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Epigenetic control of neurotransmitter expression in olfactory bulb interneurons

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Abstract

Defining the molecular mechanisms that underlie development and maintenance of neuronal phenotypic diversity in the CNS is a fundamental challenge in developmental neurobiology. The vast majority of olfactory bulb (OB) interneurons are GABAergic and this neurotransmitter phenotype is specified in migrating neuroblasts by transcription of either or both *glutamic acid decarboxylase 1 (Gad1)* and *Gad2*. A subset of OB interneurons also co-express dopamine, but transcriptional repression of *tyrosine hydroxylase (Th)* suppresses the dopaminergic phenotype until these neurons terminally differentiate. In mature OB interneurons, GABA and dopamine levels are modulated by odorant-induced synaptic activity-dependent regulation of *Gad1* and *Th* transcription. The molecular mechanisms that specify and maintain the GABAergic and dopaminergic phenotypes in the OB are not clearly delineated. In this report, we review previous studies and present novel findings that provide insight into the contribution of epigenetic regulatory mechanisms for controlling expression of these neurotransmitter phenotypes in the OB. We show that HDAC enzymes suppress the dopaminergic phenotype in migrating neuroblasts by repressing *Th* transcription. In the mature interneurons, both *Th* and *Gad1* transcription levels are modulated by synaptic activity-dependent recruitment of acetylated histone H3 on both the *Th* and *Gad1* proximal promoters. We also show that *HDAC2* has the opposite transcriptional response to odorant-induced synaptic activity when compared to *Th* and *Gad1*. These findings suggest that *HDAC2* mediates, in part, the activity-dependent chromatin remodeling of the *Th* and *Gad1* proximal promoters in mature OB interneurons.

Keywords

dopamine; GABA; tyrosine hydroxylase; glutamic acid decarboxylase; olfactory bulb; adult neurogenesis

1. Introduction

The central nervous system is characterized by its large phenotypic diversity as defined by the partially overlapping expression patterns of neurotransmitters, neuroactive peptides,

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transmembrane receptors, ion channels and calcium binding proteins. Unique combinations of these molecular factors create distinct functional properties that are essential for the circuits in which specific neurons participate. Elucidating the regulatory mechanisms controlling the expression of genes that encode these molecular features is fundamental not only for understanding the normal development of these circuits, but also for gaining insight into the etiology of nervous system diseases.

The main olfactory bulb (OB) is a well-established brain region to investigate molecular mechanisms that control development of neuronal phenotypic diversity. The OB is the initial processing center for odorant sensory information transduced by olfactory receptor neurons in the olfactory epithelium. Odorant information is relayed from olfactory receptor neurons to several cortical regions via mitral/tufted cells in the OB. Both olfactory receptor neurons and mitral/tufted cells have a glutamatergic neurotransmitter phenotype. Transmission of odorant information by these neurons is modulated by inhibitory GABAergic interneurons in the glomerular and granule cell layers of the OB (Figure 1A).

A distinguishing feature of the OB is that the interneurons are continuously generated throughout life. Most embryonic interneuron progenitors originate in the lateral ganglionic eminences, whereas post-natal and adult progenitors are generated within the subventricular zone (SVZ) of the lateral ventricles. In both the embryo and adult, progenitors migrate tangentially towards the OB through the rostral migratory stream (RMS) (Figure 1B). Once in the OB, progenitors migrate radially to their final positions, terminally differentiate and either successfully integrate in the circuitry or undergo apoptosis. Progenitor production in the mouse peaks approximately at post-natal day 4 and continues throughout adulthood at a decreased rate (Hinds, 1968). Disruption in either production or migration of neural progenitors in adult mice impairs olfactory perceptual learning (Moreno et al., 2009), long-term odorant learning and memory (Lazarini et al., 2009; Sultan et al., 2010), odorant based fear conditioning (Valley et al., 2009) and social behaviors (Feierstein et al., 2010).

Studies in the mouse OB indicate that nearly all interneurons express the GABAergic phenotype (Kiyokage et al., 2010; Panzanelli et al., 2007; Parrish-Aungst et al., 2007), but are not otherwise homogenous. A subset of periglomerular interneurons co-express the neurotransmitter dopamine, whereas other subsets are characterized by the co-expression of either neuro-active peptides or calcium binding proteins (Kosaka et al., 1998; Parrish-Aungst et al., 2007; Toida, 2008). Previous studies have suggested that combinatorial expression patterns of transcription factors are critical for specifying the many different OB interneuron phenotypes (Allen et al., 2007; Brill et al., 2008).

In addition to combinatorial codes of transcription factors, epigenetic regulatory mechanisms are required to coordinate the complex patterns of gene transcription necessary for the maturation of specific OB interneuron phenotypes. Chromatin remodeling and DNA methylation are well established epigenetic mechanisms active in regulating neural stem cell proliferation and progenitor migration in the SVZ and RMS (Fasano et al., 2009; Ferron et al., 2011; Lim et al., 2009; Molofsky et al., 2003; Sheikh et al., 2012; Wu et al., 2010). By contrast, their role in regulating gene transcription during OB interneuron maturation is less understood. Here we review previous studies and provide novel evidence for epigenetic regulation of induction and maintenance of gene transcription necessary to express the GABA and dopaminergic neurotransmitter phenotypes in OB interneurons.

2. Materials and methods

2.1 Animal procedures

All procedures were carried out under protocols approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and conformed to NIH guidelines. Mice were housed in humidity-controlled cages at 22°C under a 12:12 hour light:dark cycle and provided with food and water ad libitum. *Th*-GFP transgenic mice expressing an enhanced green fluorescent protein (GFP) reporter driven by a 9kb portion of the *Th* promoter on a C57BL/6J background were obtained from Dr. Kazuto Kobayashi (Matsushita et al., 2002). For mice subjected to unilateral naris closure, one nostril of the mice (aged 6-8 weeks) was surgically closed using a spark-gap cautery under pentobarbital anesthesia. Details of the naris occlusion procedure have been previously published (Baker et al., 1993; Liu et al., 1999). Slice cultures were prepared from 2-day old *Th*-GFP mice and all studies examining the effect of HDAC inhibitors on either endogenous *Th* gene or *Th*-GFP transgene have been described previously (Akiba et al., 2010).

2.2 Gene expression analysis by quantitative PCR

RNA was isolated from adult wild-type C57BL/6J mouse OBs using an RNA miniprep kit (Qiagen) following the manufacturer's protocol. Reverse transcription and first strand cDNA synthesis reactions were conducted using SuperScript II first strand synthesis kit (Life Technologies). Quantitative PCR (qPCR) reactions for *Th*, *HDAC2* and β -*Actin* were performed using SYBR Green Master Mix (Life Technologies) with the following primer sets: *Th* 5'-CACTCCCTGTCAGAGGAGCC-3' and 5'-ATGAAGGGCAGGAGGAATGC-3'; *HDAC2* 5'-CAACAGATCGCGTGATGACCGTCT-3' and 5'-GACAGCATAGTATTTTCCCTTTCCAGCA-3'; β -*Actin* 5'-AGATGACCCAGATCATGTTTGAGACC-3' and 5'-GGAGTCCATCACAATGCCTGTGGT-3'. *Gad1* gene expression was measured using Taqman Gene Expression Assay primer set Mm00725661.s1 (Applied Biosystems) with the TaqMan Universal PCR Master Mix (Applied Biosystems). All qPCR reactions were carried out on a 7500 Fast Real-time PCR System (Applied Biosystems). Expression levels for all genes were measured in triplicate from two mice and normalized to β -*Actin* expression levels. Expression levels are reported as the mean with error bars representing the standard deviation. Data were analyzed using two-tailed Student T-tests for each gene, and differences were considered significant at $p < 0.01$.

2.3 Chromatin immunoprecipitation (ChIP) experiments

Olfactory bulbs were dissected and fixed for 20 minutes in PBS and 1% formaldehyde, after which the tissue was rinsed in PBS and then placed in lysis buffer (20mM Tris pH 8.1, 150mM NaCl, 0.5% Triton X-100 and 0.1% SDS). Tissue in the lysate suspension was crushed with a Dounce homogenizer before sonication with a Misonix 3000 sonicator (Misonix Inc). Following sonication, cellular debris was removed via centrifugation and lysate was then pre-cleared with Protein A/G Sepharose beads (Santa Cruz). The lysate was then divided into two equivalent samples before adding 1 μ g of either rabbit pan-acetyl Histone H3 (Millipore 06-599) or, as a negative control, rabbit IgG (Millipore PP64). Antibody/lysate solutions were incubated with gentle rocking overnight at 4°C, before Protein A/G Sepharose was added to precipitate antibody-protein-DNA complexes. The Protein A/G Sepharose beads were then removed from the lysate via centrifugation and washed twice with lysis buffer, twice with wash buffer (20mM Tris pH 8.1, 150mM NaCl, 0.5% Triton X-100 and 0.1% SDS, 2mM EDTA), once with LiCl buffer (0.25M LiCl, 10mM Tris pH 8.1, 1mM EDTA, 1% NP-40, 1% deoxycholate), twice with TE (10mM Tris-EDTA pH 8.0, 1mM EDTA), and then placed in elution buffer (0.1M NaHCO₃ pH 8.0,

1%SDS, 0.33M NaCl). The eluted samples were incubated overnight in 5M NaCl at 65°C before the DNA was isolated using Qiaquick PCR clean-up spin kits (Qiagen). DNA concentrations of both immunoprecipitated and input samples were estimated using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) and the manufacturer's protocol. The immunoprecipitated DNA containing the proximal promoter regions of either *Th* or *Gad1* was measured by qPCR on a 7500 Fast Real-time PCR System (Applied Biosystems) with the following primer sets: *Th* proximal promoter, 5'-CGTGTCTAGGGCGGAGGGTGA-3' and 5'-GCCAGGCAGGCGGCCTCTTA-3'; *Gad1* proximal promoter, 5'-TTCCCTTGCCCTCACCCAACATCG-3' and 5'-ACGGGAGAGCCTGAAGAAGGGAGA-3'. For each mouse, qPCR analysis of all samples was measured in triplicate. Data are reported as the mean of three separate ChIP experiments, each using a different mouse, with error bars representing the standard deviation. Data were analyzed using two-tailed Student T-tests, and differences were considered significant if $p < 0.01$.

2.4 Immunohistochemical analysis

Mice were anesthetized with an overdose of pentobarbital (100 mg/kg) and perfused transcardially with phosphate buffered 4% formaldehyde, generated from paraformaldehyde, post-fixed for 1-2 hours at room temperature or overnight at 4°C and then cryoprotected in 30% sucrose. Sections were cut on a sliding microtome at 30µm thickness, then blocked with 1% bovine serum albumin in PBS and incubated overnight with primary antisera. Antibodies and the dilutions used were: rabbit pan-acetyl Histone H3 (Millipore 06-599) at 1:500; rabbit HDAC2 (Epitomics 1603-1) at 1:500; mouse betatubulin (Millipore/Covance MMS-435P) at 1:2,000; rabbit anti-TH (produced in our laboratory as described in Joh et al., 1973) at 1:25,000. Antigens were visualized either by fluorescence with donkey anti-rabbit AlexFluor 594 (Life Technologies) at 1:400 dilution or colorimetric detection using diaminobenzadine. *In situ* hybridizations were performed as previously described (Saino-Saito et al., 2004). Sections were imaged on both a Nikon 80i Eclipse fluorescence microscope and a Zeiss Meta 510 scanning laser confocal microscope.

3. Results and Discussion

3.1 Regulation of the GABAergic phenotype

The GABAergic phenotype is established early in the development of OB interneurons. Canonical biosynthesis occurs through the single-step conversion of glutamate to GABA by either Glutamic Acid Decarboxylase 1 (GAD1) or GAD2 enzymes. *In situ* hybridization studies and analysis of transgenic reporters in rodents indicate that neuroblasts in the SVZ and RMS transcribe either or both *Gad1* and *Gad2* genes (De Marchis et al., 2004; Plachez and Puche, 2012; Wang et al., 2003). Although the *Gad* genes are transcribed, GAD2 protein is not detected and GAD1 protein, if present, is at low levels in migrating progenitors (De Marchis et al., 2004; Plachez and Puche, 2012; Wang et al., 2003). This lack of GAD protein is paradoxical since GABA is both produced and released by progenitors as they migrate through the SVZ and RMS (Bolteus and Bordey, 2004; Liu et al., 2005). Resolution of this paradox comes from findings that show progenitors can generate GABA by an alternative biosynthetic pathway involving putrescine (Sequerra et al., 2007). In mature OB interneurons, by contrast, GAD1/2 translation is derepressed and GABA is generated by the canonical biosynthetic pathways

Terminal differentiation of OB interneurons requires integration into neuronal circuitry. Synaptic activity levels within OB circuits, however, are strongly modulated by odorant-induced activation of olfactory receptor neurons. Odor deprivation, both short (1-2 month) and long-term (12 months), enhances the sensitivity of mitral/tufted cells to odorant re-

exposure, suggesting that inhibition by OB interneurons is depressed by decreased olfactory stimulation (Wilson and Sullivan, 1995). Consistent with these findings, a recent report showed that chemically-induced suppression of synaptic activity in the OB reduces the amplitude of inhibitory post-synaptic currents and decreases GAD1 expression levels in the glomerular layer (Lau and Murthy, 2012). Together with studies showing GAD1 protein levels are reduced in response to odorant-deprivation (Parrish-Aungst et al., 2011), these findings indicate that synaptic activity-dependent control of GAD1 expression levels and GABA biosynthesis is an important modulator of inhibitory transmission in the OB.

The molecular mechanisms that regulate *Gad1* expression in the OB are not established, but epigenetic mechanisms are integral to the control of *Gad1* transcription in other brain regions (Dong et al., 2005; Guidotti et al., 2011; Kundakovic et al., 2009; Ramirez and Gutierrez, 2001). Recent studies in our laboratory using mice subjected to unilateral naris occlusion have shown that reduced *Gad1* transcription levels in the OB resulting from odor deprivation are associated with a reduced occupancy of acetylated histone H3 on the *Gad1* promoter (Figure 2A-C). Acetylated histone H3 is a marker of chromatin environments that are supportive of transcription and these findings indicate that the local chromatin environment of the *Gad1* promoter in the OB is regulated by odorant-induced synaptic activity. The reduction of acetylated-H3 on the *Gad1* promoter is not a consequence of a general reduction in H3 acetylation caused by odor deprivation since immunofluorescence analysis of the OB in mice with unilateral naris occlusion display no significant decrease in the intensity of staining and no alteration in the distribution of acetylated-H3 (Figure 2D).

Taken together, the current evidence indicates that a modified GABAergic phenotype is established early in migrating progenitors. The mature phenotype is not fully expressed until translational repression mechanisms are suppressed as part of the terminal differentiation process. Epigenetic mechanisms modulate GAD1 levels in mature OB neurons by synaptic activity-dependent chromatin remodeling of the *Gad1* gene promoter.

3.2 Regulation of the dopaminergic phenotype

In the glomerular layer, a subset of OB interneurons co-express dopamine and GABA. These dopaminergic neurons are readily identified by the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine biosynthesis. Pre-synaptic release of dopamine by glomerular layer interneurons inhibits both excitation of mitral/tufted cells by olfactory receptor neurons and excitation of interneurons by mitral/tufted cells (Davila et al., 2003; Ennis et al., 2001; Hsia et al., 1999). Recent reports suggest that OB dopaminergic neurons also modulate inter-glomerular connectivity (Kiyokage et al., 2010). Together, these studies suggest that OB dopaminergic neurons are important for both maximizing the dynamic range for odorant detection threshold and improving odorant discrimination.

Similar to GAD1, translational regulatory mechanisms spatially restrict TH protein expression to only a subset of cells that transcribe *Th* (Saino-Saito et al., 2004). Both *in situ* hybridization and *in vivo* analysis of *Th*-GFP reporter gene expression show that *Th* is transcribed in both the superficial granule cell and glomerular layers, but TH protein expression is limited to only the glomerular layer (Figure 3A). *Th*-expressing superficial granule cells are reported to co-express NeuN, a marker of mature neurons, suggesting that these cells are terminally differentiated (Kohwi et al., 2005). Belluzzi and colleagues, however, have shown that these cells have electrophysiological properties that are intermediate to migrating progenitors and mature dopaminergic neurons in the glomerular layer, suggesting that they are a reservoir of immature dopaminergic neurons that have not yet completed their migration to the glomerular layer (Pignatelli et al., 2009).

Studies with cultured primary OB neurons indicate that membrane depolarization and activation of L-type calcium channels is sufficient to induce *Th* transcription (Cigola et al., 1998; McMillian et al., 1994; Puche and Shipley, 1999). Progenitors in either SVZ or RMS, however, do not transcribe the endogenous *Th* gene (Saino-Saito et al., 2004), despite evidence that they are both depolarized and have L-type calcium channels activated by ambient glutamate in the SVZ and RMS (Darcy and Isaacson, 2009). The molecular mechanisms that mediate this developmental stage-specific repression of *Th* transcription are not fully established, but we have previously shown that histone deacetylase (HDAC) enzymes are required (Akiba et al., 2010). Treatment with Class I and II HDAC inhibitors (including Trichostatin A, Scriptaid, valproic acid and sodium butyrate) either in forebrain slice cultures or in vivo was sufficient to induce *Th* promoter activity in the RMS (Figure 3B,C). These studies also found that HDAC inhibitors induced *Th* transcription in SVZ-derived primary neural progenitor cultures (Figure 3D,E). Together, these findings indicate that transcription factors necessary to initiate *Th* transcription are present in the migrating neuroblasts, but HDAC activity prevents these proteins from initiating *Th* transcription.

Our previous studies also found that HDAC inhibitors enhanced *Th* expression levels in the glomerular layer, suggesting that HDAC-mediated mechanisms continue to regulate *Th* transcription in newly differentiated and mature dopaminergic neurons. *Th* expression in the OB glomerular layer is dependent on odorant-mediated synaptic activity (Baker et al., 1983; Saino-Saito et al., 2004). Using mice subjected to unilateral naris occlusion, we have recently found that this activity-dependent transcription is associated with differential occupancy of acetylated H3 on the *Th* promoter (Figure 3F). This finding reveals that activity-dependent control of *Th* transcription and dopamine production in mature OB interneurons is mediated, in part, by chromatin remodeling of the *Th* promoter. Thus, in both neuroblasts and mature neurons epigenetic mechanisms mediated by HDAC enzymes are critical for regulating expression of the OB dopaminergic phenotype.

The HDAC enzymes responsible for activity-dependent chromatin remodeling of the *Th* and *Gad* promoters have not been established, but recent studies in our laboratory suggest that HDAC2 may play a pivotal role. Previous studies have demonstrated that HDAC2 is preferentially expressed in the terminally differentiated neurons and is critical for the maturation of adult OB interneurons (Jawerka et al., 2010; MacDonald and Roskams, 2008). In agreement with these previous studies, we have found that nearly all dopaminergic OB interneurons in adult *Th*-GFP transgenic mice (Matsushita et al., 2002) co-express HDAC2 (Figure 4A-C). Interestingly, in mice subjected to unilateral naris occlusion, we have also found that odorant-induced synaptic activity levels differentially modify *HDAC2* and *Th* mRNA levels in the OB (Figure 4D), suggesting that HDAC2 is a negative regulator of the dopaminergic phenotype in mature OB interneurons.

3.3 Similarities in epigenetic regulation of *Gad1* and *Th* in the OB

Since *Th* is preferentially co-expressed with *Gad1* in the OB (Parrish-Aungst et al., 2007), a reasonable speculation is that transcription of both *Th* and *Gad1* are regulated by a similar activity-dependent chromatin-remodeling mechanism involving HDAC2. This shared mechanism could be mediated by a common set of transcription factor proteins that bind both the *Th* and *Gad1* promoter regions and recruit HDAC2. Several putative transcription factor binding sites are present in both the *Th* and *Gad1* promoter regions, with some sites found in both genes (Kessler et al., 2003; Kobayashi et al., 2003; Schimmel et al., 1999; Szabo et al., 1996; Yanagawa et al., 1997; Yang et al., 1998). Although a shared regulatory mechanism in the OB has yet to be demonstrated, possible co-regulation by the neural restrictive silencer element (NRSE) is a high priority candidate for further investigation. The NRSE can recruit multi-protein complexes that contain HDAC2 to repress neuronal gene expression (Ballas et al., 2001; Roopra et al., 2000) and NRSEs are present in the proximal

promoter regions of both *Th* and *Gad1* (Katarova et al., 1998; Kessler et al., 2003; Kim et al., 2003; Szabo et al., 1996). The ability of membrane depolarization to abrogate NRSE-mediated repression of some neuronal genes is also consistent with a potential role in regulating the synaptic activity-dependent transcription of *Th* and *Gad1* in the OB (Ballas et al., 2005).

Epigenetic regulation of neuronal gene transcription typically involves both covalent modifications to histones and DNA methylation (reviewed in Hsieh and Gage, 2005). The findings discussed in this report provide insight into the role that histone modifications have in regulating the GABAergic and dopaminergic phenotypes in the OB, but the role of DNA methylation in the chromatin remodeling of either *Th* and *Gad1* promoter remains to be established. Methylation of the *Gad1* promoter in cortical interneurons reduces transcription and this methylation is modulated by both H3 acetylation levels and activation of metabotropic glutamate receptors (Dong et al., 2007; Matrisciano et al., 2011). Since progenitors of both cortical and OB interneurons are derived from the embryonic ganglionic eminences, similar mechanisms may also mediate methylation of *Gad1* in OB GABAergic interneurons. By contrast, the role of DNA methylation in regulating *Th* transcription within the brain is largely unexplored, although differences in *Th* promoter methylation levels between neural and non-neural cell lines have been reported (Aranyi et al., 2005; Kilbourne et al., 1991; Yang et al., 2011).

Th and *Gad1* transcription appear coordinated in mature OB interneurons, but expression of these genes in migrating neuroblasts is clearly not. The molecular basis for this differential transcriptional regulation in progenitors is not understood, but an important component may be the developmental stage-specific expression of epigenetic regulatory proteins. In olfactory receptor neuron development, HDAC 1/2, methyl CpG binding protein 2 (MeCP2), methyl binding domain (MBD) 2, and DNA methyl transferase (DNMT) 3a/b proteins are co-expressed in developmental stage-specific patterns (MacDonald et al., 2005; MacDonald and Roskams, 2008; Macdonald et al., 2010). These stage-specific combinatorial co-expression patterns are thought to be critical for differentially expressing genes at distinct stages of maturation. Further studies are necessary to establish whether similar combinatorial co-expression patterns of epigenetic regulatory proteins also control the development and maintenance of neurotransmitter phenotypes in the OB.

3.4 Developmental role for regulating timing of neurotransmitter phenotype expression

The restriction of both the mature GABAergic and dopaminergic phenotypes to terminally differentiated neurons in the OB may be related to the role that these neurotransmitters have as non-synaptic modulators of neurogenesis in the SVZ and RMS. Progenitor proliferation and migration rates in these regions are modulated by neurotransmitters generated locally (GABA and glutamate) and distantly in other brain regions (dopamine and serotonin) (reviewed in Bovetti et al., 2011; Young et al., 2011). GABA acts as a negative regulator of both proliferation and migration (Platel et al., 2008a). By contrast, glutamate released by astrocyte-like cells in the SVZ stimulates proliferation and decreases migration (Castiglione et al., 2008; Di Giorgi Gerevini et al., 2004; Gandhi et al., 2008; Platel et al., 2008b). Together, GABA and glutamate function as local modulators of neurogenesis. In addition to these local signals, serotonin and dopamine released by terminals projecting from the Raphe nuclei and midbrain, respectively, stimulate neurogenesis in the SVZ (Baker et al., 2004; Banasr et al., 2004; Brezun and Daszuta, 1999; Freundlieb et al., 2006; Hoglinger et al., 2004; O'Keefe et al., 2009b). In the case of dopamine, the loss of midbrain dopaminergic inputs either through deafferentation or chemical lesions reduces SVZ progenitor proliferation rates and suggest that OB interneuron neurogenesis is influenced by the behavioral and movement control functions of the midbrain (O'Keefe et al., 2009a).

Regulation of progenitor proliferation and migration in the SVZ by distant brain regions indicates a need to tightly coordinate local biosynthesis of neurotransmitters. Given the large number of neuroblasts generated daily in the SVZ (Lois and Alvarez-Buylla, 1994), unrestrained production and release of neurotransmitters by neuroblasts could overwhelm regulatory signals from distant regions. Dopamine production by neuroblasts, for example, would likely increase progenitor proliferation rates, but diminish or screen out the input from midbrain neurons. The functional consequences of either a sustained increase in progenitor proliferation or a loss of midbrain input, however, are difficult to predict. By contrast, increased ambient concentrations of GABA in the SVZ and RMS could depress proliferation and migration to the point where the numbers of progenitors are insufficient to replace aging OB interneurons. Reductions in either the production or migration of neural progenitors in adult mice has been shown to impair some olfactory- based cognitive functions (reviewed in Lazarini and Lledo, 2011).

4. Conclusions

Epigenetic mechanisms are integral for establishing and maintaining dopaminergic and GABAergic phenotypes in OB interneurons. HDAC enzymes repress *Th* transcription and suppress the dopaminergic phenotype in migrating neuroblasts until progenitors terminally differentiate in the OB. In the mature interneurons, both *Th* and *Gad1* transcription levels are modulated by odorant-induced synaptic activity that results in the differential recruitment of acetylated histone H3 on both the *Th* and *Gad1* proximal promoters. The inverse transcription response of *HDAC2* to odorant-induced synaptic activity, as compared to *Th* and *Gad1*, suggests that HDAC2 mediates, in part, the activity-dependent chromatin remodeling of the *Th* and *Gad1* proximal promoters.

Although epigenetic regulatory mechanisms have a clear role in establishing and maintaining neurotransmitter phenotypes in the OB, there are several important features of this regulation that require further exploration. The role of promoter DNA methylation, in particular, is largely unexplored in OB neurogenesis and is likely an important regulatory mechanism for chromatin remodeling that is coordinated with covalent modifications of histones. Elucidation of these mechanisms will also require establishing the developmental stage-specific roles of epigenetic regulatory proteins during OB neurogenesis. As discussed above, studies in olfactory receptor neuron development indicate that complex combinatorial co-expression patterns of these proteins delineate specific transitions in the neuronal maturation process (Macdonald et al., 2010). In the OB dopamine phenotype, for example, further studies are necessary to establish whether HDAC1 and HDAC2 have distinct developmental roles in regulating *Th* transcription. These two enzymes have complementary expression patterns during development of OB interneurons: HDAC2 is preferentially expressed in mature neurons and HDAC1 is expressed in neuroblasts and glia (MacDonald and Roskams, 2008). HDAC1 may be responsible for keeping *Th* expression repressed when migrating neuroblasts are depolarized, whereas HDAC2 may be required for mediating synaptic activity-dependent regulation of *Th* transcription in mature neurons. Together, the data discussed here provide insight into the role that epigenetic mechanisms have for coordinating both developmental signals and synaptic activity levels to establish and maintain neurotransmitter phenotypes in the OB.

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Abbreviations

acH3	acetylated Histone H3
ChIP	chromatin immunoprecipitation
GABA	gamma aminobutyric acid
GAD	glutamic acid decarboxylase
HDAC	histone deacetylase
NRSE	neural restrictive silencer elements
OB	olfactory bulb
qPCR	quantitative polymerase chain reaction
RMS	rostral migratory stream
SVZ	subventricular zone
TH	tyrosine hydroxylase
TSA	Trichostatin A

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Highlights

HDAC activity represses dopamine phenotype in migrating olfactory bulb progenitors

Th and Gad1 levels in mature olfactory bulb neurons are synaptic activity dependent

Synaptic activity remodels Th and Gad1 proximal promoter chromatin environments

HDAC2 expression in mature olfactory bulb neurons is reduced by synaptic activity

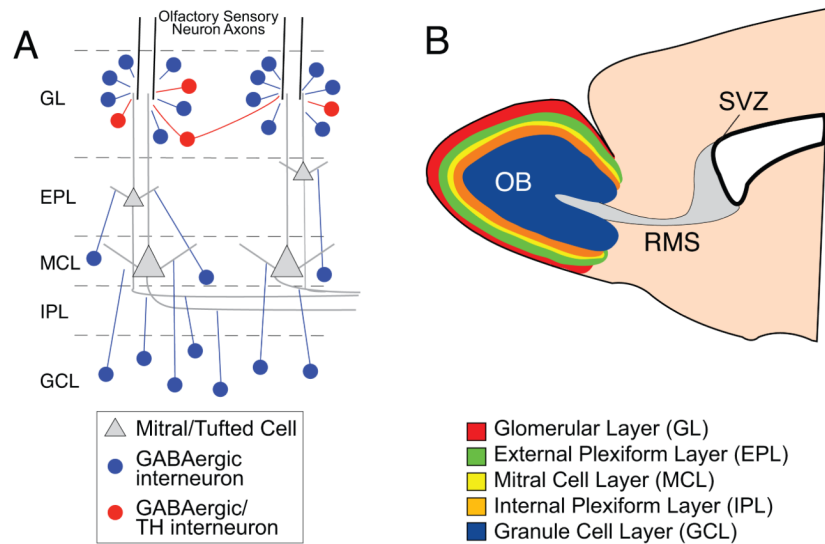


Figure 1. Laminar organization and circuit diagram for the olfactory bulb. **A**, a cartoon summary of the key synaptic connections in the main olfactory bulb based on studies in mice. Olfactory receptor neuronal axons terminate in the glomerular layer where they form axo- and dendro-dendritic connections with both the mitral and tufted cells and inhibitory periglomerular layer interneurons. The mitral/tufted cells are the primary output cells and their axons project to other cortical regions. The transmission of odorant sensory information for higher level processing in the cortex is modulated by inhibitory neurons in the granule cell layer. **B**, a cartoon of the mouse forebrain showing the position of the individual laminae of the main olfactory bulb (OB), rostral migratory stream (RMS) and subventricular zone (SVZ).

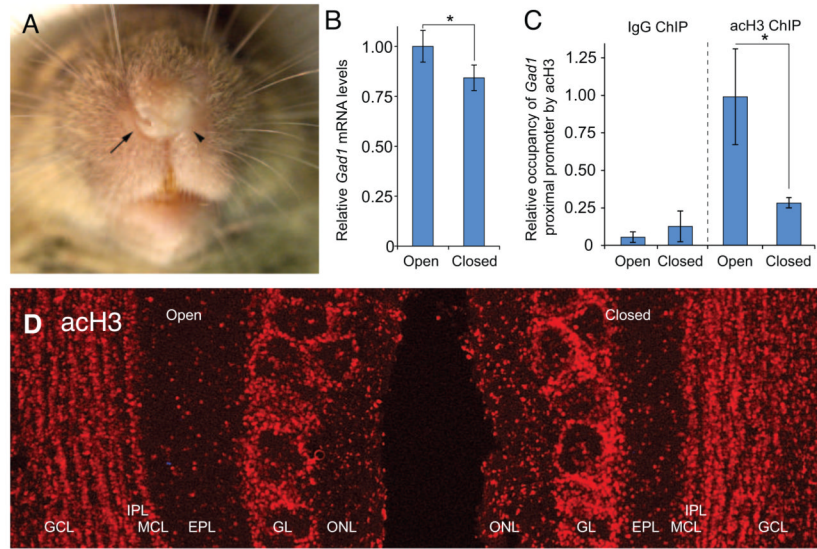


Figure 2.

Odorant-mediated regulation of *Gad1* transcription in the olfactory bulb. **A**, a mouse subjected to odorant deprivation by unilateral naris occlusion. The open and closed nares are indicated by the arrow and arrowhead, respectively. **B**, qRT-PCR analysis reveals *Gad1* mRNA levels are reduced in the olfactory bulb that is ipsi-lateral to the closed nares (closed) when compared to the bulb corresponding to the unobstructed contra-lateral nares (open). **C**, chromatin immunoprecipitation (ChIP) shows that occupancy of pan-acetylated Histone H3 (acH3) on the *Gad1* proximal promoter is dramatically reduced in the closed bulb relative to the open bulb. For comparison, negative control ChIP experiments with antibodies to rabbit IgG are also shown. **D**, immunofluorescence analysis for acH3 in the olfactory bulbs of mice subjected to unilateral naris occlusion shows that the reduced stimulation produced by odor deprivation does not diminish total levels of acH3 in the bulb ipsi-lateral to the naris closure (closed). Rather, there appears to be a slight increase in the acH3 intensity in this bulb. Thus, the changes in acH3 occupancy on the *Gad1* proximal promoter observed in **C** are the result of mechanisms that specifically target the *Gad1* promoter.

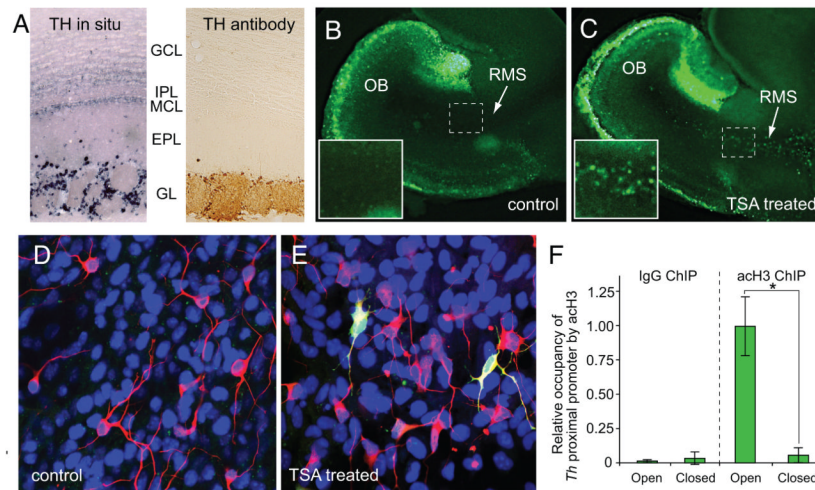


Figure 3.

Histone deacetylase activity suppresses *Th* transcription in the rostral migratory stream (RMS) and olfactory bulb (OB). **A**, in situ hybridization in the adult OB shows strong levels of *Th* transcription in the glomerular layer (GL) as well as weak expression levels in the mitral and superficial granule cell layers (MCL and GCL, respectively). There is scattered transcription in the external plexiform layer (EPL), but no expression in the internal plexiform layer (IPL). In contrast to the in situ hybridization studies, immunohistochemical analysis with antibodies to TH protein reveal that protein expression is limited to only the glomerular layer. **B** and **C**, GFP expression in neonatal forebrain slice cultures from *Th*-GFP mice after 24 hour treatment with either vehicle (control) or 1.2 μ M trichostatin A (TSA), respectively. The presence of the HDAC inhibitor, TSA, permits GFP expression within migrating progenitors in the RMS. The slices in **B** and **C** were cultured with depolarizing conditions (25mM KCl), which induces GFP expression in the glomerular layer. The presence of TSA, however, substantially increases GFP expression levels in the glomerular layer relative to the control slices. Insets show higher magnifications images of boxed areas. **D** and **E**, primary neural progenitor cultures from *Th*-GFP mice 24 hours after treatment with either vehicle (control) or 1.2 μ M trichostatin A (TSA), respectively. The neuronal progenitor marker, β -tubulin III (red) is expressed by several cells in both sets of cultures. By contrast, only TSA-treated cultures show *Th*-GFP co-expressed in a subset of neuronal progenitors (yellow cells). **F**, in mice subjected to unilateral naris occlusion, chromatin immunoprecipitation (ChIP) experiments show that occupancy of pan-acetylated Histone H3 (acH3) on the *Th* proximal promoter is drastically reduced in the bulb ipsi-lateral to nares closure (closed) relative to the bulbs that are contra-lateral (open). For comparison, negative control ChIP experiments with antibodies to rabbit IgG are also shown.

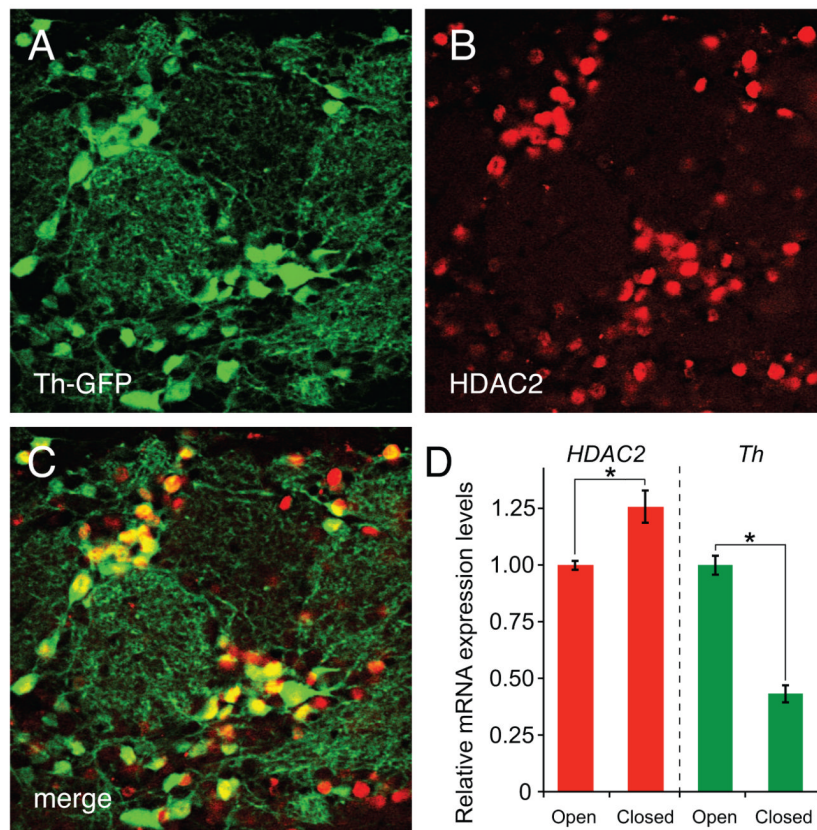


Figure 4. HDAC2 expression in olfactory bulb dopaminergic neurons. **A-C**, in the OB of adult *Th*-GFP mice, immunofluorescence analysis reveals that nearly all GFP-expressing dopaminergic neurons (green) also co-express HDAC2 (red). Regions with *Th*-GFP and HDAC2 co-expression appear as yellow. **D**, in mice subjected to unilateral naris occlusion, qRT-PCR analysis shows *Th* and *HDAC2* transcription levels have opposite responses to synaptic activity. In bulbs contra-lateral to naris closure (open), where odorant-mediated synaptic activity is highest, *Th* expression is greatest and *HDAC2* is depressed. By contrast, *Th* expression is depressed and *HDAC2* is maximal in the olfactory bulb ipsi-lateral to naris closure (closed) where synaptic activity is lowest.

Table 1

Gene and protein expression for GABAergic and dopaminergic rate-limiting biosynthetic enzymes (Gad1/2 and Th, respectively) in relationship to HDAC1/2 protein levels during OB interneuron development. Expression levels are indicated as strong (+++), modest (++), low (+), weak (+/-) and not expressed (-). Odorant-induced synaptic activity dependent expression is indicated by "AD." For the GAD and TH proteins, protein expression levels do not necessarily directly correspond to levels of the respective neurotransmitters present in the cell. For GAD, in particular, there are alternative biosynthetic pathways for GABA biosynthesis in progenitor cells (see section 3.1 for details).

Cell Type	Gad1		Gad2		TH		HDAC1	HDAC2
	mRNA	Protein	mRNA	Protein	mRNA	Protein		
Migrating Neuroblast	+++	+/-	+++	-	-	-	+++	+/-
Immature OB Neuron	+++	+/-	+++	-	+	-	++	++
Terminally Differentiated OB Neuron	+++	+++ (AD)	+++	+++	+++	+++ (AD)	+/-	+++
References	De Marchis et al., 2004; Planchez and Puche, 2012; Wang et al., 2003		Baker et al., 1983; Saino-Saito et al., 2004		Jawerka et al., 2010; MacDonald and Roskams, 2008			