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Regional Distribution of Protein Kinases in Normal and Odor-deprived Mouse Olfactory Bulbs

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Abstract

Unilateral naris closure produced dramatic down-regulation of tyrosine hydroxylase (TH) gene expression in periglomerular dopaminergic neurons in the olfactory bulb. To explore molecular mechanisms of TH gene regulation, the present study investigated the regional distribution of protein kinase A (PKAα), protein kinase C (PKCα), and CaM kinases II (CaMKIIα, β) and IV (CaMKIV) in the normal olfactory bulb and in response to odor deprivation. Strong PKAα immunostaining was found in the glomerular, granule cell, external plexiform and olfactory nerve layers. PKCα staining was strong in granule cell and external plexiform layers but weak in the glomerular layer. Whereas CaMKIV was primarily found in granule cells, CaMKII was present in the glomerular, external plexiform, mitral cell and granule cell layers. No change in immunoreactivities of these kinases occurred in the olfactory bulb ipsilateral to naris closure. The expression of PKAα, PKCα and CaMKII, but not CaMKIV, in periglomerular cells suggests that these three kinases may play a role in TH gene regulation in the olfactory bulb. The lack of change in kinase protein levels after naris closure also suggests that any involvement of these kinases in TH gene expression in the olfactory bulb must be through altered kinase activity and not protein levels.

Introduction

Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, is highly expressed in periglomerular dopaminergic neurons of the mammalian olfactory bulb. TH gene expression in the olfactory bulb is regulated by afferent innervation. For example, unilateral odor deprivation or olfactory deafferentation resulted in a dramatic reduction of TH message and protein levels in the ipsilateral olfactory bulb (Brunjes *et al.*, 1985; Stone *et al.*, 1990; Baker *et al.*, 1993; Cho *et al.*, 1996). *In vitro* studies have demonstrated that activation of protein kinase A (PKA; also called cAMP-dependent protein kinase) and protein kinase C (PKC) strongly induced TH gene expression in neuroblastoma cells (Carroll *et al.*, 1991; Kim *et al.*, 1993a,b). Analyses of TH gene revealed that the proximal 5′ upstream region of the promoter contains the *cis*-acting elements CRE (cAMP-response element) and AP1 (activating protein 1), which are activated by the PKA and PKC signaling pathways respectively (Harrington *et al.*, 1987; Lewis *et al.*, 1987; Icard-Liepkalns *et al.*, 1992; Kim *et al.*, 1993a). Moreover, calcium/calmodulin-dependent protein kinases, including CaM kinases II (CaMKII) and IV (CaMKIV), have been shown to regulate transcription of genes containing the CRE motif in their promoters (Sheng *et al.*, 1990, 1991; Enslen *et al.*, 1994; Matthews *et al.*, 1994).

Previous reports (Ouimet *et al.*, 1984; Erondu and Kennedy, 1985; Saito *et al.*, 1988; Ito *et al.*, 1990; Nakamura *et al.*, 1995) demonstrated the presence of PKC, CaMKII and CaMKIV in the rodent olfactory bulb but did not provide a detailed delineation of their distributions in this brain region. Biochemical studies (Elkabes *et al.*, 1993) demonstrated a reduced PKC activity in the olfactory bulb ipsilateral to naris closure. Not defined were the anatomical substrates for PKC changes in the olfactory bulb and, specifically, the relevance to TH gene expression in the glomerular layer. Any change in protein level of the kinases may have significant impact on the signal transduction in neurons. For instance, monocular deprivation resulted in up-regulation of CaMKII gene expression in ocular dominance columns of the primary visual cortex originally innervated by the deprived eye (Hendry and Kennedy, 1986; Benson *et al.*, 1991). Dark rearing also produced an increase in CaMKII gene transcription in the visual cortex (Neve and Bear, 1989). Thus, CaMKII is thought to play an important role in maintenance of the homeostasis of visual signal transduction (Neve and Bear, 1989).

To establish a role for specific protein kinases in signal processing and gene regulation in the mammalian olfactory bulb, the present study investigated the laminar and cellular distribution of PKAα, PKCα, CaMKIIα, CAMKIIβ and CaMKIV in the adult mouse olfactory bulb. Specifically, the

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present study sought to determine the correlation between these major kinases and TH expression in periglomerular dopaminergic neurons. Also studied were changes in expression of the kinases in response to naris closure, a treatment that reduces odorant stimulation of the olfactory system and results in down-regulation of TH expression.

Materials and methods

Animals

Adult male CD-1 mice were purchased from Charles River Breeding Laboratory (Kingston, NY) and housed under a 12:12 h light:dark cycle. Under pentobarbital anesthesia (30 mg/kg Nembutal), animals were subjected to unilateral naris closure by means of a spark-gap electrocautery. Closure was confirmed visually at the time of sacrifice and studies were carried out at least 2 months post-closure. All procedures were performed under protocols approved by the Cornell University Institutional Animal Care and Use Committee and conformed to NIH guidelines.

Materials

Rabbit anti-PKA catalytic subunit α (PKAα; cat. sc-903, 1:5000 dilution), rabbit anti-PKC α isoform (PKCα; cat. sc-208, 1:10 000), and goat anti-CaMKIV (cat. sc-1546, 1:1000) were purchased from Santa Cruz Biotechniques (Santa Cruz, CA). Mouse monoclonal anti-CaMKII α subunit (CaMKIIα; cat. 1481703, 1:10 000) was purchased from Boehringer Mannheim (Indianapolis, IN). Mouse monoclonal anti-CaMKII β subunit (CaMKIIβ; cat. 13232012, 1:5000) was purchased from Gibco-BRL (Gaithersburg, MD). Polyclonal TH antisera (1:25 000 dilution) were prepared in the laboratory (Joh *et al.*, 1973).

Immunocytochemistry

Mice were perfused under deep pentobarbital anesthesia with saline containing 0.5% sodium nitrite and 10 units/ml of Heparin followed by 4% buffered (0.1 M sodium phosphate, pH 7.2) formaldehyde. Brains were removed, post-fixed for 1 h and then infiltrated overnight with 30% sucrose. Frozen sections (40 μm) were obtained on a sliding microtome and incubated with specific antibodies overnight at room temperature followed by incubation with the appropriate biotinylated secondary antisera, the Vector Elite kit (Vector Laboratories; Burlingame, CA) and with 3,3′-diaminobenzidine–HCl (0.05%) and hydrogen peroxide (0.003%) as chromogen (Cho *et al.*, 1996).

Results

TH immunoreactivity was localized to the glomerular layer of the mouse olfactory bulb (Figure 1A,A′). TH staining was strong in both cell bodies and processes of periglomerular neurons in the olfactory bulb with normal afferent innervation (Figure 1A′). Unilateral naris closure dramatically decreased TH immunoreactivity in the olfactory bulb ipsilateral to the closure. PKAα immunoreactivity was found in all layers of the olfactory bulb (Figure 1B,B′). Strong, often punctate, PKAα staining occurred in granule and periglomerular cell somata as well as in processes within the inner part of the external plexiform layer (Figure 1B′). Mitral cell staining was weak. Ascending dendrites of granule cells were also stained (Figure 1B′, inset). In contrast to TH, odor deprivation had no effect on PKAα immunoreactivity in any layer of the olfactory bulb ipsilateral to naris closure.

Strong PKCα staining was found in the plasma membrane of granule cells and processes within the external plexiform layer (Figure 2A,A′). PKCα immunoreactivity was weak in scattered periglomerular neurons and the glomerular neuropil, and absent from the mitral cell bodies as well as the olfactory nerve layer. Strong CaMKIIα staining occurred in the somata of periglomerular, tufted, mitral and granule cells (Figure 2B,B′). CaMKIIα staining was modest in the external plexiform layer, in which the apical and lateral dendrites of mitral cells were clearly seen (Figure 2B′). In contrast, CaMKIIα staining was very weak within the glomerular neuropil, suggesting that the processes of periglomerular cells and the apical tufts of mitral cells were weakly stained (Figure 2B′). Unilateral naris closure did not affect PKCα or CaMKIIα immunoreactivity in the olfactory bulb (Figure 2A,B). CaMKIIβ and CaMKIIα exhibited a similar distribution pattern in the olfactory bulb. Naris closure did not affect CaMKIIβ immunoreactivity in any layer (data not shown).

CaMKIV was localized primarily to the nuclei of a subpopulation of granule cells in the mouse olfactory bulb (Figure 2C,C′). Ascending dendrites of granule cells were also weakly stained (Figure 2C′, inset). Interestingly, CaMKIV was absent from the glomerular layer. Odor deprivation did not affect CaMKIV immunoreactivity in the olfactory bulb. CaMKIV staining in granule cells of the olfactory bulb on the naris-closed side appeared to be stronger than that on the open side. This was likely due to a higher cell-packing density in the granule cell layer. Prolonged naris closure caused shrinkage of the ipsilateral bulb, particularly the granule cell and external plexiform layers (Benson *et al.*, 1984). A comparison between CaMKIV and the three other kinases showed that these major protein kinases have distinct cellular and regional distribution in the mouse olfactory bulb (Table 1).

Discussion

The present study demonstrated that the investigated kinases showed distinct patterns of expression in the mouse olfactory bulb. CaMKIV immunoreactivity was limited to the granule cell layer while PKAα, PKCα and CaMKIIα,β exhibited a more widespread distribution. Strong PKAα immunostaining occurred in the glomerular, external plexi-

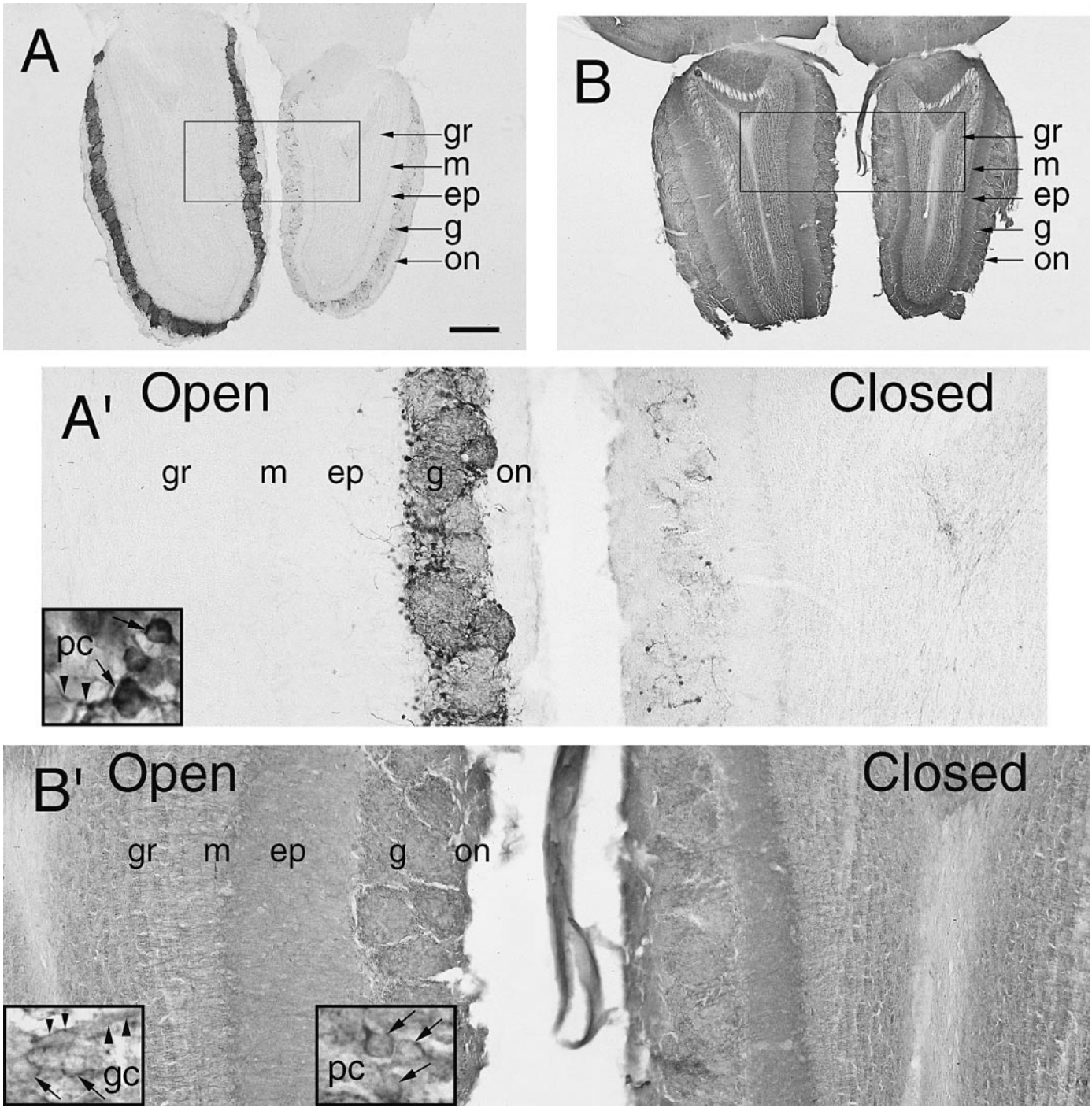

Figure 1 Light micrographs showing the localization of TH **(A, A′)** and PKAα **(B, B′)** in the mouse olfactory bulb. (A′) and (B′) are higher magnifications of the boxed areas in (A) and (B), respectively. Insets show representatives of neurons (arrows) and processes (arrowheads). The bulb on the left side of each panel represents the olfactory bulb that receives normal afferent innervation. Closed, bulb ipsilateral to naris closure; ep, external plexiform layer; g, glomerular layer; gc, granule cell; gr, granule cell layer; m, mitral cell layer; on, olfactory nerve layer; Open, bulb contralateral to naris closure; pc, periglomerular cell. Bar = 500 μm (A, B), 100 μm (A′, B′) and 12 μm (insets).

form and granule cell layers. PKCα immunoreactivity was strong in the external plexiform and granule cell layers but weak in the glomerular layer. CaMKIIα was highly expressed in the external plexiform, mitral and granule cell layers. CaMKIIα staining was strong in the somata of periglomerular cells but weak in the glomerular neuropil.

The presence of PKAα, PKCα and CaMKIIα in the periglomerular cells implied that these kinases may be

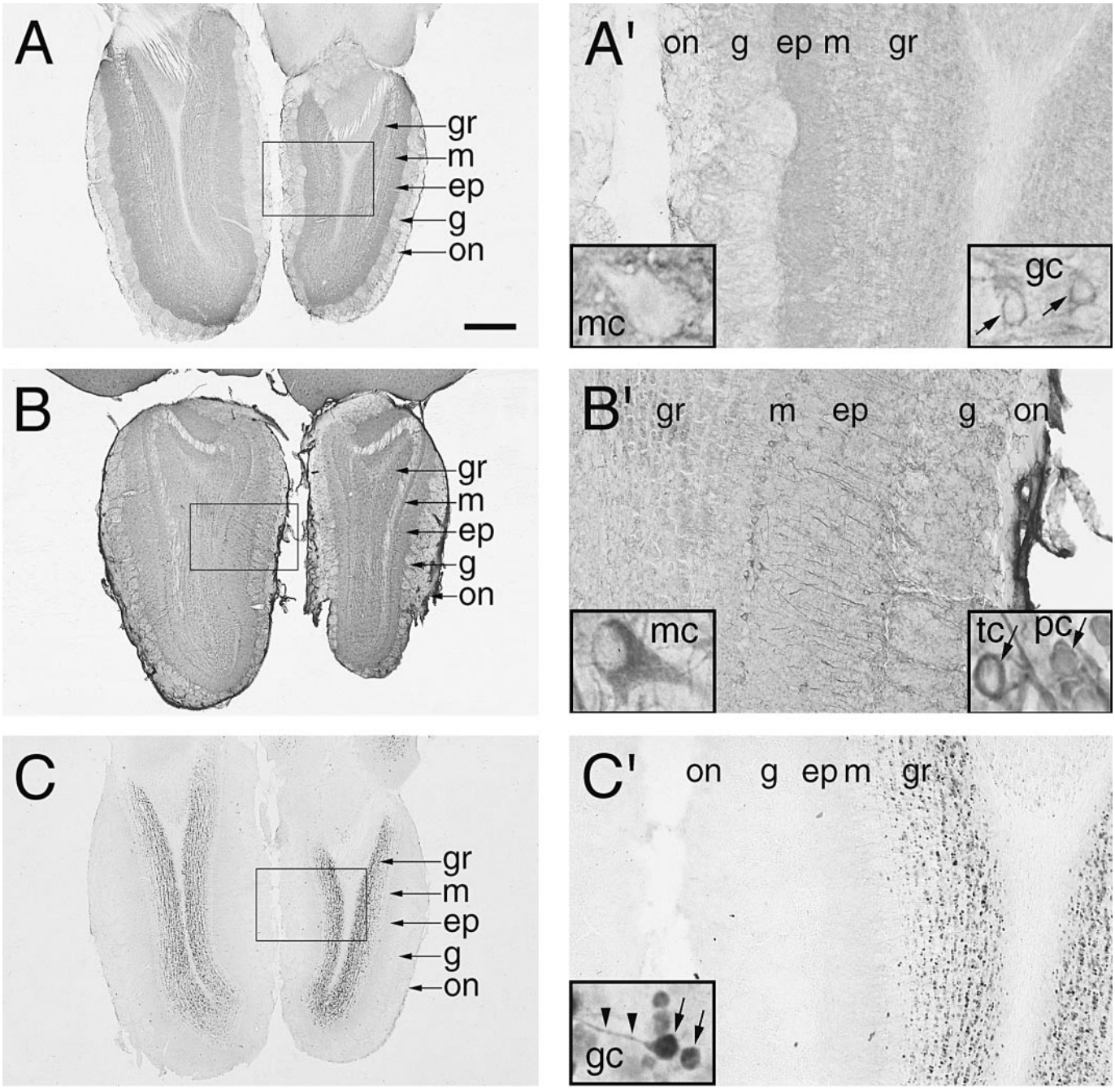

Figure 2 Light micrographs showing the localization of PKCα **(A, A′)**, CaMKIIα **(B, B′)** and CaMKIV **(C, C′)** in the mouse olfactory bulb. (A′), (B′) and (C′) are higher magnification of the boxed areas in (A), (B) and (C), respectively. Insets show representatives of neurons (arrows) and processes (arrowheads). The bulb on the left side of panels (A), (B) and (C) represents the olfactory bulb that receives normal afferent innervation. ep, external plexiform layer; g, glomerular layer; gc, granule cell; gr, granule cell layer; m, mitral cell layer; mc, mitral cell; on, olfactory nerve layer; pc, periglomerular cell; tc, tufted cell. Bar = 500 μm (A, B and C), 100 μm (A′, B′ and C′) and 12 μm (insets).

involved in TH gene regulation in the olfactory bulb. This was supported by a recent report (Trocme *et al.*, 1998) that both CRE and AP1 motifs were essential for expression of reporter genes driven by TH promoter in the adult olfactory bulb of transgenic mice. There are at least two mechanisms by which kinases may regulate gene expression. First, an increase or decrease in kinase protein levels may determine the availability of the kinases to their substrates, thus directly affecting transcription of target genes, including TH. For example, overexpression of PKA in neuroblastoma cells was shown to increase TH gene transcription (Kim *et al.*, 1993b). Secondly, alterations in kinase activity may determine the ability of the kinases to phosphorylate substrates, thus indirectly regulating gene expression. Acti-

Table 1 Regional distribution of protein kinases in the mouse olfactory bulb

	Olfactory nerve	Glomerular neuropil	Periglomerular cell	External plexiform	Mitral cell	Granule cell
PKAα	+	+	+++	+++	+	+++
PKCα	–	+	+	+++	–	+++
CaMKIIα	–	+	+++	+	+++	+++
CaMKIV	–	–	–	–	–	+++

+s indicates level of immunoreactivity: + weak, ++ moderate and +++ strong staining; – indicates little or no staining.

vation of PKA and PKC by forskolin and phorbol ester treatments, respectively, reportedly enhanced TH gene transcription *in vitro* (Carroll *et al.*, 1991; Icard-Liepkalns *et al.*, 1992). The present study demonstrated that little or no change in kinase expression occurred in the bulb ipsilateral to naris closure, suggesting that, if these kinases regulate TH expression in the olfactory bulb, it must be through altered activity and not protein levels.

CaMKII is a polymeric enzyme that is composed primarily of α and β subunits in α:β ratios of 3:1 and 1:4 in the adult rodent forebrain and cerebellum, respectively (Bennett *et al.*, 1983; McGuinness *et al.*, 1985). Although the two subunits differ in their molecular weights, they have similar enzyme activities and kinetic properties. CaMKIIα and CaMKIIβ exhibited a similar distribution pattern in the olfactory bulb. CaMKIIβ expression did not change in response to naris closure. A variety of isoforms also exist for PKC and PKA. The present study illustrated the regional distribution of PKAα and PKCα in the mouse olfactory bulb. The distribution of other isoforms of the two kinases was not examined. Therefore, the current studies do not rule out the possibility that those isoforms may be involved in the regulation of TH gene expression.

A previous report (Elkabes *et al.*, 1993) showed that PKC activities were decreased in particulate and soluble fractions of the olfactory bulb ipsilateral to naris closure, supporting the notion that PKC is involved in down-regulation of TH gene expression in the bulb after odor deprivation. However, it was not clear if the decrease in PKC activity occurred in periglomerular cells. The present findings that PKCα immunoreactivity in the periglomerular cells remained unchanged after naris closure did not rule out the possibility that a decrease in PKC activity may occur in these cells. *In vitro* studies (Icard-Liepkalns *et al.*, 1992) showed that activation of PKC produced strong AP1-protein binding activity and enhanced TH gene transcription. The transcription factors c-Fos and Fos-B, which bind to the AP1 motif as Fos/Jun heterodimers, were found in periglomerular cells of the normal olfactory bulb and were significantly down-regulated by odor deprivation (Liu *et al.*, 1999). Therefore, alterations in PKC activity in response to odorant stimulation may modulate expression of immediate early genes, thus regulating TH gene transcription through the AP1 motif.

In the olfactory bulb, CaMKIV was found only in granule cells, indicating that this kinase is unlikely to regulate TH gene expression in periglomerular dopaminergic neurons. Because CaMKIV was found to phosphorylate CRE-binding protein upon activation and enhance gene transcription (Enslen *et al.*, 1994; Matthews *et al.*, 1994), it is possible that CaMKIV regulates TH gene expression in other brain regions. Indeed, CaMKIV immunoreactivity has been found in noradrenergic neurons in the locus ceruleus, but not in dopaminergic neurons in the substantia nigra (unpublished data). Further studies will determine if CaMKIV plays a major role in the regulation of TH expression in the locus ceruleus.

Conclusions

The variations in laminar and cellular distribution of the protein kinases in the mouse olfactory bulb suggest that they participate in different aspects of olfactory signal processing and gene regulation. The expression of PKAα, PKCα and CaMKIIα,β, but not CaMKIV, in the periglomerular cells suggests that these three major kinases might play a role in regulating TH gene expression in the olfactory bulb in response to odorant stimulation. However, the lack of change in protein levels of the kinases in the glomerular layer of the olfactory bulb ipsilateral to naris closure indicates that odor-induced neuronal activity does not modulate the kinase gene expression and that any role in TH gene regulation may be indirect through changes in kinase activity.

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