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EFFECTS OF LONG-TERM METHYLPHENIDATE TREATMENT IN ADOLESCENT AND ADULT RATS ON HIPPOCAMPAL SHAPE, FUNCTIONAL CONNECTIVITY AND ADULT NEUROGENESIS

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Abstract—Methylphenidate (MPH) is a widely prescribed stimulant drug for the treatment of attention deficit hyperactivity disorder (ADHD) in children and adolescents. Its use in this age group raises concerns regarding the potential interference with ongoing neurodevelopmental processes. Particularly the hippocampus is a highly plastic brain region that continues to develop postnatally and is involved in cognition and emotional behavior, functions known to be affected by MPH. In this study, we assessed whether hippocampal structure and function were affected by chronic oral MPH treatment and whether its effects were different in adolescent or adult rats. Using behavioral testing, resting-state functional MRI, post-mortem structural magnetic resonance imaging (MRI), and immunohistochemistry, we assessed MPH's effects on recognition memory, depressive-like behavior, topological features of functional connectivity networks, hippocampal shape and markers for hippocampal neurogenesis and proliferation. Object

recognition memory was transiently impaired in adolescent treated rats, while in animals treated during adulthood, increased depressive-like behavior was observed. Neurogenesis was increased in adolescent treated rats, whereas cell proliferation was decreased following adult treatment. Adolescent treated rats showed inward shape deformations adjacent to ventral parahippocampal regions known to be involved in recognition memory, whereas such deformations were not observed in adult treated animals. Irrespective of the age of treatment, MPH affected topological features of ventral hippocampal functional networks. Thus, chronic oral treatment with a therapeutically relevant dose of MPH preferentially affected the ventral part of the hippocampus and induced contrasting effects in adolescent and adult rats. The differences in behavior were paralleled by opposite effects on adult neurogenesis and granule cell proliferation.

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Key words: methylphenidate, hippocampus, neurogenesis, MRI, memory, depressive-like behavior.

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that is nowadays commonly diagnosed in children and adolescents and often persists into adulthood (Kessler et al., 2006; Pastor and Reuben, 2008). Widely prescribed psychostimulants such as methylphenidate (MPH) provide effective treatment, but their potential interference with normal brain development is unclear and raises concerns regarding its possible long-term consequences (Andersen and Navalta, 2004; Marco et al., 2011). In rodents, early-life exposure to MPH has been associated with an increased anxiety and depressive-like behavior (Bolaños et al., 2003; Carlezon et al., 2003; Wiley et al., 2009) and with impaired learning and memory performance in hippocampal dependent tasks (Gomes et al., 2010). Since the hippocampus is critically involved in learning and memory (Squire, 1992; Cohen and Eichenbaum, 1993; Bannerman et al., 2004) and implicated in aspects of depression (De Kloet et al., 2005) and anxiety disorders (Revest et al., 2009; Canteras et al., 2010) and displays considerable ongoing plasticity (Lucassen et al., 2010), psychostimulant-induced hippocampal alterations may

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Abbreviations: ANOVA, analysis of variance; CA, cornu ammonis; DCX, doublecortin; FDR, false discovery rate; fMRI, functional MRI; FOV, field of view; FST, forced swimming test; GE, gradient-echo; MANOVA, multivariate analysis of variance; MPH, methylphenidate; MRI, magnetic resonance imaging; NOR, novel object recognition; PBS, phosphate-buffered saline; SE, standard errors of the mean; TBS, tris-buffered saline; TE, echo time; TR, repetition time.

be implicated in the therapeutic potential as well as the long-term risks of this type of medication (Britton and Bethancourt, 2009).

Therapeutic-level doses of MPH have been shown to increase hippocampal noradrenaline levels (Kuczenski and Segal, 2002) and noradrenergic fiber density in the dentate gyrus (Gray et al., 2007), while juvenile treatment was found to attenuate aspects of neurogenesis during specific stages of adulthood (Lagace et al., 2006). However, it remains unclear to what extent hippocampal alterations arise from MPH's interference with ongoing neurodevelopmental processes during adolescence. Adult neurogenesis is a novel form of structural plasticity that decreases with age (Heine et al., 2004) and has been implicated in hippocampal function (Trouche et al., 2009; Deng et al., 2010). Also, cocaine, D-amphetamine and methamphetamine were shown to modulate cell proliferation in the hippocampus (Eisch and Harburg, 2006).

In this study we therefore assessed hippocampal structural and functional alterations in rats that received chronic oral MPH treatment either during adolescence or adulthood. We hypothesized that adult exposure to MPH would impair recognition memory and enhance depressive-like behavior, parallel to reduced neurogenesis and topological alterations of hippocampal functional networks, and that these changes would be more pronounced when animals were treated during adolescence.

Behavioral tests, functional and structural magnetic resonance imaging (MRI) and immunohistochemistry were combined to analyze MPH's lasting effects on: (1) hippocampus-mediated recognition memory, and depressive-like behavior in the forced swimming test (FST); (2) topological features of cerebral and hippocampal functional connectivity networks; (3) hippocampal shape, and (4) adult neurogenesis and cell proliferation in the dentate gyrus of the hippocampus.

EXPERIMENTAL PROCEDURES

Animals

All experiments were carried out in accordance with Dutch and French regulations governing animal welfare and protection, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Male Wistar rats (Harlan) were treated with methylphenidate HCl (MPH) (oral gavage, dissolved in 0.9% saline solution) or vehicle, daily around 12PM for 21 days from post-natal day (P)25 ± 0 (adolescent) or P65 ± 4 (adult) onward. Behavioral tests were performed after a 1- or 5-week washout period following treatment with 0, 2, or 5 mg/kg MPH in 120 animals ($n = 10$ per group) (Experiment A). Based upon our findings in behavioral tests, in a separate series of experiments, *in vivo* MRI was performed after a 1 week washout-period following treatment with 0 or 5 mg/kg MPH in 64 animals ($n = 16$ per group) (Experiment B1). A subset of dissected brains was subsequently used for postmortem MRI and immunohistochemical analysis of neurogenesis markers (Experiment B2).

The MPH dosing regimen used in the present study was chosen to mimic clinically relevant, therapeutic doses and route of administration in rats (Gerasimov et al., 2000; Grund et al., 2007) which had been shown before to induce approximately a four-fold increase in extracellular norepinephrine in the hippocampus (Kuczenski and Segal, 2002).

Experiment A: Behavior and cognition

Novel object recognition. The novel object recognition (NOR) test (Ennaceur and Delacour, 1988) was performed in a black wooden open-field box (100 × 100 × 60 cm) (Levallet et al., 2009). The objects to discriminate were a glass flask filled with sand and a small ceramic statue (10–15 cm high, available in quadruplicate). The first two days were devoted to habituation sessions to the apparatus and the procedure. The animals from the same cage ($n = 3$) were placed together in the testing box for ten min. From the third to the fifth day, each animal was placed in the box for 3 min, and an object (different from those used for the test) was placed in the center of the open field for the fourth and the last habituation session, in order to get the animal used to the presence of an object. The sixth day (corresponding to the first day after the end of the washout period) was devoted to the test itself, and comprised two sessions. The acquisition session (3 min) was performed by exposing the animal to two identical objects before being replaced in the home cage. Four hours later, a retention session (3 min) was performed with a familiar object (previously explored during the acquisition session) and a novel one. Object combinations (familiar vs. novel) were alternated between animals. All the sessions were video-taped and analyzed afterward. Exploration of each object at each session, defined as directing the nose to the object at a distance less than 2 cm and/or touching the object with the nose or forepaws, was collected. The exploration times of the familiar and the novel object were compared, and the recognition index (time spent exploring novel object – time spent exploring familiar object/total exploration time) at the retention session was calculated.

FST. Depressive-like behavior was assessed by a modified version of the FST (Castro et al., 2010; Bouet et al., 2011), initially designed by Porsolt et al. (1977). Briefly, it consisted of a single trial with the rat placed in a vase full of water (25 °C) for 6 min and measuring immobility, swimming, and climbing times from videotapes. The same rats were used that were exposed to object recognition before. Depth of the water was fixed at 40 cm and the diameter of the glass cylinder was 30 cm. Rats did not have the possibility to touch the bottom of the glass cylinder with their tail. Immobility was defined as the adoption by the animal of an oblique position (body angle of approximately 45° with regard to the horizontal plane of water) that was otherwise only associated with slow movements of the rat paws to remain on surface. It was performed on the third day after the end of the washout period.

Experiment B1: Cerebral functional connectivity and morphometry

In vivo MRI. *In vivo* brain status was assessed on a 4.7T horizontal bore MR system (Agilent, Santa Clara, CA, USA) with the use of a Helmholtz volume coil (90 mm diameter) and an inductively coupled surface coil (25-mm diameter) for signal excitation and detection, respectively. Anesthesia was induced with 5% isoflurane for endotracheal intubation and tail vein cannulation. During MRI, isoflurane delivery was discontinued and anesthesia was maintained with 60 mg/kg/h propofol (20 mg/ml) i.v., following a 15 mg/kg bolus. One animal died during the transition to propofol. Animals were ventilated with a mixture of O₂/air (1:7). Blood oxygen saturation and heart rate were monitored using a pulse oximeter (8600V, Nonin Medical, Plymouth, MN, USA), and end-tidal CO₂ with a capnograph (Multinex 4200, Datascope Corporation, Paramus, NJ, USA). Body temperature was maintained at 37.0 ± 0.5 °C.

Anatomical images were obtained with a 3D gradient-echo (GE) sequence (repetition time (TR)/echo time (TE) = 6/2.25 ms; 40° flip angle; four averages; 256 × 128 × 128 matrix; field of view (FOV) = 60 × 40 × 40 mm). Ten minutes of resting-state functional MRI (fMRI) was performed with a T₂-weighted single-shot GE echo planar imaging (EPI) sequence (TR/TE = 600/25 ms; 50° flip angle; 64 × 64 matrix; FOV = 32 × 32 mm; 13 × 1.5 mm coronal slices; 1000 images).

Animals were imaged during the day with a median time of day of anesthesia induction of 2:30 PM (range: 8:02 AM–10:59 PM). These acquisitions were part of a 3-h scan protocol, at the end of which animals also received an intravenous injection of D-amphetamine (1 mg/kg) as part of a 45-min pharmacological MRI paradigm. Immediately after MRI, animals were euthanized with pentobarbital (300 mg/kg, i.p.) and transcardially perfused with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. After overnight post-fixation in the skull, the brains were removed and stored in PBS with 0.05% sodium azide.

Postmortem MRI. Dissected brains ($n = 12$ per group) were scanned on a 9.4T horizontal bore MR system equipped with a 90 mm-diameter 1000 mT·m⁻¹ gradient coil (Agilent, Santa Clara, CA, USA), using a transmit and received a birdcage coil (Millipede, Agilent, Santa Clara, CA, USA). Brains were placed in a custom-made holder and immersed in a non-magnetic oil (Fomblin, Solvay Solexis, Weesp, The Netherlands). Anatomical images with an isotropic voxel size of 93.75 μm were acquired (3D GE; TR/TE = 6.87/3.34 ms; 15° flip angle; 64 averages; 320 × 160 × 200 matrix; FOV = 30 × 15 × 18.75 mm) for volume and shape analysis of the hippocampus.

Image analysis. Image intensity non-uniformity correction was performed (Sled et al., 1998), and brain masks were obtained using the Brain Extraction Tool as implemented in FSL (Jenkinson et al., 2012). Using

elastix (Klein et al., 2010), within-subject images were aligned and subsequently registered non-rigidly to an anatomical reference image. Resting-state fMRI pre-processing was performed as described before (van Meer et al., 2010).

The complex hippocampal network was analyzed using a graph theory perspective (Bullmore and Sporns, 2009, 2012; Rubinov and Sporns, 2010; Stam and van Straaten, 2012). For each subject, a weighted undirected graph $G = (V, W)$ was constructed, with a collection of nodes V and a collection of edge weights W . A node represented a voxel in the hippocampus with an associated time-varying signal. Edges between each pair of nodes were weighted by the correlation coefficient between their signals. Whereas analysis of binary graphs requires an arbitrary threshold to separate strong from weak edge connections, we computed measures that utilize all edges in a weighted manner, except for edges that have negative weights or represent self-connections (Rubinov and Sporns, 2010). Brain graphs have been found to exhibit scale-free topologies, in which the existence of hubs (i.e., superconnected nodes) may orchestrate efficient information transfer across the network. Eigenvector centrality is a measure of the hub-like property of a node (Bonacich, 1987) and eigenvector centrality mapping was performed to measure the hub-like property of each node in voxel-based networks (Lohmann et al., 2010). Furthermore, we calculated global measures of (1) segregated/modular information processing, i.e. the clustering coefficient (average of local clustering coefficients) (Onnela et al., 2005); (2) integrated/distributed information processing, i.e. the characteristic path length (average of all shortest path lengths) (Ponten et al., 2009); (3) the balance between global and local efficiency of parallel information processing, i.e., ‘small-worldness’ (Watts and Strogatz, 1998; Bassett and Bullmore, 2006); and (4) the average centrality over all nodes in the network. Clustering coefficients and characteristic path lengths were normalized based on 50 instances of surrogate random networks obtained through random edge weight permutations for each subject’s graph (Stam et al., 2009). Each network measure was independently tested for effects of age and treatment using linear modeling (analysis of variance (ANOVA)) in three regions of interest (dorsal hippocampus, ventral hippocampus, and cerebrum). *P*-values resulting from voxel-wise ANOVA tests of the centrality effect maps were false discovery rate (FDR)-adjusted to correct for multiple comparisons.

Additionally, the effects of age and treatment on functional connectivity with the dorsal and ventral hippocampus were assessed with a seed-based analysis of resting-state fMRI data. After registration with a 3D reconstruction of a stereotaxic rat brain atlas (Paxinos and Watson, 2005), bilateral gray matter regions were projected onto each subject’s functional time series. Regions of interest included cortical areas (prefrontal, frontal, orbitofrontal, visual, auditory, temporal, parietal, motor, somatosensory, and retrosplenial cortices) and subcortical areas (hippocampus, insula, caudate-putamen, globus pallidus, amygdala, thalamus, substantia nigra, dorsal raphe nuclei, and ventral tegmental area).

Functional connectivity was defined as the Fisher z' -transformed correlation coefficient of the signals between two regions.

A template image for postmortem morphometry analysis was iteratively refined by applying (1) non-rigid registration of subject images to the current template using ANTS (Avants et al., 2011), (2) averaging of the affine transformations (Woods, 2003) and deformation fields, (3) resampling subject images to the new template space, and (4) averaging the resampled images to construct the updated template.

A point-based morphometry model (Styner et al., 2004) was used to quantitatively assess variations in hippocampal shape, as previously described (Otte et al., 2012). Briefly, the hippocampal formation, encompassing the cornu ammonis (CA)-fields 1–3, dentate gyrus, and subiculum (Witter and Amaral, 2004), in the left and right hemisphere were manually outlined on the template image and separately projected onto individual subject images. Right-sided delineations were flipped onto the left side, and all delineations were converted to surface meshes. An area-preserving, distortion-minimizing spherical parameterization was computed to obtain 1002 coordinates per surface. Procrustes analysis was applied to spatially align hippocampal shapes, providing point correspondence for all mesh vertices across subjects, and between the left and right hippocampi, thus enabling a direct statistical analysis per vertex between groups by means of a multivariate analysis of variance (MANOVA), with the coordinates as the dependent variables and group as the independent variable. Reported p -values were FDR-adjusted to account for multiple comparisons.

Experiment B2: Neurogenesis and cell proliferation

Immunohistochemistry. In a randomly selected subset of the brains collected from experiment B1 ($n = 6$ –8 per group), doublecortin (DCX) immunohistochemistry was used to estimate the number of immature neurons, a commonly used marker to estimate the rate of neurogenesis. When perfusion had been suboptimal or when otherwise tissue damage was obvious, brain samples were excluded. Ki-67 immunohistochemistry was used to quantify the number of proliferating cells in the dentate gyrus of the hippocampus. Following perfusion fixation, fixed brains were cryoprotected by saturation in 30% sucrose in phosphate buffer (PBS, 0.1 M, pH 7.4) after which they were coronally sectioned in a one-in-ten series at 40 μ m on a freezing microtome (R. Jung AG, Heidelberg, Germany). After sectioning, the tissue sections were washed twice in PB to remove remaining sucrose and were then stored in PBS with 0.01% sodium azide at 4 °C until further use.

In total 23 animals were included (P25 + vehicle: $n = 5$; P25 + MPH: $n = 5$; P65 + vehicle: $n = 6$; P65 + MPH: $n = 7$). For immunocytochemical staining for DCX, free-floating tissue sections were washed with tris-buffered saline (TBS) and between incubations unless specified otherwise. Endogenous peroxidase

activity was blocked with 0.5% hydrogen peroxide in TBS for 15 min. Following blocking aspecific sites with 2% milk powder in TBS for 30 min, sections were incubated without intermittent washing with goat anti-DCX antibody (c-18, Santa Cruz) in 0.1% Triton X-100 and 0.25% gelatin in TBS for 1 h at room temperature and overnight at 4 °C. The next day, sections were incubated in primary antibody for 2 h with biotinylated donkey-anti goat (Jackson Laboratories, 1:500) followed by a 2 h amplification step with 1:800 avidin-biotin complex (ABC Elite kit, Vectastain). Thereafter, biotinylated tyramide (1:500) was incubated for 30 min in the presence of 0.01% hydrogen peroxide after which a final ABC incubation was performed for 1.5 h. Sections were washed once in TBS followed by three washes with TB before immunoreactivity was visualized by using a standard peroxidase di-amino-benzidine (DAB) reaction. After the reaction had been stopped, the sections were mounted, dehydrated in an alcohol-xylene series and coverslipped using Entellan.

The number of DCX-positive cells was quantified unilaterally using the optical fractionator method in a 1-in-10 series of brain sections that were stereologically sampled throughout the rostral-to-caudal dentate gyrus from an ad random starting point onward (10–11 sections per subject). Next, given possible differences in the roles of, and inputs to, the different hippocampal anatomical levels, rostral (four sections) and caudal (four sections) levels were compared separately, while the supra and infrapyramidal layers were compared too (Wu and Hen, 2014; Strange et al., 2014). The large amounts of DCX cells yielded a low variation, and also for practical reasons, quantification was performed unilaterally.

In total 29 rats were included for Ki-67 immunocytochemistry (P25 + vehicle: $n = 6$; P25 + MPH: $n = 8$; P65 + vehicle: $n = 7$; P65 + MPH: $n = 8$). Free floating sections were mounted on Superfrost slides and dried overnight. Sections were washed once in distilled water for 5 min to remove excess buffer salts. Antigen retrieval was performed in 0.01 M citrate buffer pH 6.0 for 5 min at 800 W, 5 min 460 W and 5 min 280 W, after sections were allowed to cool down for 20 min. Endogenous peroxidase was inactivated using 1.5% hydrogen peroxide in TBS for 15 min. Sections were blocked using 2% milk powder in TBS for 30 min and, without prior washing, subsequently incubated in rabbit anti-Ki67p antibody (Novocastra) in 0.5% Triton X-100 and 0.25% gelatin in TBS for 1 h at room temperature and overnight at 4 °C. The next day, sections were incubated for 2 h in goat anti-rabbit antibody (1:200, Vector Laboratories) in primary antibody incubation mix. The rest of the day proceeded as described above for DCX immunohistochemistry. The number of Ki-67-positive cells was lower per tissue section and was quantified bilaterally using a standard light microscope across 11–12 sections per hippocampus in the hilus and the infra- and suprapyramidal blades of the dentate gyrus. Later, also here, the four most rostral and four most caudal sections were compared separately.

Statistics

A linear model, univariate or multivariate ANOVA or a linear mixed model analysis was performed to test for effects of (1) age (P25–P65), (2) treatment (vehicle, or 2 and 5 mg/kg MPH), and (3) age-by-treatment interaction. Linear mixed model analysis was performed in *R* (www.r-project.org) using *nlme*, or *lme4* and *languageR* packages. Multiple general linear hypothesis testing was performed using the *glht* package. Following non-independent tests, *p*-values were FDR-adjusted. Behavioral and immunohistochemistry data are presented in bar graphs that display mean and standard error of the mean. Where appropriate, standard errors of the mean (SE) obtained from linear model fits are reported. Otherwise, means $\pm$ standard deviations of data are reported.

RESULTS

Behavior and cognition

Novel object recognition. The exploration times during the acquisition and retention phases of the novel object recognition test are shown in Fig. 1A. First, we tested the effects of MPH dose, age, washout period, and object, on the object exploration times during the retention phase. Exploration times were significantly greater for the novel object as compared to the familiar object (age effect, $F_{1,115} = 74.0$, $p < 0.001$), and decreased with washout period ($F_{1,116} = 25.8$, $p < 0.001$). MPH dose (0, 2, or 5 mg/kg) did not exert a significant overall effect ($F_{1,116} = 0.6$, $p = 0.443$), but the interaction of object by dose ($F_{1,115} = 6.1$, $p = 0.015$) and object by age ($F_{1,115} = 3.9$, $p = 0.049$) was significant. Together, these interaction effects could indicate that the difference in exploration times between the objects was smaller in treated adolescents as compared to controls (-31.9% , $SE = 13.1\%$, $Z = -2.4$, $p = 0.032$), which was not found in adult treated animals (-4.4% , $SE = 18.7\%$, $Z = -0.2$, $p = 0.816$).

Second, we tested the retention index (Fig. 1B), i.e. the difference between novel and familiar object exploration times normalized with respect to the total exploration times. Longer exploration times for the novel object as compared to the familiar object resulted in a retention index that was significantly longer than 0.5, corresponding to random exploration ($t = 5.5$, $p < 0.001$). However, the retention index was not significantly different between age groups ($F_{1,111} = 0.05$, $p = 0.832$), dose ($F_{1,111} = 0.61$, $p = 0.438$), and washout periods ($F_{1,111} = 0.17$, $p = 0.680$). As a result, post-hoc tests did not reveal significant differences with vehicle-treated animals in adolescents ($Z = -1.2$, $p = 0.443$) or adults ($Z = -0.1$, $p = 0.940$).

FST. Overall immobility times during the 6 min FST (Fig. 2) were significantly longer in adult animals as compared to adolescent animals ($F_{1,112} = 5.0$, $p = 0.028$). There was neither an overall significant effect of dose, nor of its interactions with age and washout. However, pairwise tests contrasting 2 and

5 mg/kg MPH treatment with vehicle revealed that following 1-week washout, adult treated animals showed increased immobility upon treatment ($+28.3\%$, $SE = 10.6\%$, $t = 2.675$, $p = 0.030$). No significant differences were observed after a 5-week washout period in adults (-3.2% , $SE = 7.6\%$, $t = -0.416$, $p = 0.903$), and also not in adolescents following short washout (-0.72% , $SE = 12.3\%$, $t = -0.059$, $p = 0.953$) and long washout (-9.2% , $SE = 10.1\%$, $t = -0.911$, $p = 0.724$).

Functional connectivity and morphometry

Resting-state fMRI functional connectivity. The global brain network measures are summarized in Table 1. MPH treatment had a significant effect on the average centrality of the voxels within the dorsal hippocampus ($+9.2\%$, $SE = 4.4\%$, $F_{1,56} = 4.1$, $p = 0.049$), while in the ventral hippocampus treatment resulted in an increased clustering coefficient ($+5.2\%$, $SE = 2.5\%$, $F_{1,56} = 4.2$, $p = 0.045$), shorter characteristic path lengths (-2.0% , $SE = 0.9\%$, $F_{1,56} = 4.9$, $p = 0.030$), and increased small-worldness ($+7.1\%$, $SE = 3.2\%$, $F_{1,56} = 4.8$, $p = 0.033$).

Clustering coefficient decreased with age in the dorsal hippocampus ($F_{1,56} = 6.0$, $p = 0.017$), but increased with age in the ventral hippocampus ($F_{1,56} = 4.7$, $p = 0.035$). Characteristic path length was higher in adults as compared to adolescents in the dorsal hippocampus ($F_{1,56} = 10.01$, $p = 0.002$). Dorsal small-worldness was larger in adults ($F_{1,56} = 8.3$, $p = 0.006$), while ventral small-worldness was larger in adolescents ($F_{1,56} = 4.1$, $p = 0.047$).

Centrality maps confined to the dorsal and ventral hippocampus did not reveal significant effects of age or treatment at the voxel level.

Complex graph measures of cerebral networks were not significantly different between adults and adolescents, and between treatment conditions. However, the centrality maps showed a significant difference between adolescents and adults (Fig. 3A). In the bilateral striatum, adult centrality values were increased as compared to values in adolescents, while in the caudal part of the dorsal hippocampus, the adult centrality values were slightly lower as compared to adolescents (Fig. 3B).

Seed-based analysis also detected a significant effect of age in the functional connectivity between the dorsal hippocampus and the caudate-putamen ($F_{1,59} = 24.1$, $p = 0.005$), which decreased to -0.21 ± 0.20 in adults as compared to 0.03 ± 0.19 in adolescent animals. There were no significant differences associated with treatment or age by treatment interaction.

Parametric shape analysis of hippocampus. Overall, hippocampal volume increased with age ($+4.9\%$; $SE = 2.0\%$; $F_{1,44} = 6.1$, $p = 0.018$). We found an overall effect of age on hippocampus shape, with the significantly displaced vertices showing an average outward displacement of 0.100 ± 0.209 mm (Fig. 4, first and third rows). The age-related distances of outward

Fig. 1. Novel object recognition. (A) Bar graphs represent mean and standard error of the mean of the exploration times of two objects for adolescent and adult treated animals for the novel object recognition test. During the acquisition phase, animals were familiarized with two identical objects (left and right). A novel object was presented next to the familiar object during the retention phase. Animals were treated with 0, 2, or 5 mg/kg methylphenidate (MPH) during adolescence (P25) or adulthood (P65). The test was executed following a short (1 week) or a long (5 weeks) washout period. A significant effect of object by dose ($F_{1,115} = 6.1$, $p = 0.015$) and object by age ($F_{1,115} = 3.9$, $p = 0.049$) was found, indicating that the average difference between exploration times of the novel and familiar objects was 31.9% smaller ($SE = 13.1\%$, $p = 0.032$) following treatment in adolescents. (B) The ability to discriminate a familiar from a novel object during the retention phase of the novel object recognition test was calculated using the retention index. A value above 0.5 indicates increased exploration of the novel object (i.e., positive retention of the familiar object). The test was performed in rats treated with 0 (vehicle), 2, and 5 mg/kg methylphenidate during adolescence (P25) or adulthood (P65) and following a short (1 week) or a long (5 weeks) washout period. The retention index was not significantly affected by age, treatment dose, or washout period.

deformations were on average 0.221 ± 0.153 mm and were most pronounced in the lateral side toward the temporal pole, while inward deformations were most pronounced at the medial side and displaced on average -0.117 ± 0.085 mm (Fig. 4, bottom row).

Significant age-by-treatment interaction effects, though small on average, indicated three regions comprising more than a single vertex where treatment induced larger deformations in adolescents than in adults (Fig. 4, second row). In the temporal CA1-region adjacent to the perirhinal and entorhinal cortex, adolescents showed an inward deformation upon treatment (-0.213 ± 0.106 mm) while adults showed a very small increase (0.049 ± 0.041 mm). In the dorsal subiculum adjacent to the primary visual cortex,

treatment resulted in an outward deformation in adolescents (0.114 ± 0.020 mm) but not in adults (0.023 ± 0.010 mm). Finally, mostly in the right-sided caudoventral subiculum, adolescents showed a large inward deformation following treatment (-0.257 ± 0.053 mm) as compared to adults (0.059 ± 0.015 mm).

Neurogenesis and cell proliferation

Neurogenesis. The numbers of DCX-positive cells are plotted in Fig. 5. In the rostral dentate gyrus, cell number showed a significant effect of age ($F_{1,19} = 11.8$, $p = 0.003$), with 28.5% fewer cells counted in adult treated rats ($SE = 6.3\%$, $t = -4.5$, $p = 0.001$).

Fig. 2. Forced swimming test: immobility times. Graph plots the times of immobility during a 6-min forced swimming test in rats treated with 0 (vehicle), 2, or 5 mg/kg methylphenidate when adolescent (P25) or adult (P65). Longer immobility times are considered depressive-like behavior. The test was performed following a short (1 week) or a long (5 weeks) washout period. Immobility times increased with age, as both treatment during adulthood, and testing after the long washout period, resulted in prolonged immobility ($F_{1,112} = 5.0$, $p = 0.028$). After the short washout period, adult treated animals showed increased immobility following methylphenidate treatment as compared to vehicle treatment (+28.3%, $SE = 10.6\%$, $t = 2.675$, $p = 0.030$).

Table 1. Global properties of hippocampal and cerebral functional networks

Region	Parameter	Group Mean $\pm$ standard deviation				Statistics F (p -value)		
		P25 vehicle	P25 MPH	P65 vehicle	P65 MPH	Age	Treatment	Age $\times$ Treatment
Dorsal hippocampus	Clustering coefficient	1.81 $\pm$ 0.16	1.81 $\pm$ 0.15	1.69 $\pm$ 0.19	1.71 $\pm$ 0.17	6.0 (0.017)*	0.0 (0.853)	0.0 (0.950)
	Characteristic path length	0.69 $\pm$ 0.02	0.69 $\pm$ 0.03	0.72 $\pm$ 0.03	0.71 $\pm$ 0.02	10.1 (0.002)*	0.5 (0.468)	0.6 (0.447)
	Small-worldness	2.62 $\pm$ 0.29	2.63 $\pm$ 0.31	2.37 $\pm$ 0.34	2.41 $\pm$ 0.30	8.3 (0.006)*	0.1 (0.728)	0.0 (0.825)
	Centrality	0.44 $\pm$ 0.14	0.52 $\pm$ 0.05	0.49 $\pm$ 0.04	0.50 $\pm$ 0.05	0.6 (0.459)	4.1 (0.049)*	2.1 (0.156)
Ventral hippocampus	Clustering coefficient	0.49 $\pm$ 0.15	0.52 $\pm$ 0.05	0.53 $\pm$ 0.06	0.54 $\pm$ 0.04	1.7 (0.193)	1.0 (0.332)	0.2 (0.656)
	Characteristic path length	1.86 $\pm$ 0.26	1.97 $\pm$ 0.19	1.98 $\pm$ 0.16	2.07 $\pm$ 0.13	4.7 (0.035)*	4.2 (0.045)*	0.0 (0.874)
	Small-worldness	0.70 $\pm$ 0.04	0.69 $\pm$ 0.02	0.69 $\pm$ 0.01	0.68 $\pm$ 0.02	2.7 (0.107)	4.9 (0.030)*	0.5 (0.466)
	Centrality	2.67 $\pm$ 0.48	2.89 $\pm$ 0.36	2.87 $\pm$ 0.28	3.05 $\pm$ 0.26	4.1 (0.047)*	4.8 (0.033)*	0.1 (0.820)
Cerebrum	Clustering coefficient	1.67 $\pm$ 0.20	1.64 $\pm$ 0.21	1.64 $\pm$ 0.17	1.64 $\pm$ 0.17	0.1 (0.754)	0.1 (0.798)	0.1 (0.705)
	Characteristic path length	0.73 $\pm$ 0.04	0.72 $\pm$ 0.04	0.74 $\pm$ 0.03	0.73 $\pm$ 0.03	1.3 (0.265)	0.4 (0.517)	0.1 (0.812)
	Small-worldness	2.32 $\pm$ 0.41	2.29 $\pm$ 0.44	2.23 $\pm$ 0.31	2.26 $\pm$ 0.34	0.4 (0.526)	0.0 (0.958)	0.1 (0.756)
	Centrality	0.40 $\pm$ 0.09	0.42 $\pm$ 0.12	0.45 $\pm$ 0.09	0.44 $\pm$ 0.11	2.0 (0.162)	0.0 (0.844)	0.2 (0.624)

Weighted graphs were constructed for the dorsal hippocampus, ventral hippocampus, and cerebrum. Voxels represented nodes, and the edges between nodes were weighted by the correlation coefficient of their resting-state time-varying signals. Negative and self-referential edges were excluded. The global measures that characterize these graphs are here summarized. Animals were treated with 0 (vehicle) or 5 mg/kg methylphenidate (MPH) during adolescence (P25) or adulthood (P65). Independent ANOVA tests were performed to test for effects of age, treatment, and age by treatment interaction.

* $p < 0.05$.

Caudally, the number of DCX-cells showed an effect of age ($F_{1,19} = 12.5$, $p = 0.002$), treatment ($F_{1,19} = 10.8$, $p = 0.004$), and age-by-treatment ($F_{1,19} = 5.9$, $p = 0.025$), mainly because of a 49.9% increase in adolescent treated animals ($SE = 15.2\%$, $t = 3.3$, $p = 0.012$), with no measurable differences in adult animals. The total number of DCX⁺ cells in the dentate

gyrus also showed an age-effect ($F_{1,19} = 24.1$, $p < 0.001$) and 28.4% fewer counts were present in adults ($SE = 4.9\%$, $t = -5.8$, $p < 0.001$). No effects of age or treatment were detected in the middle slices. In addition, the volume of the dentate gyrus did not significantly vary with age or treatment in any of these regions.

Fig. 3. Centrality maps from cerebral graph analysis. Centrality maps were obtained from resting-state fMRI-based graphs limited to cerebral voxels. Significant ($p < 0.05$, false discovery rate (FDR)-adjusted) effects of age were observed for voxels in the caudal dorsal hippocampus, and in the striatum (panel A). The differences between adult and adolescent centrality values are displayed in panel B.

Cell proliferation. The numbers of Ki67-positive cells are plotted in Fig. 6. Suprapyramidal cell counts showed a significant effect of age ($F_{1,25} = 5.3$, $p = 0.030$) because of a 28.6% lower count in adults ($SE = 9.2\%$, $t = -3.1$, $p = 0.027$). There was no significant treatment effect ($F_{1,25} = 2.0$, $p = 0.167$). Infrapyramidal cells showed an effect of age by treatment ($F_{1,25} = 4.9$, $p = 0.036$), reflecting an opposite treatment effect in adults as compared to adolescents (-43.7% , $SE = 11.3\%$, $t = -3.9$, $p = 0.004$). No significant age and treatment effects were found in the hilus of the dentate gyrus. Fig. 7 shows representative images of Ki-67 and DCX immunocytochemistry in the hippocampal dentate gyrus.

DISCUSSION

As the hippocampus mediates learning and memory processes and is involved in emotional and affective behavior, we assessed whether structure and function of the hippocampus are affected by chronic oral MPH administration, and whether effects induced by periadolescent exposure differ from those during adulthood. In our analyses of resting-state fMRI and neurogenesis, we specifically addressed different parts

of the hippocampus, since its dorsal and ventral (or septal and temporal) regions are implicated in separate functions (Moser and Moser, 1998; Fanselow and Dong, 2010). The dorsal hippocampus contributes to cognitive functions, i.e. spatial learning and memory function (Bannerman et al., 2004), while the ventral hippocampus is involved in anxiety, emotional memory and stress regulation (Barkus et al., 2010). Cognitive processes critically depend upon the dorsal hippocampus-subiculum complex which projects to retrosplenial and anterior cingulate cortices (Fanselow and Dong, 2010). On the other hand, the targets of the ventral hippocampus and ventral subiculum include the amygdala and medial prefrontal cortex, enabling control over emotional and affective processes (Fanselow and Dong, 2010).

Periadolescent treatment affects recognition memory, neurogenesis, and hippocampal shape

The object recognition test revealed that the difference in time spent exploring the novel vs. the familiar object decreased following adolescent treatment with 2 and 5 mg/kg MPH. This is in line with earlier observations that locomotor response of adult rats to a novel environment was reduced after repeated

Fig. 4. Hippocampus shape analysis. Statistical analysis of the hippocampus shape using vertex-wise MANOVA tests revealed a significant ($p < 0.05$, false discovery rate (FDR)-adjusted) effect of age and age by treatment. Each row displays four different views with vertex values averaged between the left and right hippocampi. The vertices of the models in first two rows are color-coded according to the normalized p -value (Z) for a significant effect of age (top row) and age by treatment (second row). The average distances of vertices from the adolescent (P25) vehicle-treated hippocampus to the adult (P65) vehicle-treated hippocampus are depicted in the bottom row. Septal (S) and temporal (T) poles, and medial (M) and lateral (L) sides, are indicated in each view on the top row. The numbers in the second row indicate three distinct regions: (1) an inward deformation in adolescent treated animals (-0.213 ± 0.106 mm; adults: 0.049 ± 0.041 mm) in the temporal CA1-region adjacent to the entorhinal (Ent) and perirhinal (PRh) cortex; (2) an outward deformation in adolescent treated animals (0.114 ± 0.020 mm; adults: 0.023 ± 0.010 mm) in the dorsal subiculum (Sub) region adjacent to the primary visual cortex (V1); and (3) an inward deformation in adolescent treated animals (-0.257 ± 0.053 mm; adults: 0.059 ± 0.015 mm) in the caudal part of the ventral subiculum adjacent to the medial entorhinal cortex.

2 mg/kg i.p. administration of MPH during adolescence (Bolaños et al., 2003). Similarly, long-term treatment with 3 and 5 mg/kg oral MPH impaired performance on the object recognition task (LeBlanc-Duchin and Taukulis, 2007), although it has also been reported that recognition memory is unaffected by 2 and 5 mg/kg oral MPH treatment in rats during periadolescence and young adulthood (Bethancourt et al., 2009). It should be noted that our findings of small and transient impairments in object recognition were not expressed as significant differences on the recognition index, which may be ascribed to the limited power of statistical tests on these derived indices.

Alternatively, the discrepancy between our findings and those of Bethancourt et al. may lie in the different time intervals (twice daily for 7 days vs. daily for 21 days in the present study) between the acquisition and retention tests (Bethancourt et al., 2009), and hence in

the extent to which the hippocampus is involved in task performance, in addition to different ages P21–25 and P64 in this study vs. P27 and 71 respectively, washout periods (18 days vs. 7 days in the present study), although both studies used male Wistar rats as well as oral MPH. Recognition memory is putatively subserved by two distinct but integrated networks, i.e. the hippocampal and the perirhinal systems (Brown and Aggleton, 2001). Notably, as hippocampal lesions not necessarily impair recognition memory function directly, animal studies have shown the hippocampus, perirhinal cortex, and medial prefrontal cortex, to be involved in recognition memory tasks (Broadbent et al., 2004; Winters et al., 2004; Barker and Warburton, 2011). It is assumed that in the absence of learning associations from temporal or spatial contextual cues, recognition memory function is chiefly served by the perirhinal cortex (Brown and Aggleton, 2001; Barker and Warburton, 2011).

Fig. 5. Estimated number of DCX⁺ cells in the dentate gyrus. To assess the level of adult neurogenesis, the total number of doublecortin (DCX)-immunoreactive cells was estimated using the optical fractionator method in the rostral, medial, and caudal dentate gyrus. Animals were treated with 0 (vehicle) or 5 mg/kg methylphenidate during adolescence (P25) or adulthood (P65). The number of DCX⁺ cells showed a significant decline with age (-28.5% , $SE = 6.3\%$, $t = -4.5$, $p = 0.001$). The number of DCX⁺ cells in the caudal dentate gyrus was significantly increased in adolescent treated animals only ($+49.9\%$, $SE = 15.2\%$, $t = 3.3$, $p = 0.012$).

Fig. 6. Number of Ki67⁺ cells in the dentate gyrus. Cell proliferation was measured by counting the number of cells expressing Ki67 immunoreactivity in the infrapyramidal and suprasyramidal blades of the subgranular zone and granular cell layer, and in the hilus, of the dentate gyrus. Animals were treated with 0 (vehicle) or 5 mg/kg methylphenidate during adolescence (P25) or adulthood (P65). The suprasyramidal blade showed a significantly lower number of Ki67⁺ cells in adults as compared to adolescents (-28.6% ; $SE = 9.2\%$, $t = -3.1$, $p = 0.027$). In the infrapyramidal blade, methylphenidate had an opposite effect on the number of Ki67⁺ cells in adults as compared to adolescents (-43.7% , $SE = 11.3\%$, $t = -3.9$, $p = 0.004$).

Although, we cannot fully exclude a contribution of gavage-related stress exposure on the acquisition phase of the object recognition object task, it is very unlikely stress has contributed significantly to our results. First, the volume used for gavage administration was low, in accordance to animal welfare guidelines aimed to avoid excessive stress responses in rats. Second, our protocol includes a chronic daily administration of MPH during 21 consecutive days, before both the short (7 days – 1 week) and the long (35 days – 5 week) washout period. As with any

repeating stimulus, this long-lasting protocol will likely have caused adaptation. Indeed, based on the literature, a first gavage is known to increase blood pressure, heart rate and corticosterone levels (Turner et al., 2011), but these parameters returned to normal after the fourth gavage in a repeated protocol (Ökva et al., 2006). Thus, after our short washout period (7 days), stress levels are expected to have returned to baseline levels and indeed, in our hands the animals adjusted quickly to repeated gavage drug administration. Third, a shorter washout delay – with more recent

Fig. 7. Representative images of Ki-67 and DCX immunocytochemistry in the hippocampal dentate gyrus. (A); overview of the dentate granular cell layer (gcl) in blue, with Ki-67 positive cells in black (arrows), reflecting proliferation. (B); higher magnification of a cluster of 4 Ki-67 immunopositive cells. (C); overview of the granular cell layer with numerous DCX-immunopositive cells in brown, reflecting neurogenesis. (D); higher magnification of DCX-immunopositive cells with their cell bodies (arrow) located at the border zone of the granular cell layer and the hilus (h) and their distinct protrusions passing through the GCL into the molecular layer (m).

memories of the stressful event – would be expected to lead to a decreased exploration time rather than to an increase – as we observed in our study. Moreover, all animals had learned the test.

The results from our shape analysis of the hippocampal formation further implicate parahippocampal neocortical areas in the impairment in recognition memory after treatment during adolescence, since the most pronounced locally inward deformations of the hippocampus were found in the mediotemporal CA1 region that is adjacent to the entorhinal and perirhinal cortices. Of course, the vertex displacements on a boundary representation of the hippocampal formation provide only an aggregate view of differences in the underlying microstructural composition of adjacent structures. Hence, it remains unclear whether presently observed inward deformations of the hippocampus in adolescent treated rats represent a loss of volume of the CA1-region, or conversely, may have resulted from local tissue compression because of expansion of adjacent white matter or neocortical areas. Testing this hypothesis would require a longitudinal/sequential approach during which the temporal development of such an effect could be accurately measured. Unfortunately, this is not possible in the present tissue that represents only two time points and concerns terminal experiments.

Moreover, adolescent exposure to MPH significantly increased hippocampal neurogenesis in the caudal, but not the rostral, part of the dentate gyrus, as indicated by an increased number of DCX⁺ cells whereas Ki67-based estimates of cell proliferation were unaltered. Evidence from *in vitro* experiments with low doses of MPH suggests that differences in DCX⁺ cell numbers could indicate enhanced survival (Bartl et al., 2010). Our findings

seem thus to be consistent with an increased survival of adult-born neurons after chronic low-dose D-amphetamine treatment in mice during adolescence and early adulthood (Dabe et al., 2013). In contrast, periadolescent i.p. treatment with 2 mg/kg MPH was found not to affect adult granule cell proliferation, while the number of adult-born cells in the temporal hippocampus that survived during a 4-week period was reduced (Lagace et al., 2006). A likely explanation for these divergent observations is that reduced cell survival and novelty-induced locomotor responses were found between 7 and 11 weeks after cessation of drug exposure (Lagace et al., 2006), whereas in our study neurogenesis and proliferation was measured 1 week after termination of treatment.

Although the precise relationship between neurogenesis and memory function remains elusive, the context, type, and timing of the memory task are known to be important (Shors, 2004; Sahay and Hen, 2007; Deng et al., 2010), as well as the tools, timing, and survival of the specific newborn cell population under study. The observed increase in the number of DCX⁺ cells could represent a maladaptive response to chronic stimulant treatment, and as such, may reflect structural changes underlying the drug's long-term effects on cognition. A similar mechanism has been proposed to underlie the sustained social avoidance response in stressed mice, which show lastingly increased levels of adult hippocampal neurogenesis after stress induction (Lagace et al., 2010).

Adult treatment increases depressive-like behavior and reduces cell proliferation

Immobility times measured from the FST suggest that long-term drug treatment in adult rats might increase

depressive-like behavior, at least after a short (1 week) washout period. In adolescents, immobility times were not affected after a prolonged abstinence from MPH, whereas periadolescent exposure to MPH was associated with depressive-like effects, at least based on increased immobility in the FST in rats when tested during adulthood (Bolaños et al., 2003; Carlezon et al., 2003). Thus, increased neurogenesis in adolescents could possibly have contributed to alleviating depressive-like responding in the FST.

We further observed that infrapyramidal cell proliferation was reduced following adult treatment, whereas neurogenesis was not significantly different from vehicle-treated controls. This is in line with experimental studies suggesting that, similar to the effects of opiates, chronic exposure to psychostimulants such as cocaine and amphetamines decreases proliferation, but does not affect neuronal differentiation (Eisch and Harburg, 2006).

The presently observed reduction in proliferating cells may, at least partly, contribute to the concomitantly increased depressive-like behavior in the FST. Previously, MPH exposure has been linked to a heightened sensitivity for stressful and anxiogenic situations, which could be reversed with antidepressants (Bolaños et al., 2008). Moreover, decreased adult neurogenesis has been associated with depressive illness since one of its main risk factors, i.e. chronic stress, induces depressive-like symptoms while it reduces neurogenesis at the same time (Oomen et al., 2007; Lucassen et al., 2010). Also, antidepressants depend on neurogenesis to exert their effects (Santarelli et al., 2003; Sahay and Hen, 2007), and transgenic neurogenesis-deficient mice display prolonged stress responses and increased depressive-like behavior (Snyder et al., 2011; but see Lucassen et al., 2012).

MPH affects ventral hippocampus functional connectivity

Summary statistics of voxel-based functional connectivity graph measures showed small treatment effects in dorsal and ventral hippocampal networks. In the ventral hippocampus, a higher clustering and shorter average path lengths both contributed to an increased small-world index following treatment, while only in the dorsal hippocampus, the average node centrality was higher following treatment.

The small-world property is considered a hallmark of complex networks that enables efficient parallel information transfer among locally segregated computation units (Watts and Strogatz, 1998). Various neuropathologies have been associated with a deviation from 'normal' values on the small-world index in the direction of topologies that are either more random (i.e., low clustering and short paths) or more regular (i.e., high clustering at the cost of long paths) (Bullmore and Sporns, 2009). Although it remains poorly understood what the functional significance of such shifts are, since control values are not independent of the graph topology (Van Wijk et al., 2010) and methodology used to construct the graphs in the first place (Telesford et al., 2011), one could

argue that an increased small-world index points toward a topological architecture that enables more efficient operation of the ventral hippocampal network.

Our results suggest an increased susceptibility of the ventral domain of the hippocampus to the effects of long-term MPH treatment, which may be related to regional differences in rates of cell differentiation and maturation. Indeed, along the septotemporal axis, the number of proliferating progenitor cells in the dentate gyrus peaks toward the temporal pole (Lagace et al., 2006; Noonan et al., 2010) while over the course of 4 weeks their differentiation into mature neurons proceeds at a slower pace in the temporal, as compared to the septal, domain of the dentate gyrus (Snyder et al., 2012).

Functional connectivity, volume, neurogenesis, and proliferation change with age

We further found that the small-world index in both dorsal and ventral hippocampal networks decreased with age, because of increased path lengths and reduced clustering coefficients. This could be the result of relatively large hippocampal volumes and altered hippocampal shapes in the adult treated group as compared to the adolescent treated group. Also, adult hippocampal neurogenesis may provide support for sustained small-world properties (Manev and Manev, 2000) and smaller small-world values in adult treated animals may then be the result of the general age-related decline in neurogenesis (Heine et al., 2004), as exemplified by decreased DCX expression in the rostral and medial dentate gyrus, and reduced Ki67 expression in the suprapyramidal blade. Nonetheless, adult-born hippocampal granule cells can account for as much as 40% of the total granule cell population (Snyder and Cameron, 2012) and the small-world index could therefore also signal a shift in the relative size of the granule cell populations. The population of adult-born granule cells has properties that are qualitatively different from the developmentally generated neurons (Gould and Gross, 2002), as it has been suggested that they remain highly plastic and even necessary for learning performance in, e.g., the water maze test when mature (Lemaire et al., 2012).

We applied eigenvector centrality mapping (Bonacich, 1987; Lohmann et al., 2010), which revealed that the hub-like properties of striatal and hippocampal voxels undergo changes in opposite directions during aging. Whereas the centrality of striatal voxels in the cerebral network increases, hippocampal centrality values decrease. Small-world networks often exhibit scale-free properties (Barabasi, 1999), where a power law distribution of the node connectivities indicates the existence of a small number of hub nodes. Such a hierarchical organization has been observed in brain networks (Bassett et al., 2008; Sporns et al., 2011) and has also been confirmed for *in vitro* recordings of hippocampal CA3 neurons (Li et al., 2010).

In developing networks, a scale-free organization implies that new nodes preferentially link to existing hubs (Barabasi, 1999), which can be hindered by aging

(Amaral et al., 2000). Moreover, it has been shown that GABAergic interneurons have a critical function as hubs involved in organizing network activity in the developing hippocampus (Bonifazi et al., 2009). However, in the present study the average dorsal and ventral hippocampal centrality values were not significantly different between the two age groups.

Study limitations

Resting-state fMRI was performed in anesthetized rats. General anesthetics exert their effects through potentiation of γ -aminobutyric acid (GABA)_A receptor-mediated inhibitory neurotransmission in the brain, which predominantly alters thalamic activity (Franks, 2008; Peltier and Shah, 2011). This suggests that anesthesia selectively affects connections, thereby altering the composition of large-scale brain networks (Liang et al., 2012). Although it has been found that global measures of brain network topology did not differ between the anesthetized and awake state in rats (Liang et al., 2012), anesthesia-induced changes in network organization potentially reshape the inputs that drive hippocampal signaling. It cannot be ruled out that alterations in GABAergic signaling properties during brain maturation (McCutcheon et al., 2012; Thomases et al., 2013) may thereby lead to different effects of anesthesia on hippocampal functional connectivity in adolescent and adult brains. It should also be noted that statistical dependency is highly likely when performing voxel-wise testing of eigenvector centrality maps, since the eigenvalue centrality value of hub nodes depend by definition on centrality values of other hub-like nodes (Hayasaka and Laurienti, 2010).

As we used endogenous markers to quantify cell proliferation and neurogenesis, direct comparisons cannot be made with studies that employed exogenous pulse-labeling approaches with e.g., thymidine-analogs like 5-bromo-2'-deoxyuridine (BrdU), in addition to differences in design and survival times (e.g., Lagace et al., 2006).

As DCX is exclusively expressed in young migratory neurons, and not in the stem cell or astrocyte population (Brown et al., 2003; Couillard-Despres et al., 2005), it has been shown that DCX reliably reflects experimentally induced changes in the rate of adult neurogenesis for a period of up to 30 days (Couillard-Despres et al., 2005). Nevertheless, the present increase in numbers of DCX⁺ cells, indicative of more new, immature neurons in animals that were chronically treated during adolescence, does not necessarily imply their survival into mature neurons, and/or their functional synaptic integration into hippocampal circuits.

Ki67 is a nuclear protein expressed in cells engaged in cell cycle (Scholzen and Gerdes, 2000) that has been validated as an endogenous marker for cell proliferation in the dentate gyrus (Kee et al., 2002), with estimates of about 50% of the Ki67⁺ cells representing the number of added cells over the last 24 h (Lazic, 2012). Different parts of the cell cycle may experience differential sensitivity for external disturbances (Lagace et al., 2010) such as MPH's action, or in response to alterations in dopaminergic signaling from midbrain dopaminergic neurons that

control progenitor cell proliferation and survival (Park and Enikolopov, 2010).

Finally, although we cannot exclude that MPH's effects on cell proliferation could also result from changes associated with glial cells and/or vasculature, it is known that the majority of the newly born hippocampal cells during adulthood gives rise to neurons (Cameron et al., 1993; Cameron and McKay, 2001; Kee et al., 2002).

CONCLUSION

In sum, long-term MPH exposure exerted different behavioral effects in adolescent and adult rats; object recognition memory was impaired in adolescent treated rats, while increased depressive-like behavior was only observed in rats treated during adulthood. Furthermore, irrespective of treatment timing, we observed effects on various measures of hippocampal function and structure which were most pronounced in the ventral (temporal) region.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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