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

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Adult neurogenesis not essential in learning

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Abstract

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 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 knock-out 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.

Introduction

New neurons are produced in the brains of adult animals, including humans, throughout the 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; Shors et al. 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, Tab.1). The reason 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 (discussed in 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 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 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 the major structures present, though some of them smaller. Also, neurogenesis outside the central nervous system seems to be unaffected in these mice (Kowalczyk et al., 2004). All 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 learn trace fear-conditioning, Morris water maze (MWM), novel object recognition (NOR) and also learn 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+ (DCX+) immunoreactive cells in the dorsal hippocampal

dentate gyrus of 2-3-month old WT (n = 6) and D2 KO (n = 9) mice (Fig. 1). DCX is an intermediate filament protein expressed in young, postmitotic, less than 1 month 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 to 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.

D2 KO mice show no deficits in basic sensorimotor tests and normal procedural learning

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*, *elevated-plus maze* and *chocolate-search task*. We assessed sensorimotor performance because there are no systematic data 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 employed assessed coordinated behaviors, orienting reactions, forelimb weakness, postural abnormalities, climbing, and locomotion of 6-8-month 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. Towards 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 case of *walking initiation*, *turning in an alley* and all *bridges*. D2 KO mice showed improvement in performance between day 1 and 3 in all these tasks. These differences indicate procedural learning during the course of these tests in both WT and KO animals (Fig. 2). In 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 months 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 3 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 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 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 it 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 saw dust of a cage. As expected D2 KO mice demonstrated impairment in this olfaction-dependent task. It took the mutant mice longer times 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 ($F_{1,12} = 11.64$; $p < 0.01$) 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 months old, but only when the chocolate was buried at 10 mm ($p < 0.01$) or at 15 mm ($p < 0.05$) but not when 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 when later 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 months, received 3 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 months), where all experiments were performed using only one conditioning chamber (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 days after the conditioning (Fig. 5B). When freezing levels before training and after 36 days 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 days ($F_{1,16} = 391.41$, $p < 0.001$ for WT, n = 9; $F_{1,24} = 526.31$, $p < 0.001$ for D2 KO mice, n = 13; all mice aged 3-4 months).

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 employed 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 months old, which received ten 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 ten CS presentations (with no US). Freezing was measured during the CS (15 s tone), during trace (30 s after tone) and during former US (+/-5 s, *i.e.*, 10 s 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 +/-5 s (10 trails) groups for all 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 have 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 (compare Shors et al., 2002, Fig. 3) was compared between the mice from WT and KO groups, out of intervals shown (CS1-3, trace 1-3, +/- 5 s 1-3, Fig. 6A) the only difference was observed for all three times when the shock had occurred (+/- 5 s 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 months old. Again, in a new context KO animals displayed higher levels of freezing at the onset of former US +/- 5 s in all the first three trials (not shown). To sum up, not only were D2 KO mice, lacking neurogenesis, not deficient in trace fear conditioning, but they 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 months 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); Mann-Whitney test, U = 9.0, $p < 0.01$ (Fig. 6B).

Mice were also contextually conditioned using different trace protocol with only one CS-US pairing, based on previously published protocol (Misane et al. 2005). During training WT (n = 7) and D2 KO (n = 7) mice, 6-8 months old, after 150 s of adaptation, were presented with noise (30 s), followed by trace (30s) and a footshock (2 s duration, 0.5 mA). During testing, 1 hour and 7 days later, mice were placed in different context where, after 120 s of adaptation they were presented with the noise for 120 s; 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 measure (genotype) and dependent measure (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 hour and 7 days 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 (reviewed in Leuner et al., 2006) though new neurons of the hippocampal dentate gyrus seem to be preferentially engaged (Kee et al. 2007).

D2KO 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 travelled ($F_{1,26} = 25.87$; $p < 0.001$ 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 months, were spending significantly more than 25% time in the target quadrant (50.0 ± 7.2% for WT, 42.8 ± 5.8% for KO D2, one-tail analysis, a = 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 (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, the longer the 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 s of established contacts with the objects. No difference between D2 KO (n = 8) and WT (n = 6), all 4-5 months 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 individual mouse's visits to 4 corners and 8 bottles (2 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 nosepoke. In Place Preference test, animals from both groups were trained to associate sweet water with a specific corner within the cage with either all the gates open (Place Preference with Open Gates) or with the necessity to perform nosepokes (Place Preference with Nosepokes) 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 nosepokes were analyzed (Fig. 8). Regarding the number of visits during different types of training regiments, there was an effect of training ($F_{15,90} = 33.55$; $p < 0.001$), 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 months 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 Nosepokes**, there was an effect of training for all animals and separately for WT and KO mice ($p < 0.001$, Friedman, n = 17). Again, both WT and KO mice learned the task as both groups visited the correct corner more often than before training (Wilcoxon test, $p < 0.001$). Some of these mice were subsequently tested in **Place Avoidance** paradigm; there was an effect of training for all animals and separately for WT (n = 10) and KO (n = 10) mice ($p < 0.001$, Friedman). Both WT and KO groups learned the task as both groups visited the corner with air-puffs less often during the first 24 hours of the procedure than before training (Wilcoxon test, $p < 0.01$). Analysis of the number of nosepokes in the same group (n = 17) revealed an effect of training ($p < 0.001$), also for both WT ($p < 0.001$) and KO mice ($p < 0.001$, all Friedman tests) separately. The number of nosepokes was higher in correct corners during the first 24 hours of Place Preference with Open Gates and than with Nosepokes for both WT and KO animals (Wilcoxon test, $p < 0.001$).

Discussion

Evaluation of 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 reduced number of neuronal progenitors, which makes this mouse model a unique example of transgenic approach in limiting neural progenitors numbers (compare e.g., Garcia et al. 2004; Zhang et al. 2008). The observed reductions in BrdU+ cells were substantial and greater in case of DCX+ and 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). Importantly, the reduction of neurogenesis in D2 KO mice is maintained throughout the aging, as in both 2-3-month old and 9-10-month old animals, the number of BrdU+ cells is reduced by about 90%. These results confirm our previous observations where 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, therefore implies no “lesion effect” of ablating new or immature neurons, that 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 the 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. saw no deficits in their model. Along these lines, there is 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 employed 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 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 on overall with particularly marked reductions in the sizes of neocortex, hippocampus, and cerebellum (see Kowalczyk et al. 2004). Hence, there is likely to be substantial compensation during development which may cause changes in other brain systems that compensate for these deficits *e.g.* use extra-hippocampal structures to perform tasks that are otherwise hippocampus-dependent. We can not 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 employing 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 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 dimentions) 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 different way of designing experiments, according to which it would be beneficial to: i. find an appropriate behavioral task that

tests temporal associations in rodents (Aimone et al. 2006), ii. test conflicts of several tasks (with highly similar, sequentially learned events) or contexts (Wiskott et al. 2006; Becker and Wojtowicz 2007; Aimone et al. 2009), iii. use testing at later time-points (not days but weeks) (*e.g.*, (Aimone et al. 2006). These are ideas that we plan to test in the future. The later testing has already proved successful in showing the deficits of mice with limited adult neurogenesis (*e.g.*, Imayoshi et al. 2008; Jessberger et al. 2009).

Therefore, our results and conclusions are only an attempt to resolve the contradictions surrounding the role of new neurons in learning and memory. However, the very fact that the mutant mouse with several smaller brain structures, lacking adult hippocampal neurogenesis, still learns a lot, is in favor of the hypothesis that adult brain neurogenesis is not obligatory in learning.

Available literature suggest neurogenesis not to be critical to many of hippocampally-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 in the Tab. 1. From there, one may conclude that the cited literature is contradictory. In particular, there is a number of papers often showing one subtle deficit in one of the behavioral tasks employed 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 (compare Zhang et al., 2008, Fig. 4g). Notably, this effect was apparently not observed using a different group (Zhang et al., 2008, Fig. S16a Suppl.). 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 while 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 be yet not 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 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 in mouse models of anxiety-like behavior 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 authors 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 (Saxe et al. 2007) to, paradoxically, improve memory. 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+ s). It did not escape our attention that in our cue-trace fear conditioning, where D2 KO mice showed better memory than WT controls, the delay (trace) of 30 s was also used.

Conclusion

In conclusion, we have found D2 KO mice, lacking adult brain neurogenesis, learn surprisingly well several behavioral tasks. 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 and colleagues, 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 one week 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); 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, St. Louis, MO, prepared in PBS, pH 7.4) for: i. 2 consecutive days, twice daily, 2 h apart and killed 3 days after the last injection (for morphometric analysis of BrdU+ DCX+ cells, Fig 1) or ii. for 5 days, once daily, and killed 2 h after the last injection (for BrdU+ cells counting after trace fear conditioning).

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 2M 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% TritonX-100, Sigma-Aldrich, St. Louis, MO, USA) and blocked for 1 h in blocking solution (10% normal donkey serum from Vector Laboratories, Burlingame, CA, USA, 0.1% Triton X-100, in PBS). Then sections were incubated overnight with rat anti-BrdU primary antibody (1:200, Accurate Chemical & Scientific Corp., Westbury, NY, USA) in PBS-TX (1%) and then washed in PBS-TX. Next, samples were incubated with anti-rat Alexa Fluor 488 secondary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h and washed in PBS-TX. For double labeling, sections were blocked for 1 h with 10% rabbit donkey serum, 0.1% Triton X-100 in PBS and incubated overnight with goat anti-doublecortin primary antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Then sections were washed in PBS-TX and incubated with anti-goat Alexa Fluor 555 secondary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h and washed in PBS-TX. For nuclear staining TO-PRO-1 (Molecular Probes/Invitrogen, Carlsbad, CA) was added to the last wash. Sections were mounted on slides coated with poly-L-lysine, air-dried and embedded in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). For morphometic 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 disectors (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 were performed as described for rats (Markowska et al. 1994) with modifications) to assess coordinated behaviors, orienting reactions, forelimb weakness, postural abnormalities, climbing, locomotion, and sense of smell. Each test was given once a day for three consecutive days, with a maximum of 120 s 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 x 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, approximately 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 s. The flat bridges were wooden planks of three different widths: 1, 2, and 4 cm. The round bridge was a dowel, 0.5, 1, 2 cm in diameter.

Open field. The apparatus was a wooden floor (59.5 x 59.5 cm) surrounded by walls (34 cm high) painted grey. 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 3 virtual zones: thigmotaxic, middle and center; each 12 cm wide. Data was 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, latency of the first occurrence in the middle zone.

Elevated plus maze. An elevated plus maze apparatus was composed of wood painted grey and placed 63 cm above the floor. The apparatus consisted of four arms of equal size (30 cm x 5.5 cm): the two

opposing arms were enclosed by 17-cm high walls and the other two arms were surrounded by a 5 mm high non-transparent tape. 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 floor of the apparatus was cleaned with ethanol after each session to remove odor cues. The plus maze apparatus was divided virtually into 3 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 x 20 x 14 cm) following 2-5 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 the animals would immediately (< 5 s) 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. Then, they were 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 s which was regarded as 100%.

Fear conditioning equipment. We employed a computerized fear conditioning system (MED-VFC-MS, Med Associates Inc., St. Albans, VT) consisting of two constantly illuminated soundproof chambers, each surrounding a conditioning box (32 x 25 x 25 cm) with stainless steel grid floor connected to a shock generator, metal sides, and clear Plexiglas back-wall and door. The grid floor could be covered by 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 fans. Freezing, defined as the lack of movement besides 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 descirbed (Saxe et al. 2006) over 3 days. On day 1, mice were placed in the conditioning chamber and received three pairings between a tone (20 s, 80 dB, 2 KHz) and a coterminating shock (1 s, 0.7 mA). The intertrial interval was of 125 s, and the first tone presentation commenced 120 s after the mouse was placed into the chamber (box A). The chamber was cleaned with 70% isopropanol 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 s at 120 and 290 s 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 s of the first tone presentation (tone-elicited freezing), then for the 1 min before the second tone presentation and during the 20 s 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 the CS was not presented. Mice were placed into the chambers 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 6 consecutive days. On the first 3 days, mice were removed from their homecages and individually handled for 2 minutes 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. One day following the completion of handling, mice were trained. During training mice were placed in the conditioning chamber for 7 minutes. After 2 minutes, when basic freezing was scored, they were

presented with 5 unsignaled footshocks (2 s duration, 0.75 mA, 1 minute apart). During testing, 36 days later, mice were placed back in the conditioning chamber for 2 minutes, 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 inter-trial interval of 208 s. For each trial, a noise conditioning stimulus (tone, 15 s, 82 dB) was followed by a 30 s trace interval followed by a footshock unconditioned stimulus (US) (2 s, 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 s) and during the trace interval (30 s after CS offset) and during 10 s around the time when US had begun during training (+/- 5 s since the beginning of US onset during training) was measured in all 10 trials.

1 CS-US pairing protocol: The protocol was designed based on Misane et al. (2005). During training mice were placed into the box A, after 150 s of adaptation they were presented with noise (30 s) and a footshock (2 s duration, 0.5 mA). The tone offset and footshock onset were separated in time by trace

interval of 30 s. The mice were removed from the fear conditioning box 30 s after shock termination in order to avoid aversive association with the handling procedure. The fear conditioning box was thoroughly 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 days after training. Box B was an identically sized cage with a plain floor (no shock grid) with a triangle shaped walls introduced. Box B was cleaned with 2% acetic acid in the same way as the fear conditioning box. A 120-s exposure to a novel context without stimulation (pre-CS phase) was followed by a 120-s period of tone presentation (CS phase), and freezing was recorded.

Morris water maze (MWM). MWM was performed as described (Widy-Tyszkiewicz et al. 1993; Giese et al. 1998). The mice received one session of four trials each day for 11 days. The pool was 1.4 m in diameter; the platform was 10 cm x 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 s 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 (Chromotrack, 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 x 30 cm x 50 cm) and allowed to explore freely for 5 minutes. 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 - the cage with objects B. All the objects were cleaned with ethanol after each session to remove odor cues. 24 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, Switzerland (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 6 to 12 in the experimental room for 3-7 days before the adaptation to the cage started. The IntelliCage consists of a large standard rat cage 20.5 cm high, 40 cm x 58 cm at the top and 55 cm x 37.5 cm at the base. In each corner, a triangular learning chamber is located with 2 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 diameter, 20 mm deep into outer ring) which 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 nosepokes (Nosepoke Adaptation; plain water in all corners). In **Place Preference with Nosepokes** test, mice received sugar water (10% sucrose) in one of the corners; it was the least preferred corner during Nosepoke Adaptation; other corners were not available. Then mice were trained in **Place Avoidance**: during 2 days the animals received punishing air-puffs (1 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. Nonparametrical statistical tests were used where data still violated the assumptions for ANOVA.

Several dependent groups were evaluated using Friedman nonparametric 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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Figure Legends

Figure 1. Deficient neurogenesis in the adult granule cell layer of D2 KO mice DG. (*A*) Representative coronal sections of the granule cell layer, visualized by nuclear marker (TO-PRO-1, blue), stained with immature neuronal marker doublecortin (DCX, red) and proliferation marker BrdU (green) 3 days after 2-day BrdU treatment of WT (n = 6) and D2 KO mice (n = 8). Scale bar, 100 μm. Please note the reduction of BrdU+ cells and DCX+ cells (arrow) in D2 KO mice. (*B*-*E*) Morphometic analysis of BrdU+ and BrdU+ DCX+ cells in the granular layer. (*B*) Total BrdU+ cells in the dorsal hippocampus. (*C*) Total BrdU+ DCX+ cells in the dorsal hippocampus. (*D*) BrdU+ cells normalized to the volume of the granular cell layer. (*E*) BrdU+ DCX+ cells normalized to the volume of the granular cell layer. The results are presented as means ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, GZ, granule cell layer; SGZ, subgranular zone.

Figure 2. Normal procedural learning of D2 KO mice in sensorimotor tests. Selected results of bridge crossings are shown. Please note the differences in performance between day 1 and day 3 for both WT (n = 7) and D2 KO mice (n = 7). The results are presented as means ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 3. Lack of difference between D2 KO (n = 10) animals and WT controls (n = 11) in Open Field (*A*) and Elevated Plus Maze (*B*). Abbreviations: **tgm** - thigmotactic zone, **mid** - middle zone, **ctr** - center zone, **csd** - closed arms, **opn** - open arms. The results are presented as means ± SEM; **$p < 0.01$, ***$p < 0.001$; more information in the *Results*.

Figure 4. D2 KO mice are impaired in olfaction. Mutant mice (n = 7) took longer times than their WT siblings (n = 7) to find pieces of chocolate buried in wood shavings at 10 and 15 mm but not when just under the surface of the shavings. The results are presented as means ± SEM; *$p < 0.05$, **$p < 0.01$.

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 minutes). 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 days after training ($^{***}p < 0.001$, ANOVA). The results are presented as means ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$.

Figure 6. (*A*) D2 KO mice showed context fear conditioning and enhanced trace fear conditioning. Both KO (n = 10) and WT (n = 11) animals showed little basal freezing in the training context (day 1, context A) as well as enhanced freezing in the same context after training with ten CS-US pairings (day 3, context A, $^{*}p < 0.05$, $^{**}p < 0.01$). When moved to a novel context, both WT and D2 KO animals displayed low freezing levels (day 3, context B) but these levels went up when CS was presented (the first three trials are shown; day 3, context B, noise), during 30 s following CS (day 3, context B, trace), and between 25-30 s following the end of CS, *i.e.*, former US +/- 5 s (day 3, context B, US time). During these latter intervals D2 KO mice froze more than WT ($^{*}p < 0.05$, $^{(*)}p = 0.05$, Mann-Whitney). The results are presented as means ± SEM. (*B*) Deficit of adult neurogenesis in D2 KO mice examined in the tests presented in the previous panel – *A*. (*C*) D2 KO mice exhibited normal levels of cue-trace fear conditioning after one CS-US pairing. Both WT (n = 7) and mutant (n = 7) mice showed little basal freezing in training context and upon the CS presentation before US (day 1, box A). When moved to a novel context, both groups displayed low freezing levels (day 1, after 1 hour, box B) which went significantly up as CS was presented; similar situation took place a week later (day 8, box B); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.01$, Duncan. The results are presented as means ± SEM.

Figure 7. D2 KO mice show hippocampus-dependent learning. (*A*) Average path lengths to the platform in subsequent days of Morris water maze training, n = 14 (WT) and 13 (D2 KO). (*B*) Time spent in the target quadrant (TQ) as compared with other quadrants, $^{**}p < 0.01$, Wilcoxon. (*C*) In the novel object recognition task, D2 KO mice (n = 8), as well as their WT siblings (n = 6), take longer to investigate a **new** object than the **old** one, $^{*}p < 0.05$, $^{***}p < 0.001$, one-way ANOVA. The results are presented as means ± SEM.

Figure 8. D2 KO mice show normal learning in the IntelliCage system. (*A*) Following 24-hour adaptation (Ad) with the gates opened (average for the least frequented corner is shown, this corner was chosen for Place Preference (PP) with Open Gates), mice (n = 17 for both genotypes) learned to visit more often the corner with sweetened water (results from 24 hours are shown). (*B*) Mice were then trained to perform nosepokes (Nosepoke Adaptation, NA), average for the least frequented corner is shown, this corner was chosen for Place Preference with Nosepokes (PP). The mice learned to find sugar water in correct corners - results from the first 24 hours are shown. (*C*) Some of the mice (n = 10 for both genotypes) then learned to avoid air-puffs (Place Avoidance, PA) delivered in the formerly preferred corner (PC), $^{**}p < 0.01$, $^{***}p < 0.001$. The results are presented as means ± SEM.

wild type
D2 knock-out

A

GZ
SGZ

BrdU

DCX

TO-PRO-1

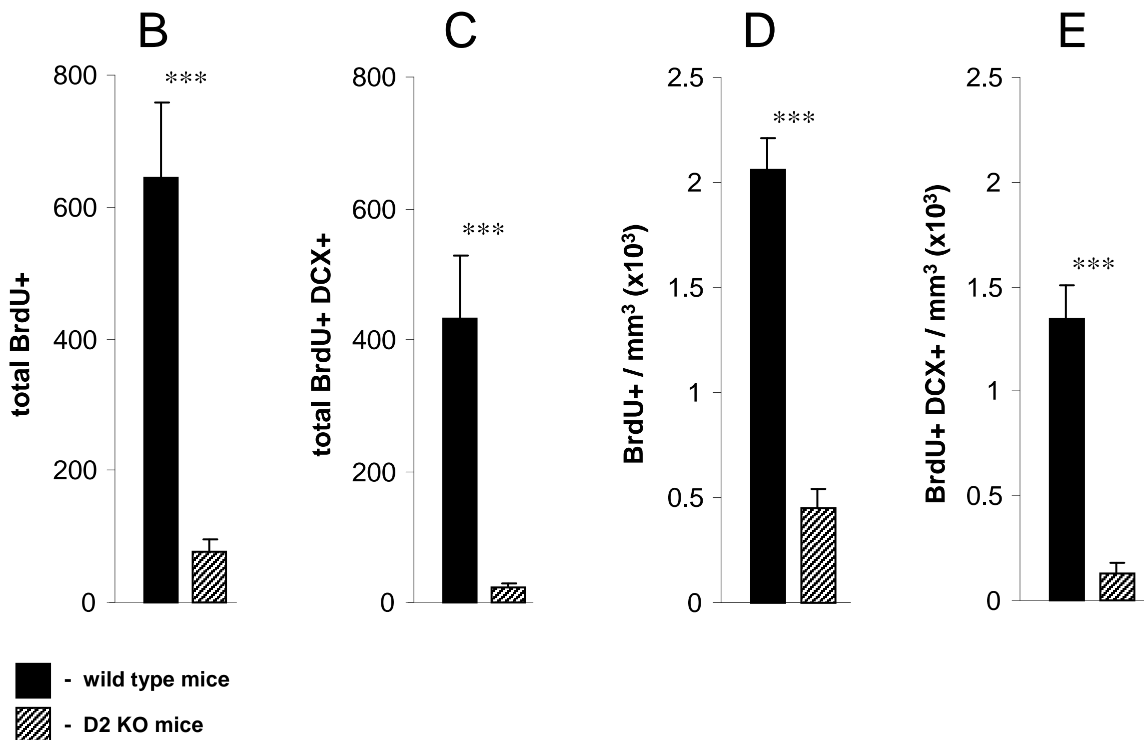
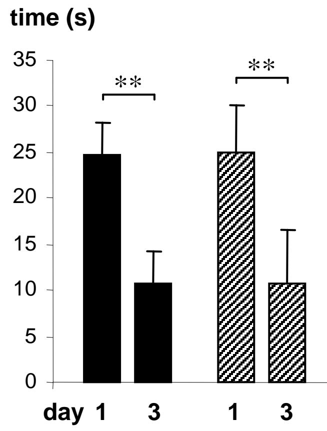
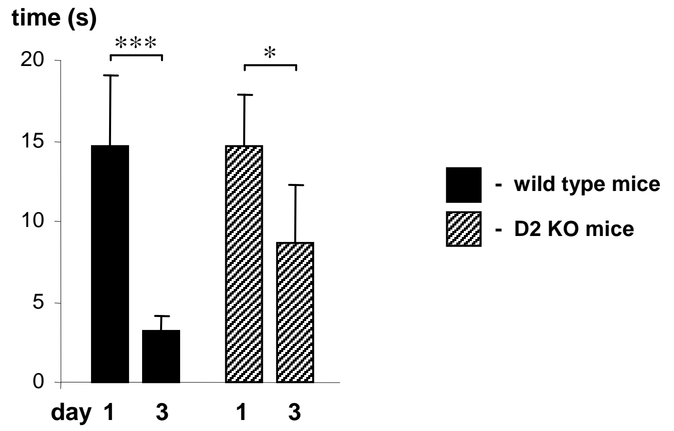


Fig. 1., Jaholkowski et al., *Learning & Memory*





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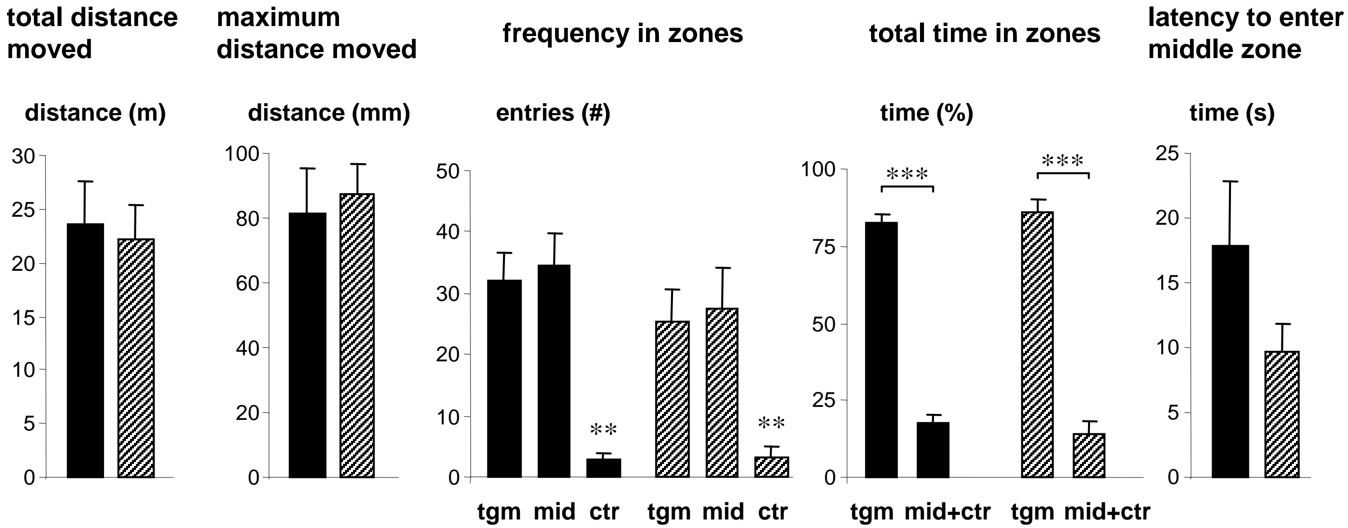

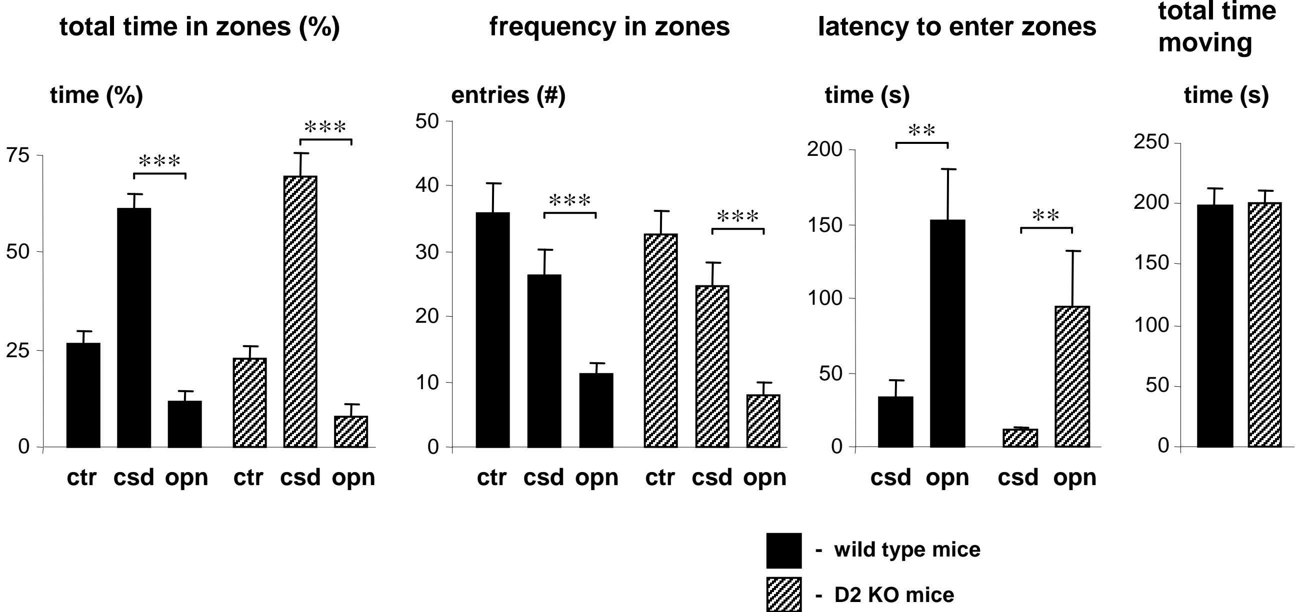

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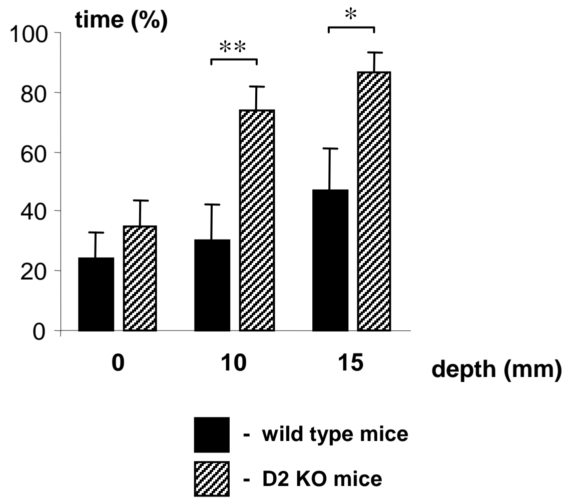

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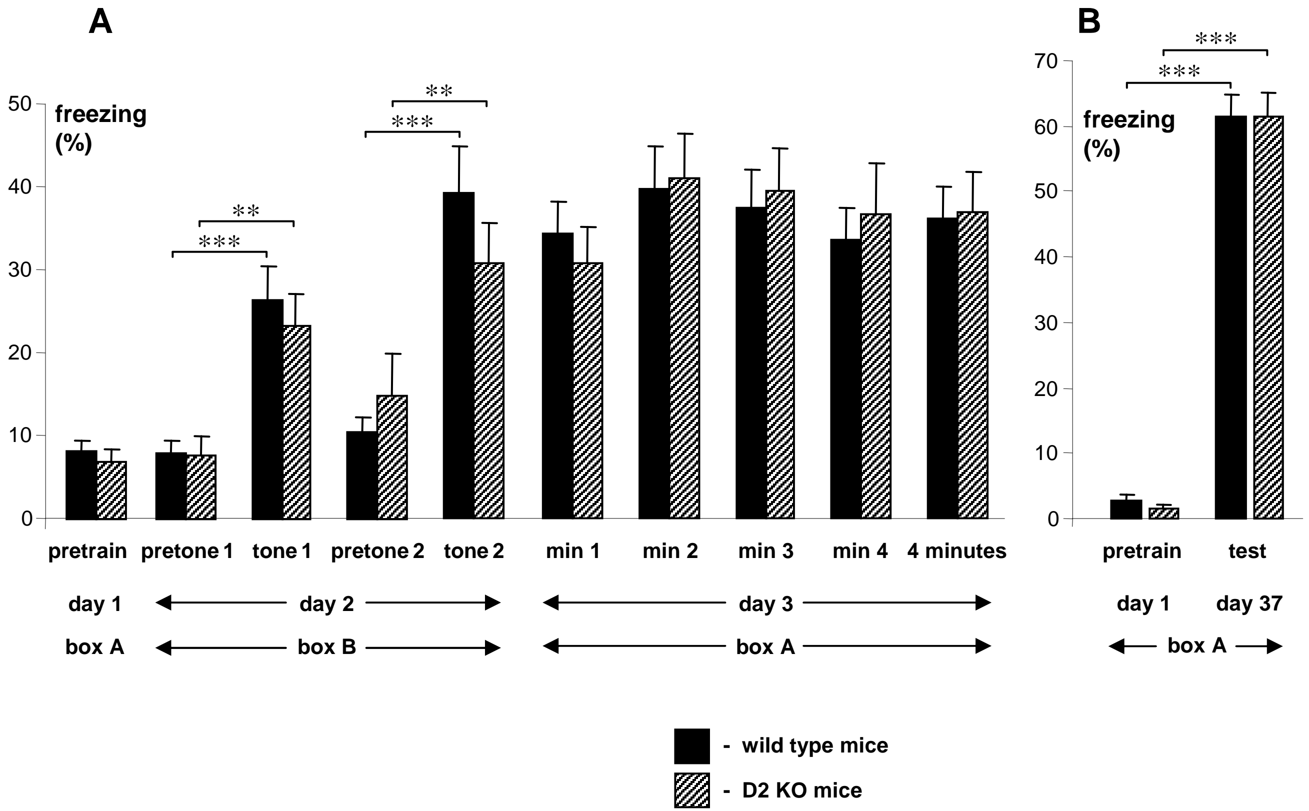

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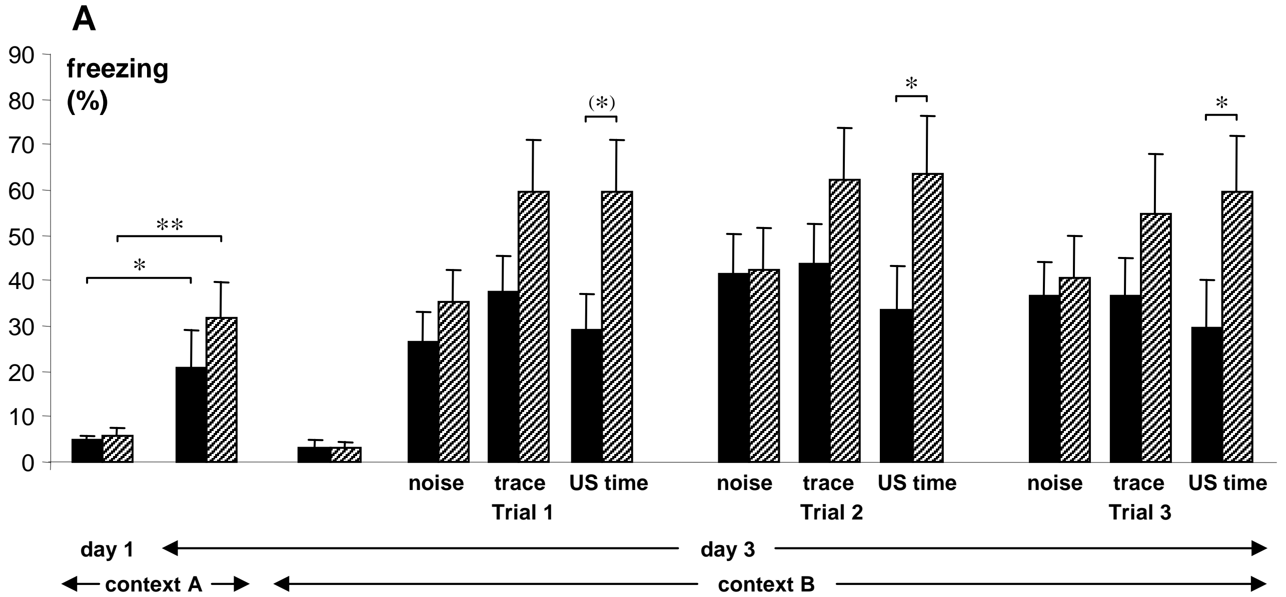
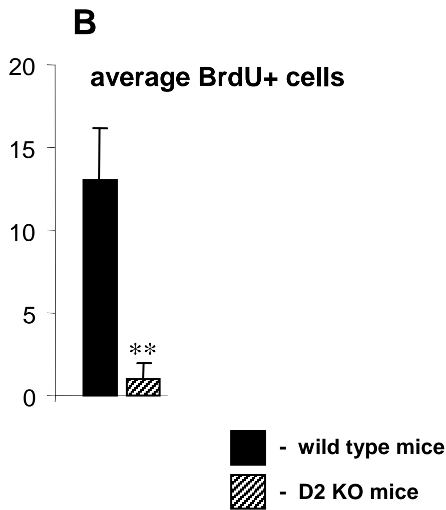




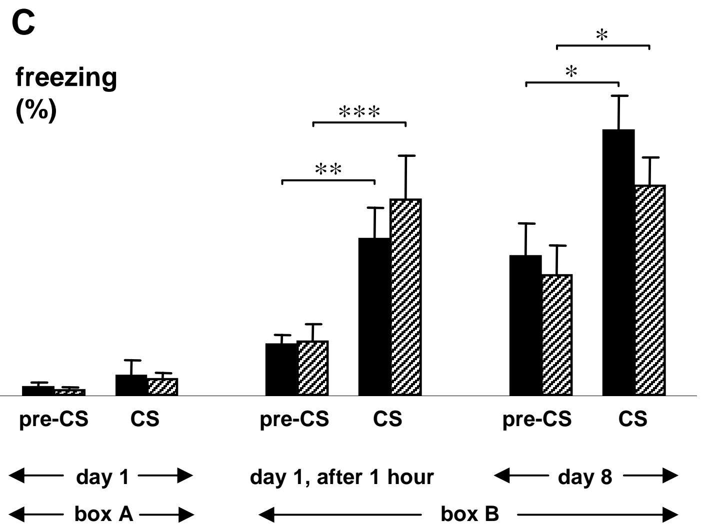
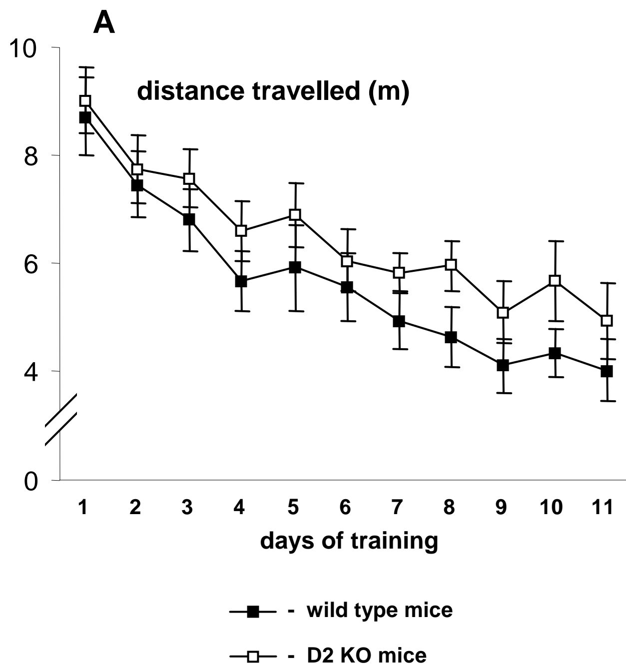
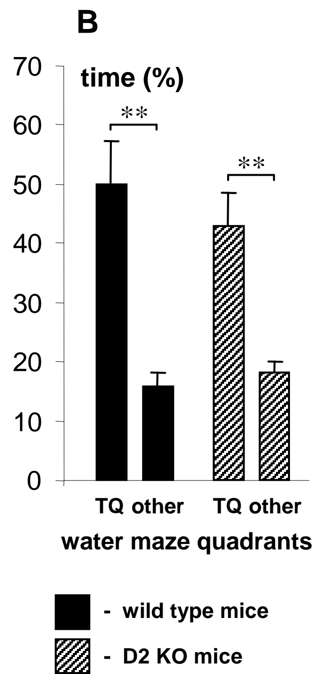


Fig. 6., Jaholkowski et al., *Learning & Memory*





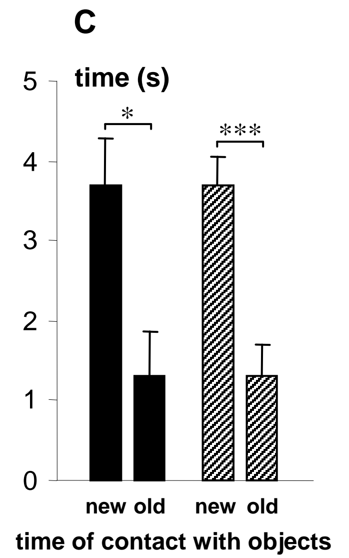
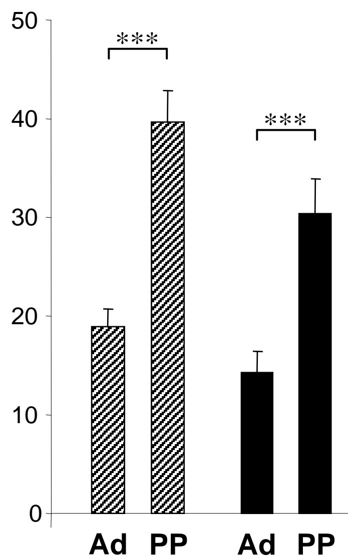
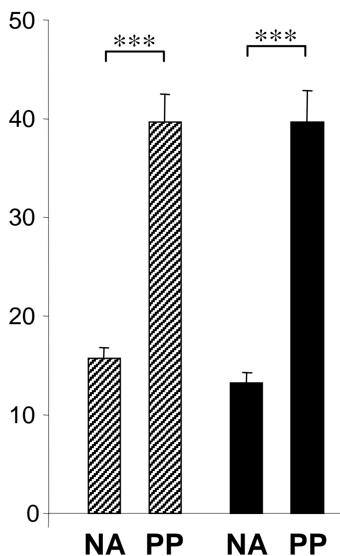
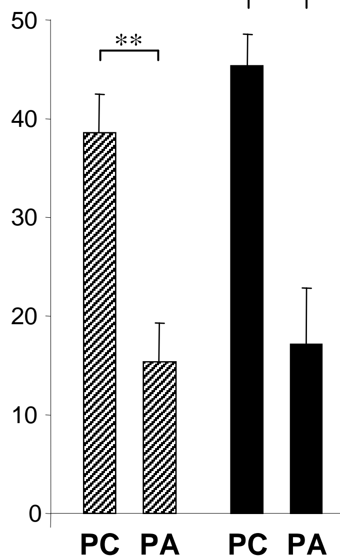


Fig. 7., Jaholkowski et al., *Learning & Memory*







- wild type mice
- D2 KO mice

Fig. 8., Jaholkowski et al., *Learning & Memory*

Table 1. Experiments cited in the text, involving ablation of adult neurogenesis and its effects on learning and memory. Abbreviations used: HML/NI – high-memory load / no intertrial interference, LML/HI – low-memory load / high interference, LML/LI – low-memory load / limited interference, MWM – Morris water maze, NMTS – non-matching-to-sample, NOR – novel object recognition, STFP – social transmission of food preference.

Behavioral model	Ablation method	Species	Effect	Reference
MWM	irradiation	mouse	deficit	(Rola et al. 2004)
	irradiation	mouse	no deficit	(Meshi et al. 2006)
	irradiation and genetic ablation	mouse	no deficit	(Saxe et al. 2006)
	genetic ablation	mouse	deficit, no deficit with visible-platform pre-training	(Zhang et al. 2008)
	antimitotic toxin	rat	no deficit	(Shors et al. 2002)
	irradiation	rat	no deficit	(Madsen et al. 2003)
	irradiation	rat	no deficit @ 1 wk; deficit @ 2/4 wks	(Snyder et al. 2005)
	lentivirus	rat	deficit @ >2 wks	(Jessberger et al. 2009)
Barnes maze	irradiation	mouse	no deficit	(Rola et al. 2004)
	genetic ablation	mouse	deficit	(Imayoshi et al. 2008)
Fear Conditioning				
Cue Fear Conditioning	irradiation and genetic ablation	mouse	no deficit	(Saxe et al. 2006)
	genetic ablation	mouse	no deficit	(Imayoshi et al. 2008)
	genetic ablation	mouse	no deficit	(Zhang et al. 2008)
	irradiation	rat	no deficit	(Winocur et al. 2006)
Context Fear Conditioning	irradiation and genetic ablation	mouse	deficit	(Saxe et al. 2006)
	genetic ablation	mouse	deficit	(Imayoshi et al. 2008)
	genetic ablation	mouse	no deficit	(Zhang et al. 2008)
	antimitotic toxin	rat	no deficit	(Shors et al. 2002)
	irradiation	rat	deficit	(Winocur et al. 2006)
Trace Cue Fear Conditioning	antimitotic toxin	rat	deficit	(Shors et al. 2002)

Eyeblink conditioning				
trace -	antimitotic toxin	rat	deficit	(Shors et al. 2001)
delay -	antimitotic toxin	rat	no deficit	(Shors et al. 2001)
NMTS				
basic rule	irradiation	rat	no deficit	(Winocur et al. 2006)
long intervals	irradiation	rat	deficit	(Winocur et al. 2006)
short intervals	irradiation	rat	no deficit	(Winocur et al. 2006)
Eight-Arm Radial Maze				
HML/NI	irradiation and genetic ablation	mouse	no dificit	(Saxe et al. 2007)
LML/HI	irradiation and genetic ablation	mouse	improvement	(Saxe et al. 2007)
LML/LI	irradiation and genetic ablation	mouse	improvement	(Saxe et al. 2007)
NOR	irradiation	mouse	no deficit	(Rola et al. 2004)
	irradiation	rat	no deficit	(Madsen et al. 2003)
	antimitotic toxin	rat	no deficit of enrichment-induced memory improvement @ 1h, deficit @ 1/2 days	(Bruel-Jungerman et al. 2005)
	lentivirus	rat	deficit	(Jessberger et al. 2009)
Place recognition	irradiation	rat	deficit	(Madsen et al. 2003)
Y maze	irradiation and genetic ablation	mouse	no deficit	(Saxe et al. 2006)
Novel location	irradiation	mouse	no deficit	(Rola et al. 2004)
STFP	lentivirus	rat	no deficit	(Jessberger et al. 2009)