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Genetic disruption of Met signaling impairs GABAergic striatal development and cognition

Gabriela J. Martins^{1,2}, Mondona Shahrokh², and Elizabeth M. Powell^{1,2,3}

¹Program in Neuroscience, University of Maryland School of Medicine, Baltimore, Maryland 21201, USA

²Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, Maryland 21201, USA

³Department of Psychiatry, University of Maryland School of Medicine, Baltimore, Maryland 21201, USA

Abstract

The largest structure of the basal ganglia, the striatum, modulates motor activity and cognitive function and is composed of GABAergic projection neurons and interneurons. To better understand the mechanisms underlying the development of the striatal neurons and their assembly into functional circuits, we used a mouse with a targeted conditional *Met* mutation in post-mitotic cells of the ventral telencephalon. Characterization of the ontogeny of the striatal neuronal populations demonstrated that disruption of Met signaling specifically altered the GABAergic interneurons. Medium spiny neurons and cholinergic interneurons were largely unaffected. Mice lacking Met signaling have increased numbers of striatal GABAergic interneurons in the lateral sensorimotor areas with distinct behavioral deficits. Motor function and memory formation and consolidation appeared intact, but procedural learning on the cued task of the Morris water maze was delayed. *MET* is a susceptibility gene in Tourette Syndrome and autism, which are human disorders with impaired procedural learning. This study reveals how a striatal targeted disruption in Met signaling after generation of striatal neurons produces behavioral phenotypes shared by Tourette Syndrome and autism, linking the human genetics with the mechanism underlying the disorders.

Keywords

GABAergic interneuron; behavior; Morris water maze; hepatocyte growth factor; Met tyrosine kinase receptor; striatum; reversal learning; cerebral cortex

The striatum is the major input center for the basal ganglia, processing inputs from nearly all cerebral cortical areas, thus modulating motor, emotional, and cognitive behaviors (Calabresi et al., 1997). The primary striatal neuronal population, the GABAergic medium spiny neurons (MSNs), projects to the globus pallidus and substantia nigra (Gerfen, 1992,

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Corresponding Author: Elizabeth M. Powell, PhD University of Maryland School of Medicine Department of Anatomy & Neurobiology HSF II S251 20 Penn St. Baltimore, MD 21201 Office: 410-706-8189 Fax: 410-706-2512 epowe001@umaryland.edu.

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Parent and Hazrati, 1995). The interneurons which modulate signaling of the projection neurons within the striatum are composed of four major classes: a group of cholinergic neurons and three GABAergic populations that express parvalbumin, calretinin, or somatostatin (Kawaguchi et al., 1995). Alterations in striatal circuitry have been implicated in various mental disorders such as Parkinson's disease, obsessive compulsive disorder, autism spectrum disorder, and Gilles de la Tourette Syndrome (TS) (Calabresi et al., 1997, Marsh et al., 2004, Kalanithi et al., 2005, Leckman et al., 2006, Voelbel et al., 2006). Genetic linkage studies have revealed a locus on human chromosome 7q31 as a susceptibility region for autism and TS (Kroisel et al., 2001, Díaz-Anzaldúa et al., 2004, Campbell et al., 2006). One of the genes found in the loci is the *MET* proto-oncogene which encodes for the receptor of the hepatocyte growth factor/scatter factor (HGF/SF). The current study establishes the function of Met in striatal ontogeny and control of procedural learning.

The striatum derives from the ganglionic eminence, a transient embryonic structure that generates the striatal projection neurons and the majority of mammalian forebrain GABAergic interneurons, including those found in the striatum, cerebral cortex, hippocampus and amygdala (Marin and Rubenstein, 2003). A myriad of transcription factors, environmental cues, axonal guidance molecules, and growth factors influence basal ganglia neuronal ontogeny (Marin and Rubenstein, 2001, Powell et al., 2001). In the nervous system, HGF/SF and Met, participate in cellular proliferation, differentiation, migration, and survival (Birchmeier and Gherardi, 1998). Reduced HGF/SF-Met signaling has been shown to lead to deficits in GABAergic interneurons in the cerebral cortex (Powell et al., 2003) and in the hippocampus (Martins et al., 2007). In the embryonic forebrain, HGF/SF and Met are expressed early in proliferative zones and then during neuronal migration and maturation (Birchmeier and Gherardi, 1998, Powell et al., 2001). The tyrosine kinase domain of Met was excised using Cre-*loxP* recombination controlled by the transcription factor *Dlx5/6* (Stenman et al., 2003), rendering Met a dominant negative receptor. Using this strategy, Met activity was eliminated selectively from post-mitotic cholinergic and GABAergic neurons of the ventral telencephalon to assess the function of Met signaling on striatal interneuron development. In summary, our data indicate that the loss of Met function in post-mitotic neurons of the GE leads to specific alterations of forebrain GABAergic interneurons and correlates with specific cognitive deficits. Met signaling has been previously linked to neurodevelopmental disorders (Levitt et al., 2004, Campbell et al., 2008); here we further show that this targeted loss of Met function leads to a phenotype that could be used as a model to better understand the biological underpinnings of disorders of the basal ganglia (Marsh et al., 2004, Kalanithi et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

All procedures conformed to NIH guidelines and were approved by the University of Maryland Institutional Animal Care and Use Committee. Founder mice, FvB-Tg(*Dlx5/6-Cre*)^{Kc} (K. Campbell, Cincinnati Children's Hospital Medical Center; (Stenman et al., 2003)) and B6;129-*Met*^{tm1/Sst} mice (S. Thorgeirsson, NCI; (Huh et al., 2004)) were generous gifts from collaborators and backcrossed onto the C57Bl/6J line obtained from Jackson Laboratory (Bar Harbor, ME, USA) for at least 8 generations. In this manuscript, the B6;FvB - Tg(*Dlx5/6-Cre*)^{Kc} mice are abbreviated as *Dlx5/6*^{Cre}, and the B6;129-*Met*^{tm1/Sst} mice as *Met*^{fx}. Mice used in these experiments were male littermates from matings between non-sibling heterozygotes. For analysis, data from *Met*^{+/+}:*Dlx5/6*^{0/0}, *Met*^{fx/fx}:*Dlx5/6*^{0/0}, and *Met*^{+/+}:*Dlx5/6*^{Cre} mice (n >= 3 for each genotype) were combined as no significant difference was observed between these mice, both behaviorally and anatomically – they are

referred to as “Control” mice throughout the paper. For all behavioral testing, mice were allowed to acclimate to the facility for at least 1h.

Immunohistochemistry

Adult (older than postnatal day 90) brains were cut at 50µm on a freezing sliding microtome (American Optical Company, Buffalo, NY). Tissue was then processed for immunohistochemistry using standard lab protocols (Martins et al., 2007) with the following primary antibodies: rabbit anti-calbindin (1:2000; Swant, Bellinzona, Switzerland), mouse anti-parvalbumin (1:2500; Sigma, St. Louis, MO), rabbit anti-somatostatin-28 (1:2000; Peninsula Labs/Bachem, San Carlos, CA), rabbit anti-calretinin (1:2500; Swant, Bellinzona, Switzerland), rabbit anti-mu-opioid receptor (MOR 1:10,000; Chemicon, Temecula, CA); goat anti-ChAT (1:5000; Chemicon, Temecula, CA); mouse anti-nNOS (1:2500; Zymed, Carlsbad, CA), rabbit anti-NPY (1:40,000; Peninsula Labs/Bachem, San Carlos, CA). For visualization of primary antibodies, slices were incubated with either biotinylated secondary antibody or Cy3-conjugated donkey anti-rabbit (1:3000; Jackson ImmunoResearch Laboratory, West Grove, PA). Images of brain sections were obtained using PowerPhase digital camera (Phase-one, Copenhagen, Denmark).

Immunoblots

Tissue samples were homogenized in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate buffered saline) with added protease inhibitors (1 mM PMSF, 10 µM pepstatin, 10 µM leupeptin and 1 µg/ml aprotinin) and stored at –80°C until used. The protein concentration of each sample was determined with the Micro-BCA assay (Pierce, Rockford, IL, USA). The samples were mixed with Laemmli sample buffer and boiled for 1 minute. For each sample, 20 µg total protein was separated on a 10% bis-acrylamide SDS gel, transferred to a nitrocellulose membrane, and blocked for 1 h with 5% milk (Carnation) in phosphate-buffered saline (PBS). Primary antibodies were diluted in 3% milk in PBS and the membranes incubated for 12 to 24 h at 4°C. Antibodies were diluted 1:500 for detection of Met (Santa Cruz Biotech) and 1:3000 for β-actin (Sigma). The membranes were washed several times with PBS and incubated with the appropriate peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove PA, USA, 1:20,000 dilution in 3% milk/PBS). Following several washes, the bound antibody was visualized using the ECL kit (Amersham Pharmacia Biotech, Inc, Piscataway, NJ, USA) and X-omat blue film (Kodak, Rochester, NY, USA). Films were scanned with a UMAX Astra 1200S scanner and labeled using Adobe Photoshop.

Cell counts

For cell counts in the cerebral cortex and striatum, 9 control brains (representing *Met^{+/+}:Dlx5/6^{0/0}*, *Met^{fx/fx}:Dlx5/6^{0/0}*, and *Met^{+/+}:Dlx5/6^{Cre}* mice) and 4 *Met^{fx/fx}:Dlx5/6^{Cre}* brains (n = 3 for amygdala) were analyzed using the NeuroLucida system (MicroBrightField, Inc; Williston, VT). Unbiased stereological cell counts were obtained from at least 3 sections spaced 400 µm apart from somatosensory cortex (from Bregma level 1.10mm extending caudally to –1.94mm; Paxinos and Franklin, 2001), orbitofrontal cortex (OFC; from Bregma level 2.86mm extending caudally to 2.20mm), visual cortex (between Bregma levels –2.70mm and –4.48mm), and 5 sections along the striatum (Bregma level = 1.54mm and extending caudally to –1.06mm). For cortical regions a dissector of 100 µm × 100 µm was used, for striatal medium spiny neurons: 50 µm × 50 µm, and for striatal interneurons, 350 µm × 350 µm. Volume was estimated using the Cavalieri method. Statistical significance was determined for counts from each region and for each antigenic marker using a Student's *t*-test analysis with a significance level of *P* < 0.05 in Microsoft Excel.

For analysis of cell distribution, images of the entire dorsal striatum were captured at 5 bregma levels (1.5, 1.0, 0.5, 0.0, -0.5, and -1.0 mm), representing the entire rostral-caudal extent. The images were analyzed with ImageJ (<http://rsb.info.nih.gov/ij/>) and the position of each immunoreactive cell was recorded using the 'Analyze Particles' macro. Landmarks for the striatum included the corpus callosum for the lateral boundary and the edge of the lateral ventricle for the medial boundary. The corpus callosum defined the dorsal boundary and the ventral boundary was determined by cytoarchitecture and presence of the patch-matrix patterning. The lateral distribution of cells was compared between 9 control and 4 *Met^{fx/fx}:Dlx5/6^{Cre}* brains using a distance of 200 μ m over the entire medial to lateral distance, at each bregma level. Distributions were compared using the Kolmogorov-Smirnoff test. For data presentation (Fig 3H), the data was grouped into three regions, medial (location 0 – 800 μ m from the ventricle), intermediate (800 – 1400 μ m from the ventricle), and lateral (1400 – 2200 μ m from the ventricle).

Morris water maze

The Morris water maze was performed in a rubber tub (Aquatic Systems) with water maintained at 25°C (Holmes et al., 2002, Wahlstena et al., 2005, Vorhees and Williams, 2006) and colored with white tempera paint (Crayola) to obscure the platform. The 10cm in diameter platform was placed 15cm from the edge of the tub in the center of each randomly selected quadrant. Large colored shapes (a circle, a triangle, a rhombus, and a star) were used as the cues for the mice to navigate through the maze. Animals were pre-trained with a 30s free swim, 30s on the submerged platform, and two trials where the mouse had to find the platform and remain on it for 10s, (Wolff et al., 2002, Wahlstena et al., 2005, Vorhees and Williams, 2006). During training, the animals underwent four trials a day until the average latency for the mice to successfully find the platform was less than 10s. For the probe test, the platform was removed and the time a mouse spent in each quadrant was recorded for a total of 60s. To test reversal learning, the platform location was changed to the quadrant opposite from that in previous trials. A Spatial Cued test was given to test striatal function, whereby the hidden platform was indicated by the presence of a flag (Whishaw et al., 1987, Devan et al., 1996) but no external cues surrounding the maze. The mice were allowed to explore the maze for a maximum of 60s before being removed. Data is reported as the mean $\pm$ the standard error of the mean (SEM), for a group of 13 control (*Met^{+/+}:Dlx5/6^{0/0}*, *Met^{fx/fx}:Dlx5/6^{0/0}*, and *Met^{+/+}:Dlx5/6^{Cre}* alleles) mice and 7 *Met^{fx/fx}:Dlx5/6^{Cre}* mice. Statistical significance between genotypes of percent time spent in each quadrant during the probes and the latency to reach the cued platform was determined using a two-way ANOVA with Fisher's least-significant difference *post-hoc* comparison in Statistica (StatSoft Inc., Tulsa, OK).

Open Field

Exploratory activity (Crawley, 2000, Holmes et al., 2002, Bothe et al., 2004) was assessed in a TruScan automated open field (Mouse *TruScan* Activity Arena; Coulbourn Inst., Allentown, PA) that was evenly illuminated by overhead fluorescent white room lighting. Each mouse (18 control and 15 *Met^{fx/fx}:Dlx5/6^{Cre}*) was individually placed in the center of the arena and left to freely explore for 10min. The number of horizontal and vertical beam breaks was taken as measures of horizontal and vertical activity, respectively. Data was collected by TruScan 2.0 software and analyzed in Microsoft Excel using a Student's *t*-test. Data is reported as the mean $\pm$ SEM.

Light/Dark Exploration

The light dark exploration test was conducted as previously described (Mathis et al., 1994, Homanics et al., 1999, Holmes et al., 2002, Powell et al., 2003). Mice were placed in the TruScan arena (Mouse *TruScan* Activity Arena; Coulbourn Inst., Allentown, PA) as for the

Open Field test, but with a partition dividing the arena into a light (maintained at ambient light) and dark (a dark box with only a small opening at floor level allowing for mouse entry) half. Mice were individually placed in the center of the light compartment and allowed to freely explore the arena for 10min. A Student's *t*-test was performed in Microsoft Excel to statistically analyze differences in the amount of time spent in the dark compartment between control and *Met^{fx/fx}:Dlx5/6^{Cre}* mice. Data is reported as the mean $\pm$ SEM, for a group of 18 control mice and 15 *Met^{fx/fx}:Dlx5/6^{Cre}* mice.

Elevated Plus Maze

The elevated plus-maze test was conducted (Crawley, 2000, Holmes et al., 2002, Powell et al., 2003, Wahlsten et al., 2003) on an apparatus designed for mice, comprised of two open arms (30.5 x 6.5cm) and two closed arms (with 15.5cm walls) at 90° to each other (San Diego Instruments). Each mouse was evaluated individually and the amount of time a mouse spent in each arm (as defined by all four paws in an arm) during a 5min session was recorded. These times were calculated as the percentage of the test session spent in an open or closed arm [(time in open arms/session duration) $\times$ 100]. The data were analyzed using an ANOVA with a Tukey's highest-significant difference *posthoc* comparison in Statistica (StatSoft Inc., Tulsa, OK). Data is reported as the mean $\pm$ SEM, for a group of 12 control mice and 9 *Met^{fx/fx}:Dlx5/6^{Cre}* mice.

Reversal Learning Task

A reversal task was performed as described in previous reports (Colacicco et al., 2002, Bissonette et al., 2008). Food deprived mice were trained to dig in bowls of scented media to retrieve cereal rewards. At the start of each trial, the mouse was placed in the testing arena to explore two bowls with combinations of odors and digging media until digging in one bowl to signify a choice. The bait was a piece of Honey Nut Cheerio cereal (~5 mg), and the cues, either olfactory (odor) or somatosensory and visual (texture of the digging medium which hides the bait) were altered and counterbalanced. All cues were presented in identical small animal food bowls (All Living Things Nibble Bowls, PetSmart) that were identical in color and size. Digging media was mixed with the odor (0.01% by volume) and Honey Nut Cheerio powder (0.1% by volume). On the first day of training, the mice were given 4 consecutive trials with the baited food bowl to ascertain they could reliably dig. All mice were able to dig for the reward. Mice were tested through a series of discriminations where the exemplar pair was changed, but the dimension (odor or medium) of the correct choice remained the same. The dimension was relevant if its attributes predicted outcome. For example, if odor was the relevant dimension, then the mouse was required to choose the correct odor from each pair and ignore the attributes of the digging medium. In this example, the digging medium is considered the irrelevant dimension.

The baited bowl was randomly presented on either side of the testing cage, and the relevant exemplar was randomly presented with the irrelevant exemplars. The trial was stopped if the mouse did not dig within 3 min in the testing cage. Stopped trials were uncommon (< 3% of all trials). A criterion of eight consecutive correct trials was required to complete each test. Reversal trials consisted of reversing the rewarded cue with the unrewarded cue in the exemplar pair. Data are reported as the mean number of trials to criterion $\pm$ SEM. A two-way ANOVA was used to determine statistical significance between groups and discriminations, followed by Neuman-Keuls *post-hoc* analysis using the Statistica software package (Statsoft, Inc, Tulsa, OK). Statistical significance was considered as $P < 0.05$, and denoted by asterisks.

RESULTS

Expression patterns of interneurons in the adult mouse

In the rodent striatum, *Dlx5/6* is expressed by medium spiny neurons and interneurons (Wu and Parent, 2000), and therefore Met activity was likely lost in all populations. Expression of a GFP reporter gene in the *Met^{fx/fx}·Dlx5/6^{Cre/Z/EG}* demonstrates many cells undergoing recombination in the embryo (Fig 1A-B). We confirmed loss of the Met receptor in the adult by PCR and immunoblot (Fig 1C-D), which demonstrates downregulation of receptor levels after recombination in the striatum. Downregulation of the levels of the mutant Met is in agreement with previous reports (Huh et al., 2004, Judson et al., 2009).

The GABAergic MSNs compose 90-95% of the neurons in the dorsal striatum (Gerfen, 1992, Kawaguchi et al., 1995). MSNs are born in the lateral GE, in contrast to the interneuron populations which arise from the medial and caudal GE (GABAergic) and the preoptic area (cholinergic) (Marin and Rubenstein, 2003, Stenman et al., 2003). The expression of *Dlx5/6* in postmitotic cells in the GE and preoptic area (Stenman et al., 2003) was predicted to alter Met-dependent ontogeny throughout the dorsal striatum. However, the striatum of the *Met^{fx/fx}·Dlx5/6^{Cre}* mice appeared grossly normal as compared to control mice using cresyl violet staining (Fig. 2A-B). Furthermore, there was no difference in estimated volume between mutant and control mice (Fig. 2C). CB-immunoreactive cells reflect 75-85% of the neurons of the striatum (Liu and Graybiel, 1992). The total number of CB⁺ cells was not significantly different in control and *Met^{fx/fx}·Dlx5/6^{Cre}* mice (Fig. 2D-F; *t*-test, *P* = 0.15). Similarly, overall distribution of CB⁺ cells appeared to be normal across genotypes. These data indicate that Met does not regulate postmitotic MSNs.

In the adult, the numbers and distribution of striatal cholinergic interneurons, identified by their expression of choline acetyltransferase (ChAT; Fig. 3A-B, G), were similar in both control and mutant mice (*t*-test, *P* = 0.21). The non-overlapping expression of PV, SST, or CR defined the majority of dorsal striatal GABAergic interneurons (Kawaguchi et al., 1995). The embryonic loss of Met function led to a 68% increase in PV expressing neurons in the *Met^{fx/fx}·Dlx5/6^{Cre}* mice (Fig. 3D, G) relative to control mice (Fig. 3C, G; *t*-test, *P* = 0.0005). Similarly, there was a 39% increase in SST⁺ cells in *Met^{fx/fx}·Dlx5/6^{Cre}* mice (Fig. 3F-G; *t*-test, *P* = 0.003). The increased numbers of striatal GABAergic interneurons are consistent with the estimated numbers of GABAergic interneurons that are missing from the cerebral cortex. CR expressing neurons, however, were not different between control and *Met^{fx/fx}·Dlx5/6^{Cre}* mice (*t*-test, *P* = 0.46). Dorsal striatal interneurons are also identified with the markers neuronal nitric oxide synthase (NOS), neuropeptide Y (NPY) and CB expression, however, these markers overlap with each other and with SST, and thus the numbers of cells are included in the analysis of the SST expressing population (Kawaguchi et al., 1995). Even so, the populations of NOS and NPY expressing cells were normal in *Met^{fx/fx}·Dlx5/6^{Cre}* mice relative to control mice (data not shown). Striatal CB⁺ interneurons cannot be distinguished from CB⁺ medium spiny neurons. There was no difference in the number of CB⁺ cells between control and *Met^{fx/fx}·Dlx5/6^{Cre}* mice (Fig. 2D-F). In summary, embryonic loss of Met function specifically increased the PV⁺ and SST⁺ GABAergic striatal interneuron populations.

Examination of the pattern of striatal interneurons showed differences at more caudal portions of the dorsal striatum. At bregma levels = 1.5 and 1.0 (based on Paxinos and Franklin, 2001), the distributions of PV⁺ cells were similar, *P* > 0.22, Kolmogorov-Smirnoff test, (Fig. 2H). At bregma levels = 0.5, 0.0, -0.5 and -1.0, the distributions were statistically different, *P* < 0.001 (Fig. 2H). In the *Met^{fx/fx}·Dlx5/6^{Cre}* mice fewer cells of the population were located in the medial (associative) regions and more cells were observed in the lateral

(sensorimotor) areas. In absence of Met signaling, the numbers and distribution of striatal interneurons were altered.

Overall, cerebral cortical formation in *Met^{fx/fx};Dlx5/6^{Cre}* mice appeared normal as detected by cresyl violet with both control and *Met^{fx/fx};Dlx5/6^{Cre}* mice having normal lamination and cell distribution. Similarly, volumes of the cerebral cortex obtained in somatosensory cortex, visual cortex, and orbitofrontal cortex were comparable between control and *Met^{fx/fx};Dlx5/6^{Cre}* mice. GABAergic interneurons comprise a relatively small fraction of the total cells in the neocortex, and the disruption of this population during development, therefore, is likely to go unnoticed when using traditional histological methods. Therefore, the majority of adult cerebral cortical GABAergic interneurons can be identified by three non-overlapping populations that express the markers parvalbumin (PV), somatostatin (SST), or calretinin (CR) (Kawaguchi et al., 1995). All three populations arise from the GE, and begin to express Dlx5/6 after their final cell cycle (Stenman et al., 2003). The inactivation of Met in the *Met^{fx/fx};Dlx5/6^{Cre}* mice led to a selective 28% loss of PV⁺ cells in primary somatosensory cortex (Fig. 4B, E) relative to control (Fig. 4A, E; *t*-test, *P* = 0.014). This deficit extended to rostral brain regions as seen in the orbitofrontal cortex (Fig. 4C-E) where there was a 31% reduction in PV⁺ cells in *Met^{fx/fx};Dlx5/6^{Cre}* mice (Fig. 4D-E) relative to control mice (Fig. 4C and E; *t*-test, *P* = 0.009). More caudally, in visual cortex, there was no difference in the number of PV⁺ interneurons between control (117,548 ± 29,635) and *Met^{fx/fx};Dlx5/6^{Cre}* mice (104,431 ± 32,774; *P* = 0.185). In all cortical regions, the SST and CR subpopulations of GABAergic interneurons were unaffected (unpublished observations). The amygdala showed normal levels of PV⁺ and SST⁺ neurons (Fig. 5A-D) as did the olfactory bulbs (unpublished observations). In summary, the embryonic perturbation affected frontal and parietal areas.

In summary, despite the same developmental expression of Dlx5/6 in cortical and striatal interneuron precursors (Stenman et al., 2003), the embryonic disruption of Met function specifically alters the PV⁺ and SST⁺ GABAergic interneurons. In the *Met^{fx/fx};Dlx5/6^{Cre}* mice, ~30% fewer PV⁺ cells were observed in the cerebral cortex. The cells appeared to remain in the striatum as increased numbers of PV⁺ and SST⁺ subpopulations supporting the hypothesis that in the absence of Met signaling, interneurons are delayed and remain at the source.

Alterations to forebrain GABAergic populations do not affect open field activity and measures of anxiety

The Open Field Test (Fig. 6A) (Crawley, 2008) assesses locomotor activity, hyperactivity, and exploratory behaviors. The percentage of time spent exploring and the total distance traveled in the Open Field test were measurements of activity. Control mice moved around the arena an average of 60% (± 3.7 s.e.m) of the duration of the test and covered 1,744 cm (± 236.3 cm; Fig. 6A black bar). Similarly, *Met^{fx/fx};Dlx5/6^{Cre}* mice explored 56 ± 4.2% of the time and covered 1,416 ± 148.0 cm (Fig. 6A white bar; *t*-test *P* = 0.392). In the home cage, all mice had normal gait and motor activity. Therefore, changes in GABAergic expression did not alter exploratory activity in the *Met^{fx/fx};Dlx5/6^{Cre}* mice.

Alterations in GABA levels have been shown to modulate anxiety (Partyka et al., 2007, Depino et al., 2008). However, neither the loss of PV⁺ GABAergic cells in the cerebral cortex nor their significant increase in the striatum appear to alter anxiety levels in *Met^{fx/fx};Dlx5/6^{Cre}* mice (Fig. 6B). In the Open Field, the ratio of time spent at the edges versus the center of the arena can be used as an initial measure of anxiety. Both groups spent similar amounts of time near the margin of the arena versus the center (data not shown). The Light-Dark Choice test (Fig. 6B) is used as a measure of relative anxiety in mice (Crawley, 2000, Crawley, 2008). Control mice spent about 30% of the duration of the test in the light

compartment and $70 \pm 7\%$ in the dark. *Met^{fx/fx}:Dlx5/6^{Cre}* mice spent similar amounts of time in each compartment (36% in the light and $64 \pm 10\%$ in the dark; $P = 0.30$) relative to controls, suggesting comparable levels of anxiety.

The Elevated Plus Maze is another paradigm to investigate anxiety (Crawley, 2000, Crawley, 2008). The open arms of the maze are considered to be an anxiety-producing stimulus that mice will tend to avoid. Control mice spent $72 \pm 3.1\%$ of their time exploring or sitting in the closed arms, $9 \pm 1.2\%$ in the center, and $18 \pm 2.6\%$ in the open arms (Fig. 6C, black bars). *Met^{fx/fx}:Dlx5/6^{Cre}* mice performed similarly, spending $71 \pm 5.0\%$ of the time in the closed arms, $14 \pm 3.7\%$ in the center, and only $14 \pm 3.0\%$ in the open arms (Fig. 6C, white bars). ANOVA revealed a significant main effect on location [$F(2,60) = 64.34$, $P < 0.0001$] (a preference for the closed arm) but not on genotype [$F(1,60) = 2.25$, $P = 0.114$], further indicating that alterations in interneuron number in *Met^{fx/fx}:Dlx5/6^{Cre}* mice did not influence anxiety levels.

The decreased numbers of interneurons in the OFC indicated a possible deficit in reversal learning (Bissonette et al.). Both groups of mice readily learned the discriminations (Fig. 6D). ANOVA revealed significant effects of task [$F(1,28) = 32.81$, $P < 0.0001$] and of genotype [$F(1,28) = 11.44$, $P = 0.002$]. Posthoc analysis showed similar performances for control and *Met^{fx/fx}:Dlx5/6^{Cre}* mice in learning the discriminations ($P = 0.288$). On the reversal discriminations, the *Met^{fx/fx}:Dlx5/6^{Cre}* mice required significantly more trials than control mice ($P < 0.001$). The loss of cortical interneurons impaired reversal learning but not general locomotion or anxiety.

Delayed striatal-mediated behavior in *Met^{fx/fx}:Dlx5/6^{Cre}* mice

The striatum mediates planning and modulation of movement pathways, but it is also involved in cognition (Pisa and Cyr, 1990, Reading et al., 1991, Packard and Knowlton, 2002, Clarke et al., 2008). The Morris water maze tests hippocampal-mediated spatial learning and striatal-mediated procedural learning (Packard et al., 1994, Devan et al., 1996, Packard and Teather, 1997, Wahlstena et al., 2005, Vorhees and Williams, 2006). The significant increase in interneurons in the *Met^{fx/fx}:Dlx5/6^{Cre}* striatum prompted testing of performance in the Morris water maze. First, mice were trained to locate the hidden platform based on external cues to test their spatial memory. Within 3 days all mice were able to find the platform in 10s or less (Fig. 7A). *Met^{fx/fx}:Dlx5/6^{Cre}* mice had a longer latency to locate the platform on the first 2 days of training (Fig. 7A white bars; [$F(1,42) = 5.77$, $P = 0.006$]) when compared to control littermates. However, acquisition of this task involves various circuits including both hippocampal and striatal function (Devan et al., 1996, Teather et al., 2005, Vorhees and Williams, 2006). The platform was subsequently removed and mice were allowed to explore the maze for 1 min (probe test) to directly assess hippocampal function. The time each mouse spent exploring each quadrant was measured. Based on the spatial cues, mice should spend the majority of their time around the quadrant where the platform had been (Devan et al., 1996, Vorhees and Williams, 2006). Following the probe test, the mice were retrained with the platform now hidden in another (opposite) quadrant and subsequently probed for their spatial learning ability (reversal test). Even though the *Met^{fx/fx}:Dlx5/6^{Cre}* mice have fewer hippocampal PV⁺ neurons (Martins et al., 2007), the performance on the probe test (Fig. 7B) and reversal probe tests (Fig. 7C); which evaluate hippocampal-mediated spatial memory (Devan et al., 1996, Vorhees and Williams, 2006), was the same for all groups. All mice showed a greater than chance preference for the correct quadrant, ([$F(3,68) = 20.758$, $P < 0.0001$] for probe; [$F(3,68) = 16.614$, $P < 0.0001$] for reversal probe). No effect of genotype was observed. These data indicate normal hippocampal-mediated spatial learning.

Several double-dissociation studies have demonstrated the importance of the striatum on basic stimulus-response learning. Lesions to the striatum resulted in longer latencies for rodents to reach the visible (cued) platform (Packard et al., 1994, Devan et al., 1996, Devan et al., 1999, Packard and Knowlton, 2002, Teather et al., 2005, Rossato et al., 2006, Vorhees and Williams, 2006). Therefore, spatial cues were removed and a flag (cue) was placed directly on the platform (cued platform) to assess striatal-mediated procedural learning (Devan et al., 1996, Devan et al., 1999, Vorhees and Williams, 2006). The platform and the mouse were placed in a random quadrant at the start of each trial. Normal procedural learning allows mice to quickly escape onto the platform once they locate the flag (Devan et al., 1996, Devan et al., 1999, Vorhees and Williams, 2006). Consistent with a striatal defect, the *Met^{fx/fx}:Dlx5/6^{Cre}* mice showed increased latency performance on the cued-platform test (Fig. 7D, white bars). There was an effect on genotype for latencies to reach the platform on the cued test [$F(1,41) = 6.686$, $P = 0.013$]. On the first day of the cued test, all mice were able to navigate to the platform in approximately 9s (9 ± 0.5 s for control mice and 9 ± 1.1 s for *Met^{fx/fx}:Dlx5/6^{Cre}* mice). On the second day, *Met^{fx/fx}:Dlx5/6^{Cre}* mice took more than 4s longer (8.5 ± 1.5 s) than control mice (4 ± 0.5 s). This delay in latency performance was decreased by day 3, but reflects a similar pattern seen during training (Fig. 7A) and resulting in a significant interaction between genotype and trial day [$F(2,41) = 4.701$, $P = 0.015$]. In summary, *Met^{fx/fx}:Dlx5/6^{Cre}* mice had normal hippocampal-mediated spatial memories but demonstrated a specific delay in striatal-dependent cued learning relative to control mice.

DISCUSSION

The loss of the Met receptor kinase domain in embryonic postmitotic Dlx5/6-expressing cells altered the ratios of adult cerebral cortical and striatal GABAergic interneurons. The subpopulation of PV⁺ interneurons in the cerebral cortex were diminished by ~30% in rostral areas, but the PV⁺ and SST⁺ striatal interneurons were increased by ~70% and ~40%, respectively. The GE is also the source of GABAergic interneurons in the amygdala and olfactory bulbs, yet these structures were not significantly affected by the loss of Met signaling. Behavioral characterization of the *Met^{fx/fx}:Dlx5/6^{Cre}* demonstrated delayed procedural learning and reversal learning, with otherwise normal activity and emotionality. These deficits are common endophenotypes reported in patients with Tourette Syndrome, obsessive compulsive disorder, or autism. The underlying causes of these disorders are likely due to multiple genetic and environmental interactions. This study demonstrates how specific interruption in a subset of developing neurons can produce abnormal ontogeny and subsequent altered neural circuitry.

The regional nature of the anatomical deficits reflects the embryonic origins. The PV⁺ and SST⁺ interneurons arise from the medial GE, whereas the CR⁺ cells are generated in the caudal GE (Anderson et al., 2001, Nery et al., 2002). The inactivation of Met did not alter cells from the caudal GE, as observed by normal numbers of CR⁺ in the cerebral cortex and striatum and also the unchanged interneuron profiles in the amygdala or the caudal GE-derived hippocampal interneurons (Martins et al., 2007). Similarly, the derivatives of the lateral GE, the medium spiny neurons and the olfactory interneurons were unaffected. Finally, the numbers of the striatal ChAT⁺ cells from the Dlx5/6-expressing preoptic area were similar in control and mutant mice. These data lead to the conclusion that loss of the autophosphorylation domain in Met only altered PV⁺ and SST⁺ interneuron populations in the cerebral cortex and striatum, implying a specific role for Met in GABAergic neuronal ontogeny.

Multiple mechanisms may have lead to the cortical and striatal interneuron disturbances. Since Dlx5/6 is expressed mainly in post-mitotic cells (Stenman et al., 2003), the loss of Met in Dlx5/6⁺ cells was unlikely to affect cell proliferation, and birthdating and

immunohistochemical studies revealed similar cell numbers in both control and mutant mice (data not shown). Alternatively, loss of Met signaling embryonically could be altering cell specification and fate, prompting interneurons destined for the dorsal telencephalon to remain ventrally in the striatum. Another explanation for the decreased cerebral cortical PV⁺ neurons may be increased cell death, yet, no changes in activated caspase 3 were observed (data not shown). Met signaling has been previously implicated in regulating interneuron migration from the GE into the cerebral cortex (Powell et al., 2001). Therefore, in the conditional *Met* mutant mouse, delayed migration from the GE would lead to decreased cerebral cortical interneurons with a concomitant apparent increase in striatal interneurons. However, the increase in striatal interneurons is not uniform, with fewer cells in the associative regions and greater numbers in sensorimotor regions. The abnormal distribution of PV⁺ interneurons in the *Met^{flx/flx}:Dlx5/6^{Cre}* mouse, especially the increased number at the corticostriatal border, supports the delayed migration hypothesis.

Deficits in inhibitory neurotransmission are associated with increased anxiety-related behaviors (Mathis et al., 1994, Chiu et al., 2005, Partyka et al., 2007, Depino et al., 2008). Alterations to cortical GABA levels in *Met^{flx/flx}:Dlx5/6^{Cre}* mice did not show any differences in anxiety levels or stereotyped movements relative to control mice (Fig. 5) despite reductions in PV⁺ cells in somatosensory and orbitofrontal cortex (Fig. 4). Normal performance in open field and anxiety tests suggest that the approximately 30% loss of PV⁺ neurons, (or a ~15% loss of all GABA⁺ interneurons) is not sufficient to impair these functions.

Developmental changes to GABAergic number in the striatum can lead to relatively mild behavioral phenotypes (Van de Berg et al., 2003), and we observed a permanent deficit in cued learning in the *Met^{flx/flx}:Dlx5/6^{Cre}* mice. One possible explanation is the increase in striatal GABAergic interneurons in mainly lateral regions and the overall abnormal distribution of interneurons disrupts the processing of signals in the basal ganglia loops, in particular when a stimulus-response is transformed into a habit (Balleine and O'Doherty). The PV⁺ striatal interneurons inhibit MSNs and gate cortical input to a more 'fine-tuned' signal. Similarly, SST⁺ interneurons modulate signals propagating between MSNs by acting presynaptically to regulate GABA release from the interconnected MSN terminals (Lopez-Huerta et al., 2008). Our data suggest that increased inhibition provided by the PV⁺ and SST⁺ interneurons in the *Met^{flx/flx}:Dlx5/6^{Cre}* striatum reduce or interrupt MSN output to alter striatal function.

The dorsolateral striatum receives afferent inputs predominantly from the sensorimotor cortex. Lesions to the dorsolateral striatum disrupt sensorimotor function (Pisa and Schranz, 1988) and simple stimulus-response learning (Reading et al., 1991, Packard, 2009). The ectopic interneurons in the *Met^{flx/flx}:Dlx5/6^{Cre}* mice (68% increase PV⁺ and 39% increase in SST⁺ neurons Fig. 3) are predominantly observed in the dorsolateral striatum. The delayed cued learning in the Morris water maze implies that permanent consequences of embryonic perturbations in Met signaling prompt abnormal distributions of forebrain GABAergic interneurons and retard procedural learning.

The dorsomedial striatum receives afferent inputs predominantly from the visual and medial prefrontal cortical regions. Lesions to the dorsomedial striatal area disrupt reversal learning (Pisa and Cyr, 1990, Clarke et al., 2008), short-term memory (DeCoteau et al., 2004), place navigation (Whishaw et al., 1987, Brown and Robbins, 1989). We observed impaired reversal learning, but normal set-shifting in a two-cup choice task (Fig. 6, and unpublished observations), suggesting some disruption in dorsomedial striatal function. Hence, the local striatum interneurons may modulate stimulus-response and response-outcome associations in dorsal striatum.

Altered cortico-striatal anatomy and function is an emerging theme in TS and ADHD patients. Young adults (mean age of 28 years) with TS and children (mean age 11 years) with ADHD demonstrated decreased cortical inhibition as measured by transcranial magnetic stimulation (Ziemann et al., 1997, Moll et al., 2000). Deficits in motor and cognitive function have been observed in the human sensory-motor and orbitofrontal cortical areas (Stern et al., 2008). The loss of PV⁺ cortical interneurons in the *Met^{flx/flx}:Dlx5/6^{Cre}* mice and impaired reversal learning parallel the human phenotype, however, data regarding the specific interneuron profiles in patients is not currently available. But, PV⁺ interneurons were found to be decreased in the caudate and putamen in TS patients with severe and persistent disease (Kataoka et al., Kalanithi et al., 2005, Leckman et al., 2006) along with deficits in habit learning (Marsh et al., 2004, Leckman et al., 2006). The loss of both PV⁺ and ChAT⁺ populations in the human patients suggest an early developmental perturbation, during cell proliferation, that may be followed by subsequent changes in cell migration or maturation. Whereas, in the *Met^{flx/flx}:Dlx5/6^{Cre}* mouse, Met signaling was reduced after neuronogenesis. The defects observed in the *Met^{flx/flx}:Dlx5/6^{Cre}* mouse may represent the migration and maturation aspects of the developmental aberrations that may lead to TS-associated symptoms.

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List of abbreviations

ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
CB	Calbindin
ChAT	Choline acetyltransferase
CR	Calretinin
Ctx	Cerebral cortex
E	Embryonic
GE	Ganglionic Eminence
HGF/SF	Hepatocyte growth factor/scatter factor
IZ	Intermediate zone
MOR	Mu opioid receptor
MSN	Medium spiny neuron
NOS	nitric oxide synthase
NPY	neural peptide Y
OFC	orbitofrontal cortex
P	Postnatal
TS	Tourette syndrome
PV	Parvalbumin

SST

Somatostatin

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Fig. 1.

Distribution of Met after recombination. *Met^{fx/fx};Dlx5/6^{Cre}* were bred with *Z/EG* reporter mice to visualize the pattern of recombination. (A) Coronal section at E15.5 demonstrating cells in the GE and migrating into the intermediate zone (IZ) of the overlying cerebral cortex (Ctx). The box outlined is shown in (B) demonstrating migratory phenotype of the cells, with long process in the direction of migration (arrows). (C) PCR of genomic DNA from adult demonstrates recombination in the mutant strium (lane 2, 450 bp product), but not in the control strium (lane 2, 300 bp product). Genomic DNA from tail of a *Met^{fx/+};Dlx5/6^{Cre}* mouse (lane 3) shows all bands (300 bp wildtype allele, 380 bp floxed allele, and 450 bp recombined allele, marked with white arrows), due to recombination in skin. Lane 4 is the reaction in absence of DNA template. (D) Immunoblot of Met expression in adult mice showing decreased levels in strium after expression of *Dlx5/6* and recombination and similar levels in cerebellum. Immunoblot for β -actin was used as a loading control. Bar = 200 μ m.

Fig. 2.

Normal overall striatal anatomy is observed with decreased HGF/SF-Met signaling. Cresyl violet staining demonstrates normal striatal formation in control (A) and *Met^{fx/fx};Dlx5/6^{Cre}* mice (B). (C) Overall striatal volume is also the same between control (black bars) and *Met^{fx/fx};Dlx5/6^{Cre}* mice (white bars). Calbindin immunohistochemistry in control (D) and *Met^{fx/fx};Dlx5/6^{Cre}* (E) mice shows no differences in the number (F) of striatal medium spiny neurons, as estimated using unbiased stereology. Scale bar = 400 μ m for images A and B and 100 μ m for images D and E.

Fig. 3.

Embryonic loss of Met function leads to specific perturbations in adult striatal interneuron populations. (A and B) ChAT⁺ cholinergic interneurons arise from Dlx5/6-expressing cells. *Met^{flx/flx};Dlx5/6^{Cre}* mice (B) show no differences in the number of ChAT⁺ cells (G white bars) relative to control brains (A and G black bars). (C and D) Parvalbumin immunohistochemistry demonstrates a 68% increase in cell number in *Met^{flx/flx};Dlx5/6^{Cre}* mice (D and G white bars) relative to control mice (C and G black bars). (E and F) Analysis of SST⁺ cells indicates a 39% increase in overall cell number in *Met^{flx/flx};Dlx5/6^{Cre}* mice (F and G white bars) as compared to control mice (E and G black bars). (H) Distribution of PV⁺ cells throughout the medial-lateral extent of the adult dorsal striatum. At bregma levels = 0.0 and 1.0, the *Met^{flx/flx};Dlx5/6^{Cre}* mice had fewer cells in the medial regions and more cells in the lateral regions. Asterisks denote statistical differences between the distributions at $P < 0.001$. Scale bar in B = 100 μ m; * $P < 0.05$.

Fig. 4.

Altered parvalbumin expression patterns in the adult cerebral cortex. Loss of Met function yielded a deficit of PV⁺ cells in somatosensory cortex (A and B) and orbital frontal cortex (OFC) (C and D). Images show PV immunoreactivity in the control (A and C) and *Met^{fx/fx};Dlx5/6^{Cre}* mice (B and D). (E) Counts of the parvalbumin immunoreactive neurons in the cerebral cortex demonstrate *Met^{fx/fx};Dlx5/6^{Cre}* mice have a 28% reduction in the somatosensory region and a 31% reduction in the orbital frontal area. Scale bar in B, D = 100 μ m; * P < 0.05.

Fig. 5. PV⁺ and SST⁺ interneurons are unchanged in the amygdala. PV⁺ cells have similar patterns in control (A) and *Met^{fx/fx}:Dlx5/6^{Cre}* (B) mice. SST⁺ cells exhibited similar distributions in control (C) and *Met^{fx/fx}:Dlx5/6^{Cre}* mice (D). Scale bar = 100 μ m.

Fig. 6.

Abnormal interneuronal anatomy does not alter overall mouse behavior and anxiety, but impairs decision making. (A) Open field activity in control (black bar) and *Met^{fx/fx}:Dlx5/6^{Cre}* mice (white bar) indicates that both groups of mice travel the same distance the arena during the 10 min session. (B) In the Light/Dark test for anxiety, *Met^{fx/fx}:Dlx5/6^{Cre}* mice (white bar) spend an equal amount of time in the dark of the arena as compared to control mice (black bar). (C) Similarly, in Elevated Plus Maze, control and *Met^{fx/fx}:Dlx5/6^{Cre}* mice spend comparable amounts of time on each of the areas: closed arm, open arm or the center. (D) In a reversal learning task, the *Met^{fx/fx}:Dlx5/6^{Cre}* required more trials to reach criterion. * $P < 0.05$

Fig. 7.

Altered striatal anatomy leads to delayed performance on the Morris water maze. (A) The graph shows the latency to locate the hidden platform during spatial learning training. Both control (black bars) and *Met^{fx/fx}:Dlx5/6^{Cre}* mice (white bars) learn to locate the hidden platform with the same success criterion; the *Met^{fx/fx}:Dlx5/6^{Cre}* mice require longer times on days 1 and 2, suggesting impaired learning. (B and C) Graphs show percent time spent in each quadrant. Probe trials on day 4 (B) indicate normal hippocampal function in the *Met^{fx/fx}:Dlx5/6^{Cre}* mice (white bars), as they successfully searched for the hidden platform (labeled as 'Platform' on graph) for a duration comparable to control mice (black bars). The dashed line represents the percent time in a quadrant if all quadrants were searched equally (25%). Spatial reversal learning (C) also appears to be normal in the *Met^{fx/fx}:Dlx5/6^{Cre}* mice (white bars) as they spend about the same time in the new target quadrant (Platform) as compared to control mice (black bars). (D) The cued learning task demonstrates abnormal striatal function in *Met^{fx/fx}:Dlx5/6^{Cre}* mice (white bars) which have longer latencies to locate the cued platform during days 2 and 3 of testing relative to control mice (black bars). **P* < 0.05.