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MeCP2 deficiency disrupts axonal guidance, fasciculation, and targeting by altering Semaphorin 3F function

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Abstract

Rett Syndrome (RTT) is an autism spectrum disorder that results from mutations in the transcriptional regulator methyl-CpG binding protein 2 (*MECP2*). In the present work, we demonstrate that MeCP2 deficiency disrupts the establishment of neural connections before synaptogenesis. Using both *in vitro* and *in vivo* approaches, we identify dynamic alterations in the expression of class 3 semaphorins that are accompanied by defects in axonal fasciculation, guidance, and targeting with MeCP2 deficiency. Olfactory axons from *Mecp2* mutant mice display aberrant repulsion when co-cultured with mutant olfactory bulb explants. This defect is restored when mutant olfactory axons are co-cultured with wild type olfactory bulbs. Thus, a non-cell autonomous mechanism involving Semaphorin 3F function may underlie abnormalities in the establishment of connectivity with *Mecp2* mutation. These findings have broad implications for the role of *MECP2* in neurodevelopment and RTT, given the critical role of the semaphorins in the formation of neural circuits.

Introduction

Methyl-CpG binding protein 2 (MeCP2) is a multifunctional protein that induces transcriptional repression by recruitment of co-repressors and chromatin remodeling (reviewed in (Chahrour and Zoghbi, 2007; Francke, 2006; Klose and Bird, 2006). Mutations in the *Mecp2* gene have been linked to human diseases such as Rett Syndrome (RTT, MIM 312750), a neurodevelopmental disorder associated with mental retardation, autistic behavior, and loss of previously acquired milestones, including purposeful hand use and expressive language (Amir et al., 1999). Alterations in MECP2 expression have also been detected in other autism spectrum disorders (ASD) as well as in nonsyndromic mental retardation (Chahrour and Zoghbi, 2007). The observed correlation between MECP2 expression/function and neurological disorders clearly indicates a requirement for MECP2

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in the normal development of the nervous system. However, the mechanisms whereby MECP2 mutation disrupts neurodevelopment remain unclear.

To elucidate the role of MeCP2 in neurodevelopment, mouse models that either lack MeCP2 or express a mutant form of MeCP2 have been generated. These animals share many of the features of the human disorder (Chen et al., 2001; Guy et al., 2001; Pelka et al., 2006; Shahbazian et al., 2002). Studies in such animal models indicate that MeCP2 functions in maturation/terminal differentiation of CNS neurons (Kishi and Macklis, 2004; Matarazzo et al., 2004; Smrt et al., 2007), axonal and dendritic growth and morphology (Belichenko et al., 2009a; Cusack et al., 2004; Jugloff et al., 2005; Larimore et al., 2009; Zhou et al., 2006), synaptic formation and function (Asaka et al., 2006; Chao et al., 2007; Dani et al., 2005; Fukuda et al., 2005; Moretti et al., 2006; Nelson et al., 2006) and neurotransmission (Chao et al., 2007; Dani et al., 2005; Medrihan et al., 2008; Nelson et al., 2006). In addition, the absence of MeCP2 in mouse models is accompanied by deficits in learning, memory (Moretti et al., 2006; Pelka et al., 2006) and social behavior (Moretti et al., 2005). The current view proposes that MeCP2 dysfunction affects synaptic plasticity via failure of dendritic function. However, the molecular basis for these defects remains unclear, and the effect that MeCP2 deficiency may have on axonal function has not been explored.

Our laboratory has used the olfactory system as a developmental model. We demonstrated that MeCP2 expression correlates with the maturation of olfactory sensory neurons (OSNs) and precedes synaptogenesis (Cohen et al., 2003). We also found that both MeCP2 deficiency and dysfunction impair OSN terminal differentiation at the time of synaptogenesis (Matarazzo et al., 2004; Palmer et al., 2008). These acute and transient defects in synaptogenesis are followed by a separate and persistent phase of chronic compromise in synaptic architecture. Since the olfactory system undergoes continuous neurogenesis, sampling this epithelium from patients allowed us to perform *in vivo* analyses not possible to do from brain. Our studies of olfactory nasal biopsies from RTT patients have revealed similarities in the neurodevelopmental defects found in RTT patients and mouse models of RTT, validating the olfactory system as a model to study RTT and MeCP2 dysfunction (Ronnett et al., 2003). Using this system, we identified functional groups of proteins that are dysregulated in *Mecp2*-null mice; in particular, a group of proteins involved in cytoskeletal arrangements and axonal guidance (Matarazzo and Ronnett, 2004).

Therefore, we hypothesized that MeCP2 deficiency alters axonal guidance events during development to ultimately compromise the establishment of neural connections and synaptic function. Using the olfactory system for both *in vitro* and *in vivo* approaches, we identify a novel function for MeCP2 in axonal guidance, fasciculation, and targeting, and a role for class 3 Semaphorins in these defects. These findings have broad implications for the role of MeCP2 in neurodevelopment and RTT, given the critical role of semaphorins in the formation and maintenance of neural circuits (Yazdani and Terman, 2006).

Results

Laminar targeting is altered in the main olfactory bulb (MOB) from *Mecp2*-null mice

OSN axons enter the MOB forming the olfactory nerve layer (ONL) and terminating in regions of neuropil called glomeruli, where they form synapses with mitral and tufted cells. The MOB has a distinct lamellar structure, each layer containing a different cell type (Mori et al., 1999). During the first postnatal weeks, axons from OSNs target the glomerular layer but can also be found overshooting the external plexiform layer (EPL). By P10-20, this excessive axonal growth is normally refined and the adult pattern of glomerular innervation is attained (Tenne-Brown and Key, 1999). We have previously shown that MeCP2 is expressed in all MOB cell layers, and that its expression increases during development

(Cohen et al., 2003). Using the *Mecp2*-null mouse model, we also demonstrated that individual glomeruli became smaller in the absence of MeCP2 and had abnormal axonal projections (Matarazzo et al., 2004).

Therefore, we hypothesized that MeCP2 deficiency affects neural connectivity during the formation of olfactory circuits. To assess this, coronal sections of MOB from WT and *Mecp2*-null mice were immunostained with antibodies against olfactory marker protein (OMP) to visualize mature olfactory axons. At P14, we observed that olfactory axons extend into deeper layers of the *Mecp2*-null OB when compared to WT (Fig. 1A, B, B'). We quantified this defect by counting the percentage of glomeruli that have overgrowing axons (Fig. 1C). Thus, 19% of glomeruli showed overgrowing axons in WT mice; however, the same defect was found in a significantly larger number of glomeruli in *Mecp2*-null mice (40%, $p < 0.01$). By P28, no differences in axonal projections were observed ($p = 0.34$) and both groups showed a normal laminar distribution. These results indicate that MeCP2 deficiency induces a delay in the establishment of laminar targeting in the MOB and suggest that MeCP2 is involved in the formation of olfactory circuits.

Targeting of M72 olfactory axons is not affected in *Mecp2*-null mice

Axons from OSNs form specific and highly stereotyped projections to higher-order neurons in the OB. While OSNs expressing one type of odorant receptor (OR) are dispersed in the OE, their axons converge onto specific clusters of glomeruli in the OB (Ressler et al., 1994; Vassar et al., 1994). Considering that *Mecp2* deficiency alters the laminar targeting in the MOB, we sought to evaluate whether the targeting of specific OR populations is also affected. Thus, we examined the convergence of a subset of olfactory axons that expresses the OR M72. For this, M72-IRES-tauLacZ mice (M72-LacZ mice) (Mombaerts, 1996) were bred with *Mecp2* heterozygous mice to generate double mutants. M72-LacZ mice (WT and *Mecp2*-null littermates) were then subjected to whole mount X-gal staining to visualize the trajectories of M72 axons. As shown in Fig. 2A and D, M72 axons converge into one or more distinct glomeruli at the medial and lateral sides of each OB, as previously described (Zou et al., 2004).

In order to uncover possible differences in M72 axonal targeting, we used whole mount images to measure the distance to each M72 glomerulus from either the rostral and lateral OB tip (for each lateral M72 glomerulus) or from the rostral and dorsal OB tip (for each medial M72 glomerulus), as indicated in Fig. 2A and D. The results are expressed as the ratio of the distance to each M72 glomerulus divided the total length of the OB for each axis, in order to normalize for the reduced OB size of *Mecp2*-null mice (Matarazzo et al., 2004). As shown in Fig. 2B, C, E and F we did not find defects in the axonal targeting for this specific population, as M72 glomeruli converge to the same positions in the OB of WT and *Mecp2*-null littermates at P14.

The establishment of the olfactory glomerular map is complex and involves a hierarchy of guidance cues, OR-mediated mechanisms as well as activity-dependent responses (Cho et al., 2009; Mombaerts, 2006). While *Mecp2*-deficiency affects the initial formation of the MOB lamellar structure, the convergence of axons for a specific OR population appears preserved at early development. Thus, our results indicate that *Mecp2* deficiency alters the formation of olfactory circuits in a selective manner during development.

Mecp2-null mice show defective vomeronasal nerve (VNN) fasciculation

Most mammals possess a secondary olfactory system in which pheromone responsive sensory neurons (located in the vomeronasal organ, VNO) project their axons to the accessory olfactory bulb (AOB) located at the dorsocaudal surface of the MOB (Buck,

2000). We assessed the expression of MeCP2 in the accessory olfactory system and found that it is present in all VNO and AOB neurons at P14 and P28 (Fig. 3). VNO axons coalesce and form the VNN, which travels on the medial surface of the MOB before reaching its target. To evaluate the condition of VNO axons in the absence of MeCP2, coronal and sagittal sections of MOB from WT and *Mecp2*-null mice at several postnatal ages were stained with *Erythrina cristagalli* (EC) lectin to visualize all axons in the VNN (Cloutier et al., 2002). While VNO axons in WT mice are grouped into two large bilateral nerves, *Mecp2*-null mice show defasciculation of the VNN into multiple smaller bundles of axons distributed along the dorsoventral axis of the MOB (Fig. 4A, B). In a sagittal view, WT VNO axons coalesce into a tight fascicle as they grow along the medial surface of the MOB. Again, the VNN from *Mecp2*-null animals is organized into several smaller fascicles (Fig. 4C,D). Although most of these VNO axons reach the AOB, some fibers terminate erratically along the nerve pathway (Fig. 4D, arrowhead). VNN defasciculation was observed in all *Mecp2*-null animals analyzed at P14 (n=5), and was still present in 80% of *Mecp2*-null mice examined at P28 (n=5). These results indicate that MeCP2 plays a role in regulating axonal pathfinding and fasciculation of VNO axons during development and establish that in the absence of MeCP2 defects in connectivity occur early on, before axons establish synaptic contacts.

Zonal targeting of VNO axons is altered in *Mecp2*-null mice

VNO axons segregate into different regions within their target (AOB). Neurons located in the apical portion of the VNO express the G protein α subunit $G_{i2-\alpha}$, and project their axons to glomeruli restricted to the anterior half of the AOB. In contrast, sensory neurons located in the basal region express G protein α subunit $G_{o\alpha}$ and project their axons to the posterior half (Jia and Halpern, 1996). As shown in Fig. 3A-B, MeCP2 is expressed in both populations of VNO neurons. To evaluate the effect of MeCP2 deficiency on the zonal targeting of VNO axons, sagittal sections of AOB were stained with antibodies against $G_{i2-\alpha}$ or $G_{o\alpha}$ to visualize VNO axons that project to the anterior or posterior half of the AOB, respectively. The anterior-posterior border of the AOB was defined by *Bandeiraea simplicifolia* (BS) lectin and $G_{o\alpha}$ staining (Cloutier et al., 2004). As shown in Fig. 5A-A', all $G_{i2-\alpha}^+$ axons innervate the anterior region of the AOB in WT mice, as expected. However, in *Mecp2*-null mice, $G_{i2-\alpha}^+$ projections were also detected in the posterior half of the AOB (Fig. 5B-B'). To determine the extent of mis-targeting, we counted the number of $G_{i2-\alpha}^+$ axon bundles that project into the posterior AOB (Fig. 5C). At P14, *Mecp2*-null mice show a significantly greater number of $G_{i2-\alpha}^+$ bundles innervating the posterior AOB in comparison to WT littermates. Again, this defect was not apparent at P28. Targeting of $G_{o\alpha}$ projections to the posterior AOB was normal in *Mecp2*-null mice (data not shown). Interestingly, zonal segregation of $G_{i2-\alpha}$ and $G_{o\alpha}$ expression in the VNO was also unaltered in *Mecp2*-null mice (Fig. 5D-G). Similar to the defect observed in MOB, these data suggest that MeCP2 is required for the proper zonal targeting of olfactory axons during development.

Mecp2-null mice have abnormal levels of class 3 semaphorins (Sema3s) and receptors

Our proteomics studies in *Mecp2*-null mice revealed differences in the expression of proteins involved in cytoskeletal rearrangements and axonal guidance (Matarazzo and Ronnett, 2004). One of these proteins was CRMP-2 (collapsin receptor mediator protein-2), whose expression was decreased 2-fold at P14 in the olfactory system of *Mecp2*-null mice. CRMP-2 mediates the intracellular response to Sema3s (Schmidt and Strittmatter, 2007). Sema3s (Sema3A-Sema3G) are secreted axon guidance proteins that, in conjunction with the receptor proteins class A plexins (plexinA) and neuropilins (Npn), are expressed in the olfactory system and are required for the proper connectivity of main and accessory olfactory axon (Cloutier et al., 2002; Cloutier et al., 2004; Schwarting et al., 2000; Schwarting et al., 2004; Taniguchi et al., 2003; Walz et al., 2007; Walz et al., 2002).

Therefore, we hypothesized that MeCP2 deficiency may affect the expression of *Sema3*'s and/or associated receptor and signaling molecules, which could underlie the defects in axonal targeting we describe here (Figs. 1, 4-5). To assess this, we determined the mRNA expression levels of components of the *Sema3* signaling pathway in WT and *Mecp2*-null olfactory tissues by real time RT-PCR. It has been proposed that *in vivo* *Sema3A* acts via the Npn-1–PlexinA4 receptor complex, while Npn-2–PlexinA3 mediates *Sema3F* effects (Suto et al., 2005; Yaron et al., 2005). Activation of plexinA-Npn receptor complexes triggers intracellular signalling cascades that converge upon the cytoskeleton (Zhou et al., 2008).

As shown in Fig. 6A-B, the expression levels of some components of the *Sema3* pathway are dysregulated in the olfactory system in *Mecp2*-null mice. Consistent with our proteomics results, these changes in expression were dynamic during development and tissue-specific. *Sema3F*–Npn-2–PlexinA3 pathway was the most dramatically affected (Fig. 6A). *Sema3F* mRNA levels were markedly up regulated in *Mecp2*-null olfactory epithelia (OE) at P1, and down-regulated about 10-fold in *Mecp2*-null MOB at P1 and P7, with levels normalizing by P14. *Npn-2* expression was increased (>2-fold) in *Mecp2*-null OE at P1 and P7, and also in *Mecp2*-null MOB at P14. *PlexinA3* appeared significantly up regulated at all time points in both the OE and MOB. Although the *Sema3A*–Npn-1–PlexinA4 pathway was also affected by MeCP2 deficiency, expression changes were less robust and more restricted. *Sema3A* and *Npn-1* mRNA increased (>2-fold) in *Mecp2*-null OE only at early time points, while *PlexinA4* levels were similar to WT tissues at all times. Again, the intracellular mediator *CRMP2* increased significantly in OE and MOB at P7, and appeared down regulated in *Mecp2*-null OE at P14 (Fig. 6A), in line with our proteomics data (Matarazzo and Ronnett, 2004). In addition, we determined the expression of the same genes in accessory olfactory tissues (VNO and AOB) (Fig. 6B). Among all the genes tested, *Npn-1* shows a modest increase (<2-fold) at P7, whereas *PlexinA3* expression showed a greater increase (>2-fold) in accessory olfactory tissues at both P7 and P14.

Thus, *Mecp2*-null mice show developmental and regional differences in the expression of *Sema3*s as well as *Sema3* receptor and signaling molecules. These results suggest that MeCP2 controls (directly or indirectly) the expression of *Sema3*s and their receptors during development.

Mecp2*-null olfactory axons demonstrate normal outgrowth and response to *Sema3*s *in vitro

The role of semaphorins as guidance cues for growing axons is well established (Tran et al., 2007; Yazdani and Terman, 2006). Considering that *Mecp2*-null olfactory tissues differ in the expression of *Sema3*s signaling components, we evaluated whether the axonal response to *Sema3*s was altered *in vitro*, via classic collagen matrix assays (Cloutier et al., 2002; Pasterkamp et al., 2003). OE and VNO explants from E16 *Mecp2*-null or WT mice were cultured in the proximity of 293 cell aggregates transfected with *Sema3A*-AP (*Sema3A*), *Sema3F*-AP (*Sema3F*), or AP-Tag-4 vector (as control). Following 2-4 days in culture, the explants were stained using anti-TUJ1 antibodies. In our culture conditions, WT OE axons were repelled by *Sema3A* but not *Sema3F*; conversely, WT VNO axons responded to *Sema3F* but not to *Sema3A* (data not shown). Therefore, although the transcripts for both Npns and Plexins can be found in both olfactory tissues, they appear to show differential chemotropic responses. Thus, in subsequent experiments we tested OE and VNO responses to *Sema3A* or *Sema3F*, respectively.

When VNO explants derived from either WT or *Mecp2*-null mice were cultured next to *Sema3F*-secreting cells, most of the axons grew away from the aggregates (Fig. 7B and D). As expected, this effect was not present when explants were cultured next to AP control cells (Fig. 7A and C). The extent of axonal repulsion was scored blindly using the criteria

presented in Fig. 7E, and the percentage of explants assigned to each score is represented in Fig. 7F. Both WT and *Mecp2*-null VNO axons avoided *Sema3F* at similar levels (Fig. 7B, D, and F). Similarly, OE explants from WT or *Mecp2*-null mice were cultured in the proximity of *Sema3A*-transfected cells or control AP. Again, the repulsion response was comparable in WT and *Mecp2*-null tissues (Fig. 8A-E). It is important to consider that *Sema3s* levels are high in this kind of assay, so small changes in *Sema3* sensitivity might not be detected. To assess this, we tested the response of WT and *Mecp2*-null axons to lower levels of *Sema3F* (50% and 10% from original levels), and the responses remained similar in both groups (data not shown). In addition, we did not observe differences in neurite outgrowth from WT or *Mecp2*-null olfactory explants (Fig. 8F). Altogether, these results indicate that *Mecp2*-null olfactory axons are intrinsically capable of responding to *Sema3A* and *Sema3F*, and suggest that non-cell autonomous defects mediate the axonal guidance disturbances seen in *Mecp2*-null mice.

MOB-derived guidance cues are deficient in *Mecp2*-null mice

VNO axons cross the medial MOB as they travel to their target, the AOB. It is known that repellent cues from the MOB (such as *Sema3F*) play a role in the fasciculation of the VNN and in maintaining the segregation of main and accessory olfactory projections (Cloutier et al., 2002; Walz et al., 2007). Our results show that *Mecp2*-null VNO axons can intrinsically respond appropriately to Semaphorin 3F (Fig. 7). Therefore, it is possible that changes in the expression of guidance cues in the MOB are responsible for the defective VNN fasciculation we observed *in vivo* (Fig. 4B,D). In fact, we found that *Sema3F* levels are significantly down regulated (~10-fold) in the MOB from *Mecp2*-null mice (Fig. 6A). To test this hypothesis, we performed co-cultures of VNO with MOB by positioning WT or *Mecp2*-null VNO explants next to either WT or Null MOB explants in the four possible combinations (Fig. 9). When VNO explants from WT or *Mecp2*-null mice were co-cultured with WT MOB, VNO axons were repelled by the MOB as anticipated, based upon previous studies (Cloutier et al., 2002). However, when VNO explants from either WT or *Mecp2*-null mice were cultured in the proximity of *Mecp2*-null MOB, repulsion in response to MOB tissue was not observed (Fig. 9A-E).

In addition, we sought to confirm that the repellent effect elicited by the MOB on VNO axons involves the action of the *Sema3F*-*Npn-2* pathway, as previously reported for rat explants (Cloutier et al., 2002). For this purpose, VNO_{WT}/MOB_{WT} co-explants were established, and anti-*Npn-2* or rabbit IgG were added to the culture media (Cloutier et al., 2002). The addition of anti-*Npn-2* reduced the MOB repellent effect in comparison to control (Fig. 9F). This result establishes that components of the *Sema3* pathway, including the *Npn-2* receptor, are involved in the guidance of VNO axons in our *in vitro* system. Altogether, these results suggest that the observed alterations in connectivity within the olfactory circuit in *MeCP2* deficiency are due to non-cell autonomous defects in axon targeting mediated by *Sema3* signaling pathways.

Discussion

In the present study we demonstrate that 1) *MeCP2* deficiency disrupts the establishment of neural connections well before synaptogenesis; 2) *MeCP2* deficient mice have defects in axonal guidance, fasciculation, and targeting; 3) guidance defects are accompanied by temporal/regional alterations in the expression of class 3 Semaphorins and receptors; and 4) non-cell autonomous dysfunction of *Sema3F* signaling is responsible for some of the defects in connectivity observed with *Mecp2* mutation. These findings have broad implications for the role of *MeCP2* in neurodevelopment, the pathogenesis of RTT, and ASDs in general, since recent studies speculate that defective functional connectivity could be the basis for ASDs (Geschwind and Levitt, 2007).

MeCP2 is required to establish an accurate spatio-temporal pattern of olfactory connections

Early evidence from postmortem studies supported the hypothesis that RTT is a disorder of dendritic refinement and synaptic plasticity (Johnston et al., 2001). Therefore, most efforts have addressed the role of MeCP2 in dendritic development and function. However, our present study reveals an additional role for MeCP2 in the establishment of neural circuits by controlling axonal guidance events (axonal pathfinding, fasciculation and targeting).

We observed that axonal pathfinding is disrupted in the accessory olfactory system of *Mecp2*-null mice. Specifically, the VNN is defasciculated and shows abnormal trajectories toward its target (Fig. 4B, D). Similar defects were evident in olfactory biopsies from RTT patients, where axon bundles from OSNs showed altered fasciculation (Ronnett et al., 2003). Interestingly, defective pathfinding has also been observed in the hippocampus of *Mecp2*-null mice, where projections from immature neurons were abnormally oriented at early postnatal times (Smrt et al., 2007). Moreover, recent studies demonstrate that cortical axons from *Mecp2*-null mice also show marked defects in trajectory and orientation (Belichenko et al., 2009b). Altogether, these observations suggest that MeCP2 is required to form a proper spatio-temporal pattern of connections during the development of neural circuits.

In addition, we found zonal targeting defects in both AOB and MOB from *Mecp2*-null mice. $Gi2\alpha^+$ VNO axons are improperly oriented and extend into the posterior AOB at early postnatal ages (Fig. 5B-B'), whereas the MOB undergoes defective laminar targeting, i.e., mature *Mecp2*-null olfactory axons projected into deeper OB layers (Fig. 1). A possible explanation for the observed defects is that *Mecp2*-null axons have intrinsic elongation defects. To test this possibility, we measured neurite outgrowth using *in vitro* cultures of OE and VNO explants derived from E16 WT and Null mice. Contrary to previous reports indicating that MeCP2 deficiency altered neurite elongation/complexity *in vitro* (Cusack et al., 2004; Jugloff et al., 2005), we did not detect differences in the extent of growth between the two groups (Fig. 8F). This demonstrates that olfactory axons from *Mecp2*-null mice do not have intrinsic defects in neurite growth or extension.

Another possibility is that some of the targeting defects that we found occur as a consequence of the OSN maturational delay that we previously reported (Matarazzo et al., 2004). However, it has been shown that the maturational rate of OSNs is dependent upon and secondary to the status of connectivity with their target, the MOB. Thus, OSN maturational rate can be altered when connectivity is compromised, as in lesioning of the MOB (Carr and Farbman, 1992). It is possible then, that defective signals derived from the target could underlie the guidance defects we observed. Our results support this latter possibility since we demonstrate that the *Mecp2*-null MOB fails to provide adequate guidance cues to OSN axons (Fig. 6A and 9), and suggest that defects in connectivity involves non-cell autonomous mechanisms. Interestingly, a recent study in a female model of RTT establishes that *Mecp2* deficiency lead to both cell autonomous and non-autonomous defects of dendritic morphology (Belichenko et al., 2009a). Also, the pathology found in the CNS from RTT postmortem tissues (Armstrong, 2001) support a non-cell autonomous mechanism of pathogenesis, which is important when considering therapeutic interventions.

The question arises whether early disturbances in axonal guidance have consequences for later development. It is well established that developing neural circuits exhibit sensitive/critical periods during which the effect of experience is key in shaping the architecture and determining the level of plasticity that the neural circuit will display (Hensch, 2005). Thus, it is possible that even transient defects in guidance during sensitive/critical periods could alter the normal timing and patterning of circuitry formation, leading to dysfunction or altered

plasticity in the long term. We have previously reported that *Mecp2*-null mice show a transient delay in neuronal maturation at P14 and here we describe axonal guidance defects at the same developmental time. Although these abnormalities are not apparent later on, a chronic defect in the size and compartmentalization of synaptic structures (glomeruli) does persist in these animals (Matarazzo et al., 2004). Interestingly, different studies characterizing animal models of *Mecp2* mutation during development indicate that the morphological abnormalities detected are temporally dynamic or transient in nature (Chao et al., 2007; Dani et al., 2005; Matarazzo et al., 2004; Matarazzo and Ronnett, 2004; Palmer et al., 2008). However, their contribution to the pathogenic mechanism is difficult to address *in vivo*. Experiments using a mouse model in which *Mecp2* expression can be activated at different times during development will allow these studies in the future (Guy et al., 2007).

Our findings reveal that defects in connectivity occur with MeCP2 deficiency during the formation of neural circuits, and even though transient, could contribute to the pathogenesis of the neurological defects seen in RTT.

MeCP2 regulates *Sema3* pathways during development

Our proteomic studies (Matarazzo and Ronnett, 2004) led us to hypothesize that MeCP2 deficiency might affect *Sema3*s pathways, which could explain the observed axonal guidance defects (Fig. 1, 4 and 5). Indeed, our findings show that *Mecp2*-null mice have abnormal levels of expression of *Sema3*s and their receptors in the developing olfactory system (Fig. 6). Recent reports suggesting that *Mecp2* could act either as a transcriptional repressor or activator (Chahrour et al., 2008), may explain the observed variability in expression changes during development (Fig. 6). *Sema3F*, *Npn-2* and *PlexinA3* showed the most striking differences in expression. *Sema3F*-*Npn-2* signaling is required for fasciculation of the VNN as it courses past the MOB. It is also essential for the segregation of projections within the main and accessory olfactory systems and for the establishment of an adult pattern of laminar targeting in the MOB (Cloutier et al., 2002; Cloutier et al., 2004; Walz et al., 2007; Walz et al., 2002). Interestingly, our results indicate that these processes are altered in *Mecp2*-null mice (Fig. 1, 4 and 5). *PlexinA3* mRNA expression is up regulated at most times and in most tissues tested, and it is proposed to mediate *Sema3F* effects *in vivo* (Suto et al., 2005; Yaron et al., 2005). On the other hand, the expression of *Sema3A* and *Npn-1* was altered at early postnatal ages (P1) in the main olfactory system from *Mecp2*-null mice, yet expression of *PlexinA4*, a transducer of *Sema3A* action (Yaron et al., 2005), was unchanged (Fig. 6). *Sema3A* plays a role in the spatial arrangement of the MOB glomerular map (Pasterkamp et al., 1998; Schwarting et al., 2000; Schwarting et al., 2004; Taniguchi et al., 2003). We did not detect defects in the convergence of M72-expressing olfactory axons in the MOB from *Mecp2*-null mice at this early age (Fig. 2). Additional studies assessing axonal targeting for other olfactory receptors are required to confirm whether the topographic map is affected in *Mecp2*-null mice. It has been proposed that the establishment of the topographic map in the OB requires a combination of guidance cues, activity-dependent and other receptor-mediated mechanisms that will determine the final position of the glomeruli (Cho et al., 2009; Mombaerts, 2006). Although still unclear, the hierarchy of cues that contribute to form the topographic map seems different for the laminar structure formation in the OB. We found that laminar targeting in the MOB is affected by *Mecp2* deficiency at early ages, while topographic mapping appears normal. Our results therefore suggest that *Mecp2* deficiency affects some of these processes selectively, possibly by regulating the expression of different cohorts of guidance cues during development.

To understand the consequences of changes in guidance cue expression, we evaluated the axonal response to semaphorins *in vitro*. Our results show that despite the differential expression of guidance receptor molecules (Fig. 6), *Mecp2*-null olfactory axons preserve the ability to respond to *Sema3F* or 3A (Fig. 7, 8). Then, we evaluated whether the axonal

response to endogenous cues secreted along the axonal trajectory was altered. We observed that when either WT or Null VNO axons were cultured in the proximity of the Mecp2-null MOB, the expected chemorepellent response was significantly diminished (Fig. 9A-E), indicating that cues normally secreted from the MOB are deficient in MeCP2 absence. Consistent with previous studies (Cloutier et al., 2002), we showed that the repellent effect in our system is dependent upon *Sema3F*-*Npn-2* signaling (Fig. 9F) and, more important, *Sema3F* levels are markedly reduced in Mecp2-null MOB at P1 and P7 (Fig. 6A). These results suggest that the expression changes we observed in the *Sema3F*-*Npn-2* pathway in Mecp2-null mice account for the defective response observed *in vitro*, and possibly for the fasciculation and targeting defects found *in vivo*.

Thus, we identified a deficiency in *Sema3F* function, as well as deficits in neural circuit formation in Mecp2-null mice during early development. Interestingly, *Sema3F*^{-/-} and *Npn-2*^{-/-} mice show similar defects in axonal fasciculation and targeting of olfactory axons (Cloutier et al., 2002; Cloutier et al., 2004; Walz et al., 2007; Walz et al., 2002). Furthermore, *Sema3F*^{-/-} and *Npn-2*^{-/-} mice present deficits in neural circuit formation and are prone to seizures (Gant et al., 2008; Sahay et al., 2005), resembling other aspects of the Mecp2-null mouse model. It is well established that the expression of guidance cues is dynamic and precisely regulated by the decisions occurring at a particular time and place. This is achieved mainly through transcriptional regulation of guidance molecules and receptors (Yu and Bargmann, 2001). Therefore, our results suggest that a failure in transcriptional regulation of guidance cues could account for the defects in laminar targeting, fasciculation and zonal segregation we found in Mecp2-null mice.

The present study reveals a novel role for MeCP2 in the development of neural connectivity. We propose that MeCP2 affects axonal guidance and the establishment of neural circuits by regulation of guidance cue expression and non-cell autonomous mechanisms. We also define a defect in *Sema3* responses associated with MeCP2 deficiency. Having narrowed down the possible semaphorins involved to a few candidates from the *Sema* family, as well as the affected developmental time points, our future studies will investigate how Mecp2 regulate the expression of candidate genes from this pathway. Considering the crucial role that these molecules play in the development of neural circuits, our results have important implications for understanding the pathogenic mechanism underlying RTT and autism, and support existing hypotheses that ASDs are disorders of neural connectivity (Geschwind and Levitt, 2007).

Methods

Mice

We used a Mecp2-null mouse model generated by the Cre *LoxP* recombination system to delete exon 3 of *Mecp2* (Chen et al., 2001). Mice were generously provided by Dr. R. Jaenisch and maintained on a Balb/c background. M72-IRES-tauLacZ transgenic mice were obtained from Dr Peter Mombaerts (Mombaerts et al., 1996), and were crossed with the Mecp2-null mice to generate double mutants. All the experiments were performed using hemizygous *Mecp2* males (Mecp2-null) and WT littermate controls. Animal procedures were done in accordance with the Johns Hopkins University Institutional Animal Care and Use Committee, and guidelines from the National Institute of Health.

Immunohistochemistry

Olfactory tissues comprising olfactory epithelia (OE), vomeronasal organ (VNO), OB (olfactory bulb) and accessory olfactory bulb (AOB) were harvested from wild type (WT) and Mecp2-null male mice at post-natal (P) day 14 and 28, after cardiac perfusion using

0.1M phosphate-buffer (PB), pH 7.4, followed by 4% paraformaldehyde-PB. Sagittal and coronal sections (18 μ m) were prepared, and IHC was performed as previously described (Cloutier et al., 2002; Cohen et al., 2003). Primary antibodies used include: Methyl-CpG binding protein 2 (rabbit anti-Mecp2; 1:500) (Cohen et al., 2003), olfactory marker protein (OMP; 1:3000; Wako Pure Chemical Industries), G-protein subunit Gai2 (Gi2 α ; 1:300; Wako Pure Chemical Industries) and G-protein subunit Go α (Go α ; 1:200; Medical and Biological Laboratories Co.). Secondary antibodies include: Cy3 donkey anti-rabbit IgG (1:500), and FITC donkey anti-rabbit (or mouse) IgG (1:50) (both from Jackson Labs). Lectin histochemistry was performed using Fluorescein-*Erithrina cristagalli* lectin (EC lectin; 1:500, Vector Labs) and Biotinylated-*Bandeirae simplifolia* lectin (BS lectin; 1:2000, Sigma) followed by Cy3-Streptavidin, as previously described (Cloutier et al., 2002). Data were obtained from at least 4 animals from 3 litters per genotype per time point. Images were collected using either a Zeiss Axioskop with a digital camera (AxioCam; Zeiss) or a Zeiss Axiovert 200 scanning confocal microscope equipped with a Kr/Ar laser. In the last case, serial digital images at $\sim$ 1 μ m intervals were collected and z-stacks projected for image reconstruction.

Whole-mount stainings

OE and OB were dissected from P14 WT and Mecp2-null mice. Tissues were fixed in 4% PFA in 0.1 M phosphate buffer (PB) pH 7.4, for 30 min, and rinsed with PB. In order to reveal activity of the enzymatic reporter β -galactosidase (β -gal), the tissues were incubated in a mix of 5 mM potassium-ferricyanide, 5 mM potassium-ferrocyanide and 0.5 mg/ml X-gal in PBS at 37°C O/N. Images were collected in a Zeiss Axioskop with a digital camera (AxioCam; Zeiss).

Real Time RT-PCR

Total RNA samples were prepared from five to six individual male mice per genotype per age. Tissues were immediately frozen in liquid nitrogen and homogenized on dry ice. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Genomic DNA was digested with 1 unit of DNase I (Invitrogen). cDNA was produced using the ThermoScript RT-PCR kit (Invitrogen). Real-time PCR was carried out on an iCycler (Bio-Rad) by using a reaction mixture with SYBR Green as the fluorescent dye (Bio-Rad), a 1/10 vol of the cDNA preparation as template, and 250 nM of each primer (sequences available upon request). The cycle used for PCR was as follows: 95°C for 180 sec (1 time); 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec (40 times); and 95°C for 60 sec (1 time). After completion, samples were subjected to a melting-curve analysis to confirm the amplification specificity. The change in fluorescence of SYBR Green dye was monitored in every cycle and the threshold cycle (CT) was calculated above the background for each reaction. For each cDNA sample, a ratio between the relative amounts of target gene and GAPDH was calculated to compensate for variations in quantity or quality of starting mRNA, as well as for differences in reverse transcriptase efficiency. The fold change in the target gene relative to the GAPDH endogenous control gene was determined by: fold change = $2^{-\Delta(\Delta C_T)}$ where $\Delta C_T = C_{T,target} - C_{T,GAPDH}$ and $\Delta(\Delta C_T) = \Delta C_{T,KO} - \Delta C_{T,WT}$. RT-PCRs were run separately for each mouse in triplicate, and data were analyzed for statistical differences by the Mann-Whitney *U* test using PRISM 4.0 software (GraphPad, San Diego).

Explants cultures and collagen gel co-cultures

OE and VNO explants were prepared as described (Cloutier et al., 2002; Pasterkamp et al., 2003; Williams-Hogarth et al., 2000), with modifications. Briefly, VNO and OE from E16 embryos (WT or Mecp2-null) obtained from timed pregnant mice were dissected in Leibovitz (L15) media (Gibco). VNO tissues were incubated in pancreatin: trypsin 0.25%

EDTA (1:1, Sigma) for 30 min on ice and the digestion stopped by the addition of 10% FBS-L15 media (Cloutier et al., 2002). Explants (250-350µm) were obtained using fine forceps and were cultured in a mixture of rat tail collagen (Sigma) and Matrigel (Collaborative Biomedical Products). For some experiments, explants were co-cultured next to aggregates of 293-EBNA cells transfected with expression plasmids for either alkaline phosphatase (AP), human *Sema3A*-AP or human *Sema3F*-AP (kind gifts of Dr. A Puschel). In order to test different levels of Semas, we performed parallel experiments in which the cell aggregates were prepared using 50% or 10% of the initial amount of 293-EBNA cell transfected with AP or Semas-AP. Semas levels were then verified by determination of alkaline phosphatase activity in the supernatant. In another set of experiments, VNO explants were co-cultured next to OB explants from P5-P7 day old mice as described (Cloutier et al., 2002). Explants were cultured in NB/B27 medium in a 5% CO₂, 37°C, 95% humidity incubator for 48-96hrs. To visualize neuronal processes, cultures were fixed in 4% PFA and incubated with anti-βIII tubulin antibody (TUBJ1; 1:500, Chemicon), followed by Cy3-conjugated donkey anti-rabbit antibody (1:500, Jackson Lab). Individual neurites are difficult to identify in our cultures, therefore the overall area covered by them was measured using OpenLab™ software (Improvision, US). This gives an estimate of neurite length and numbers (de Castro et al., 1999). For co-culture experiments, the repulsive effect was quantified blind by assigning a score from 1-4 to each explant, 4 being the highest observed repulsion (Cloutier et al., 2002).

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Fig. 1. Mecp2-null mice show defective MOB laminar targeting
(A-C) Coronal MOB sections obtained from WT **(A)** or Mecp2-null mice **(B)** were immunostained with anti-OMP antibodies to visualize mature olfactory axons that innervate the MOB. Olfactory nerve layer (ONL); Glomerular layer (GL); External plexiform layer (EPL). **(B)** Arrowheads indicate axons overshooting the EPL in Null mice, which are shown in more detail in B' (inset). **(C)** Percentage of glomeruli that show aberrant projections in MOB from WT and Mecp2-null mice at P14 and P28. For quantification, 3-4 mice per genotype per age were used. From each mouse, four MOB sections at similar rostro-caudal levels were analyzed blind for genotype and a total of 200 glomeruli per mice were assessed (** $p < 0.01$, Mann-Whitney t test). Scale Bar: 50 μ m.

Fig. 2. Axonal convergence of M72 axons is preserved in Mecp2-null mice

(A and D) Whole-mount staining of MOB at P14 showing representative M72 glomeruli. (A) Whole-mount view of lateral M72 glomeruli showing the method of quantification. Scale Bar: 400μm. (B) Ratio between the distances of lateral M72 glomeruli from the rostral tip of the OB (μm) divided by the total distance from the rostral tip to the caudal tip (μm). (C) Ratio between the distances of lateral M72 glomeruli from the lateral tip of the OB (μm) divided by the total distances from the lateral border to the medial border (μm). (D) Whole-mount view of a medial M72 glomerulus. Scale Bar: 400μm (E) Ratio between the distances of medial M72 glomeruli from the rostral tip of the OB (μm) divided by the total distances from the rostral tip to the caudal tip (μm). (F) Ratio between the distances of medial M72 glomeruli from the dorsal tip of the OB (μm) divided by the total distances from the dorsal tip to the ventral tip (μm). Each bar is the average of 16 glomeruli obtained from 4 animals per genotype. No significant changes were found in the relative position of M72 glomeruli from WT and Mecp2-null mice (Mann-Whitney t-test).

Fig. 3. Mecp2 expression in the accessory olfactory system

(A-B) Coronal sections of VNO from P14 (A-B) WT mice were incubated with polyclonal anti-Mecp2 (red) as previously indicated (Cohen et al., 2003). DAPI (blue) was used for nuclei labelling. Mecp2 was detected in the cell body of all receptor neurons (RC) from the VNO but not in the supporting cell layer (SC). (C-D) Similarly, sagittal sections from P14 AOB were stained for Mecp2 and the expression was restricted to neuronal bodies located in the glomerular layer (GL), periglomerular layer (PL) and granular layer (GrL), and absent from the nerve layer (NL). A similar expression pattern was detected in accessory olfactory tissues from P28 mice (not shown). Top: dorsal. Scale Bar: 100µm.

Fig. 4. Mecp2-null mice show defective vomeronasal nerve (VNN) fasciculation
(A-D) Coronal and sagittal sections of MOB were obtained from WT and Mecp2-null mice at P14 and stained with EC lectin to label VNO projections. WT mice VNO axons are organized into large fascicles (**A**, arrowheads), while they form several smaller bundles in Mecp2-null mice (**B**, arrowheads). Top: dorsal. Scale bar: 100 μ m. On a sagittal view, WT VNO fibers coalesce on the medial surface of the OB as they extend caudally to the AOB (**C**), whereas Mecp2-null projections show distorted trajectories and some axons terminate erratically in the OB (**D**, arrow). Top: caudal. Scale bar: 200 μ m. n=5 mice per genotype, per age.

Fig. 5. Zonal targeting of vomeronasal axons is altered in Mecp2-null mice

(A-C) Sagittal sections of MOB were stained with anti-Gi2-α (green) to visualize VNO fibers that project to the anterior half of the AOB. The anterior-posterior border of the AOB (arrows) was defined using BS lectin (red), which binds to the posterior AOB. (A-A') In WT mice, all Gi2-α⁺ projections innervate the anterior region of the AOB. (B-B') Several improperly targeted Gi2-α⁺ axon bundles were detected deep in the posterior half of the AOB in Mecp2-null mice (arrowheads). Top: caudal. The number of Gi2-α⁺ bundles (per section) that innervate the posterior AOB is represented graphically in (C). Quantification was performed from 4-5 mice per genotype per age. From each animal, 6 sagittal sections showing clear anterior/posterior AOB were examined (6 AOB sections per side). Therefore, each bar represents the average of 12 sections per animal, analyzed in 4-5 animals, per genotype per age (***p* < 0.01, Mann-Whitney *t* test). Scale Bars: 250 μm. (D-G) Coronal sections of VNO from P14 day old WT and Mecp2-null mice were incubated with anti-Gi2-α (D, E) or anti-Goα (F, G). The sections were contrasted with DAPI to visualize all the cells in the VNO. Cell bodies of Gi2-α-expressing neurons are located in the apical VNO in both WT and Mecp2-null mice (D, E). Similarly, Go-expressing cell bodies are restricted to the basal region of the VNO in both WT and Mecp2-null mice (F, G). The pattern of staining for these markers is complementary, and the boundaries display an undulating shape as previously reported (Jia and Halpern, 1996). Scale bar: 100 μm.

Figure 6. Mecp2-null mice show differential expression of guidance molecules
mRNA levels of *Sema3A*, *Neuropilin-1*, *PlexinA4*, *CRMP2*, *Sema3F*, *PlexinA3*, and *Neuropilin-2* were determined in WT and Mecp2-null mice by real-time RT-PCR. (A) OE and OB or (B) VNO and AOB were obtained from 5-6 animals per group at P1, P7 and P14, and cDNA was prepared individually from each mouse. White bars represent WT mice and black bars Mecp2-null mice. Results are represented as a ratio between the relative amounts of target gene and GAPDH. WT results were normalized to 1. Real time-PCR reactions were run separately in triplicates for each mouse and data analyzed by unpaired Mann–Whitney *t* test with 95% confidence intervals. **p* < 0.05; ***p* < 0.01, ****p* < 0.001.

Figure 7. Mecp2-null olfactory axons respond normally to Semaphorin 3F *in vitro*
VNO explants (E16) from WT (A,B) or Mecp2-null mice (C,D) were co-cultured next to aggregates of 293 cells transfected with AP-Tag-4 vector (left panels) or Sema3F-AP (right panels). Explants were stained with TUJ1 (red), scored blindly using the criteria presented in E, and the percentage of explants assigned to each score was represented graphically (F). Data was analyzed by Kruskal-Wallis followed by Dunn's multiple comparison tests with 95% confidence intervals. (a) $p < 0.001$ with respect to WT-AP and Null-AP and (b) $p < 0.001$ with respect to WT-AP and Null-AP. Scale Bar: 100 μ m.

Figure 8. Sema3A-repellent effect and axonal outgrowth are not impaired in OE explants from Mecp2-null mice

(A-D) E16 OE explants from WT or Mecp2-null mice were cultured in the proximity of aggregates of 293 cells transfected with AP-Tag-4 vector or Sema3A-AP. Explants were stained for β III-tubulin (TUJ1, green) and the extent of repulsion was scored blindly using the criteria shown in A-D. Scale Bar: 100 μ m. E) The percentage of explants assigned to each score represented graphically. Data was analyzed by Kruskal-Wallis followed by Dunn's multiple comparison tests with 95% confidence intervals. (a) $p < 0.001$ with respect to WT-AP and Null-AP and (b) $p < 0.001$ with respect to WT-AP and Null-AP; F) Axonal outgrowth was determined in embryonic OE explants cultured alone by measuring the ratio between the area of axonal growth (determined by TUJ1 staining) and the area of the explant ($p > 0.05$, Mann-Whitney t test).

Figure 9. Mecp2-null OB fails to repel VNO axons

(A-D) E16 VNO explants from WT or Mecp2-null mice were co-cultured in the proximity of WT or Mecp2-null MOB explants (P5-P7) as indicated in the methods and labeled for β III-tubulin. Panels A-D show representative images for each condition. Scale Bar: 100 μ m. (E). The repulsive response is reduced only when VNO axons are cultured in the proximity of Mecp2-null MOB. Data was analyzed by Kruskal-Wallis followed by Dunn's multiple comparison tests with 95% confidence intervals. (a) $p < 0.01$ with respect to WT_{OB}-WT_{VNO} and WT_{OB}-KO_{VNO}; (b) $p < 0.05$ with respect to WT_{OB}-WT_{VNO} and WT_{OB}-KO_{VNO}. (F) Similarly, WT VNO explants were co-cultured with WT MOB explants (P5-P7) in the presence of 100 μ g of anti-Npn-2 or rabbit IgG as a control. The MOB repellent effect is reduced by the addition of anti-Npn-2 (* $p < 0.05$, Mann-Whitney t test).