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MeCP2 is required for activity-dependent refinement of olfactory circuits

Alicia L. Degano[1,2,5,*], **Min Jung Park**[1,2,*], **Judy Penati**[1,2], **Qun Li**[1,2], and **Gabriele V. Ronnett**[1,2,3,4,6]

[1]The Center for Metabolism and Obesity Research, The Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA

[2]Department of Neuroscience, The Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA

[3]Department of Neurology, The Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA

[4]Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA

[5]Departamento de Química Biológica, Facultad de Ciencias Químicas. CIQUIBIC-CONICET, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

[6]Daegu Gyeongbuk Institute of Science and Technology, Daegu, Republic of Korea

Abstract

Methyl CpG binding protein 2 (MeCP2) is a structural chromosomal protein involved in the regulation of gene expression. Alterations in the levels of MeCP2 have been related to neurodevelopmental disorders. Studies in mouse models of MeCP2 deficiency have demonstrated that this protein is important for neuronal maturation, neurite complexity, synaptogenesis, and synaptic plasticity. However, the mechanisms by which MeCP2 dysfunction leads to neurodevelopmental defects, and the role of activity, remain unclear, as most studies examine the adult nervous system, which may obfuscate the primary consequences of MeCP2 mutation. We hypothesize that MeCP2 plays a role during the formation and activity-driven maturation of neural circuits at early postnatal stages. To test this hypothesis, we use the olfactory system as a neurodevelopmental model. This system undergoes postnatal neurogenesis; axons from olfactory neurons form highly stereotyped projections to higher-order neurons, facilitating the detection of possible defects in the establishment of connectivity. *In vivo* olfactory stimulation paradigms were

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Correspondence address: Gabriele V. Ronnett, MD, PhD, The Johns Hopkins University School of Medicine, 855 N. Wolfe Street, Rangos Bldg., Suite 470, Baltimore, MD 21205, USA, Phone: +1-410-614-6484, Fax: +1-410-614-8033, gronnett@jhmi.edu. Alicia Degano, PhD, Haya de la Torre S/N, Pabellón Argentina, Ala Derecha 1er piso, Ciudad Universitaria, X5000HUA Córdoba, Argentina, Phone: +54-351-535-3855 (ext. 3418), Fax: +54-351-535-3855 (ext. 3406), adegano@fcq.unc.edu.ar.
*Both authors contributed equally to this work

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used to produce physiological synaptic activity in gene-targeted mice in which specific olfactory circuits are visualized. Our results reveal defective postnatal refinement of olfactory circuits in *Mecp2* knock out (KO) mice after sensory (odorant) stimulation. This failure in refinement was associated with deficits in the normal responses to odorants, including brain-derived neurotrophic factor (BDNF) production, as well as changes in adhesion molecules known to regulate axonal convergence. The defective refinement observed in *Mecp2* KO mice was prevented by daily treatment with ampakine beginning after the first postnatal week. These observations indicate that increasing synaptic activity at early postnatal stage might circumvent the detrimental effect of MeCP2 deficiency on circuitry maturation. The present results provide *in vivo* evidence in real time for the role of MeCP2 in activity-dependent maturation of olfactory circuitry, with implications for understanding the mechanism of MeCP2 mutations in the development of neural connectivity.

Keywords

olfactory circuitry; MeCP2; neurodevelopment; autism spectrum disorder; Rett Syndrome

Introduction

Methyl CpG binding protein 2 (MeCP2) is a structural chromosomal protein involved in the regulation of gene expression. Mutations in the gene encoding MeCP2 result in Rett Syndrome (RTT, [OMIM] 312750), a pervasive neurodevelopmental disorder with a frequency of 1/10,000–1/15,000 per live births. RTT is one of few Autism Spectrum Disorders (ASDs) in which the cause is identified as a single gene mutation. Clinical manifestations include disruptions in motor and cognitive development, seizures, motor stereotypies, and features of autism (Amir et al., 1999; Chahrour et al., 2008; Moretti and Zoghbi, 2006; Van den Veyver and Zoghbi, 2001). Abnormal levels of MeCP*2* are associated with other neurodevelopmental disorders, as well as neuropsychiatric disorders (Chahrour et al., 2008). Therefore, the role of MeCP2 in the nervous system has been an area of special interest.

Studies in mouse models of MeCP2 deficiency/dysfunction suggest that this protein is important for several processes including neuronal maturation, neurite complexity, dendritic morphology, synaptogenesis and synaptic plasticity (Ballas et al., 2009; Belichenko et al., 2009a; Belichenko et al., 2009b; Cusack et al., 2004; Jugloff et al., 2005; Larimore et al., 2009; Maezawa and Jin,; Maezawa et al., 2009; Tropea et al., 2009; Wood et al., 2009). While these studies examine mostly symptomatic (~8 week old) mouse brain, and thus make inferences about the early developing brain, few real time developmental studies have been performed to show the consequences of MeCP2 deficiency during the actual formation and maturation of neural circuits. Developmental studies are crucial for elucidating these questions, as findings in the symptomatic brain may reflect compensatory changes, and not the primary defect incurred by MeCP2 dysfunction.

We have extensively validated the olfactory system for modeling the neurodevelopmental defects occurring with *Mecp2* mutations in mice and humans (Cohen et al., 2003; Matarazzo

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et al., 2004; Matarazzo and Ronnett, 2004; Ronnett et al., 2003). The olfactory epithelium (OE) contains olfactory sensory neurons (OSNs), which are bipolar cells, extending an apical dendrite and an axon through the basal lamina to the olfactory bulb (OB). The olfactory axons enter to the OB and terminate in region of neuropil called glomeruli, where they form synapses with mitral and tufted cells, the second order neurons (DeMaria and Ngai, 2010; Munger et al., 2009; Zarzo, 2007). Axons from OSNs form specific and highly stereotyped projections to these higher-order neurons. In the adult, all OSNs expressing the same odorant receptor (OR) "converge" to terminate in a few glomeruli in each OB (Ressler et al., 1994a, b; Vassar et al., 1994), and all OSN axons terminating in these glomeruli express only one type of OR, and are thus termed "homogeneous" (Zou et al., 2004). Both these characteristics of mature glomeruli, convergence and homogeneity, are established during postnatal development through the process of glomerular, and thus synaptic refinement. At early postnatal ages, postnatal day 10 (P10), axons expressing a specific OR initially target multiple glomeruli located in both medial and lateral halves of the OB, while in older animals (>P40) the projection of these axons becomes restricted to a single glomerulus (Kerr and Belluscio, 2006; Zou et al., 2004). This refinement of convergence is dependent on olfactory stimulation during a critical period in development. Absence of sensory stimulus (i.e., naris occlusion) interferes with glomerular maturation at later postnatal ages (Zou et al., 2004), while specific odorant stimulation accelerates glomerular maturation for those glomeruli that receive input from OSNs expressing the cognate receptors for that odorant (Kerr and Belluscio, 2006). The ability to examine and quantify glomerular maturation as an indicator of effective sensory activity renders the olfactory circuit useful in detection of possible defects in the course of the establishment of connectivity and refinement.

Using this system, we previously demonstrated that MeCP2 deficiency/dysfunction results in defects in axonal guidance and in the targeting of incoming OSN axons into the developing glomeruli, which causes a delay in OSN terminal differentiation and persistent abnormalities of synaptic structures within the OB. These defects were described during early postnatal development under standard *in vivo* housing conditions (Degano et al., 2009; Matarazzo et al., 2004; Palmer et al., 2008; Palmer et al., 2012). However, a major aspect of MeCP2 function is that it is regulated by synaptic activity (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006); the role of sensory activity in the consequences of MeCP2 deficiency during early postnatal stages, when sensory activity is critical, is unknown. Here, we use an *in vivo* olfactory (odorant) sensory stimulation paradigm to provide physiological synaptic activity to specific circuits in order to evaluate the consequences of *Mecp2* deficiency on activity-dependent maturation of olfactory circuits. These results may have broad implications for understanding molecular pathophysiology and behavioral deficits that occur with *Mecp2* mutations, and for the clinical findings in ASDs.

Results

Activity-dependent glomerular refinement is impaired in *Mecp2* KO mice

Postnatal glomerular refinement is dependent on sensory stimulation during a critical period of development. Absence of sensory stimulus (i.e., naris occlusion) leads to a decrease in

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mature glomeruli (Zou et al., 2004), while odorant stimulation using odorants known to activate specific ORs accelerates glomerular maturation for those axons whose cells express that OR (Kerr and Belluscio, 2006). Therefore, we determined whether sensory activity (odorant stimulation) could have an effect on the refinement of connectivity, as visualized by the maturation of glomerular targeting in the setting of *Mecp2* deficiency.

To visualize a specific subpopulation of olfactory axons for the purpose of tracking their paths and targeting within the OB, we used M71-IRES-tauGFP and M72-IRES-taulacZ mice that were bred with female *Mecp2* heterozygous mice to generate double mutants (see Experimental Methods) (Potter et al., 2001; Vassalli et al., 2002; Zou et al., 2004). OBs from double mutants M72-WT or M72-*Mecp2* KO mice were processed for whole mount X-gal staining at P14, P28 and P49. This assay permits tracking of the trajectories of M72 axons into the OB and onto specific glomeruli by β-galactosidase expression. Representative images of whole mount staining for M72 mice are shown in Fig. 1A–C. M72 axons from either WT or *Mecp2* KO mice converge onto distinct glomeruli in a highly stereotypic manner (Degano et al., 2009; Mombaerts, 1996; Mombaerts et al., 1996a; Potter et al., 2001; Zou et al., 2004), and the process of glomerular refinement can be assessed by counting the number of glomeruli per half bulb at times during development.

We utilized a protocol for odorant stimulation established by others (Kerr and Belluscio, 2006); acetophenone is an agonist for M71 (Bozza et al., 2002) and M72 receptors (Feinstein and Mombaerts, 2004), and was used for odorant stimulation experiments. Acetophenone (1% diluted in mineral oil) was applied to the nipples of the lactating dam daily from birth to P14, P28 or P49. The control group was treated in the same way, but mineral oil vehicle was used instead of odorant. M72-WT and *Mecp2* KO mice were treated with acetophenone or mineral oil; X-gal staining of whole mounts was prepared at the indicated times, and the numbers of M72 glomeruli were quantified as shown in Fig. 1A and B (arrows).

At P14, WT and KO mice treated with acetophenone showed a similar number of glomeruli per half bulb (Fig. 1D). By P28, WT mice had started to refine olfactory projections, or reduce the number of glomeruli targeted by M72 axons, as demonstrated by the decrease in glomerular numbers compared to P14 mice; this decrease became even more significant by P49 (Fig. 1D). In contrast, M72-*Mecp2* KO mice showed no reduction in the number of M72 glomeruli throughout development, displaying significantly higher glomerular numbers when compared with WT mice of the same ages. These results indicate that *Mecp2* KO mice are not able to refine the number of glomeruli in response to odorants to generate a mature circuitry. It is noteworthy that the chronic treatment with vehicle alone did not induce significant glomerular refinement in both WT and *Mecp2* KO mice (Fig. 1E). Therefore, while no differences in axonal convergence were seen between both groups in the absence of sensory stimulation, a significant difference in the refinement of M72 glomeruli was visualized once sensory activity was introduced at early postnatal stages.

We also evaluated the effect of MeCP2 deficiency on the homogeneity of glomeruli by assessing the maturation of a specific subpopulation of olfactory axons as they converge within glomeruli through immunohistochemical analysis. A mature glomerulus is only

innervated by axons from OSNs expressing the same type of OR (Treloar et al., 2002; Treloar et al., 1999). We used M71-GFP double mutants to quantify the numbers of homogeneous and heterogeneous glomeruli in M71-WT and M71-*Mecp2* KO mice. Coronal sections of OB were double-labeled with antibody to olfactory marker protein (OMP) that is expressed in, and therefore identifies, all mature olfactory axons, while antibody to GFP only labels M71-expressing axons. Glomeruli that show a homogeneous overlap of these two markers are considered as mature M71 glomeruli (Fig. 1F, top panel). In contrast, we defined an heterogeneous glomeruli when it showed distinct $OMP^{+}GFP^{-}$ islands with a size 10% of the total glomerular area, as represented in Fig. 1F (bottom panel), and according to Zou et al (2004). These heterogeneous glomeruli are innervated by axons from OSNs expressing ORs other than M71, and are therefore immature. Using confocal microscopy, we evaluated the number of homogeneous and heterogeneous glomeruli in WT and *Mecp2* KO mice treated with acetophenone from birth. For this, we performed serial analysis for individual M71 (GFP positive) glomeruli in each half bulb, without considering the total count per half bulb. Four M71 glomeruli were assessed in 4 to 5 WT and *Mecp2* KO mice at P49. From this analysis, we found a frequency of 0.63 ± 0.13 heterogeneous glomeruli in P49 *Mecp2* KO mice, and 0.25 ± 0.13 ($p < 0.05$, Chi-square test) in WT mice at the same age. Thus, *Mecp2* KO mice at P49 still displayed significantly more heterogeneous glomeruli than did WT mice (Fig. 1F). These results indicate that the activity-driven maturation of individual M71 glomeruli is also impaired in the absence of MeCP2.

Activity-driven competition for survival is impaired in OSNs from *Mecp2* KO mice

In the olfactory system, as in other neuronal systems, activity fuels a competition for survival among neurons, with the survivors having received activity-related survival cues (Zhao and Reed, 2001; Zhou et al., 2006). In addition to defects in glomerular maturation, deprivation of sensory activity by naris occlusion also leads to defects in the normal competition for survival, and therefore to increased survival of OSNs (Zhou et al., 2006; Zou et al., 2004). Therefore, maturation is associated with an increased rate of turnover (cell death) of OSNs within the OE. We have previously demonstrated a delay in OSN terminal differentiation in *Mecp2* KO mice (Matarazzo et al., 2004), which could in turn alter the rate of activity-dependent OSN turnover in these animals. To address this, we examined OSN proliferation and death by evaluating the numbers of cells expressing Ki-67 (a marker of proliferation) or activated caspase-3 (a cell death marker for OSNs) in the OE after mice were exposed to olfactory stimulation from P1 until P14 or P28 (Cowan and Roskams, 2004).

The number of proliferating cells was higher in younger mice irrespective of genotype (Fig. 2A–C). We only detected a slight increase of $Ki67^{+}$ cells in KO mice after odorant stimulation at P28 (Fig. 2C). Considering that we have previously described a delay of terminal differentiation at P14 in *Mecp2* KO mice, showing lower numbers of mature neurons (Matarazzo et al, 2004), it is possible to suggest that the small increase in proliferation observed at P28 is a compensatory response for that delay.

The analysis of activated caspase-3 positive cells indicated that there was a greater level of cell death in WT mice exposed to odorant stimulation compared to that found in KO mice

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exposed to odorants; *Mecp2* KO mice displayed similar numbers of caspase-3 positive cells with and without stimulation (Fig. 2D–E). Conversely, we noticed a slight decrease or no change in cell death at P28 (Fig. 2F). To our knowledge, there are no studies for time course of cell death after odorant stimulation. It is possible to speculate that odorant stimulation elicits signals for cell death initially but that are not necessarily sustained with the chronic odorant exposure we are using, possibly through a mechanism similar to the process of odor adaptation (Kurahashi and Menini, 1997)

These results indicate that in response to activity, OSNs in WT mice participate in activity-induced competition for survival, which drives the remodeling of axonal projections in this and others systems (Katz and Shatz, 1996; Zhao and Reed, 2001). In contrast, OSNs from *Mecp2* KO mice do not display activity-induced increased competition that would result in increased cell death, and would derive in improved remodeling and functionality.

Mecp2-deficient mice display abnormal activity-induced responses

The process of glomerular refinement is dependent on odorant receptor activity (Imai et al., 2006). Interestingly, it has been shown that MeCP2 is phosphorylated in response to synaptic activity and regulates gene expression, such as BDNF production (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). Therefore, we evaluated the integrity of activity-derived responses in the OB by assessing BDNF expression, since this is a well-characterized model for activity-dependent gene expression, and a target of MeCP2 (Chen et al., 2003; Zhou et al., 2006). For this, both groups of animals were exposed to a mix of odorants or vehicle overnight, and then OB tissues were processed for real time RT-PCR to determine BDNF levels. Given that MeCP2 expression increases developmentally with maturation and reaches its ceiling expression by P49 in the OB (Cohen et al., 2003), we decided to performed acute odorant stimulation studies at that age, in order to discern the effect of MeCP2 deficiency at the time it is normally highly expressed.

WT mice showed an increase in BDNF levels after olfactory stimulation, but this increase was absent in *Mecp2* KO mice (Fig. 3A). Importantly, there were no significant changes in the expression levels of key signaling molecules, including adenylyl cyclase-3 (AC3), cyclic nucleotide gated channel 2 (CNGA2), or in the expression levels of M72-LacZ olfactory receptor in OE from M72-WT and M72-*Mecp2* KO mice (Fig. 3B–D). These findings indicate that the initial events of odorant detection (odorant signal transduction) appear unaffected by the loss of MeCP2; however, in response to activity, MeCP2 deficiency affects downstream events in the transduction pathway, such as activity-driven signaling at the primary synapse in the OB.

Recent work has established that the convergence of axons expressing the same type of OR is dependent upon OR- and CNGA2-evoked signaling cascades that regulate the expression of the homophilic adhesive proteins Kirrel-2/Kirrel-3 and the repulsive proteins ephrin-A5/Eph-A5 (Oztokatli et al., 2012; Serizawa et al., 2006). Kirrel 2 and 3 are adhesion molecules showing complementary patterns of expression in OE and OB, and can act as ligands and receptors on OSN like-axons. Their potential role is to segregate like-axons via homophilic interactions at the axon terminals (Gerke et al., 2005; Minaki et al., 2005; Schneider et al., 1995). Similarly, Eph receptor tyrosine kinases and their ligands, the ephrins, have been

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implicated in the activity-dependent formation of the sensory map in the OB (Cutforth et al., 2003; St John et al., 2002). Therefore, we determined the expression levels of the aforementioned molecules in olfactory tissues from WT and *Mecp2* KO mice after 4 hours of odorant stimulation. OB and OE tissues were processed for real time RT-PCR using primers for Kirrel 2, Kirrel 3, EphA5 and ephrin-A5 as previously described (Henion et al., 2011). Kirrel 3 and ephrin-A5 mRNA levels were similar in WT and *Mecp2* KO under all conditions (Fig. 4B, D, F, and H). In contrast, after acute odorant stimulation, Kirrel 2 and EphA5 expression was decreased in the OB of *Mecp2* KO mice (Fig. 4E, G), whereas the levels were increased in the OE from the same mice (Fig. 4A, C) in comparison with WT littermates. Thus, in the absence of MeCP2, abnormal levels of Kirrel 2 and Eph-A5, known regulators of olfactory axon sorting, were found to correlate with the increase in heterogeneity seen in the glomeruli of these mice. These results suggest that MeCP2 may play a role in the transcriptional regulation of these molecules in response to sensory (odorant) activity.

MeCP2 deficiency leads to an imbalance between excitatory and inhibitory neurotransmission in the OB

Current evidence indicates that MeCP2 deficiency induces an imbalance in excitatory/inhibitory (E/I) transmission, resulting in either hyper-excitation or excessive inhibition (Calfa et al., 2011; Chao et al., 2010; Dani et al., 2005; Medrihan et al., 2008; Monteggia and Kavalali, 2009; Zhang et al., 2008). What is unclear is whether these observations are primary or compensatory in the natural history of MeCP2 deficiency. The regulation of glutamatergic transmission at individual glomerulus in the OB is crucial for refinement of axonal projections and for the maturation of the olfactory map (Biju et al., 2008; Yu et al., 2004). On this basis, we investigated whether such an imbalance occurs in the OB of MeCP2 deficient mice, which could contribute to the observed defective glomerular maturation. We determined the expression levels of vesicular glutamate transporters (VGLUT) 1, which is widely used as a marker for glutamatergic terminals (Bellocchio et al., 2000). We also quantified the expression of glutamic vesicular gamma-aminobutyric acid (GABA) transporters (VGAT), a marker of GABAergic innervations (Chaudhry et al., 1998).

The levels of VGLUT1, and VGAT were determined in OB from 7 week-old *Mecp2* WT and KO mice chronically exposed to vehicle or acetophenone by IHC (Fig. 5). This approach allows monitoring the effect of odorant stimulation on the glomerular layer of the OB (olfactory synapse). We observed lower VGLUT1 expression in *Mecp2* KO mice treated with vehicle, compared with WT mice treated with vehicle at P49 (Fig. 5 A,C,E). Interestingly, chronic odorant stimulation with acetophenone induced increased expression of VGLUT1, as described in other systems (Erickson et al., 2006); however, the levels reached in KO mice were still lower than the WT treated with odorant (Fig. 5 B,D,E). Conversely, the expression of a GABAergic marker (VGAT) was similar in WT and *Mecp2* KO OB treated with Vehicle or Odorant (Fig. 5F–J).

These data suggest that MeCP2 deficiency induces an imbalance in glutamatergic/GABAergic innervation in the OB, resulting in reduced excitation in *Mecp2* KO

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symptomatic mice. Considering that lower glutamatergic neurotransmission (decreased VGLUT1 expression) has been previously reported in the cortex from symptomatic *Mecp2* KO mice (Chao et al, 2007), we propose that this is the primary event for defective E/I balance at the synapse.

In order to confirm this hypothesis, tyrosine hydroxylase (TH)-expressing periglomerular cells (PGCs) were examined. Dopaminergic PGCs contribute to the presynaptic inhibition of OSNs and participate in plasticity of the glomerular circuitry (Hsia et al., 1999; Sawada et al., 2011). Odorant-induced activity has been postulated to regulate the expression of the dopaminergic phenotype within PGC of the OB; either removal of afferent innervation or naris occlusion causes a dramatic decrease in the activity and expression of TH in PGCs (Baker, 1990; Baker et al., 1993; Stone et al., 1990). Thus, TH expression can be analyzed as an indicator of incoming activity into the OB. We performed IHC for TH in OB from 7 week-old *Mecp2* WT and KO mice chronically exposed to vehicle or acetophenone (Fig. 6). As we hypothesized, we found lower TH expression levels in the glomerular layer (Fig. 6 C,E) as well as fewer TH^+ PGCs in the OB from KO mice at P49 (Fig. 6F, J) compared with WT littermates (Fig. 6A, E, F, J). We also confirmed this result by real time RT-PCR (data not shown). Similarly as shown for VGLUT1 expression, the chronic treatment with acetophenone induced a significant increase in TH labeling and TH^+ cells in the WT, indicating increased incoming activity to the OB (Fig. 6 B, E, G, J). Conversely, the increase in TH levels after chronic odorant stimulation was not observed in *Mecp2* KO mice (Fig. 6 D, E, I, J).

It is important to mention that, while TH^+ cells are replaced postnatally from the SVZ (Lledo et al., 2008), the lower numbers found here may be not due to defects in the replacement, since previous evidence from our group and others suggest that MeCP2 is not critical for either embryonic or postnatal neurogenesis (Kishi and Macklis, 2004; Matarazzo et al., 2004; Smrt et al., 2007). Overall, these results indicate that incoming activity from OSN axons into the OB is abnormal in the absence of MeCP2, resulting in ineffective or reduced excitatory input at the level of the primary synapse.

Ampakine treatment improves circuitry refinement in *Mecp2* KO mice

Since our results suggest that MeCP2 deficiency affected the levels of excitatory/inhibitory innervation at the primary synapse in the OB, we tested whether modulating neurotransmission *in vivo* could remediate connectivity defects incurred by MeCP2 deficiency. Ampakines are a diverse family of small molecules that positively modulate α-amino-3-hydroxy5methyl-4-isoxazolepropionic acid (AMPA) receptors and thereby enhance glutamatergic transmission (Lynch, 2006). In fact, ampakine CX546 [1- (1,4-benzodioxan-6-yl-carbonyl) piperidine] has been successfully used in mouse models of RTT (Ogier et al., 2007). In contrast, valproic acid (VPA) is an anticonvulsant and mood-stabilizing drug widely used in the treatment of epilepsy (Davis et al., 1994; Jeong et al., 2003; Laeng et al., 2004; Mattson et al., 1978; Yuan et al., 2001). VPA works by suppressing repetitive neuronal firing through inhibition of voltage-sensitive sodium channels, and by increasing brain concentrations of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter in the CNS (Laeng et al., 2004). To evaluate whether boosting

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either excitatory or inhibitory neurotransmission could improve connectivity refinement in *Mecp2* KO mice, we utilized a chronic treatment paradigm using these drugs *in vivo*.

Mecp2 WT and KO mice were exposed to chronic odorant stimulation from P1 with 1% acetophenone application. At P7, we started each treatment, which consisted of daily subcutaneous (s.c.) injection of CX546 or VPA or the corresponding vehicles, as described in Experimental methods. Considering that the number of M72 glomeruli are significantly reduced (odorant-induced maturation) in WT mice by P28 (Fig. 1), we applied each treatment daily until P28, at which time animals were euthanized and assessment of glomerular numbers was performed. We monitored body weight daily during the treatments, and no significant changes were observed among WT and *Mecp2* KO groups at the tested doses (data not shown). Our results revealed that chronic treatment with CX546 from P7 decreased the number of M72 glomeruli present in *Mecp2* KO OB to the numbers seen in WT mice of the same age. Interestingly, a reduction of M72 glomerular number was observed also in CX546-treated WT mice (Fig. 7A, WT:Odorant vs. WT:Vehicle p=0.0591, Two-way ANOVA). Since it has been reported that ampakine treatment increases the levels of glutamatergic activity and BDNF levels (Lynch, 2006; Lauterborn et al., 2000; Mackowiak et al., 2002), it is not surprising that the treatment may accelerate glomerular maturation in WT mice. In contrast, VPA injected daily at the indicated dose did not affect glomerular numbers in any of the tested groups (Fig. 7B). Altogether, our results indicate that defective excitatory input is the primary event incurred by MeCP2 deficiency, and that increasing excitatory neurotransmission at early postnatal ages may be an effective way of overcoming the detrimental effects of MeCP2 deficiency on the maturation of connectivity in response to activity.

Discussion

In this study, we demonstrate that MeCP2 enables synaptic activity to influence the refinement and maturation of neuronal connections during critical periods of development. Specifically, MeCP2 is required for activity-driven competition for survival; its absence is associated with abnormal expression of Kirrel 2 and EphA5, known adhesion molecules and regulators of olfactory axon sorting. The primary defect in the E/I imbalance seen with MeCP2 deficiency appears to be defective excitatory transmission, which is compensated by decreasing inhibitory activity, and which can be averted by increasing excitatory transmission during the critical period.

We employed the developing olfactory system and an *in vivo* sensory (odorant) stimulation model to provide physiological synaptic activity to evaluate the consequences of MeCP2 deficiency on activity-dependent maturation and refinement of neuronal circuits. The development of precise connectivity in the olfactory circuit relies on guidance molecules whose expression is regulated by OR-derived transduction signals and neuronal activity (Chesler et al., 2007; Imai and Sakano, 2009; Imai et al., 2006; Imai et al., 2009). We define a novel link by which MeCP2 and neuronal activity act in concert to regulate the postnatal refinement of the olfactory map. Our results show that MeCP2 deficiency leads to defective connectivity of the olfactory circuit, with increase in heterogeneous and supernumerary glomeruli that failed to be refined during development. This alteration in glomerular

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refinement was associated with decreased incoming activity to the primary synapse in the OB, alterations in BDNF and adhesion molecules expression in response to olfactory activity (Fig. 8). These results may have broad implications for understanding molecular pathophysiology and behavioral deficits that occur with *Mecp2* mutations, and for the clinical findings in ASDs.

We have used the olfactory system to model neurodevelopmental defects of MeCP2 dysfunction (Degano et al., 2009; Matarazzo and Ronnett, 2004; Palmer et al., 2008; Ronnett et al., 2003). OSNs expressing the same OR converge their axons to a specific set of glomeruli in the OB, generating a stereotypic olfactory map (Mombaerts, 1996; Mombaerts et al., 1996a; Mombaerts et al., 1996b; Ressler et al., 1994a; Vassar et al., 1994). During early development, some glomeruli are innervated by axons of neurons that do not express the same receptor. These “heterogeneous” glomeruli disappear with age, in a process of glomerular refinement that is dependent on sensory activity (Kerr and Belluscio, 2006; Zou et al., 2004). These attributes were exploited to address fundamental and unresolved questions regarding the roles of MeCP2 during active phases of neurodevelopment, a time during which patients with *Mecp2* mutations demonstrate the onset of symptoms.

Previously, we demonstrated that MeCP2 deficiency/dysfunction induces a delay in OSN terminal differentiation, defective axonal guidance and abnormalities of olfactory synapses. Importantly, although *Mecp2* KO mice showed alterations in laminar targeting in the OB and the accessory olfactory bulb (AOB) during the first postnatal weeks, axonal convergence to glomeruli in the right dorso-ventral and anterior-posterior (A-P) position was preserved compared to control WT mice in the absence of sensory activity for M72 populations of axons (Degano et al., 2009). The alterations detected in *Mecp2* KO mice were associated with changes in Sema3F signaling, but no significant differences were found in the expression of molecules that regulate A-P localization, i.e., Sema 3A and Np1 (Degano et al., 2009; Imai and Sakano, 2008). A major aspect of MeCP2 function is that it is regulated by synaptic activity (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006), but the consequences of MeCP2 deficiency during early postnatal stages, when sensory activity is critical, were unknown. In the present work, we evaluated the role of activity in the refinement of olfactory circuits induced by odorant stimulation in the absence of MeCP2. We used a transgenic mouse model that permitted visualization of subpopulations of OSN expressing the OR M72 or M71 in the context of MeCP2 deficiency. While WT mice responded to chronic stimulation by refining the number of M72 glomeruli during the first postnatal weeks (Fig. 1), no decrease in M72 glomerular number was observed during further development in *Mecp2* KO mice. Similarly, the percentage of heterogeneous/immature M71 glomeruli was higher in MeCP2 deficient mice, consistent with the lack of maturation of this circuitry. These data suggest that MeCP2 deficiency disrupts activity dependent responses needed for circuitry refinement.

We evaluated the rate of OSNs turnover in WT and *Mecp2* KO mice chronically exposed to vehicle or acetophenone (Fig. 2). Our results showed that in response to activity, OSNs in WT mice participate in activity-induced competition for survival (increased cell death), which would result in improved remodeling and functionality (Katz and Shatz, 1996; Zhao and Reed, 2001). In contrast, OSNs from *Mecp2* KO mice do not display activity-induced

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increased competition that would result in lack of glomerular refinement. Interestingly, Kerr and Belluscio (2006) performed similar odorant stimulation experiments and found no alterations in neuron proliferation and survival. However, they examined a transgenic OR, rI7-M71, generated by receptor "swap", in which the rat *I7* coding sequence replaces the mouse *M71* coding sequence at the *M71* locus (Bozza et al., 2002). In our experiments, we assessed the endogenous M71 glomeruli, and the OSNs expressing it may have different threshold for survival than the transgenic rI7-M71. In this sense, a recent publication examined the expression patterns of fifteen OR genes in mice after 4 weeks of unilateral naris closure. The cell density in the sensory deprived side was either: significantly lower, similar or significantly higher than in the open side, according to the OR they expressed. Therefore, this study suggests that sensory stimulation may have differential effects on OSNs expressing different OR genes (Zhao et al., 2013).

The current view for the olfactory map development indicates that OR-dependent cAMP signaling and neuronal activity determines the expression levels of axon guidance and adhesion molecules that influence glomerular positioning and axon sorting(Imai and Sakano, 2009; Imai et al., 2006; Imai et al., 2009; Serizawa et al., 2006). In this respect, members of the Kirrel family of adhesion molecules and ephrins have been proposed to play a role in the segregation of OR-like axons via homophilic or repulsive interactions (Brown et al., 2000; Serizawa et al., 2006). Considering MeCP2 is a transcriptional regulator, we hypothesize that the lack of glomerular refinement in MeCP2 deficiency could be due to alterations in the expression of key molecules for odor signaling and axon sorting. The expression levels of the OR M72, AC3, and CNGA2 were not affected in *Mecp2* KO OSNs (Fig. 3B–D), indicating that the main components for odor-induced signaling pathway in the OE that could influence activity-induced circuit refinement were preserved. However, acute olfactory stimulation experiments showed that the expression of adhesion molecules involved in axon sorting was altered in *Mecp2* KO mice (Fig. 4). In particular, Kirrel2 and EphA5 levels appeared up-regulated in *Mecp2* KO OE, while the OB showed decreased levels of these molecules after olfactory stimulation. Likewise, Kirrel2 and EphA5 appear down regulated in CNGA2 KO mice, where the odor-evoked cation influx is disrupted, causing the absence of sensory activity (Oztokatli et al., 2012; Serizawa et al., 2006). These observations are consistent with a disruption of activity-dependent responses at the synapse level in the OB from *Mecp2* KO mice, while OSNs expressing higher than normal levels might reflect a compensatory response for the lack of activity reaching the target. Indeed, we demonstrated here that odorant stimulation elicited an increase in the expression of the glutamatergic marker VGLUT1 as well as TH expression in the olfactory synapse of WT mice, that was absent or minimal in *Mecp2* KO mice (Figs. 5 and 6). Altogether, our results suggest that the lack of glomerular maturation in the absence of MeCP2 could derive from deficient glutamatergic/excitatory inputs reaching the OB after odorant stimulation.

In addition, our results demonstrate that MeCP2 is required for normal levels of BDNF expression in the OB after olfactory stimulation (Fig. 3A). The function of BDNF in the OB has been controversial. Studies using BDNF promoter–mediated β-galactosidase expression demonstrated that BDNF is mainly expressed in a subset of GABAergic periglomerular and external tufted cells in the OB (Clevenger et al., 2008). These results are consistent with a

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proposed role for BDNF in the modulation of activity-dependent competition in axonal branching among OSNs within the glomeruli. In addition, mice deficient for the BDNF receptor p75 neurotrophin receptor (NTR) display aberrant axonal branching that results in the formation of extraneous glomeruli (Tisay et al., 2000). Thus, it is possible that lack of activity-induced BDNF in the OB from *Mecp2* KO mice is mediating the lack of glomeruli refinement.

BDNF is a target of MeCP2 transcriptional regulation and its levels increase after activity-induced phosphorylation of MeCP2 (Martinowich et al., 2003). Interestingly, low levels of BDNF have been shown in cortex from symptomatic *Mecp2* KO mice (Chang et al., 2006) and these results were explained by reduced cortical activity observed in this mouse model (Dani et al., 2005). Our present results show that BDNF levels were similar in the OB from WT and *Mecp2* KO symptomatic mice, although BDNF failed to increase after olfactory stimulation (Fig. 3A). In the absence of MeCP2, the actual BDNF levels seem to be dependent upon the local level of activity and the CNS region.

Interestingly, it has been shown that increasing BDNF levels in nodose ganglia cells by ampakine treatment *in vivo*, can improve breathing frequency in *Mecp2* KO mice (Ogier et al., 2007). Ampakines are modulators of AMPA-type glutamate receptors, and therefore increase excitatory transmission (Nagarajan et al., 2001). In addition to their acute action in excitatory transmission, ampakines have the ability to increase BDNF expression *in vitro* and *in vivo* (Lauterborn et al., 2000; Mackowiak et al., 2002). Our present results show that treatment with ampakines from P7 prevents the defective refinement of the olfactory system observed in the absence of MeCP2. In this sense, we observed a decrease of dopaminergic PGCs in the OB from symptomatic *Mecp2* KO mice, indicative of reduced excitatory input at the level of the primary synapse (Fig. 6). However, we detected no changes in the total numbers of GABAergic PGCs (VGAT-positive cells) in *Mecp2* KO mice (Fig. 5F–J) and moreover, increasing GABAergic function via VPA treatment had no effect on olfactory maturation (Fig. 7B). These results imply that defective excitatory input is the primary event incurred by MeCP2 deficiency, and that increasing excitatory neurotransmission at early postnatal ages may be an effective way of circumvent the detrimental effect of MeCP2 absence on the maturation of olfactory connectivity. Treatment with ampakines during early postnatal age may maintain the proper E/I balance, and improve the expression of guidance and adhesion molecules important for glomerular maturation, as well as BDNF (Lauterborn et al., 2000; Mackowiak et al., 2002). BDNF has proven to be important for glomerular maturation (Tisay et al., 2000), as well as it regulates vesicular glutamate transporters expression (Melo et al., 2013). Future work will explore the mechanism underlying the effect of ampakine treatment on the olfactory circuit maturation in *Mecp2* KO mice.

Neuronal activity induces the phosphorylation of MeCP2 in neurons *in vitro* and *in vivo,* and this modification was required for MeCP2-dependent regulation of dendritic patterning, spine morphogenesis, and activity-dependent expression of several genes, like BDNF (Chen et al., 2003; Martinowich et al., 2003; Tao et al., 2009a; Tao et al., 2009b; Zhou et al., 2006). It is possible that MeCP2 plays a similar role in OSNs in response to odorant stimulation. Our results support a role for MeCP2 in modulating signaling pathways downstream of the receptive events of odorant signal transduction, possibly by modulating

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the expression of essential molecules for the postnatal refinement of the olfactory circuit like BDNF and adhesion molecules.

Conclusions

Neuronal activity contributes to the organization of the nervous system through axonal remodeling and regulation of cell survival. In this report, we define a novel link by which MeCP2 and neuronal activity act in concert to regulate the development and maintenance of precise neural connections. In addition, we present an *in vivo* model for testing potential treatments to improve activity-dependent maturation of neural circuits.

Experimental methods

Mice

Mouse use was conducted in accordance with Institutional Animal Care and Use Committee at the Johns Hopkins University, and all applicable guidelines from the National Institute of Health "Guide for the Care and Use of Laboratory Animals" were followed. *Mecp2* heterozygous mice were provided by Dr. Rudolf Jaenisch (*Mecp2* KO mice) (Chen et al., 2001). M71-IRES-tauGFP (M71 mice) or M72-IRES-taulacZ (M72 mice) gene-targeted male mice on a mixed 129/SvEv/C57BL/6 background were bred with female *Mecp2* heterozygous mice on a BALB/c background to generate double mutants(Conzelmann et al., 2001; Royal and Key, 1999). The double mutants (M71-GFP homozygous/Mecp2 heterozygous or M72-LacZ homozygous/Mecp2 heterozygous) were obtained in the 3rd generation and afterwards they were kept in the same mixed 129/SvEv-C57BL/6-BALB/c background. Females M71-GFP/*Mecp2* heterozygous were then bred with males M71-GFP WT and all the analyses were carried out using mice after the 8th generation of inbred crosses. We only used males M71-GFP *Mecp2* WT or KO and M72-LacZ *Mecp2*WT or KO to avoid complications of X-inactivation. This approach allowed us to track a subset of axons destined for convergence onto a very limited group of glomeruli and therefore to test the effect of MeCP2 deficiency in the connectivity of this circuitry.

Odorant stimulation

M71 or M72 mouse pups (*Mecp2* WT or KO littermates) were raised with or without odorant stimulation. Control group was treated with mineral oil (Sigma) and odorant group with 1% acetophenone (Sigma) diluted in mineral oil. Chronic odorant stimulation was conducted by applying 1% acetophenone daily to the beddings and the nipples of the lactating mother. The treatments started at P1 (postnatal day 1) and continued until the mice were sacrificed at P14, P28 or P49. For acute stimulation of odorant, *Mecp2*WT and KO mice were exposed to a mixture of 3 odorants at concentrations that we previously described (Moon et al., 1999). Six to eight weeks old male WT and KO mice were used for acute odorant treatment.

Ampakine treatment

CX546 (1-(1,4-benzodioxan-6-yl-carbonyl) piperidine, Sigma) was applied subcutaneously daily at a dose of 40mg/kg in 16.5% 2-hydroxypropyl-β-cyclodextrin (vehicle), starting at

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P7 and continued until the mice were sacrificed at P28. Vehicle was diluted to 16.5% in sterile water and filtered through a 0.22μm filter.

Valproate treatment

Valproic acid sodium salt (Sigma) was injected daily subcutaneously at a dose of 30mg/kg in saline starting at P7 and continued until the mice were sacrificed at P28. Control group was injected with saline.

Immunohistochemistry

Olfactory tissues comprising OE and OB were harvested from male *Mecp2* WT and KO mice at P14, P28 and P49, after perfusion with ice cold PBS and 4% PFA (Sigma). Coronal 15 μm sections were prepared, and IHC performed according to our established protocols. Primary antibodies include: Ki67 (Chemicon,1:200), active caspase-3 (BD, Biosciences, 1:2000), TUJ1 (Covance, 1:1000), olfactory marker protein (OMP, Wako Pure Chemical Industries, 1:3000), β-galactosidase (Chemicon, 1:500), Green Fluorescent Protein (GFP, Chemicon, 1:2000), Tyrosine Hidroxylase (TH, Chemicon, 1:250); VGLUT1 (Chemicon, 1:1000), VGAT (Synaptic Systems, 1:2500). Secondary antibodies (Jackson ImmunoResearch) include Cy3 donkey anti-rabbit IgG (1:500), Alexa633 anti-goat (1:1000), FITC donkey anti-rabbit or -mouse IgG (1:50) to facilitate double or triple labeling. Images from OE were collected using a Zeiss Axioskop with a digital camera (Axiocam; Zeiss). Cell counts and OE length measurements were performed using Openlab 3.0.9 software (Improvision Inc., Lexington, MA). Serial images of individual glomeruli were taken in a Pascal 5 confocal microscope (Carl Zeiss, Germany). The measurement for fluorescence intensity of axonal terminals and cell counting for double-labeled OB neurons in Figs. 5 and 6 were performed with Image-J software (NIH). Fluorescence intensity from the WT-vehicle group was considered 100% and the other groups were then normalized accordingly.

X-gal staining

For whole mounts, dissected OE and OB were fixed in 4% PFA in 0.1 M phosphate buffer pH 7.4, for 30 min, and rinsed with PBS. To reveal activity of the enzymatic reporter β-galactosidase (β-gal), bulbs were incubated in 5 mM potassium-ferricyanide, 5 mM potassium-ferrocyanide and 0.5 mg/ml X-gal in PBS at 37°C overnight (Zou et al., 2004).

Real time quantitative RT-PCR

Real time quantitative RT-PCR was performed as previously described (Degano et al., 2009). Briefly, tissues were collected and immediately frozen in liquid nitrogen and homogenized using a FastPrep® Instrument (MP Biomedicals). Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Genomic DNA was digested with 1 U of DNase I (Invitrogen). cDNA was produced using the TaqMan RT-PCR kit (Invitrogen). Real time PCR was carried out on an iCycler (BioRad) by using a reaction mixture with SYBR Green as the fluorescent dye (BioRad), a 1/10 vol of the cDNA preparation as template, and 250 nM of each primer (Henion et al, 2011). The cycle used for

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PCR was as follows: 95 °C for 180 s (1 time); 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s (40 times); and 95 °C for 60 s (1 time).

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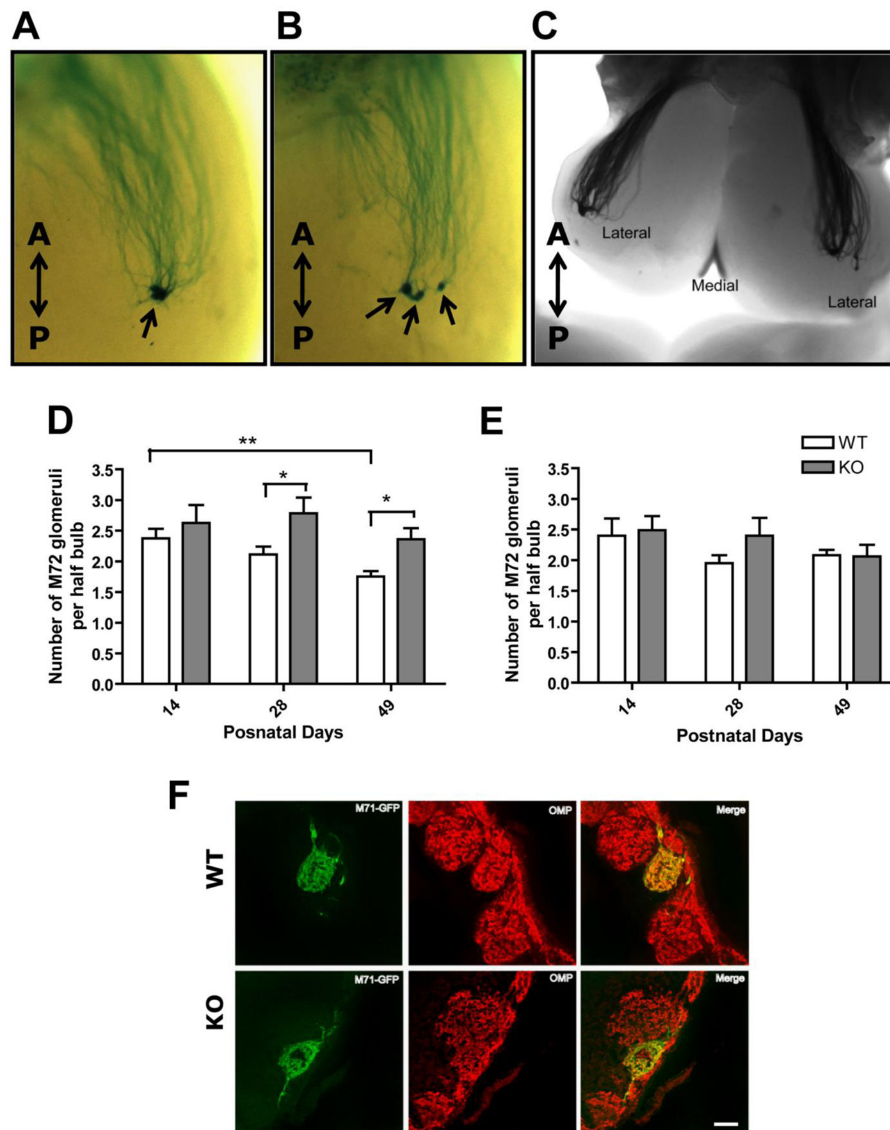

Fig. 1. Activity-dependent refinement of olfactory circuits induced by odorant stimulation is abnormal in the absence of *Mecp2*

(A–B) Representative pictures of M72 glomeruli in OB whole-mount staining with X-gal from *Mecp2* WT (A) and KO mice (B) at P28. **(C)** Whole-mount view of lateral and medial glomeruli. **(D)** The activity-dependent glomerular refinement is disrupted in *Mecp2* KO mice. Male WT and *Mecp2* KO mice were exposed to chronic olfactory stimulation with 1% acetophenone from P1 to P14, P28 or P49.OBs were collected and processed for whole-mount X-gal labeling and the number of M72+ glomeruli per bulb was assessed as shown in Fig. 1A–C. Results are expressed as number of glomeruli per half bulb, mean ± s.e.m.; n=6–9 mice/group; * $p<0.05$, ** $p<0.01$, unpaired t-test. **(E)** The baseline for glomerular refinement is not significantly different in *Mecp2* KO and WT mice. Male WT and *Mecp2* KO mice were treated with mineral oil from P1 to P14, P28 or P49. OBs were collected and processed as explained **D**. Results are expressed as number of glomeruli per half bulb, mean ± s.e.m.; n=6–9 mice/group; unpaired t-test. **(F)** P49 *Mecp2* KO mice show more heterogeneous/immature glomeruli than WT mice. Maturation of M71-positive glomeruli was assessed in coronal sections of OB double immunostained for OMP (red) and GFP (green). Representative pictures from mature or homogeneous M71 glomeruli (top panel) and immature or heterogeneous M71 glomeruli (bottom panel). Scale Bar: 50 µm.

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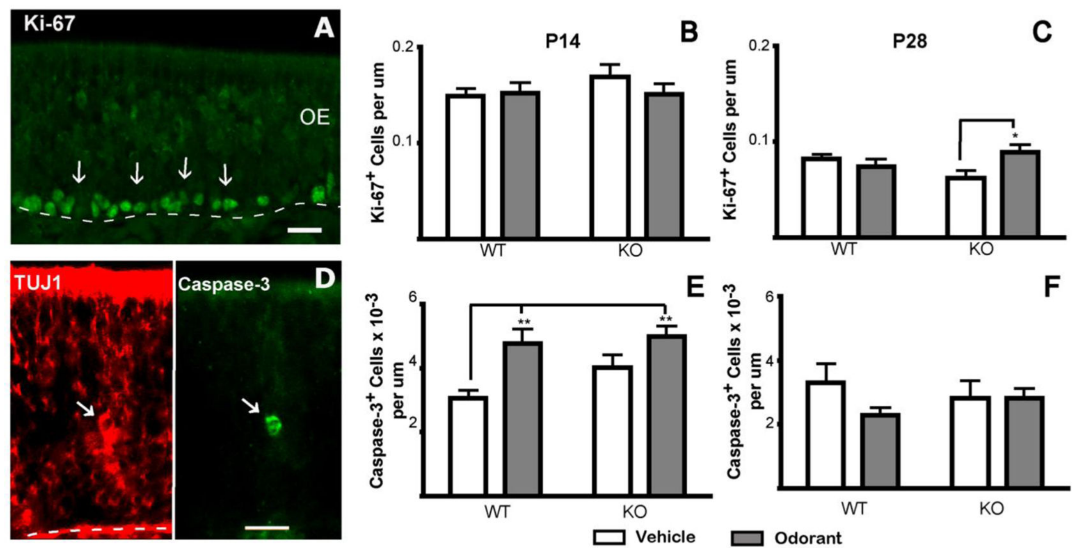

Fig. 2. OSN from *Mecp2* KO mice show impaired activity-driven competition for survival

WT and *Mecp2* KO mice were exposed to chronic olfactory stimulation from birth. At P14 or P28, mice were sacrificed and olfactory tissues processed for immunohistochemistry using Ki-67 **(A)** or active Caspase-3 antibodies (green), TUJ1 (red) **(D)**. Both images are representatives from WT mice; Scale Bar: 20 μm. In each case, positive OSNs were visualized in the olfactory epithelia lining the septum and the number of positive cells per um of OE was calculated and represented **(B, C, E, F)**. *Mecp2* KO OSNs do not show significant increment in cell death in response to odorants, indicative of a lack of competition for survival. *p<0.05, **p<0.01; two-way ANOVA with Tukey *post hoc* test.

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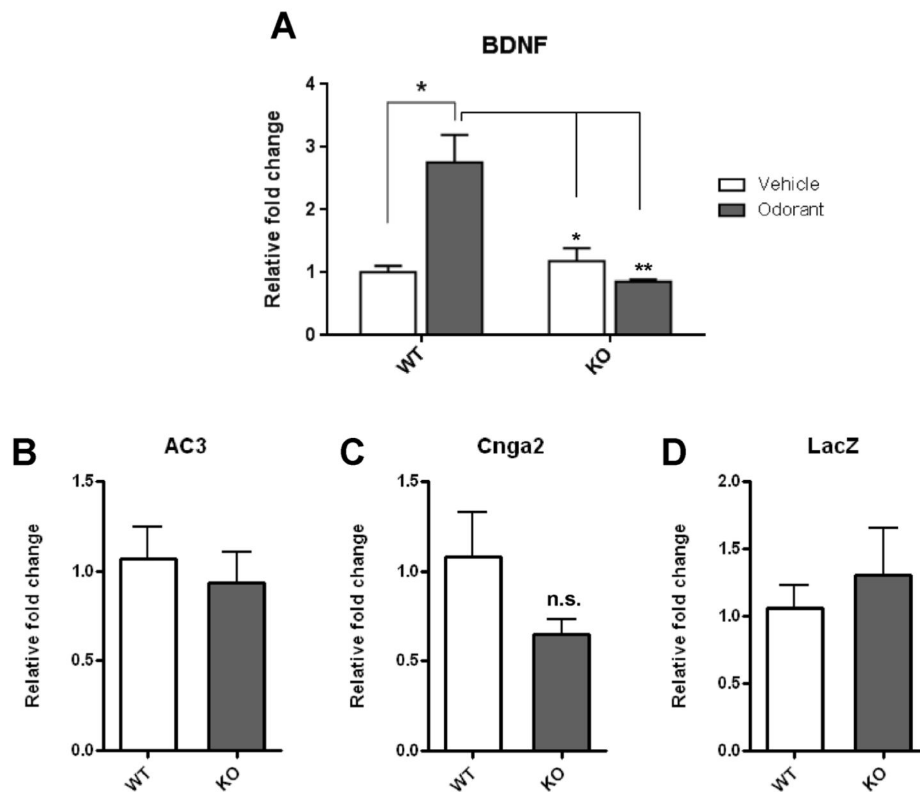

Fig. 3. Odorant-induced BDNF expression is abnormal in OB from *Mecp2 KO* mice, without changes in the expression of odor-transduction molecules in OSNs

(A) The expression level of BDNF was quantified after odorant stimulation in WT and *Mecp2* KO mice at P49. OB tissues were processed for real time RT-PCR using primers as noted. *p<0.05; **p<0.01; two-way ANOVA with Tukey *post hoc* test. All graphs represent mean ± s.e.m; n=5–6 mice/group.**(B–D)** OE from WT and *Mecp2* KO mice at P49 were processed for real time RT-PCR, and specific primers were used to quantify mRNA levels for M72 olfactory receptor, AC3 and CNGA. We observed no significant changes in the level of expression of the tested genes between the two groups, unpaired-t-test.

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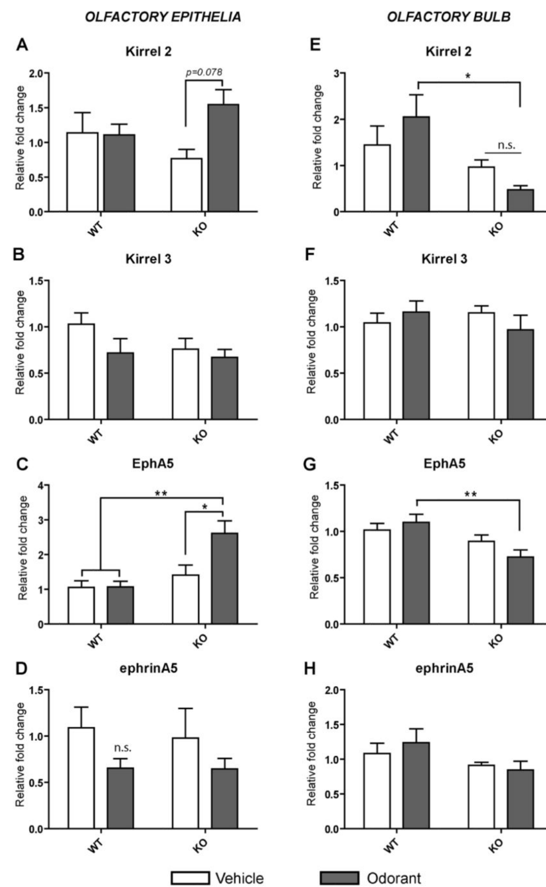

Fig. 4. Differential activity-dependent expression of adhesion molecules in OB and OE from *Mecp2* KO mice

The expression levels of adhesion molecules were quantified after 4 hrs odorant stimulation in WT and *Mecp2 KO* mice. OB tissues **(A–D)** or OE tissues **(E–H)** obtained from P49 old mice were processed for real time RT-PCR using specific primers for *Kirrel* 2, *Kirrel* 3, *ephrinA5* and *Eph-A5* (see methods). Each column represents the mean fold change ± s.e.m; n=5–6 mice/group. *p<0.05; **p<0.01; two-way ANOVA with Tukey *post hoc* test.

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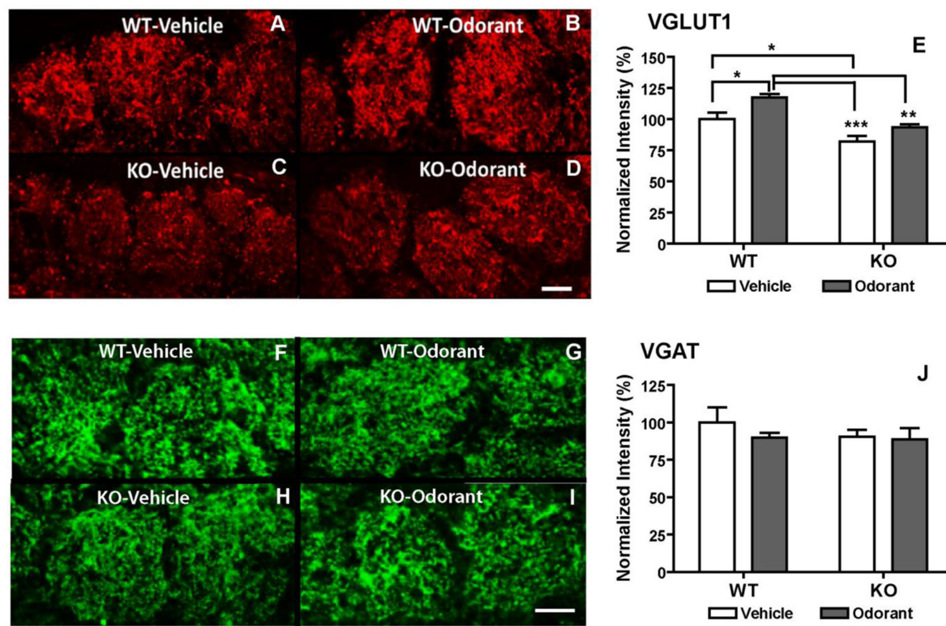

Fig. 5. Excitatory/inhibitory imbalance in the olfactory circuit of *Mecp2* KO mice

IHC was performed in OB sections from 7 week-old *Mecp2* WT and KO mice chronically exposed to vehicle or acetophenone. The expression of vesicular glutamate transporter (VGLUT) 1 (**A–D**) and vesicular GABA transporter (VGAT) (**F–I**) were used as markers of glutamatergic and GABAergic synapses, respectively. Scale Bar: 20 μm. **(E, J)** Measurements for fluorescence intensity of axonal terminals were performed with Image-J software (NIH). Fluorescence intensity from the WT-vehicle group was considered 100% and the other groups were then normalized accordingly. Each column represents the mean percentage ± s.e.m; n=4–5 mice/group. *p<0.05, **p<0.01, ***p<0.001; two-way ANOVA with Tukey *post hoc* test.

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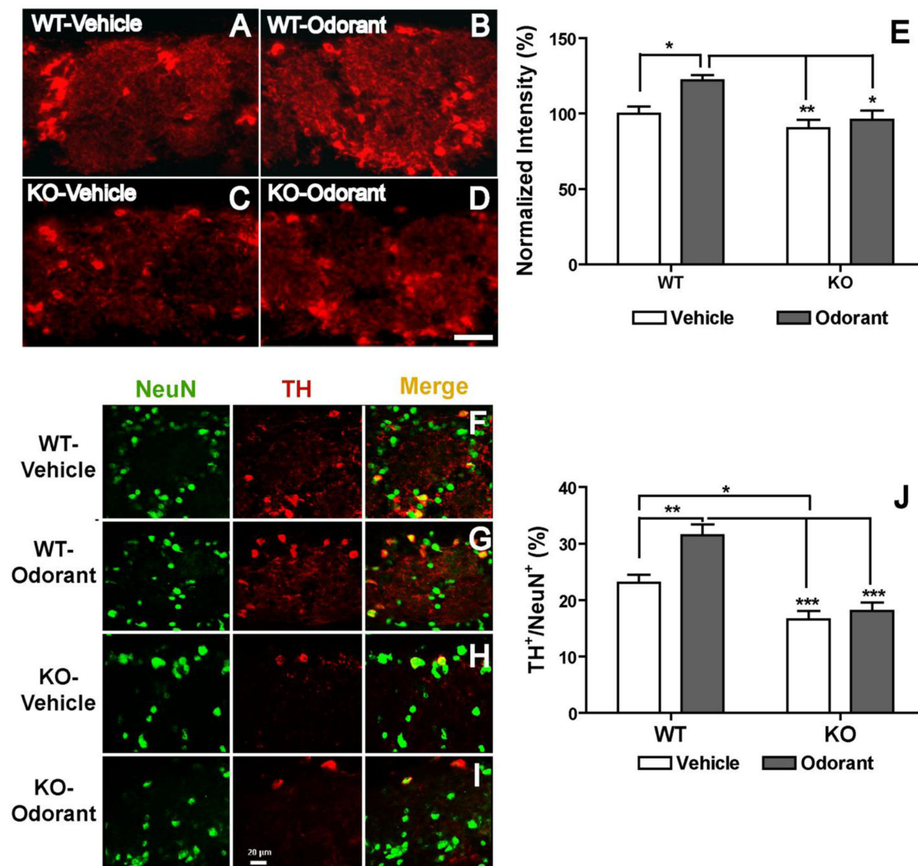

Fig. 6. Decreased tyrosine hydroxylase (TH) expression and TH$^+$ periglomerular cells in OB from *Mecp2* KO mice

(A–D) TH immunoreactivity in OB from 7 week-old *Mecp2* WT and KO mice chronically exposed to vehicle or acetophenone (odorant). Scale Bar: 20 μm. **(E)** Measurements for fluorescence intensity of axonal terminals were performed with Image-J software (NIH). Fluorescence intensity from the WT-vehicle group was considered 100% and the other groups were then normalized accordingly. *p<0.05, **p<0.01; two-way ANOVA with Tukey *post hoc* test. Each column represents the mean percentage ± s.e.m; n=4–5 mice/group. **(F–I)** Immunolabeling of TH$^+$ (red) and NeuN$^+$ (green) periglomerular cells (PGCs) in OB from 7 week-old *Mecp2* WT and KO mice chronically exposed to vehicle or odorant. Scale Bar: 20 μm. **(J)** Cell counting for TH$^+$ PGCs was performed with Image-J software (NIH) and represented as percentage of NeuN$^+$ PGCs. Each column represents the mean percentage ± s.e.m; n=4–5 mice/group. *p<0.05, **p<0.01, ***p<0.001; two-way ANOVA with Tukey *post hoc* test.

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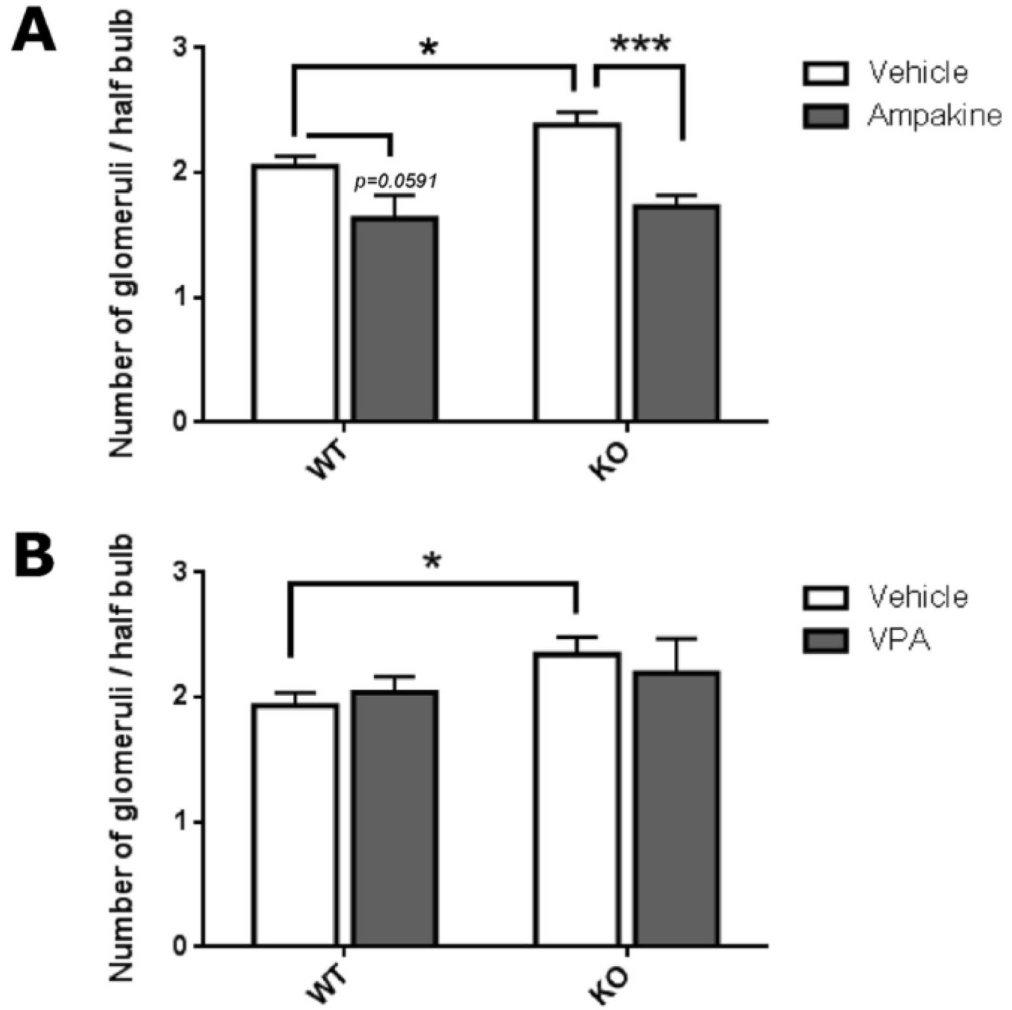

Fig. 7. Assessment of olfactory circuit refinement after pharmacological modulation of glutamatergic activity

(A) Increased excitation by ampakine improves glomerular organization in *Mecp2* KO mice. *Mecp2* WT and KO mice were treated with vehicle or ampakine daily for 21 days from P7 and with 1% acetophenone from birth. * $p<0.05$; ***$p<0.0001$; unpaired t-test; n=8. All graphs represent mean ± s.e.m. **(B)** Increased inhibition by valproic acid does not improve glomerular organization in *Mecp2* KO mice. *Mecp2* WT and KO mice were treated with vehicle or VPA daily for 21 days from P7 and with 1% acetophenone from birth. *$p<0.05$, ***$p<0.001$; unpaired t-test; n=8–10. All graphs represent mean ± s.e.m.

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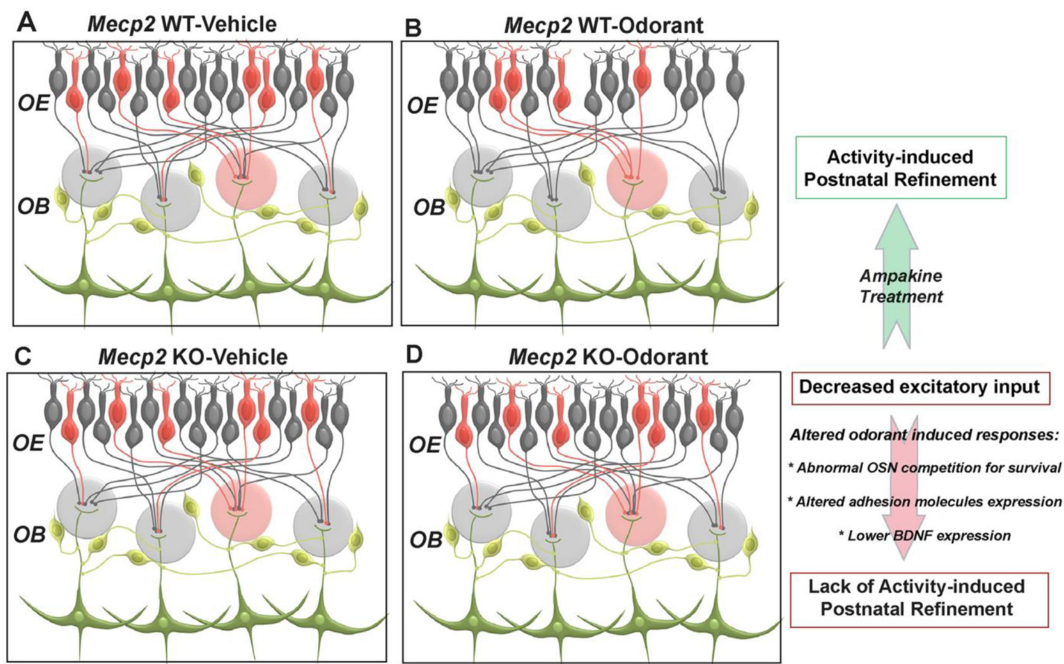

Fig. 8. Activity-dependent refinement is impaired in the absence of MeCP2

During the first postnatal days, axons expressing a specific OR initially target multiple glomeruli (**A**), while in older animals (>P40) the projection of these axons becomes restricted to a single glomerulus (**B**). This refinement of convergence is accelerated by odorant stimulation: WT animals chronically treated with odorant, reach significant higher glomerular refinement than WT mice treated with vehicle (**A–B**). Conversely, in the absence of MeCP2, there is a lack of glomerular refinement even after chronic odorant treatment (**C–D**). We showed that this lack of refinement was accompanied by abnormal odorant-elicited responses for OSN survival, BDNF and adhesion/repulsion molecules expression and a significant reduction in the excitatory input to the OB. Since increasing excitatory responses with ampakines improved glomerular maturation in *Mecp2* KO mice, we propose that defective excitatory input is the primary event incurred by MeCP2 deficiency leading to abnormal circuitry maturation.