RESEARCH ARTICLE

Differential Effects of Social and Physical Environmental Enrichment on Brain Plasticity, Cognition, and Ultrasonic Communication in Rats

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ABSTRACT

Environmental enrichment (EE) exerts beneficial effects on brain plasticity, cognition, and anxiety/depression, leading to a brain that can counteract deficits underlying various brain disorders. Because the complexity of the EE commonly used makes it difficult to identify causal aspects, we examined possible factors using a 2 × 2 design with social EE (two vs. six rats) and physical EE (physically enriched vs. nonenriched). For the first time, we demonstrate that social and physical EE have differential effects on brain plasticity, cognition, and ultrasonic communication. Expectedly, physical EE promoted neurogenesis in the dentate gyrus of the hippocampal formation, but not in the subventricular zone, and, as a novel finding, affected microRNA expression levels, with the activity-dependent miR-124 and miR-132 being upregulated. Concomitant improvements in cognition were observed, yet social deficits were seen in the emission of prosocial 50-kHz ultrasonic vocalizations (USV) paralleled by a lack of social approach in response to them, consistent with the intense world syndrome/theory of autism. In contrast, social EE had only minor effects on brain plasticity and cognition, but led to increased prosocial 50-kHz USV emission rates and enhanced social approach behavior. Importantly, social deficits following physical EE were prevented by additional social EE. The finding that social EE has positive whereas physical EE has negative effects on social behavior indicates that preclinical studies focusing on EE as a potential treatment in models for neuropsychiatric disorders characterized by social deficits, such as autism, should include social EE in addition to physical EE, because its lack might worsen social deficits. J. Comp. Neurol. 524:1586–1607, 2016.

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Environmental enrichment (EE) is a combination of "inanimate and social stimulation" (Rosenzweig et al., 1978), which is widely used to study experience-dependent changes in rodent brain and behavior. In the laboratory, EE is typically composed of groups of rodents living in large housing cages with objects that are periodically changed to stimulate curiosity and exploration, often in combination with running wheels. EE is thought to improve well-being by helping animals to fulfill some of their ethological needs, while reducing

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the stress of captivity, and is probably the best translational model to investigate positive life experiences acting either as protective or curative factors in neurological and psychiatric disorders (for reviews, see Nithianantharajah and Hannan, 2006; van Praag et al., 2000).

Early studies showed that EE leads to increased brain weight and cortical thickness (Bennett et al. 1969; Diamond et al., 1976; Renner and Rosenzweig, 1986; Rosenzweig et al., 1978), possibly due to dendritic and synaptic growth (Faherty et al., 2003; Leggio et al., 2005; Rampon et al., 2000b). Furthermore, EE promotes adult hippocampal neurogenesis (Kempermann et al., 1997) and the integration of newborn cells into functional circuits (for review, see Kempermann et al., 2010). Such cellular changes are associated with altered expressions of genes involved in synaptic function and cellular plasticity, including increased levels of brain-derived neurotrophic factor (BDNF) and postsynaptic density protein 95 (PSD95) (Kuzumaki et al., 2010; Rampon et al., 2000a), consistent with enhanced glutamatergic signaling and synaptic strength, as revealed by long-term potentiation (Foster and Dumas, 2001; Green and Greenough, 1986). In addition to glutamate, EE affects various other neurotransmitter systems (for review, see Solinas et al., 2010), particularly mesocorticolimbic dopamine (DA; Bezard et al., 2003; Neugebauer et al., 2004), resulting, for instance, in altered psychomotor responses to amphetamine (Bardo et al., 1995; Bowling and Bardo, 1994; Bowling et al., 1993; Cain et al., 2012; Gill et al., 2012).

Regarding cognition, EE leads to improved learning and memory as assessed by spatial mazes (Bennett et al., 2006; Kempermann et al., 1997; Leggio et al., 2005; Nilsson et al., 1999) and novel object recognition (Bruel-Jungerman et al., 2005; Rampon et al., 2000b). EE also affects exploratory behavior and habituation learning (Brenes et al., 2009; Elliott and Grunberg, 2005; Neugebauer et al., 2004; Zimmermann et al., 2001), whereas olfactory social discrimination learning is unchanged (Peña et al., 2006; Rampon et al., 2000b). In addition to learning and memory, EE is known to reduce anxiety- and depression-related behavior (Brenes et al., 2008, 2009; Roy et al., 2001; Schneider et al., 2006). Furthermore, several studies showed that EE can reverse deficits in models of brain dysfunction, including brain damage, dementia, and aging (Bezard et al., 2003; Speisman et al., 2013; van Dellen et al., 2000; Will et al., 1976; Wolf et al., 2006). It has thus been hypothesized that EE leads to a brain that can counteract or compensate for deficits underlying various brain dysfunctions (for review, see Nithianantharajah and Hannan, 2009; van Praag et al., 2000).

In contrast, relatively few studies have examined the effects of EE on social behavior. This is surprising because brain plasticity processes, particularly hippocampal neurogenesis, have been repeatedly linked to social behaviors. Thus, social experiences strongly affect adult neurogenesis, with social defeat (Becker et al., 2008; Czéh et al., 2007) and isolation (Lu et al., 2003; Stranahan et al., 2006) having negative effects, while mimicking rough-and-tumble play through tickling (Wöhr et al., 2009) and mating (Leuner et al., 2010; Spritzer et al., 2009) can lead to increased adult neurogenesis. The few available EE studies mostly found increased social behavior following EE (Green et al., 2010; Laviola et al., 2004; Morley-Fletcher et al., 2003; Neugebauer et al., 2004; Schneider et al., 2006), yet others reported no effects or inconsistent findings (Peña et al., 2006; Renner and Rosenzweig, 1986).

One type of social behavior, namely rodent communication, has not yet been investigated in EE research, but can be studied by means of ultrasonic vocalizations (USV), which serve as socially relevant and situation-dependent affective signals in rats (for review, see Brudzynski, 2013; Wöhr and Schwarting, 2013). The so-called 50-kHz USV are typical for appetitive juvenile social interactions, especially rough-and-tumble play and tickling, or adult mating (Burgdorf and Panksepp, 2001; Burgdorf et al., 2008; Knutson et al., 1998; Panksepp and Burgdorf, 2000; Sales, 1972; Schwarting et al., 2007; Wöhr et al., 2009). Rats also emit 50-kHz USV when being separated from conspecifics, suggesting that they serve a communicative function, namely, to maintain or (re)establish social contact (Schwarting et al., 2007; Wöhr et al., 2008). Such an affiliative function was confirmed by means of playback experiments, showing that 50-kHz USV elicit social approach behavior in recipients, in both males (Wöhr and Schwarting, 2007, 2009, 2012) and females (Willadsen et al., 2014). Importantly, social approach behavior in response to 50-kHz USV is paralleled by phasic dopamine (DA) release in the nucleus accumbens (Willuhn et al., 2014), supporting the idea of a perception-and-action-loop because 50-kHz USV can be triggered by electrical stimulation of the mesolimbic DA system (Burgdorf et al., 2000) and by DAergic psychostimulants, such as amphetamine (for review, see Rippberger et al., 2015).

The present study was designed to determine the differential effects of social and physical EE during adolescence on brain plasticity, cognition, and ultrasonic communication in rats. In general, the complexity of EE commonly used in the laboratory makes it difficult to identify specific factors causing such changes. Therefore, the exact causes of the EE effects are the subject

of considerable speculation (for review, see Kempermann et al., 2010). Typically, EE consists of a combination of both social and physical factors, hindering the assessment of their single contributions, particularly when animals raised under EE conditions are housed in groups, whereas controls are housed individually, i.e., exposed to social isolation, which is known to have adverse effects on brain and behavior (Fone and Porkess, 2008). Only recently have studies trying to separate out these components been conducted, mainly focusing on social isolation effects on locomotor activity, anxiety-related behavior, and spatial cognition, or body weight gain and feeding (Elliott and Grunberg, 2005; Schrijver et al., 2002; Zaias et al., 2008).

Here, we used a 2 $\times$ 2 experimental design with the factors social (two vs. six rats per cage) and physical (enriched vs. nonenriched cages) EE, resulting in four experimental housing conditions: standard control (CO: two rats in a nonenriched cage), social enrichment (SE: six rats in a nonenriched cage), physical enrichment (PE: two rats in an enriched cage), and physical plus social enrichment (PESE: six rats in an enriched cage). This allowed us to separate out the contribution of individual EE components, namely, social EE and physical EE. Importantly, we employed an EE protocol without the often used running wheels, which allowed us to exclude the possibility that potential EE effects might simply be attributed to running exercise. As measures for experience-dependent brain plasticity we assessed hippocampal neurogenesis, i.e., cell proliferation (proliferating cell nuclear antigen [PCNA]) and survival (5-bromodeoxyuridine [BrdU]) in the dentate gyrus (DG), the immediate early gene and transcription factor c-fos, the cAMP response binding protein1 (CREB1), and changes in microRNA (miRNA) expression, focusing on miRNAs that are activity-regulated and involved in postnatal neuronal development and plasticity (miR-124, -132, and -137; for review, see Schratt, 2009; McNeill and Van Vactor, 2012). At the behavioral level, we studied cognition by means of a habituation learning paradigm and the place object recognition test (PORT), both strongly associated with the brain plasticity processes assessed. Finally, we determined whether EE leads to changes in social behavior and ultrasonic communication, including both sender and receiver, by means of our established 50-kHz USV radial maze playback paradigm (for review, see Seffer et al., 2014).

MATERIALS AND METHODS

Animals

Forty-eight juvenile male Wistar rats (HsdCpb:WU; Harlan-Winkelmann, Horst, The Netherlands) were group-housed (six per cage) on postnatal day (PND) 21 and kept in a climate-controlled room with a 12:12-hour light–dark schedule (lights on at 07:00 hours). On PND22–25, they were handled for 4 consecutive days. Because emission of 50-kHz ultrasonic vocalizations (USV) is characterized by considerable interindividual variability (Schwarting et al., 2007; Wöhr et al., 2009), experimental groups were counterbalanced according to interindividual 50-kHz USV emission rates by means of the cage test, a simple and reliable test for assessing 50-kHz USV (Schwarting et al., 2007; Natusch and Schwarting, 2010; Wöhr et al., 2008). The cage test was conducted on 2 consecutive days (PND26–27) for 5 minutes each. Based on the average number of 50-kHz USV, rats were split into the four experimental housing conditions, resulting in groups that did not differ in spontaneous emission of 50-kHz USV ($P >$ 0.050). All animal work was conducted according to the relevant national and international guidelines.

Experimental housing conditions

A modified version of our routine EE protocol without running wheels was implemented (Brenes et al., 2009). Rats (n = 12 per condition) were kept for 5 weeks (PND33–69) in one of the following conditions: standard control (CO: two rats in a nonenriched cage), social enrichment (SE: six rats in a nonenriched cage), physical enrichment (PE: two rats in an enriched cage), and physical plus social enrichment (PESE: six rats in an enriched cage). Briefly, PESE rats were housed in commercial pet cages (85 cm length $\times$ 46 cm width $\times$ 75 cm height), which consisted of two rectangular wooden platforms (46 $\times$ 23 cm each) placed at 30 cm and 51 cm above the cage floor, respectively, two wooden stairs, three metal food dispensers, and a stainless steel grid with a bottle of water placed over it. All other groups were housed in normal polycarbonate Macrolon type IV cages (size: 380 $\times$ 200 $\times$ 590 mm, plus high stainless steel covers). The PE/PESE cages contained the following items: bedding, pieces of towel paper, roll papers, grass nests (three for the PESE cage and one for each PE cage), wood sticks, feeding balls filled with towel paper and food pellets, cardboard boxes, and nonchewable plastic, glass and metal objects. Except for grass nests, bedding, and towel papers, enriching items were only temporarily available in the cages. Two or three times a week, half of these objects were either relocated or replaced by a new set of similar objects. As a nutritional stimulus, 10 g of sweetened puffed wheat cereals (Smacks, Kellogg's) were randomly hidden at different places inside the PE/PESE cages once or twice a week. In all groups, food (Altromin, Lage, Germany) and water (0.0004% HCL-solution) were available ad libitum. Except for behavioral testing and cage cleaning, animals

remained undisturbed. Cage cleaning, including changes of bedding material, was carried out at least once a week.

Behavioral characterization

Once a week during 4 consecutive weeks, rats were tested in the cage test and open field paradigms (PND41, PND48, PND56, and PND63). During the fourth week, playback of prosocial 50-kHz USV (PND64–65) and place object recognition test (PORT; PND66-67) were conducted. In the fifth week, the rats were treated with 2.5 mg/kg (intraperitoneally [i.p.]) amphetamine (PND68–69; the results of this experiment are reported elsewhere). At about 45 minutes after amphetamine injection, rats were transcardially perfused. In all behavioral tests, equipment was thoroughly cleaned with 0.1% acetic acid solution between subjects. Test order was counterbalanced within and across testing days.

Cage test

Our routine cage test protocol was used (Schwarting et al., 2007; Natusch and Schwarting, 2010; Wöhr et al., 2008). Briefly, a given rat was separated from conspecifics and individualized in a clean cage (type III) with fresh bedding (Tapvei) for a 5-minute 50-kHz USV recording session. For USV detection, an ultrasonic microphone was mounted centrally at 35 cm above the floor of the cage, which was illuminated by dim red light (~7 lx). Locomotor activity (the number of cage-halves crossed with at least three paws) and rearing behavior were scored off-line from videotapes.

Open field

As previously described (Schwarting et al., 2007; Wöhr and Schwarting, 2008), open field activity was automatically monitored (TruScan, Photo beam Sensor-E63-22, Coulbourn Instruments, Allentown, PA) for 10 minutes in two acrylic boxes (40 × 40 × 40 cm) equipped with two grids of infrared sensor beams mounted horizontally 2.5 cm and 14.5 cm above the floor that measured locomotion (distance traveled in m) and rearing (n), respectively. For USV detection, an ultrasonic microphone was mounted centrally at 45 cm above the floor of the box. Open field testing was conducted immediately after the cage test under dim red light (~7 lx).

Playback of prosocial 50-kHz USV

Testing for social approach behavior in response to playback of prosocial 50-kHz USV was performed by using our established 50-kHz USV radial maze playback paradigm (Wöhr and Schwarting, 2007, 2009, 2012; for review, see Seffer et al., 2014). Acoustic stimuli presented were: 1) natural 50-kHz USV recorded from an adult male Wistar rat during exploration of a cage containing scents from a cage mate (Fig. 5A); and 2) time- and amplitude-matched white noise, which served as a control for novelty-induced changes in behavior (Fig. 5B). Both stimulus types were presented at ~69 dB (measured from a distance of 40 cm) for 1 minute with a sampling rate of 192 kHz in a 16-bit format. Playback of acoustic stimuli was monitored by two ultrasonic microphones. Each rat was exposed to both stimulus types in a counterbalanced manner with an inter-stimulus interval of 10 minutes after being habituated to the elevated radial arm maze for 15 minutes. Testing was performed under dim red light (~10 lx) in a testing room with no other rats present.

Place object recognition test (PORT)

The test apparatuses were two open field chambers (60 × 60 × 60 cm; Eckart et al., 2012) illuminated with red light (~10 lx) and equipped with a white plastic disc (5 cm diameter each) attached to the middle of one of the walls at 40 cm above the box floor to facilitate spatial orientation. The test consisted of three parts, i.e., habituation, sample trial, and test trial. On the first day (habituation trial), animals were allowed to habituate to the empty open fields for 5 minutes. Twenty-four hours later they were exposed to two identical objects for 5 minutes (sample trial), either two silver iron cylinders (5 cm in diameter, 8 cm high) or two solid glass pillars (6 cm in diameter, 8 cm high). Objects and spatial location were counterbalanced across groups. In the sample trial, objects were placed in the back corners of the box, with the objects situated 15 cm away from the walls. During the intertrial interval animals were returned to their home cages for 30 minutes. Afterwards, rats were again allowed to explore the objects (test trial), but now one of them was displaced to one of the front corners of the open field. Object exploration was scored whenever the rat's nose touched the object or when it was directed toward it within a distance of 2 cm. Climbing onto an object was not recorded as exploration unless the snout of the rat was directed towards it by less than 2 cm. The relative amount of time spent exploring the displaced object as compared with the nondisplaced one was taken as a memory index [exploration time displaced object/(exploration time displaced object + exploration time stationary object) × 100]. Object exploration, locomotor activity (the number of lines crossed with at least three paws), and rearing behavior were manually scored off-line from videotapes.

Ultrasonic recording and analysis

USV were monitored with UltraSoundGate Condenser Microphones (CM16; Avisoft Bioacoustics, Berlin,

Germany) and recorded with Avisoft Recorder 2.7 software (sampling rate: 214,285 Hz; format: 16 bit). High-resolution spectrograms (frequency resolution: 0.488 kHz, time resolution: 0.512 ms) were obtained after a fast Fourier transformation (512 FFT-length, 100% frame, Hamming window, 75% time window overlap), by using the Avisoft SASLabPro 4.38 software. Experienced observers manually counted the numbers of USV, with USV emitted within a frequency range of 20–32 kHz being considered as 22-kHz USV and USV between 32 and 96 kHz as 50-kHz USV (Schwarting et al., 2007). If two 50-kHz USV elements were at least 0.048 seconds apart, two independent 50-kHz USV were counted. Any change in peak frequency higher than 5 kHz either within a single 50-kHz USV element, as the zigzag shape in TRILL calls, or between two or more overlapped 50-kHz USV elements, as in STEP calls, was considered as a modulation in peak frequency (frequency-modulation [FM]). Therefore, a FLAT call was scored when peak frequency changes within a single call element were equal to or lower than 5 kHz. However, the difference between the start and the end peaks could be higher than 5 kHz, i.e., in 50-kHz USV with a flat shape in either an upward or downward direction. When a fundamental flat 50-kHz USV had at least one short flat element overlapping at the start and/or at the end of the 50-kHz USV, a STEP was counted. At least one of these short steps had to be 5 kHz higher than the fundamental call. TRILL calls were considered when at least one frequency-modulation occurred or when at least two frequency peaks in opposed directions were 5 kHz apart within a single call.

Neuronal characterization

In addition to the behavioral tests, the rate of newborn cells in the subventricular zone (SVZ) and the DG of the hippocampal formation was investigated. To this aim, animals were treated with BrdU (Sigma, St. Louis, MO), which allows the identification of cells that undergo division when BrdU is systemically available, i.e., during the injection period (Höglinger et al., 2004). BrdU was dissolved at 5 mg/ml in 0.9% NaCl and injected i.p. at doses of 100 mg/kg from PND48–52 (see Fig. 1A; 5 consecutive days according to Wöhr et al., 2009). Solutions were prepared freshly each day. The last BrdU dose was at least 15 days prior to the day of perfusion (PND69). This interval is thought to give labeled cell sufficient time to differentiate and mature. In addition, we used an endogenous marker of cell division, PCNA, as a punctual measure of ongoing cell proliferation occurring only at the time of perfusion (Höglinger et al., 2004). Finally, the expression of c-Fos and CREB1 mRNA and different miRNAs was determined by the isolation of RNA from whole-brain slices. The expression of c-Fos and CREB1 mRNA was used as an indirect marker of neural activity, whereas changes in precursor (pre-) and mature miR-124 and miR-132 were taken as indicative of brain plasticity processes induced by EE. As a control, pre-miR-137 was used, which was not expected to vary with treatments.

Tissue preparation and immunohistochemistry

Rats were sacrificed ~45 minutes after amphetamine administration (PND69) with 100 mg/kg pentobarbital i.p. (Merial, Hallbergmoos, Germany) and perfused transcardially using 0.9% saline followed by 4% (wt/vol) paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4; Wöhr et al., 2009). Postfixed (4% paraformaldehyde, 24 hours, 4°C), cryoprotected (20% sucrose, 48 hours, 4°C), and snap-frozen (methylbutane, −30°C, 2 minutes) brains were cut on a freezing microtome (CM33050S, Leica, Wetzlar, Germany) in 40-μm coronal sections. Sections were collected in 10 regularly spaced series, and stored in 0.1 mol/L PBS containing 0.02% (wt/vol) sodium azide at 4°C. For immunohistochemistry, free-floating sections were incubated successively for 30 minutes with 0.1% H_2O_2 in 0.1 mol/L PBS to block endogenous peroxidase activity, and for 30 minutes with 3% vol/vol donkey serum in 0.1 mol/L PBS to inhibit nonspecific binding. Samples were pretreated with 2 N HCl (37°C, 20 minutes) and neutralized with borate buffer (pH 8.5, 10 minutes, room temperature) for BrdU detection or with 70% ethanol (−20°C, 30 minutes) for PCNA detection prior to incubation with the respective primary antibodies, i.e., anti-BrdU or anti-PCNA antibody (both from DAKO, Glostrup, Denmark; Cat# M0744, RRID:AB_10013660 and Cat# M0879, RRID:AB_2160651, respectively; 1:500 and 1:1,000, 4°C, 12 hours). All antibodies were diluted in 0.1 mol/L PBS with 2.5% donkey serum and 0.15% Triton X-100 (Sigma). Sections were then incubated for 1 hour at room temperature with the appropriate biotinylated secondary antibody (donkey anti-rat IgG# 712-005-150 or donkey anti-mouse IgG# 715-005-150, Dianova, Hamburg, Germany) in 0.1 mol/L PBS with 3% donkey serum and 0.15% Triton X-100 (Sigma). The avidin–biotin method was used to amplify the signal (ABC kit, Vector, Burlingame, CA) and 3,3'-diaminobenzidine tetrachloride/$NiCl_2$ was used to visualize bound antibodies. To exclude nonspecific labeling, the primary antibodies were omitted.

Image analysis

Immunolabeled cells were counted stereologically on every 10th section of regularly spaced, 40-μm-thick

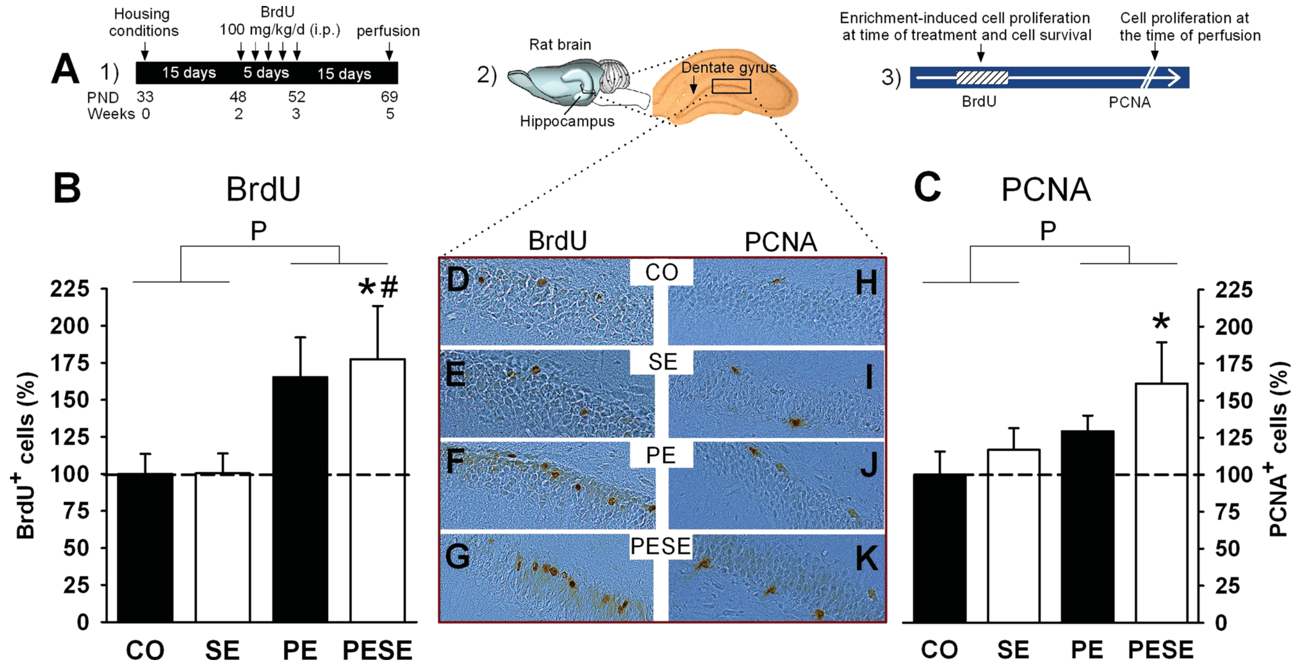

Figure 1. Effects of social and physical environmental enrichment (EE) on cell proliferation and survival in the dentate gyrus (DG) of the hippocampal formation. **A:** 1) Experimental design showing time-points of i.p. BrdU administion (PND, postnatal days). 2) Illustration of rat brain and hippocampal formation; 3) Schematic overview on the information provided by each cellular marker. **B:** Effect of housing conditions on the number of $BrdU^{+}$ cells in the DG. **C:** Effect of housing conditions on the number of $PCNA^{+}$ cells in the DG. **D–G:** Representative coronal hippocampal sections immunostained for BrdU. **H–K:** Representative coronal hippocampal sections immunostained for PCNA. Data are shown as mean + SEM. *P* values: P $P \leq 0.050$ main effect physical enrichment;* $P \leq 0.050$ versus CO; # $P \leq 0.050$ versus SE. CO, standard control (two rats in a nonenriched cage); SE, social enrichment (six rats in a nonenriched cage); PE, physical enrichment (two rats in an enriched cage); PESE, physical plus social enrichment (six rats in an enriched cage).

sections using a semiautomatic stereology system (Imager.M2 Axio microscope, Zeiss, Oberkochen, Germany and Stereoinvestigator, MBF Bioscience, Williston, VT) in the SVZ (2.2 to −0.4 mm in relation to bregma) and the subgranular zone of the DG (−4.1 to −5.5 mm; Paxinos and Watson, 1998), covering its entire dorsoventral extension. The rate of cell proliferation was expressed as $BrdU^{+}$ or $PCNA^{+}$ cells per μm^3.

RNA isolation

The remaining slices of the immunohistochemistry analysis were stored in antifreeze solution at −20°C. For each animal, four whole-brain slices including the hippocampual formation (−4.1 to −5.5 mm) were taken for RNA isolation. The total RNA was isolated using Recover-All-Total-Nucleic-Acid-Isolation-Kit (Ambion, Carlsbad, CA; Cat# AM1975, RRID:nif-0000-3092) as described in the manual. TurboDNase (Ambion; Cat# AM2238, RRID:nif-0000-3092) was used to remove DNA. RNA concentrations were determined by measuring absorbance (A_{260}) on the NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE; RRID:nlx_152478).

Quantitative real-time PCR

As previously reported (Siegel et al., 2009; Fiore et al., 2009), quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA; RRID:nlx_144442) using iTaq SybrGreen Supermix with ROX (Bio-Rad, Hercules, CA; Cat# 1725852, RRID:nif-0000-30176) for mRNAs and pre-miRNAs (primer sequences are available on request) and TaqMan MicroRNA Assays (U6 snRNA and has-mir132; Applied Biosystems; Cat# 4440887 and Cat# 4427975, RRID:nlx_144442, respectively) for the detection of mature miRNAs. U6 was used as qRT-PCR normalization control and the average of triplicate CT values from each sample was used to calculate the relative RNA amount ($2^{-\Delta CT}$). Brain samples were taken from two independent cohorts of rats (n = 24 per cohort), with each cohort including subjects of all experimental groups. Consequently, qRT-PCR analysis was performed in two independent runs. Detection of c-fos, CREB1, pre-miR-124, pre-miR-137, and mature miR-132 varied between both cohorts (c-fos: $F_{1,48} = 8.818$, $P = 0.005$; CREB1: $F_{1,48} = 7.106$, $P = 0.011$; pre-miR-124: $F_{1,48}$

$= 68.575$, $P < 0.001$; pre-miR-137: $F_{1,36} = 18.688$, $P < 0.001$; mature miR-132: $F_{1,48} = 108.638$, $P < 0.001$). Importantly, however, variability between cohorts did not affect the effects of social and physical EE, because no interactions between cohort and experimental housing conditions, i.e., social and physical EE, were detected (all $P > 0.050$).

Data analysis and statistics

The particular contribution of either social or physical EE was determined by comparing groups of two versus six rats irrespective of physical EE (effect of social EE) and groups of rats with or without physical EE irrespective of social EE (effect of physical EE), respectively, by two-way analyses of variance (ANOVAs; social EE $\times$ physical EE). For all cage test and open field parameters, three-way ANOVAs for repeated measures (4 PND $\times$ social EE $\times$ physical EE) were conducted. One-way ANOVA analysis followed by protected least significant difference (LSD) Fisher post hoc test was used for single group comparisons when appropriate. ANOVAs or paired *t*-tests were used to compare 50-kHz USV subtypes and behavior in the playback experiment. Cohort was used as an additional factor in the statistical analyses of c-fos, CREB1, and miRNA expression levels. For all statistical tests the level of significance was defined as $P \leq 0.050$. Data are expressed as mean $\pm$ standard error of the mean (SEM).

RESULTS

Physical but not social EE promoted adult hippocampal neurogenesis

EE is known to induce several neuromorphological and neurochemical changes underlying improved learning and memory, with increased hippocampal neurogenesis being one of the most prominent (for review, see Kempermann et al., 2010). The first aim of the present study was to better understand the factors underlying EE effects on hippocampal neurogenesis. After two weeks of exposure to one of our four experimental housing conditions, rats received daily i.p. injections of BrdU for 5 consecutive days to label newly generated cells. Two weeks after the last injection, the number of surviving cells was examined. In addition, the number of cells expressing PCNA protein was quantified to assess the level of cell proliferation occurring at the time of perfusion (Höglinger et al., 2004; Fig. 1A).

EE significantly affected cell proliferation and/or survival in the DG of the hippocampal formation, as assessed by BrdU (physical EE: $F_{1,46} = 8.497$, $P = 0.006$; all other $P > 0.050$; Fig. 1B) and PCNA (physical EE: $F_{1,44} = 4.075$, $P = 0.050$; all other $P > 0.050$; Fig. 1C). As compared with CO rats, the number of $BrdU^{+}$ cells in the DG was enhanced by 65% in PE rats and by 77% in PESE rats, whereas no change was observed in SE rats. When comparing individual housing conditions, PESE rats had significantly more $BrdU^{+}$ cells than CO and SE rats, but not PE rats (LSD: all $P < 0.050$; all other $P > 0.050$; for exemplary hippocampal slices see Fig. 1D–G). Similar results were obtained for PCNA. The number of $PCNA^{+}$ cells in the DG was enhanced by 29% in PE rats and by 61% in PESE rats, as compared with CO rats, whereas only a minor increase (16%) was observed in SE rats. Group comparison revealed that PESE rats had significantly more $PCNA^{+}$ cells than CO rats, but not SE and PE rats (LSD: $P < 0.050$; all other $P > 0.050$; for exemplary hippocampal slices see Fig. 1H–K). Importantly, EE effects were specific to the DG, because no differences in the level of either $BrdU^{+}$ or $PCNA^{+}$ cells were detected in the subventricular zone (SVZ; all $P > 0.050$).

Together, the data show that physical EE without running wheels led to enhanced cell proliferation and/or survival in the DG, whereas social EE had no net effects on the levels of $BrdU^{+}$ and $PCNA^{+}$ cells by its own. However, social EE appeared to have a minor additive effect to physical EE, because $PCNA^{+}$ cells in particular tended to be higher in PESE than in PE rats.

Physical but not social EE increased c-fos, CREB1, and activity-dependent miRNA expression

Upregulation of immediate early genes, such as c-fos or zif-268, has been repeatedly detected in brains of rats exposed to EE (Rampon et al., 2000a; Solinas et al., 2009), whereas miRNAs have been rarely studied (Kuzumaki et al., 2011). Complementing the analysis of cell proliferation and survival, c-fos and CREB1 mRNA expression levels were measured as markers of functional activity in brain slices containing the hippocampal formation. In addition, miRNA levels were determined in the same samples. The short noncoding miRNAs act as post-transcriptional regulators of gene expression by complementary binding to the 3′ untranslated region of target mRNAs and mediate experience-dependent changes in brain plasticity (for review, see Schratt, 2009). We measured expression of the activity-dependent precursor and/or mature miR-124 and miR-132, both of which are known to positively regulate dendritic morphogenesis, synaptic plasticity, and/or neurogenesis (Magill et al., 2010; Luikart et al., 2011, 2012; Remenyi et al., 2010; Vo et al., 2005; Wayman et al., 2008). Thus, we anticipated higher expression levels in enriched rats, as EE is known to enhance

dendritic spine number and density (Faherty et al., 2003; Leggio et al., 2005; Rampon et al., 2000b). As a control, pre-miR-137 was selected, because its overexpression inhibits dendritic morphogenesis rather than increasing it (Silber et al., 2008; Szulwach et al., 2010; Smrt et al., 2010; Fig. 2A).

EE significantly affected c-fos expression levels (physical EE: $F_{1,48} = 5.097$, $P = 0.029$; all other $P > 0.050$; Fig. 2B). In comparison with CO rats, c-fos expression was enhanced by 21% in PE rats and by 30% in PESE rats, whereas only a minor increase of 3% was observed in SE rats. Likewise, CREB1 was significantly affected by EE (physical EE: $F_{1,48} = 11.201$, $P = 0.002$ and social $\times$ physical EE: $F_{1,48} = 4.418$, $P = 0.042$; all other $P >$ 0.050; Fig. 2C). As compared with CO rats, CREB1 levels were strongly enhanced by 52% in PE rats, whereas relatively moderate increases of 13% and 25% were observed in SE and PESE rats, respectively. Individual group comparisons showed that PE rats had significantly more CREB1 expression than CO and SE rats, but not PESE rats (LSD: all $P < 0.050$; all other $P >$ 0.050). At the miRNA level, the activity-dependent pre-miR-124 and pre-miR-132 were affected by EE (physical EE: $F_{1,48} = 5.297$, $P = 0.027$ and $F_{1,48} = 5.271$, $P =$ 0.027, respectively; all other $P > 0.050$; Fig. 2D,E). As compared with CO rats, levels of pre-miR-124 and pre-miR-132 were enhanced by 25% and 26%, respectively, in PE rats, and by 20% and 27%, respectively, in PESE rats, whereas only minor increases of 3% and 7%, respectively, were observed in SE rats. Levels of pre-miR-137 were not affected by EE (all $P > 0.050$; Fig. 2F). In addition to pre-miR, mature levels of miR-132 were assessed, which were strongly affected by EE (physical EE: $F_{1,48} = 6.580$; $P = 0.014$; all other $P >$ 0.050; Fig. 2G). As compared with CO rats, miR-132 levels were enhanced by 17% in PE rats, and by 38% in PESE rats, whereas only a minor increase of 7% was observed in SE rats, again indicating strong effects of physical but not social EE. A comparison of individual housing conditions revealed that PESE rats had significantly higher miR-132-levels than CO and SE rats, but not PE rats (LSD: all $P < 0.050$; all other $P > 0.050$).

Together, these data show that physical EE without running wheels led to enhanced neuronal activity levels, as assessed by the immediate early gene c-fos and CREB1, and changes in miRNA expression levels, with the activity-dependent precursor and/or mature miR-124 and miR-132 being upregulated, whereas pre-miR-137, the negative regulator of neuronal maturation, was not affected. As for cell proliferation and survival, social EE had no net effects on c-fos, CREB1, and miRNA expression levels by its own. However, social EE appears to have a minor additive effect to physical EE: particularly the mature miR-132 tended to be higher in PESE than in PE rats. Increased expression levels of activity-dependent miRNAs in rats exposed to physical EE are in line with enhanced hippocampal cell proliferation, survival, and activation following physical EE, suggesting that experience-dependent changes induced by EE could involve miRNA regulation.

Physical but not social EE improved learning and memory

Besides brain plasticity, EE is known to affect behavior, with improved learning and memory being the most consistent outcome (for reviews see: Nithianantharajah and Hannan, 2006; van Praag et al., 2000). To test whether the changes in brain plasticity following physical EE are associated with changes in cognitive functions, we first assessed habituation learning. To this aim, a given rat was individually exposed to a cage with clean bedding, referred to as cage test. Immediately after the cage tests the rat was individually tested in an adjacent open field. Both tests were conducted once per week for four weeks whereas rats were exposed to the experimental housing conditions (Fig. 3A).

EE significantly affected locomotor activity in both tests, i.e., cage test and open field. In the open field, physically enriched rats, i.e., PE and PESE, displayed significantly lower levels of locomotor activity than non-physically enriched rats, i.e., CO and SE (physical EE: $F_{1,44} = 18.867$, $P < 0.001$), but also social EE led to reduced locomotor activity (social EE: $F_{1,44} = 8.619$, $P = 0.005$; all other $P > 0.050$), probably reflecting reduced psychomotor activation following social and physical EE (Fig. 3B). A comparison of housing conditions over the four open field tests showed that PESE rats consistently displayed significantly less locomotor activity than CO, SE, and PE rats, with PE rats also displaying significantly reduced locomotor activity during the last open field test, as compared with CO but not SE rats (LSD: all $P < 0.050$; all other $P > 0.050$). In support of habituation learning, locomotor activity in the open field decreased during testing (time: $F_{3,176} =$ 308.410, $P < 0.001$; Fig. 3C) and with repeated testing over weeks (week: $F_{3,176} = 18.858$, $P < 0.001$; Fig. 3D). Within-session habituation was enhanced by physical EE (time $\times$ physical EE: $F_{3,176} = 2.477$, $P =$ 0.009), but not social EE (all $P > 0.050$; Fig. 3C). Between-session habituation, in contrast, was not affected seperately by the individual experimental housing conditions, namley social and physical EE (all $P >$ 0.050; Fig. 3D). Despite an overall similar tendency toward reduced locomotor activity with repeated

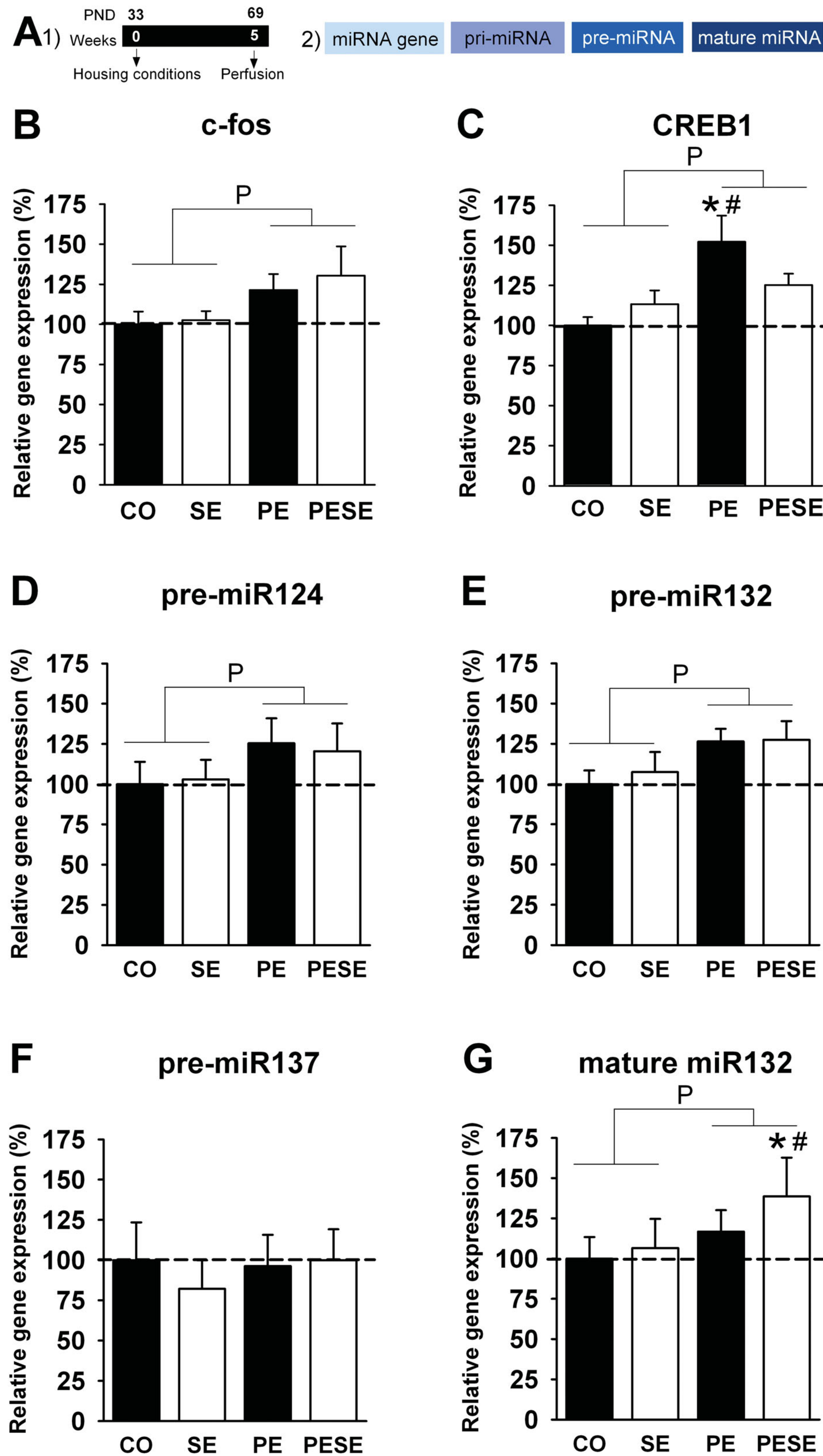

Figure 2. Effects of social and physical environmental enrichment (EE) on c-fos mRNA, CREB1 mRNA, and microRNA (miRNA) expression. **A:** 1) Experimental design showing when brain samples were obtained (PND, postnatal days). 2) Simplified schematic overview on miRNA biogenesis. **B:** Expression of c-Fos mRNA. **C:** Expression of CREB1 mRNA. **D:** Expression of pre-miR-124. **E:** Expression of pre-miR-132. **F:** Expression of pre-miR-137. **G:** Expression of mature miR-132. Data are shown as mean + SEM. *P* values: P $P \leq 0.050$ main effect physical enrichment; * $P \leq 0.050$ versus CO; # $P \leq 0.050$ versus SE. For group abbreviations see Figure 1.

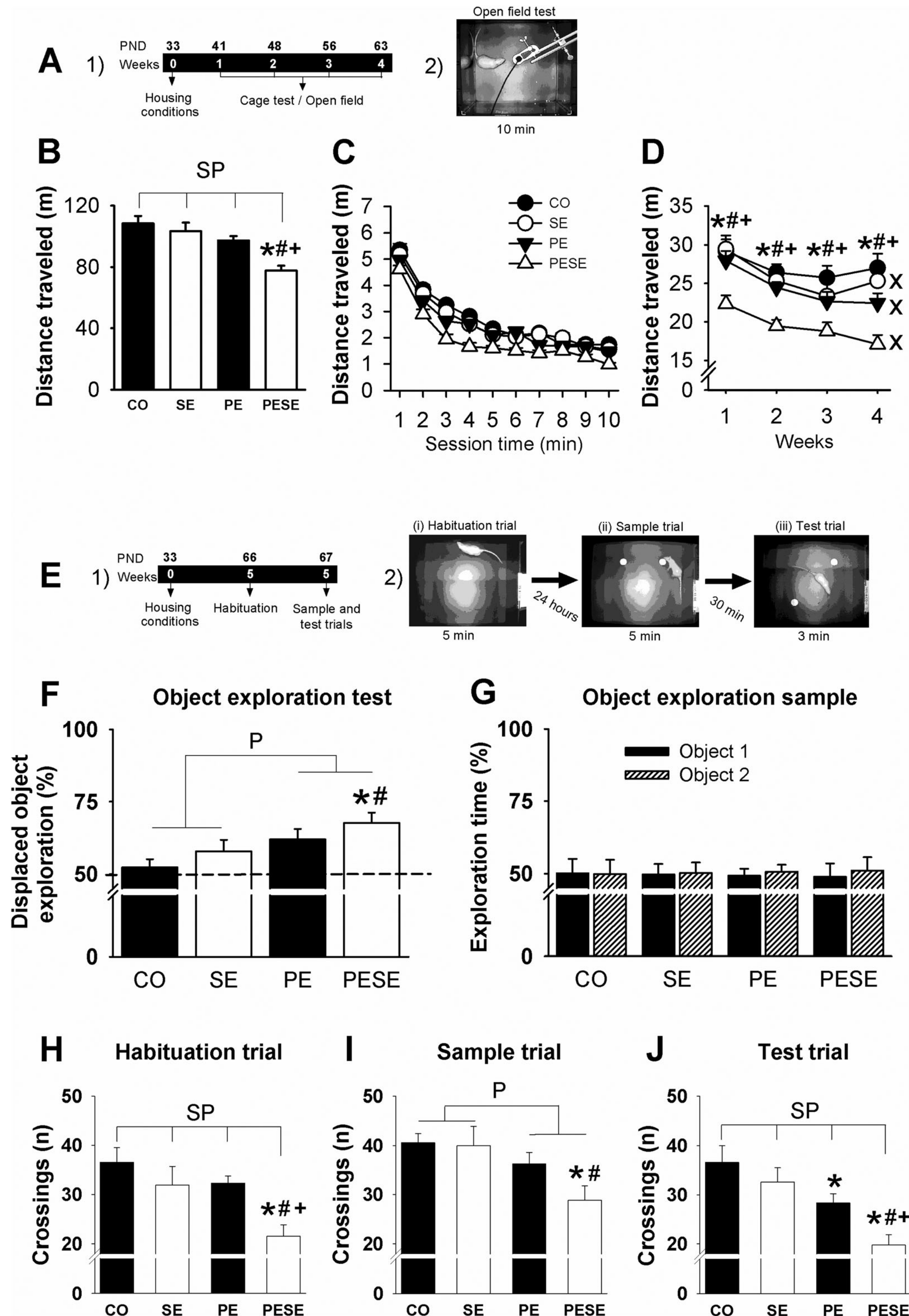

Figure 3. Effects of social and physical environmental enrichment (EE) on habituation learning and declarative memory. **A:** 1) Experimental design showing when open field and cage tests were performed (PND, postnatal days). 2) Picture illustrating the open field test. **B:** Cumulative locomotion. **C:** Locomotion per minute over the 10-minute session (all weeks averaged). **D:** Locomotion over weeks. **E:** 1) Experimental design showing when the object recognition trials took place. 2) Pictures illustrating the testing arena during the different trials. **F:** Percentage of time spent exploring the displaced object. **G:** Time spent exploring the two objects in the sample trial expressed as percentages of total exploration time. **H:** Locomotion on habituation trial. **I:** Locomotion on sample trial. **J:** Locomotion on test trial. Data are shown as mean + SEM. S $P \leq 0.050$ main effect social enrichment; P $P \leq 0.050$ main effect physical enrichment; * $P \leq 0.050$ versus CO; # $P \leq 0.050$ versus SE; + $P \leq 0.050$ versus PE; X $P \leq 0.050$ versus week 1. For group abbreviations see Figure 1.

testing, however, reductions in locomotor activity from the first to the fourth open field tests were weak in CO rats and did not reach statistical significance (t_{11} = 1.176, P = 0.264), but were pronounced in SE (t_{11} = 4.268, P = 0.001), PE (t_{11} = 3.758, P = 0.003), and PESE (t_{11} = 3.983, P = 0.002) rats. Similar findings were obtained in the cage test (not shown). Faster within- and between-session habituation in cage test and open field in PE and PESE rats probably reflects enhanced habituation learning in rats exposed to physical EE during adolescence.

We also assessed place object recognition by means of the PORT, a test for hippocampus-dependent declarative-like episodic memory (for review, see Dere et al., 2007) known to be improved by EE (Bennett et al., 2006; Kempermann et al., 1997; Leggio et al., 2005; Nilsson et al., 1999). Rats were first exposed to two identical objects, termed the sample trial. After a delay of 30 minutes, they were again allowed to explore the objects, but in this trial, one of two objects was displaced to a different location, termed the test trial (Fig. 3E). The relative amount of time spent exploring the displaced object, as compared with the nondisplaced one, was taken as the memory index.

EE significantly affected performance in the PORT. Physically enriched rats, i.e., PE and PESE, spent significantly more time exploring the displaced object than nonphysically enriched rats, i.e., CO and SE, indicating enhanced place object learning in rats exposed to physical but not social EE during adolescence (physical EE: $F_{1,44}$ = 8.215, P = 0.006; all other $P > 0.050$; Fig. 3F). Comparing housing conditions showed that PESE rats spend significantly more time exploring the displaced object than CO and SE, but not PE rats (LSD: all $P < 0.050$; all other $P > 0.050$). Importantly, enhanced performance in the PORT was not due to differences in the motivation to explore objects, because rats did not differ in object exploration and preference during the sample trial (all $P > 0.050$; Fig. 3G). This also shows that differences in locomotor activity between experimental housing conditions, as observed in cage test and open field, but also in the PORT (not shown in detail; Fig. 3H–J), are unlikely to be due to a general decrease in the motivation to explore.

Together, these data show that physical EE without running wheels led to improved learning and memory, as assessed by habituation learning and place object recognition, whereas social EE had no net effects by its own. However, social EE appeared to have a minor additive effect to physical EE, because in both the habituation learning and the PORT, PESE rats tended to outperform PE rats. This pattern is consistent with the changes in brain plasticity following physical EE, namely, increased hippocampal cell proliferation, survival, and activation, along with enhanced levels of activity-dependent miRNAs.

Physical EE reduced, whereas social EE enhanced, the relative number of prosocial 50-kHz USV

To test whether social and physical EE differentially affect rat social ultrasonic communication, rats were separated from conspecifics and singly exposed to a cage once per week for 4 weeks; then separation-induced 50-kHz USV emission rates were assessed (Fig. 4A). Importantly, we differentiated between call subtypes, namely, FLAT and FM calls. For example, 50-kHz USV occur when rats are separated from conspecifics while being isolated in an empty cage, with FLAT calls, a subtype characterized by a nearly constant frequency, being most prominent (Schwarting et al., 2007; Wöhr et al., 2008). In contrast, FM 50-kHz USV, such as STEP and TRILL, are less prominent there, but occur at high rates in appetitive situations, such as rough-and-tumble play in juveniles (Burgdorf et al., 2008; Knutson et al., 1998).

EE significantly affected the emission of 50-kHz USV in the cage test. Physically enriched rats, i.e., PE and PESE, displayed significantly lower 50-kHz USV emission rates than nonphysically enriched rats, i.e., CO and SE (physical EE: $F_{1,44}$ = 9.362, $P < 0.004$; all other $P > 0.050$; Fig. 4B). When comparing housing conditions in the first, second, third, and fourth cage tests, we found PE and PESE rats to emit significantly fewer 50-kHz USV than CO but not SE rats, with the exception of the first test, in which only a trend was observed (LSD: all $P < 0.050$; all other $P > 0.050$). In contrast to locomotor activity, there was no general decrease in 50-kHz USV emission with repeated testing over weeks (week: $F_{3,132}$ = 1.903, P = 0.132; Fig. 4C). In fact, changes in 50-kHz USV emission were dependent on housing conditions (week × physical EE: $F_{3,176}$ = 2.913, P = 0.037; all other $P > 0.050$) and even increased over weeks from the first to the fourth test in CO rats (t_{11} = 2.520, P = 0.028), but were unchanged in SE (t_{11} = 1.411, P = 0.186), PE (t_{11} = 0.045, P = 0.965), and PESE (t_{11} = 1.670, P = 0.123) rats.

EE also significantly affected the 50-kHz USV profile in the cage test. FLAT calls were the most prominent type in all experimental housing conditions and occurred with an overall occurrence rate of 78% as compared with STEP (15%) and TRILL (7%). Socially enriched rats, i.e., SE and PESE, emitted significantly more FLAT and less FM calls, particularly STEP but not TRILL, than the nonsocially enriched counterparts, i.e., CO and PE (social EE: $F_{1,44}$ = 10.451, P = 0.003; $F_{1,44}$

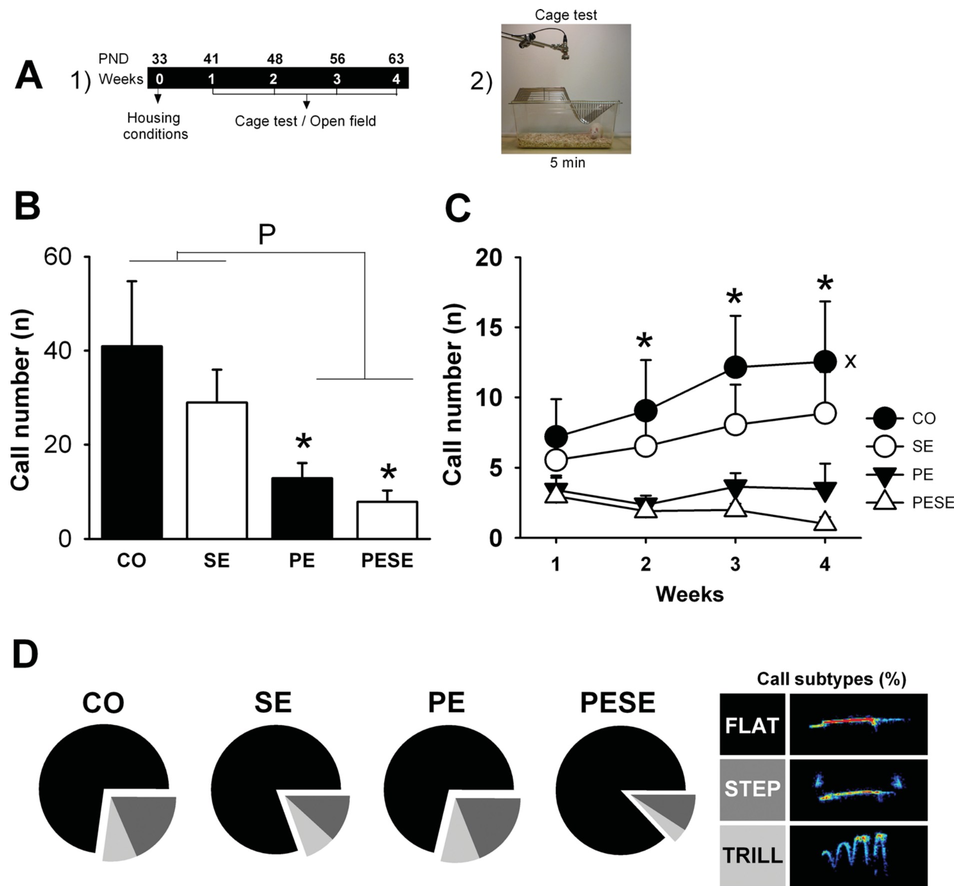

Figure 4. Effects of social and physical environmental enrichment (EE) on spontaneous 50-kHz ultrasonic vocalizations (USV) in the cage test. **A:** 1) Experimental design showing when open field and cage tests were performed (PND, postnatal days). 2) Picture illustrating the cage test. **B:** Cumulative spontaneous 50-kHz USV. **C:** Spontaneous 50-kHz USV over weeks. **D:** Spontaneous 50-kHz USV profiles. Each area represents the group mean of a given 50-kHz USV subtype, expressed as the percentage of all 50-kHz USV. For significant differences in the 50-kHz USV profile charts see main text. Data are shown as mean + SEM. P $P \leq 0.050$ main effect physical enrichment; * $P \leq 0.050$ versus CO; X $P \leq 0.050$ versus week 1. For group abbreviations see Figure 1.

= 12.472, $P = 0.001$; $F_{1,44} = 2.301$, $P = 0.136$; respectively; Fig. 4D). In contrast, physical EE had no effect (all $P > 0.050$). PESE rats emitted significantly more FLAT but fewer STEP calls than CO and PE but not SE rats (LSD: all $P < 0.050$; all other $P > 0.050$). Aversive 22-kHz USV were only rarely observed (average call number per rat/cage test: <0.1 22-kHz USV) and occurred significantly less often than 50-kHz USV in all experimental housing conditions (all $P < 0.050$), with experimental housing conditions not differing from each other (all $P > 0.050$). Similar findings were obtained in the open field (not shown), despite the fact that USV emission was relatively low, which is probably due to the short intertest interval between cage test and open field and the fact that 50-kHz USV emission is typically inhibited when animals are confronted with a mild stress context, such as open fields without bedding material (Wöhr et al., 2008; Natusch and Schwarting, 2010).

Together, these data show that physical EE without running wheels led to reduced emission of 50-kHz USV in cage test and open field, without changing the 50-kHz USV profile. In contrast, social EE had no net effects on emission rates by its own, but led to an increase in the relative amount of FLAT calls. This indicates that 50-kHz USV emission rate and qualitative features of 50-kHz USV can be independently modulated by social and physical EE.

Physical EE reduced, whereas social EE enhanced, social approach behavior in response to playback of prosocial 50-kHz USV

Despite the increasing interest in understanding ultrasonic communication in rodents, most USV studies focus on the emission of USV by the sender, whereas the behavioral responses elicited in the receiver in

response to it are rarely studied. By using a playback paradigm, we have repeatedly shown that 50-kHz USV elicit social approach behavior in the recipients (Wöhr and Schwarting, 2007, 2009, 2012). Because EE affected the emission of 50-kHz USV serving prosocial functions, we asked whether social approach elicited by playback of such 50-kHz USV (Wöhr and Schwarting, 2007, 2009, 2012) is also affected by social and physical EE. To this aim, rats were tested for behavioral changes in response to playback of prosocial 50-kHz USV (Fig. 5A) or time- and amplitude-matched white noise (Fig. 5B). The latter stimulus served as a control for novelty-induced changes in behavior.

EE significantly affected social approach behavior elicited by playback of 50-kHz USV. In line with previous studies (Wöhr and Schwarting 2007, 2009, 2012), rats not exposed to physical EE, i.e., CO and SE rats, displayed locomotor inhibition in response to time- and amplitude-matched white noise (CO: $t_{11} = 3.306$; $P = 0.007$; SE: $t_{11} = 5.099$; $P < 0.001$; Fig. 5C), whereas playback of 50-kHz USV led to an increase in locomotor activity, reflecting the induction of social exploration (CO: $t_{11} = 2.524$; $P = 0.028$; SE: $t_{11} = 2.681$; $P = 0.021$; Fig. 5D). When the direction of the 50-kHz USV-induced social exploration seen in CO and SE rats was analyzed by measuring the time spent on proximal and distal arms (Fig. 5E), significant preferences toward the arms proximal to the speaker producing 50-kHz USV were found in both groups (CO: $t_{11} = 3.373$; $P = 0.003$; SE: $t_{11} = 5.105$; $P < 0.001$; Fig. 5F). In addition, when the time spent on proximal arms in the 5 minutes before and during playback of 50-kHz USV was compared, significant increases in the time spent on proximal arms during playback were found in both groups (CO: $t_{11} = 2.470$; $P = 0.031$; SE: $t_{11} = 3.787$; $P = 0.003$; Fig. 5F).

In rats exposed to physical EE only, i.e., PE rats, time- and amplitude-matched white noise led to a similar reduction in locomotor activity as seen in rats not exposed to physical EE, i.e., CO and SE rats ($t_{11} = 8.612$; $P = 0.001$; Fig. 5C). However, in contrast to the latter, PE rats did not show social exploration when exposed to 50-kHz USV ($t_{11} = 1.421$; $P = 0.182$; Fig. 5D). In line with the lack of an induction of social exploratory behavior, no preference toward proximal arms was found when the time spent on proximal and distal arms was compared ($t_{11} = 1.578$; $P = 0.143$; Fig. 5F). Also, the time spent on proximal arms before and during playback of 50-kHz USV did not differ ($t_{11} = 0.713$; $P = 0.490$; Fig. 5F). This behavioral response pattern indicates deficits in social exploratory and approach behavior in PE rats when exposed to prosocial 50-kHz USV despite intact acoustic information processing.

Deficits in social exploration and approach induced by exposure to physical EE were prevented when rats were exposed to social EE in addition to physical EE, i.e., PESE rats. With the exception of a lack of locomotor inhibition in response to time- and-amplitude white noise ($t_{11} = 1.095$; $P = 0.297$; Fig. 5C), PESE rats displayed a behavioral response pattern very similar to that of CO and SE rats and showed social exploration when exposed to 50-kHz USV ($t_{11} = 3.530$; $P = 0.005$; Fig. 5D). As in CO and SE rats, social exploration was directed toward the ultrasonic speaker producing 50-kHz USV, as reflected in a significant preference toward proximal arms in comparison with distal arms ($t_{11} = 5.413$; $P < 0.001$; Fig. 5F) and an increased time spent on proximal arms during playback when compared with the 5 minutes before ($t_{11} = 4.162$; $P = 0.031$; Fig. 5F). The prosocial effects of social EE were also reflected in a significant difference in the time spent proximal during playback of 50-kHz USV between rats exposed to social EE, i.e., SE and PESE rats, and rats not exposed to social EE, i.e., CO and PE rats (social EE: $F_{1,48} = 9.683$; $P = 0.003$; Fig. 5F), in the absence of an effect of physical EE (all $P < 0.050$). Such an effect was not seen for locomotor activity in response to time- and amplitude-matched white noise (all $P > 0.050$) and 50-kHz USV (all $P > 0.050$). Preferences toward the ultrasonic speaker producing time- and amplitude-matched white noise were not detected (all $P > 0.050$), and the groups did not differ in their behavioral profile before playback of time- and amplitude-matched white noise and 50-kHz USV (all $P > 0.050$). Two exemplary track profiles during playback of prosocial 50-kHz USV are shown for each experimental housing condition in Figure 5G.

Together, these data show that social EE led to enhanced social exploration and approach in response to 50-kHz USV, whereas physical EE led to social deficits, as indicated by a lack of social exploration and approach in PE rats when exposed to 50-kHz USV. Importantly, however, deficits in social exploration and approach due to physical EE were rescued by exposing rats to social EE in addition, i.e., in PESE rats. This indicates that enhanced affiliative behavior provided by rearing rats in larger groups during adolescence has positive effects on the rats' social development that counteract or compensate for deficits induced by physical EE. This view is supported by the fact that rearing rats in larger groups also favored the emission of FLAT calls with presumably prosocial functions. Observed effects were specific to playback of prosocial 50-kHz USV because they were not observed in response to

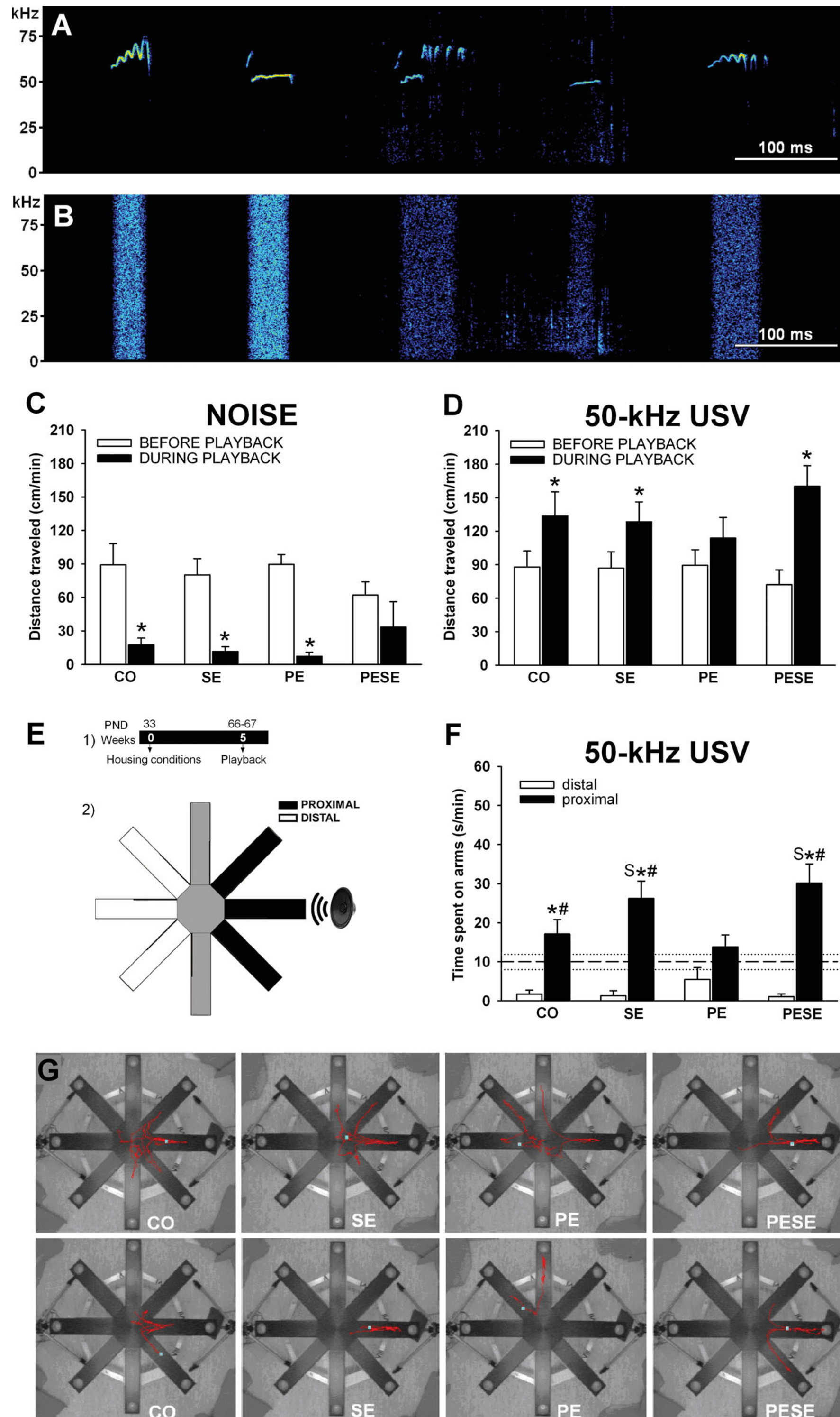

Figure 5. Effects of social and physical environmental enrichment (EE) on approach in response to playback of prosocial 50-kHz ultrasonic vocalizations (USV). Exemplary spectrograms of acoustic stimuli used for playback. **A:** Natural 50-kHz USV. **B:** Time- and amplitude-matched white noise. **C:** Locomotor activity before and during playback of time- and amplitude-matched white noise. **D:** Locomotor activity before and during playback of prosocial 50-kHz USV. **E:** 1) Experimental design showing when the playback experiment was performed (PND, postnatal days); 2) Illustration of the radial maze used for playback. **F:** Time spent on proximal and distal arms in response to prosocial 50-kHz USV. The time spent on proximal arms before playback of prosocial 50-kHz USV is shown as dashed (mean) and dotted lines (SEM). **G:** Two exemplary track profiles during playback of prosocial 50-kHz USV are shown for each experimental group. Data are shown as mean + SEM. S $P \leq 0.050$ main effect social enrichment; * $P \leq 0.050$ versus before; # $P \leq 0.050$ versus distal. For group abbreviations see Figure 1.

our acoustic control, time- and amplitude-matched white noise.

DISCUSSION

EE is known to exert a variety of beneficial effects on brain and behavior, including enhanced brain plasticity, improved learning and memory, and reduced anxiety- and depression-like behavior, and hence it was hypothesized that EE leads to a brain that can counteract or compensate for deficits underlying various neurological and psychiatric disorders (for reviews, see Nithianantharajah and Hannan, 2006; van Praag et al., 2000). In general, however, the complexity of EE commonly used in the laboratory makes it difficult to identify specific factors causing the observed changes. The aim of the present study was to better understand these factors by using a 2 × 2 design with the factors social EE (two vs. six rats per cage) and physical EE (enriched vs. non-enriched cages), all without running wheels. We showed that physical EE but not social EE leads to enhanced cell proliferation and/or survival in the DG of the hippocampal formation, increased neuronal activity levels, as assessed by the immediate early gene c-fos and CREB1, and changes in miRNA expression levels, with the activity-dependent precursor and/or mature miR-124 and miR-132 being upregulated, whereas pre-miR-137 was not affected. Consistent with enhanced plasticity, we found that physical EE leads to improved learning and memory, as assessed by habituation learning and place object recognition. Social EE, in contrast, had no net effect on brain plasticity, and no evidence for changes in learning and memory was obtained, yet PESE rats often outperformed PE rats, indicating an additive effect of social EE. Furthermore, the numbers of 50-kHz USV emitted in cage test and open field were not affected by social EE, whereas physical EE consistently led to reduced prosocial 50-kHz USV emission. In contrast, however, social EE, but not physical EE, increased the relative amount of FLAT calls, indicating that 50-kHz USV emission rate and qualitative features of 50-kHz USV can be independently modulated by the two EE factors. Whereas social EE, i.e., rearing rats in larger groups, favored the emission of FLAT calls with presumably prosocial functions, physical EE reduced the propensity to emit them. Furthermore, social EE enhanced social exploration and approach in response to 50-kHz USV, whereas physical EE led to social deficits, as indicated by a lack of social exploration and approach in PE rats when exposed to such 50-kHz USV. Importantly, deficits in social behavior induced by physical EE were rescued by exposing rats to social EE in addition to physical EE, i.e., in PESE rats. This indicates that enhanced affiliative behavior provided by rearing rats in larger groups during adolescence has positive effects on the rats' social development that counteract or compensate for deficits induced by physical EE. Together, our data show that social and physical EE have differential effects on brain plasticity, cognition, and ultrasonic communication in rats.

Effects of social and physical EE on brain plasticity and cognition

Adult hippocampal neurogenesis

Among the many effects of EE on brain plasticity processes, increased hippocampal neurogenesis is probably one of the most prominent (for review, see Kempermann et al., 2010). Whereas many studies attributed enhanced hippocampal neurogenesis following EE to a combination of several factors, others see exercise as the main contributor. For instance, it has been suggested that the effects of EE on hippocampal neurogenesis are exclusively due to exercise, because cell proliferation and survival were found to be enhanced only when running wheels were accessible (Kobilo et al., 2011; Mustroph et al., 2012). Our data suggest, however, that wheel running exercise might be sufficient but not necessary for the induction of hippocampal neurogenesis, because enhanced cell proliferation and/or survival were observed under physical EE conditions without running wheels, in agreement with recent reports in mice and rats (Freund et al., 2013; Speisman et al., 2013).

Enhanced neurogenesis in physical EE without running wheels suggests that other factors play important roles as well, such as the social environment (Freund et al., 2013; Speisman et al., 2013). It is well known that social isolation has adverse effects on cognition (Fone and Porkess, 2008) and it has thus been hypothesized that the social component of EE may play a role in hippocampal neurogenesis, yet specific studies have been missing (for review, see Kempermann et al., 2010). In support of this hypothesis, however, it was shown that adult hippocampal neurogenesis correlates with individual differences in establishing territories in large social groups housed in enriched environments (Freund et al., 2013). Our present data show that social EE alone did not lead to significant net enhancement of cell proliferation and/or survival when compared with non-socially enriched animals, yet the effects of physical EE varied according to the number of animals per cage, with animals raised in larger groups displaying higher cell proliferation and/or survival rate. In fact, when housing conditions were compared, only PESE

rats differed significantly from CO rats. This is possibly due to higher social activity in cages with more animals, in line with a study by Fabel et al. (2009) showing that exercise mainly enhances cell proliferation, whereas EE leads to a increase in cell survival (for review, see Kempermann et al., 2010). Thus, one potential reason for the enhanced cell proliferation despite the lack of running wheels is social EE and perhaps the exercise associated with it, for instance in the form of rough-and-tumble play behavior (Burgdorf et al., 2008; Knutson et al., 1998). In fact, we recently found that mimicking rough-and-tumble play enhances hippocampal cell proliferation (Wöhr et al., 2009), whereas social isolation has negative effects (Lu et al., 2003; Stranahan et al., 2006).

Expression of miRNAs

Consistent with the EE effects on neurogenesis, we further showed for the first time distinct contributions of social and physical EE to miRNA expression levels positively associated with dendritic spine morphogenesis, synaptic plasticity, and/or neurogenesis (Magill et al., 2010; Luikart et al., 2011, 2012; Remenyi et al., 2010; Vo et al., 2005; Wayman et al., 2008). Specifically, the activity-dependent precursor and/or mature miR-124 and miR-132 were enhanced by EE, in contrast to pre-miR-137 which was selected as a control miRNA, because its overexpression inhibits dendritic morphogenesis rather than increasing it (Silber et al., 2008; Szulwach et al., 2010; Smrt et al., 2010).

The enhancement of miR-132 expression following EE in brain samples including the hippocampal formation is in accordance with findings showing that acute experience-dependent neural activation induces a transient increase in pre-miR-132 expression (Nudelman et al., 2010). The activity-dependent miR-132, known to be regulated by BDNF and CREB1, has been shown to promote mature spine morphology by targeting the spine inhibitor GTPase p250GAP (Remenyi et al., 2010; Vo et al., 2005; Wayman et al., 2008). Also, the neurotrophic action of BDNF is thought to be directly mediated by miR-132 (Luikart et al., 2011, 2012; Numakawa et al., 2011). In addition, dendritic branching and synaptic integration of newborn hippocampal neurons is modulated by miR-132 (Magill et al., 2010; Luikart et al., 2011, 2012). Therefore, it is tempting to speculate that EE effects on cell proliferation and/or survival were at least partly mediated by miR-132 through BDNF and CREB1 downstream activation in the hippocampal formation. This view is supported by the fact that also CREB1 was upregulated by physical EE. The upregulation of miR-132 induced by physical EE occurred at the level of the precursor and its effect endured upon the expression of the mature miRNA. Pre-miR-132 has been considered as a rapid response gene because its induction phase parallels that of c-fos (Nudelman et al., 2010; Vo et al., 2005). In line with this idea, we found that physical EE induced both c-fos and pre-miR-132 expression to a similar degree. The fact that miR-132 can be rapidly induced but with a persistent expression, suggests that miR-132 may act as a signal-dependent switch that regulates neuronal homeostasis over the long term (Vo et al., 2005). This can account for the differences found between precursor and mature miRNA levels, with social stimulation again having a modulatory effect on brain plasticity changes induced by physical EE. As for hippocampal cell proliferation and survival, social EE had no net effects on miRNA expression levels by its own. However, social EE appeared to have a minor additive effect to physical EE, with only PESE rats differing significantly from CO rats when housing conditions were compared. Importantly, our observation that environmental factors, namely, EE, exert prominent effects on miRNA expression levels is in line with our recent finding that social isolation induces alterations in miRNA expression functionally linked to cognitive impairment (Valluy et al., 2015). However, enhanced miR-132 expression following EE contrasts with a recent study by Kuzumaki et al. (2011) in which no effects of EE on miR-132 expression levels were found. Besides the fact that we used rats as compared with mice, conflicting results are possibly due to the fact that we used juvenile animals, whereas Kuzumaki et al. (2011) studied older animals, as it is known that miR-132 expression levels peak around adolescence (Miller et al., 2012; Nudelman et al., 2010).

Cognition

Physical EE without running wheels led to improved habituation learning and place object recognition, whereas social EE had no net effects by its own. However, social EE appeared to have a minor additive effect to physical EE in both paradigms, because only PESE rats differed from controls in the single-group comparisons. Importantly, the pattern of results is consistent with the changes in brain plasticity following physical EE, namely, increased hippocampal cell proliferation, survival, and activation, along with enhanced levels of activity-dependent miRNAs. Our findings are in line with enhanced hippocampus-dependent learning following EE, as assessed in the Morris water maze, radial arm maze, and novel object recognition (Bennett et al., 2006; Bruel-Jungerman et al., 2005; Kempermann et al., 1997; Leggio et al., 2005; Nilsson et al., 1999; Rampon et al., 2000b). Also, the result of enhanced habituation learning following EE is consistent with the literature

(Brenes et al., 2009; Elliott and Grunberg, 2005; Neugebauer et al., 2004; Zimmermann et al., 2001).

Effects of social and physical EE on separation-induced 50-kHz USV emission

Rodent USV serve important communicative and affective functions (for review, see Brudzynski, 2013; Wöhr and Schwarting, 2013), and our findings show for the first time that the emission of 50-kHz USV depends on both social and physical EE. High rates of 50-kHz USV typically occur during social interactions, such as rough-and-tumble play in juveniles (Burgdorf et al., 2008; Knutson et al., 1998) or mating in adulthood (Burgdorf et al., 2008; Sales, 1972). However, rats also emit 50-kHz USV when being separated from conspecifics, with FLAT calls being most prominent (Schwarting et al., 2007; Wöhr et al., 2008). Such 50-kHz USV serve communicative functions as social contact calls to (re)establish or maintain social proximity, as revealed by means of playback experiments (Willadsen et al., 2014; Wöhr and Schwarting, 2007, 2009, 2012). Here, we showed that physical EE led to an overall reduced emission of separation-induced 50-kHz USV in both tests, i.e., cage test and open field, whereas social EE had no net effects by its own. However, social but not physical EE led to an increase in the relative amount of FLAT calls. This indicates that 50-kHz USV emission rate and qualitative call features can be independently modulated by social and physical EE. Whereas a combination of motor, cognitive, and sensory stimulation reduced the propensity to call, rearing rats in larger groups favored the emission of FLAT calls with presumably prosocial functions. The latter is in line with enhanced social behavior in rodents reared under social EE conditions (Green et al., 2010; Laviola et al., 2004; Morley-Fletcher et al., 2003; Neugebauer et al., 2004; Schneider et al., 2006) and might reflect increased social competence, namely, the ability to perceive and process social information to optimize behavior in a given social context (Taborsky and Oliveira, 2012). Emission of 50-kHz USV after separation from conspecifics may signal a positive affective state reflecting the receptiveness to engage in social interactions, while reducing the likelihood of intraspecific aggression.

The inhibitory effects of physical EE on USV emission may be explained by two nonmutually exclusive factors: 1) physical EE may reduce the salience of stimuli eliciting 50-kHz USV associated with social exploration, in agreement with current and previous data of enhanced habituation in enriched rats (Brenes et al., 2009; Elliott and Grunberg, 2005; Neugebauer et al., 2004; Zimmermann et al., 2001); and 2) physical EE may induce an extinction-like effect by accelerating learning that no social encounter follows the emission of separation-induced 50-kHz USV. In support of the latter, we have recently shown that social approach normally occurs during first exposure to playback of 50-kHz USV, but not in response to a second exposure even 1 week later, an effect that can be blocked by post-trial treatment with the amnesic drug scopolamine (Wöhr and Schwarting, 2012). This indicates that a particular type of learning and memory for social acoustic stimuli is active during ultrasonic communication, which might also encompass 50-kHz USV emission and not just the behavioral response to them.

Effects of social and physical EE on social approach in response to 50-kHz USV

As outlined above, it has repeatedly been shown that EE can lead to a number of beneficial effects, especially in case of models of brain dysfunctions, and our present data on adult hippocampal neurogenesis, miRNA expression levels, and learning and memory are consistent with that view. However, our results obtained in the USV playback paradigm seem to contrast with such an auspicious view. On the one hand, social EE had positive effects, because it not only enhanced the relative amount of prosocial 50-kHz USV, i.e., FLAT calls, but it also led to enhanced social exploration and approach in response to such signals. Physical EE, on the other hand, caused social deficits, as indicated by a lack of social exploration and approach in PE rats when exposed to 50-kHz USV. This finding is consistent with the intense world syndrome/theory of autism of Markram and Markram (2010; see also Markram et al., 2007), who hypothesized that the core neurophysiological pathology of autism is “excessive neuronal information processing and storage in local circuits of the brain, which gives rise to hyper-functioning of brain regions most affected. Such hyper-functioning in different bran regions is proposed to cause hyper-perception, hyper-attention, and hyper-memory that could potentially explain the full spectrum of symptoms in autism” (Markram et al., 2007). In line with this idea, physical EE might have led to “hyper-plasticity” and “hyper-reactivity” (increased levels of c-fos, CREB1, and miRNAs), together with “hyper-memory” (improved learning and memory in open field and object recognition), but reduced social functioning in terms of both sender (reduced prosocial 50-kHz USV emission) and receiver (lack of social approach behavior in response to 50-kHz USV playback). Of note, whereas the reduction in prosocial 50-kHz USV emission following physical EE was seen in two independent behavioral test

paradigms, namely, cage test and open field, lack of social approach behavior in response to 50-kHz USV playback was seen in only one behavioral test paradigm; a replication appears to be needed. Also, it would be interesting to see how direct social interactions are affected by physical EE.

Although our findings are in line with the intense world syndrome/theory of autism, they might appear surprising because EE has successfully been used to reverse social deficits in animal models of autism (Schneider et al., 2006) and fragile X syndrome (Oddi et al., 2014; Restivo et al., 2005). It was also found to ameliorate the negative effects of prenatal stress and inflammation on social behavior (Connors et al., 2014; Laviola et al., 2004; Morley-Fletcher et al., 2003). Schneider et al. (2006) therefore proposed EE as an "important tool for the treatment of autism." Our results, however, suggest exactly the opposite, namely, that exposure to physical EE results in social deficits. Importantly, the observed deficits were specific for behavioral changes in response to playback of prosocial 50-kHz USV, because no effects were observed in response to our acoustic control, time- and amplitude-matched white noise. Although there are many potential reasons for the observed discrepancy, it is particularly relevant to highlight the fact that we used normal healthy rats in our study and not a model characterized by brain dysfunction. This difference might actually be of importance because reversal of deficits by EE were mostly obtained in models with severe cognitive impairments (Oddi et al., 2014; Restivo et al., 2005, Speisman et al., 2013; Will et al., 1976; Wolf et al., 2006). It would therefore be of interest to see whether physical EE also exerts beneficial effects in a model displaying social deficits but intact cognitive functions. Furthermore, it is important to highlight that the deficits in social exploration and approach induced by physical EE were prevented when rats were exposed to social EE in addition to physical enrichment, i.e., in PESE rats. This indicates that enhanced affiliative behavior provided by rearing rats in larger groups during adolescence has positive effects on the rats' social development that counteract or compensate for deficits induced by physical EE. In fact, Oddi et al. (2014) observed improved social functioning in the fragile X mouse model following social EE, and in the study by Schneider et al. (2006), reversal of autism-related behavioral deficits induced by valporate acid was obtained in rats exposed to social and physical EE, and not just physical EE. Moreover, Schneider et al. (2006) found that exposure to social and physical EE during early development results in more rough-and-tumble play behavior in juveniles and higher levels of social exploratory behavior in adulthood, irrespective of whether the animals were treated with valporate acid before or not. This means that the combination of both factors, i.e., social and physical EE, has positive effects on social behavior, and this is exactly what was found in the present study, in which the highest levels of social approach were seen in PESE rats, i.e., rats exposed to both social and physical EE.

As outlined above, increased social contact provided by living in larger groups during adolescence appears to enhance social competence (Taborsky and Oliveira, 2012). In fact, early social EE has been found to affect social behavior in different domains. For example, it is known to increase social grooming and other forms of social interaction (Green et al., 2010; Laviola et al., 2004; Morley-Fletcher et al., 2003; Neugebauer et al., 2004; Schneider et al., 2006). Also, mice reared in a communal nest, an early form of social EE, more promptly achieve the social status of either the dominant or the subordinate, display a marked propensity to interact socially, and show a pronounced emotional response that is modulated by the social context (Branchi et al., 2006; for review, see Branchi, 2009). The emission of USV and appropriate responses to such USV is an important part of the social repertoire of rodents. For instance, rats prefer conspecifics that emit abundant 50-kHz USV over those calling at lower rates (Panksepp et al., 2002). Rough-and-tumble play, usually associated with high levels of 50-kHz USV (Burgdorf et al., 2008; Knutson et al., 1998), is altered in deaf (Siviy and Panksepp, 1987) and devocalized (Kisko et al., 2015) rats. Similarly, devocalization of male rats disrupts sexual behavior by reducing proceptive and receptive behavior in females, yet such deficits are reversed by playback of male USV (Thomas et al., 1982; White and Barfield, 1990). The present study is in line with these findings and shows for the first time that variation in the level of social stimulation provided during adolescence alters social approach in response to 50-kHz USV.

Methodological considerations

The two physical EE conditions, namely, PE and PESE, as used in the present study, differed in the size of the cage. This difference in cage size is explained by the fact that the space available per rat and number of enrichment items were proportional to the number of rats housed together to avoid crowding. It is unlikely that the size of the cage had a prominent impact on the results obtained. For most significant physical EE effects, these two groups did not differ from each other, indicating that differences in cage dimensions and number of enrichment items were largely irrelevant

for the measures determined, except for some minor additive effects seen in PESE rats. The only experiment in which the two physical EE conditions clearly differed from each other was the prosocial 50-kHz USV playback experiment. However, considering that PE rats displayed a lack of approach behavior in response to prosocial 50-kHz USV, whereas PESE rats displayed enhanced social approach behavior, one can attribute these differences to the presence of additional conspecifics in the PESE condition and not to the size of the cage, as opposite effects are unlikely to be explained by even more enrichment items in the PESE as compared with the PE condition.

CONCLUSIONS

In summary, we showed that social and physical EE have differential effects on brain plasticity, cognition, and ultrasonic communication in rats. Physical EE led to enhanced brain plasticity, as revealed by means of cell proliferation and survival in the DG of the hippocampal formation, as well as c-fos, CREB1, and miRNA expression levels. Concomitant improvements were observed in learning and memory paradigms, yet social deficits were seen in the emission of prosocial 50-kHz USV and paralleled by a lack of social approach in response to them, consistent with the intense world syndrome/theory of autism. In contrast, social EE had only minor effects on brain plasticity and cognition, but led to increased relative emission rates of prosocial 50-kHz USV and enhanced social approach behavior. Importantly, social deficits following physical EE were prevented by additional social EE. This shows that social and physical EE can have independent, additive, or even opposite effects, depending on the biological or behavioral domain studied. The finding that social EE has positive whereas physical EE has negative effects on social behavior is highly relevant for enrichment research. For example, it indicates that preclinical studies focusing on EE as a potential treatment in models for neuropsychiatric disorders characterized by social deficits, such as autism, should include social EE in addition to physical EE, because its lack might worsen social deficits. A better understanding of such differential effects may help to uncover specific factors underlying beneficial and adverse effects of EE in distinct behavioral domains.

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ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. J.B., R.S., and M.W. conceived and designed the experiments; J.B., M.L., and M.W. performed the experiments; J.B. and M.W. analyzed the data; G.H., G.S., R.S., and M.W. contributed reagents/materials/analysis tools; J.B., R.S., and M.W. wrote the manuscript; J.B., M.L., G.H., G.S., R.S., and M.W. gave final approval of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

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