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Developmental Connectivity and Molecular Phenotypes of Unique Cortical Projection Neurons that Express a Synapse-Associated Receptor Tyrosine Kinase

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Abstract

The complex circuitry and cell-type diversity of the cerebral cortex are required for its high-level functions. The mechanisms underlying the diversification of cortical neurons during prenatal development have received substantial attention, but understanding of neuronal heterogeneity is more limited during later periods of cortical circuit maturation. To address this knowledge gap, connectivity analysis and molecular phenotyping of cortical neuron subtypes that express the developing synapse-enriched MET receptor tyrosine kinase were performed. Experiments used a Met^{GFP} transgenic mouse line, combined with coexpression analysis of class-specific molecular markers and retrograde connectivity mapping. The results reveal that MET is expressed by a minor subset of subcerebral and a larger number of intratelencephalic projection neurons. Remarkably, MET is excluded from most layer 6 corticothalamic neurons. These findings are particularly relevant for understanding the maturation of discrete cortical circuits, given converging evidence that MET influences dendritic elaboration and glutamatergic synapse maturation. The data suggest that classically defined cortical projection classes can be further subdivided based on molecular characteristics that likely influence synaptic maturation and circuit wiring. Additionally, given that MET is classified as a high confidence autism risk gene, the data suggest that projection neuron subpopulations may be differentially vulnerable to disorder-associated genetic variation.

Key words: autism, connectivity, cortical neuron diversity, met receptor tyrosine kinase, synapse development

Introduction

The proper assembly of cortical circuits, and ultimately their enduring higher order functions, requires the appropriate progression of neurodevelopmental events that occurs across a diverse and dynamic cellular landscape. Much progress has

been made toward understanding the prenatal diversification of cortical neuronal subtypes (Greig et al. 2013; Lodato and Arlotta 2015), but current knowledge of cortical neuron diversity is rather limited during the period of circuit assembly and maturation. A growing list of genes has been implicated in

dendritic development and in the formation of synapses (de Wit and Ghosh 2016). Yet, the function of only a small number of these genes has been investigated in the context of the neuronal diversity of the cortex to determine whether their site of action on cortical circuit development is broad, or more targeted to individual cortical neuron subtypes (Fazzari et al. 2010; Harwell et al. 2012). Importantly, genes implicated in the development, maturation and function of synapses have been associated with neurodevelopmental disorders such as autism spectrum disorder (ASD) and schizophrenia (Voineagu et al. 2011; Zoghbi and Bear 2012; Horváth and Mirnics 2014; Schizophrenia Working Group of the Psychiatric Genomics 2014). A major goal, therefore, is to determine the contribution that individual synapse-enriched genes make to the development and function of specific cortical circuits, leading to a more advanced understanding of cortical circuit development and selective disorder-related vulnerability. One well-studied gene in this regard is the schizophrenia-associated receptor tyrosine kinase, *ErbB4*. Neuregulin signaling through *ErbB4* selectively regulates the development of excitatory synaptic input onto parvalbumin interneurons (Fazzari et al. 2010), and thereby dictates the timing of critical period plasticity (Gu et al. 2016; Sun et al. 2016), and contributes to schizophrenia-related behavioral phenotypes (Del Pino et al. 2013).

Human genetic evidence indicates that developing excitatory projection neurons are a primary site of convergence for ASD risk gene expression (Parikshak et al. 2013; Willsey et al. 2013; Caubit et al. 2016). The pleiotropic MET receptor tyrosine kinase is relevant in this regard, as MET is a high confidence autism risk gene (Eagleson et al. 2017). Activation of MET by its only known ligand, hepatocyte growth factor (HGF), regulates pyramidal neuron dendritic elaboration, spine morphology, and glutamatergic synapse maturation in the neocortex and hippocampus (Judson et al. 2010; Qiu et al. 2014; Eagleson et al. 2016; Peng et al. 2016). There is basic knowledge of the regional and laminar neocortical expression patterns of MET, consisting of expression in subsets of excitatory neurons and exclusion from inhibitory interneurons (Judson et al. 2009; Eagleson et al. 2011). However, fundamental understanding of circuit maturation and vulnerability related to MET function requires a more precise definition of MET expression in relation to the remarkable diversity of excitatory cortical neuron subtypes (Harris and Shepherd 2015; Sorensen et al. 2015; Tasic et al. 2016).

Here, a new transgenic reporter mouse that expresses GFP under the control of part of the *Met* genomic locus was used to map the developmental expression of MET onto anatomically and molecularly defined classes of cortical projection neurons. There is highly selective expression of the receptor among glutamatergic projection neuron classes. Moreover, the characterization reveals previously unknown heterogeneity among the large intratelencephalic (IT) and subcerebral classes. The data reflect diversity in the molecular mechanisms governing the maturation of projection neuron subtypes, which is likely to confer differential disorder vulnerability across developing cortical circuits.

Materials and Methods

Animals

The *Met*-EGFP BAC transgenic mouse line was rederived from the *Met*-EGFP Bacterial Artificial Chromosome (BX139) obtained from the GENSAT repository. The mouse line was rederived on a FVB background. Founder mice were backcrossed with C57Bl/

6J mice for 2 generations. Male mice homozygous for the *Met*-EGFP transgene were generated and crossed with female C57Bl/6J mice to produce heterozygous *Met*^{GFP} pups used for all experiments. All animal procedures used in this study were in accordance with the guidelines of the Institutional Animal Care and Use Committee at Children's Hospital Los Angeles. Mice were housed on a 13:11 h light-dark cycle and were provided with food and water ad libitum.

Retrograde Tracing

Met^{GFP} reporter mice (age 12 days postnatal) were anesthetized with vaporized isoflurane (5% induction, 1.5–2% maintenance) and stabilized in a Narishige SG-4N small animal head holder. The respiratory rate was continuously monitored to assess anesthesia depth, and mice were maintained at 37 °C for the duration of the surgical procedure through a TCAT-2 temperature controller (Physitemp Instruments Inc.). Through stereotaxic guidance, a picospritzer connected to a pulled glass pipette (28 µm tip diameter) was used to inject 50–100 nL of cholera toxin subunit B (CTB), Alexa Fluor Conjugate (Invitrogen) into the desired cortical or subcortical target (see Supplementary Table S1 and Figure S3). The needle was left in place for 5 min following injection, and then retracted slowly to minimize contamination of unintended brain regions along the needle tract. To minimize discomfort, mice were given a subcutaneous injection of the non-steroidal anti-inflammatory drug ketoprofen (5 mg/kg) immediately before the surgery, and provided with ibuprofen (0.2 mg/mL) in the drinking water. After 2 days of recovery, on postnatal day 14, mice were transcardially perfused with 4% paraformaldehyde dissolved in phosphate buffered saline (PBS) and tissue was processed for immunohistochemical analysis as described below.

In Situ Hybridization

Fresh frozen brain tissues were prepared by submerging rapidly dissected brains in ice cold isopentane following isoflurane (P7 and P14) or hypothermia (P0) induced deep anesthesia. Brains were stored at –80 °C until sectioning. Twenty micrometer cryosections were prepared at –11 °C for multiplexed fluorescent in situ hybridization with RNAscope probes as described (Wu et al. 2016; Kast et al. 2017). For DIG in situ hybridization, cryosections were prepared at 25 µm. Sections were stored at –80 °C until in situ hybridization was performed as described (Wu et al. 2016).

Immunohistochemistry

Early postnatal (postnatal day 0) mouse brains were dissected in room temperature PBS, transferred to 4% paraformaldehyde dissolved in PBS (pH 7.4), and incubated at 4 °C for 12–18 h. Mice aged 7 days postnatal or older were perfused transcardially with 4% paraformaldehyde dissolved in PBS. Following perfusion, brains were immediately removed, transferred to 4% paraformaldehyde, and incubated at 4 °C for 12–18 h. Following overnight fixation, brains were incubated sequentially in 10%, 20%, and 30% sucrose dissolved in PBS for 12–24 h each. Next, brains were embedded in Clear Frozen Section Compound (VWR International) and placed on a weigh boat floating in liquid nitrogen. Once frozen, embedded brains were stored at –80 °C until cryosectioning. Twenty micrometer coronal cryosections were collected at –20 °C and then stored at –80 °C until immunocytochemical analysis. For immunostaining, sections

were warmed at room temperature for 10 min, dried in a hybridization oven at 55°C for 15 min, and then incubated in PBS for 10 min. Blocking and permeabilization were done by incubating sections in PBS containing 5% normal donkey serum and 0.3% Triton X-100 for 1 h at room temperature. Sections were incubated subsequently in primary antibodies diluted in 0.1% Triton X-100 in PBS overnight at room temperature. Sections were washed 5 times for 5 min each with 0.2% Tween 20 in PBS. Sections were incubated in Alexa Fluor conjugated secondary antibodies (1:500) diluted in 0.1% Triton X-100 in PBS for 1 h at room temperature. Sections were washed 3 times for 5 min each with 0.2% Tween 20 in PBS. Sections were then incubated in 950 nM DAPI in PBS for 8 min, and then subjected to 2 additional 5 min PBS washes. Sections were mounted in Prolong Gold antifade reagent (Life Technologies), and the mounting medium cured for at least 24 h before collecting confocal microscopy images. Primary antibodies used in this study were as follows: guinea-pig anti-ppCCK (1:500; T. Kaneko lab), rabbit anti-PCP4 (1:3000; J. Morgan lab), mouse monoclonal anti-Satb2 (1:500; Abcam #ab51502), rat anti-Ctip2 (1:500; Abcam #ab18465), chicken anti-GFP (1:500; Abcam #ab13970), and goat anti-Foxp2 (1:100; Santa Cruz #sc-21069).

Colocalization Analyses

All images used for cell counting and colocalization analyses were collected through a 20x/0.8NA Plan-APOCHROMAT objective lens mounted on a Zeiss LSM 700 confocal microscope with refractive index correction. The barrel field of primary somatosensory cortex, and specific layers thereof, was identified through the DAPI signal. Optical sections were collected at 1 AU and 2 μ m z-steps through the entire thickness of each 20 μ m section. For each animal, the percentage of colocalized cells was calculated from 3 sections spaced a minimum of 200 μ m apart, which corresponded to the anterior, middle, and caudal aspects of the posteromedial barrel subfield of the primary somatosensory cortex.

IMARIS (Bitplane) image analysis software was used to analyze colocalization of immunocytochemical signals in a 3D rendering of each confocal z-stack. For markers that displayed a continuous and smooth immunocytochemical signal across the entire nucleus or cell soma, the “spots” tool in IMARIS was used to identify “positive” cells in a semiautomated fashion. The average diameter of each signal was manually input into the spots tool to aid in identifying “positive” objects. A K-means clustering algorithm ($K = 3$) was applied to the quality score of the spots with local background subtraction. The 2 clusters with the highest quality scores were deemed positive for the signal of interest. After identification of the cells positive for each signal, the positive cells were further filtered to exclude cells that were partially outside of the tissue section and thus did not contain an intact nucleus. This was accomplished by eliminating cells that fell below threshold for the DAPI signal.

The fluorescent markers (CTB, ppCCK, and PCP4) that did not satisfy the criterion of a smooth and continuous signal required for accurate identification using the automated IMARIS “Spots” tool were manually identified and assigned a “Spot” with a 10 μ m diameter centered on the DAPI positive nucleus associated with the signal.

To identify colocalization of fluorescent signals, the “co-localize spots” tool was used in IMARIS. The “distance threshold” (maximum distance between the center of each spherical “spot”) was set to the radius of the largest spot in each pair of colocalized signals.

Results

The spatial and temporal expression patterns of *Met* transcript and protein in the developing mouse forebrain were mapped previously through a combination of in situ hybridization, immunocytochemistry, and western blot analysis (Judson et al. 2009). This initial characterization revealed a dynamic temporal expression pattern in the neocortex that began late prenatally, peaked at postnatal day 7, and progressively declined to reach low levels by postnatal day 35. Here we employed a *Met*-EGFP reporter mouse (*Met*^{GFP} from here on) that we rederived using a bacterial artificial chromosome (BAC) in the GENSAT collection (Gong et al. 2003).

To assess the fidelity of GFP expression in the cortex of *Met*^{GFP} reporter mice, GFP protein and *Met* transcript were mapped in cryosections of *Met*^{GFP} and wild-type mouse brains, respectively. The laminar distribution of GFP and *Met* appeared nearly identical at all ages and within all cortical regions examined (Fig. 1A,B). GFP expression was observed in many radially migrating neurons at P0, before the superficial layers were fully distinguishable (Figs 1A and 2A). Notably, at P7 and P14, once migration was complete and all 6 layers were readily discerned, the highest density of *Met* and GFP-expressing neurons occurred in the supragranular layers of cortex, with sparser labeling of neurons located in infragranular layers. Additionally, there was a marked absence of GFP and *Met* expression in layer 4 from postnatal day 7 onward (Figs 1 and 2). Within the infragranular layers, there was a greater density of GFP and *Met* positive cells in layer 6 compared with layer 5 (Figs 1 and 2B,C). There also were GFP-expressing neurons positioned immediately above the white matter in the subplate at P0 and P7, but this GFP-positive population became sparser by P14 (Fig. 2A–C, see Supplementary Fig. S2). To further ensure that GFP expression in the *Met*^{GFP} reporter mouse provides a high-fidelity reporter of *Met* expression at the cellular level in the cortex, multiplexed fluorescent in situ hybridization was performed to compare *Met* and GFP transcript expression in the P14 *Met*^{GFP} cortex. There was near perfect coexpression of the two transcripts by individual neurons within each layer of the primary somatosensory cortex (Fig. 1C). The faithfulness of GFP labeling in cortex representing *Met*-expressing neurons is consistent with the analyses of *Met*⁺ neurons located in other brain regions, including the caudal subnucleus of the dorsal raphe (Kast et al. 2017) and the dorsal motor vagal nucleus (Kamitakahara et al. 2017).

Having validated that GFP expression in this transgenic line provides a reliable proxy for *Met* expression, immunocytochemistry was next employed to characterize the molecular profile of GFP-expressing neurons across postnatal cortical development. The analysis focused on primary somatosensory cortex at P0, P7, and P14. At these ages, most GFP⁺ neurons in each infragranular layer coexpressed the IT neuron-enriched transcription factor *Satb2* (see Supplementary Fig. S1). Additionally, a minor subset of GFP⁺ neurons in the infragranular layers that coexpressed the corticofugal neuron marker *Ctip2* at P7 and P14, but not at P0 (see Supplementary Fig. S1). Interestingly, most of the GFP and *Ctip2* double-positive neurons coexpressed *Satb2* (see Supplementary Fig. S1C). Together, these findings suggest that most *Met*-expressing neurons in each layer belong to the IT neuron class, but that some corticofugal neurons also express *Met*. To assess colocalization in the context of the total *Satb2*-expressing and total *Ctip2*-expressing neuron populations in layers 5 and 6, quantitative analyses determined the proportion of the total *Satb2*⁺ and *Ctip2*⁺ populations that expressed GFP in each layer (Fig. 2). Less than half of each

Figure 1. The Met^{GFP} BAC transgenic mouse is a high-fidelity reporter of endogenous *Met* expression in the developing cortex. (A) In situ hybridization reveals endogenous *Met* transcript pattern in coronal sections of P0, P7, and P14 wild-type mouse forebrain. (B) GFP immunocytochemistry demonstrates the unique expression pattern of the GFP reporter in coronal sections of P0, P7, and P14 Met^{GFP} mouse forebrain. Note the labeling of callosal, commissural, and corticofugal axons by GFP, reminiscent of endogenous MET protein transport during development. (C) Multiplexed fluorescence in situ hybridization shows colocalization of GFP and *Met* transcripts in the posteromedial barrel field of the somatosensory cortex at P14. Yellow circles denote cells that coexpress *Met* and GFP transcripts. Scale bar = 50 μ m.

population in layer 5 and layer 6 expressed GFP at each age (Fig. 2D,E). More $Satb2^{+}$ than $Ctip2^{+}$ neurons appeared to express GFP at each age. Notably, none of the $Ctip2^{+}$ neurons in layer 5 expressed GFP at P0, but limited $Ctip2$ and GFP colocalization was observed at P7 and P14 (Fig. 2D). Additionally, in layer 6, there was a transient increase in the proportion of $Satb2^{+}$ neurons that expressed GFP from P0 ($13.0 \pm 2.7\%$) to P7 ($35.33\% \pm 2.2\%$), which decreased by P14 ($17.8\% \pm 1.9\%$; Fig. 2E).

Several recent studies have reported that *Satb2* expression can be detected in a subset of corticothalamic and subcerebral neurons, albeit at lower levels than callosal projections neurons (McKenna et al. 2015; Molyneaux et al. 2015; Harb et al. 2016). Thus, to complement and extend the initial molecular profiling of GFP neurons with a more direct measure of connectivity, the expression of GFP was mapped onto specific projection neuron types by retrograde neuroanatomical tracing. Given the higher density of GFP-expressing neurons in layer 6 compared with layer 5 (Fig. 2), initial tracing experiments focused on individual layer 6 projection neuron subtypes.

Layer 6 of primary sensory cortex is predominantly comprised of 2 projection neuron classes: corticothalamic and corticocortical neurons (Thomson 2010; Petrof et al. 2012). CTB was injected into the ventral posteromedial nucleus of the thalamus or the primary motor cortex of Met^{GFP} mice on postnatal day 12 to label corticothalamic or corticocortical neurons, respectively (Fig. 3A,B). Colocalization of GFP and CTB was quantitated on postnatal day 14. Within layer 6 of primary somatosensory cortex approximately 15.6% ($\pm 2.3\%$) of motor cortex-projecting corticocortical neurons expressed GFP (Fig. 3C). Moreover, if the analysis was restricted to retrogradely

labeled neurons in layer 6A, the percentage of corticocortical neurons that were GFP $^{+}$ increased to 26.1% ($\pm 2.6\%$). In striking contrast, approximately 1.3% ($\pm 0.6\%$) of the layer 6 corticothalamic neurons labeled by injection of CTB into the ventrobasal thalamus were GFP $^{+}$ (Fig. 3C).

To complement the retrograde tracing analysis, coimmunocytochemistry with antibodies against GFP, PCP4, and the pre-processed form of CCK (ppCCK) was performed. In the adult rat, PCP4 and CCK label separate populations of layer 6 cell types that correspond to corticothalamic and corticocortical neurons, respectively (Watakabe et al. 2012). In layer 6 of the mouse somatosensory cortex at postnatal day 14, strong and largely non-overlapping expression of ppCCK and PCP4 was observed throughout Layer 6A, with some coexpression in layer 6B/subplate, as reported in the adult rat. In the mouse, there was minimal expression of either molecule in layer 6 at P0 or P7 (data not shown). Approximately 36.3% ($\pm 4.4\%$) of ppCCK-positive neurons coexpressed GFP, whereas only 4% ($\pm 0.7\%$) of PCP4-positive neurons coexpressed GFP at P14 (Fig. 3E). When examining colocalization as a fraction of the total layer 6 GFP population, 74.4% ($\pm 1.1\%$) of layer 6 GFP neurons coexpressed ppCCK. In comparison, only 11.2% (1.2%) of GFP-positive layer 6 neurons coexpressed PCP4 (Fig. 3F). These data are consistent with the connectivity phenotyping, leading to the conclusion that GFP is expressed primarily in layer 6 corticocortical neurons.

Given the Met^{+} IT population enrichment evident at P14, it was important to determine whether the expression of *Met* in layer 6 was restricted to corticocortical neurons throughout development, or whether expression included layer 6 corticothalamic neurons early in development, but was refined over

Figure 2. Met co-expression with transcriptional regulators of cortical neuron subtype specification. (A) GFP immunocytochemistry at P0 shows laminar distribution of GFP-expressing cells (green). Asterisks denote GFP-expressing neurons in the subplate at this age. (A') High magnification of layer 5 inset shows colocalization of GFP with Satb2 (cyan, white arrowheads), but not Ctip2 (magenta) at P0. (A'') High magnification of layer 6 inset reveals patterns of colocalization of GFP with Satb2 and Ctip2 at P0. (B) GFP immunocytochemistry shows laminar distribution of GFP-expressing cells at P7. Asterisks denote GFP-expressing neurons in the subplate at this age. (B') High magnification of layer 5 inset illustrates colocalization of GFP with Satb2 and Ctip2 (white arrows) at P7. (B'') High magnification of layer 6 inset illustrates colocalization of GFP with Satb2 and Ctip2 at P7. (C) GFP immunocytochemistry at P14 shows laminar distribution of GFP-expressing cells. Note the sparse distribution of GFP-expressing neurons in the subplate. (C') High magnification of layer 5 inset illustrates colocalization of GFP with Satb2 and Ctip2 at P14. (C'') High magnification of layer 6 inset illustrates colocalization of GFP with Satb2 and Ctip2 at P14. (D) Quantification of the percentage of layer 5 Satb2+ or Ctip2+ neurons that coexpressed GFP at P0, P7, and P14. (E) Quantification of the layer 6 Satb2+ or Ctip2+ neurons that coexpressed GFP at P0, P7, and P14. Error bars represent SEM. Scale bars: 100 μ m A, B, and C; 20 μ m A', A'', B', B'', C', and C''.

time. This was an open question because of the greater density of GFP neuron labeling in layer 6 at P7 than P14 (Fig. 2E), which could reflect expression in corticothalamic neurons at earlier developmental stages. Given that robust ppCCK and PCP4 expression could not be detected earlier than P14, a different

labeling strategy was employed to distinguish corticocortical and corticothalamic neurons during early postnatal development. A cre-dependent tdTomato reporter line, Ai14, was crossed to the Ntsr1-cre driver mouse line, which selectively and comprehensively labels layer 6 corticothalamic neurons

Figure 3. Met expression in layer 6 is enriched in corticocortical neurons throughout development. (A) Retrograde tracing of layer 6 corticothalamic neurons by injection of CTB into the VPM thalamus, combined with GFP (green) immunocytochemistry. Boxed area is shown as higher magnification images (2-channel and 1-channel images, respectively), and reveals that GFP expression (white arrows) is excluded from CTB-labeled (magenta) corticothalamic neurons. (B) Retrograde labeling of layer 6 ipsilateral corticocortical neurons by CTB injection into primary motor cortex. White asterisk denotes CTB-labeled, GFP⁻ corticocortical neuron in layer 6B. White box is shown at higher magnification in 2- and 1-channel images. Note colocalization (white arrowheads) of CTB and GFP in primary somatosensory cortex. (C) Quantification of the percentages of retrogradely traced layer 6 corticothalamic and corticocortical neurons in primary somatosensory cortex that express GFP at P14. (D) GFP, ppCCK, and PCP4 immunocytochemistry at P14 shows colocalization of GFP and ppCCK, but not GFP and PCP4 in layer 6 of the primary somatosensory cortex. (E) Quantification of the percentages of layer 6 ppCCK and PCP4 expressing layer 6 neurons that coexpress GFP. (F) Quantification of the percentage of layer 6 GFP neurons that coexpress PCP4 or ppCCK. (G) Ntsr1-cre; tdTomato (magenta) and GFP (green) immunocytochemistry reveal minimal colocalization of GFP and tdTomato-labeled corticothalamic neurons at P0, P7, and P14. (H) Quantification of the percentage of layer 6 GFP neurons that express Ntsr1-cre driven tdTomato. (I) Quantification of the percentage of Ntsr1-cre; tdTomato expressing neurons that coexpress GFP. Error bars represent SEM. Scale bars: 50 μ m A, B low-magnification images; 20 μ m A, B high-magnification images, D, and G.

with tdTomato (Bortone et al. 2014; Kim et al. 2014; Oh et al. 2014). Next, this corticothalamic reporter line was crossed with the Met^{GFP} reporter mouse and colocalization of tdTomato and GFP was determined at P0, 7, and 14. Less than 6% overlap between the two reporters was observed at each time point (Fig. 3G–I), suggesting that Met expression in somatosensory cortex is excluded from most layer 6 corticothalamic neurons throughout postnatal development. This cell-type selectivity appears to occur in at least some non-sensory regions of the cortex, as GFP is excluded from nearly all molecularly defined or retrogradely labeled corticothalamic neurons of the anterior cingulate and retrosplenial cortices at P0, P7, and P14 (see Supplementary Fig. S4).

Of the sparse tdTomato and GFP double-positive cells, many were positioned at the very bottom of layer 6, corresponding to layer 6B. Colocalization of GFP and tdTomato was thus quantified in 50 μ m bins extending radially from the white matter (see Supplementary Fig. S2). Greater colocalization was observed in the bins 50 μ m and 100 μ m from the white matter. Though still debated, neurons in this deepest compartment, layer 6B, are believed to be the surviving fraction of the developmentally transient subplate (Marx et al. 2015). A considerable fraction of these subplate/layer 6B neurons were GFP⁺ at P0 and P7, but few were labeled at P14 (Fig. 2, see Supplementary Fig. S2), consistent with previous reports of Met transcript expression in subplate neurons during early postnatal development (Judson et al. 2009; Eagleson et al. 2011).

Having demonstrated that Met expression is restricted to IT neurons in layer 6A, we wondered whether expression of Met is restricted to this projection class in more superficial layers. The pyramidal neurons in layer 5 can be categorized into two broad projection classes: IT and pyramidal tract (PT) neurons (Harris and Shepherd 2015). To differentially label layer 5 IT and PT neurons in primary somatosensory cortex, CTB was injected into the primary motor cortex and cerebral peduncle of P12 Met^{GFP} mice, respectively (Fig. 4A,B). GFP immunocytochemistry was then performed on cryosections prepared at P14 and colocalization of GFP and CTB was quantified in retrogradely labeled layer 5 IT and PT neurons. Approximately 31.3% ($\pm$ 4.6%) of the layer 5 IT neurons expressed GFP, whereas 22.4% ($\pm$ 2.1%) of the PT neurons expressed GFP (Fig. 4C). Notably, the percentages of IT and PT neurons that express Met at P14, determined here by retrograde tracing, are consistent with the fraction of Satb2 and Ctip2-expressing neurons that coexpressed GFP at P14 (Fig. 2D,E). Thus, in contrast to the class-specific expression pattern observed in layer 6, Met expression in layer 5 extends across projection classes to include both IT and PT neurons. Colocalization of GFP and CTB in layer 5 corticothalamic neurons, which represent a subset of PT neurons (Bourassa et al. 1994), was also quantified following injection of CTB into the ventrobasal thalamus. A similar fraction ($25.8 \pm 5.9\%$) of layer 5 corticothalamic neurons were GFP⁺ (Fig. 4C,D). It is important to note that the labeling of layer 5 corticothalamic neurons is likely due to uptake by fibers passing through Vpm in route to POm, as layer 5 corticothalamic neurons do not typically terminate in Vpm thalamus (Bourassa et al. 1994; Deschenes et al. 1994; Frangeul et al. 2016; Grant et al. 2016).

All projection neurons in the supragranular layers can be classified as IT neurons. Some of these neurons project axons ipsilaterally to form association projections, others project contralaterally to form commissural projections, and only a minor subset of neurons in layers 2/3 maintain dual projections to both ipsilateral and contralateral cerebral hemispheres (Mitchell and Macklis 2005). To measure the percentage of

ipsilateral and contralateral projection neurons in layers 2/3 that express Met, CTB was injected into the ipsilateral motor cortex or the contralateral somatosensory cortex of Met^{GFP} mice on postnatal day 12 and colocalization of CTB and GFP within layer 2/3 of primary somatosensory cortex was quantified at P14. Approximately 40.3% ($\pm$ 3.5%) of the layer 2/3 neurons projecting to the ipsilateral primary motor cortex expressed GFP, whereas 46.1% ($\pm$ 4.5%) of the layer 2/3 contralateral projection neurons expressed GFP (Fig. 5A–C). As in infragranular layers, the data are consistent with subsets of MET-expressing supragranular subclasses.

Discussion

The present study of the molecular and connectivity phenotypes of GFP-expressing cortical neurons in the Met^{GFP} reporter mouse reveals a unique categorization of neuronal subtypes that may underlie mechanistic differences in the maturation of discrete cortical circuit components. Specific subpopulations of glutamatergic cortical neurons, including most layer 4 neurons and layer 6 corticothalamic neurons, do not express Met during development. Moreover, even within the projection subclasses that contained higher percentages of Met-expressing neurons, many neurons did not express Met. The differential expression of Met therefore reflects diversity across and within traditional cortical projection neuron classes during postnatal circuit wiring (Figure 6).

The use of the Met^{GFP} reporter mouse enabled efficient quantification of the expression of Met across defined cortical projection populations. This would not be possible by localization of endogenous receptor protein, which is concentrated in the plasma membrane. The use of high-fidelity transgenic reporter mice has similarly enabled the profiling of cortical neuron subtypes that express other membrane bound and secreted proteins (Harwell et al. 2012; Wu et al. 2016).

MET Is Enriched in Early Postnatal Layer 6 Corticocortical Neurons

Met is expressed by a relatively large fraction of layer 6 neurons in the first two weeks after birth, but exhibits remarkably limited colocalization with layer 6 corticothalamic markers. Many molecules have been described as markers of layer 6 corticothalamic neurons (Watakabe et al. 2012; Bortone et al. 2014; Molyneaux et al. 2015; Sorensen et al. 2015; Galazo et al. 2016). By comparison, there are few genes with selective expression in layer 6 corticocortical neurons (Bai et al. 2004; Watakabe et al. 2012; Sorensen et al. 2015) despite the abundance of this layer 6 neuron subtype (Zhang and Deschênes 1997; Petrof et al. 2012). To our knowledge, Met is the first gene that is segregated to a subtype of layer 6 corticocortical neurons in the first postnatal week of life. In the adult rat, ppCCK and PCP4 were found to label layer 6 corticocortical and corticothalamic neurons, respectively (Watakabe et al. 2012). However, in the developing mouse, neither of these genes is expressed at detectable levels in layer 6A of somatosensory cortex until after the first postnatal week. In this regard, three key findings reported here support the conclusion that Met expression is the earliest developmental marker of layer 6 corticocortical neurons reported to date. First, there was greater colocalization of GFP with ppCCK than PCP4 at P14. Second, GFP neurons were retrogradely labeled from injections placed in distant cortical locations, but not in the thalamus. Third, despite the presence of substantial numbers of GFP-expressing neurons in

Figure 4. *Met* is expressed in subsets of layer 5 intratelencephalic and PT neurons. (A) Retrograde tracing of PT projection neurons by injection of CTB (magenta) into the rostral cerebral peduncle combined with GFP immunocytochemistry (green). White box is shown at higher magnification in 2- and 1-channel images, revealing that a subset of PT neurons express GFP (arrowheads). (B) Retrograde tracing of intratelencephalic neurons by injection of CTB (magenta) into ipsilateral motor cortex combined with GFP immunocytochemistry (green). White box is shown at higher magnification in 2- and 1-channel images, revealing that a subset of layer 5 ipsilateral corticocortical neurons express GFP (green). (C) Retrograde tracing of layer 5 corticothalamic neurons by injection of CTB (magenta) into the VPM thalamus combined with GFP immunocytochemistry (green). White box is shown at higher magnification in 2- and 1-channel images, revealing colocalization in layer 5 of primary somatosensory cortex (arrowheads). (D) Quantification of the percentages of PT and intratelencephalic neurons in primary somatosensory cortex that express GFP. Error bars represent SEM. Scale bars: 50 μ m low-magnification images in A, B, and C; 20 μ m high-magnification images in A, B, and C.

layer 6 at P0 and P7, these neurons do not coexpress markers of corticothalamic neurons. The observation that only a subset of the layer 6 corticocortical neurons express *Met* at P14 (Fig. 3C,E) may relate to diversity within this neuron class that has been observed at the transcriptome level (Tasic et al. 2016). *Met*

expression might be more ubiquitous across layer 6 corticocortical neurons at P7, but *Met* expression at P14 is more limited. This heterogeneity may be functionally relevant, possibly contributing to a novel mechanism of differential synaptic maturation of *MET*⁺ neurons within this class (see below).

Figure 5. *Met* is expressed in subsets of ipsilateral and contralateral layer 2/3 projection neurons. (A) Retrograde tracing of callosal projection neurons by injection of CTB (magenta) into the contralateral somatosensory cortex combined with GFP immunocytochemistry (green). White box is shown at higher magnification in 2- and 1-channel images, revealing that a subset of layer 2/3 contralateral projection neurons express GFP (arrowheads). (B) Retrograde tracing of ipsilateral projection neurons by injection of CTB (magenta) into the ipsilateral motor cortex combined with GFP immunocytochemistry (green). White box is shown at higher magnification in 2- and 1-channel images, revealing that a subset of layer 2/3 contralateral projection neurons express GFP (arrowheads). (C) Quantification of the percentages of contralateral and ipsilateral projection neurons that express GFP. Error bars represent SEM. Scale bars: 50 μ m low-magnification images in A and B; 20 μ m high-magnification images in A and B.

Layer 6 corticocortical neurons are characterized by larger and more complex dendritic and local axonal arbors than corticothalamic neurons in the same layer (Zhang and Deschènes 1997; Velez-Fort et al. 2014). As HGF-stimulated activation of MET causes an increase in dendritic and axonal outgrowth in cortical and hippocampal neurons (Judson et al. 2010; Qiu et al. 2014; Eagleson et al. 2016; Peng et al. 2016), it is possible that the morphological differences between layer 6 corticocortical and corticothalamic neurons depend, at least in part, upon differential MET signaling. Direct dendritic arbor measures of intracellularly filled, retrogradely identified layer 6 corticocortical neurons in conditionally deleted *Met*^{-/-} mice could address this hypothesis.

MET Is Enriched in “Driver-Like” Corticothalamic Neurons

The finding that, in multiple cortical regions analyzed, most layer 6A corticothalamic neurons do not express *Met* was surprising, given our previous report that MET-immunoreactivity in the thalamic neuropil is eliminated following *Emx1*^{cre}-mediated deletion of *Met* from cortical structures (Judson et al. 2009). While the possibility remains that, in cortical regions not examined here, layer 6A corticothalamic neurons express *Met*, two features of the expression patterns observed in the current study inform a new understanding of *Met* expression by a specific subset of corticothalamic neurons. First, *Met* expression was abundant in subplate neurons in the first postnatal week (Fig. 2), a period during which many thalamus-projecting neurons are present in the subplate (Grant et al. 2012). Second, we identified a subset of layer 5 corticothalamic neurons that express *Met* (Fig. 4C).

Under normal conditions, both layer 5 and layer 6B corticothalamic populations form prominent terminal projections in higher order thalamic nuclei, including the posterior medial and lateral posterior nuclei, but provide limited input to first-order thalamus (Bourassa et al. 1994; Deschenes et al. 1994; Frangeul et al. 2016; Grant et al. 2016; Roth et al. 2016). In contrast, layer 6A corticothalamic neurons provide “modulatory” input to first- and second-order thalamic nuclei (Sherman and Guillery 2002; Sherman 2016). The segregation of corticothalamic neurons that project to hierarchically distinct thalamic nuclei and the unique “driver-like” synapses formed between layer 5 corticothalamic neurons and relay neurons in higher order thalamus are thought to be critical for proper corticocortical communication (Theyel et al. 2010; Sherman 2016). In this context, *Met* expression seems enriched in the subsets of corticothalamic neurons that function as drivers, rather than modulators, of cortico-thalamocortical communication. Interestingly, in the primate, MET protein is abundant in the inferior and lateral subdivisions of the pulvinar, a higher order thalamic nucleus, whereas it is absent from the dorsal lateral geniculate and other first-order thalamic nuclei (Judson et al. 2011). This pattern may reflect conservation of MET expression among specific corticothalamic subpopulations, with expression largely restricted to those neurons that project to second-order thalamus, which are positioned outside of layer 6A (Sherman 2016).

Heterogeneity of Intratelencephalic and PT Neurons

Within layer 5, subsets of corticofugal and intratelencephalic neurons express *Met*, which differs from the intratelencephalic-specific expression observed in layer 6. Despite this laminar

Figure 6. Summary of *Met* expression across cortical projection neuron classes. (A) Line drawing modified with permission from Franklin and Paxinos (2008) mouse stereotaxic atlas illustrating the projection populations analyzed by retrograde tracing and molecular characterization. (B) Quantitative summary of the percentage of each layer- and projection-defined neuron population observed to be *Met*-expressing.

difference, the observation that *Met*-positive neurons account for less than half of any projection population was consistent across layers and projection types. This indicates that within individual laminar- and projection-defined subclasses, there is a molecularly distinct profile that may relate to distinct functions that have not been reported to date. It will be important to decipher whether additional anatomical and molecular features distinguish the *MET*⁺ from the *MET*[−] neurons within each of the projection subtypes. One possibility relates to heterogeneous *Met*-expression among layer 5 PT neuron populations, which can be further subcategorized depending on whether they extend axons into the spinal cord (corticospinal neurons) or terminate in brainstem motor nuclei (corticobulbar neurons). Recently, Harb et al. (2016) reported that these two subcerebral projection subtypes can be partially distinguished based on their propensity for expression of *Satb2*. In their study, approximately 30% of PT projection neurons labeled by injection of retrograde tracer into the cerebral peduncle (which labels corticobulbar and corticospinal populations) coexpressed *Satb2* and *Ctip2*, whereas none of the neurons labeled by tracer injection at the level of the cervical spinal cord (corticospinal neurons) expressed *Satb2*. Together, their findings suggest that *Satb2* expression is enriched in corticobulbar compared with corticospinal neurons. Although the present

retrograde tracing experiments did not distinguish corticobulbar from corticospinal neurons, approximately 70% of the GFP and *Ctip2* double-positive layer 5 neurons also coexpressed *Satb2* at P7 and P14 (see supplementary Fig. S1C). This is consistent with most *Met*-expressing layer 5 PT neurons being part of the corticobulbar cell class, which may explain why only 20% of the corticofugal neurons labeled by injection of CTB into the cerebral peduncle were GFP-expressing.

Implications for Synaptic Maturation

Converging lines of anatomical, biochemical, and electrophysiological evidence point to *MET* as a modulator of synapse development (Judson et al. 2010; Qiu et al. 2011, 2014; Eagleson et al. 2016, 2017; Xie et al. 2016a). Two recent reports suggest that the temporal dynamics of *MET* expression is critical for the proper timing of glutamatergic synapse maturation (Qiu et al. 2014; Peng et al. 2016). In these studies, *MET* expression was shown to maintain synapses in an immature state. Bidirectional manipulations of *MET* receptor expression levels were shown to produce opposing shifts in several measures of synapse maturity. Only one other protein, *SynGAP1*, has been shown to have similar effects on the timing of glutamatergic synaptic maturation (Clement et al. 2012;

Araki et al. 2015). Interestingly, MET and SynGAP1 interact in cortical synaptosomal protein complexes during the peak period of cortical synapse formation (Xie et al. 2016b). In the context of the data presented here, one attractive hypothesis is that differential MET expression among anatomically defined projection populations contributes to heterogeneity in the timing of synaptic maturation within individual cortical projection neuron classes. Specifically, MET⁺ neurons in each population might mature later than their MET⁻ counterparts. Thus, the differential expression of MET may confer an advantage by widening the developmental window over which otherwise homogenous populations of projection neurons integrate into emerging circuitry. A prolonged temporal window for synaptic integration could enable flexibility in the fine tuning and consolidation of specific cortical circuits, and it could serve to protect the emerging circuitry from time-delimited environmental insults.

Circuit Vulnerability in ASDs

Major advances are being made in the search for genetic variation that contributes to neurodevelopmental disorder risk. A critical next step toward achieving a fundamental understanding of disorder pathogenesis is identifying the developing neural circuits impacted by disorder risk genes. Recent efforts, based on coexpression network analysis of developing human brain transcriptome data, have converged to implicate glutamatergic projection neurons as a primary substrate for disorder-related genetic vulnerability in ASD (Parikshak et al. 2013; Willsey et al. 2013). Yet, despite this convergence, these two studies reached different conclusions about which cortical projection neuron classes are primarily vulnerable. This may be due to differences in network building algorithms and criteria for risk gene inclusion. Greater resolution will come from well-defined, and developmentally relevant molecular phenotyping of projection-defined subsets of neurons within each cortical layer.

The finding that there is nonuniform expression of the Met receptor across and within projection neuron classes has important implications for understanding disorder-related circuit vulnerability. MET is a category 2 (strong candidate) ASD risk gene according to SFARI gene. A functional single nucleotide polymorphism in the MET promoter decreases transcription and protein expression by 50%, confers a 2.25-fold increased risk for ASD (Campbell et al. 2006, 2007), and is enriched in children with more severe social communication deficits and with cooccurring gastrointestinal disturbances (Campbell et al. 2009, 2010). Interestingly, the risk allele is associated with decreased cortical thickness in typically developing humans (Hedrick et al. 2012), and is linked to altered structural and functional connectivity within specific cortical networks that express MET in the primate brain (Judson et al. 2011; Kang et al. 2011; Mukamel et al. 2011; Rudie et al. 2012). The current data support the notion that subclasses of cortical projection neurons are differentially vulnerable to the direct influence of genetic variants that disrupt MET signaling.

Supplementary Material

Supplementary data are available at *Cerebral Cortex* online.

Authors' Contributions

R.J.K. and P.L. conceived of the study, designed experiments, and wrote the paper. R.J.K. and H.H.W. performed experiments and analyzed data.

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