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Expression of EGR-1 in a subset of olfactory bulb dopaminergic cells

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Abstract

In the adrenal medulla, binding of the immediate early gene (IEG) proteins, EGR-1 (ZIF-268/KROX-24/NGFI-A) and AP-1, to the *tyrosine hydroxylase* (*Th*) proximal promoter mediate inducible *Th* expression. The current study investigated the potential role of EGR-1 in inducible *Th* expression in the olfactory bulb (OB) since IEGs bound to the AP-1 site in the *Th* proximal promoter are also necessary for activity-dependent OB TH expression. Immunohistochemical analysis of a naris-occluded mouse model of odor deprivation revealed weak EGR-1 expression levels in the OB glomerular layer that were activity-dependent. Immunofluorescence analysis indicated that a majority of glomerular cells expressing EGR-1 also co-expressed TH, but only small subset of TH-expressing cells contained EGR-1. By contrast, granule cells, which lack TH, exhibited EGR-1 expression levels that were unchanged by naris closure. Together, these finding suggest that EGR-1 mediates activity-dependent TH expression in a subset of OB dopaminergic neurons, and that there is differential regulation of EGR-1 in periglomerular and granule cells.

Keywords

dopamine; olfactory bulb; immediate early gene; transcription; differentiation

Introduction

Tyrosine hydroxylase (TH), the rate limiting enzyme for the biosynthesis of dopamine and a reliable marker for catecholaminergic neurons, is expressed in a subset of interneurons in the glomerular layer of olfactory bulb (OB). These dopaminergic cells provide inhibitory feedback onto olfactory receptor neurons, mitral and tufted projection neurons as well as other glomerular layer interneurons (Cave and Baker 2008). TH expression in the OB is induced by afferent input from olfactory receptor neurons and synaptic activity of mitral and tufted cells (Baker et al. 1983; Baker et al. 1993). Previous studies have shown that AP-1 and CREB immediate early gene (IEG) transcriptions factor binding sites in the *Th* proximal promoter are necessary for activity-dependent TH expression in the OB (Liu et al. 1999; Trocme et al. 1998).

IEG transcription factors also regulate inducible *Th* expression in other groups of catecholaminergic neurons, including the adrenergic cells of the adrenal medulla (Sabban 1997). The zinc finger transcription factor EGR-1 (ZIF-268/KROX-24/NGFI-A) regulates *Th* transcription in the adrenal medulla by binding an SP1-like binding site in the *Th* proximal

promoter and synergistically interacting with nearby AP-1 proteins (Nakashima et al. 2003; Papanikolaou and Sabban 2000).

Although EGR-1 expression in the OB has been previously demonstrated, a potential role in the regulation of OB TH expression has not been examined. In the glomerular layer, inducible EGR-1 expression has been used to map innervation of glomeruli by olfactory receptor neurons that are responsive to specific odorants (Alonso et al. 2006; Inaki et al. 2002; Johnson et al. 1995; Mandaïron et al. 2006). Together with the established regulatory role in the adrenal medulla, these studies suggest that EGR-1 is a potential regulator of activity-dependent *Th* expression in the OB. To test this possibility, we have examined the OB of adult mice for co-expression of EGR-1 and TH.

Materials and Methods

Animals

C57BL/6J 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. For the naris occluded mouse model of odor deprivation, one nostril of adult mice (aged 6–8 weeks) was surgically closed using a spark-gap cautery under pentobarbital anesthesia. Naris occlusion was confirmed at 1 and 3 months post-operation. Details of the naris occlusion procedure have been previously published (Baker et al. 1993; Liu et al. 1999). All procedures were carried out under protocols approved by the Cornell University Institutional Animal Care and Use Committee and conformed to NIH guidelines. Animals sacrificed and examined in this study were approximately 1 year of age.

Immunohistochemistry

Localization of single antigens was performed as previously published (Saino-Saito et al. 2007). Briefly, frozen sections (40 µm) were obtained from brains fixed with phosphate-buffered (pH 7.2) 4% formaldehyde. Sections were washed in phosphate-buffered saline (PBS) before being blocked with 1% bovine serum albumin in PBS and incubated overnight with primary antisera. Antigens were visualized by incubation with appropriate biotinylated secondary antiserum (1:200) and the Vector Elite kit (Vector Laboratories) with both 3,3'-diaminobenzidine (DAB, 0.05%) and hydrogen peroxide (0.003%). Slides were dehydrated through a graded series of alcohols and cover-slipped. For double label immunofluorescence, secondary antibodies conjugated to either Alexa-488 or Alexa-594 were used (1:600, Molecular Probes/Invitrogen). Primary antibodies used were rabbit anti-TH (lot 15-2, raised in our laboratory, 1:25,000 and 1:10,000, for single and double-labeling, respectively), mouse anti-TH monoclonal (1:10,000 for double labeling; Roche), rabbit anti-EGR-1 (1:7500 and 1:1500, for DAB and immunofluorescence, respectively; sc-110X antibody from Santa Cruz Biotechnology).

Cell Counts—For cell counts of EGR-1 and TH expression in unilateral naris-occluded mouse models of odor deprivation, cells in the entire glomerular layer were counted from equivalent sections obtained from three separate mice. For cell counts to quantify the overlap of EGR-1 and TH in the glomerular layer of immunofluorescent-labeled sections, cells were counted in 200 µm glomerular lengths from equivalent sections of three adult mice. For both sets of cell counts, means and their standard deviation are reported.

Results

Immunohistochemical analysis of adult mice revealed strong nuclear EGR-1 expression in the granule cells of both the granule and mitral cell layers (Figure 1A). However, in the glomerular

layer, EGR-1 was expressed at substantially lower levels in scattered cells (Figure 1A and inset). This pattern contrasted with TH, which was strongly expressed almost exclusively in the glomerular layer (Figure 1B).

Immunohistochemistry and cell counts of sections from the OB of unilateral naris-occluded mice demonstrated that EGR-1 expression in the glomerular layer was activity-dependent (Figure 2A and 2B). In this unilateral odor deprivation model system, the loss of odorant stimulation reduces olfactory receptor neuron synaptic activity in the OB ipsi-lateral to the closed naris and down-regulates activity-dependent gene expression, such as *Th* (Figure 2C and 2D)(Baker et al. 1993). In the glomerular layer of the OB contra-lateral to the closed naris, where olfactory receptor neuron synaptic activity remains high, EGR-1 expression was detected in scattered cells (Figure 2A"). In contrast, the number cells expressing EGR-1 was drastically reduced in the glomerular layer ipsi-lateral to the naris closure (Figure 2B), and the EGR-1 expression levels within those cells was noticeably weaker relative to the cells in contra-lateral bulb. Unlike the glomerular layer, EGR-1 expression levels in the granule cells of either the granule or mitral cell layer were unchanged by naris closure (Figure 2A).

Immunofluorescence studies revealed that EGR-1 was expressed in a subset of TH-containing cells in the OB (Figure 3). These studies found that $61\pm9\%$ of all cells expressing EGR-1 also contained TH. However, only about $25\pm7\%$ of TH-containing cells co-expressed EGR-1.

Since there was partial co-expression of EGR-1 and TH in the OB glomerular layer, immunofluorescence studies were also performed to determine if there was overlap in the cells that continue to express EGR-1 and TH in OB ipsi-lateral to naris occlusion. However, the weak expression levels of EGR-1 expression in the OB ipsilateral to the closure were undetectable by immunofluorescence (data not shown), which was not surprising since EGR-1 in the ipsi-lateral OB was only weakly detected by the significantly more sensitive diaminobenzidine (DAB) immunohistochemical method (Figure 2). Thus, it is unclear whether EGR-1 and TH co-label a distinct sub-population of periglomerular cells in the OB ipsi-lateral to naris occlusion.

Discussion

The findings in this study suggest that EGR-1 is an activity-dependent regulator of inducible TH expression within a limited subset of dopaminergic OB neurons. Within this subset, EGR-1 likely mediates activity-dependent expression of TH by a mechanism similar to what has been shown in the adrenal medulla. In this mechanism, EGR-1 expression is induced by synaptic activity, and EGR-1 bound to the *Th* proximal promoter synergistically interacts with IEG transcription factors bound to nearby AP-1 site (Nakashima et al. 2003; Papanikolaou and Sabban 2000). The co-expression of EGR-1 in only a subset of TH-containing cells suggests that there is molecular heterogeneity among the OB dopaminergic neurons. The functional significance of such heterogeneity has yet to be demonstrated, but may indicate that subgroups of these neurons are derived from different lineages as recent studies have suggested (Inoue et al. 2007; Vergano-Vera et al. 2006).

Previous studies with transgenic mice have shown that the 9kb upstream regulatory region of *Th* gene can direct either LacZ or GFP expression in superficial granule cells (Min et al. 1996; Saino-Saito et al. 2004). Although EGR-1 is strongly expressed in the superficial granule cells, preliminary studies examining EGR-1 expression in *Th*-GFP mice have indicated that there is little overlap between GFP and EGR-1 in superficial granule cells (data not shown). However, this lack of co-expression is difficult to interpret since these superficial granule cells lack TH protein and *Th*-driven reporter gene expression is not activity-dependent (Baker et al.

2001), even though these cells have been reported to be differentiated neurons (Kohwi et al. 2005).

The weak EGR-1 expression levels in the OB glomerular layer was unexpected since robust EGR-1 expression in the OB glomerular layer has been used to map the innervation of olfactory receptor neurons (Alonso et al. 2006; Inaki et al. 2002; Johnson et al. 1995; Mandairon et al. 2006). In these mapping studies, animals were exposed to deodorized air for several hours before exposure to odorants. The sustained exposure to deodorized air minimizes olfactory receptor neuron synaptic activity and was thought to account for the absence of EGR-1 expression in the remaining OB glomerular layer not stimulated by the experimental odorants. However, the findings in this study indicate that EGR-1 is expressed at low levels throughout the OB glomerular layer under normal physiological conditions, which further suggests that high EGR-1 expression levels in OB glomerular layer are transient and require strong odorant activity.

The finding that EGR-1 expression levels in the OB granule cells were not sensitive to naris closure suggests that there is differential regulation of EGR-1 expression in granule and periglomerular cells. Two possible mechanisms are that either EGR-1 expression in the granule cell layer is not activity-dependent or synaptic activity from centrifugal inputs that specifically contact granule cells is sufficient to maintain EGR-1 expression when olfactory receptor neuron activity is absent. This OB laminar-specific differential regulation of EGR-1 expression is similar to the IEG c-FOS. In the glomerular layer, c-FOS expression is dependent on olfactory receptor neuron activity, but robust c-FOS expression in the granule cell layer is maintained even under conditions of naris occlusion (Liu et al. 1999). Together, these findings suggest that there are lamina-specific regulatory mechanisms for IEG expression in the OB. Elucidating these regulatory mechanisms will be critical for the understanding activity-dependent TH expression and dopaminergic neuron differentiation in the OB.

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Figure 1.

EGR-1 and TH expression in horizontal OB sections from an adult mouse. **A**, EGR-1 is strongly expressed in the nuclei of granule cells in the granule and mitral cell layers (gcl and m, respectively). In the glomerular layer (gl), weak nuclear EGR-1 expression was observed in scattered cells (see inset). **B**, TH was primarily expressed in the periglomerular cells.

Abbreviations: granule cell layer (gcl), internal plexiform layer (ipl), mitral cell layer (m), external plexiform layer (epl), and glomerular layer (gl). Bar = 300 μm for **A** and **B**, and 100 μm for the insets.

Figure 2.

Activity-dependent EGR-1 and TH expression in horizontal sections from the OB of a naris closed mouse model of odor deprivation. **A**, in the glomerular layer, EGR-1 expression is drastically reduced ipsi-lateral to the closed naris (cf. A' vs. A''). By contrast, EGR-1 expression in granule cells is unchanged by naris closure. **B**, cell counts of EGR-1 expressing glomerular cells confirm that odor-mediated synaptic activity is critical for EGR-1 expression in the OB glomerular layer. **C** and **D**, immunohistochemistry and cell counts, respectively, demonstrate that TH expression levels in the glomerular layer are strongly dependent odorant activity (cf. C' vs. C''). Abbreviations: granule cell layer (gcl), internal plexiform layer (ipl), mitral cell

layer (m), external plexiform layer (epl), and glomerular layer (gl). Bar = 250 μm for **A** and **C**, and 25 μm for the insets.

Figure 3.

Co-expression of EGR-1 and TH in the OB glomerular layer of an adult mouse.

Immunofluorescence of nuclear EGR-1 (red) and cellular TH (green) in periglomerular cells.

Although many cells with EGR-1 (arrowheads) co-express TH, most cells containing TH lack EGR-1. Bar = 15 μ m.