Supplementary Materials for

Emergence of Individuality in Genetically Identical Mice

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Materials and Methods

Animals and experimental groups

60 female C57BL/6N mice were obtained from a commercial breeder at 4 weeks of age. The breeder follows a strict "International Genetic Standardization (IGS) Program" (http://www.criver.com/SiteCollectionDocuments/rm_rm_r_IGS.pdf), including a sophisticated pyramidal breeding scheme and SNP-based genetic testing to certifying genetic authenticity. We aimed at receiving mice from as many different litters as possible.

The animals were tagged with RFID transponders (see below) and acclimatized for 1 week in groups of ten. At the start of the experiment, the mice were randomly assigned to three groups and put into the respective housing (described in detail below): 40 animals were held in enrichment (ENR), 8 animals constituted the baseline group and 12 animals served as control group (CTR). Animals of all groups were provided with bedding and nesting material as well as food and water ad libitum and were held in a constant light/dark cycle of 12h.

The ENR and CTR animals spent a total of 105 days in their enclosures. Three weeks before the end of the experiment, they received intraperitoneal injections of thymidine analog BrdU (5-Bromo-2′-deoxyuridine; 50 mg/kg body weight), one per day for three consecutive days. The baseline group was injected with BrdU at the start of the experiment and was killed 3 weeks later (see experimental schedule, Fig. 1B). The interval of 3 weeks between labeling with BrdU and perfusion ensured that only the cells which had successfully differentiated into neurons were taken into the analysis (see histology section below).

Preceding perfusion, the animals were deeply anesthetized with a mixture of ketamine and xylazine (100 mg/kg body weight and 10 mg/kg body weight in saline solution). The mice were transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde. The brains were removed from the skull, dissected, and postfixed in 4% paraformaldehyde in phosphate buffered saline. After 24h, the brains were transferred to

30% sucrose in 0.1 M phosphate buffer, pH 7.4, where they were kept until they had sunk.

All experiments were carried out according to national laws and institutional guidelines and were approved by the appropriate authorities at Westfälische Wilhelms-Universität Münster (reference no. 8.87-50.10.36.08.250).

Enclosures and RFID tracking system

The 40 ENR animals were kept in a large enriched enclosure which had a 1.75 x 1.75 m ground area and was 2 m in height [see also a detailed general description in (*30*)]. The sides and top were made from wire mesh with a door in the front. Inside, there were five levels connected via acrylic glass tubes so that the mice could move freely to all levels. The total area available to the animals was approx. 5 m^2. On each level, water and food was provided ad libitum. The two topmost levels each had a nesting box, which the mice could enter through an opening in the side and a acrylic glass tube (see Fig. 1A). On all levels, objects were distributed to enrich the environment of the animals (e.g. plastic and cardboard tubes, plastic flower pots, wooden scaffolds, toys, etc.).

The mice had the opportunity to leave the enclosure in the case of high social pressure via a tube in the enclosure's front. The tube led to a water basin which a mouse had to swim through in order to get to an emigration cage (Fig. 1A). The mice's aversion to swimming kept them from investigating the emigration cage under normal circumstances. None of the animals emigrated during the period of the experiment.

The radio frequency identification (RFID) system served to track the animals in their entire time in the enclosure. The transponders used in this project were passive integrated transponders (diameter: 2.12 mm; length: 11.5 mm) which were subcutaneously injected into the animals' necks (see Fig. 1A) under anaesthesia with Isoflurane.

Throughout the enrichment enclosure, 20 RFID ring antennas, were systematically placed so that any change of the levels as well as the use of water sources and nesting boxes could be detected (Fig. 1A). The control group, as well as the baseline group, were housed as groups of 4 in standard-size type 3 cages. In order to record a basic activity

measure of the control group animals, each of the control cages had an antenna fixed next to a hole in a dividing wall. The wall separated the access to food from access to water. The two baseline group cages had neither dividing walls nor antennas.

The cleaning and maintenance procedures were as follows:

- Once a week, all animals were removed from their respective enclosure, while cleaning and maintenance work was carried out.
- Once a week all animals were given a small amount of dry oat flakes.
- CTR and baseline group cages were changed once a week.
- ENR enclosure:
    - Bedding was replaced in the nesting boxes every other day.
    - Additional bedding was replaced where necessary once a week.
    - Objects destroyed by the mice were replaced (such as cardboard tubes etc.) where necessary once a week.
    - Once a month, the enclosure was completely cleaned and bedding replaced.
    - Over the whole experimentation time, the ENR enclosure was entered only by one person wearing overshoes.
- Some small amount of the old bedding was distributed over the fresh one in order to increase familiarity and, thus, decrease stress for the animals when re-entering the enclosures
- Food and water sources were cleaned and refilled once a week or when necessary.

Whenever a mouse approached close proximity of an antenna, the passive integrated transponder in the mouse's neck transmitted a unique ten-digit number identifying the mouse. Data acquisition was carried out with software especially developed for these purposes by the Institute for Geoinformatics in Münster, and a MySQL database was employed for data storage. Before the experiment was started, cage geometry and antenna positions were digitally stored. Upon an antenna contact, an event was stored in the database containing a unique, numeric identifier for the event, the numeric mouse identifier, a numeric antenna identifier, and a timestamp in millisecond resolution.

Histology

After tissue fixation, the brains were collected and cut into 40 µm coronal sections on a dry-ice-cooled copper block on a sliding microtome and cryoprotected. To visualise and quantify the amount of surviving newly synthesized cells, the sections were subjected to a staining procedure using an antibody against BrdU as follows: in order to block endogenous peroxidase reactions, the free-floating brain sections were pre-treated with 0.6% H_2O_2 for 30 minutes. All intermediate washing steps were done with tris-buffered saline (TBS), pH 7.4. DNA was denatured in 2N HCl for 30 minutes at 37°C. The sections were subsequently treated with TBS++ (TBS supplemented with 0.2% TritonX-100 and 10% donkey serum) for 2h in order to block unspecific antibody binding. The rat anti-BrdU antibody (AbD Serotec) was diluted 1:500 in TBS+ (TBS with 0.2% TritonX-100 and 3% donkey serum) and the sections were incubated overnight at 4°C. After washing out the primary antibody and a blocking step in TBS++, a 2-hour incubation with the biotinylated secondary antibody (donkey anti-rat, Jackson Immunoresearch), diluted 1:250 in TBS+, followed. ABC reagent was applied for 1h according to the manufacturer's instructions. Diaminobenzidine (DAB) was used as a chromogen at the concentration of 0.25 mg/ml in TBS with 0.01% H_2O_2 and 0.08% nickel chloride followed by rinsing with tap water and TBS.

The stained sections were mounted onto glass slides, dried and subsequently cleared in Neo-Clear®, coverslipped with Neo-Mount® and the amount of surviving BrdU-labeled cells in the subgranular zone of the dentate gyrus in the hippocampus was assessed. All labeled cells in the dentate gyrus were counted in every 6th hippocampus-containing section. Both hemispheres were counted and the resulting cell number was multiplied by six in order to estimate the total amount of surviving cells per brain.

To phenotype the new cells, triple immunohistochemistry for BrdU, astrocytic marker S100ß and neuronal marker NeuN were performed. After DNA denaturation and blocking of unspecific binding (see above), the primary antibodies were applied in the following concentrations: rat anti-BrdU 1:500 (AbD Serotec), rabbit anti-S100ß 1:1000 (Abcam) and mouse anti-NeuN 1:100 (Chemicon). The incubation took place overnight at 4°C. Secondary antibodies were fluorochrome-coupled anti-rabbit, anti-mouse, and

anti-rat (1:250, Jackson Laboratories). Sections were mounted and coverslipped with Aqua Poly/Mount®. Data on astrocytic differentiation are presented in Fig. S1.

All quantitative analyses were done in a blinded fashion and by the same researcher using a Zeiss Apotome. At least 100 randomly selected BrdU-positive cells per animal were phenotyped. Absolute numbers of BrdU-positive cells were determined using a modified version of the optical fractionator method, as described elsewhere (*31*). Multiplying the number of BrdU-positive cells with the ratio of the phenotypes yielded the absolute numbers of new cells per dentate gyrus.

Reduction, Aggregation, and Statistical Analysis of Behavioral Data

Over the course of the experiment, the RFID-based tracking system recorded more than 7.6 million events, each describing date and time of a mouse changing its location from the tuning range of one to that of a different antenna in the cage. Several data reduction and aggregation steps were taken to permit a quantitative analysis of this vast amount of information. First, given that mice are nocturnal animals, only night segments (i.e. dark phases in the cage from 8 PM to 8 AM) were considered. Next, discrete time series were generated for each mouse and night segment. Time series were discretized to blocks of 5*s* length; for each block, the antenna contact last observed within the five-second-segment was recorded. This procedure resulted in an ordinal time series with 8640 segments per night for each mouse. The domain of the resulting discrete time series is the set of antenna identifiers. From the observed frequencies in these time series, we estimated probabilities of a mouse *i* being at antenna *j* at day *t*, denoted as $p_{i,j,t}$. These probabilities are the basis for computing the roaming distribution, and, by implication, the roaming entropy (RE), which is defined as the Shannon entropy of the roaming distribution of mouse *i* at day *t*:

$$\mathrm{RE}_{i,t} = \sum_{j=1}^{k} \left(p_{i,j,t} \log p_{i,j,t} \right) / \log(k) \qquad (1)$$

In Equation 1, k is the number of antennas in the cage ($k = 20$). Dividing the entropy by the factor $\log(k)$ scales the RE to the range from zero to unity. RE quantifies differences in territorial coverage. It is at minimum for mice that remain at the same place over the course of a night, and reaches its maximum for mice that spend equal amounts of time at

each antenna. Visiting fewer antennas or spending more time at fewer locations decreases RE, whereas visiting more antennas and spending less time in limited areas of the environment increases RE.

Whenever the experimental logbook registered an anomaly, the particular day, the day before and the day after were excluded from the analysis. Anomalies included computer failure (e.g., antenna failure or computer reboots) or human interactions (e.g., cage cleaning and maintenance) that disturbed the animals and resulted in increased RE measurements. This led to the exclusion of 53 days of observation. The reduced set of undisturbed observations comprised 43 days. These 43 observations were aggregated into four time periods, each representing the average RE over a time span of 24 calendar days. Cumulative Roaming Entropy (cRE) was obtained by cumulatively summing RE over time at the level of the blocks of 24 days. cRE was modeled as a three-factor growth curve model with the following structural equation (*32*):

$$\mathrm{cRE}_{i,t} = Intercept_i + t \cdot Slope_i + 2^{t-1} \cdot Exp_i + \epsilon_{i,t} \quad (2)$$

In Equation 2, $\mathrm{cRE}_{i,t}$ represents the measurement of cRE for individual *i* at time *t*, the $Intercept_i$, the $Slope_i$, and the Exp_i are latent variables defining the intercept, the linear change score, and the exponential change score, respectively, of individual *i* across four repeated measures T_1 ($t = 0$) to T_4 ($t = 3$). The error term $\epsilon_{i,t}$ is the mouse- and time-specific residual error. Intercept, linear change, and exponential change are assumed to be normally distributed with variances σ_I^2, σ_S^2, and σ_E^2 around their means μ_I^2, μ_S^2, and μ_E^2, respectively. The intercept and linear slope are allowed to covary, whereas intercept and exponential slope, and linear slope and exponential slope are assumed to be independent. The $\epsilon_{i,t}$ are assumed to be normally distributed with identical variance σ_I^2 across time and mean zero, and to be independent of intercept, linear and exponential change, as well as of each other in time. The exponential term models an exponential deviation from a linear growth process with a rate of change that is assumed constant across individuals.

Calculations were also performed without prior cleaning of the data (except for detection failures). In this case, the correlation between cRE and adult neurogenesis (analogous to Fig. 2D) was $r = 0.45$ ($p = 0.0035$). With no cleaning whatsoever the correlation was still $r = 0.44$ ($p = 0.0043$).

Statistics

Data analysis was performed with custom-made scripts in the statistical programming language R (*33*). Group differences were considered at the conventional threshold of $p = 0.05$ and were analyzed using ANOVA and Student's *t* test (unpaired, two-tailed). The structural equation model was estimated with **Ω**nyx (*34*) and estimates were confirmed with Mplus (*35*).

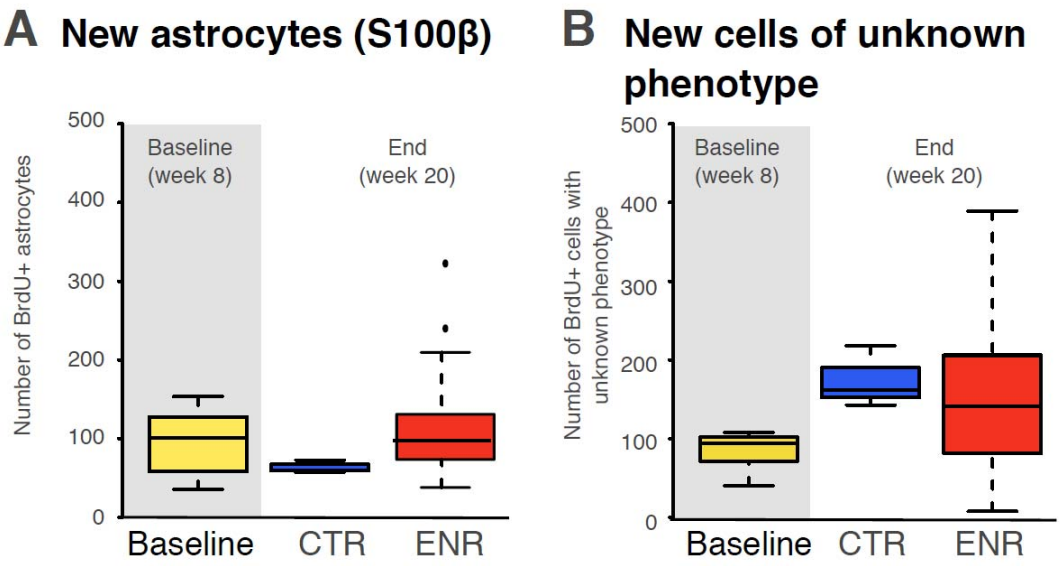

Fig. S1.

The ENR condition also effected the generation of new S100β-positive astrocytes (A) but not that of cells that were BrdU-positive but neither S100β- nor NeuN-positive (B). With age, CTR had a tendency of producing lower numbers of S100b-positive cells compared to baseline (t = 1.989; p = 0.08) and towards more undefined cells (t = 3.698; p = 0.05). ENR significantly increased astrogenesis but not the number of cells with undetermined phenotype..

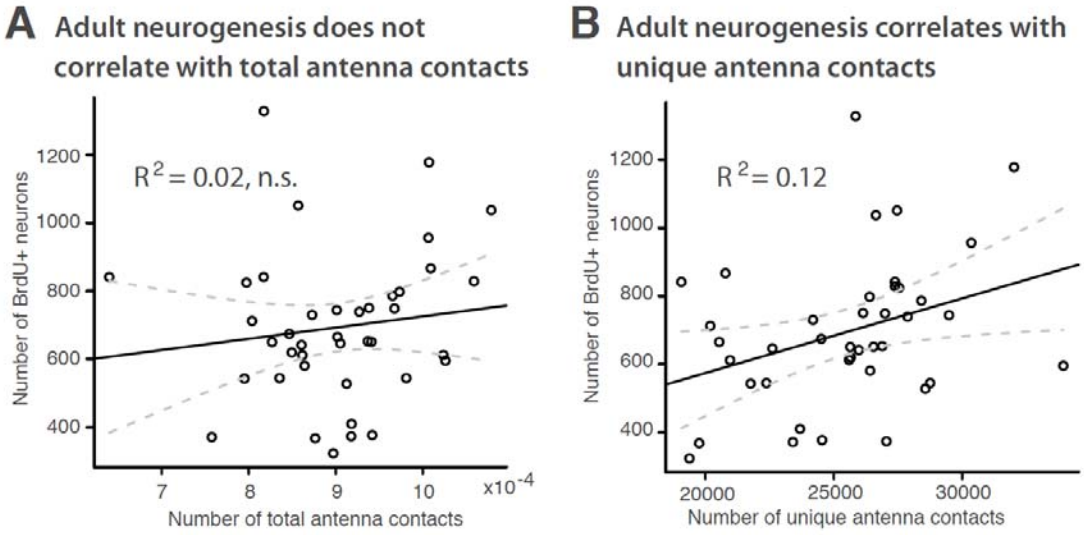

Fig. S2

Individual differences in adult hippocampal neurogenesis were not significantly correlated with the total number of recorded antenna contacts as marker for locomotion (r = 0.133; t = 0.829; p = 0.412). The number of unique, non-repetitive antenna contacts as a proxy of distance traveled showed a significant, but weaker association with adult neurogenesis than RE (r = 0.345; t = 2.268; p = 0.029).

Movies S1 and S2

The animations show the temporal development of the roaming distribution and its entropy for two selected mice from the enriched environment. In the upper part of the animation, the cage is shown from a bird's eye view. Boundaries of cage, levels, and nesting boxes are drawn in white. Antenna positions are depicted as black boxes. The heat map projected onto the cage visualizes the roaming distribution, i.e. the probability of the respective mouse being on a specific location. Low probabilities are shown in blue, medium probabilities in green, and high probabilities in orange. In the lower part of the animation, the temporal development of the roaming entropy, i.e. the entropy of the roaming distribution, is shown. S1 depicts an animal with low decreasing roaming entropy. S2 shows a mouse with high stable roaming entropy.

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