New hippocampal neurons are not obligatory for memory formation; cyclin D2 knockout mice with no adult brain neurogenesis show learning

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The role of adult brain neurogenesis (generating new neurons) in learning and memory appears to be quite firmly established in spite of some criticism and lack of understanding of what the new neurons serve the brain for. Also, the few experiments showing that blocking adult neurogenesis causes learning deficits used irradiation and various drugs known for their side effects and the results obtained vary greatly. We used a novel approach, cyclin D2 knockout mice (D2 KO mice), specifically lacking adult brain neurogenesis to verify its importance in learning and memory. D2 KO mice and their wild-type siblings were tested in several behavioral paradigms, including those in which the role of adult neurogenesis has been postulated. D2 KO mice showed no impairment in sensorimotor tests, with only sensory impairment in an olfaction-dependent task. However, D2 KO mice showed proper procedural learning as well as learning in context (including remote memory), cue, and trace fear conditioning. Morris water maze, novel object recognition test, and in a multifunctional behavioral system—IntelliCages. D2 KO mice also demonstrated correct reversal learning. Our results suggest that adult brain neurogenesis is not obligatory in learning, including the kinds of learning where the role of adult neurogenesis has previously been strongly suggested.

New neurons are produced in the brains of adult animals, including humans, throughout their lifespan (Altman 1963; Eriksson et al. 1998). Since one of the sites of adult brain neurogenesis is hippocampal formation, a brain structure involved in learning and memory, new neurons were expected to be involved in these phenomena. Following years of research, adult brain neurogenesis, along with synaptic strengthening, synaptic elimination/weakening, and synaptogenesis are now regarded as four major types of plasticity required for formation and retention of memories (Bruel-Jungerman et al. 2007). However, the very evidence for the role of adult hippocampal neurogenesis in learning and memory remains limited, and in most cases, indirect (Leuner et al. 2006). Most of these studies show either a correlation between the number of new neurons and learning abilities or the influence of learning on the number of new neurons. Only a few experiments demonstrate the effects of new neuron depletion on learning and memory (Shors et al. 2001, 2002; Madsen et al. 2003; Rola et al. 2004; Bruel-Jungerman et al. 2005; Snyder et al. 2005; Saxe et al. 2006; Winocur et al. 2006; Zhang et al. 2008; Jessberger et al. 2009; Table 1). The reason for this is a lack of proper methods to selectively reduce adult brain neurogenesis without affecting other aspects of brain function. For example, irradiation (Wojtowicz 2006) and antimitotic drugs (Dupret et al. 2005) can induce nonspecific effects on performance or brain function, raising the possibility of false-positive results (for review, see Leuner et al. 2006).

We showed before (Kowalczyk et al. 2004) that cyclin D2, a protein involved in cell cycle regulation, is the only cyclin D (out of D1, D2, and D3) expressed in wild-type (WT) hippocampal neuronal progenitors expanded in vitro into neurospheres. Furthermore, careful analysis of the brains of cyclin D2 knockout mice (D2 KO mice) failed to reveal BrdU-positive neurons in the dentate gyrus of the hippocampal formation. We were also unsuccessful in increasing the number of newly generated cells in the hippocampus by a variety of stimulations, including introducing the mice to a novel environment, or even by a local brain injury. Notably, while adult brain neurogenesis of D2 KO mice is missing, their developmental neurogenesis allows for the formation of the brain, with all of the major structures present, though some of them are smaller. Also, neurogenesis outside of the central nervous system seems to be unaffected in these mice (Kowalczyk et al. 2004). All of this allows testing D2 KO mice to determine the importance of adult brain neurogenesis in learning and memory paradigms.

Our data, presented herein, show that D2 KO mice exhibit proper procedural learning as well as learning trace fear conditioning. Morris water maze (MWM), novel object recognition (NOR), and also learning in a multifunctional behavioral system, IntelliCage. D2 KO mice also demonstrate correct reversal learning in MWM and IntelliCage. We conclude that hippocampal adult neurogenesis is, in general, not crucial in learning.

Results

D2 KO mice show reduced adult brain neurogenesis

To determine that our mice had reduced hippocampal neurogenesis, we examined BrdU+ (marker of dividing cells) and doublecortin+...
With WT controls (1D) and 90.4% (0.001; Fig. 1B) and 94.9% reduction of BrdU KO mice, these reductions were 78.4% (normalized to the volume of the granule cell layer (smaller in D2

Before examining the possible effects of the lack of adult brain neurogenesis on learning of D2 KO mice, we analyzed the overall behaviors of these animals in sensorimotor tests, open field, locomotion of 6–8-mo-old WT (n = 6) and D2 KO (n = 9) mice (Fig. 1). DCX is an intermediate filament protein expressed in young, postmitotic, <1-mo-old neurons (Couillard-Despres et al. 2005). Cyclin D2 deletion resulted in 87.9% reduction of BrdU+ cells ($F_{1,13} = 62.33; P < 0.001$; Fig. 1B) and 94.9% reduction of BrdU+ DCX+ cells compared with WT controls ($F_{1,13} = 64.33; P < 0.001$; Fig. 1C). When normalized to the volume of the granule cell layer (smaller in D2 KO mice), these reductions were 78.4% ($F_{1,13} = 89.71; P < 0.001$; Fig. 1D) and 90.4% ($F_{1,13} = 76.14; P < 0.001$; Fig. 1E), respectively.

**Table 1.** Experiments cited in the text involving ablation of adult neurogenesis and its effects on learning and memory

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| (HML/NI) High memory load/no internal interference; (LML/HL) low memory load/high interference; (LML/LI) low memory load; limited interference; (MWM) Morris water maze; (NMTS) nonmatching to sample; (NOR) novel object recognition; (STFP) social transmission of food preference.

(DCX+) immunoreactive cells in the dorsal hippocampal dentate gyrus of 2–3-mo-old WT (n = 6) and D2 KO (n = 9) mice (Fig. 1). Determining whether the intensity of neurogenesis alters sensorimotor skills. Also, we assumed that this information would help to interpret the expected detrimental effects of the impaired adult brain neurogenesis on cognitive tasks. That is, if the lack of neurogenesis did not alter sensorimotor skills, then any potential changes in performance of the cognitive tasks would more likely be due to altered memory and not due to nonmnemonic aspects of behavior. The tasks used assessed coordinated behaviors, orienting reactions, forelimb weakness, postural abnormalities, climbing, and locomotion of 6–8-mo-old WT (n = 7) and D2 KO (n = 7) animals (Markowska et al. 1994). Each test was given once a day for three consecutive days and performance of both investigated groups was compared each day. Also, the comparison of the scores between days was assessed as a measure of procedural learning. Toward this end, we used two-way ANOVA with independent measure (genotype) and dependent measure (day) for each test. We found no significant differences between mutants and WT mice (no effect of genotype, $P > 0.05$). When investigating WT and
KO mice separately, there was an effect of day in most of the tasks, e.g., for KO mice in the case of walking initiation, turning in an alley and over all bridges. D2 KO mice showed improvement in performance between day 1 and day 3 in all of these tasks. These differences indicate procedural learning during the course of these tests in both WT and KO animals (Fig. 2). In the case of turning on an inclined screen and wire suspension, there was no effect of day in either of the groups. Taken together, the mice lacking adult brain neurogenesis were not impaired in the performance of the sensorimotor tasks and showed normal procedural learning.

D2 KO mice show normal anxiety levels in open field and elevated plus maze

In order to determine whether basic emotional responses and anxiety-related behaviors were affected in D2 KO mice, they were subjected to open field and elevated plus maze. In both tests, we observed no differences in behavior between D2 KO mice (n = 10) and WT littermate controls (n = 11), all 4-6-mo old, using two-way ANOVA with independent measure (genotype) and dependent measure (parameter, see Materials and Methods). In open field (Fig. 3A), testing exploratory behavior and emotional responses to a novel environment, there was no effect of genotype as far as: total distance moved (P = 0.78), maximum distance moved without stopping (P = 0.73), frequency in three zones (P = 0.39), time in thigmotaxic zone vs. other zones combined (P = 1.00), and latency to the first visit to the middle zone (P = 0.30). There was an effect of the number of visits in a zone (F(2,38) = 188.09; P < 0.001); this effect was present for both WT and KO animals (Friedman analysis, P < 0.001) with the center less frequented than thigmotaxic and middle zones in both genotypes (Wilcoxon test, P < 0.01). There was also an effect of time in a zone (F(1,19) = 186.83; P < 0.001), with longer times spent in the thigmotaxic zone by both WT (F(1,20) = 265.02; P < 0.001) and KO mice (F(1,18) = 143.74; P < 0.001). In elevated plus maze (Fig. 3B), where a longer time spent in the closed arms is regarded as indicating anxiety-like behavior, we found no effect of genotype in the total time spent in each zone (P = 0.36) with an effect of zone (F(2,38) = 82.38; P < 0.001) only. The effect of zone was there for both WT (F(2,20) = 46.64; P < 0.001) and KO animals (F(2,18) = 35.63; P < 0.001) with both groups spending more time in the closed arms (Duncan, P < 0.001). There was also no effect of genotype in the frequency of passing through each zone (P = 0.48), only with an effect of zone again (F(2,38) = 56.89; P < 0.001); the effect of zone was there for WT (F(2,20) = 26.01; P < 0.001) and KO mice (F(2,18) = 28.58; P < 0.001), with animals from both groups frequenting the closed arms more often (P < 0.001, Duncan). Similarly, we found no difference between both groups in the latency of the first occurrence in each zone (P = 0.07); there was an effect of zone (F(1,19) = 23.17; P < 0.001); animals from both groups entered the closed arms faster (P < 0.01, Wilcoxon). Finally, there was no difference between groups in the total time spent moving (P = 0.93). These results indicate that the emotional state of D2 KO and control mice did not differ in their anxiety-like behaviors under the conditions used.

D2 KO mice display impairment in olfaction-dependent task

Since D2 KO mice have smaller olfactory bulbs, we expected these mice to have impaired olfaction. In order to verify this, and following published protocols (Alberts and Galef 1971), we designed a test based on the drive of mildly food-deprived animals to look for hidden pieces of chocolate buried in the sawdust of a cage. As expected, D2 KO mice demonstrated impairment in this olfaction-dependent task. It took the mutant mice longer to find...
deeply hidden pieces of chocolate (Fig. 4). Using two-way ANOVA with independent measure (genotype) and dependent measure (experiment; deepness), we found an effect of genotype ($F_{1,121} = 11.64; P < 0.01$) and of experiment ($F_{2,24} = 8.71; P < 0.01$), while post-hoc analysis showed differences between D2 KO (n = 7) and WT mice (n = 7), all 6–8-mo old, but only when the chocolate was buried at 10 mm ($P < 0.01$) or at 15 mm ($P < 0.05$), but not when it was just under the surface of the wood shavings ($P = 0.48$).

**D2 KO mice show normal cue, context, context-remote, and improved trace-cue fear conditioning**

In fear conditioning, mice form an association between a neutral, conditioned stimulus (CS, e.g., context or noise cue), and an aversive, fearful, unconditioned stimulus (US, electric footshock), so that later, when CS alone is presented, it elicits the conditioned response, i.e., freezing behavior. Acquisition of the tone–US association requires the amygdala and was shown not to require adult neurogenesis (LeDoux et al. 1990; Anagnostaras et al. 1999; Saxe et al. 2006; Imayoshi et al. 2008; Zhang et al. 2008), while acquisition of a context–US association usually requires both the hippocampal formation and amygdala (Phillips and LeDoux 1992; Frankland et al. 1998; Daumas et al. 2005) and was shown to be influenced by ablation of adult neurogenesis (Saxe et al. 2006). We closely followed the protocol described in this latter publication. D2 KO (n = 19) and WT (n = 22) mice, aged 4–6 mo, received three electric shocks paired with tone. The next day, the mice were tested in a different context with two tone presentations. On the third day, mice freezing was evaluated in the training context for 4 min. Freezing was also measured on the first day in the training context before CS presentation as well as on the second day—before and during tone presentations (Fig. 5A). There was an effect of training when levels of freezing were compared in all tested animals as well as for WT and KO mice separately (Friedman test, $P < 0.001$). There was no difference between WT and KO groups in any of the treatments ($P > 0.05$, Mann-Whitney test). Both groups showed normal cue fear conditioning, since there was a difference between freezing levels before and during the tone presentations, $P < 0.001$ (Wilcoxon tests) for WT, $P < 0.01$ (Wilcoxon tests) for D2 KO mice. Also, both groups showed normal context conditioning—both WT and KO mice froze more in the context where they received shock ($P < 0.001$; Wilcoxon tests). Very similar results were obtained for a second group of animals (WT, n = 27; D2 KO, n = 17, all aged 4–6 mo), where all experiments were performed using only one conditioning chamber (data not shown).

The formation of new contextual fear memories involves the hippocampal formation. These memories are eventually transferred to other brain regions such as the anterior cingulate cortex (Frankland et al. 2004). In order to test whether storage and transfer of contextual fear memory is disrupted in mice lacking adult brain neurogenesis, we followed the protocol used by Frankland et al. (2004) and tested context fear memory in D2 KO mice 36 d after the conditioning (Fig. 5B). When freezing levels before training and after 36 d were compared with ANOVA, we noticed no effect of genotype ($F_{1,20} = 0.46; P = 0.50$) with a strong effect of treatment ($F_{1,20} = 891.94; P < 0.001$). Mice from both groups were freezing more after 36 d ($F_{1,16} = 391.41; P < 0.001$ for WT, $n = 9$; $F_{1,14} = 526.31; P < 0.001$ for D2 KO mice, $n = 13$; all mice aged 3–4 mo).

In trace fear conditioning, a time gap is introduced between CS and US. In such conditions, forming CS–US association requires hippocampal formation (McEchron et al. 1998; Huerta et al. 2000) and was shown to require adult neurogenesis (Shors et al. 2002). To determine the influence of the lack of adult brain neurogenesis in D2 KO mice on learning of context and trace fear conditioning, we used a protocol as close as possible to the one described for rats (Shors et al. 2002). We used D2 KO (n = 10) and WT (n = 11) mice, all 6–7-mo old, which received 10 trace CS–US pairings, with tone as CS. Three days later the level of freezing was evaluated in the same context, in a novel context, and following 10 CS presentations (with no US). Freezing was measured during the CS (15-sec tone), during trace (30 sec after tone), and during former US ($\pm 5$ sec, i.e., 10 sec around the onset of US). D2 KO mice showed context fear conditioning and enhanced trace fear conditioning (Fig. 6A). There was an effect of training when levels of freezing were compared between basal, context, novel context, cue (tone; 10 trials), trace (10 trials), and US ($\pm 5$ sec (10 trials) groups for all of the animals ($P < 0.001$, Friedman test) as well as for WT ($P < 0.001$) and KO mice ($P < 0.001$, Friedman test) separately. Both WT ($P < 0.05$) and KO animals ($P = 0.01$, Wilcoxon test) froze more in the context where they had been conditioned. In a new context, WT and D2 KO animals froze more during and following multiple presentations of CS ($P < 0.01$, Wilcoxon). When freezing in the new context during the first three trials (cf. Shors et al. 2002; Fig. 3) was compared between the mice from WT and KO groups, out of intervals shown (CS 1–3, trace 1–3, $\pm 5$ sec 1–3; Fig. 6A), the only difference was observed for all three times when the shock had occurred ($\pm 5$ sec 1–3) during training. Please note that in all three cases KO animals showed higher freezing at the most “appropriate” time, suggesting that they knew exactly when to “expect” US. Similar results were obtained from a separate group of WT (n = 9) and KO animals (n = 11), all 5–6 mo old. Again, in a new context, KO animals displayed higher levels of freezing at the onset of former US $\pm 5$ sec in all of the first three trials (data not shown). To sum up, not only were D2 KO mice lacking neurogenosis not deficient in trace fear conditioning, but they also showed improved learning. Additionally, we confirmed the deficit of adult brain neurogenesis in the mice trained in trace fear conditioning (the first group). BrdU injections were performed after behavioral analysis was completed; mice were then at 9–10 mo of age. The number of cells detected per slice in the granular...
layer of the dentate gyrus from WT (13.1 ± 3.2) was much higher than in KO (1.0 ± 1.0; 92% reduction); Mann-Whitney test, \( U = 9.0, P < 0.01 \) (Fig. 6B).

Mice were also contextually conditioned using different trace protocols with only one CS–US pairing, based on previously published protocols (Misane et al. 2005). During training, WT \((n = 7)\) and D2 KO \((n = 7)\) mice, 6–8-mo old, after 150 sec of adaptation, were presented with noise (30 sec), followed by trace (30 sec), and a footshock (2 sec, 0.5 mA). During testing, 1 h and 7 d later, mice were placed in a different context where, after 120 sec of adaptation, they were presented with the noise for 120 sec; freezing was recorded. The results (Fig. 6C) show that trace fear conditioning, with one CS–US pairing, is not affected in D2 KO mice. Two-way ANOVA with independent measures (genotype) and dependent measures (treatment) showed no significant differences between mutants and WT mice (no effect of genotype, \( P > 0.05 \)) with a strong effect of the treatment \( (F_{(5,60)} = 49.02; P < 0.001) \). This effect was also strong for WT \( (F_{(5,30)} = 25.02; P < 0.001) \) and D2 KO \( (F_{(5,30)} = 24.25; P < 0.001) \) mice analyzed separately, while post-hoc analysis showed differences between pre-tone and tone freezing at 1 h and 7 d after training in both groups (Fig. 6C).

D2 KO mice show normal learning and memory in Morris water maze (MWM)

We wanted to assess the result of missing adult brain neurogenesis in D2 KO mice on spatial learning and memory of the hidden version of MWM task (Morris 1984), which is hippocampus dependent (Morris et al. 1982; Schenk and Morris 1985). During numerous trials of MWM procedure, mice learn the spatial position of the submerged platform in a circular pool using distant cues of the experimental room. During probe trial testing, the platform is removed and mice are allowed to search around the water maze while the time spent in different quadrants of the pool is measured. The role of adult neurogenesis in learning of MWM remains unresolved (for review, see Leuner et al. 2006), though new neurons of the hippocampal dentate gyrus seem to be preferentially engaged (Kee et al. 2007).

D2 KO mice learned the task. During the training period, both groups of mice learned the position of the platform as shown by a decrease in distances traveled \( (F_{(1,20)} = 25.87; P < 0.001) \) for WT, \( (F_{(1,24)} = 18.97; P < 0.001) \) for KO mice) over days of training (Fig. 7A). At probe trial, both WT \((n = 14)\) and D2 KO \((n = 13)\) mice, all aged 3–4 mo, were spending significantly more than 25% of the
time in the target quadrant (50.0 ± 7.2% for WT, 42.8 ± 5.8% for KO D2, one-tail analysis, α = 0.01 significance level), which was significantly more than in all the other quadrants (P < 0.01 for WT and KO mice, Wilcoxon tests) or in quadrants adjacent to the target one (P < 0.05 for WT and KO mice, Wilcoxon tests; Fig. 7B). The mice showed no difference between groups in the visible platform test and during reversal learning (data not shown).

D2 KO mice show memory in novel object recognition (NOR)

To determine the effect of no adult brain neurogenesis on performance of recognition memory, the mice were subjected to NOR procedure. This task, exploiting animals’ innate preference for novelty is being increasingly utilized. In this task, longer time spent with a novel object is a measure of memory of an old, familiar object. NOR was shown to be impaired by hippocampal damage (Clark et al. 2000; Clark and Martin 2005). New neurons in the dentate gyrus may participate in this task (Jessberger et al. 2009).

The animals from both groups learned the task (Fig. 7C), as repeated measures ANOVA showed an effect of an object (F(1,12) = 13.20; P < 0.01) concerning the total duration of contacts during the first 5 sec of established contacts with the objects. No difference between D2 KO (n = 8) and WT (n = 6), all 4–5 mo old groups was noted, as there was no effect of genotype (P = 0.48). One-way ANOVA showed differences between the time spent with new and old objects, both in WT (F(1,10) = 8.87; P < 0.05) and KO groups (F(1,14) = 20.29; P < 0.001).

D2 KO mice show learning in IntelliCages

Finally, D2 KO mice and WT controls were tested in a newly developed automated learning apparatus, IntelliCage system (Galsworthy et al. 2005; Knapska et al. 2006; www.newbehavior.com), a group-housing cage that is also a recording and testing apparatus, automatically gathering data of an individual mouse’s visits to four corners and eight bottles (two bottles per corner). Each corner can harbor one animal at a time. The access to water bottles can be free (Open Gates) or controlled by motorized doors opening after a nose poke. In the Place Preference test, animals from both groups were trained to associate sweet water with a specific corner within the cage with either or all of the gates open (Place Preference with Open Gates) or with the necessity to perform nose pokes (Place Preference with Nose pokes) to open the gates. Then, some of the animals were trained to avoid certain corners that greeted them with an air puff in a form of aversive learning (Place Avoidance). Therefore, the IntelliCage system allowed us to test spatial, operant, and aversive learning of D2 KO mice.

D2 KO mice showed learning in all tasks tested when both visits and nose pokes were analyzed (Fig. 8). Regarding the number of visits during different types of training regiments, there was an

Figure 5. (A) D2 KO mice showed normal cue and context conditioning memory. Both KO (n = 19) and WT (n = 22) showed little basal freezing in the training context (day 1, box A), where tone was paired with shock, as well as enhanced (P < 0.001, Wilcoxon) freezing in the same context (day 3, box A, 4 min). When moved to a novel context, both WT and mutant animals displayed low freezing levels (day 2, box B, pretone 1, pretone 2), which went up when tone was presented (tone 1, tone 2; [**] P < 0.01, [***] P < 0.01, Wilcoxon). There was no difference between WT and KO groups in any of the treatments. (B) D2 KO mice (n = 13), like WT mice (n = 9), exhibited robust levels of freezing in remote retention test of context conditioning memory. WT and KO mice had higher levels of freezing when introduced to the context (box A) 36 d after training ([***] P < 0.001, ANOVA). The results are presented as means ± SEM.
Effect of training \((F_{1,90} = 33.55; P < 0.001)\), with no effect of genotype \((F_{1,6} = 1.40; P = 0.28)\). Regarding the number of visits during Place Preference with Open Gates, there was an effect of training for all animals and separately for WT \((n = 17)\) and KO \((n = 17)\) \((P < 0.001, Friedman)\), and both WT and KO animals (all 4–6-mo old) learned the task as both groups visited the correct corner more often than before training (Wilcoxon test, \(P < 0.001\)). Similarly, as far as the number of visits during Place Preference with Nose pokes, there was an effect of training for all animals and separately for WT and KO mice \((P < 0.001, Friedman)\). Again, both WT and KO mice learned the task as both groups visited the correct corner more often than before training (Wilcoxon test, \(P < 0.001)\).

**Discussion**

**Evaluation of the D2 KO mouse model**

D2 KO were shown to have no adult brain neurogenesis (Kowalczyk et al. 2004), which was confirmed in this study by a reduced number of neuronal progenitors, and which makes this mouse model a unique example of transgenic approach in limiting neural progenitor numbers (cf., e.g., Garcia et al. 2004; Zhang et al. 2008).

The observed reductions in BrdU+ cells were substantial and greater in the case of BrdU+ DCX+ cells. Also, it was argued before that faintly DCX-stained cells cannot always be taken as evidence for neuronal presence (Nacher et al. 2001; Koketsu et al. 2003).
Presented as means in a theoretical work (Cecchi et al. 2001). The role of adult neurogenesis is robust in the rostral instance where adult neurogenesis supplies the tissue with cells, contributes to neuronal replacement, and is therefore critical for tissue maintenance. Our transgenic approach gave an extensive depletion of new neuronal replacement, and is therefore critical for tissue maintenance.

Importantly, the reduction of neurogenesis in D2 KO mice is maintained throughout aging, as in both 2–3-mo-old and 9–10-mo-old animals the number of BrdU+ cells is reduced by almost 90%. These results confirm our previous observations in which virtually no BrdU+ NeuN+ cells could be detected in D2 KO mice (Kowalczyk et al. 2004). Together, the data suggest that cyclin D2 is essential for maintaining the neural stem-cells pool and the associated neurogenesis in adult brains. Additionally, our mouse model does not involve any interventional approach, and therefore implies no “lesion effect” of ablating new or immature neurons which might account for observed deficits seen after irradiation, drug-treatment, and even genetically induced elimination of new cells (e.g., Imayoshi et al. 2008).

We showed that sensorimotor aspects of performance and procedural learning were not affected in D2 KO mice. Secondly, the mutant mice did not differ from control animals in their emotional state; anxiety-related and exploratory behavior under the conditions used in the open field and elevated plus maze tests. Only in the olfaction-dependent task did the mutant mice display impairments when the food was hidden deeper—it took them significantly longer to find a piece of chocolate in wood shavings. These results are in line with those obtained by Imayoshi et al. (2008), who showed fundamental differences of the role of adult neurogenesis in the hippocampal formation vs. the olfactory bulb, where neurogenesis supplies the tissue with cells, contributes to neuronal replacement, and is therefore critical for tissue maintenance. Our transgenic approach gave an extensive depletion of new cells in the olfactory bulb, which resulted in functional deficits. It is likely that the supply of new cells is required for odor detection rather than discrimination, as Imayoshi et al. (2008) saw no deficits in their model. Along these lines, there are a growing number of instances where adult neurogenesis is robust in the rostral migratory stream/olfactory bulb, but absent in the dentate gyrus of mammals (Amrein et al. 2007; Bartkowska et al. 2008). The role of neurogenesis in the olfactory bulb’s functioning was also proposed in a theoretical work (Cecchi et al. 2001).

**Principal finding**. D2 KO mice in learning and memory paradigms

To test whether the impaired adult brain neurogenesis, seen in cyclin D2-deficient mice, compromises the animals behavior, we used several behavioral paradigms, i.e., context fear conditioning, trace fear conditioning, novel object recognition, the hidden-platform version of the Morris water maze, Place Preference, and Place Avoidance tests in IntelliCages. D2 KO mice showed as much learning as the control mice in all of these protocols. Moreover, mutant mice showed enhanced learning in trace fear conditioning.

**Limitations of the approach employed, future directions**

We realize that our mouse model and, hence, our findings have limitations. The mutant mouse used is a constitutive knockout; therefore, it lacks cyclin D2 throughout the body and through all of development. As a result, the phenotype of the KO mice is not limited to adult brain neurogenesis. The mice have smaller brains overall, with particularly marked reductions in the sizes of neocortex, hippocampus, and cerebellum--temporal compensation during development, which may cause changes in other brain systems that compensate for these deficits, e.g., using extrahippocampal structures to perform tasks that are otherwise hippocampus dependent. We cannot also exclude that some number of neurons do appear in D2 KO mice, and that these neurons fulfill some specific functions of novel neurons. Finally, the tasks used in the study may not include those that new neurons are actually required for.

Along these lines, theoretical publications using computational modeling suggest both new roles and new investigational approaches concerning new neurons. According to some investigators, new neurons are needed for temporal coding of events, i.e., to encode the occurring of the events at the same time vs. temporally separated occurrence of several weeks (Aimone et al. 2006) by a mechanism referred to as “pattern integration,” which, in general, may allow distinct memories to be encoded as more similar to each other (Aimone et al. 2009). Additionally, owing to the addition of new neurons, DG can encode new memories in familiar contexts (familiar dimensions), while treating novel contexts differently (Aimone et al. 2009). Others hypothesize that new neurons are necessary to help the dentate gyrus to avoid the negative side effects of differentiating between new (but composed of known patterns) and novel (requiring extension of known patterns) stimuli when adapting to new environments—a problem described as catastrophic interference (Wiskott et al. 2006). Again, others hypothesize that new neurons, which often form clusters of cells, might be important for binding together elements that occur at different times, but are part of the same context, and that such contextual memory has an impact on mood, which explains the connection between neurogenesis and depression (Becker and Wojtowicz 2007). These theories imply a different way of designing experiments, according to which it would be beneficial to: (1) find an appropriate behavioral task that tests temporal associations in rodents (Aimone et al. 2006), (2) test conflicts of several tasks (with highly similar, sequentially learned
**Available literature suggests neurogenesis not to be critical to many of hippocampal-dependent learning paradigms**

There is growing literature dealing with adult neurogenesis and its role in learning of mice and rats. Clearly, one may expect a species difference in this regard. Therefore, we have compiled results of a number of studies on this topic and presented them in Table 1. From there, one may conclude that the cited literature is contradictory. In particular, there are a number of studies often showing one subtle deficit in one of the behavioral tasks used and several “no deficits” in other tasks (e.g., Shors et al. 2002; Zhang et al. 2008). The latter work showed only a minor effect on between-platform latencies during water maze acquisition (cf. Fig. 4G in Zhang et al. 2008). Notably, this effect was apparently not observed using a different group (Supplemental Fig. S1A from Zhang et al. [2008]). To date, the field failed to name a single adult brain-neurogenesis-dependent task, e.g., some researchers claim to demonstrate that MWM is adult neurogenesis dependent, and show that context fear conditioning is not (Zhang et al. 2008); others prove, vice-versa, that context fear conditioning depends on the presence of new neurons, whereas MWM does not (Saxe et al. 2006); while others again fail to show the significance of new neurons in any of the two models reporting other forms of fear conditioning to be neurogenesis dependent (Shors et al. 2002). The differences reported are subtle, while at the same time the ill side effects of the treatments used (irradiation, cytostatic drugs, tamoxifen) are well known (Hayashi and McMahon 2002; Dupret et al. 2005; Wojtowicz 2006) or might not yet be fully known. Moreover, these manipulations do result in either removing a subset of dentate gyrus cells or in affecting their connectivity. Finally, with a large number of laboratories and scientists performing numerous behavioral tests using mice with affected neurogenesis, there is a possibility that the type I statistical error could also play a role in mistakenly reporting some significant differences. Therefore, the only sound conclusion from up-to-date literature could be that either adult brain neurogenesis is not necessary for memory formation, or its role appears to be marginal.

Our results argue against the crucial role of adult brain neurogenesis in learning and memory. These results go along with the observations of others questioning the significance of adult neurogenesis in learning. For example, Meshi and colleagues (2006) show that the newborn granule cells do not mediate the behavioral effects of environmental enrichment, including improved spatial learning. These authors exposed mice to focal X-irradiation and housed them in an enriched environment and then tested their anxiety-like behaviors and spatial learning. Housing of adult mice in an enriched environment improved their spatial learning in the Morris water maze during both acquisition and probe trial. Local hippocampal irradiation blocked adult hippocampal neurogenesis but did not attenuate any behavioral effects mediated by the enriched environment. The investigators conclude that the effects of enrichment on spatial learning, habituation to an unfamiliar environment, and conflict-based anxiety do not require adult hippocampal neurogenesis in their experimental conditions (Meshi et al. 2006).

Reducing adult neurogenesis was also reported to, paradoxically, improve memory (Saxe et al. 2007). Ablating adult neurogenesis, using two independent methods—a focal hippocampal irradiation and a genetic elimination of neural progenitor cells—caused an improvement of hippocampal-dependent working memory when repetitive information was presented in a single day using trials with a long temporal delay (30 sec). It did not escape our notice that in our cue-trace fear conditioning, where D2 KO mice showed better memory than WT controls, the delay (trace) of 30 sec was also used.

**Conclusion**

In conclusion, we have found that D2 KO mice, lacking adult brain neurogenesis, learn several behavioral tasks surprisingly well. It appears that adult brain neurogenesis is not indispensable for some kinds of learning, notably those formerly suggested to be adult neurogenesis dependent. It is possible that new neurons are preferentially used in learning (Kee et al. 2007) but can be successfully replaced by older neurons. Some studies, like the one...
by Meshi et al. (2006), report results against the role of adult brain neurogenesis in learning. We further confirm these observations using mice lacking adult-generated granule cells.

Materials and Methods

Mice
Cyclin D2 mutant mice were generated before (Sicinski et al. 1996) and kept under C57BL/6 background. They were crossed once with Balb/c mice, and the lines were kept as cyclin D2 heterozygotes (+/−). Their homozygous progeny: −/− (KO) and +/+/WT littermates were used in all experiments. The animals were kept under a natural light/dark cycle in Plexiglas cages with water and food provided ad libitum, usually single caged at least 1 wk before the experiment. To minimize animal suffering, the rules established by the First Warsaw Ethical Committee on Animal Research and based on the Animal Protection Act of the Polish Republic were strictly followed. The age of animals was carefully matched between WT and D2 KO mice within each group and is always indicated in the Results section. The groups were n = 6 or larger (always indicated); the number of males and females was always balanced between WT and KO groups. Experimenters were always unaware of the genotype of the mice. BrdU administration: Dividing cells were labeled by intraperitoneal injection of BrdU (50 mg/kg body weight, Sigma, prepared in PBS at pH 7.4) for: (1) two consecutive days, twice daily, 2 h apart, and killed 3 d after the last injection (for morphometric analysis of BrdU+DCX+ cells; Fig. 1), or (2) for 5 d, once daily, and killed 2 h after the last injection (for BrdU+cells counting after trace fear conditioning; Fig. 6B).

Immunohistochemical analysis
BrdU detection was performed as described elsewhere (Kowalczyk et al. 2004) with modifications. Mice were perfused with ice-cold saline, followed by 4% paraformaldehyde, 0.8% picric acid in PBS, pH 7.4. The brains were removed and stored in the same fixative at 4°C overnight and then in 30% sucrose at 4°C. Brains were then frozen and 50-μm thick hippocampal formation cryostat sections were cut at −20°C. The slices were washed in PBS and incubated with 2 M HCl for 30 min at 37°C. Then, pH was neutralized by incubation with 0.1 M boric acid (pH 8.5) for 10 min at room temperature. Next, slices were again washed in PBS and PBS-TX (0.1% Triton X-100, Sigma-Aldrich) and blocked for 1 h in blocking solution (10% normal donkey serum from Vector Laboratories, PBS-TX). Then, sections were incubated overnight with rat anti-BrdU primary antibody (1:200, Accurate Chemical and Scientific Corp.) in PBS-TX (1%) and washed in PBS-TX. Next, samples were incubated with anti-rat Alexa Fluor 488 secondary antibody (1:200, Santa Cruz Biotechnology) for 1 h and washed in PBS-TX. For double-labeling, sections were blocked for 1 h with 10% rabbit donkey serum in PBS-TX and incubated overnight with goat anti-doublecortin primary antibody (1:200, Santa Cruz Biotechnology). Then, sections were washed in PBS-TX and incubated with anti-goat Alexa Fluor 555 secondary antibody (1:200, Santa Cruz Biotechnology) for 2 h and washed in PBS-TX. For nuclear staining, TO-PRO-1 (Molecular Probes/Invitrogen) was added to the last wash. Sections were mounted on slides coated with poly-L-lysine, air-dried, and embedded in Vectashield mounting medium (Vector Laboratories). For morphometric analysis, the numbers of BrdU+ and/or DCX+ cells within the dentate gyrus were estimated following disector principle (Gundersen 1986). The measurements were performed in every sixth section, selected by systematic-random sampling out of the complete set of coronal serial sections of each brain. The labeled cells were counted by optical confocal dissectors (30-μm thick) according to Bergman and Ulfhake (1998). The number of BrdU+ nuclei after trace fear conditioning in the granular zone of the hippocampal dentate gyrus was determined using fluorescent microscope.

Sensorimotor tests
Sensorimotor tests were performed as described for rats (Markowska et al. [1994] with modifications) to assess coordinated behaviors, orienting reactions, forelimb weakness, postural abnormalities, climbing, and locomotion. Each test was given once a day for three consecutive days, with a maximum of 120 sec for each test during each day.

Walking initiation
The mouse was placed on a table, and the time for the mouse to move 10 cm was recorded.

Turning in an alley
The mouse was placed facing the back wall of an alley (3-cm wide, 20-cm long, with high walls). The time taken by the mouse to turn around and to face the open end of the alley was recorded.

Turning on an inclined screen
The mouse was placed in the center of a horizontal wire mesh screen (35 × 35 cm) 60 cm above a foam cushion. Then, immediately, the screen was inclined to 45° with the mouse facing downward. The time taken by the mouse to turn to face upward was recorded.

Wire suspension
The mouse was placed hanging by its front paws in the middle of a 45-cm long horizontal wire (2 mm diameter) between two escape platforms with hiding chambers, −60 cm above a foam cushion. The time until the mouse fell from the wire was recorded; otherwise the times to climb the wire, reach the platform, and enter the chamber were recorded.

Bridges
Each bridge was a wooden plank or dowel (50-cm long) suspended between two platforms with hiding chambers, 60 cm above a foam cushion. Each mouse was placed in the middle of the bridge. If the mouse fell, the latency to reach a platform was recorded as 120 sec. The flat bridges were wooden planks of three different widths: 1, 2, and 4 cm. The round bridge was a dowel, 0.5, 1, or 2 cm in diameter.

Open field
The apparatus was a wooden floor (59.5 × 59.5 cm) surrounded by walls (34-cm high) painted gray. The animals' behavior was monitored by a video camera placed above the center of the apparatus. The mice were put individually in one corner of the open field facing the corner and were allowed to explore freely for 5 min. The floor of the apparatus was cleaned with ethanol after each session. To analyze the behavior, the open field apparatus was divided into three virtual zones: thigmotaxis, middle (each 12-cm wide), and center. Data were analyzed by an EthoVision system (Noldus Information Technology), and the following parameters were counted: total distance moved, maximum distance moved without stopping, total time spent in each zone, frequency of passing through each zone, and latency of the first occurrence in the middle zone.

Elevated plus maze
An elevated plus maze apparatus was composed of wood painted gray and placed 63 cm above the floor. The apparatus consisted of four arms of equal size (30 cm × 5.5 cm): The two opposing arms were enclosed by 17-cm high walls (closed arms) and the other two arms were surrounded by 5-mm high nontransparent tape (open arms). The walls were connected by a common central square platform (5.5 cm). The mice were put individually in the center of the plus maze facing the open arms and were allowed to explore freely for 5 min. The apparatus was cleaned with ethanol after each session to remove odor cues. The plus maze apparatus was divided virtually into three zones: closed arms, open arms, and center. In each of the zones the behavior was analyzed by EthoVision, considering the latency of the first occurrence in each zone, total time spent moving, total time spent, and frequency of passing through each zone.
Chocolate-search task
The animals were trained to look for, find, and eat pieces (5 mm in diameter) of chocolate in a new cage (26 × 20 × 14 cm) following 24 h of food deprivation. The mice were placed in one of the corners of the cage, facing the wall. The training took several attempts until all of the animals would immediately (<5 sec) start to bite at the chocolate located at various locations within the cage, first placed on the bottom of the cage, then on the surface of a layer of wood shavings. They were then presented with the pieces of chocolate buried under 0 mm (simply covered) and later under 10 and 15 mm of wood shavings. The time between being placed inside the cage and finding the piece of chocolate was recorded. During each trial, the wood shavings were replaced and a clean cage was used. The mice were allowed to search for 180 sec, which was regarded as 100%.

Fear conditioning equipment
We used a computerized fear conditioning system (MED-VFC-MS, Med Associates Inc.) consisting of two constantly illuminated soundproof chambers, each surrounding a conditioning box (32 × 25 × 25 cm) with a stainless-steel grid floor connected to a shock generator, metal sides, and a clear Plexiglas back wall and door. The grid floor could be covered by a Plexiglas plate, the back walls of the boxes could be marked with different stripe patterns of detachable panels. Chambers (boxes A and B) were located in adjacent rooms, cleaned with different solutions, and ventilated with a built-in fan. Freezing, defined as the lack of movement beside respiration and heartbeat, was measured by the manufacturer’s software according to the instructions.

Cue and context fear conditioning
The fear conditioning procedure was conducted as described (Saxe et al. 2006) over 3 d. On day 1, mice were placed in the conditioning chamber and received three pairings between a tone (20 sec, 80 dB, 2 KHz) and a coterminating shock (1 sec, 0.7 mA). The intertrial interval was of 125 sec, and the first tone presented commenced 120 sec after the mouse was placed into the chamber (box A). The chamber was cleaned with 70% isopropenol between each mouse and scented by a paper towel dabbed with mint solution placed underneath the chamber floor. On day 2, the procedure and context were changed in several ways to test conditioned fear of the tone CS in the absence of contextual cues associated with shock. The back wall of the chamber (box B) was sheltered by different white and black stripes, while the grid floor was covered with a white Plexiglas floor; the chamber was scented with lemon; the ventilation fan was not operated; the experimenter wore a different style of gloves; chambers were cleaned with a nonalcoholic disinfectant between runs. Each mouse was placed into the chamber for 5.5 min. The tone was presented twice for 20 sec at 120 and 290 sec into the session. No shocks were administered. Freezing was scored for the 1 min before the first tone presentation (pretone freezing) and during the 20 sec of the first tone presentation (tone-elicted freezing), then for 1 min before the second tone presentation and during the 20 sec of the second tone presentation. On day 3, mice were tested for conditioned fear of the training context (box A). The testing procedure and context were identical to those used on day 1, except that the CS was not presented. Mice were placed into the chamber for 4 min. The entire session was scored for freezing.

Contextual conditioning for remote memory
The fear conditioning procedure was conducted as described previously (Frankland et al. 2004). Prior to contextual fear conditioning, D2 KO mutant and WT littermate control mice were handled for six consecutive days. On the first 3 d, mice were removed from their home cages and individually handled for 2 min in the vivarium. In order to habituate the mice to the general procedures used during training and testing, on days 4–6 mice were taken to the room housing the contextual fear conditioning apparatus where they were handled individually. One day following the completion of handling, mice were trained. During training, mice were placed in the conditioning chamber for 7 min. After 2 min, when basic freezing was scored, they were presented with five unsignaled footshocks (2-sec duration, 0.75 mA, 1 min apart). During testing, 36 d later, mice were placed back in the conditioning chamber for 2 min, and freezing was recorded.

Trace fear conditioning
10 CS–US pairings protocol
The protocol was designed following Shors et al. (2002) experiments on rats. Box A was cleaned with ethanol (70%) and ventilated with a built-in fan. On the back wall of the freeze-monitor box was a detachable panel with alternating black and white lines in a horizontal pattern. Box B was located in an adjacent room and additionally changed by altering the stripes, covering the grid floor with a white Plexiglas floor, using a differently scented cleaning solution, and turning off the fan. The mice were acclimated to the training chamber (context A) for 30 min with no stimuli presented. After 2 min, a baseline measurement of movement was recorded for 3 min. The following day, the mice were returned to the same freeze-monitor box to which they had been acclimated (context A). After 2 min, they were exposed to 10 trials of paired stimuli using a trace paradigm with an intertrial interval (ITI) of 208 sec. For each trial, a tone-eliciting stimulus (CS, tone, 15 sec, 82 dB) was followed by a 30-sec trace interval, followed by a footshock unconditioned stimulus (US, 2 sec, 0.4 mA) delivered through the grid floor of the freeze-monitor box. One day later, the mice were placed in the conditioning chamber (context A) for 5 min and no conditioning stimuli were presented. After 2 min, movements over 3 min were recorded as a measure of fear associated with the training context. The mice were then returned to their home cages for 30 min. They were then placed in a novel testing chamber (context B). After 2 min, movements over 3 min were recorded in the novel context. Then, 10 CS were delivered with an ITI of 4 min (no US). The amount of movement during the noise (15 sec) and during the trace interval (30 sec after CS offset), and during 10 sec around the time when US had begun during training (±5 sec since the beginning of US onset during training) was measured during all CS presentations.

One CS–US pairing protocol
The protocol was designed based on Misane et al. (2005). During training, mice were placed into box A; after 150 sec of adaptation they were presented with noise (30 sec) and a footshock (2-sec duration, 0.5 mA). The tone offset and footshock onset were separated in time by a trace interval of 30 sec. The mice were removed from the fear conditioning box 30 sec after shock termination in order to avoid aversive association with the handling conditioning procedure. They were then returned to their home cages and cleaned with 70% ethanol before the placement of each mouse. Tone-dependent memory was tested in a novel context (box B) 1 h and 7 d after training. Box B was an identically sized cage with a plain floor (no shock grid) with triangle-shaped walls introduced. Box B was cleaned with 2% acetic acid in the same way as the fear conditioning box. A 120-sec exposure to a novel context without stimulation (pre-CS phase) was followed by a 120-sec period of tone presentation (CS phase), and freezing was recorded.

Morris water maze (MWM)
MWM was performed as described (Widy-Tyszkiwetz et al. 1993; Giese et al. 1998). The mice received one session of four trials each day for 11 d. The pool was 1.4 m in diameter; the platform was 10 cm × 10 cm. During the transfer test, on day 12, the platform was removed from the pool and the animals were allowed to swim for 60 sec while the time spent in each quadrant was measured. In the visible platform version of the task, all animals were given one session of four trials, each time with the marked platform located in a different place. Data were recorded using an HVS image analyzing system (Chronotrack, San Diego Instruments).

Novel object recognition (NOR)
During three consecutive days of habituation, each mouse was placed individually in a large plastic cage (30 cm × 30 cm × 50 cm)
and allowed to explore freely for 5 min. The cage contained two identical objects situated in opposite corners (southwest and northeast)—either two objects A (plastic tube) or two objects B (pyramid). Half of the mice explored the cage with objects A, whereas the other half explored the cage with objects B. All of the objects were cleaned with ethanol after each session to remove odor cues. Twenty-four hours after the third habituation session, each mouse was placed in the southwest corner of the same cage containing one of the familiar objects and a novel object in the northeast corner. Data were analyzed by a TechView program, and the time when a mouse's head was within 2 cm of any object was classified as object exploration. The time spent exploring the new object in comparison to the familiar one was defined as an object recognition memory.

IntelliCages

The mice were tested in a novel automated learning apparatus, an IntelliCage system, from NewBehavior AG (Galsworthy et al. 2005; Knapska et al. 2006). A week before the experiment the mice were exposed to isoflurane anesthesia and injected with a glass-covered microtransponder (11.5 mm length, 2.2 mm diameter; Trovan, ID-100) with a unique code. Then, the animals were housed in groups of six to 12 in the experimental room for 3–7 d before the adaptation to the cage started. The IntelliCage consists of a large standard rat cage 20.5 cm high, 40 cm × 58 cm at the top and 55 cm × 37.5 cm at the base. In each corner, a triangular learning chamber is located with two bottles of water. To drink, only one mouse at a time can go inside a plastic ring (outer ring: 50 mm diameter; inner ring: 30 mm deep into outer ring) that ends with two 13-mm holes (one on the left, one on the right side) giving access to water-bottle nipples.

Procedure

Following their introduction to the cages, the mice were allowed to explore with free access to water in all corners (Simple Adaptation). Then, for several days the mice received "Place Preference with Open Gates" with one corner with sweet water (10% sucrose) for the whole group and other corners with plain water available. The corner with sugared water was the least preferred corner during Simple Adaptation. Following Place Preference, the holes were closed by small motorized doors and the mice learned to open the gates by nose pokes (Nose poke Adaptation; plain water in all corners). In Place Preference with Nose pokes test, mice received sugar water (10% sucrose) in one of the corners; it was the least preferred corner during Nose poke Adaptation; other corners were not available. Mice were then trained in Place Avoidance: During 2 d, the animals received punishing air puffs (one bar) when entering the corner that was the most preferred during the Place Preference test.

Data analysis

All data are represented as means with standard error of the means (SEM). The minimal level of significance was P < 0.05. The effects of behavioral experiments and BrdU+ cell numbers were evaluated with ANOVAs. In cases where the data did not meet the assumptions for ANOVA, the results were transformed using Box-Cox transformation (JMP3.2.6; SAS Institute, Inc.). Significant main effects or interactions were followed up with post-hoc analysis (Duncan), where appropriate. Nonparamometrical statistical tests were used where data still violated the assumptions for ANOVA. Several dependent groups were evaluated using Friedman non-parametric analysis followed by individual Wilcoxon comparisons and Mann-Whitney tests (for independent groups). The calculations were made using STATISTICA (data analysis software system), version 7.1. (StatSoft, Inc., 2005).

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