HIPPOCAMPUS 26:646–657 (2016)

Large-Scale Phenotyping Links Adult Hippocampal Neurogenesis to the Reaction to Novelty

R. Maarten van Dijk,[1,2,3†] Stanley E. Lazic,[4†] Lutz Slomianka,[1] David P. Wolfer,[1,2,3] and Irmgard Amrein[1,2*]

ABSTRACT: The discovery of adult-born neurons in the hippocampus has triggered a wide range of studies that link the new neurons to various behavioral functions. However, the role of new neurons in behavior is still equivocal. Conflicting results may be due to the difficulty in manipulating neurogenesis without off-target effects as well as the statistical approach used, which fail to account for neurogenesis-independent effects of experimental manipulations on behavior. In this study, we apply a more comprehensive statistical and conceptual approach. Instead of between-group analyses, we consider the within-group relationships between neurogenesis and behavior (ANCOVA and mediation analysis) in a large-scale experiment, in which distinct age- (3 and 5 months) and strain- (DBA and C57) related differences in basal levels of neurogenesis in mice are compared with a large number (~1,500) of behavioral read outs. The analysis failed to detect any association between anxiety and motor impulsivity with neurogenesis. However, within-group adult hippocampal neurogenesis is associated with the reaction to novelty. Specifically, more neurogenesis is associated with a longer latency to explore and a lower frequency of exploratory actions, overall indicative of a phenotype where animals with more neurogenesis were slower to explore a novel environment. This effect is observed in 5-months-old, but not in 3-months-old mice of both strains. An association between the reaction to novelty and adult neurogenesis can have a major impact on results from previous studies using classical behavioral experiments, in which animals are tested in a—for the animal—novel experimental set-up. The neurogenesis–novelty association found here is also a necessary link in the relation that has been suggested to exist between neurogenesis and psychiatric disorders marked by a failure to cope with novelty. © 2015 Wiley Periodicals, Inc.

KEY WORDS: behavior; IntelliCage; exploration; anxiety; mouse

INTRODUCTION

Behavioral outcomes of the functional integration of adult-born granule cells into the existing circuitry (Shors et al., 2002; Santarelli et al., 2003; Snyder et al., 2005; Clelland et al., 2009; Akers et al., 2014) of the hippocampal dentate gyrus remain debatable because covariation between neurogenesis and behavior is rarely estimated (Lazic, 2010; Lazic et al., 2014). In the majority of studies, the link between behavior and neurogenesis is assessed by a group-level comparison. For example, the experimental group has both an increase in neurogenesis and a better behavioral performance. This does not directly test whether differences in neurogenesis are responsible for differences in behavioral performance, and it does not take into account alternative explanations such as off-target effects of experimental manipulations (Lazic, 2012). If a link between behavior and neurogenesis exists, then this link should extend to the within-group relationship between neurogenesis and behavior. This relationship is amenable to statistical testing (Lazic, 2012). When tests could be performed based on published data, these additional analyses show little or no evidence for a link between behavior and neurogenesis, assigning the between-group effect to unknown mechanisms other than adult neurogenesis (Lazic, 2012; Lazic et al., 2014). In addition, meta-analyses showed only small and nonsignificant contributions of neurogenesis to behavioral outcomes (Groves et al., 2013; Lazic et al., 2014).

Although focusing on within-group relations, this study initially aimed at a traditional comparison of neurogenesis and key measures of two behavioral traits: motor impulsivity and anxiety. Motor impulsivity was selected because previous studies have shown differences in impulsivity in the two strains that were used in this study (Gubner et al., 2010; Pinkston and Lamb, 2011) and strain differences in neurogenesis (Kempermann and Gage, 2002; Huang et al., 2010; Clark et al., 2011). Furthermore, both strains show age-related changes in impulsivity (Pinkston and Lamb, 2011) and age-related differences in neurogenesis (Kempermann et al., 1998; Ben Abdallah et al., 2010; Overall et al., 2013). Also, exercise, which transiently increases hippocampal neurogenesis (van Praag

[1] **Institute of Anatomy, University of Zürich, Switzerland;** [2] **Neuroscience Center Zurich, University of Zurich and ETH Zurich, Zürich, Switzerland;** [3] **Institute of Human Movement Sciences and Sport, Department of Health Sciences and Technology, ETH Zurich, Zürich, Switzerland;** [4] **In Silico Lead Discovery, Novartis Institutes for Biomedical Research, Basel, Switzerland**

Additional Supporting Information may be found in the online version of this article.

R.M.D. S.E.L. contributed equally to this work.

Grant sponsor: Swiss National Science Foundation (SNSF); Grant number: 31003A_141244/1.

*Correspondence to: Irmgard Amrein, Division of Functional Neuroanatomy, Institute of Anatomy, University of Zurich-Irchel, Winterthurerstr. 190, CH-8057 Zurich, Switzerland. E-mail: i.amrein@anatom.uzh.ch

Received 25 June 2015; Revised 23 October 2015; Accepted for publication 2 November 2015.

DOI 10.1002/hipo.22548

Published online 5 November 2015 in Wiley Online Library (wileyonlinelibrary.com).

et al., 1999; Brown et al., 2003; Klaus and Amrein, 2012), has been shown to alter impulsive behavior in mice and rats (Binder et al., 2004; García-Capdevila et al., 2009). Together, these studies suggest the presence of age and strain differences in neurogenesis and impulsivity and a relationship in which animals with low neurogenesis, either due to age or strain, are more impulsive compared to their high neurogenesis counterparts. Such a relationship, which would be taken as evidence for neurogenesis effects on a behavior, would be amenable to within-group testing that our study aims at. In addition, the relationship between motor impulsivity and hippocampal neurogenesis has not been directly assessed previously. In contrast, the relationship between anxiety and neurogenesis has been studied extensively, but with equivocal outcomes. Studies have shown both a negative relationship with neurogenesis, where anxiety is increased when neurogenesis is impaired (Bergami et al., 2008; Revest et al., 2009) as well as the opposite relationship, where increased neurogenesis led to an increase of anxious behavior (Fuss et al., 2010a,b; Onksen et al., 2012). We were motivated to assess anxiety by a reassessment (Lazic et al., 2014) that did suggest the presence of within-group relations between neurogenesis and anxiety that were able to explain group differences in behavior. To take full advantage of the findings presented in earlier work, we tested both traits using two strains known for their differences in these two behavioral traits as well as for their different baseline levels of neurogenesis (Kempermann and Gage, 2002; Gubner et al., 2010; Pinkston and Lamb, 2011). Strains were tested at two ages, further differentiating both baseline levels of neurogenesis as well as behavioral performance (Ingram et al., 1981; Lhotellier and Cohen-Salmon, 1989; Ben Abdallah et al., 2010).

Subsequent to the assessment of the key behavioral readouts of impulsivity and anxiety tests, we undertook an exploratory study to assess whether behavioral outcomes beyond the key readout of impulsivity and anxiety are associated with neurogenesis. To this purpose, we used a very large number (1,953) of behavioral variables obtained from the IntelliCage system (Galsworthy et al., 2005; Vannoni et al., 2014) that was used to conduct the behavioral tests and all variables (41) recorded during light/dark box anxiety testing. Given the exploratory nature of this part of our study, several steps were taken to control the false positive rate and to increase reproducibility. First, within-group associations between neurogenesis and behavior were estimated rather than between group differences (Lazic, 2010, 2012; Lazic et al., 2014). Second, quantification of cell numbers used as proxies for adult neurogenesis was done blind and behavioral assessments were largely automated, reducing the chance of experimenter bias. Third, the use of two strains at two ages allowed us to assess the generalizability of the results (Richter et al., 2010). Fourth, to reduce the number of false-negative results, a relatively large number of animals was used. Fifth, both the false discovery rate (FDR) and permutation-based *P*-values were used to control for multiple testing across all behavioral variables. Finally, when arbitrary but important analytic decisions were made, such as selecting a threshold for the definition of highly correlated behavioral variables, a sensitivity analysis was performed by using different thresholds and checking whether this affected the overall conclusions.

MATERIALS AND METHODS

Animals

Fifty-six C57BL/6 and 50 DBA 8-week-old female mice were given 1 week to acclimatize to the housing facility (lights on: 20:00–08:00, room temperature: 23°C, standard type III mouse cages, free access to standard mouse food, water, and nesting materials). To avoid batch effects, all animals were ordered at the same time and obtained at the same age. One week before testing, radiofrequency identification transponders (Planet ID GmbH, Essen, Germany) were implanted subcutaneously in the dorso-cervical region under isoflurane inhalation anesthesia. During the testing, animals were group housed in the IntelliCage (NewBehavior AG, Zurich, Switzerland) with shelters and free access to standard mouse food. Females were chosen due to the likely disruption of behavioral testing by aggressive behaviors arising in group-housed males (Van Loo et al., 2001). Mice were assigned randomly to test or control groups at 9 or 17 weeks of age. Both age groups were housed in standard type III mouse cages until their introduction into the IntelliCage experiment. Both ages represent the adult time window within which resident granule cell numbers and the declines in neurogenesis and cell death are stable (Ben Abdallah et al., 2010). Strains were mixed in the IntelliCage (up to 14 mice/cage). Test groups were assessed for general exploration, motor impulsivity, and anxiety. The control groups were also housed in the IntelliCage, but only exploration in the IntelliCage and anxiety in the light/dark box was assessed (Fig. 1).

Behavior

Each IntelliCage was equipped with four conditioning chambers fitted in the cage corners. Each chamber contained two drinking bottles (accessible via motorized doors) and a motorized valve for delivery of air puffs. The duration of a visit to a corner was determined by the transponder reading and a temperature sensor detecting the presence of the animal. The number and duration of nosepokes were recorded using IR-sensors. Licking episodes were monitored using lickometers (duration of the episode, number of licks, and total contact time). During the first 7 days (test groups) or 30 days (control groups) in the IntelliCage, all animals had free access to all bottles. Data of the first 24 h were used to assess habituation in the new environment. The number of corner visits, licks, and nosepokes over the rest of the free adaptation week were used as measures of activity.

The protocol used to test *motor impulsivity* in the IntelliCage was taken from Kobayashi et al. (2013). To obtain access to

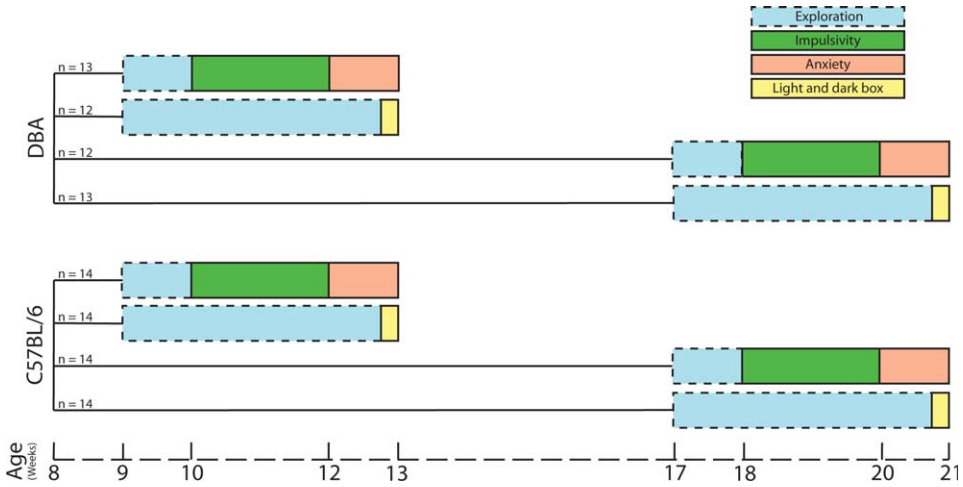

FIGURE 1. Overview of behavioral experiments. Two strains of female mice were used: C57BL/6 and DBA. For both strains, one group was tested at 9 weeks of age and the second group at 17 weeks of age. All four groups underwent the same series of behavioral experiments: exploration assessment, motor impulsivity test, and the anxiety test. To control for environmentally induced, test-dependent factors that might influence neurogenesis, each of the four groups was paired with a control group housed under the same condition as the test groups. Controls were monitored for exploration for the entire test period and tested 5 min before the end of the experiment in the light and dark box. All animals were sacrificed immediately after finishing their final behavioral experiment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

water, the animal had to nosepoke one of the two doors, designating that door for testing and initiating a random delay period (0.5, 1.5, or 2.5 s). After the delay period, the door to the water bottle on the designated side would open for 5 s. If the animals nosepoked during the delay period (premature nosepokes), the door to the water bottle would not open after the delay during this trial. Any subsequent visit to any corner would initiate a new trial. Successful trials required the animals to wait a random delay before gaining access to water. The task lasted for 7 days. It was preceded by two training phases (training phase 0 for 3 days and training phase 1 for 7 days). In T0, a nosepoke immediately opened the door. In T1, a nosepoke started a random delay after which the door opened whether a premature nosepoke was made or not. The number of premature nosepokes made, the percentage of successful trials, and the latency to start drinking after the door opened were used as principal behavioral readouts to assess motor impulsivity.

Subsequent to the testing for impulsivity, a protocol was run to test *anxiety* in the IntelliCage that was adapted from Safi et al. (2006). Test animals were assigned to one corner and trained to drink during two daily sessions (11:00–12:00 and 16:00–17:00). The test lasted for 7 days. During the last drinking session, each animal received a single air puff of 2 bar at their first lick contact. Latency and frequency of visits and licks after the aversive stimulus were used to assess anxiety. Animals were sacrificed immediately following the IntelliCage anxiety test.

Each control animal was removed from the intellicage and tested for anxiety in the light/dark box for 5 min according to standard protocols (Madani et al., 2003). Immediately after the test, the animals were sacrificed. Time spent in the different zones as well as number of rearings, transitions, and grooming episodes were used as principal readouts of anxiety.

Data generated by the IntelliCagePlus Controller and Analyzer applications were exported for further analysis. The IntelliCagePlus Controller software records the locations, beginnings, durations, and ends of all visits and nosepokes. Nosepokes are assigned to visits and all visits are assigned to an individual mouse. For each individual nosepoke, the software records the number and duration of the associated licks. The recorded data was used to calculate the variables (Supporting Information) used in the full behavioral assessments.

Histology and Cell Counts

Perfusion and tissue processing were performed as described previously (Ben Abdallah et al., 2010) followed by immunohistochemistry. For epitope retrieval, free-floating sections were either heat treated for 40 min (Ki67, 95°C) or shortly microwaved (DCX) in target retrieval solution (pH 6.0; DAKO, Glostrup, Denmark). To inhibit endogenous peroxidase activity, sections were incubated in 0.6% H_2O_2 for 30 min. After preincubation in 2% normal serum in Tris-buffered saline with 0.25% Triton (TBS-T) for 60 min at RT, the sections were incubated overnight with the primary antibody Ki67 (polyclonal rabbit-anti-Ncl Ki-67, Novocastra, UK, 1:2000) or DCX (polyclonal goat-anti-doublecortin, Santa Cruz, CA, 1:500). Incubation in secondary antibody (goat anti rabbit 1:300 for Ki67 and rabbit anti goat 1:300 for DCX) was followed by incubation with ABC solution (Vectastain, Vector Laboratories, Burlingame, CA). Finally, sections were stained with 3,3′-diaminobenzidine (DAB) and mounted. Between these steps, sections were rinsed in 3 changes of buffer (TBS-T between

primary and secondary antibodies; otherwise TBS) for 10 min. DCX-stained sections were counterstained with hematoxylin solution, and all sections were dehydrated and cover-slipped.

The total number of DCX+ cells were estimated with the optical Fractionator (West et al., 1991) using the StereoInvestigator software (v10.50, MBF Bioscience, Burlington, Vermont) with a 63× lens (ZEISS Plan-Apochromat 1.40 Oil DIC). Providing cell number estimates at a definable level of precision (Gundersen et al., 1999; Slomianka and West, 2005) that are independent of cell size, shape, orientation, and distribution. DCX+ cells were counted in a frame of 40 × 40 μm with a step size of 100 μm throughout the entire hippocampus. Total cell numbers (N) were calculated using the formula: $N = \sum Q \cdot \frac{1}{\text{asf}} \cdot \frac{1}{\text{ssf}}$, where $\sum Q$ is the total number of cells counted, asf is the area sampling fraction, and ssf is the section sampling fraction. Cells in the top focal plane were excluded from the counts. On average, 14 sections were assessed per animal in which a mean of 212 cells were counted.

Ki67+ cells were counted exhaustively in every fifth section omitting cells in the top focal plane. Total cell number estimates were calculated by multiplying the cell counts by the section sampling fraction. On average, 13 sections were assessed per animal in which a mean of 498 cells were counted.

Fourteen animals (7 C57BL/6 and 7 DBA, all young group) had to be removed from the experiment prior to analysis for DCX due to a freezer failure, resulting in a total group size of 106 animal for testing behavioral relationships with Ki67+ cells (control group: young C57BL/6: 14, adult C57BL/6: 14, young DBA: 12, adult DBA: 13, test group: young C57BL/6: 14, adult C57BL/6: 14, young DBA: 13, adult DBA: 13) and 92 animals for the testing of relationships with DCX+ cells (control group: young C57BL/6: 14, adult C57BL/6: 14, young DBA: 12, adult DBA: 13, test group: young C57BL/6: 7, adult C57BL/6: 14, young DBA: 6, adult DBA: 12). All quantification was done blind to the identity of the animals.

Statistical Analysis

Statistical analyses were conducted with R version 3.0.3. All graphical representations were prepared using the R package ggplot2 (Wickham, 2009).

To test for differences in cell numbers between strains and ages, a two-way ANOVA was used with Tukey's post-hoc test.

Prior to testing for relationships between behavior and neurogenesis, the R package caret (Kuhn, 2014) was used to identify and remove near-zero-variance and highly correlated behavioral variables ($-0.9 > r > 0.9$). Variables were removed in order of mean absolute correlations, in which variables with the highest mean (also meaning the least independent contribution to variance) were removed first until no variables above the cut-off point were left. This preprocessing step was used to remove redundant variables so that fewer analyses are conducted and a less-stringent correction for multiple testing is required. Of the 1,993 recorded variables, 536 were removed. The remaining 1,457 behavioral variables were Box–Cox transformed to ensure approximate normality.

The relationships between behavior and neurogenesis were tested using an ANCOVA. Main effects and all possible interactions between neurogenesis, age, and strain were included in the model. Benjamini–Hochberg correction was used to adjust P values across all behavioral variables (Benjamini and Hochberg, 1995). For the additional statistical analyses, the stepwise selection of the included interaction effects was performed using the R package MASS (Venables and Ripley, 2002). The stepwise selection starts with a full model including all main and interaction effects after which, based on the best-fitting Akaike information criterion (AIC), interaction effects are removed. Selection is done in a bidirectional way, where for each removed interaction, both including and excluding all possible interactions are tested to obtain the best overall AIC. When performing the stepwise selection, the hierarchical order of the effects was maintained, such that lower order main and interaction effects were not removed if higher order interactions involving those factors were present.

A permutation test was run on the full interaction model where the values for the neurogenesis markers were randomly permutated and tested in 5,000 iterations. The distribution of P-values obtained from the permutated data represents a distribution that would be expected if there are no associations between neurogenesis and behavior (null distribution). The number of significant effects observed in the original data was then tested against that of the null distribution. The P values for all main effects as well as all interaction effects were tested in the permutated test.

A mediation analysis was performed using the R package mediation (Tingley et al., 2014). The strain differences were treated as the independent variable and the neurogenesis markers as the mediator variable.

A power analysis was not conducted because there was no primary outcome with a specific hypothesis being tested. Sample size for each group was based on published data (Fuss et al., 2010a) in which, postpublication, a within-group analysis suggested a significant association between neurogenesis and behavior. No individual data points were excluded. The experimental units were the individual animals.

RESULTS

Neurogenesis, Impulsivity, and Anxiety

Estimates of the total numbers of proliferating Ki67+ cells and differentiating DCX+ neurons varied predictably with strain and age (Fig. 2), with DBA mice having 60–65% fewer Ki67+ ($F(1,103) = 543$, $p < 0.001$) and DCX+ ($F(1,89) = 252.5$, $p < 0.001$) cells than C57BL/6 mice. The age difference of 8 weeks caused a 40–50% decline in Ki67+ ($F(1,103) = 171.5$, $P < 0.001$) and DCX+ ($F(1,89) = 139.9$, $P < 0.001$) cell number estimates in both strains. Strain and age interacted significantly in the raw cell numbers (Ki67+ ($F(1,102) = 80.4$, $P < 0.001$) and DCX+ ($F(1,88) = 60.7$,

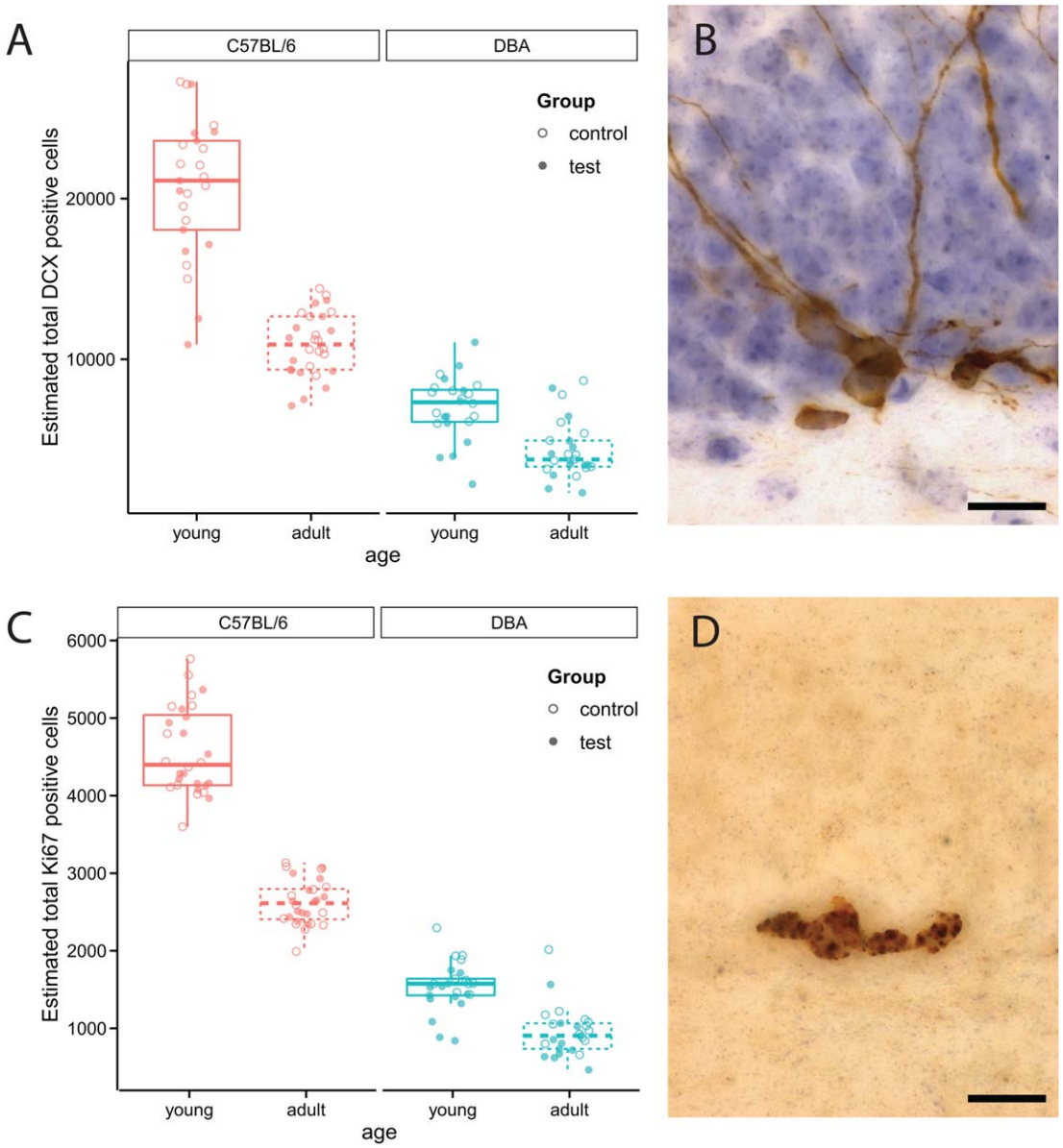

FIGURE 2. Total cell numbers for the neurogenesis markers DCX and Ki67. (A & C) Boxplots with distribution of estimated unilateral total cell numbers for (A) young neurons marked by DCX and (C) proliferating cells marked by Ki67. Both neurogenesis markers decrease significantly with age and show significant differences between the two strains (all comparisons $P < 0.001$), no significant differences were found between the experimental and control group. (B & D) Representative images of stains for (B) DCX and (D) Ki67, scale bars: 10 μm. Figures are stack composites of 30 images for the Ki67 figure and 16 images for the DCX figure, covering a section thickness of 15 μm for Ki67 and 8 μm for DCX. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$P < 0.001$) but not when using log-transformed cell numbers (Ki67+ ($F(1,102) = 0.405$, $P = 0.526$) and DCX+ ($F(1,88) = 0.339$, $P = 0.562$) indicating that both strains follow a similar exponential decline with age that agrees with previous exponential models (Ben Abdallah et al., 2010; Knoth et al., 2010; Amrein et al., 2011). Each individual group differed significantly from any other group (all comparisons $P < 0.001$). No differences were observed in either Ki67+ ($P = 0.418$) or DCX+ ($P = 0.698$) cell number estimates between animals undergoing testing in the IntelliCage and control animals housed in the IntelliCage without behavioral testing, showing that behavioral testing by itself did not alter cell numbers. Coefficients of error of cell number estimates for strain and age groups ranged from 0.06 to 0.13. Variability contributed by the estimation procedure was a minor source of the total variability of cell number estimates in the strain and age groups (CE^2/CV^2 range: 0.05–0.26).

Strong strain differences exist in motor impulsivity and anxiety. *Impulsivity*: DBA mice make more premature nosepokes per incorrect visit ($F(1,35) = 50.43$, $P < 0.001$), have fewer correct visits ($F(1,35) = 6.49$, $P = 0.015$) and respond slower to rewards ($F(1,35) = 22.68$, $P < 0.001$). All animals make

significantly more errors with increasing delay periods ($F(1,52) = 51.64$, $P < 0.001$). Errors appear to increase linearly with the delays used in this study (0.5, 1.5, and 2.5 s). No differences are seen between the learning curves of the different age groups and strains. Despite strong strain differences in neurogenesis and behavior, Ki67+ or DCX+ cell numbers did not associate with any of the main impulsivity variables (Supporting Information, Fig. 1). The lack of significant within-group covariation strongly suggests that neurogenesis does not impact behavior but instead covaries across groups with the unknown factor that is responsible for the behavioral differences. *Anxiety*: DBA mice spend significantly more time in the dark zone ($F(1,49) = 522.94$, $P < 0.001$), made fewer light/dark transition ($F(1,49) = 380.25$, $P < 0.001$) and covered less area in the light zone ($F(1,49) = 84.99$, $P < 0.001$). The area covered in the light zone was the only variable affected by age ($F(1,49) = 4.06$, $P = 0.049$). Similar to impulsivity testing, neurogenesis was not associated with the main anxiety variables (Supporting Information, Fig. 1). Strain, age, or neurogenesis did not impact the anxiety variables in the IntelliCage.

Large-Scale Phenotyping: Neurogenesis and Novelty

Due to the lack of neurogenesis effects in the designed behavioral assessments, we explored effects on 1,457 variables (full overview in Supporting Information, S6) measured in the IntelliCage, which allowed a more comprehensive coverage of the behavioral phenome. In addition to variables extracted from the tests for motor impulsivity (training phase 0, 424 variables; training phase 1, 379 variables; test phase, 379 variables) and anxiety (IntelliCage anxiety test: 303 variables; light/dark box: 40 variables), the 7-day exploration phase prior to testing was subdivided (according to Vannoni et al. (2014)) into the initial 8 h of exploration to assess habituation (44 variables) and the remaining days grouped to assess activity and explorative behavior (424 variables). All remaining variables were tested for both main effects and interaction effects against both neurogenesis markers. Figure 3A shows all resulting *P*-values for the estimated main and interactions effects with DCX (for Ki67, see Supporting Information, Fig. 2). Figure 3B shows the results after correcting for false discovery rate using the Benjamini and Hochberg correction (Benjamini and Hochberg, 1995). After correction, 27 out of 1,457 variables show either a main or interaction effect with either of the two neurogenesis markers (Supporting Information, Table 1). Two notable observations can be made concerning the 27 significant associations. First, there are no main effects of neurogenesis on behavior; instead, all findings show an interaction with neurogenesis and the age of the animals. Second, the majority (18 out of 27) of significant relationships between neurogenesis and behavior are found in the exploratory phases in the IntelliCage, and all show an association with specifically the marker for young neurons, DCX. The variables showing a relationship with the marker for proliferating cells, Ki67, exhibit a less uniform phenotype that, in contrast to the novelty reaction seen with DCX, is not robust to changes in the statistical model used. Furthermore, the effects are only seen in form of triple interactions, i.e. they occur in only one strain and age. Finally, no relation is seen between behavior and DCX for these variables, i.e., the relation between behavior and proliferation is not translated into a relation between young neurons and behavior.

To further investigate the neurogenesis-by-age interaction, the data was split according to the two age groups, and each age was analyzed separately. The results from this separate analysis show a main effect of neurogenesis on 41 behavioral variables. Again, the vast majority (35 out of 41) of behavioral variables that show a significant association were recorded in the exploration phase and were only found in the older animals. A closer inspection of the variables in the exploration phase shows that they can be categorized into two groups: "latency" variables, measuring the time the animals take to perform a visit, poke, or lick in a particular corner for the first time and "frequency" variables, measuring the frequency of visits, pokes, or licks performed during the exploration phase. One representative variable of each of the two categories was chosen to illustrate the relationship between neurogenesis and the specific behavioral phenotype (Fig. 4). In animals of both strains, higher numbers of DCX+ cells are associated with a longer latency to start drinking specifically in the older animals (Fig. 4A, young animals $P = 0.62$, adult animals $P < 0.0001$), and higher numbers of DCX+ cells are associated with a decrease in the frequency of nosepokes in older animals (Fig. 4B, young animals $P = 0.40$, adult animals $P = 0.001$). These trends are consistent in all the behavioral variables found to be significant in the exploration phase (Supporting Information, Fig. 3). Taken together, within the older animals, more neurogenesis is associated with lower reactivity toward the novel environment.

Confirmation by Alternative Analyses

In addition to the described approach, several alternative parameter settings and models were used to assess the robustness of the obtained results to the method of analysis.

Mediation analysis—All 41 behavioral variables which showed an association with neurogenesis were tested in a mediation analysis. This analysis was used to assess the degree to which neurogenesis contributes to the overall behavioral effect compared to alternative mechanisms. The mediation analyses was performed separately for each age group, the strain differences were treated as the independent variable and the neurogenesis markers as the mediator. The overall results agree with the previous analyses where after false discovery rate adjustment, the majority of reported variables in the exploration phase were dependent on the number of DCX+ cells specifically in the older animals, in which more DCX+ cells are associated with slower exploration. The outcomes of the mediation analysis for two variables are presented in Figure 4 (for all 41 variables, see Supporting Information, Fig. 4).

Alternative correlation coefficient cut-off points—In addition to a correlation coefficient cut-off of 0.9, the analysis was run

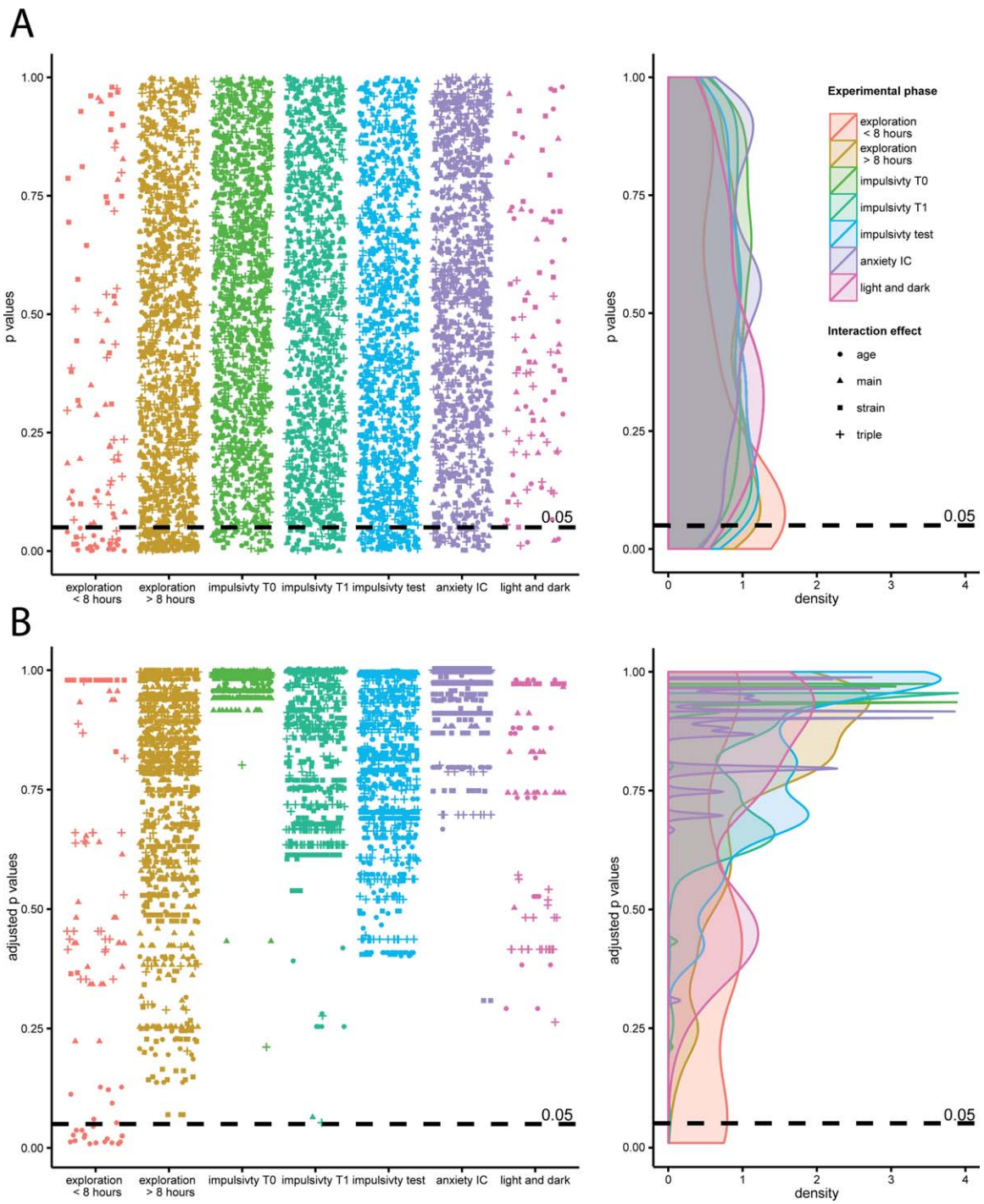

FIGURE 3. P-value distribution of the associations between DCX and all behavioral variables. A total of 1993 variables were extracted from the behavioral experiments, of which 1457 were tested in an ANCOVA model to estimate the main effects of strain, age, and DCX as well as all possible interaction effects. Resulting *P*-values of the DCX estimate are plotted (A) with the distribution of the *P*-values (*y*-axis) for each experimental phase (*x*-axis) with matching *P*-value density plot (right) showing values per experimental phase. After adjustment for false discovery rate (B) 27 behavioral variables show an interaction with either DCX or Ki67 (Supporting Information, Fig. 2), the majority of these interactions can be seen in the exploration phase, more specifically the period when the animals were introduced into the IntelliCage. No main effects are found, all significant variables show an interaction with the age group of the mice. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

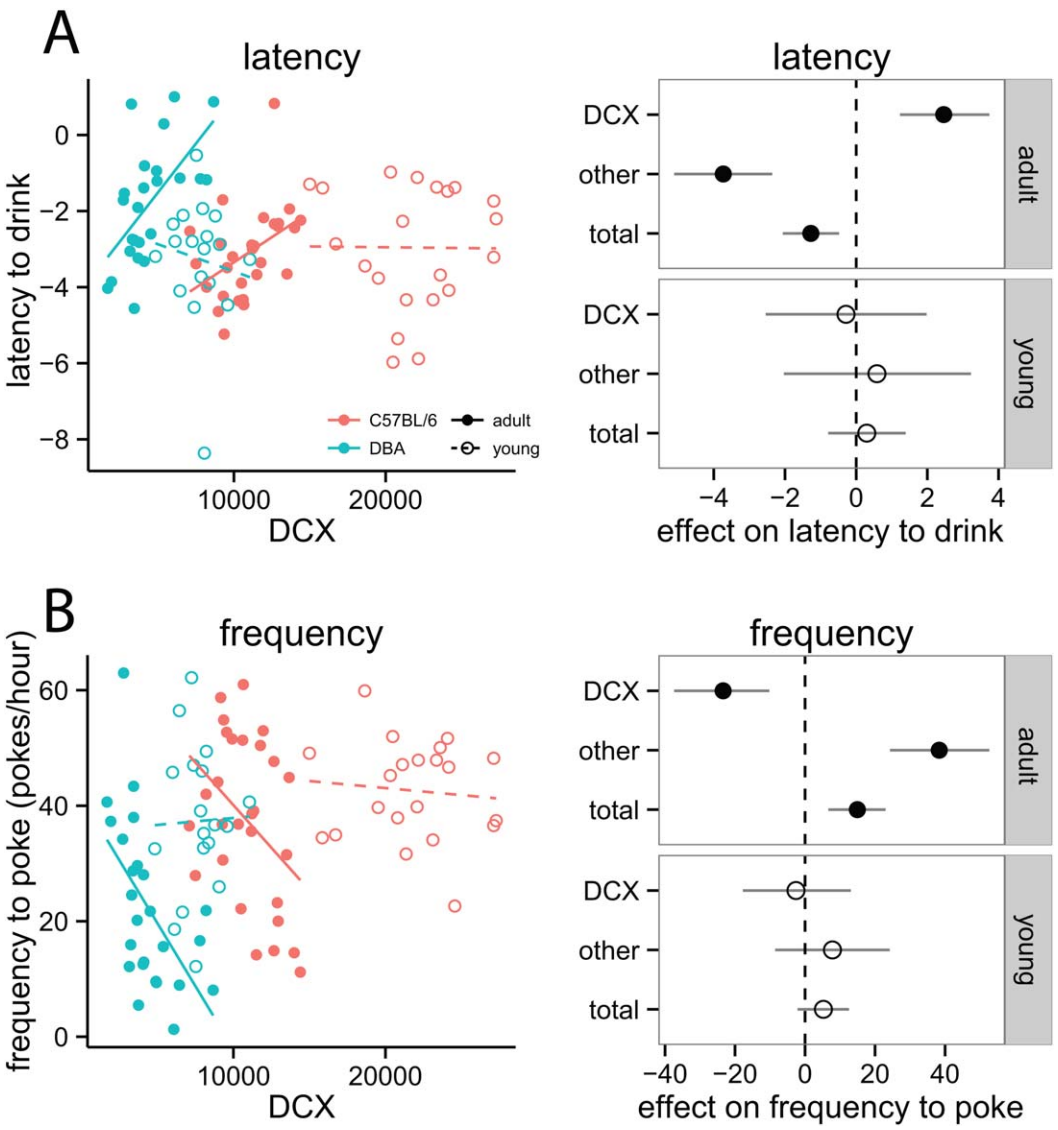

FIGURE 4. Effect of neurogenesis on novelty reactivity. The 27 behavioral variables found to be significantly associated with neurogenesis consist largely of variables measuring the exploration in the novel environment, the variables can be grouped into two categories (A) "latency" variables, measuring the time the animals take to perform a visit, poke, or lick in a particular corner for the first time and (B) "frequency" variables, measuring the frequency of visits, pokes, or licks performed during the exploration phase. For each of the two categories, one representative variable was selected and plotted in a scatter plot and matching mediation analyses. Neurogenesis is associated with an increased latency to drink after introduction into the IntelliCage (A, DCX age interaction $P = 0.005$, separate main effect analyzed per age group: DCX in young animals $P = 0.62$; DCX in adult animals $P < 0.0001$) and a decreased frequency of nosepokes (B, DCX age interaction $P = 0.01$, separate main effect analyzed per age group: DCX in young animals $P = 0.40$; DCX in adult animals $P = 0.001$). The mediation analyses show how neurogenesis has a significant effect on the total behavioral effect observed, the effect is only seen in the older animals for both the latency (A, Neurogenesis (DCX) in young animals $P = 0.77$, in adult animals $P < 0.001$) and the frequency variable (B, Neurogenesis (DCX) in young animals $P = 0.79$, in adult animals $P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with cut-off points of 0.8 and 0.5 or with no cut-off point. Similar overall outcomes were found for all cut-offs, i.e., variables found in the exploration phase were the only ones consistently showing an association with DCX+ cell number estimates. Here, again, in older animals, higher numbers of DCX+ cells are associated with slower exploration.

Assessing the differences in power—In the exploration phases, both control and test animals were tested ($n = 106$) after which impulsivity and anxiety were assessed using the test animals ($n = 53$). Effect sizes in the different experimental phases were compared to assess whether the lack of significant results in the impulsivity and anxiety phases were due to the decrease in statistical power. To allow direct comparison of effect sizes, all behavioral data was normalized. The distribution of effect sizes in all but the first exploration phase follows a normal distribution around 0, indicating that effect sizes follow a distribution

that can be expected by chance. This is not likely to change when the number of animals increases. However, for the first exploration phase, all variables show either a positive or a negative effect of DCX (deviation from normality $P < 0.001$; Supporting Information, Fig. 5). In addition to this validation, a so-called accuracy in parameter estimation (AIPE) was performed to estimate a total number of animals needed to replicate our results in a similar experimental set-up. Based on the median within-group errors across all behavioral variables and an alpha level of 0.05, we calculate that approximately 30 animals per group would be required to estimate the main or interaction effect of neurogenesis to within one standard deviation precision (Kelley et al., 2003). In other words, the 95% CI for an estimate will be ± 0.5, meaning that a standardized effect of 0.5 will just be significant. A power analysis was performed to assess the number of animals needed to replicate the results we observed in the adult animals (Fig. 4) in a simple, planned comparison. To obtain 80% power, 11 animals per group are suggested to replicate the results of the latency results while 25 animals per group are suggested for the frequency results.

Stepwise selection of interaction effects—In order to select the best-fitting model for each behavioral variable, the analysis was run using a bidirectional stepwise selection of interaction effects as opposed to the full interaction model. Similar to the full model, the majority of behavioral variables associated with DCX+ cell numbers are found in the exploration phases describing the same response to the novel environment, i.e., in older animals, higher numbers of DCX+ cells are associated with slower exploration.

Permutation test—In order to account for correlations between behavioral variables, a permutation test was performed. The test was performed on the *P*-values obtained from the full interaction model, where both the *P* values for the main effects as well as all possible interaction effects were tested. The experimental phases where the results of the permutated data and the actual data are significantly different are the first exploration phases (DCX age interaction $P < 0.001$), second exploration phase (DCX strain interaction $P = 0.04$, DCX main effect $P = 0.03$), impulsivity phase training phase 0 (Ki67 triple interaction $P = 0.01$), test phase (Ki67 age interaction $P = 0.03$) and light and dark box (Ki67 age interaction $P = 0.01$).

The common denominator which can be observed in these alternative analyses is one confirming the original analysis, where specifically in the older animals more DCX+ cells are associated with variables measuring exploration. Within the group of older mice, higher numbers of DCX+ cells are associated with a lower reactivity to the novel environment.

DISCUSSION

We tested different groups of mice with large differences in basal adult hippocampal neurogenesis due age and genetic differences between strains. By using these naturally occurring differences in neurogenesis, we avoid acute off-target effects of chemical, genetic, or environmental manipulations of adult neurogenesis (Lazic, 2012). The pre-existing, neurogenesis-independent differences between strains and ages remain and are accounted for by adjusting for overall strain and age effects on behavior and testing the within-group association between neurogenesis and behavior.

We did not observe associations between adult neurogenesis and the vast majority of behavioral readouts. The findings in the anxiety tests are particularly interesting given that the nature of reported relationships are ambiguous (Bergami et al., 2008; Revest et al., 2009; Fuss et al., 2010b). Our findings support a recent meta-analysis (Groves et al., 2013) that did not find an overall effect of neurogenesis on commonly used anxiety testing paradigms, attributing this phenotype to factors other than adult neurogenesis that differ between the groups tested. Novelty within the experimental setup as one such factor is discussed below.

The association we found is that between neurogenesis and the reactivity to novelty, i.e., in older animals, an increase of DCX+ cells is associated with slower exploration. Lesions studies have shown a hippocampal involvement in novelty processing (Belzung, 1992; Lee et al., 2005). Also, rats living in an enriched environment, which increases neurogenesis, display lower levels of locomotor and novelty-induced activity compared to controls (Bowling et al., 1993; Zambrana et al., 2007) leading to the proposal of a role of neurogenesis in coping with novelty (Kempermann, 2002). Direct evidence for a relationship between adult neurogenesis and reactivity to novelty comes from findings in rats, in which individual differences in the reactivity to novelty correlate with differences in neurogenesis (Lemaire et al., 1999). Furthermore, a recent study investigating the link between adult neurogenesis and anxiety did not find an effect of reduced adult neurogenesis. However, when the test was repeated in a novel environment, an effect of reduced neurogenesis was observed (Deng and Gage, 2015). Subsequent assessments of habituation to the novel environment (Fig. 7 in Deng and Gage, 2015) found a higher activity in the novel environment in animals with low neurogenesis, compared to the less active mice with high neurogenesis. If the reactivity of the animal in a novel environment is a key component in detecting a behavioral effect of adult neurogenesis, then this would affect not only classical anxiety tests, but would extend to a range of behavioral tests where animals are taken out of their home cage to be tested in an experimental set-up. Another classical behavioral paradigm that has been linked to adult neurogenesis is contextual fear conditioning (CFC). Several studies have reported impaired CFC in animals with reduced neurogenesis (Winocur et al., 2006; Ko et al., 2009) while others did not observe any effect of neurogenesis (Zhang et al., 2008; Groves et al., 2013). Trying to reconcile these inconsistencies, Drew et al. (2010) tested the link between neurogenesis and CFC in series of different alterations of the CFC test. Their results show an interaction between freezing and neurogenesis, where animals with reduced

neurogenesis freeze less than wild-type mice, but only when the environment and stimulus are novel to the animal. In our study, the animals were tested in their home (Intelli)cage, allowing us to separate the effects of neurogenesis on the reaction to the novel environment and the actual behavioral testing paradigms, which were performed after the animals became habituated to the cage. The studies linking novelty and adult neurogenesis have used either both sexes (Deng and Gage, 2015) or males (Lemaire et al., 1999; Drew et al., 2010) suggesting that this relationship is not sex-specific.

Changes in the reaction to novelty as a consequence of a dysregulation of neurogenesis have previously been suggested to influence the onset of anxiety and depression-related psychiatric disorders in humans (Kempermann, 2002). Along the same lines, a link was suggested between neurogenesis and mood disorders, post-traumatic stress disorder, borderline personality, and schizophrenia, which are all characterized by an altered stress response to novel contextual changes (Chambers and Conroy, 2007). Our results provide the evidence for interactions between novelty reaction and neurogenesis that are part of these hypotheses.

The decrease of neurogenesis associated with age is well established (Kuhn et al., 1996; Ben Abdallah et al., 2010; Amrein et al., 2011) and this decrease in neurogenesis has often been linked to a decrease in performance in typical hippocampus-dependent tasks, such as the water maze (Gil-Mohapel et al., 2013) and pattern discrimination (Drapeau and Abrous, 2008). Again, results are equivocal as other studies found either no effect or an opposing relationship (Bizon and Gallagher, 2003; Merrill et al., 2003; Bizon et al., 2004). Our results indicate that, specifically in older animals, the level of neurogenesis associates with the way a novel environment was explored, whereas this relation was not seen in the younger animals. Previous studies that show an effect of neurogenesis on hippocampal-dependent tasks often use group-level correlations. They may not be representative of the within-group associations described here and are therefore difficult to compare to our data. In our own data, between-group differences can show a reversed relationship compared to the within-group analyses (Simpson's effect: Simpson, 1951), i.e., C57BL/6 mice with overall more neurogenesis have a higher frequency of pokes compared to DBA mice (Fig. 4) even though high neurogenesis decreases visit frequency within groups. Also, the within-group relation between neurogenesis and behavior may depend on the stage at which neurogenesis is being assessed (proliferation or long-term integration; Bizon and Gallagher, 2003; Bizon et al., 2004). Finally, our study was not aimed at changes in behavior during aging, i.e., a relative small age difference of 8 weeks during early adulthood was chosen to differentiate the level of neurogenesis and not behavior. While we took several steps to control the false-positive rate in the exploratory part of this experiment, neither the association between neurogenesis and novelty nor the age-specific effects were predicted in advance. These results therefore require replication, but also introduce novelty as a factor that needs to be considered when planning experiments and interpreting those already published.

Cell numbers are one first estimate of the functional importance of a cell population within the network that it belongs to. A more comprehensive assessment of morphological or physiological parameters and their interactions may allow the definition of associations between neurogenesis and behavior that go beyond those reflected in cell numbers. When combined with the large-scale behavioral phenotyping, cell numbers are, however, able to provide a differentiated view on structure–function relationships, and this view may well be extended from neurogenic to resident cell populations.

Acknowledgment

We thank Stefanie Engler, Ann-Kristina Fritz, and Shihhui Huang for expert technical assistance.

REFERENCES

Ben Abdallah NMB, Slomianka L, Vyssotski AL, Lipp HP. 2010. Early age-related changes in adult hippocampal neurogenesis in C57 mice. Neurobiol Aging 31:151–161.

Akers KG, Martinez-Canabal A, Restivo L, Yiu AP, De Cristofaro A, Hsiang H-L, Wheeler AL, Guskjolen A, Niibori Y, Shoji H, Ohira K, Richards BA, Miyakawa T, Josselyn SA, Frankland PW. 2014. Hippocampal neurogenesis regulates forgetting during adulthood and infancy. Science 344:598–602.

Amrein I, Isler K, Lipp H-PP. 2011. Comparing adult hippocampal neurogenesis in mammalian species and orders: Influence of chronological age and life history stage. Eur J Neurosci 34:978–987.

Belzung C. 1992. Hippocampal mossy fibres: Implication in novelty reactions or in anxiety behaviours? Behav Brain Res 51:149–155.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc 57:289–300.

Bergami M, Rimondini R, Santi S, Blum R, Götz M, Canossa M. 2008. Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc Natl Acad Sci USA 105:15570–15575.

Binder E, Droste SK Ohl F., Reul JMHM. 2004. Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice. Behav Brain Res 155:197–206.

Bizon JL, Gallagher M. 2003. Production of new cells in the rat dentate gyrus over the lifespan: Relation to cognitive decline. Eur J Neurosci 18:215–219.

Bizon JL, Lee HJ, Gallagher M. 2004. Neurogenesis in a rat model of age-related cognitive decline. Aging Cell 3:227–234.

Bowling SL, Rowlett JK, Bardo MT. 1993. The effect of environmental enrichment on amphetamine-stimulated locomotor activity, dopamine synthesis and dopamine release. Neuropharmacol 32: 885–893.

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG. 2003. Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. Eur J Neurosci 17:2042–2046.

Chambers RA, Conroy SK. 2007. Network modeling of adult neurogenesis: Shifting rates of neuronal turnover optimally gears network learning according to novelty gradient. J Cogn Neurosci 19:1–12.

Clark PJ, Kohman RA, Miller DS, Bhattacharya TK, Brzezinska WJ, Rhodes JS. 2011. Genetic influences on exercise-induced adult hippocampal neurogenesis across 12 divergent mouse strains. Genes Brain Behav 10:345–353.

Clelland CD, Choi M, Romberg C, Clemenson GD, Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker Ra, Gage FH, Bussey TJ. 2009. A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325:210–213.

Deng W, Gage FH. 2015. The effect of immature adult-born dentate granule cells on hyponeophagial behavior is related to their roles in learning and memory. Front Syst Neurosci 9:34.

Drapeau E, Abrous DN. 2008. Stem cell review series: Role of neurogenesis in age-related memory disorders. Aging Cell 7:569–589.

Drew M, Denny C, Hen R. 2010. Arrest of adult hippocampal neurogenesis in mice impairs single-but not multiple-trial contextual fear conditioning. Behav Neurosci 124:446–454.

Fuss J, Ben Abdallah NMB, Hensley FW, Weber K-JJ, Hellweg R, Gass P. 2010a. Deletion of running-induced hippocampal neurogenesis by irradiation prevents development of an anxious phenotype in mice. PLoS One 5:1–9.

Fuss J, Ben Abdallah NMB, Vogt MA, Touma C, Pacifici PG, Palme R, Witzemann V, Hellweg R, Gass P. 2010b. Voluntary exercise induces anxiety-like behavior in adult C57BL/6J mice correlating with hippocampal neurogenesis. Hippocampus 20:364–376.

Galsworthy MJ, Amrein I, Kuptsov Pa, Poletaeva II, Zinn P, Rau A, Vyssotski A, Lipp H-P. 2005. A comparison of wild-caught wood mice and bank voles in the Intellicage: Assessing exploration, daily activity patterns and place learning paradigms. Behav Brain Res 157:211–217.

García-Capdevila S, Portell-Cortés I, Torras-Garcia M, Coll-Andreu M, Costa-Miserachs D. 2009. Effects of long-term voluntary exercise on learning and memory processes: Dependency of the task and level of exercise. Behav Brain Res 202:162–170.

Gil-Mohapel J, Brocardo PS, Choquette W, Gothard R, Simpson JM, Christie BR. 2013. Hippocampal neurogenesis levels predict WATERMAZE search strategies in the aging brain. PLoS One 8: 1–19.

Groves JOJ, Leslie I, Huang GG-J, McHugh SBS, Taylor A, Mott R, Munafò M, Bannerman DM, Flint J. 2013. Ablating adult neurogenesis in the rat has no effect on spatial processing: Evidence from a novel pharmacogenetic model. PLoS Genet 9:e1003718-

Gubner NR, Wilhelm CJ, Phillips TJ, Mitchell SH. 2010. Strain differences in behavioral inhibition in a Go/No-go task demonstrated using 15 inbred mouse strains. Alcohol Clin Exp Res 34:1353–1362.

Gundersen HJG, Jensen EBV, Kiêu K, Nielsen J. 1999. The efficiency of systematic sampling in stereology - Reconsidered. J Microsc 193:199–211.

Huang G-J, Smith AL, Gray DHD, Cosgrove C, Singer BH, Edwards A, Sims S, Sim S, Parent JM, Johnsen A, Mott R, Mathis D, Klenerman P, Benoist C, Flint J. 2010. A genetic and functional relationship between T cells and cellular proliferation in the adult hippocampus. PLoS Biol 8:e1000561-

Ingram DK, London ED, Reynolds MA, Waller SB, Goodrick CL. 1981. Differential effects of age on motor performance in two mouse strains. Neurobiol Aging 2:221–227.

Kelley K, Maxwell SE, Rausch JR. 2003. Obtaining power or obtaining precision. Delineating methods of sample-size planning. Eval Heal Prof 26:258–287.

Kempermann G, Gage FH. 2002. Genetic determinants of adult hippocampal neurogenesis correlate with acquisition, but not probe trial performance, in the water maze task. Eur J Neurosci 16:129–136.

Kempermann G, Kuhn HG, Gage FH. 1998. Experience-induced neurogenesis in the senescent dentate gyrus. J Neurosci 18:3206–3212.

Kempermann G. 2002. Regulation of adult hippocampal neurogenesis - Implications for novel theories of major depression. Bipolar Disord 4:17–33.

Klaus F, Amrein I. 2012. Running in laboratory and wild rodents: Differences in context sensitivity and plasticity of hippocampal neurogenesis. Behav Brain Res 227:363–370.

Knoth R, Singec I, Ditter M, Pantazis G, Capetian P, Meyer RP, Horvat V, Volk B, Kempermann G. 2010. Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One 5:e8809-

Ko H-G, Jang D-J, Son J, Kwak C, Choi J-H, Ji Y-H, Lee Y-S, Son H, Kaang B-K. 2009. Effect of ablated hippocampal neurogenesis on the formation and extinction of contextual fear memory. Mol Brain 2:1-

Kobayashi Y, Sano Y, Vannoni E, Goto H, Suzuki H, Oba A, Kawasaki H, Kanba S, Lipp H-P, Murphy NP, Wolfer DP, Itohara S. 2013. Genetic dissection of medial habenula-interpeduncular nucleus pathway function in mice. Front Behav Neurosci 7:17-

Kuhn HG, Dickinson-Anson H, Gage FH. 1996. Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027–2033.

Kuhn M. 2014. caret: Classification and Regression Training. http://CRAN.R-project.org/package=caret

Lazic SE, Fuss J, Gass P. 2014. Quantifying the behavioural relevance of hippocampal neurogenesis. PLoS One 9:e113855-

Lazic SE. 2010. Relating hippocampal neurogenesis to behavior: The dangers of ignoring confounding variables. Neurobiol Aging 31: 2169–2171.

Lazic SE. 2012. Using causal models to distinguish between neurogenesis-dependent and -independent effects on behaviour. J R Soc Interface 9:907–917.

Lee I, Hunsaker MR, Kesner RP. 2005. The role of hippocampal subregions in detecting spatial novelty. Behav Neurosci 119:145–153.

Lemaire V, Aurousseau C, Le Moal M, Abrous DN. 1999. Behavioural trait of reactivity to novelty is related to hippocampal neurogenesis. Eur J Neurosci 11:4006–4014.

Lhotellier L, Cohen-Salmon C. 1989. Genetics and senescence. I. Age-related changes in activity and exploration in three inbred strains of mice. Physiol Behav 45:491–493.

Van Loo PL, Mol JA, Koolhaas JM, Van Zutphen BF, Baumans V. 2001. Modulation of aggression in male mice: Influence of group size and cage size. Physiol Behav 72:675–683.

Madani R, Kozlov S, Akhmedov A, Cinelli P, Kinter J, Lipp H-P, Sonderegger P, Wolfer DP. 2003. Impaired explorative behavior and neophobia in genetically modified mice lacking or overexpressing the extracellular serine protease inhibitor neuroserpin. Mol Cell Neurosci 23:473–494.

Merrill Da, Karim R, Darraq M, Chiba Aa, Tuszynski MH. 2003. Hippocampal cell genesis does not correlate with spatial learning ability in aged rats. J Comp Neurol 459:201–207.

Onksen JL, Briand LA, Galante RJ, Pack AI, Blendy JA. 2012. Running-induced anxiety is dependent on increases in hippocampal neurogenesis. Genes Brain Behav 11:529–538.

Overall RW, Walker TL, Leiter O, Lenke S, Ruhwald S, Kempermann G. 2013. Delayed and transient increase of adult hippocampal neurogenesis by physical exercise in DBA/2 mice. PLoS One 8: e83797-

Pinkston JW, Lamb RJ. 2011. Delay discounting in C57BL/6J and DBA/2J mice: Adolescent-limited and life-persistent patterns of impulsivity. Behav Neurosci 125:194–201.

Van Praag H, Kempermann G, Gage FH Praag H., Van. 1999. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.

Revest J-M, Dupret D, Koehl M, Funk-Reiter C, Grosjean N, Piazza P-V, Abrous DN. 2009. Adult hippocampal neurogenesis is involved in anxiety-related behaviors. Mol Psych 14:959–967.

Richter SH, Garner JP, Auer C, Kunert J, Würbel H. 2010. Systematic variation improves reproducibility of animal experiments. Nat Methods 7:167–168.

Safi K, Neuhäusser-Wespy F, Welzl H, Lipp H-P. 2006. Mouse anxiety models and an example of an experimental setup using unconditioned avoidance in an automated system. Cogn Brain Behav 10: 475–488.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R. 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805–809.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E. 2002. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578–584.

Simpson E. 1951. The interpretation of interaction in contingency tables. J R Stat Soc Ser B 13:238–241.

Slomianka L, West MJ. 2005. Estimators of the precision of stereological estimates: An example based on the CA1 pyramidal cell layer of rats. Neuroscience 136:757–767.

Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM. 2005. A role for adult neurogenesis in spatial long-term memory. Neuroscience 130: 843–852.

Tingley D, Yamamoto T, Hirose K, Keele L, Imai K. 2014. Mediation: R package for causal mediation analysis. J Stat Softw 59:1–38.

Vannoni E, Voikar V, Colacicco G, Sánchez MA, Lipp H-P, Wolfer DP. 2014. Spontaneous behavior in the social homecage discriminates strains, lesions and mutations in mice. J Neurosci Met 234:26–37.

Venables WN, Ripley BD. 2002. Modern Applied Statistics with S. Fourth. New York: Springer.

West MJ, Slomianka L, Gundersen HJ. 1991. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231: 482–497.

Wickham H. 2009. ggplot2: Elegant graphics for data analysis. New York: Springer.

Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S. 2006. Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296–304.

Zambrana C, Marco EM, Arranz L, De Castro NM, Viveros MP, De La Fuente M. 2007. Influence of aging and enriched environment on motor activity and emotional responses in mice. Ann N Y Acad Sci 1100:543–552.

Zhang C-L, Zou Y, He W, Gage FH, Evans RM. 2008. A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 451:1004–1007.