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Reward circuitry is perturbed in the absence of the serotonin transporter

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ABSTRACT

The serotonin transporter (SERT) modulates the entire serotonergic system in the brain and influences both the dopaminergic and norepinephrinergic systems. These three systems are intimately involved in normal physiological functioning of the brain and implicated in numerous pathological conditions. Here we use high-resolution magnetic resonance imaging (MRI) and spectroscopy to elucidate the effects of disruption of the serotonin transporter in an animal model system: the SERT knock-out mouse. Employing manganese-enhanced MRI, we injected Mn^{2+} into the prefrontal cortex and obtained 3D MR images at specific time points in cohorts of SERT and normal mice. Statistical analysis of co-registered datasets demonstrated that active circuitry originating in the prefrontal cortex in the SERT knock-out is dramatically altered, with a bias towards more posterior areas (substantia nigra, ventral tegmental area, and Raphé nuclei) directly involved in the reward circuit. Injection site and tracing were confirmed with traditional track tracers by optical microscopy. In contrast, metabolite levels were essentially normal in the SERT knock-out by *in vivo* magnetic resonance spectroscopy and little or no anatomical differences between SERT knock-out and normal mice were detected by MRI. These findings point to modulation of the limbic cortical–ventral striatopallidal by disruption of SERT function. Thus, molecular disruptions of SERT that produce behavioral changes also alter the functional anatomy of the reward circuitry in which all the monoamine systems are involved.

Introduction

The serotonin transporter (SERT) regulates serotonin levels in the synaptic cleft through active uptake from the extracellular space (Li, 2006) and is encoded by a single gene in mouse (Bengel et al., 1997). SERT is the target of a large class of psychoactive drugs used in a number of anxiety disorders, as well as drugs of abuse such as cocaine and methylenedioxymethamphetamine (MDMA). Moreover, SERT is the principal regulator of the entire serotonergic system (Murphy et al., 2004) and dysregulation of SERT gene expression is implicated as a risk factor for a number of affective disorders (Gainetdinov and Caron, 2003; Murphy et al., 2004; Murphy et al., 2001).

Mouse knock-outs for SERT and the other two monoamine transporters, dopamine transporter (DAT) and norepinephrine transporter (NET), have been used extensively to study the pharmacological, behavioral, and anatomic consequences of disruption (Caron, 1999; Dykstra et al., 2003; Gainetdinov and Caron, 2003; Gainetdinov et al., 2002; Hall et al., 2004; Kita et al., 2003; Numachi et al., 2007; Reith, 2005; Rocha, 2003; Torres and Caron, 2005; Uhl, 2003; Uhl et al., 2002; Xu et al., 2000; Yamashita et al., 2006). Single and multiple knock-outs of the monoamine transporters have been especially useful in investigations aimed at linking the molecular actions and behavioral consequences of drugs of abuse (Sora et al., 2001; Uhl and Lin, 2003). These studies have generated a wealth of information about specific aspects of these model systems at the molecular level (*e.g.* up/down regulation of monoamine receptors in response to uptake inhibition, altered concentrations of monoamine metabolites and related molecules), and at the behavioral level (*e.g.* conditioned place preference, locomotor response, drug induced response) (Homberg et al., 2007; Li et al., 2003; Numachi et al., 2007; Rocha, 2003; Shen et al., 2004).

Here we explore brain circuitry in SERT knock-out mice to link molecular alterations to anatomical and behavioral observations. SERT knock-out mice exhibit avoidance and hyperarousal and are more vulnerable to stress than wild-type mice (Adamec et al., 2008). In addition, SERT mice display an initial impairment of food- and cocaine-self-administration (Thomsen et al., 2009). A number of behaviors, including addiction, anxiety, aggression, and affective disorders such as depression, have been linked to anatomical brain regions, specifically the limbic cortical–ventral striatopallidal circuitry (Berton and Nestler, 2006; Everitt and Robbins, 2005; Murphy and Lesch, 2008; Nelson and Trainor, 2007; Robbins and Everitt, 2002). The prefrontal cortex (PFC) is believed to perform executive functions in this circuit (Berton and Nestler, 2006; Robbins and Everitt, 2002) where it has been implicated in working memory, affect, temperament, attention, response initiation and management of autonomic control and emotion (Canli et al., 2001; Groenewegen and Uylings, 2000; Groenewegen et al., 1997; Hagen et al., 2002; Zald et al., 2002). The PFC is also densely innervated by serotonergic neurons arising in

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the median Raphé nuclei of the brain stem (Puig et al., 2004). Recent work in monkey demonstrates that injection of Mn^{2+} into the PFC traces expected pathways deeper into the brain (Simmons et al., 2008). How these pathways might be altered by a loss of SERT activity remains an open question.

Due to the importance of the PFC and its connections, and the expected involvement of serotonin in this circuit, we chose to exploit the SERT knock-out mouse to probe circuitry originating in the PFC with and without SERT activity. After stereotaxic injection of nanoliter volumes of $MnCl_2$ into the PFC of knock-out and normal control mice, we followed the time course of Mn^{2+} uptake, transport, and accumulation over the first 24 h post-injection by sequential high-resolution MRI. We also employed MRS to compare metabolite levels in living brains of normal versus SERT knock-out mice. After *in vivo* MR imaging, we fixed the brains and used diffusion tensor imaging to obtain additional structural information and then processed them for histology and analysis by microscopy. Co-injection of fluorescent tracer with the Mn^{2+} allowed definitive identification of the injection site, confirming its location and lack of damage at the cellular level. Detection of this conventional fluorescent tracer at distant sites was examined to verify the MEMRI results.

Finally, we adopted a non-biased comprehensive approach to identify all connections traced by Mn^{2+} throughout the brain after PFC injection. Whole brain MRI data sets from both genotypes at all time points were co-registered into the same 3D space (Kovacevic et al., 2005; Lee et al., 2005) using a straightforward linear and nonlinear alignment (Bearer et al., 2007b; Tyszka et al., 2006). Image alignment allows an automated voxel-wise comparison of 3D MR images (Hammers et al., 2003; Kassubek et al., 2004; Lee et al., 2005; Mechelli et al., 2005; Toga and Mazziotta, 2002). This allows identification of those voxels with statistically significant intensity changes across time and between cohorts (Bearer et al., 2007b; Cross et al., 2004). By comparing the intensities between one time point and the next, we detected the pathway of the Mn^{2+} as it progressed along neuronal circuits in each genotype. This allowed us to probe changes in the reward/addiction circuitry (limbic cortical–ventral striatopallidal) due to loss of SERT activity.

Materials and methods

Animals

Mice were obtained from Taconic Farms, Inc (Hudson, NY). Ten serotonin transporter (SERT) knock-out mice (Taconic: B6.129-Slc6a4^{tm1Kpl} N10) and ten normal mice (C57Bl/6NTac) were used in this study. Mice were female between the ages of 19 and 23 weeks. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the California Institute of Technology.

Stereotaxic injections

Stereotaxic injection procedure was similar to that employed by Bearer et al. (2007b). Mice were anesthetized by spontaneous inhalation of 1% isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). 5 nl of 600 mM $MnCl_2$ with 0.5 mg/ml 3k rhodamine dextran-amine (RDA) (Molecular Probes/Invitrogen, Eugene, OR) was injected unilaterally into the right prefrontal cortex (coordinates x −0.5 mm (lateral), y 1.0 mm (anterior–posterior A–P with Bregma = 0), z 1.0 mm (dorso–ventral D–V with brain surface = 0) (Paxinos and Franklin, 2001)) over 5 min using a quartz micropipette (1 mm OD quartz capillary pulled to approximately 80 μm OD tip). The animal then received 0.2 ml glycopyrrolate (0.02 mg/kg) and 0.1 ml dextrose (5%) subcutaneously; with 0.2 ml lactated Ringer's solution (10 ml/kg) IP. It was then immediately placed in the MR scanner under 0.8% isoflurane anesthetic.

Preparation for ex vivo imaging

From 1 to 10 days after *in vivo* imaging, animals were sacrificed and brains fixed via transcardiac perfusion with 4% paraformaldehyde (PFA) in phosphate buffered saline, pH 7.2–7.4, as previously described (Tyszka et al., 2006). After overnight rocking in 4% PFA at 4 °C the mouse head was cleaned of skin, lower jaw, ears and cartilaginous nose tip and then rocked in 50 ml 0.01% sodium azide in PBS for 7 days at 4 °C. The head was then transferred to a 5 mM solution of gadoteridol (Prohance®, Bracco Diagnostics Inc, Princeton NJ) and 0.01% sodium azide in PBS and rocked for 7 days at 4 °C prior to MR imaging.

Magnetic resonance imaging and spectroscopy

Each animal was scanned before the stereotaxic injection; and beginning at 0:38 ± 0:14, 1:20 ± 0:14, 2:00 ± 0:16, 2:44 ± 0:14, 4:08 ± 0:14, and 22:51 ± 1:02 h post-injection. Times are averages over all animals ± standard deviation. We use the midpoint of each 40 minute scan as the "scan time" and for convenience call these the 1 h, 1 h 40 m, 2 h 20 m, 3 h, 4 h 20 m and 24 h time points. An 11.7T 89 mm vertical bore Bruker BioSpin Avance DRX500 scanner (Bruker BioSpin Inc, Billerica, MA) equipped with a Micro2.5 gradient system was used to acquire all mouse brain images and spectroscopic data with a 35 mm linear birdcage RF coil. For *in vivo* imaging the animal's head was secured in a Teflon stereotaxic unit within the RF coil to minimize movement and aid in reproducible placement. Temperature and respiration were continuously monitored during data acquisition and remained within normal ranges. We employed a 3D RARE imaging sequence (Hennig et al., 1986) with RARE factor of 4, 4 averages, $TR/TE_{eff} = 250$ ms/24 ms; matrix size of 160 × 128 × 78; FOV 16 mm × 12.8 mm × 7.8 mm; yielding 100 μm isotropic voxels with 40 minute scan time. The short TR provides T_1 weighting to emphasize the location of the paramagnetic Mn^{2+}, while the relatively long effective TE (24 ms) provides T_2 weighting that aids in providing contrast between different anatomical features.

All *in vivo* mouse brain magnetic resonance spectroscopy (MRS) experiments were conducted using Point Resolved Spectroscopy (PRESS) (Bottomley, 1987) with a short echo time TE of 7.267 ms, recycle time of 2.3 s, a spectral width of 7 kHz, 4000 data points in each free induction decay signal (FID), and 128 averages. The sequence was preceded by a VAPOR water suppression module (Tkáč et al., 1999) interleaved with outer volume saturation. Optimized second order shimming was done with the Fastmap routine (Gruetter, 1993) in a 5 mm cube centered in the striatum. The PRESS spectra were then recorded inside a 2 mm^3 volume (8 μl) at the center of the volume used for shimming.

For *ex vivo* imaging, two intact fixed heads were secured in a Teflon® holder and submerged in a perfluoropolyether (Fomblin®, Solvay Solexis, Inc, Thorofare, NJ) within a 50 ml vial and imaged. The ambient bore temperature was maintained at 4 °C by thermostatically controlled airflow. Diffusion weighted images were acquired using a conventional pulsed-gradient spin echo (PGSE) sequence (TR/TE = 300 ms/11.9 ms, 256 × 150 × 120 matrix, 25.6 mm × 15 mm × 12 mm FOV, 100 μm isotropic voxel size, 1 average, $\delta = 3$ ms, $\Delta = 5.2$ ms, Gd = 1125 mT/m, nominal b-factor = 3370 s/mm^2). An optimized six point icosahedral encoding scheme (Hasan et al., 2001) was used for diffusion weighted acquisitions with a single unweighted reference image for a total imaging time of 14.5 h.

Histology

Brains were fixed in pairs (SERT/normal) at different time points (1, 2, 4, 6, 8, 11, 12, 13, 16 and 21 days) after injection to allow histologic determination of transport differences. After fixation and *ex vivo* MR imaging, brains were dissected from the calvarium and sent to Neuroscience Associates (NSA, Knoxville, TN) for gelatin embedding