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Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis

Deborah R.S. Cohen,[a,1] Valéry Matarazzo,[a,1] Amy M. Palmer,[a] Yajun Tu,[a] Ok-Hee Jeon,[a,c] Jonathan Pevsner,[a,c] and Gabriele V. Ronnett[a,b,*]

[a] *Department of Neuroscience, The Johns Hopkins School of Medicine, Baltimore, MD 21205, USA*
[b] *Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*
[c] *Department of Neurology, The Kennedy Krieger Institute, Baltimore, MD 21205, USA*

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Abstract

Rett syndrome, a neurodevelopmental disorder hypothesized to be due to defective neuronal maturation, is a result of mutations in the *mecp2* gene encoding the transcriptional repressor methyl-CpG binding protein (MeCP2). We utilized the olfactory system, which displays postnatal neurogenesis, as a model to investigate MeCP2 expression during development and after injury. MeCP2 expression increased postnatally, localizing to mature olfactory receptor neurons (ORNs) and sustentacular supporting cells. The timing of MeCP2 expression was defined by using detergent ablation (to remove the ORNs) and unilateral olfactory bulbectomy (to remove the ORN target), both of which increase neurogenesis. MeCP2 expression in the ORNs reached prelesioning levels as cells matured after ablation, whereas expression was not completely restored after bulbectomy, in which functional synaptogenesis cannot occur. Thus, MeCP2 expression correlates with the maturational state of ORNs, and precedes synaptogenesis. Identifying the time window of MeCP2 expression should help further clarify the biological defects in Rett syndrome.

Introduction

Neuronal development is a complex process that requires transitions between cellular programs for precursor proliferation, differentiation of neuronal precursors, and survival. Transcription factors coordinate the changes in the protein expression profiles that accompany these transitions (Shirasaki and Pfaff, 2002). Transcriptional repressors additionally silence the expression of proteins that may interfere with progression through these cellular programs (Johnson and McKnight, 1989). Evidence for the importance of transcriptional regulators during neuronal development comes from Rett syndrome (RTT) (Amir et al., 1999; Dragich et al., 2000).

RTT is an X-linked neurodevelopmental disorder affecting primarily females that results in profound mental retardation (Woodyatt and Ozanne, 1992) and motor dysfunction (Nomura and Segawa, 1990). Onset of symptoms generally occurs between 6 and 18 months of age, a critical time in development (Dunn, 2001). A number of studies point to a persistence of an immature nature of the nervous system in Rett patients. In the cortex, reduction in MAP2 staining has been found in RTT, suggesting abnormal development of dendrites (Kaufmann et al., 1995; 1997). There is also reduced dendritic branching (Armstrong et al., 1995; Belichenko et al., 1997), fewer neurons in layers II–III than V–VII, and a paucity of dendritic spines in pyramidal neurons, further suggesting a defect in synaptic development. In situ hybridization and immunohistochemical studies indicate the presence of MeCP2 primarily in mature neuronal populations (Akbarian et al., 2001; Coy et al., 1999; LaSalle et al., 2001; Shahbazian et al., 2002).

RTT is due to mutations in the *mecp2* gene (Amir et al., 1999). MeCP2 belongs to a family of transcriptional repres-

* Corresponding author. Departments of Neuroscience and Neurology, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA. Fax: +1-410-614-8423.
E-mail address: gronnett@jhmi.edu (G.V. Ronnett).
[1] These authors contributed equally to this study.

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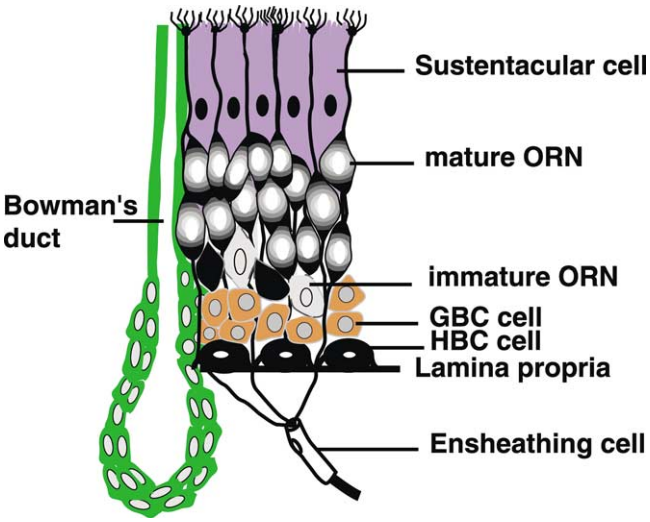


Fig. 1. Schematic of olfactory epithelium illustrating the disposition of the different cell types within the epithelium. Abbreviations: GBC, globose basal cell; HBC, horizontal basal cell; ORN, olfactory receptor neuron.

sors. It was first characterized by its binding activity toward methyl-CpG DNA base pairs (Lewis et al., 1992; Meehan et al., 1992); hence, the name methyl-CpG binding protein 2. MeCP2 binds to DNA in a protein complex that consists of histone deacetylases 1 and 2 and sin3A (Jones et al., 1998). Methylation is developmentally regulated, and is one mechanism whereby certain developmental programs proceed due to the silencing of certain genes. Recently, the developmentally regulated methylation of the GFAP (glial fibrillary acidic protein) promoter has been demonstrated (Takizawa et al., 2001). In embryonic mouse brain, the methylation status of this promoter is crucial in determining the timing of glial differentiation.

Understanding the cellular dysfunction that results from a mutation in a transcriptional regulator such as MeCP2 may be quite daunting, due to the large number of genes potentially affected. The role of MeCP2 may be clarified using the olfactory system, an excellent neurodevelopmental model (Graziadei and Graziadei, 1979a). The olfactory epithelium consists of several layers containing three principal cell types (Fig. 1). The basal cell layer contains horizontal and globose basal cells, some of which function as neuronal progenitor or precursor cells (Caggiano et al., 1994). Above this layer reside immature and mature olfactory receptor neurons (ORNs), while sustentacular supporting cells are found most apically. Basal cells undergo robust rates of neurogenesis throughout life, providing access to the process of neurogenesis postnatally (Caggiano et al., 1994). As ORNs mature, they move up through the epithelium, permitting determination of neuronal age by position and the expression of stage-specific markers (Bakalyar and Reed, 1990; Calof and Chikaraishi, 1989; Calof et al., 1998; Hartman and Margolis, 1975; Jones and Reed, 1989; Roskams et al., 1998; Verhaagen et al., 1990a). The availability of two injury models in the olfactory system that hyperinduce neurogenesis is also highly advantageous. Detergent lavage of the olfactory epithelium in the nasal cavity destroys cells of the olfactory epithelium except for the basal (stem) cells, which then repopulate the olfactory epithelium (Cummings et al., 2000). In contrast, olfactory bulbectomy removes the target of ORNs permanently, such that regenerating neurons cannot make contact with their target to form functional synapses (Schwob et al., 1992).

Using the olfactory system, we addressed a number of key issues regarding MeCP2 expression. We investigated whether MeCP2 expression is developmentally regulated, and which cell populations expressed MeCP2. Furthermore, we determined the timing of MeCP2 expression by using the aforementioned injury models, and whether MeCP2 expression is contingent upon synapse formation.

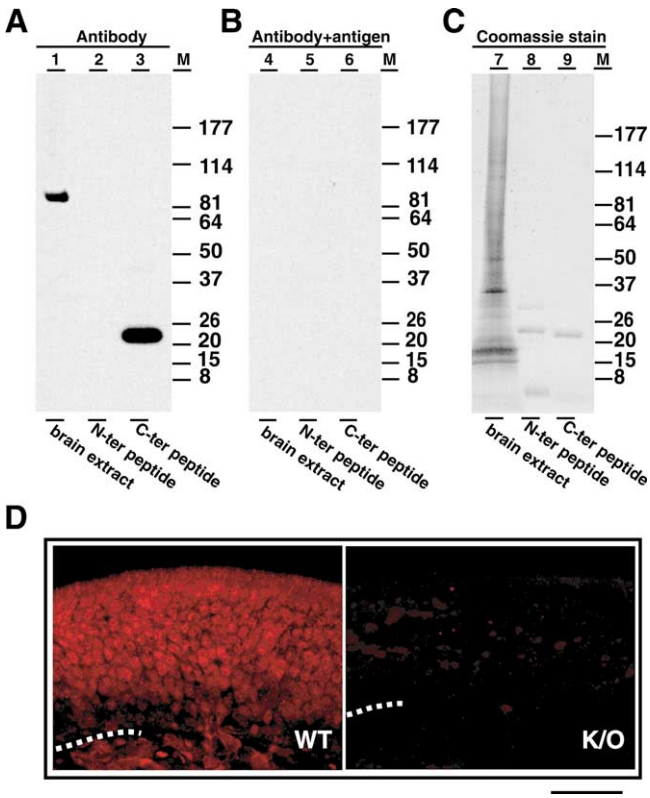
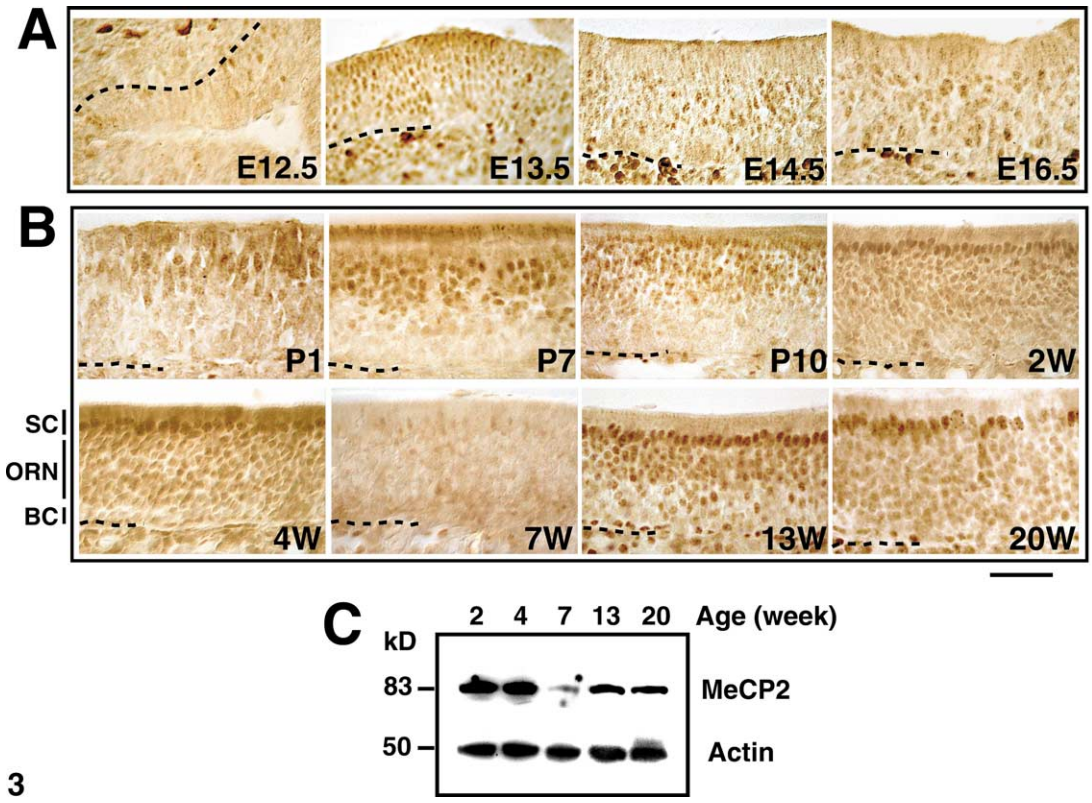


Fig. 2. Western blot demonstrating the specificity of affinity-purified anti-MeCP2 antibody. (A) A rabbit affinity-purified anti-peptide antiserum recognized a single band at the expected molecular weight of 84 kDa in a nuclear extract from human brain (lane 1). The antibody did not recognize an amino-terminal construct of recombinant MeCP2 (lane 2), but it did recognize a recombinant carboxyl-terminal fragment containing the peptide antigen (lane 3). (B) The specificity of the antiserum was confirmed by blocking experiments. The same protein samples were analyzed as in A, but the antibody was preincubated with excess peptide and no signal was detected (lanes 4–6). (C) The proteins electrophoresed in A and B were visualized by staining with Coomassie Blue (lanes 7–9). Nuclear extract (15 μg) and 1 μg of each recombinant protein were used in all panels. Abbreviation: M, molecular mass markers (Invitrogen). (D) The specificity of the MeCP2 antibody was also confirmed by immunohistochemical staining of mouse wild type and MeCP2 knockout tissue sections of the olfactory epithelium (OE). Scale bar: 50 μm.



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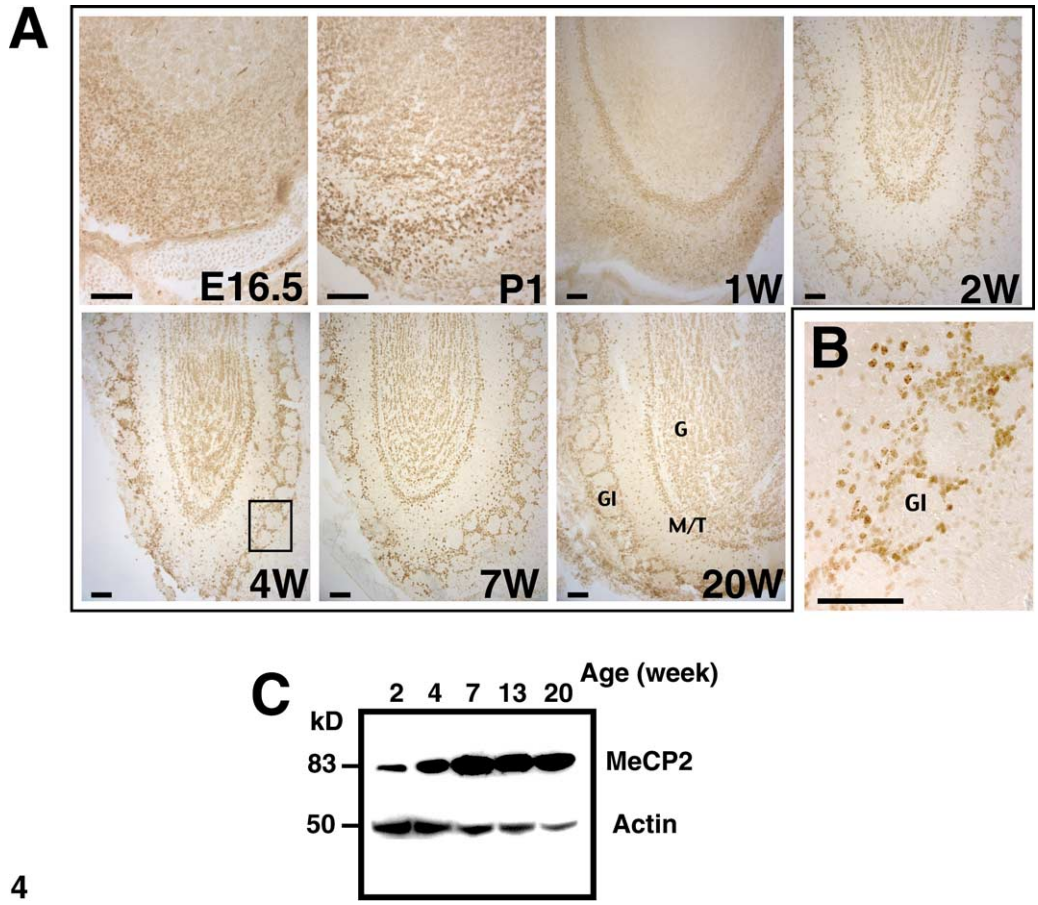

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Fig. 3. MeCP2 expression in embryonic and postnatal mouse olfactory epithelium. (A) Embryonic coronal sections of the olfactory epithelium at various ages were immunostained for MeCP2. (B) Postnatal coronal sections containing the nasal septum at various ages were immunostained for MeCP2. (C) Western blot analysis of MeCP2 expression in the postnatal olfactory epithelium. Scale bar: 50 μm. Dashed line: lamina propria. Abbreviations: E, embryonic; BC, basal cells; ORN, olfactory receptor neurons; P, postnatal day; SC, sustentacular cells; W, week.

Fig. 4. MeCP2 expression in mouse olfactory bulb. (A) Coronal sections of olfactory bulb (OB) stained with αMeCP2 at various ages. (B) High magnification of the glomeruli at 4 weeks postnatal stained with α-MeCP2. (C) Immunoblot analysis of MeCP2 expression in the OB. Scale bar: 100 μm. Abbreviations: E, embryonic; G, granule; GL, glomeruli; M/T, mitral/tufted cells; P, postnatal day; W, week.

Results

Generation and characterization of an antibody to MeCP2

We generated a rabbit antiserum to MeCP2 using a peptide antigen corresponding to the carboxyl-terminal 15-amino acid residues of human (and rodent) MeCP2 for these studies. The antiserum was affinity purified, and tested for its specificity by Western blotting (Fig. 2). While the predicted molecular mass of MeCP2 protein is 52 kDa, the previously reported mass of the protein based on its relative mobility by gel electrophoresis was 84 kDa (Lewis et al., 1992). We detected a band with a relative mobility of 84 kDa by Western blot (Fig. 2A, lane 1), confirming published reports. Our antiserum detected a recombinant fusion protein corresponding to the carboxyl terminus of the protein (Fig. 2A, lane 3), but not the amino terminus (Fig. 2A, lane 2). The specificity of the antiserum was further confirmed by blocking with excess peptide, which eliminated the signal from brain extract and recombinant protein (Fig. 2B). Fig. 2C displays a gel identical to those used in Fig. 2A and B, but which was stained with Coomassie Blue to visualize protein loading. Fig. 2D is an immunohistochemical control of the antiserum demonstrating the absence of specific staining in a section of olfactory epithelium obtained from a mouse with targeted deletion of the gene for *mecp2.*

Expression of MeCP2 in the mouse olfactory system

The spatiotemporal pattern of expression of a protein may provide insight into its function. To address the function of MeCP2 in neural development, we examined the developmental expression pattern of MeCP2 in the olfactory epithelium as well as the olfactory bulb, the target of the ORNs, using immunohistochemistry and Western blot analysis. Furthermore, we used cell type- and developmental stage-specific markers to identify the ages and types of cells that expressed MeCP2.

Developmental expression profile in the mouse olfactory epithelium

A number of embryonic (E) and postnatal (P) ages were analyzed by immunohistochemistry for MeCP2 expression (Fig. 3A and B, respectively). MeCP2 immunoreactivity was nuclear, as anticipated (Akbarian et al., 2001; Shahbazian et al., 2002). A few MeCP2-immunoreactive nuclei were visible by E12.5 and increased with embryonic age. Postnatally (Fig. 3B), MeCP2 expression increased from P0 to P28 (4 weeks). At 7 weeks after birth, staining was markedly and consistently reduced. MeCP2 expression thereafter increased, as seen at 13 weeks, and this level of expression persisted through 20 weeks. Western blot analysis (Fig. 3C) of P2-, 4-, 7-, 13-, and 20-week olfactory epithelial tissue for MeCP2 confirmed the changes in expression levels seen by immunohistochemistry, i.e., tissue obtained from 7-week-old animals contained the least amount of MeCP2.

The distribution of MeCP2 within layers of the olfactory epithelium was the second feature that varied with age. At embryonic ages, MeCP2 protein was not expressed in a laminar fashion, as immunopositive nuclei were located throughout the epithelium. This is consistent with the lack of organization of the embryonic olfactory epithelium into discrete layers, as it is in the adult (Cuschieri and Bannister, 1975). Postnatally, however, MeCP2-expressing cells were located primarily in the regions containing sustentacular cells and mature ORNs. Cells in regions containing predominantly immature neurons and basal cells displayed little immunoreactivity. Immunoreactivity in sustentacular cells was not distinct until P10. After P10, the sustentacular cells became the population of cells with the highest level of MeCP2 immunoreactivity. The pattern of MeCP2 expression in the epithelium changed at 4 weeks. At this time, MeCP2 immunoreactivity was associated with cells deeper in the epithelium, still sparing the most basal regions. This was also evident at 13 weeks, and very prominent at 20 weeks. This pattern is consistent with the decrease in rate of neurogenesis and the establishment of a mature epithelium with age.

The third feature that varied with age was the subcellular staining pattern of MeCP2. At embryonic ages, nuclear staining was punctate. In contrast, immunostaining became uniformly distributed in nuclei postnatally. In addition, expression levels were variable between cells; some cells stained intensely while others stained lightly. These results indicated that MeCP2 expression was dynamic in cells of the neuronal lineage as well as in sustentacular cells in the developing olfactory epithelium, and generally increased with age. This suggested that MeCP2 was associated with neuronal maturation.

Developmental expression profile in the mouse olfactory bulb

ORN axons enter the bulb to form the olfactory nerve layer, and terminate in regions of neuropil called glomeruli, where they form synapses with mitral and tufted cells. The bulb has a distinct lamellar structure, each layer containing a different cell type (for review, see Mori et al., 1999). We performed MeCP2 immunohistochemistry on the olfactory bulbs obtained from animals of various ages, primarily postnatal, because much of synaptogenesis and bulb development occurs postnatally (Hinds 1968a, 1968b), and also because MeCP2 is hypothesized to act during synaptogenesis (Johnston et al., 2001) (Fig. 4A). We examined a late embryonic age and several postnatal ages (essentially the same as those shown for the olfactory epithelium). Overall, at every age, staining appeared to be consistently stronger in the bulb than in the olfactory epithelium. MeCP2 expression increased with age up until 7 weeks, although the change

appeared less dramatic than in the olfactory epithelium. At E16.5 and P1, many positive nuclei were observed throughout the layers of the developing olfactory bulb. By P7, the layers became more delineated, with expression present in the developing mitral and glomerular layers. Expression continued to increase and became more distinct as the glomerular, mitral/tufted, and granule cell layers developed. Staining was strong, but heterogeneous in periglomerular cells that surround the glomeruli (Fig. 4B). The increase in expression with increasing postnatal age was confirmed by Western blot of olfactory bulb tissue (Fig. 4C). Thus, MeCP2 expression increases with maturation of the olfactory bulb coincident with synaptogenesis, which occurs within the first two postnatal weeks.

Double-labeling immunohistochemistry for MeCP2 and developmental markers

To confirm that MeCP2 expression correlated with neuronal maturation, we performed double-labeling immunohistochemistry on mouse olfactory epithelium for MeCP2 and stage-specific markers expressed by ORNs. Proliferating neuronal daughter cells and postproliferative immature ORNs located basally in the epithelium express neuron-specific tubulin (NST). Mature ORNs express olfactory marker protein (OMP), a cytoplasmic protein found in mature ORNs (Hartman and Margolis, 1975; Monti-Graziadei et al., 1977). As ORNs mature, NST expression decreases and OMP expression increases (Lee and Pixley, 1994; Roskams et al., 1998). OMP expression correlates with synaptogenesis (Verhaagen et al., 1990b). Double labeling with MeCP2 and NST (Fig. 5A) or MeCP2 and OMP (Fig. 5B) was performed on olfactory tissue from P2-week-old animals. In Fig. 5A, NST-immunoreactivity is visualized by fluorescein (green), and was seen to label cell bodies located predominately in the lower one-third of the epithelium (small arrows) and dendrites extending apically. In contrast, OMP immunoreactivity (Fig. 5B, green) was associated with cell bodies in the upper two-thirds of the epithelium (small arrows), sparing cells and processes in the lower one-third. The distribution of these markers is consistent with previous reports (Hartman and Margolis, 1975; Monti-Graziadei et al., 1977).

MeCP2 immunoreactivity was visualized using Cy3 (Fig. 5A and B, red). There were relatively few MeCP2-positive nuclei seen in the lower one-third of the epithelium, and even fewer that colocalized with NST (Fig. 5A, large arrow). An example of an NST-positive/MeCP2-negative cell is shown in the left inset, lower right corner of Fig. 5A. Examples of NST-positive/MeCP2-negative cells are shown by large arrows and in the right inset of Fig. 5A. Some of the MeCP2-positive cells were actually located close to the basal lamina, a region in which basal cells, early neuronal precursors, and horizontal cells are found (Fig. 5A, asterisks) (Huard et al., 1998). In contrast, the majority of MeCP2 immunoreactivity was associated with OMP-positive cells (Fig. 5B). An example of an OMP-positive/MeCP2-positive cell is shown in the inset of Fig. 5B. These results confirm that MeCP2 expression correlates with neuronal maturity and functional synaptogenesis.

Expression of MeCP2 in regenerating olfactory epithelium post injury

Several injury models exist to accelerate the rate of neurogenesis in the postnatal olfactory epithelium (Farbman, 1997). Surgical removal of the olfactory bulb (bulbectomy) removes the ORN target, resulting in retrograde degeneration of ORNs, and increasing the rates of neurogenesis and maturation (Carr and Farbman, 1992; Carr and Farbman, 1993; Costanzo and Graziadei, 1983; Schwartz Levey et al., 1991). Cycles of neurogenesis and cell death continue, since functional synaptogenesis cannot occur, and neurons are deprived of target-derived support. Alternatively, detergent lavage of the naris ablates the olfactory epithelium, essentially removing the superficial layers of the olfactory epithelium, increasing neurogenesis, while sparing the olfactory bulb (Cummings et al., 2000). We followed MeCP2 expression at various time points as the epithelium recovered from these injury paradigms to better define the timing of MeCP2 expression in relationship to neuronal maturation.

Chemical ablation

Mice received intranasal detergent lavage as described in Materials and Methods. Over a period of weeks, the olfactory epithelium regenerated, as reported by others (Cummings et al., 2000). During the first stage (first week post ablation), mature and immature ORNs and their processes in the bulb degenerate. This signals basal cell proliferation, which gives rise to GAP-43-positive ORNs (immature neurons; Margolis et al., 1991). This population matures over a number of weeks, and by 7 weeks post lesion, the normal complement of immature and mature (OMP-positive) ORNs has been restored with the appropriate connections to the bulb. We utilized this injury model to follow the ontogeny of MeCP2 expression in the context of the repopulation of the olfactory epithelium and reestablishment of functional contacts with the bulb.

Fig. 6A shows MeCP2 expression in the mature ORNs and sustentacular cells of a 7-week-old control animal. Neural cell adhesion molecule (N-CAM) is expressed in immature and mature cells of the ORN lineage (Calof and Chikaraishi 1989); sustentacular cells and basal cells do not express N-CAM, thereby allowing us to determine which cell types express MeCP2 during regeneration. As expected, N-CAM and MeCP2 colocalized to neuronal cells of the control epithelium, while N-CAM was absent from the most apical layer of nuclei, where sustentacular cells are found (Fig. 6E).

One week post ablation, much of the epithelium has

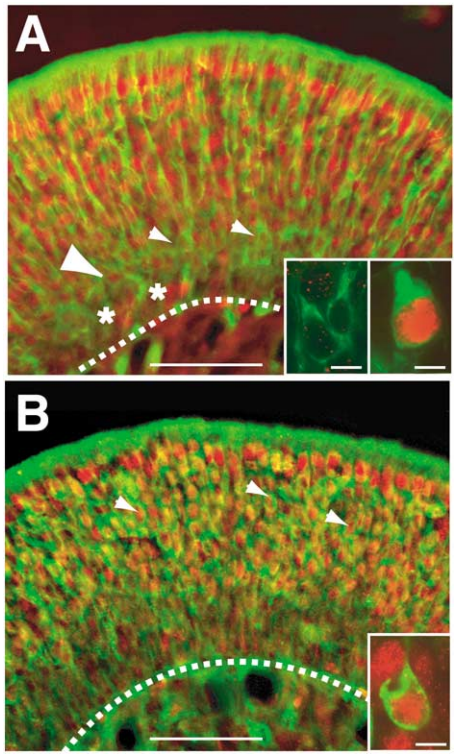

Fig. 5. Double-labeled immunofluorescence of MeCP2 (red) and olfactory marker protein (OMP) or neuron-specific tubulin (NST) (green) in 2-week mouse olfactory epithelium. (A) MeCP2 does not predominantly colocalize with NST. Small arrows and left inset (high magnification) indicate examples of MeCP2-negative NST cell bodies; large arrow and right inset (high magnification) indicate MeCP2-positive NST cell bodies. Asterisks indicate basal cell staining. (B) MeCP2 colocalizes with OMP (arrow indicates representative OMP cell body). Inset (high magnification) shows and example of a double-labeled olfactory receptor neuron. Scale bars: 50 μm (A and B) and 5 μm (insets). Dashed line: lamina propria.

disappeared, and MeCP2 expression has decreased in the remaining cells (Fig. 6B), with the exception of some apical cells and several prominently staining cells located immediately above the basal lamina (Fig. 6B, arrows). These MeC2-positive cells were not neuronal, as N-CAM and MeCP2 immunoreactivities did not colocalize in these cells (Fig 6F). Considering the stratification of the olfactory epithelium, the apical cells are likely sustentacular cells. The identity of the basal cell type expressing MeCP2 at 1 week post ablation was determined by double labeling sections with MeCP2 and BS-1, a lectin binding specifically to horizontal basal cells (HBCs) (Fig. 6I, arrows) (Huard et al., 1998). Moreover, these MeCP2-positive basal cells did not appear to be dividing precursor cells, as there was no double labeling of cells with MeCP2 and phospho-histone H3 antibody, a mitosis marker (Fig. 6J, arrows). Consequently, we conclude that the basally located cells were HBCs.

At 2 weeks post ablation, the thickness of the olfactory epithelium had increased as neurons continued to repopulate the epithelium. MeCP2 expression was prominent in regions containing sustentacular and horizontal basal cells (Fig. 6C). In fact, MeCP2 expression in sustentacular cells was striking. N-CAM expression was increased, yet MeCP2 expression was still only detected in the epithelial layers containing sustentacular and basal cells (Fig. 6G). By 3 weeks post ablation, the epithelial thickness appeared restored (Fig. 6D), and MeCP2 immunoreactivity colocalized with N-CAM-positive cells in the epithelium (Fig. 6H). The intensity of MeCP2 immunostaining also increased in both the neuronal and nonneuronal (sustentacular cell) populations. In contrast to the dynamic changes observed in the olfactory epithelium, no change in MeCP2 expression was observed in the olfactory bulbs examined at any of the time points studied (data not shown). These data indicate that MeCP2 expression did correlate with the maturation of neuronal cells. However, the timing of MeCP2 expression with respect to synaptogenesis remained unclear.

Unilateral bulbectomy

To determine whether MeCP2 expression can occur in the absence of the establishment of a synaptic connection, MeCP2 immunoreactivity was examined in rat following unilateral bulbectomy (Fig. 7). Degeneration of mature ORNs is maximal around 3 days post lesioning, while neurogenesis peaks a few days later. Subsequently, new mature neurons appear, but fail to survive due to their inability to synapse in the bulb (Schwob et al., 1992). Two weeks after bulbectomy, a new balance is reached between cell death and proliferation, both of which are elevated above control rates (Carr and Farbman, 1992; 1993; Costanzo and Graziadei, 1983; Schwartz Levey et al., 1991). Thus, in contrast to chemical ablation, there are futile cycles of cell birth, maturation, and cell death, so that normal cell dynamics are never attained.

Fig. 7A and B show the normal variations in the pattern of MeCP2 expression in the olfactory epithelium of a control 4–5-week-old rat. In a given coronal section, variations in the patterns of expression of MeCP2 were observed. In some regions (Fig. 7A), nuclear staining was present throughout the mature layer of the olfactory epithelium. In others (Fig. 7B), there was often a different pattern of MeCP2 expression, such that cells in the middle one-third of the epithelium contained less immunoreactivity. These patterns may reflect the normal difference in the level of neurogenesis present in regions of the epithelium, which varies considerably across the epithelium (Graziadei and Graziadei, 1979b)

Fig. 7C–E shows the pattern of MeCP2 expression on the side of the epithelium ipsilateral to the bulbectomy at 3 days, 1 week, and 2 weeks post lesioning, while Fig. 7F–H shows the patterns of expression contralateral to the lesioned side. Most notably, the variability seen in MeCP2 expression in control animals was less apparent on both sides of the epithelium in lesioned animals (Fig. 7C–E). Three days post lesioning on the side ipsilateral to the lesion, MeCP2 immunostaining was only observed in the sustentacular cells and most mature ORNs (Fig. 7C). In

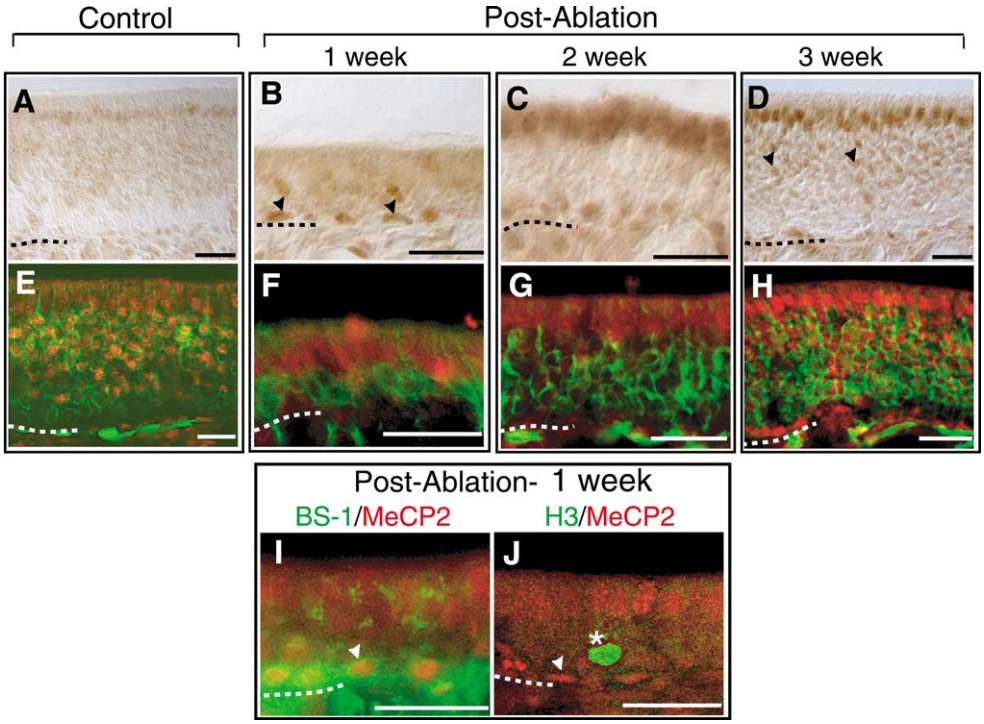

Fig. 6. MeCP2 expression in mouse olfactory epithelium after chemical ablation. Prelablated olfactory epithelium (A and E) from 7-week-old mice shows representative MeCP2 staining. Following chemical ablation of 7-week-old mice, expression of MeCP2 was observed at 1 week (B and F), 2 weeks (C and G), and 3 weeks post ablation (D and H). MeCP2 appears early in the basal cells (B, arrowhead) and during recovery MeCP2 expression gradually appears in maturing cells (C and D, arrowhead). MeCP2 (red) double labeled with NCAM (green), before ablation (E) and following ablation (F–H). To identify which population of basal cells was MeCP2 positive after 1-week recovery (B), 1-week postablated tissue was double labeled with either BS-1 (I) or H3 (J). BS-1 labels the horizontal basal cell population while H3 is a mitosis marker of the M phase. Scale bar: 25 μm (A–D). Dashed line: lamina propria.

addition, there was expression in the basal cell layer. One week after bulbectomy, expression increased ipsilateral to the lesion (Fig. 7D), becoming more abundant in the mature ORN and sustentacular cell layers, but remaining relatively constant in the basal layer. MeCP2 expression appeared to decrease at 2 weeks post bulbectomy, although the distribution remained much the same (Fig. 7E). Contralateral to the lesion, MeCP2 expression remained at a relatively constant level at every time point, and many positive nuclei were observed throughout the upper two-thirds of the epithelium as expected (Fig. 7F).

As in the ablation experiment, we utilized N-CAM, BS-1, and H3 double immunostaining with MeCP2 to determine the phenotypes of cells expressing MeCP2 post lesioning (Fig. 7I and J). N-CAM/MeCP2 double labeling indicated that only the most apical N-CAM-positive cells expressed MeCP2, consistent with induction of MeCP2 expression with maturation (Fig. 7I). Using MeCP2/BS-1 double labeling, we identified the MeCP2-positive cells located basally as horizontal basal cells (Fig. 7J). These basal cells were not neuronal precursors undergoing proliferation, as MeCP2/phospho-histone H3 double labeling failed to reveal any colocalization (Fig. 7K). Thus, MeCP2 expression can occur in maturing neurons in the absence of functional synaptogenesis, although it does not attain prelesioning levels.

The bulbectomy model was used to investigate the timing of MeCP2 expression in mature neurons with respect to synapse formation (Fig. 8). OMP is a marker of mature ORNs and is expressed prior to synaptogenesis, although synaptogenesis is required for the survival of mature OMP-positive ORNs (Schwob et al. 1992). Thus, olfactory neurons that repopulate the epithelium after bulbectomy do develop OMP expression, although they cannot survive long term. We performed MeCP2 and OMP double labeling at 3 days, 1 week, and 2 weeks after unilateral bulbectomy (Fig. 8). The number of OMP-positive neurons was decreased on the ipsilateral side (Fig. 8A–C) compared to the noninjured (Fig. 8D–F, contralateral) side at all times. At 3 days, there are relatively few MeCP2- or OMP-positive cells (Fig. 8A); these OMP-positive cells represent residual degenerating cells. At 1 week, the number of MeCP2- and OMP-positive cells increased (Fig. 8B). There were, however, a large number of MeCP2-positive cells that did not express OMP. Apically, these were sustentacular cells and in the middle layer these MeCP2 expressing cells were likely immature cells. At 2 weeks, the number of OMP-positive cells decreased, reflecting the death of OMP-expressing mature neurons, while cells expressing MeCP2 had increased (Fig. 8C). These data indicate that MeCP2 expression occurs before synaptogenesis and prior to terminal differentiation.

Discussion

Mutations in MeCP2 cause RTT, a devastating neurodevelopmental disease (Rett, 1986). Defining the timing of MeCP2 expression is important to identify the stages of development when neurons are vulnerable to MeCP2 dysfunction. In this study, we utilized the olfactory epithelium as a neurodevelopmental model to investigate the expression of MeCP2 during development and in response to neuronal injury. MeCP2 was expressed during embryogenesis, and expression increased postnatally. MeCP2 expression was predominantly in mature neuronal cells, although significant immunostaining was visualized in sustentacular cells. After injury, MeCP2 expression increased during repopulation of the olfactory epithelium, suggesting that in addition to a developmental role, MeCP2 may be important in regulating gene expression changes in response to injury. Of importance for understanding the underlying cell biological basis of RTT, the unilateral bulbectomy paradigm was instrumental in narrowing MeCP2 expression to a time window preceding terminal differentiation and synaptogenesis.

The spatiotemporal patterns of MeCP2 expression within the olfactory epithelium were dynamic and correlated with neuronal maturation but not neurogenesis. From embryonic ages to P10, neurogenesis remains quite robust (Legrier et al., 2001), and MeCP2 expression was low in the basal portion of the epithelium. MeCP2 immunoreactivity could be detected sporadically in cells of the developing olfactory epithelium as early as E12.5, and increased subsequently during embryogenesis in a dispersed pattern, consistent with the dispersed localization of developing neurons within the embryonic olfactory epithelium. Postnatally, a more mature spatial organization into a lamellar pattern becomes evident in the epithelium, and concomitantly, MeCP2 expression became associated with the most apical (mature) cell nuclei after P1. By 2 weeks post natal, the rate of neurogenesis has slowed, and MeCP2-positive nuclei occupied all except the most basal layer of the epithelium, which still contains dividing progenitor cells and immature neurons (Fung et al., 1997). These data thus indicate that the temporal expression of MeCP2 expression and timing of neurogenesis are not coincident.

Interestingly, there was a transient and consistent decrease in MeCP2 expression in ORNs at 7 weeks post natal, which was confirmed by Western blot analysis. In contrast to the expression of MeCP2 in the epithelium, MeCP2 expression in the olfactory bulb steadily increased. MeCP2 was found in the mitral/tufted cell layer, the granule cell layer, and in periglomerular cells. Notably, there was no drop in expression at 7 weeks post natal. The decrease in MeCP2 expression in the olfactory epithelium may be related to the onset of sexual maturity, as one might predict that significant changes in gene expression occur in the olfactory system at this time. Fluctuation of MeCP2 expression has been reported in humans, albeit at a different time of development (Itoh and Takashima, 2002). MeCP2 expression was detectable in all neurons of fetal brain until 20 gestational weeks, but disappeared in the cortex after 20 gestational weeks and in the brainstem after the perinatal period. MeCP2 reappeared in the brainstem after adolescence. Therefore, hormonal or other growth factor changes could in part influence MeCP2 expression.

The correlation of MeCP2 with neuronal maturation was confirmed by double-labeling immunohistochemistry. NST is highly expressed in daughter neurons undergoing cell division and in immature postproliferative neurons (Roskams et al., 1998). Only a small number of NST-positive cells coexpressed MeCP2. In contrast, the vast majority of OMP-positive cells coexpressed MeCP2. Even within the mature ORN population, however, there was variability in expression levels, likely indicating the developmental state of the cell, i.e., whether it just matured or was an older neuron. Such a correlation with maturity and the observation of heterogeneous neuronal populations is consistent with recent studies in both mouse and human cortex (LaSalle et al., 2001; Shahbazian et al., 2002). An apparent exception to this pattern was the finding of cells situated on the basal lamina that were intensely MeCP2 positive, potentially arguing against a correlation of MeCP2 with maturation. However, these cells were identified as horizontal basal cells and not mitotic progenitor cells, using BS-1 and H3 double-labeling immunohistochemistry, respectively. They may thus indeed be a more differentiated population distinct from neuronal precursors.

Collectively, these results indicate that MeCP2 expression is associated with neuronal maturation, but that it does fluctuate at specific developmental times, even in established neuronal populations. The most dramatic increase in MeCP2 immunostaining in the epithelium occurred between P7 days and P2 weeks, coincident with peak synaptogenesis (Coy et al., 1999; Hinds and Hinds, 1976a, 1976b). The intensity of immunostaining also increased in cells during this time. However, the timing of MeCP2 expression as pre- or postsynapse formation could not be determined from these data.

The injury paradigms provided a dynamic view of MeCP2 expression, confirming coincidence of expression with the maturation of neurons, and further refined the timing of expression. Detergent ablation of the olfactory epithelium removes most of the neurons, sparing basal cells and hyperinducing neurogenesis (Farbman, 1997). These new neurons reestablish connections with the bulb. Olfactory bulbectomy removes the target of the ORNs, causing retrograde degeneration of neurons and hyperinduction of neurogenesis, but in this case synaptic connections cannot be reestablished. Thus, these injury models permit us to follow a nascent population of ORNs as they mature. Furthermore, they provide complementary information as to whether MeCP2 may play a role, either pre or post synaptogenesis.

Prior to detergent ablation, MeCP2 expression was

prominent in sustentacular cells and in OMP-positive ORNs, as expected. By following time periods post ablation, MeCP2 expression was observed to reappear only once ORNs matured and presumably reestablished their functional connections. Thus, although MeCP2 expression was retained in sustentacular cells, MeCP2 immunostaining was absent from all developing neurons (visualized by N-CAM double immunostaining) until 3 weeks post ablation at which time synaptogenesis may recur (Cummings et al., 2000). No MeCP2 expression was observed in dividing cells (marked by phospho-histone H3 antibody and presumed to be globose basal cells, due to their position and round morphology). This result of a noncoincidence with neurogenesis is in agreement with the developmental data.

The first unexpected observation from the ablation study was the intense expression of MeCP2 in the horizontal basal cells, a population of cells in which MeCP2 expression was rarely observed in 7-week-old olfactory epithelium. The functional significance of this finding remains unclear, due to the lack of knowledge of the role of horizontal basal cells. Globose basal cells give rise to neuronal precursors, and after ablation are suggested to generate globose basal cells as well as HBCs (Huard et al., 1998). In contrast, HBCs appear only to self-renew in response to appropriate signals (Caggiano et al., 1994; Calof and Chikaraishi, 1989; Graziadei and Graziadei, 1979a; Schwartz Levey et al., 1991; Schwob et al., 1994), which does not provide insight into their role in olfactory homeostasis. At present, it can be suggested that after injury, MeCP2 may be necessary to initiate or prevent certain developmental programs unique to the HBC population.

The second finding was the dynamic change in MeCP2 expression in sustentacular cells following injury. There was initially a reduction in MeCP2 expression 1 week post ablation (Fig. 6B). Subsequently, expression increased (Fig. 6C and D) upon regeneration of the olfactory epithelium. Although it is possible that this change in MeCP2 expression is due to a repopulation of sustentacular cells, we cannot exclude the possibility of an actual change in expression in existing cells.

The expression and ablation data confirmed that MeCP2 is associated with neuronal maturation, and suggested a coincidence with synaptogenesis, but did not distinguish whether MeCP2 is expressed before synaptic contact occurs or as a consequence of connection with the target. The bulbectomy data, in contrast, defined for the first time the timing of MeCP2 expression. We followed the expression pattern of MeCP2 through the dynamics of neurogenesis and cell death in the olfactory epithelium reported to occur after unilateral bulbectomy (Carr and Farbman, 1992, 1993; Costanzo and Graziadei, 1983; Schwartz Levey et al., 1991).

Three days after target lesioning, there is widespread death of mature ORNs, and consequently a dramatic reduction of MeCP2 immunostaining on the side ipsilateral to the lesion. This result suggests that MeCP2 expression may decrease in apoptotic neurons as part of this cell program. After 1 week, immature neurons have reappeared, are positioned in the mature ORN layer, and displayed increasing MeCP2 immunostaining. The finding that OMP-positive neurons are generated and express MeCP2 in the absence of their target suggests that MeCP2 comes on perhaps with the initiation of a maturation program to transition from NST to OMP, but occurs before OMP-positive ORNs make synaptic contact. Finally, by 2 weeks post lesioning, the newly generated ORN population undergoes death due to the absence of their target, with a concomitant decrease in MeCP2 expression. The failure of MeCP2 to return to comparable control levels in bulbectomy is in contrast to ablation, in which the target olfactory bulb is intact, allowing for a reestablishment of synaptic contacts. In the case of bulbectomy, the olfactory epithelium cannot return to a normal state of maturity and functionality without reconnecting with its target (Margolis et al., 1991; Schwob et al., 1992).

Although the emphasis has been on changes in the neuronal population following bulbectomy, an unexpected finding was the increase in MeCP2 expression in sustentacular cells 1 week post injury and a subsequent decrease. The dynamic nature of expression in these cells suggests a possible role in regeneration. Indeed, sustentacular cells have been shown to produce neuropeptides such as neuropeptide Y known to function in regeneration/repair (Hansel et al., 2001). Thus, MeCP2 expression may be involved in gene regulation in sustentacular cells relating to a response to injury.

The temporal patterns of MeCP2 expression in the developing olfactory epithelium and bulb and in the epithelium after injury provide information regarding the processes in which MeCP2 may function. ORNs synapse directly on mitral and tufted cells (Mori et al., 1999) and on periglomerular cells (in certain species of mice). MeCP2 expression is intense in each of these mature cell populations in the bulb. This suggests, together with the observation that MeCP2 is expressed in the absence of a synaptic target, that MeCP2 may set in motion the cellular programs that make neurons competent to form synaptic contacts. It does not appear to be the case that MeCP2 expression is induced, upon synaptic contact, to subsequently set in motion synaptic maintenance programs. If so, MeCP2 expression would be absent following bulbectomy.

Being able to determine when MeCP2 is necessary during neurodevelopment provides important information regarding critical time points during which disruptions in the MeCP2 gene might induce deleterious effects on development, as seen in RTT. The data presented here suggest that MeCP2 expression is related to neuronal maturity, but occurs before synaptogenesis. Identifying this time window is useful for finding genes that are regulated by MeCP2 or necessary for normal MeCP2 function. Furthermore, the data indicate that MeCP2 expression is dynamic, and can fluctuate even in established neuronal populations, suggest-

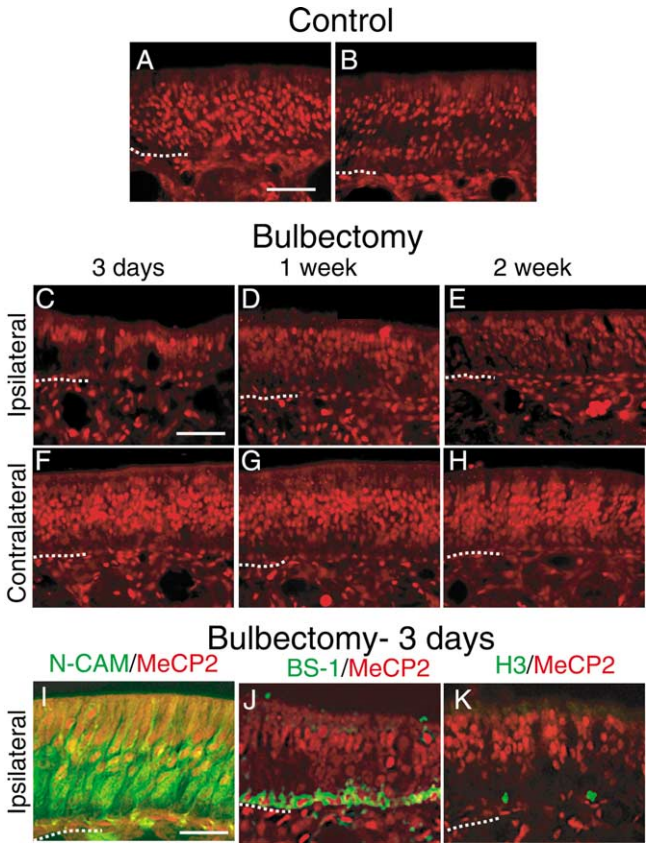

Fig. 7. MeCP2 expression in rat olfactory epithelium after unilateral bulbectomy. (A and B) Coronal section of representative MeCP2 expression throughout the olfactory epithelium in nonbulbectomized 4–5-week-old rat. Following bulbectomy, coronal sections containing both ipsilateral lesioned (C–E) and contralateral control sides (F–H) were fluorescently labeled for MeCP2 (red). MeCP2 expression at 3 days (C and F), 1 week (D and G), and 2 weeks (E and H) post bulbectomy. Note that MeCP2 is downregulated on the ipsilateral side following bulbectomy. (I) MeCP2 (red) colocalizes with N-CAM (green) 3 days post bulbectomy. (J) MeCP2 (red) colocalizes with BS-1 (green). (K) MeCP2 is not found in H3-positive cells (i.e., cells in M phase). Scale bar. 50 μm. Dashed line: lamina propria.

ing that MeCP2 could be reintroduced/expressed in neuronal populations through therapeutic manipulations.

Experimental methods

Experimental animals and tissue preparation

All experimental protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the National Institute of Health "Guide for the Care and Use of Laboratory Animals" were followed. Both male BALB/c mice and male adult Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). For immunohistochemistry, animals were anesthetized with intraperitoneal xylaket and fixed by cardiac perfusion with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS buffer. Tissue was dissected, postfixed overnight in fixative buffer at 4°C, washed in PBS, placed in 30% sucrose, and embedded in Tissue-Tek O.C.T. compound (VWR, Baltimore, MD). Coronal sections of both olfactory epithelium and bulb were cut at 18 μm using an HM500M cryostat (Zeiss, Waldorf, Germany), thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), dried for 2 h at room temperature, and stored at −80°C until used. For protein preparation, mice were killed by CO_2 asphyxiation and cervical dislocation, and then olfactory epithelium and bulb were immediately dissected and processed.

Olfactory bulbectomy

Adult Sprague-Dawley rats (75–100 g) were anesthetized with xylaket and fixed in a stereotaxic apparatus for surgery. The right olfactory bulb was exposed via a partial dorsal craniotomy and was ablated by suction. Care was taken to avoid damage to the contralateral (left) olfactory bulb. The ablation cavity was filled with Gelfoam to prevent invasion of frontal cortex neurons into this cavity, which could provide an alternative target for regenerating olfactory axons. The skin above the lesion was closed using surgical clips, and animals were allowed to recover from anesthesia under a heat lamp. Following recovery from anesthesia, rats were returned to the animal colony and maintained on a normal diet until animals were killed at 3 days, 1 week, or 2 weeks post bulbectomy.

Detergent ablation of olfactory epithelium

Seven-week old BALB/c mice, momentarily immobilized, received unilateral intranasal (right cavity) irrigation with 100 μl of 0.7% Triton X-100 in PBS, pH 7.4, using a 1-cc syringe fitted with a blunt-end, polished, 25-gauge needle. Mice were returned to the animal colony and maintained on a normal diet until animals were killed at 1 week, 2 weeks, or 3 weeks post ablation.

Antibody production

A peptide corresponding to the carboxyl terminus of human methyl-CpG binding protein 2 (MeCP2) was synthesized (Protein and Nucleic Acid Facility, Stanford University; NH2-EEPVDSRTPVTERVS-COOH), coupled to keyhole limpet hemocyanin, and injected into rabbits. Antiserum was affinity-purified using peptide antigen coupled to Affigel 10/15 (Pierce, Rockford, IL). The specificity of the antiserum was confirmed on Western blots by blocking with excess peptide, which resulted in the expected loss of immunoreactivity. For Western blotting, antisera were diluted (1:2000) and incubated overnight at 4°C. The secondary antibody was protein A-horseradish peroxidase (1:2000; Bio-Rad, Hercules, CA). The specificity of the antiserum was also confirmed by immunohistochemical staining of

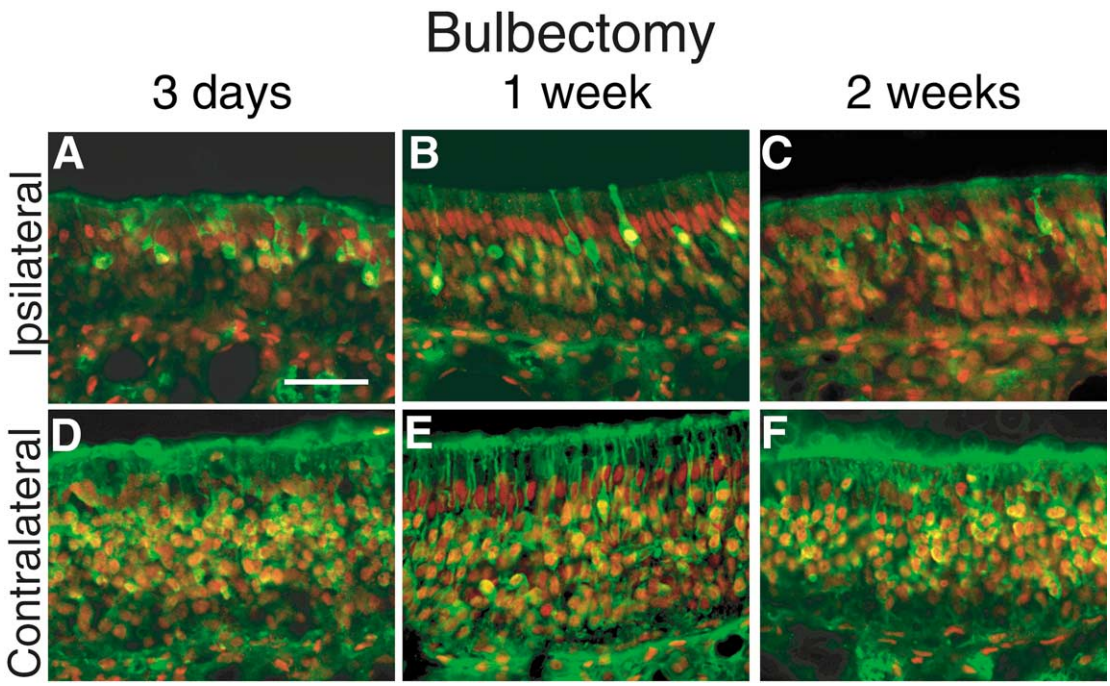

Fig. 8. MeCP2 and olfactory marker protein (OMP) double labeling in rat olfactory epithelium after unilateral bulbectomy. Following bulbectomy, ipsilateral lesioned side (A–C) and contralateral control side (D–F) coronal sections were fluorescently labeled for MeCP2 (red) and OMP (green). MeCP2/OMP expression at 3 days (A and D), 1 week (B and E), and 2 weeks (C and F) after bulbectomy. Note that OMP is downregulated on the ipsilateral side following bulbectomy. (A) MeCP2 colocalizes with OMP 3 days post bulbectomy. (B) One week after injury, MeCP2 colocalizes with more OMP-positive cells likely corresponding to maturation of new cells. (C) MeCP2 is found in few more weakly stained OMP-positive cells 2 weeks after bulbectomy, likely due to death of cells. In contrast, on the contralateral side, at each respective time point post lesioning, the OMP population spanned a good portion of the epithelium and was double labeled (D–F). Scale bar: 50 μm.

olfactory epithelial sections from the MeCP2 knockout mouse (generous gift of Rudolf Jaenisch, Whitehead Institute, Cambridge, MA), olfactory epithelium (OE), which resulted in the expected loss of immunoreactivity.

Immunohistochemistry

Following two rinses in 0.1 M PBS, sections on slides were permeabilized using 0.1 M PBS and 0.2% Triton X-100 (0.7% Triton X-100 for MeCP2 staining) for 30 min. Sections were again rinsed twice in 0.1 M PBS. For MeCP2-horseradish peroxidase (MeCP2-HRP) staining, endogenous peroxidase was quenched by using 0.5% hydrogen peroxide (1% for embryonic tissue sections) for 10 min, and then sections were rinsed twice. Sections were preincubated for 1 h in 0.1 M PBS, 4% of the corresponding serum, 0.2% Triton X-100, and then incubated overnight at 4°C in the same preincubation buffer added with the corresponding primary antibody: rabbit anti-MeCP2 (1:500), goat anti-olfactory marker protein (anti-OMP; 1:3000; gift of Frank Margolis), rabbit anti-neural cell adhesion molecule (anti-N-CAM; 1:750; Chemicon International, Temecula, CA), fluorescein-conjugated *Griffonia (bandeiraea) simplicifolia* lectin 1 (BS-1; 1:1000; Vector Laboratories, Burlingame, CA), rabbit anti-phospho-histone 3 (H3; 1:200; Upstate Biotech, Lake Placid, NY), mouse anti-β-tubulin (βIII; 1:1000; BAbCo, Richmond, CA). For double labeling, incubation of the primary antibodies was performed sequentially. Following primary antibody incubation, sections were rinsed twice in 0.1 M PBS and incubated 1 h at room temperature with the appropriate secondary antibody. For MeCP2-HRP staining, the secondary antibody was purchased from Vector Laboratories and was used at a concentration of 1:200. For fluorescent staining, all the following secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA): Cy3 donkey anti-rabbit IgG (1:500), FITC donkey anti-rabbit (or mouse) IgG (1:500), and Cy2 donkey anti-goat IgG (1:100). For double labeling using two primary antibodies originating from the same host species (rabbit), Fab fragments (Cy3 goat anti-rabbit; 1:500) of secondary antibody were used to avoid cross-reaction. Dilutions were made using 0.1 M PBS, 0.05% of the corresponding serum. The sections were rinsed twice in 0.1 M PBS and coverslipped using an anti-fading (Mowiol, Calbiochem, La Jolla, CA) mounting medium. For MeCP2-HRP staining, slides were incubated with an avidin-biotin complex for 1 h using the Vectastain Elite ABC Kit (Vector Laboratories). The peroxidase reaction was catalyzed by using 3,3′-diaminobenzidine tetrahydrochloride (25% wt/vol) and 1% hydrogen peroxide as substrate in 50 mM Tris-HCl, pH 7.4, buffer. Slides were rinsed and coverslipped using Aquapoly-

mount mounting medium (Polysciences Inc., Warrington, PA). Mounted sections were viewed on a Zeiss Axioskop, and the images were captured by using a digital camera (Axiocam; Zeiss, München-Hallbergmous, Germany).

Western blot procedure

After dissection, whole olfactory epithelium and olfactory bulb were homogenized by sonication in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Total protein levels were quantified by using a modified Bradford assay (Bio-Rad protein assay) with bovine serum albumin as a standard. The protein samples were mixed with the Laemmli solution (Laemmli, 1970) and boiled 5 min. Fifty micrograms of protein was loaded and electrophoresed on a 4–20% gradient sodium dodecyl sulfate–polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane. Blot membranes were preincubated at room temperature for 30 min in Tris-buffered saline with 0.1% Tween 20 (TBS-T), 5% (wt/vol) nonfat dry milk, and probed overnight at 4°C with rabbit anti-MeCP2 antibody diluted to 1:5000 in the preincubation solution. The membranes were washed twice for 30 min with TBS-T, and then incubated with the secondary antibody. HRP-conjugated goat anti-rabbit Ig (Chemicon International, Temecula, CA) was used as a secondary antibody at a 1:5000 dilution. A mouse anti-actin antibody (JLA 20; 1:500; Developmental Studies Hybridoma Bank, Iowa, IA) was used as a protein-loading control, and detected by an HRP-conjugated goat anti-mouse Ig as a secondary antibody (Roche, Molecular Biochemicals, Indianapolis, IN). After two washes of 30 min each with 0.1 M PBS, 0.1% Tween 20, bands were visualized by the Super Signal West Pico Chemiluminescence Substrate Kit (Pierce, Rockford, IL).

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