ($F(1)=106.342$, $p<0.001$). DCL knockdown did not affect memory extinction except for the first cue (cc1). DCL-KD animals showed a much stronger freezing response to the first cue compared to wildtype littermates (fig. 2C, $t(30)=-2.413$, $p=0.022$). In the period prior to the cue, no strong increase of freezing behaviour over time was seen (fig. 2C, $F(1.581)=2.190$, $p=0.133$).

Tail rattling

Tail rattling represents ambivalent behaviour and is seen during territorial fights between male mice. In a fight, mice show this behaviour when they are at a certain distance from the opponent. It likely represents an internal conflict between approaching or avoiding the opponent (Grant E.C. and Mackintosh J.H., 1963;Scott, 1966). During the fear conditioning paradigm, we observed numerous times tail rattling behaviour (Fig. 3), in particular in the training sessions. 76% of the wildtype animals and 24% of the DCL-KD animals showed at least 1 time this behaviour during the context period (Table 1). When the cue with electrical shock was presented 65% (wildtype) and 41% (DCL-KD) of the animals showed tail rattling behaviour. Tail rattling was significantly reduced after DCL knockdown ($F(1)=12.818$, $p=0.001$). DCL-KD animals did not show different amounts of tail rattling behaviour in cue-

or context-setting (F(1)=0.725, p=0.398). In the memory retrieval test, the amount of tail rattling behaviour was reduced in DCL-KD animals (Fig. 3B). Only wildtype animals showed high amounts of tail rattling behaviour (F(1)=10.041, p=0.002) which was only observed in the context-setting (F(1)=11.024, p=0.002). Similar effects were found during the memory extinction test (Fig. 3C). Wildtype animals showed tail rattling behaviour in the context setting, DCL-KD animals did not (F(1)=7.357, p=0.009). Hardly any tail rattling behaviour was recorded during the presence of the cue resulting in a significant difference between context and cue settings (F(1)=5.330, p=0.024).

Table 1: Percent of animals showing tail rattling behaviour.

	Context		**Cue**	
	DCL WT	DCL KD	DCL WT	DCL KD
Training	76%	24%	65%	41%
Memory retrieval	59%	12%	0%	6%
Memory extinction	59%	0%	0%	6%

Discussion

In this study, we investigated the effect of impaired neurogenesis, induced by DCL knockdown, on behavioural aspects after contextual fear conditioning. As was observed previously in the circular hole board (see chapter 3), both wildtype and DCL knockdown mice exhibited similar learning curves in the CFC. Also, all mice are capable of to acquire, retrieve and extinguish fear memory, in a manner that is not affected by DCL knockdown. However, during training, DCL-KD animals showed a stronger freezing response in the cue-setting compared to the context-setting. Wildtype littermates showed a smooth learning curve whereas in the DCL-KD animals learning became manifest in a see-saw pattern. Remarkably, in response to the first cue in both the memory retrieval and extinction phase DCL-KD mice demonstrated a strong freezing response, which was significantly different from that of wildtype mice. Furthermore, in a context setting, DCL-KD animals showed significant less tail rattling behaviour in the memory retrieval and extinction phase as compared to wildtype animals.

Recently, we showed that knockdown of DCL resulted in a strong reduction of newborn neurons, that concerned in particular post-mitotic type 3 DCX positive NPCs (see Chapter 3). Newborn neurons are known to be preferentially activated during learning tasks (Arruda-Carvalho et al., 2011;Gu et al., 2012;Kee et al., 2007;Arruda-Carvalho et al., 2011;Gu et al., 2012). However, we cannot exclude the possibility that not all NPCs are blocked by DCL knockdown and that CFC-induced learning is mediated by newborn type 3 cells that are not affected by DCL knockdown (see chapter 3, Fig. 3G). Also, newborn neurons are not exclusively activated during learning (Kee et al., 2007), which opens up the possibility that the

older and mature granule cells in the dentate gyrus did participate in CFC-induced learning in DCL-KD mice.

Since our results suggest that DCL-positive neurons do not seem to be implicated in processing of contextual fear memory, an alternative explanation might be that our CFC test was inappropriate to elucidate the biological relevance of adult hippocampal neurogenesis. Indeed, the role of adult neurogenesis in contextual fear conditioning is debated as studies so far showed contradicting results. Several studies showed reduced contextual fear memory after impaired or aberrant adult neurogenesis (Fitzsimons et al., 2013;Imayoshi et al., 2008;Latchney et al., 2013;Pan et al., 2012;Saxe et al., 2006) whereas other studies including the current one did not find such an effect (Clark et al., 2008;Drew et al., 2010;Dupret et al., 2008;Jaholkowski et al., 2009;Jedynak et al., 2012;Shors et al., 2002;Tronel et al., 2012;Zhang et al., 2008).

There are, however, small differences in the experimental design of the CFC task in the above mentioned studies that may explain the contradictory findings. While most studies used 3 tone/shock pairings during the training, the context in which the cue was presented during the memory retrieval phases appeared either familiar -as we did- or novel. However, after inspection of the individual studies it appeared that fear memory processes proceeded irrespective the nature of the context.

Another explanation for the lack of effect of DCL-induced impaired neurogenesis on CFC-induced learning might be the number cue-context pairings in the CFC paradigm used in our study. Recently, Drew and colleagues compared several fear conditioning paradigms in combination with UV-irradiation decreased adult neurogenesis (Drew et al., 2010). Like DCL knockdown, irradiation did not affect freezing behaviour after a series of tone-shock pairings. However, they found an effect when they delivered a single shock without an additional cue. In this experiment the animals received a shock after 3 minutes in the conditioning chamber. One day later, the animals were exposed again to the same context. Under these specific conditions the animals with reduced neurogenesis showed less freezing behaviour suggesting subtle involvement of newborn neurons in context recognition. Also, Tronel and colleagues (Tronel et al., 2012) concluded that adult born neurons are not required for acquisition of contextual fear memory. Instead, they found impaired adult neurogenesis to be deleterious for the ability to discriminate between changes introduced into context during extensive training. Therefore, further testing DCL-KD mice in behavioural paradigms addressing contextual discrimination, may shed light on the role of DCL in context recognition.

Next to changes in the test paradigms, also the experimental strategies to block adult neurogenesis differ in the studies mentioned above. These strategies range from destruction

of the dentate gyrus by UV radiation (Clark et al., 2008;Drew et al., 2010;Saxe et al., 2006) to the knockout of specific cell proliferation-related genes (Dupret et al., 2008;Imayoshi et al., 2008;Jaholkowski et al., 2009;Jedynak et al., 2012;Latchney et al., 2013;Tronel et al., 2012;Zhang et al., 2008). In our study, we used inducible RNA-interference technology to specifically knockdown DCL which is an approach that may be comparable with TLX (Zhang et al, 2008), BAX (Dupret et al, 2008) and D2 knockout mice (Jaholkowski et al., 2009;Jedynak et al., 2012). Similar as observed in our study, CFC-induced learning is not changed in these knockout mice with compromised adult neurogenesis.

We found a significant increased in freezing behaviour in the first cue and a striking and significant decrease in tail rattling after DCL knockdown in the retrieval and extinction phase. As these type of behaviours are anxiety-related, impaired maturation of type-3 progenitor cells induced by DCL knockdown might be involved in the neuronal networks regulating fear-related processes. Interestingly, adult hippocampal neurogenesis has been implicated in subtle context discrimination or pattern separation (Sahay et al, 2011; Clelland et al, 2009), a process, which is believed to underlie anxiety disorders such as panic disorders and the post-traumatic stress disorders (Kheirbek et al., 2013).

In conclusion

DCL-induced impaired neurogenesis does not abolish hippocampus-dependent contextual fear memory. DCL-KD animals show a stronger response to the first cue where after they behave like wildtype littermates except for tail rattling behaviour during fear. These results are highly significant. 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.

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