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The autism risk genes MET and PLAUR differentially impact cortical development

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Abstract

Over the past decade, candidate genes that increase risk for autism spectrum disorder (ASD) have been identified. However, little is known about how these findings of increased genetic risk translate into the disruptions in brain development that cause the behaviors that are characteristic of ASD. One idea has been that risk genes for ASD cause an imbalance in excitation and inhibition in circuits of the cerebral cortex, the structure that controls complex functions. Imbalances would lead to problems in information flow and, in extreme cases, cause seizure disorder, a common medical condition in children with ASD. Our laboratory has identified changes in the DNA sequence of two genes, *MET* and *PLAUR*, that are associated with increased risk for ASD. Our previous work suggested that both of these genes were involved in controlling inhibitory neuron development. Through the use of genetic mouse models and biochemical and molecular analysis, we show that *Met* can only be involved directly in the development and function of excitatory neurons. In contrast, *PLAUR* modulates the maturation of inhibitory neurons, which act as a brake on projection neuron function. We also find, however, that under certain environmental conditions, *Met* can be activated in inhibitory neurons and therefore could influence the development of this cell type under certain pathological conditions during pregnancy. Our data support a popular hypothesis that a disruption in the balance of function between excitatory projection neurons and inhibitory interneurons underlies, in part, altered cortical architecture in ASD.

Candidate risk genes for autism spectrum disorder (ASD) have been identified, but the challenge of determining their contribution to pathogenesis remains. We previously identified two ASD risk genes encoding the receptor tyrosine kinase *MET* and the urokinase plasminogen activator receptor (*PLAUR*), which is thought to modulate availability of the *MET* ligand. We also reported a role for *Met* signaling in cortical interneuron development *in vitro* and a reduction of these neurons in *uPAR* (mouse ortholog of *PLAUR*) null mice, suggesting that disruption of either gene impacts cortical development similarly. Here, we modify this conclusion, reporting that interneuron numbers are unchanged in the neocortex of *Met^{flx/flx}/Dlx5/6^{cre}* mice, in which *Met* is ablated from cells arising from the ventral telencephalon (VTel). Consistent with this, *Met* transcript is not detected in the VTel during interneuron genesis and migration; furthermore, during the postnatal period of interneuron maturation, *Met* is co-expressed in glutamatergic projection neurons, but not interneurons. Low levels of *Met* protein are expressed in the VTel at E12.5 and E14.5, likely reflecting the arrival of *Met*-containing corticofugal axons. *Met*

expression, however, is induced in E12.5 VTe1 cells after 2 days *in vitro*, perhaps underlying discrepancies between observations *in vitro* and in *Met^{flx/flx}/Dlx5/6^{cre}* mice. We suggest that, *in vivo*, Met impacts the development of cortical projection neurons, whereas uPAR influences interneuron maturation. An altered balance between excitation and inhibition has been postulated as a biological mechanism for ASD; this imbalance could arise from different risk genes differentially affecting either or both elements.

INTRODUCTION

Over the past decade, there has been rapid progress in identifying putative risk genes for autism spectrum disorder (ASD) and related neurodevelopmental disorders (Abrahams & Geschwind, 2008; Bill & Geschwind, 2009; El-Fishawy & State, 2010). Thus far, however, much less is known about the mechanisms through which these genes impact brain development and, when disrupted, how they contribute to disorder pathogenesis. We identified a common functional polymorphism in the *MET* gene promoter, which leads to decreased transcription of *MET* and significantly increases the risk for developing ASD (Campbell et al., 2006). This finding has been replicated by our own (Campbell et al., 2008) and other groups (Sousa et al., 2008; Jackson et al., 2009). The subsequent identification of copy number variants (Marshall et al., 2008) and rare functional mutations (Campbell et al., 2006) in the *MET* gene lends further support to the idea that alterations in MET signaling contribute to ASD risk. Consistent with these genetic data, *MET* transcript and MET protein expression are decreased in the temporal cortex of ASD cases compared to controls (Campbell et al., 2007).

MET is a receptor tyrosine kinase, which, when activated, can induce multiple cellular responses, including proliferation, migration, differentiation and survival, depending on the cell and environmental context (for example, (Bladt et al., 1995; Ebens et al., 1996; Beilmann et al., 2000; Birchmeier et al., 2003; Okunishi et al., 2005; Giacobini et al., 2007)). Binding of the only known endogenous ligand, hepatocyte growth factor (HGF), results in MET receptor dimerization and autophosphorylation that ultimately activates key intracellular signaling pathways, such as the PI3-kinase and ERK systems (Longati et al., 1994; Ponzetto et al., 1994; Stefan et al., 2001; Xiao et al., 2001). HGF is secreted as a single-chain pro-form that is devoid of signaling activity; pro-HGF requires proteolytic cleavage by a serine protease to acquire biological activity (Lokker et al., 1992; Kirchhofer et al., 2004). Several such proteases have been reported to activate HGF *in vitro*, including the urokinase-like plasminogen activator (uPA), whose potency is increased when bound to the urokinase-like plasminogen activator receptor (uPAR) (Naldini et al., 1992; Blasi, 1993; Mars et al., 1993). A recent study, however, suggests that uPA/uPAR may not contribute to HGF activation under physiological conditions (Owen et al., 2010). Thus, our original hypothesis (Powell et al., 2001) connecting the bioavailability of activated HGF ligand through an intact uPA and uPAR system, which in turn modulates MET signaling *in vivo*, may not be accurate. Yet both uPA and uPAR are expressed in the forebrain (Dent et al., 1993; Masos & Miskin, 1996; Del Bigio et al., 1999; Yoshida & Shiosaka, 1999; Bahi et al., 2006; Lahtinen et al., 2009) and genetic disruption leads to neurodevelopment perturbations (Meiri et al., 1994; Del Bigio et al., 1999; Powell et al., 2001; Powell et al., 2003a; Eagleson et al., 2005; Eagleson et al., 2010). Moreover, functional polymorphisms in the plasminogen activator, urokinase receptor (*PLAUR*) gene, the human homolog of the mouse *uPAR* gene, like those found in *MET*, increase the risk for ASD (Campbell et al., 2008).

Animal model systems provide a useful experimental tool for investigating potential links between ASD susceptibility genes and alterations in brain architecture, including insights into spatial and temporal dynamics of altered developmental trajectories. *In vitro* model

systems are also used to address important issues regarding the impact of altered signaling and/or environmental conditions on basic cellular processes. Our initial attempts to delineate the role of Met in cortical development utilized an *in vitro* model system. We demonstrated that HGF stimulates the migration of GABAergic interneurons that arise from cultures of explants of the ventral telencephalon (VTel; (Powell et al., 2001)), presumably through the activation of the Met receptor that is expressed on migrating VTel neurons *in vitro*. *In vivo*, consistent with our initial proposal, there is a disruption of cortical interneuron development in a region- and cell-type specific manner in adult *uPAR*^{-/-} mice (Powell et al., 2001; Powell et al., 2003a; Eagleson et al., 2005). Specifically, frontal and parietal cortical areas exhibit reduced numbers of parvalbumin (PV)-positive GABAergic neurons, while in the hippocampus, there is a loss of the somatostatin population of interneurons in the CA1 and dentate gyrus subfields. Based on these observations, we suggested that alterations in Met signaling directly impact interneuron migration, leading to alterations in the interneuron profile in the adult cortex.

More recent observations suggest a discrepancy between our initial reports and hypotheses regarding direct action of Met signaling on interneuron migration in the developing mouse forebrain. For example, detailed molecular neuroanatomical analysis of gene and protein expression indicates that the Met receptor protein is expressed at low levels *in vivo* in the developing VTel that includes the proliferative ganglionic eminences (GE) and the postmitotic neurons of the developing striatum, and that detection of protein by the western blot method is due to receptor transport by neurons projecting from the cortex into the striatum (Judson et al., 2009). Furthermore, a recent study reported normal numbers of GABAergic neurons in the hippocampus when *Met* is deleted in cells arising from the GE, suggesting that interneuron migration to the hippocampus is unaffected in the absence of Met (Martins et al., 2007). Finally, a recent report suggests that pro-HGF is not activated by uPA under biologically relevant conditions (Owen et al., 2010). In the current study, we use conditional disruption of Met signaling to examine directly the influence of Met function on neocortical interneuron development. We also use radiolabel- and double-label *in situ* hybridization to clarify Met expression by cortical interneurons throughout key prenatal periods of development. In addition, we examine the influence of tissue culture environments on Met receptor expression to decipher possible differences in the roles for Met *in vitro* and *in vivo*.

MATERIALS AND METHODS

C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Conditional Met mutant mice (*Met*^{flx/flx}/*Dlx5*/*Cre*) were generated by mating mice homozygous for a Met allele (*Met*^{flx/flx}), in which exon 16 is flanked by loxP sites (courtesy of Dr Snorri Thorgeirsson, NIH/Center for Cancer Research, Bethesda, MD; (Huh et al., 2004)), to *Dlx5*/*Cre* mice (courtesy of Dr K. Campbell, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; (Stenman et al., 2003)) that were also heterozygous for the floxed allele (*Met*^{flx/+}/*Dlx5*/*Cre*). Both breeding lines were back-crossed on the C57Bl/6 background for greater than 10 generations. The mice were genotyped via polymerase chain reaction (PCR), using the same *Met* and *cre* primers as previously described (Judson et al., 2009). Animals were provided free access to food and water and were housed in a 12 hour light:dark cycle. All research procedures using mice were approved by the Institutional Animal Care and Use Committee at Vanderbilt University or at the University of Southern California, and conform to NIH guidelines.

Immunostaining and cell counting

The tissue processing, immunostaining and cell counting procedures were performed using previously published protocols (Eagleson et al., 2005). Briefly, animals were anesthetized

with sodium pentobarbital (60mg/kg i.p.) and perfused transcardially with 4% paraformaldehyde in phosphate buffered saline (pH 7.2). Brains were equilibrated in 30% sucrose, frozen and sectioned in the coronal plane. GABAergic interneurons in the cortex and hippocampus were visualized by the immunocytochemical detection of glutamic acid decarboxylase-67 (GAD-67) in the adult (>P90), using a mouse anti-GAD67 antibody (1:2,000; MAB5406, Chemicon, Temecula, CA). Subpopulations of GABAergic interneurons were analyzed using a mouse anti-PV antibody (1:500; clone Parv-19, Sigma, St Louis, MO). Profiles of GAD-67- or PV-immunoreactive cells were counted in three areas of the neocortex, frontal (at Bregma levels +2.0, +1.5, and +0.5), parietal (at Bregma levels -0.5, -1.0, and -1.5mm) and occipital (-2.5, -3.0, and -3.5mm), using stereotaxic coordinates in wild type (n=4) and *Met^{flx/flx}/Dlx5^{cre}* (n=4) mice. For each area at each level, a 440µm strip of cortex, from the white-gray matter interface to the pial surface, was analyzed. Within the hippocampus, the number of immunoreactive cells contained within a box (440µm × 660µm) was counted at Bregma levels -2.2 and -2.7mm for CA1, CA3 and dentate gyrus. All counts were done on a Zeiss Axioplan 2 microscope using brightfield illumination. The average number of immunoreactive cells at each level in a given cortical region or hippocampal subfield was obtained from bilateral counts in a single section and the estimated number of cells calculated according to Abercrombie's formula (Abercrombie, 1946). For each region analyzed, the number of immunoreactive cells at each Bregma level was summed to give a single value for each animal. The individual counting the anatomical material was blind to genotype. Counting accuracy in the laboratory revealed <3% variance between individuals performing quantitative analysis.

In Situ Hybridization

The tissue processing, probe synthesis and hybridization procedures for radiolabeled single-label and fluorescent double-label *in situ* hybridization were performed using previously published protocol (Judson et al., 2009; Thompson et al., 2010). Briefly, brains were harvested from embryonic (E12.5 – E16.5) or postnatal (P14) mice and immediately frozen using ice-cold 2-methylbutane. Twenty micrometers sections were cut in the coronal plane through the entire telencephalon with a cryostat and collected on Superfrost Plus glass slides (VWR, West Chester, PA).

Embryonic brains were processed with a radiolabeled probe for *Met*. Following pre-treatment, each slide was hybridized with 4.2 ng of probe incorporated with ³⁵[S]-CTP, transcribed from a DNA probe template (corresponding to nucleotides 2665–4051 of NM_008591) at 55°C overnight, followed by a series of buffer washes. Slides were dipped in Kodak autoradiography emulsion (Type NTB; Carestream Health Inc, Rochester, NY) and exposed for 8 days prior to development with Kodak Developer D19 (Ted Pella Inc, Redding, CA).

To determine which sub-populations of cells in the postnatal cortex express *Met*, P14 brains were processed for fluorescent double-label *in situ* hybridization of *Met* with probes for glutamatergic projection neurons (*VGlut1*), interneurons (*Gad1*), glia (*Slc1a2*) or oligodendrocytes (*Plp1*). *Met* probe synthesis and sequence was as described above. Mouse probe templates were made by RT-PCR amplification of adult mouse cortex cDNA, using probe sequence obtained from the Allen Brain Atlas (*VGlut1*, *Slc1a2* and *Plp1*). The following mouse sequences were amplified: *Slc1a2*, nucleotides 873 to 1627 (754 base pairs) of NM_011393.2; *VGlut1*, nucleotides 1655 to 2375 (720 base pairs) of NM_182993.2; *Plp1*, nucleotides 712 to 1682 (970 base pairs) of NM_011123.2; and *Gad1*, nucleotides 1860 to 2299 (440 bp) of NM_008077.3. Custom primers (Integrated DNA Technologies, Coralville, IA) were designed for each probe: *Plp1* forward (GGGGATGCCTGAGAAGGT), reverse (TGTGATGCTTTCTGCCCCA); *VGlut1* forward (CAGAGCCGGAGGAGATGA), reverse (TTCCCTCAGAAACGCTGG); *Slc1a2* forward

(ATGATCATGTGGTACTCCCCCTC), reverse (TAGAGTTGCTTCCCTGTGGTT); and *Gad1* forward (GCTCTGATGATGGAGTCAGG), reverse (GGTCTAGGACTAAGCCACAG). Following successful PCR amplification of the mouse-derived probe targets, the DNA fragments were TA-cloned into pSTBlue-1 (Novagen Cat. No 70573, Gibbstown, NJ). Direct re-sequencing of probe templates confirmed cloning of transcript. Probes were transcribed and incorporated with digoxigenin-UTP nucleotides into *Met* antisense, or fluorescein-UTP nucleotides into *VGlut1*, *Plp1*, *Slc1a2*, or *Gad1* antisense. Each slide was hybridized with ~200ng/ml of probe overnight at 55°C, followed by a series of buffer washes. Each set of experiments included slides treated with negative-control sense strand probes (transcribed from the same DNA probe template as the experimental antisense strand probe) to ensure hybridization specificity.

Explant Cultures and Western Blot Detection of Met

Explant cultures were established from the VTel dissected from E12.5 mice, using a similar protocol to that previously published (Powell et al., 2001). At this age, most of the isolated tissue is composed of the GE and a relatively small mantle zone of postmitotic neurons. The tissue was embedded in a collagen gel (BD, Franklin Lakes, NJ) in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA). HGF (10ng/ml; R & D Systems, Minneapolis, MN) was added to both the culture medium and the collagen gel. After 48 hours in culture, explants were harvested, with those derived from the same litter pooled, frozen immediately in liquid nitrogen and stored at -80°C. In addition, VTel tissue was dissected from E12.5 and E14.5 mice, with tissue from the same litter pooled and snap-frozen in liquid nitrogen for later biochemical analyses. Western blot analysis was performed using previously published protocols (Judson et al., 2009). Briefly, tissue was sonicated in ice-cold homogenization buffer containing a protease inhibitor cocktail (Sigma). Tissue homogenates were then cleared and protein concentrations determined using the Dc protein assay (Bio-Rad, Hercules, CA). Protein samples (35µg protein per lane) were fractionated by SDS-PAGE and transferred to supported nitrocellulose membranes. For each blot, three independent samples were prepared from a) E12.5 fetuses, b) E14.5 fetuses and c) E12.5 fetuses grown for two days *in vitro* prior to harvesting. This was replicated with three additional independent samples, to give n=6 for each condition. Membranes were probed with a mouse anti-Met (1:500; sc8057, Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse anti-alpha-tubulin loading control (1:100,000; Oncogene Research Products, San Diego, CA) antibody, followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA). Immunoreactive bands were visualized with enhanced chemiluminescence reagents (GE/Amersham ECL, Arlington Heights, IL) and detected with autoradiography film (GE/Amersham). Films were imaged with a high-resolution scanner and subjected to densitometric quantification using IMAGE-J (NIH, Bethesda, MD).

Statistical analysis

Data are provided as means ± SEM. For the analysis of interneuron development in *Met^{fx/fx}/Dlx5/6^{cre}* mice, each animal is considered a separate sample. For each brain region analyzed, a two-tailed t-test was used to determine significance, with the alpha level set at 0.05. To examine potential differences in Met expression *in vivo* and *in vitro*, the total optical density of the Met immunoreactive band was determined and a ratio was calculated based on the density of the alpha-tubulin band. Tissue isolated from one litter is considered an independent sample. A one-way ANOVA was used to determine significance, with the alpha level set to 0.05. When a significant difference was observed, this analysis was complemented with a pairwise test, using a two-tailed *t*-test to identify the source of possible interactions. All analyses were implemented in Statview, version 5.0.1 (SAS Institute, Inc., Cary, NC).

Digital illustrations

Micrographs of tissue processed for immunocytochemistry or radiolabeled *in situ* hybridization were acquired with an AxioCam HRc camera (Zeiss, Jena, Germany) attached to an Axioplan II microscope (Zeiss), using Axiovision 4.1 software (Zeiss), using brightfield or dark field illumination, respectively. Micrographs of tissue processed for fluorescent double-label *in situ* hybridization were acquired using an Olympus FluoView FV1000 confocal microscope, with a pixel size of $0.31\mu\text{m} \times 0.31\mu\text{m}$. Images were adjusted for contrast and resizing. The image of the Western blot was acquired using a CCD camera coupled to a UVP BioImaging System using UVP Imager software (UVP, Upland, CA). Figures were prepared digitally in SigmaPlot 7.0 (SPSS Incorporated, Chicago, IL, USA) and Adobe Photoshop 6.01 (Adobe Systems Incorporated, San Jose, CA, USA).

RESULTS

Interneuron numbers in the adult neocortex are unaltered in the absence of Met receptor signaling

We previously described a reduction in the number of GABAergic interneurons in the adult *uPAR*^{-/-} cortex and hippocampus (Powell et al., 2003a; Eagleson et al., 2005). In our original studies, we proposed, based on *in vitro* observations and the reported role of uPA/uPAR in the activation of HGF, that reduced signaling through the Met receptor underlies the altered interneuron phenotype observed in this mouse. To test this directly, we examined interneuron numbers in *Met*^{fx/fx}/*Dlx5*^{6cre} mice, in which the targeted deletion preferentially ablates exon 16 of the *Met* gene (Huh et al., 2004), an experimental design that will greatly reduce the processed, membrane-bound form of the Met receptor (Huh et al., 2004; Judson et al., 2009) from all postmitotic cells arising from the VTel (Stenman et al., 2003). Using this *Met*^{fx/fx} line, we and others have shown that, when exon 16 is deleted, the Met receptor is unable to signal (Huh et al., 2004; Judson et al., 2009), and is thus considered kinase-dead. Our observations on the number of GAD-67 immunoreactive neurons in the *Met*^{fx/fx}/*Dlx5*^{6cre} hippocampus (Figure 1A,B) were consistent with a previous report that used GABA immunoreactivity to visualize interneurons in a mouse line using the same deletion strategy (Martins et al., 2007). Specifically, we found no significant changes in the number GAD-67 immunoreactive cells in any region of the hippocampus (Figure 1E; CA1 ($t(1,6) = 0.49$, $p > 0.05$), CA3 ($t(1,6) = -0.25$, $p > 0.05$) and dentate gyrus ($t(1,6) = -0.26$, $p > 0.05$)). We then extended our observations to the neocortex. As has been reported previously, GAD-67 immunoreactive cells can be observed in all layers of frontal, parietal and occipital cortices in wild type mice (Figure 2A) and this pattern is preserved in the conditional null mice (Figure 2B). In contrast to the *uPAR*^{-/-} cortex, where significantly fewer GAD-67 positive cells are present in more rostral neocortical regions (Eagleson et al., 2005), there are statistically indistinguishable numbers of immunolabeled cells in frontal ($t(1,6) = 0.42$, $p > 0.05$), parietal ($t(1,6) = 1.84$, $p > 0.05$) and occipital cortices ($t(1,6) = 0.18$, $p > 0.05$) of wild type and *Met*^{fx/fx}/*Dlx5*^{6cre} mice (Figure 2C). These data indicate that should signaling through the Met receptor occur in the VTel, it has no direct effect on interneuron migration to and differentiation in the neocortex and hippocampus.

It has previously been reported that there is a decrease in PV-immunoreactive neurons in the CA3 hippocampal subfield in adult *Met*^{fx/fx}/*Dlx5*^{6cre} mice (Martins et al., 2007) while other hippocampal subfields are unaffected. We failed to replicate this finding, observing no significant change in the number of parvalbumin-positive cells in any region of the hippocampus examined (Figure 1C, D, F; CA1 ($t(1,6) = -0.13$, $p > 0.05$), CA3 ($t(1,6) = -0.20$, $p > 0.05$) and dentate gyrus ($t(1,6) = -2.38$, $p > 0.05$)). In addition, there were statistically indistinguishable numbers of parvalbumin cells in frontal ($t(1,6) = 0.09$, $p > 0.05$), parietal ($t(1,6) = 0.24$, $p > 0.05$) and occipital cortices ($t(1,6) = 1.19$, $p > 0.05$).

cortices of wild type and *Met^{fx/fx}/Dlx5/6^{cre}* mice (Figure 2D, E, F), indicating that eliminating *Met* signaling from cells arising in the VTel had no effect on the differentiation or survival of this subpopulation of interneurons that migrates to dorsal pallial structures.

Interneurons do not express the *Met* receptor

Given the negative findings regarding interneuron development using the *Met^{fx/fx}/Dlx5/6^{cre}* line, we re-examined the putative expression of the *Met* receptor in the early developing VTel and in interneuron populations in the postnatal neocortex. We previously described in detail *Met* transcript and protein expression in the developing telencephalon from late fetal ages (E18.5) to adulthood (Judson et al., 2009). In the forebrain, *Met* transcript expression is observed in the neocortex, hippocampus, amygdala and lateral septum, but not in the GE and striatum, throughout late prenatal and postnatal development. However, by E18.5, the majority of interneurons destined to populate the cortex and hippocampus have migrated from the subpallium (Pleasure et al., 2000; Anderson et al., 2001). Thus, we focused our current analysis of *Met* expression to an earlier fetal period during which cortical interneurons are being generated and beginning their migration to the overlying dorsal pallium. We did not detect *Met* transcript expression in the developing forebrain at E12.5 and E14.5 (Figure 3A), although expression already is abundant in peripheral and brainstem structures, as well as the spinal cord, at this time in sections from the same embryos (Figure 3B). By E16.5, expression of the transcript in the lateral cortex and subplate is relatively high, but expression in other telencephalic structures, including the GE and mantle zone of the VTel, containing postmitotic neurons, is notably absent (Figure 3C, D, E). Together with our previous observations, these data indicate that *Met* is not expressed by proliferating progenitor cells or postmitotic neurons arising from the GE during the period of migration out of the VTel.

Our previous analysis of *uPAR^{-/-}* mice indicated that, when maintained on the congenic C57BL/6 strain, there are normal numbers of interneurons in the neocortex and hippocampus at birth (Eagleson et al., 2005), but statistically significant reductions in immunodetectable cells later postnatally. These data are consistent with the notion that maturation, rather than proliferation or migration, of interneurons may be impacted in the *uPAR^{-/-}* mouse. During the period of interneuron maturation that occurs over the first three postnatal weeks in the mouse, *Met* transcript and protein expression increases dramatically in the neocortex and hippocampus (Judson et al., 2009). To explore the possibility that interneurons are included in the cortical *Met*-expressing population, we performed double-label *in situ* hybridization analyses using a variety of cell-type specific markers together with *Met*. Although we could reliably detect *Met* transcript during the early postnatal period (Figure 4), none of these cells co-expressed *Met* with the interneuron marker *Gad1* (Figure 4A). In contrast, co-expression of *Met* and *VGlut1* reveals that glutamatergic projection neurons express this receptor (Figure 4B), consistent with our recent report of *Met* protein expression in the axons of these cells (Judson et al., 2009). Finally, *Met* expression has previously been observed in glial cells after brain trauma or *in vitro* (Shimazaki et al., 2003; Nagayama et al., 2004; Lalive et al., 2005; Kitamura et al., 2007). Expression in this population under normal physiological conditions during development has not been determined. We found that none of the cells co-express *Met* and *Plp1* or *Met* and *Slc1a2* (Figure 4C and 4D). Thus, taken together, our current and previous analyses demonstrate that cortical projection neurons, but not interneurons or forebrain glia, express *Met*. These data are consistent with our anatomical analyses showing normal interneuron development in the *Met^{fx/fx}/Dlx5/6^{cre}* mouse.

Expression of the Met receptor is upregulated in VTel cells *in vitro*

Based on our observations in the *Met^{fx/fx}/Dlx5/6^{re}* mouse and the absence of Met expression in cortical interneurons, we decided to re-assess our original *in vitro* studies. There is now evidence that expression of the Met receptor is upregulated under a variety of environmental challenges, including hypoxia and following injury, in both the central nervous system and in peripheral organs (Joannidis et al., 1994; Okura et al., 1999; Nakamura et al., 2000; Rabkin et al., 2001; Pennacchietti et al., 2003; Hayashi et al., 2005; Hara et al., 2006; Kitamura et al., 2007). We reasoned that environmental conditions conducive to inducing Met expression might occur as the VTel explants are being established. We therefore compared relative levels of Met expression under three conditions: VTel dissected at E12.5 and E14.5 and immediately snap frozen for subsequent immunoblot analysis, and those dissected at E12.5 and grown as explants for 2 days *in vitro* prior to harvesting and freezing. There was a significant effect of the source of VTel tissue on the level of Met protein expression detected by Western blot ($F(1,14) = 53.93$, $p < 0.05$; Figure 5). There was a 1.8-fold increase in Met protein expression in the VTel between E12.5 and E14.5 *in vivo* ($p > 0.05$, $df = 10$). It should be noted that the level of Met protein expression in the embryonic VTel is much less than observed at the peak of Met expression during the early postnatal period (Figure 5). Moreover, as there is no detectable *Met* transcript expression at these ages, Met protein detection likely reflects early arrival of axons, arising from Met-expressing populations from the dorsal pallium that project through the anterior commissure and internal capsule to the contralateral cortex and ipsilateral thalamus and brainstem. In contrast, there is a 4.0-fold increase in Met expression in VTel explants maintained for two days *in vitro* compared to the E12.5 VTel ($p > 0.05$, $df = 10$). As there are no contributions from axons arising from the Met-expressing dorsal pallium in the explants, this increase in Met expression indicates that subpallial cells are capable of expressing Met *in vitro* under these culture conditions.

DISCUSSION

The present study reveals that two ASD risk genes, *MET* and *PLAUR*, have distinct effects on cortical development, with *MET* impacting the development of excitatory projection neurons (Judson et al., 2009; Judson et al., 2010) and *PLAUR* influencing the maturation of inhibitory interneurons (Powell et al., 2003a; Eagleson et al., 2005). ASD is a highly heritable disorder that presents with heterogeneity in both the core triad of symptoms and co-occurring medical conditions (Tuchman & Rapin, 2002; Jyonouchi et al., 2005; Valicenti-McDermott et al., 2006; Geschwind, 2009; Levitt & Campbell, 2009). There has been remarkable progress in identifying genes that increase the risk of developing ASD (Abrahams & Geschwind, 2008; Bill & Geschwind, 2009; El-Fishawy & State, 2010), but identifying the link between genetic risk and underlying biological etiologies of the disorder has proven more elusive. Even less attention has been paid to understanding this relationship in the context of ASD heterogeneity. Converging evidence from clinical and animal studies indicate that aberrant formation of neural circuits during development may represent a common feature of developmental disorders, including ASD (Frith, 2004; Courchesne & Pierce, 2005; Geschwind & Levitt, 2007; Just et al., 2007; Minshew & Williams, 2007). Heterogeneity would then arise, in part, as a consequence of distinct genetic etiologies impacting circuit development in the brain differentially, by influencing this process at distinct temporal windows, by impacting distinct components of the circuit, or both. The present data are consistent with this hypothesis.

Met expression in cortical development

Met is a receptor tyrosine kinase with known roles in multiple cellular processes, including proliferation, migration, and survival (for example, (Bladt et al., 1995; Ebens et al., 1996;

Beilmann et al., 2000; Birchmeier et al., 2003; Okunishi et al., 2005; Giacobini et al., 2007)). In the forebrain, cell culture studies pointed to a key role for Met signaling in the migration of cortical interneurons from the GE to the overlying pallium (Powell et al., 2001). It was somewhat surprising, therefore, when a comprehensive analysis of Met protein and transcript expression during late embryonic and postnatal development in the rodent revealed patterns consistent with Met being expressed specifically in neocortical and hippocampal (CA1) projection neurons, with preferential localization of the protein to the axons, during the peak period of axon outgrowth and synapse formation (Judson et al., 2009). In contrast to the intense expression observed in the cerebral cortex, *Met* transcript could not be detected in the GE, the primary source of neocortical and hippocampal interneurons (Anderson et al., 1997; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Pleasure et al., 2000; Anderson et al., 2001), nor in the developing mantle zone of the striatum at any time (Judson et al., 2009). The current study extends these findings, demonstrating that even during earlier stages of embryonic development, when interneurons are first being generated and migrating out of the GE, *Met* transcript is absent in the GE. The lack of detection is not a technical issue, as other embryonic structures, such as the spinal cord, express detectable levels of *Met* in tissue from the same embryos. Furthermore, co-labeling analyses confirmed that, during the period when *Met* expression peaks in the neocortex, the transcript is expressed solely in glutamatergic projection neurons, but not GABAergic interneurons or glia.

Reconciling the cellular targets of Met signaling

In contrast to our initial conclusions concerning a role for Met in interneuron development, the expression patterns of the Met receptor described above suggest that disruption in Met signaling *in vivo* would impact the development of cortical projection neurons rather than interneurons. Animal models in which Met signaling is compromised allow us to examine this prediction directly. Because deletion of either *Met* or *HGF* is embryonic lethal (Bladt et al., 1995; Uehara et al., 1995), we employed a conditional deletion strategy using the loxp/cre approach. As reported here, mice in which Met is deleted from all postmitotic cells arising from the GE (*Met^{fx/fx}/Dlx5/6^{cre}* mice) have comparable interneuron numbers in the neocortex and hippocampus as wild types, indicating that even if there were low levels of Met expression in VTel neurons, the generation, migration and survival of this cell population is not compromised in the absence of Met signaling. This is consistent with a previous report that focused on interneuron development in the hippocampus of the same mouse line (Martins et al., 2007). In that report, however, a reduction in the number of cells expressing parvalbumin was observed in the CA3 region of the mutant hippocampus. In contrast, the present study demonstrates normal numbers of parvalbumin-positive cells in the *Met^{fx/fx}/Dlx5/6^{cre}* hippocampus, as well as in all neocortical regions examined. The reason for this discrepancy is not clear, but it should be noted that the data reported here are consistent with the absence of Met expression in interneurons at any stage of development. Parallel analyses in another mouse model of compromised Met signaling, in which Met is deleted from progenitor cells arising from the dorsal pallium (*Met^{fx/fx}/Emx^{cre}* mice), revealed altered dendritic and spine development in layer II-III and V pyramidal neurons (Judson et al., 2010). Combined with preliminary electrophysiological analysis of altered excitatory input onto pyramidal cells (S. Qiu, C. Anderson, P. Levitt, G. Shepherd, unpublished observations), the phenotypes resulting from deficient *Met* signaling in the neocortex are consistent with altered development of excitatory circuits.

Our original studies demonstrated that cells arising from the GE initiate a motogenic response to HGF *in vitro* (Powell et al., 2001), even though *Met* transcript cannot be detected in this population *in vivo*. Similarly, process outgrowth from cultured dorsal thalamic neurons, which also do not express *Met* transcript *in vivo* (Judson et al., 2009), is

altered in the presence of HGF (Powell et al., 2003b). Although Met protein can be detected in these structures *in vivo*, this is due to subcortical projections of Met-expressing cells residing in the dorsal pallium (Judson et al., 2009). Thus, it appears that tissue culture conditions provide a permissible environment for ectopic expression of the Met receptor in cells that normally do not have either the transcript or protein during development. The detection of Met protein in explants of GE after 2 days *in vitro* by Western blot (present study) and immunocytochemically (Powell et al., 2001), even in the absence of cortical axons, is consistent with this proposal and addresses the apparent discrepancies between our anatomical observations *in vivo* and the conclusions drawn from our *in vitro* data.

It is now evident that Met expression can be readily induced or upregulated in multiple cell types under a variety of environmental conditions. For example, Met expression is upregulated in the brain following a hypoxic event, such as occurs during an ischemic insult (Nagayama et al., 2004), or in response to injury (Okura et al., 1999; Kitamura et al., 2007). Thus, although we suggest that Met does not play a direct role in interneuron development under normal physiological conditions *in vivo*, Met signaling may nonetheless impact this population should environmental stressors, which could induce aberrant Met expression, be encountered. Such conditions may occur *in utero* or during parturition, leading to an atypical developmental trajectory of cells that do not normally express this receptor. While speculative, the combination of pre- or perinatal environmental factors that are reported to increase ASD risk (Wilkerson et al., 2002; Glasson et al., 2004; Larsson et al., 2005; Klevzon et al., 2007; van Handel et al., 2007; Kinney et al., 2008; Mann et al.), combined with having the functional *MET* risk allele, is a testable gene X environment pathogenic mechanism that would alter the balance of MET expression in different cell populations relevant to ASD pathogenesis.

uPAR signaling

Our initial hypotheses of compromised Met signaling in the *uPAR*^{-/-} mouse were based on the requirement of activation of pro-HGF by serine proteases for biological activity (Lokker et al., 1992; Kirchhofer et al., 2004). It had been proposed, based on *in vitro* assays, that uPA is an important activator of HGF, particularly when it is bound to its receptor, uPAR (Naldini et al., 1992; Blasi, 1993; Mars et al., 1993). However, a recent study provides evidence that under physiologically relevant conditions, uPA is not a significant activator of pro-HGF (Owen et al., 2010); under the same conditions, other candidate serine proteases, including matriptase and hepsin, are able to activate pro-HGF efficiently. This suggests that Met signaling may not be compromised in *uPAR*^{-/-} mice. Rather, we propose that uPAR signaling may directly impact interneuron development. Several studies have demonstrated roles for uPA/uPAR signaling independent of uPA's protease activity. Upon binding of uPA, uPAR interacts with other proteins, including integrins and G-protein coupled receptors, to modulate cell migration, adhesion, proliferation and differentiation (reviewed in (Binder et al., 2007; Tang & Wei, 2008)). Given our proposal that the interneuron deficits observed in *uPAR*^{-/-} mice are not a consequence of reduced Met signaling, a recent report that increasing HGF levels in the *uPAR*^{-/-} mouse brain, starting in the postnatal period, restores normal numbers of PV-positive cells in the adult orbitofrontal cortex (Bissonette et al., 2010) at first appears contradictory, but can be reconciled. Specifically, because the HGF receptor, Met, is expressed in projection neurons and not interneurons, we suggest that the restorative effect of HGF supplementation on interneurons is indirect, likely through a direct influence on projection neuron development. The importance of proper projection neuron development to interneuron maturation, particularly with respect to PV expression, has been demonstrated. In this context, the availability of BDNF, provided by projection neurons, is a key modulator of PV expression by cortical interneurons (Cellerino et al., 1996; Gorba & Wahle, 1999; Huang et al., 1999; Berghuis et al., 2004; Patz et al., 2004).

Several authors have postulated that an altered balance between excitation and inhibition contributes to the etiology of ASD (Casanova et al., 2003; Rubenstein & Merzenich, 2003; Levitt et al., 2004; Vaccarino et al., 2009; Rubenstein, 2010). Such an imbalance could arise from an altered developmental trajectory in either of the two major neuron populations in the neocortex, the glutamatergic projection neurons or the GABAergic interneurons, or both. The differential cellular targets of uPAR and Met in the developing forebrain provide a plausible biological link between genetic vulnerability and variability in key developmental processes that may none-the-less lead to a final common path in ASD etiology. Moreover, there is a significant gene-gene interaction between *MET* and *PLAUR* with respect to ASD risk (Campbell et al., 2008). This may reflect a greater compromised ability in those individuals with an 'ASD polymorphism' in both genes to compensate adequately for disrupting both excitatory and inhibitory developmental processes. Human genetic studies focused on ASD subpopulations stratified by endophenotype will inform future development of even more sophisticated animal models, thus allowing for the exploration of further links between genetic vulnerability and ASD heterogeneity.

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Figure 1.

Brightfield photomicrographs illustrate GAD-67 (A,B) and PV (C,D) immunoreactivity in coronal sections through the hippocampus of adult wild type (A,C) and *Met^{fx/fx}/Dlx5/6^{cre}* (B,D) mice. Quantitative analysis reveals no significant difference ($p > 0.05$) in the number of GAD-67- (E) and PV- (F) immunoreactive cells in any subfield of the hippocampus examined. Black histograms, wild type; gray histograms, *Met^{fx/fx}/Dlx5/6^{cre}*. Scale bar = 500 μ m.

Figure 2.

Brightfield photomicrographs illustrate GAD-67 (A,B) and PV (D,E) immunoreactivity in coronal sections through the parietal cortex of adult wild type (A,D) and *Met^{fx/fx}/Dlx5/6^{cre}* (B,E) mice. Quantitative analysis reveals no significant difference ($p > 0.05$) in the number of GAD-67- (C) and PV- (F) immunoreactive cells in frontal, parietal or occipital cortex. Black histograms, wild type; gray histograms, *Met^{fx/fx}/Dlx5/6^{cre}*. Scale bar = 500 μm.

Figure 3.

Darkfield photomicrographs of coronal sections from wild type mice after processing for autoradiography and emulsion dipping. At E14.5 (A) *Met* transcript can be detected in the olfactory epithelium (oe), but is absent from the telencephalon (tel). Even as early as E12.5 (B) a prominent *Met* signal can be observed in multiple peripheral structures, including the ventral horn of the spinal cord (sc) and developing limb muscle (lm). By E16.5, *Met* transcript can be readily observed in the cortical plate (CP) at both rostral (C) and caudal (D) levels, but is absent from the ventral telencephalon (VTel) at both levels. At more caudal levels, the signal is particularly intense in the cortical plate laterally, while the subplate (SP) is more prominent medially. The boxed area in D is shown at higher magnification in E. Scale bar = 1mm (A–D), 250 μ m (E).

Figure 4.

Confocal photomicrographs of coronal sections from the medial frontal cortex of postnatal day 14 wild type mice after processing for fluorescent *in situ* hybridization, illustrating double-labeling for *Met* and *Gad1* (A–C), *Met* and *Plp1* (D–F), *Met* and *Slc1a2* (G–I), or *Met* and *VGlut1* (J–L). Full arrows indicate cells with only a single label of *Met*, *Gad1*, *Plp1*, or *Slc1a2*. Arrowheads indicate cells that co-express *Met* and *VGlut1* (L). While there is double labeling of *Met* and *VGlut1*, *Met* is not co-localized with *Gad1*, *Plp1*, or *Slc1a2*. Accumulated punctate labeling over cellular profiles is readily distinguishable from more diffuse background puncta, which is similar to background labeling using the sense strand. Scale bar in panel J represents 50µm.

Figure 5.

Western blotting analysis of Met protein expression in the ventral telencephalon in wild type mice. Lane 1, E12.5; Lane 2, E14.5; Lane 3, E12.5 + 2 days *in vitro* (DIV); Lane 4, P7. Protein levels are low embryonically *in vivo*, although there is an increase in levels between E12.5 and E14.5. A more robust increase is observed when explants of E12.5 ventral telencephalon are maintained for 2 DIV. None-the-less, this expression is modest compared to that observed during the peak of Met expression at P7. A one-way ANOVA reveals a significant effect of the source of the tissue on the relative level of Met expression ($p > 0.05$).