

SEX DIFFERENCES IN MYELIN-ASSOCIATED PROTEIN LEVELS WITHIN AND DENSITY OF PROJECTIONS BETWEEN THE ORBITAL FRONTAL CORTEX AND DORSAL STRIATUM OF ADULT RATS: IMPLICATIONS FOR INHIBITORY CONTROL

D. W. BAYLESS^{a*} AND J. M. DANIEL^{a,b}

^a Department of Psychology, Tulane University, New Orleans, LA 70118, USA

^b Neuroscience Program, Tulane University, New Orleans, LA 70118, USA

Abstract—Impulsive actions and decisions often lead to undesirable outcomes. Lesion and neuroimaging studies have revealed that the orbital frontal cortex (OFC) and dorsal striatum (dSTR) play key roles in inhibitory control. It has been proposed that greater OFC input into the dSTR reflects enhanced top-down cognitive control and less impulsive responding. We previously reported a sex difference in inhibitory control, such that female rats make fewer impulsive errors than do male rats. The goal of the present study was to investigate differences in the OFC and dSTR of young adult male and female rats. In Experiment 1, we measured levels of two myelin-associated proteins, myelin basic protein (MBP) and myelin proteolipid protein (PLP), in the OFC and dSTR. Western blot data revealed that females had significantly higher levels of both MBP and PLP in the OFC but similar levels in the dSTR as compared to males. In Experiment 2, we infused the anterograde tracer, biotinylated dextran amine (BDA), into the OFC and measured the density of BDA in the dSTR. BDA was visualized using histochemistry followed by light microscopy imaging and densitometry analysis. Density of BDA in the dSTR was significantly greater in females as compared to males indicating that the projections from the OFC to dSTR may be greater in females as compared to males. Our results suggest a potential neuroanatomical sex difference that may contribute to the reported differences in inhibitory control levels of male and female rats. © 2015 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: sex difference, orbital frontal cortex, striatum, myelin, anterograde tracer, inhibitory control.

INTRODUCTION

Inhibitory control is vital for normal everyday functioning, and deficits in inhibitory control contribute to many neuropsychiatric disorders (Steel and Blaszczyński, 1998; Breedlove et al., 2007). Studies investigating the neuroanatomical circuitry of impulsivity have demonstrated the important role that the prefrontal cortex (PFC) plays in inhibitory control in humans and animals (Dove et al., 2000; Brass and von Cramon, 2002; Cardinal, 2006; Eagle and Baunez, 2010). Human subjects with damage to the PFC display impulsive actions during stop-signal tasks (Aron et al., 2003) and impulsive choices during gambling tasks (Bechara et al., 1994). It has been proposed that the PFC influences impulsivity by modulating the operations of lower brain areas involved in reward-based behaviors, such as the striatum (Galvan et al., 2006; Perry et al., 2011; Peper et al., 2012). The striatum is involved in reward associated stimulus–response behaviors (Eichenbaum, 2012). In this view, the PFC acts as a top-down modulator of these lower brain areas, integrating behaviorally relevant information and preventing the over-reliance on fixed action patterns (Perry et al., 2011; Peters and Buchel, 2011). Using functional magnetic resonance imaging (fMRI), the striatum has been shown to be hyper-responsive when individuals choose immediate small rewards over delayed large rewards (McClure et al., 2004), and the magnitude of striatal activation correlates with the amount of impulsive choices individuals make during a delay-discounting task (Hariri et al., 2006). In addition, using transcranial magnetic stimulation (TMS) to disrupt the PFC leads to an increase in preference for immediate small rewards over delayed large rewards (Figner et al., 2010), suggesting that blocking the ability of the PFC to modulate the operations of lower brain areas results in increased impulsivity. Furthermore, results using tract-based diffusion tensor imaging have revealed that lower integrity within the frontostriatal white matter tract predicts a greater increase in impulsivity as the delay for a large reward over an immediate small reward increases (Peper et al., 2012).

Studies investigating sex differences in inhibitory control and the symptomology of attention deficit

*Corresponding author. Address: Department of Anatomy, University of California San Francisco, San Francisco, CA 94158, USA. Tel: +1-415-514-4383; fax: +1-415-514-4360.

E-mail addresses: daniel.bayless@ucsf.edu (D. W. Bayless), jmdaniel@tulane.edu (J. M. Daniel).

Abbreviations: ABC, avidin–biotin peroxidase complex; ADHD, attention deficit hyperactivity disorder; ANOVA, analysis of variance; BDA, biotinylated dextran amine; dLSTR, dorsolateral striatum; dmSTR, dorsomedial striatum; dSTR, dorsal striatum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; MBP, myelin basic protein; OFC, orbital frontal cortex; PBS, phosphate-buffered saline; PFC, prefrontal cortex; PLP, myelin proteolipid protein; TTBS, 0.1% Tween/1 X Tris-buffered saline; vSTR, ventral striatum.

hyperactivity disorder (ADHD) indicate that males display greater inhibitory control deficits and are more frequently diagnosed with the hyperactive-impulsive subtype of ADHD than are females (Gershon, 2002; Rucklidge, 2010; Davies, 2014). In addition, many but not all studies examining inhibitory control in the general population indicate that males are more impulsive than are females (Kirby and Marakovic, 1996; Rosenblitt et al., 2001; Whiteside and Lynam, 2003; Fillmore and Weafer, 2004; Skinner et al., 2004; Heyman and Gibb, 2006; Reynolds et al., 2006; Li-Grining, 2007; Van et al., 2008; Moilanen et al., 2009). Our laboratory and others have reported that adult male rats make more impulsive actions than do females when a delayed response is required during the 5-choice serial reaction time task and other tests of spatial divided attention (Jentsch and Taylor, 2003; Bayless et al., 2012). In addition, our laboratory has demonstrated that prepubertal male rats make more impulsive choices for an immediate small food reward over a delayed large food reward than do females and that this sex difference is organized by neonatal exposure to estrogens and androgens (Bayless et al., 2013).

One possible neuroanatomical mechanism that could contribute to a sex difference in inhibitory control would be a difference in myelination and density of projections from the orbital frontal cortex (OFC) to dorsal striatum (dSTR), such that females as compared to males have increased OFC input into the dSTR leading to enhanced inhibitory control. The levels of myelination or white matter vary across brain regions, and sex differences in brain connectivity are reported (for review see, Gong et al., 2011). To our knowledge no study to date has examined sex differences in myelination and projections from the OFC to dSTR. Projections from the dSTR diverge into two routes: the direct pathway leading to excitation of thalamic projections to motor cortex and the indirect pathway leading to inhibition of thalamic projections to motor cortex (Mink, 1996; Graybiel, 2000). The balance between these two pathways allows for the release of desired behavioral patterns while inhibiting undesired behavioral patterns (Miller and Buschman, 2007). The excitatory glutamatergic inputs of the OFC into the dSTR are hypothesized to modulate the activity of the direct and indirect pathways of the dSTR (for review see, Eagle and Baunez, 2010). A neuroanatomical sex difference in the density of projections from the OFC to dSTR in female rats as compared to males could provide females with increased OFC input into the dSTR leading to increased inhibition of thalamic projections to motor cortex via the indirect pathway, thereby dampening motor output and enhancing the ability of females to inhibit undesirable behaviors.

The goal of the present study was to test the hypothesis that there is a sex difference in male and female rats such that myelination and density of projections from the OFC to dSTR are greater in females as compared to males. In Experiment 1, we examined sex differences in the levels of myelin-associated proteins in the OFC and dSTR via western blotting for myelin basic protein (MBP) and myelin proteolipid protein (PLP). MBP is responsible for

the adhesion of multilayered compact myelin to axons and to itself and is the second most abundant protein in central nervous system (CNS) myelin (Boggs, 2006). PLP is a hydrophobic integral membrane protein and the most abundant protein in CNS myelin (Greer and Lees, 2002). Increased levels of MBP and PLP are indicative of increased levels of myelination. In Experiment 2, in order to gain insights into a possible sex difference in the density of the projections from the OFC to dSTR, we infused an anterograde tracer into the OFC of male and female rats and measured the density of the tracer in the dSTR.

EXPERIMENTAL PROCEDURES

Experiment 1: analysis of myelin-associated protein levels in the OFC and dSTR

Animals. Nine female and eight male Long-Evans hooded rats, approximately 60 days old, were purchased from Harlan Sprague–Dawley. Animal care was in accordance with the guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Institutional Animal Care and Use Committee of Tulane University. Rats were housed in same-sex pairs in a temperature-controlled vivarium under a 12-h light/dark cycle (lights on at 7:00 a.m.). Prior to use in the present experiment, rats were used to pilot behavioral tasks for future use in the laboratory.

Vaginal cytology. To control for effects of fluctuating ovarian hormones, vaginal smears of female rats were collected by lavage each morning and analyzed daily beginning 2 weeks prior to brain dissection. To control for handling effects, males underwent sham smears during which a small amount of water was placed on the genitals using a medicine dropper. Females were sacrificed at the proestrous stage of the estrous cycle, at which time circulating estradiol levels are at their peak and vaginal cytology is characterized by large nucleated epithelial cells (Becker et al., 2005). Each male was paired with a particular female and was sacrificed on the same day as that female.

Tissue dissection and processing. At approximately 85 days old, male and female rats were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg) and killed by decapitation. Whole brains were removed and quick-frozen on dry ice. In a cryostat at -20°C , coronal sections containing the OFC and dSTR were cut using coordinates from Paxinos and Watson (1998) (OFC: AP +4.2 mm to +2.7 mm; dSTR: AP +1.7 mm to -0.4 mm). Using a scalpel and visual cues from natural boundaries such as the corpus callosum, the OFC and dSTR were dissected (as described in Spijker, 2011). Tissue samples from both hemispheres were pooled for each animal and stored at -80°C until processing. Tissue was homogenized in 20 $\mu\text{l}/\text{mg}$ lysis buffer

containing 1 mM EGTA, 1 mM EDTA, 20 mM Tris, 1 mM sodium pyrophosphate tetrabasic decahydrate, 4 mM 4-nitrophenyl phosphate disodium salt hexahydrate, 0.1 μ M microcystin, and 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). Samples were then centrifuged for 15 min at 1000 $\times g$ at 4 °C, protein concentration of supernatants was determined (Bradford Protein Assay Kit; Pierce, Rockford, IL, USA), and each sample was diluted 1:1 with Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA), mixed with 350 mM D,L-dithiothreitol, boiled for 5 min, and stored at –80 °C.

Proteins of interest. Western blotting procedures were used to assess protein levels of MBP and PLP in the OFC and dSTR. MBP consists of four major isoforms with molecular masses of 21.5, 18.5, 17.0, and 14.0 kDa (Akiyama et al., 2002). PLP consists of one major isoform with a molecular mass of 23 kDa (Greer and Lees, 2002).

Electrophoresis and immunostaining. For each MBP and PLP sample, 25 μ g of total protein was loaded and separated at 200 V on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE gels) (Bio-Rad, Hercules, CA, USA) for 60 min. Molecular weight markers (Kaleidoscope; Bio-Rad, Hercules, CA, USA) were included with each run. Proteins were transferred to nitrocellulose membranes at 100 V for 60 min. Membranes were blocked with 5% nonfat dry milk in 0.1% Tween/1 X Tris-buffered saline (TTBS) at room temperature for 60 min. Following this, membranes were cut to separate the section containing the proteins of interest from the section containing the loading control β -actin. Membranes were then incubated with primary antibodies for MBP (mouse monoclonal; 1:5000, Abcam, Cambridge, MA, USA, AB78156), PLP (mouse monoclonal; 1:1500, Millipore, Billerica, MA, USA, MAB388), or β -actin (mouse monoclonal; 1:15,000; Santa Cruz, Dallas, TX, USA) overnight at 4 °C in 1% nonfat dry milk-TTBS. Blots were washed three times for 15 min each with TTBS and incubated with 5% nonfat dry milk containing goat antimouse IgG (MBP, 1:10,000; PLP, 1:10,000; β -actin, 1:10,000; Santa Cruz, Dallas, TX, USA) conjugated to horseradish peroxidase for 1.5 h at room temperature. Blots were washed again three times for 15 min each and incubated for 1 min with the chemiluminescent substrate Pierce ECL western blotting substrate (MBP; β -actin; Fisher Scientific, Pittsburgh, PA, USA) or 5 min with the chemiluminescent substrate SuperSignal West Femto (PLP; Fisher Scientific, Pittsburgh, PA, USA) and exposed to film (Kodak Biomax MR) for varying durations to capture optimal signal intensity. Films were imaged using MCID Core imaging software (InterFocus Imaging Ltd., Cambridge, England), and optical density \times area was measured for bands of interest. All values were represented as a percentage relative to β -actin for each sample.

Statistical analyses. For PLP data, the optical density \times area values as a percentage of β -actin loading control were analyzed using a one-way analysis of variance (ANOVA). For MBP data, the optical

density \times area values as a percentage of β -actin loading control were analyzed using repeated measures ANOVAs with isoform as the within-subject factor and sex as the between-subject factor.

Experiment 2: analysis of projections from the OFC to dSTR

Animals. Six male and six female Long-Evans hooded rats, approximately 60 days old, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA). Rats were housed in same-sex pairs, and animal care was the same as described in Experiment 1.

Stereotaxic surgery and tracer injection. A week after arrival, rats underwent stereotaxic surgery to allow for bilateral microinjection of the anterograde tracer, biotinylated dextran amine (BDA), into the OFC. BDA is a widely used anterograde tracer that is selectively taken up by neurons at the site of injection (Wang et al., 2013). BDA is well transported both retrogradely and anterogradely depending on its molecular weight (Lazarov, 2013). At 10 kDa, as was used in the current experiment, BDA is anterogradely transported. BDA is biotinylated (Reiner et al., 2000). Thus, there is no need to attach a biotin-conjugated secondary to BDA in order to visualise a peroxidase reaction product after incubation with avidin–biotin peroxidase complex (ABC) followed by diaminobenzidine (DAB) during histochemistry (Lazarov, 2013). Anterograde tracer and histochemistry techniques were based upon Springer Protocols for anterograde tracing experiments (Lazarov, 2013; Wang et al., 2013).

Surgeries were conducted on anesthetized rats using appropriate aseptic techniques. Rats were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg). Buprenorphine (0.025 mg/kg, Buprenex) was administered subcutaneously as an analgesic. Rats were placed in a stereotaxic apparatus. An incision was made along the midline of the head of each rat, and the underlying fascia was scraped to the side. The local anesthetic, 0.5% lidocaine was applied to the fascia at the time of the incision. Using a small drill mounted on a stereotaxic arm, a 0.9-diameter hole was drilled through the skull. Using a Hamilton syringe, 0.5 μ l per hemisphere of 10% BDA (10 kDa) in artificial cerebrospinal fluid (aCSF) was infused bilaterally into the OFC as shown in Fig. 1 (AP +3.2 mm, ML \pm 2.5 mm from bregma, and DV –4.6 mm from the dura, coordinates from Paxinos and Watson, 1998). The skull surface was completely dried, and the entire infusion site was covered with sterile bone wax. The incision was closed with sterile absorbable sutures.

Perfusion and fixation. After a survival period of 10 days allowing for anterograde transport of the tracer to the dSTR, rats were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg). The stage of the estrous cycle after the 10-day survival period was random across females. The chest and abdominal cavity was surgically opened. Rats were injected intracardially with 0.05 ml of heparin,

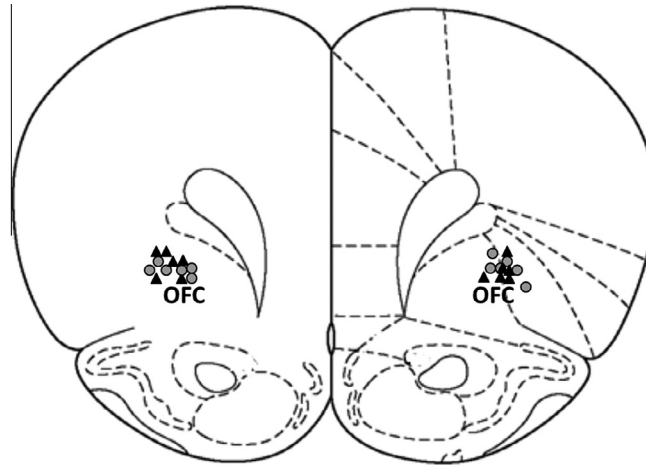


Fig. 1. Illustration of a coronal section of the rat brain at 3.2 mm anterior to bregma (Paxinos and Watson, 1998) with injection sites for individual rats indicated by gray circles for females and black triangles for males.

an anticoagulant. Rats were then perfused intracardially with 200 ml of 0.9% saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). After perfusion, brains were removed and post-fixed in a fresh fixative of 30% sucrose in 0.1 M PBS.

Tissue sectioning. Frozen 50- μ m coronal sections of the OFC from AP +4.2 mm to +2.7 mm and dSTR from AP +1.7 mm to –0.4 mm were collected in cryoprotectant (1% polyvinylpyrrolidone, 30% sucrose, 30% ethylene glycol in 0.1 M PBS) using a sliding microtome. Sections were stored in 0.5-ml vials in cryoprotectant at 4 °C until further use. OFC sections at AP +3.2 mm were mounted onto gelatin-coated slides and stained with cresyl violet to verify infusion location (see Fig. 1). Three consecutive dSTR sections at AP +0.5 mm were used for imaging and statistical analysis of BDA density.

Histochemistry. Sections were washed three times for 10 min each in 0.05 M PBS and then incubated for 60 min in 0.1% bovine serum albumin in 0.05 M PBS to block non-specific binding sites. Following blocking, sections were washed three times for 10 min each in 0.05 M PBS. Sections were then incubated for 120 min in ABC reagent (Vectastain Elite Kit, Vector Laboratories, Inc., Burlingame, CA, USA) in 0.3% Triton X in 0.05 M PBS. Following three more washes for 10 min each in 0.05 M PBS, the peroxidase reaction product was visualized by incubation in a solution containing 0.05% diaminobenzidine (DAB; Sigma–Aldrich, St. Louis, MO, USA) and 0.01% H₂O₂ in 0.1 M PBS for 7 min. Sections were then washed again three times for 10 min in 0.01 M PBS, mounted onto gelatin-coated slides, and allowed to air-dry overnight. The following day, the slides were placed in xylene for 5 min, and then coverslipped using diluted permount.

Imaging and quantification of BDA density. Sections were imaged at 20 \times magnification using a light microscope (Olympus IX71) and digital camera (Hamamatsu Camera; 0.1 s exposure) interfaced with

HC Image Software. MCID Analysis software was used to calibrate images to control for variations in background density. Optical density was measured within circular regions (1 mm diameter) of the dorsolateral striatum (dlSTR) determined by measuring 0.6 mm horizontally and 0.75 mm vertically from the corpus callosum and within circular regions (1 mm diameter) of the dorsomedial striatum (dmSTR) determined by measuring 0.6 mm horizontally from the lateral ventricle and 1 mm vertically from the corpus callosum (see Fig. 2). Fig. 3 provides example images of anterograde tracer staining in the dSTR at varying magnification levels.

Statistical analyses. Three consecutive sections at AP +0.5 mm for each rat were imaged and optical density values were averaged for statistical analysis of BDA density. The optical density values as a percentage of average female density for the left and right hemispheres of the dlSTR and dmSTR were analyzed using a repeated measures ANOVA with hemisphere as the within-subject factor and sex as the between-subject factor for each brain region.

RESULTS

Experiment 1: analysis of myelin-associated protein levels in the OFC and dSTR

MBP in OFC and dSTR. Western blots for MBP revealed four isoform bands of MBP-like immunoreactivity at approximately 21.5, 18.5, 17.0, and 14.0 kDa. As illustrated in Fig. 4A–C, analysis of protein levels of MBP in the OFC of males and females revealed a main effect of MBP isoform ($F(3,45) = 51.17, p < 0.001$) and a main effect of sex ($F(1,15) = 4.95, p = 0.042$). There was no interaction between sex and MBP isoform. There was no effect of sex on levels of β -actin loading control. Results indicate that protein levels of MBP in the OFC are significantly greater in females as compared to males.

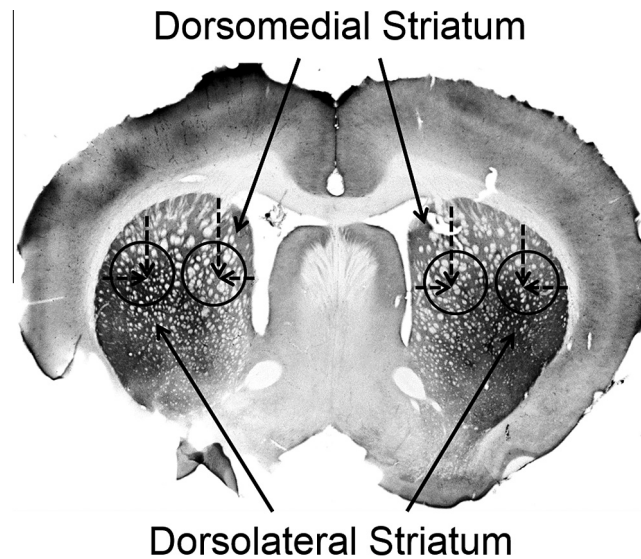


Fig. 2. Defined areas of measurement within the dorsolateral striatum and dorsomedial striatum from example female coronal section processed for biotinylated dextran amine histochemistry. Dashed arrows represent distance measurements from the corpus callosum and lateral ventricles. Circles represent 1 mm diameter circular regions of density analysis.

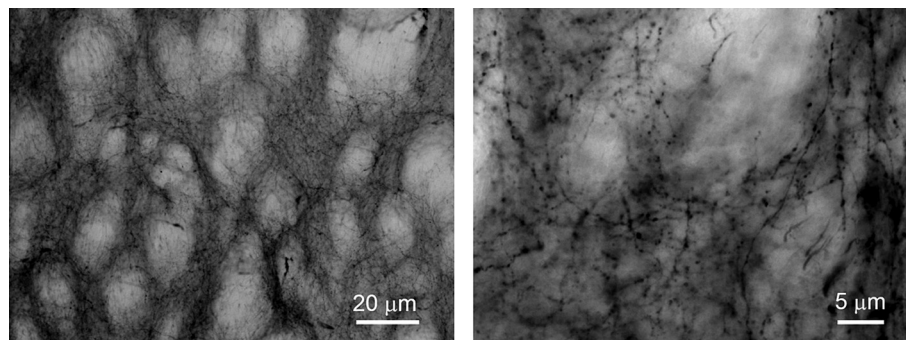


Fig. 3. Biotinylated dextran amine staining in example female dorsal striatum at varying magnification levels.

As illustrated in Fig. 4D–F, analysis of protein levels of MBP in the dSTR of males and females revealed a main effect of MBP isoform ($F(3,45) = 70.26$, $p < 0.001$), indicating that protein levels varied across the four isoforms. However, there was no main effect of sex or interaction between sex and MBP isoform. There was no effect of sex on levels of β -actin loading control. Results indicate that protein levels of MBP in the dSTR are not significantly different between males and females.

PLP in OFC and dSTR. Western blots for PLP revealed a band of PLP-like immunoreactivity at approximately 23 kDa. As illustrated in Fig. 5A, B, analysis of protein levels of PLP in the OFC of males and females revealed a main effect of sex ($F(1,15) = 5.40$, $p = 0.035$). There was no effect of sex on levels of β -actin loading control. Results indicate that protein levels of PLP in the OFC are significantly greater in females as compared to males.

As illustrated in Fig. 5C, D, analysis of protein levels of PLP in the dSTR of males and females revealed no main effect of sex. There was no effect of sex on the levels of β -actin loading control. Results indicate that protein

levels of PLP in the dSTR are not significantly different between males and females.

Experiment 2: analysis of projections from the OFC to dSTR

Anterograde tracer density in dISTR and dmSTR. As illustrated in Fig. 6A, analysis of optical density of OFC-injected anterograde tracer in the dISTR of males and females revealed a significant main effect of sex ($F(1,10) = 5.67$, $p = 0.039$), indicating that the density of projections from the OFC to dISTR is significantly greater in females as compared to males. There was no significant main effect of hemisphere, indicating that the density of the tracer was similar in the left and right hemispheres.

As illustrated in Fig. 6B, analysis of optical density of OFC-injected anterograde tracer in the dmSTR of males and females revealed a strong trend to significance for a main effect of sex ($F(1,10) = 4.57$, $p = 0.058$), suggesting that the density of projections from the OFC to dmSTR is greater in females as compared to males.

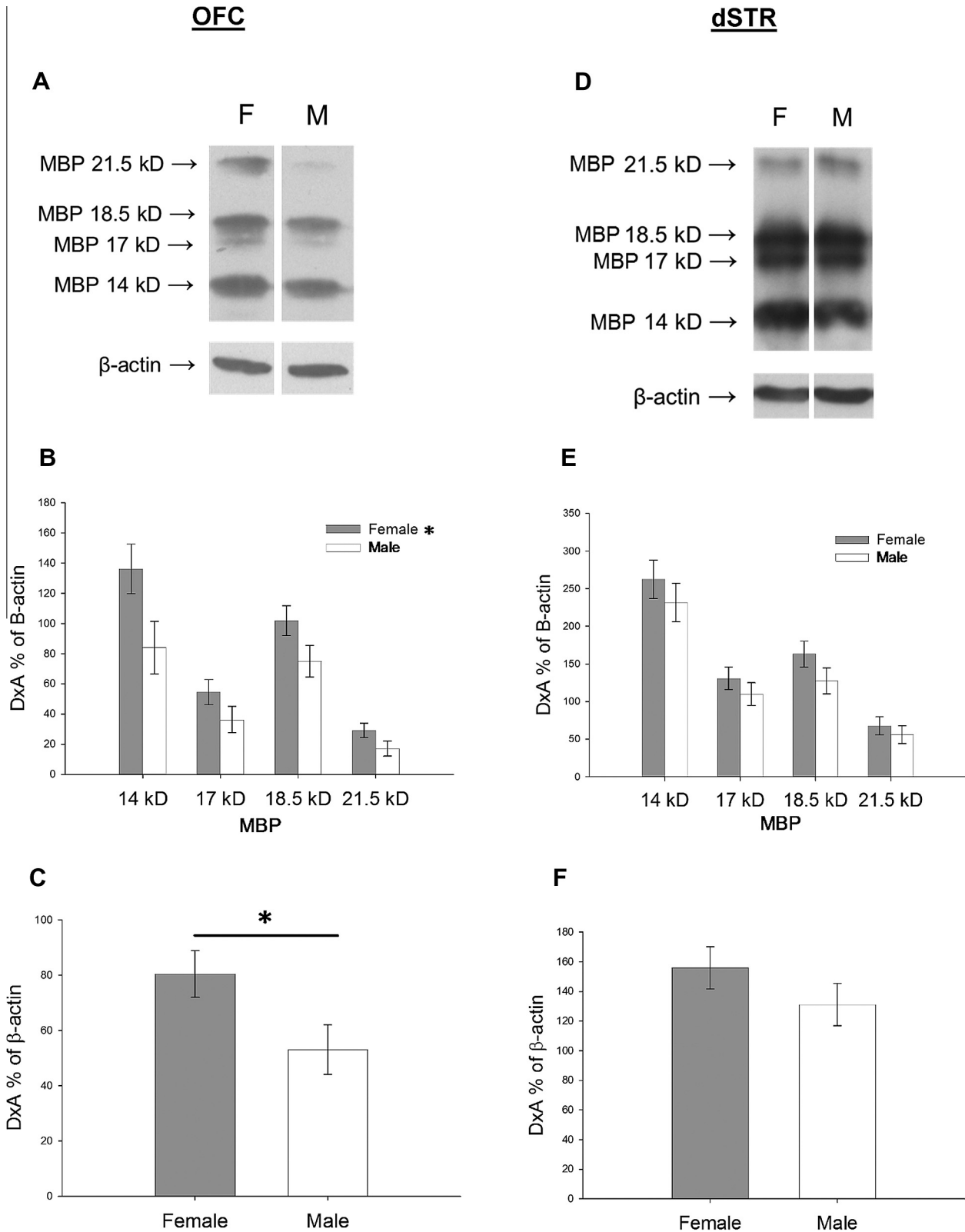


Fig. 4. Western blot data showing the effect of sex on protein levels of myelin basic protein (MBP) in the orbital frontal cortex (OFC) (A)–(C) and dorsal striatum (dSTR) (D)–(F) of male and female rats. (A) Representative blot images for MBP and the loading control β-actin in OFC samples. (B) OFC data with MBP isoforms isolated. Mean density × area ($D \times A$) (\pm SEM) expressed relative to control β-actin protein levels. Overall main effect of sex: $p < 0.05$. (C) OFC data with MBP isoforms averaged. Mean $D \times A$ (\pm SEM) expressed relative to control β-actin protein levels. Overall main effect of sex: $p < 0.05$. (D) Representative blot images for MBP and the loading control β-actin in dSTR samples. (E) dSTR data with MBP isoforms isolated. Mean $D \times A$ (\pm SEM) expressed relative to control β-actin protein levels. (F) dSTR data with MBP isoforms averaged. Mean $D \times A$ (\pm SEM) expressed relative to control β-actin protein levels.

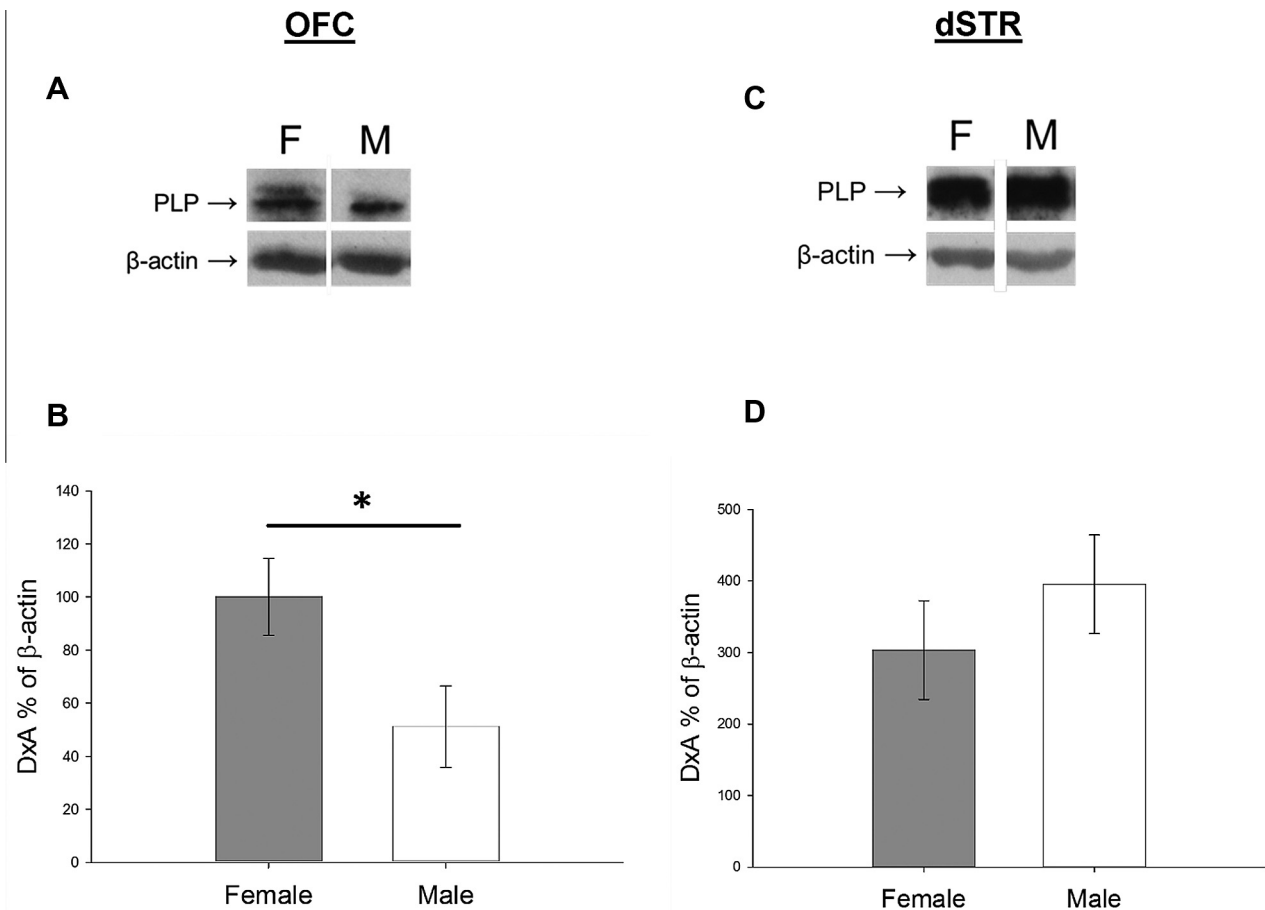


Fig. 5. Western blot data showing the effect of sex on protein levels of myelin proteolipid protein (PLP) in the orbital frontal cortex (OFC) (A) and (B) and dorsal striatum (dSTR) (C) and (D) of male and female rats. (A) Representative blot images for PLP and the loading control β -actin in OFC samples. (B) OFC data. Mean density \times area ($D \times A$) (\pm SEM) is expressed relative to control β -actin protein levels. Overall main effect of sex: $^*p < 0.05$. (C) Representative blot images for PLP and the loading control β -actin in dSTR samples. (D) dSTR data. Mean $D \times A$ (\pm SEM) expressed relative to control β -actin protein levels.

There was no significant main effect of hemisphere, indicating that the density of the tracer was similar in the left and right hemispheres.

DISCUSSION

The present experiments demonstrate that myelin-associated protein levels in the OFC and the density of an OFC-infused anterograde tracer is greater in the dSTR of female rats as compared to male rats. Protein levels of MBP and PLP were significantly greater in the OFC but not the dSTR of females as compared to males, suggesting that myelination is increased in the OFC but similar in the dSTR of females as compared to males. Furthermore, the density of an anterograde tracer injected into the OFC and measured in the dSTR was significantly greater in females as compared to males, indicating that the density of projections from the OFC to dSTR may be greater in females as compared to males. These sex differences are consistent with the possibility that females have increased OFC input into the dSTR leading to greater modulation of the reward-based stimulus–response behaviors in females as compared to males. The current findings suggest a

potential neuroanatomical mechanism that could underlie the previously reported sex difference in inhibitory control (Kirby and Marakovic, 1996; Rosenblitt et al., 2001; Jentsch and Taylor, 2003; Whiteside and Lynam, 2003; Li-Grining, 2007; Van et al., 2008; Moilanen et al., 2009; Bayless et al., 2012, 2013).

The current finding of a sex difference in the levels of myelin-associated proteins in the OFC of rats is consistent with the findings from a magnetic resonance imaging (MRI) study in humans that reported that women have more white matter voxels in frontal and parietal brain areas that correlate with intelligence scores than do men (Haier et al., 2005). In the CNS, myelin is formed by oligodendrocytes that wrap tightly around axons insulating and increasing the speed of neurotransmission (Sherman and Brophy, 2005). The ratio of the axon diameter to myelin diameter (g -ratio) is fairly consistent across myelinated neurons, meaning that larger axons have thicker myelin, and vice versa (Sherman and Brophy, 2005). Therefore, increased levels of myelination indicate either a greater number of neuronal connections or a greater average axonal diameter of neurons. Both the number of neuronal connections and axonal diameter are associated with faster, more efficient

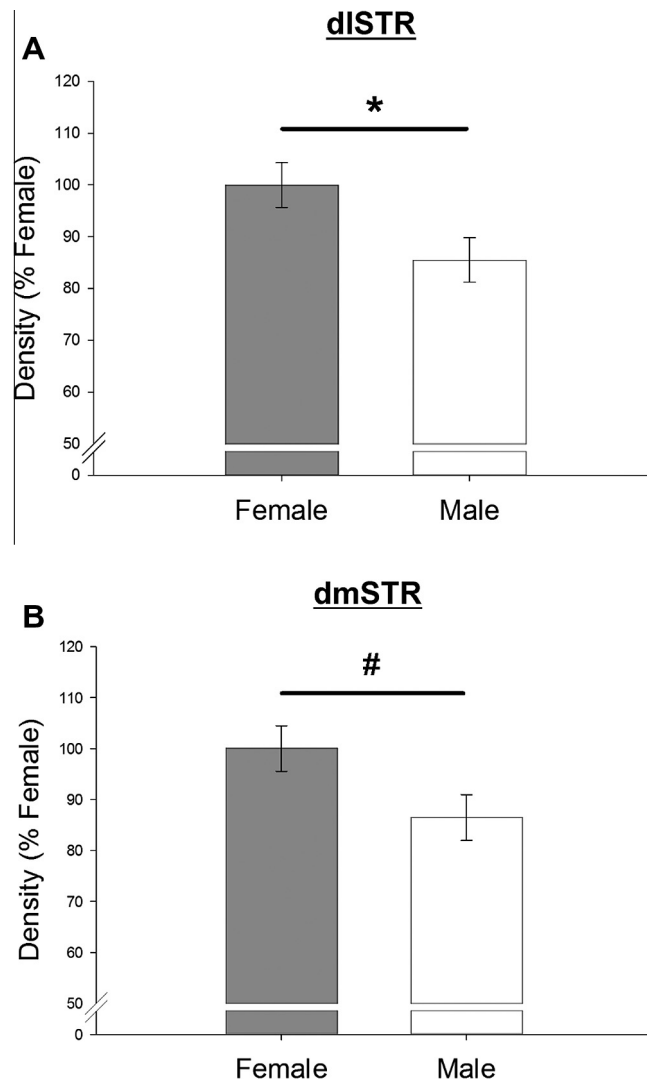


Fig. 6. (A) Biotinylated dextran amine (BDA) density in the dorsolateral striatum (dISTR) of male and female rats across hemispheres. Overall main effect of sex: $p = 0.039$. (B) BDA density in the dorsomedial striatum (dmSTR) of male and female rats across hemispheres. Overall main effect of sex: $\#p = 0.058$.

neuronal communication (Sherman and Brophy, 2005). Therefore, the current results are consistent with the hypothesis that the speed of neurotransmission within the OFC but not the dSTR is greater in female rats as compared to male rats resulting in increased OFC input into and control over the dSTR in females as compared to males. The corticostriatal projections of interest in the current study are fully myelinated, but interestingly, the dopaminergic inputs into the dSTR from the substantia nigra are mostly unmyelinated (Pissadaki and Bolam, 2013). Our western blot assays of myelin-associated protein levels in the OFC and dSTR do not provide insights into the density of projections from the OFC to dSTR. Therefore in the current experiment, we measured density levels of an OFC-infused anterograde tracer in the dSTR resulting in the novel discovery that the density of the tracer in the dSTR was greater in females as compared to males. Taken together, the increased myelin-associated protein levels in the OFC and increased anterograde tracer density in the dSTR of females as

compared to males indicate that OFC input into the dSTR may be greater in female rats than it is in male rats.

Projections from the OFC distribute widely throughout the brain, including cortical targets, such as the medial prefrontal, insular, piriform, and parahippocampal cortices, and subcortical targets, such as the dorsal and ventral striatum (vSTR), septum, amygdala, thalamus, hypothalamus, substantia nigra, ventral tegmental area, and dorsal and median raphe (Hoover and Vertes, 2011). The excitatory glutamatergic projections from the OFC to dSTR are strongly implicated in impulse control (for review see, Eagle and Baunez, 2010). Studies using the vesicular glutamate transporter type 1 as a marker of cortical innervation indicate that dSTR medium-sized spiny neurons of both the direct and indirect pathway receive cortical input (Doig et al., 2010; Huerta-Ocampo et al., 2014). The balance between the direct and indirect pathways of the dSTR allows for the release of desired behavioral patterns while inhibiting undesired behavioral patterns (Miller and Buschman, 2007). The current

findings in combination with our previous reports that female rats display increased inhibitory control as compared to males (Bayless et al., 2012, 2013) support the hypothesis that the OFC influences impulsivity by modulating the operations of lower brain areas involved in reward-based behaviors, such as the dSTR (Galvan et al., 2006; Perry et al., 2011; Peper et al., 2012). The increased density of the OFC-infused anterograde tracer in the dSTR and increased myelin-associated protein levels in the OFC in female rats as compared to males may allow for increased OFC control over the dSTR leading to increased inhibition of thalamic projections to motor cortex via the indirect pathway, thereby dampening motor output and enhancing the ability of females to inhibit undesirable behaviors.

The time course of many neurodevelopmental processes varies between the sexes (for review see, Brenhouse and Andersen, 2011). In the current study, animals were age-matched and approximately 85 days of age at the time of the experiments. The onset of puberty is typically around 35 days of age for female rats and 45 days of age for male rats (Kennedy and Mitra, 1963). Although the animals in the current study are considered adults, some neurodevelopmental processes are still ongoing in young adulthood (Brenhouse and Andersen, 2011). Therefore, it is possible that a developmental delay in the maturation of OFC projections to the dSTR in males as compared to females could contribute to the neuroanatomical sex differences reported here. Future studies are needed to fully understand the impact that developmental sex differences might have on OFC projections to the dSTR and inhibitory control. In addition, in the current study our focus was on the mechanisms of inhibitory control associated with the dSTR. The vSTR is strongly implicated in reward-seeking behavior (Dalley et al., 2008) and could also contribute to differences in impulsivity in males and females. Future research examining the vSTR in males and females will further enhance our understanding of the neurobiological underpinnings of the reported sex differences in impulsivity. Furthermore, the current study only provides a neuroanatomical sex difference and does not provide an isolated assessment of the OFC inputs into the direct or indirect pathway of the dSTR. Future studies are needed to demonstrate the causal link between the possible sex difference in density of projections from the OFC to dSTR and enhanced inhibitory control.

Previous research in our laboratory has demonstrated that the sex difference in impulsive choice behavior in prepubertal rats is organized neonatally by the actions of both androgens and estrogens (Bayless et al., 2013). It is unclear if the sex differences in myelin-associated proteins in the OFC and density of the OFC-infused anterograde tracer in the dSTR are organized by neonatal hormone levels. Future studies are needed to determine if this is the case. However, it is possible that epigenetic changes caused by neonatal activation of estrogen or androgen receptors could cause changes in the rate of apoptotic cell death during development that might produce the sex difference in the number of cells that project from the OFC to dSTR. Sex differences in cell number

could be established through neurogenesis, neuronal migration, phenotypical differentiation, or cell death. Of these neurodevelopmental events, cell death has received the majority of the support as the most common mechanism by which sex differences in cell number are established (Forger, 2009). Cell death is a naturally occurring and essential process in the brain, and over 50% of neurons born during neurodevelopment undergo apoptosis or programmed cell death during development (Oppenheim, 1991). Expression of high levels of the Bax protein promotes apoptosis, whereas expression of high levels of the Bcl-2 protein protects neurons from apoptosis (Forger, 2009). Neonatal activation of estrogen or androgen receptors could affect the development of impulsive behavior by increasing histone acetyltransferase activity at the promoter region of the Bax gene in neurons projecting from the OFC to dSTR during neonatal development in males resulting in increased gene transcription of the Bax protein and ensuing cell death of these neurons producing an increased impulsive phenotype later in life. Deletion of the Bax gene eliminates the sex difference in cell number in some sexually dimorphic brain areas (Forger et al., 2004; Gotsiridze et al., 2007), and the human Bcl-2 gene contains the sequence for the estrogen-response element (Perillo et al., 2000). So, it is possible that neonatal activation of estrogen receptors could alter the rate of cell death in specific brain areas in a sex-dependent manner. Future studies are needed to test this hypothesis and investigate the organizational role of neonatal estrogen and androgen levels in the neurodevelopmental basis of the sex differences reported here.

CONCLUSIONS

Collectively, the present experiments demonstrate that female rats as compared to males have increased levels of myelin-associated proteins in the OFC and an increased density of an OFC-infused anterograde tracer in the dSTR. These novel neuroanatomical sex differences could underlie the reported differences in inhibitory control levels of male and female rats by providing females as compared to males with increased OFC input into the dSTR leading to greater modulation of the reward-based stimulus–response behaviors of the dSTR. In addition, the sex differences reported here have implications for the understanding of sex differences in impulsivity in the general population as well as in neuropsychiatric disorders, such as ADHD, pathological gambling, and drug addiction.

Acknowledgments—This work was supported by the National Science Foundation Grant 0951008 to JMD and State of Louisiana Board of Regents Graduate Fellowship LEQSF(2009–2014)-GF-13 to DWB.

REFERENCES

- Akiyama K, Ichinose S, Omori A, Sakurai Y, Asou H (2002) Study of expression of myelin basic proteins (MBPs) in developing rat brain using a novel antibody reacting with four major isoforms of MBP. *J Neurosci Res* 68:19–28.

- Aron AR, Fletcher PC, Bullmore ET, Sahakian BJ, Robbins TW (2003) Stop-signal inhibition disrupted by damage to right inferior frontal gyrus in humans. *Nat Neurosci* 6:115–116.
- Bayless DW, Darling JS, Stout WJ, Daniel JM (2012) Sex differences in attentional processes in adult rats as measured by performance on the 5-choice serial reaction time task. *Behav Brain Res* 235:48–54.
- Bayless DW, Darling JS, Daniel JM (2013) Mechanisms by which neonatal testosterone exposure mediates sex differences in impulsivity in prepubertal rats. *Horm Behav* 64:764–769.
- Bechara A, Damasio AR, Damasio H, Anderson SW (1994) Insensitivity to future consequences following damage to human prefrontal cortex. *Cognition* 50:7–15.
- Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J, Young E (2005) Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* 146:1650–1673.
- Boggs JM (2006) Myelin basic protein: a multifunctional protein. *Cell Mol Life Sci* 63:1945–1961.
- Brass M, von Cramon DY (2002) The role of the frontal cortex in task preparation. *Cereb Cortex* 12:908–914.
- Breedlove SM, Rosenzweig MR, Watson NV (2007) Psychopathology: biological basis of behavioral disorders. In: *Biological psychology*. Sunderland, MA: Sinauer Associates. pp. 482–494.
- Brenhouse HC, Andersen SL (2011) Developmental trajectories during adolescence in males and females: a cross-species understanding of underlying brain changes. *Neurosci Biobehav Rev* 35:1687–1703.
- Cardinal RN (2006) Neural systems implicated in delayed and probabilistic reinforcement. *Neural Netw* 19:1277–1301.
- Dalley JW, Mar AC, Economidou D, Robbins TW (2008) Neurobehavioral mechanisms of impulsivity: fronto-striatal systems and functional neurochemistry. *Pharmacol Biochem Behav* 90:250–260.
- Davies W (2014) Sex differences in attention deficit hyperactivity disorder: candidate genetic and endocrine mechanisms. *Front Neuroendocrinol* 35:331–346.
- Doig NM, Moss J, Bolam JP (2010) Cortical and thalamic innervation of direct and indirect pathway medium-sized spiny neurons in mouse striatum. *J Neurosci* 44:14610–14618.
- Dove A, Pollmann S, Schubert T, Wiggins CJ, von Cramon DY (2000) Prefrontal cortex activation in task switching: an event-related fMRI study. *Brain Res Cogn Brain Res* 9:103–109.
- Eagle DM, Baunez C (2010) Is there an inhibitory-response-control system in the rat? Evidence from anatomical and pharmacological studies of behavioral inhibition. *Neurosci Biobehav Rev* 34:50–72.
- Eichenbaum H (2012) A brain system for procedural memory. In: *The cognitive neuroscience of memory*. New York: Oxford University Press. pp. 267–289.
- Figner B, Knoch D, Johnson EJ, Krosch AR, Lisanby SH, Fehr E, Weber EU (2010) Lateral prefrontal cortex and self-control in intertemporal choice. *Nat Neurosci* 13:538–539.
- Fillmore MT, Weafer J (2004) Alcohol impairment of behavior in men and women. *Addiction* 99:1237–1246.
- Forger NG (2009) Control of cell number in the sexually dimorphic brain and spinal cord. *J Neuroendocrinol* 21:393–399.
- Forger NG, Rosen GJ, Waters EM, Jacob D, Simerly RB, de Vries GJ (2004) Deletion of Bax eliminates sex differences in the mouse forebrain. *Proc Natl Acad Sci USA* 101:13666–13671.
- Galvan A, Hare TA, Parra CE, Penn J, Voss H, Glover G, Casey BJ (2006) Earlier development of the accumbens relative to orbitofrontal cortex might underlie risk-taking behavior in adolescents. *J Neurosci* 26:6885–6892.
- Gershon J (2002) A meta-analytic review of gender differences in ADHD. *J Atten Disord* 5:143–154.
- Gong G, He Y, Evans AC (2011) Brain connectivity: gender makes a difference. *Neuroscientist* 17:575–591.
- Gotsiridze T, Kang N, Jacob D, Forger NG (2007) Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: role of Bax-dependent cell death. *Dev Neurobiol* 67:355–362.
- Graybiel AM (2000) The basal ganglia. *Curr Biol* 10:R509–R511.
- Greer JM, Lees MB (2002) Myelin proteolipid protein—the first 50 years. *Int J Biochem Cell Biol* 34:211–215.
- Haier RJ, Jung RE, Yeo RA, Head K, Alkire MT (2005) The neuroanatomy of general intelligence: sex matters. *Neuroimage* 25:320–327.
- Hariri AR, Brown SM, Williamson DE, Flory JD, de WH, Manuck SB (2006) Preference for immediate over delayed rewards is associated with magnitude of ventral striatal activity. *J Neurosci* 26:13213–13217.
- Heyman GM, Gibb SP (2006) Delay discounting in college cigarette chippers. *Behav Pharmacol* 17:669–679.
- Hoover WB, Vertes RP (2011) Projections of the medial orbital and ventral orbital cortex in the rat. *J Comp Neurol* 519:3766–3801.
- Huerta-Ocampo I, Mena-Segovia J, Bolam JP (2014) Convergence of cortical and thalamic input to direct and indirect pathway medium spiny neurons in the striatum. *Brain Struct Funct* 219:1787–1800.
- Jentsch JD, Taylor JR (2003) Sex-related differences in spatial divided attention and motor impulsivity in rats. *Behav Neurosci* 117:76–83.
- Kennedy GC, Mitra J (1963) Body weight and food intake as initiating factors for puberty in the rat. *J Physiol* 166:408–418.
- Kirby KN, Marakovic NN (1996) Delay-discounting probabilistic rewards: rates decrease as amounts increase. *Psychon Bull Rev* 3:100–104.
- Lazarov NE (2013) Neuroanatomical tract-tracing using biotinylated dextran amine. *Methods Mol Biol* 1018:323–334.
- Li-Grining CP (2007) Effortful control among low-income preschoolers in three cities: stability, change, and individual differences. *Dev Psychol* 43:208–221.
- McClure SM, Laibson DI, Loewenstein G, Cohen JD (2004) Separate neural systems value immediate and delayed monetary rewards. *Science* 306:503–507.
- Miller EK, Buschman TJ (2007) Bootstrapping your brain: how interactions between the frontal cortex and basal ganglia may produce organized actions and lofty thoughts. In: Kesner RP, Martinez JL, editors. *Neurobiology of learning and memory*. Burlington, MA: Academic Press. p. 339–351.
- Mink JW (1996) The basal ganglia: focused selection and inhibition of competing motor programs. *Prog Neurobiol* 50:381–425.
- Moilanen KL, Shaw DS, Dishion TJ, Gardner F, Wilson M (2009) Predictors of longitudinal growth in inhibitory control in early childhood. *Soc Dev* 19:326–347.
- Oppenheim RW (1991) Cell death during development of the nervous system. *Annu Rev Neurosci* 14:453–501.
- Paxinos G, Watson C (1998) *The rat brain in stereotaxic coordinates*. San Diego, CA: Academic Press.
- Peper JS, Mandl RC, Braams BR, de WE, Heijboer AC, Koolschijn PC, Crone EA (2012) Delay discounting and frontostriatal fiber tracts: a combined DTI and MTR study on impulsive choices in healthy young adults. *Cereb Cortex* 23(7):1695–1702.
- Perillo B, Sasso A, Abbondanza C, Palumbo G (2000) 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol* 20:2890–2901.
- Perry JL, Joseph JE, Jiang Y, Zimmerman RS, Kelly TH, Darna M, Huettl P, Dwoskin LP, Bardo MT (2011) Prefrontal cortex and drug abuse vulnerability: translation to prevention and treatment interventions. *Brain Res Rev* 65:124–149.
- Peters J, Buchel C (2011) The neural mechanisms of inter-temporal decision-making: understanding variability. *Trends Cogn Sci* 15:227–239.
- Pissadaki EK, Bolam JP (2013) The energy cost of action potential propagation in dopamine neurons: clues to susceptibility in Parkinson's disease. *Front Comp Neurosci* 7:1–17.

- Reiner A, Veenman CL, Medina L, Jiao Y, Del MN, Honig MG (2000) Pathway tracing using biotinylated dextran amines. *J Neurosci Methods* 103:23–37.
- Reynolds B, Richards JB, de WH (2006) Acute-alcohol effects on the Experiential Discounting Task (EDT) and a question-based measure of delay discounting. *Pharmacol Biochem Behav* 83:194–202.
- Rosenblitt JC, Soler H, Johnson SE, Quadagno DM (2001) Sensation seeking and hormones in men and women: exploring the link. *Horm Behav* 40:396–402.
- Rucklidge JJ (2010) Gender differences in attention-deficit/hyperactivity disorder. *Psychiatr Clin N Am* 33:357.
- Sherman DL, Brophy PJ (2005) Mechanisms of axon ensheathment and myelin growth. *Nat Rev Neurosci* 6:683–690.
- Skinner MD, Aubin HJ, Berlin I (2004) Impulsivity in smoking, nonsmoking, and ex-smoking alcoholics. *Addict Behav* 29:973–978.
- Spijker S (2011) Dissection of rodent brain regions. In: *Neuroproteomics* (Li KW, ed), pp. 13–26. Springer.
- Steel Z, Blaszczynski A (1998) Impulsivity, personality disorders and pathological gambling severity. *Addiction* 93:895–905.
- Van LL, Westenberg PM, Crone EA (2008) A developmental study of risky decisions on the cake gambling task: age and gender analyses of probability estimation and reward evaluation. *Dev Neuropsychol* 33:179–196.
- Wang X, Deng L, Xu XM (2013) Anterograde axonal tract tracing. In: *Animals models of acute neurological injuries II*, Springer protocols handbooks (Chen J, ed), pp. 461–486.
- Whiteside SP, Lynam DR (2003) Understanding the role of impulsivity and externalizing psychopathology in alcohol abuse: application of the UPPS impulsive behavior scale. *Exp Clin Psychopharmacol* 11:210–217.

(Accepted 12 May 2015)
(Available online 19 May 2015)