Neuroscience 230 (2013) 31–38

THE EFFECTS OF VOLUNTARY RUNNING EXERCISE COINCIDENCE WITH SOCIAL ISOLATION AFTER EARLY WEANING ON MONOAMINERGIC AXONAL DEVELOPMENT

J. ISHIKAWA * AND A. ISHIKAWA

Systems Neuroscience, Department of Neuroscience, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan

Abstract—The axonal development of serotonin (5-HT)-, noradrenaline (NA)-, or tyrosine hydroxylase (TH)-containing monoaminergic neurons is affected by rearing conditions during the juvenile period. Impaired monoaminergic axonal development is implicated in the pathophysiology of emotional and cognitive dysfunction. On the other hand, exercise may have beneficial effects on emotional and learning performance in adults. We have examined whether voluntary running exercise during social isolation after early weaning (early weaning/social isolation; EI) from postnatal day (PD) 14–28 could prevent the impaired monoaminergic axonal development associated with EI. Compared with control animals reared with their dam and siblings until PD28, the EI animals showed lower density of 5-HT and NA axons in the dorsal–medial prefrontal cortex (mPFC) and basolateral nucleus of the amygdala and of NA- and TH-containing axons in the ventral-mPFC. These adverse effects of EI were not observed in rats taking part in voluntary running (EI + R) when these animals were compared to controls. The 5-HT axon density in the ventral-mPFC was significantly higher in the EI + R rats than that in the EI rats, although both these values were significantly lower than those in the control rats. The density of monoaminergic axons in the dentate gyrus and CA3 of the hippocampus was not affected by either EI or EI + R. These results suggest that the beneficial effects of voluntary running may be because of the modulation of monoaminergic axonal morphology. Our findings will hopefully provide the basis for future research into the beneficial effects of voluntary exercise during the juvenile period on brain development and emotional and cognitive performance.

Key words: exercise, axonal development, serotonin, noradrenaline, tyrosine hydroxylase.

*Corresponding author. Tel/fax: +81-836-22-2211.
E-mail address: junko-lc@yamaguchi-u.ac.jp (J. Ishikawa).

Abbreviations: 5-HT, serotonin; 6-OHDA, 6-hydroxydopamine; A, anterior; ANOVA, analysis of variance; BLA, basolateral nucleus of amygdala; DA, dopamine; DBH, dopamine β-hydroxylase; DG, dentate gyrus; DR, dorsal raphe; EI, early weaning/social isolation; IR, immunoreactive; L, lateral; LC, locus coeruleus; mPFC, medial prefrontal cortex; MR, median raphe; NA, noradrenaline; PBS-T/NGS/SA, phosphate-buffered saline containing 0.3% Triton X-100 and 0.05% normal goat serum and 0.1% sodium azide; PD, postnatal day; R, voluntary running; SERT, serotonin transporter; TH, tyrosine hydroxylase; V, ventral; VTA, ventral tegmental area.

INTRODUCTION

Monoaminergic neurons containing serotonin (5-HT), noradrenaline (NA), and tyrosine hydroxylase (TH) diffusely innervate most regions of the brain and play critical roles in a variety of brain functions including emotion and cognition (Friedman et al., 1999; Montague et al., 2004; Hohmann et al., 2007; Elliott et al., 2011; Haenisch and Bönisch, 2011; Neumann et al., 2011). The development of monoaminergic innervation is sensitive to rearing conditions during the juvenile period.

Maternal separation during the neonatal period and/or social isolation after weaning alters the density of monoaminergic axons in the rodent forebrain such as the medial prefrontal cortex (mPFC), amygdala and hippocampus (Braun et al., 2000; Whitaker-Azmitia et al., 2000; Gos et al., 2006; Kuramochi and Nakamura, 2009). Post-weaning social isolation reduces the density of 5-HT axons in the basolateral nucleus of the amygdala (BLA) and hippocampal CA3 in rats and increases the density of NA axons in the infralimbic area of the mPFC (Kuramochi and Nakamura, 2009). In the precocious rodent *Octodon degus,* maternal/parental separation (three times/day for 1 h) from postnatal day (PD) 1–21 followed by post-weaning social isolation increases or decreases the densities of 5-HT axons and TH-containing axons, depending on the subregions or layers of the PFC, amygdala and hippocampus (Braun et al., 2000; Gos et al., 2006).

Alteration of monoaminergic axon density in the PFC, amygdala and hippocampus is involved in the pathophysiology of emotional and cognitive dysfunction (Espejo, 1997; Liang, 1998; Akil et al., 2000; Austin et al., 2002; Sprague et al., 2003; Clarke et al., 2004; Wedzony et al., 2005; Clinton et al., 2006; Gonzalez and Aston-Jones, 2008; Pum et al., 2009). Early weaning, maternal separation, and post-weaning social isolation, which are widely used as juvenile stress, are also known risk factors of developing emotional and cognitive dysfunction (Frisone et al., 2002; Lee et al., 2007; Kodama et al., 2008; Ono et al., 2008; Fone and Porkess, 2008; Hulshof et al., 2011; Stiller et al., 2011; Baudin et al., 2012). In the study of Kuramochi and Nakamura (2009) described above, they also demonstrated that post-weaning social isolation induces depressive behaviors as well as alters the density of monoaminergic axons. These findings indicate that

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.11.020

alterations in the density of monoaminergic axons in the PFC, amygdala and hippocampus is involved in the mechanism that develops emotional and cognitive dysfunction induced by juvenile stress.

Exercise is well known to have beneficial effects on emotional and learning performance in adult and elderly animals, including humans (Binder et al., 2004; Greenwood et al., 2005; Greenwood and Fleshner, 2008; Jedrziewski et al., 2010; Knöchel et al., 2012), and the beneficial effects of exercise may be due, at least in part, to morphological changes of brain neurons, e.g. neurogenesis in the hippocampus (van Praag et al., 1999, 2005; Van der Borght et al., 2007; Clark et al., 2008). However, reports on the effects of exercise on developing animals are limited. Maniam and Morris (2010) found that voluntary running exercise beginning immediately after weaning at postnatal day (PD) 20 in rats attenuates anxiety- and depression-like behaviors induced by maternal separation from PD2 to PD14. To the best of our knowledge, the effects of exercise before normal weaning on the morphological development of brain neurons have not been reported.

In this study, we have examined the effect of postnatal voluntary exercise during social isolation after early weaning (early-weaning/social-isolation; EI) on the disruption of monoaminergic fiber innervation in early-weaned and socially isolated rats. Neonatal male rats were weaned at PD14 and separated from their dam and siblings until PD28, and the densities of specific monoaminergic axons were compared between early-weaned/socially isolated animals and comparable animals allowed to perform voluntary running exercise.

EXPERIMENTAL PROCEDURES

Animal subjects

Sprague–Dawley rats (Clea Japan, Tokyo, Japan) were housed in individual plastic cages (40 cm long, 25 cm wide, 25 cm high) at a constant temperature (22 °C) with free access to food and water and on a 12-h-light/dark cycle. Female rats (from 8- to 11-week-old) were housed overnight with adult male rats for mating, and vaginal smears were examined the following morning. The sperm-positive day was designated embryonic day 0. All efforts were made to minimize the number of animals used and their suffering. The experimental conditions and procedures were approved by the Committee of the Ethics on Animal Experiments at Yamaguchi University Graduate School of Medicine. All manipulations and protocols were performed according to the Guidelines for Animal Experiments at Yamaguchi University Graduate School of Medicine and in accordance with Japanese Federal Law (No. 105), Notification (No. 6) of the Japanese Government, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23), revised in 1996.

Experimental model

Male pups were weaned and isolated on PD14 and housed individually until PD28 in plastic cages with either locked (early weaning/social isolation, EI) or unlocked running wheels (+ voluntary running; EI + R) equipped with a revolution counter. The EI and EI + R rats were fed an ordinary adult diet softened by soaking it in water. Control pups were housed in a cage with their dam and two or three pups until PD28. Wheel revolution counts were noted every other day, and running distance was calculated by wheel circumference (1.0 m) × number of revolutions. Some of running wheels were kindly gifted by Dr. Hitoshi Hirano (Health Administration Center, Yamaguchi University).

Immunohistochemistry

Tissue preparation. At PD28, rats were deeply anesthetized (sodium pentobarbitone, 50 mg/kg, intraperitoneal) and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were immediately removed and immersed in 4% paraformaldehyde overnight at 4 °C, followed by 30% sucrose at 4 °C for several days.

Quantitative analysis of monoaminergic axons. Forty micron coronal brain slices were collected using a microtome, and processed for immunohistochemistry according to Ishikawa et al. (2007). Anti-mouse monoclonal antibodies against serotonin transporter (anti-SERT) (Chemicon International Inc., Temecula, CA, USA), dopamine β-hydroxylase (anti-DBH) (Chemicon International Inc., Temecula, CA, USA), and tyrosine hydroxylase (anti-TH) (Chemicon International Inc., Temecula, CA, USA) were used to visualize 5HT-, NA-, and TH-containing axons, respectively. SERT immunostaining is proposed to be more reliable than 5-HT immunostaining for visualizing 5-HT fibers (Nielsen et al., 2006). Brain slices were immersed in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 0.05% normal goat serum and 0.1% sodium azide (PBS-T/NGS/SA). Free-floating slices were incubated in 5% normal goat serum in PBS-T/NGS/SA for 3 h at 37 °C to block non-specific binding. After washing with PBS-T/NGS/SA, the sections were incubated with methanol containing 0.3% H_2O_2 for 10 min to suppress endogenous peroxidase activity. The sections were rinsed in PBS-T/NGS/SA and then incubated in anti-SERT (1:5000) and anti-TH (1:500) for 7 days at 37 °C, or in anti-DBH (1:10,000) for 48 h at 37 °C. After washing, the sections were incubated in biotinylated anti-mouse IgG (H + L) (1:200; Vector Laboratories) for 3 h, and then incubated in streptavidin–peroxidase (1:500; DAKO A/S, Glostrup, Denmark) for 1 h. Immunoreactivity was visualized using 0.01% diaminobenzidine as the chromogen and 0.6% nickel ammonium sulfate to enhance the reaction.

The densities of immunoreactive (IR) axons were measured in the following regions: dorsal-mPFC (d-mPFC), ventral-mPFC (v-mPFC), dentate gyrus (DG) and CA3 of the dorsal-hippocampus, and the BLA which corresponds to the following stereotaxic coordinates in the rat brain atlas of Paxinos and Watson: d-mPFC [anterior (A) from bregma, 3.2–3.7 mm; lateral (L) to midline, 0.2–1.0 mm; ventral (V) from bregma, 3.2–4.2 mm], v-mPFC (A, 3.2–3.7 mm; L, 0.2–1.0 mm; V, 4.8–5.4 mm), DG (P, 3.1–3.6 mm; L, 0.6–1.2 mm; V, 3.8–4.2 mm), CA3 (P, 3.1–3.6 mm; L, 2.8–3.8 mm; V, 2.8–3.4 mm) and BLA (P, 2.8–3.3 mm; L, 4.7–5.3 mm; V, 8.5–9.0 mm). Since the distribution of TH-IR fibers in the mPFC varies exceedingly depending on the distance from the midline, we analyzed the region in which a uniform distribution of the TH-IR fibers was observed: d-mPFC (A, 3.2–3.7 mm; L, 0.2–0.5 mm; V, 3.2–4.2 mm), v-mPFC (A, 3.2–3.7 mm; L, 0.2–0.5 mm; V, 4.8–5.4 mm). Stained tissue was observed under a light microscope (BX 50; Olympus, Tokyo, Japan) and images were collected with a digital camera (Camedia C-2020ZOOM; Olympus). Axons were traced manually on digital images using Photoshop 6 (Adobe Systems Inc., San Jose, CA, USA). Axon densities were measured using Image-Pro plus 5.1 (Media Cybernetics, Silver Spring, MD, USA) for a computed image analysis system. SERT-, DBH-IR axon densities in the d-mPFC and v-mPFC were analyzed in a square sampling frame (200 × 200 μm^2).