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Down-regulated GABAergic expression in the olfactory bulb layers of the mouse deficient in monoamine oxidase B and administered with amphetamine

Hsiang-Shu Yin[*,1], **Kevin Chen**[2], **Jean C Shih**[2], and **Tai-Wei Tien**[1]

[1]Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan 100, ROC

[2]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089-9121, USA

Abstract

This study explores primarily the role of the activity of monoamine oxidase B (MAOB) in the regulation of glutamic acid decarboxylase$_{67}$ (GAD$_{67}$) expression in distinct layers of main olfactory bulb (OlfB), which links the limbic system. Moreover, the response of GAD$_{67}$ was investigated to amphetamine perturbation in the absence of MAOB activity. Immunocytochemical analysis was performed on OlfB sections prepared from the adult wild type (WT) and the MAOB gene-knockout (KO) mice after receiving repeated intraperitoneal injections (2 doses/day, total 7 doses) of saline or amphetamine, 5 mg/kg. The levels of the GAD$_{67}$ immunoreactivity were approximate 25% and 38% lower in respective glomerular (GloL) and mitral cell layers (ML) of saline-treated KO mice than that of WT, whereas similar in the external plexiform or granule cell layers (GraL) of the KO and WT. In the GloL, the level of tyrosine hydroxylase was 39% lower in the KO mice than WT, implicating different dopamine content in the KO from WT. The amphetamine exposure down-regulated the levels of GAD$_{67}$ in the WT layers by 46% to 52%, and in KO layers 65% to 71%, except ML. The GraL GAD$_{67}$ level may be regulated by the activation of CREB, as the phosphorylated (p) CREB coexisted with GAD$_{67}$, and the percentage of GAD$_{67}$-expressing pCREB neurons was decreased by the amphetamine exposure. The data indicate that the activity of MAOB could modulate the regular and amphetamine-perturbed expression of GAD$_{67}$ and pCREB. Thus, interactions are suggested among the MAOB activity, GABA content of OlfB and olfaction.

Keywords

dopaminergic neurons; limbic system; neurotransmitter synthesizing enzymes; addiction

Introduction

The main olfactory bulb (OlfB) is the relay station of the peripheral olfactory sensation before it enters the cerebral cortex. After integrating the inputs from olfactory nerves and periglomerular and granule cells in the glomerula, the mitral cells send their axons as the olfactory tract to the primary olfactory cortices, which are connected with the limbic structures (Shipley and Ennis, 1996). Furthermore, the OlfB contains intrinsic dopaminergic interneurons which are among the periglomerular cells, and receives norepinephrinergic and serotonergic

[*]Correspondence: Hsiang-Shu Yin Department of Anatomy and Cell Biology College of Medicine National Taiwan University Taipei, Taiwan, 100 Republic of China hsyin@ntu.edu.tw FAX: 8862-2391-5292 TEL: 8862-2395-9284.

projections from the brain stem (Shipley et al, 1985; McLean and Shipley, 1987). Thus, the olfactory bulbectomized animals have been used as models for studying the affective disorder, depression (Kelly et al, 1997). The metabolism of those monoamines is carried out by the monoamine oxidase (MAO).

The MAO includes two isoforms, A and B; MAOA is thought to preferentially metabolize dopamine, norepinephrine and serotonin, while MAOB appears to be central for catalyzing serotonin and phenylethylamine (PEA), an endogenous trace amine, which shares structural and physiological similarities with amphetamine (Amph), a psychostimulant (Shih et al., 1999). The dopaminergic and noradrenergic neurons contain both MAOA and MAOB, with probably the MAOA activity predominant, whereas serotonergic neurons are believed to contain mainly MAOB (Hashizume et al, 2003; Levitt et al, 1982; Vitalis et al, 2002).

Interactions have been demonstrated between the GABAergic system and monoamines, such as dopamine and serotonin, in the rat basal ganglia (Giorgetti et al., 2002; Takahashi et al., 1998; Westerink et al., 1996). In addition, the MAO inhibitors, being used as antidepressant drugs, were found to interact with the GABAergic system, including the $GABA_B$ receptor (Sands et al., 2004; Tunnicliff and Malatynska, 2003). Acute administration of phenelzine, a nonselective MAO inhibitor, to the rat produced increased brain levels of GABA (Paslawski et al., 1995; Parent et al, 2000). These findings may be related to that the MAOB gene knocked-out (KO) mice showed increased reactivity to stress than the wild type (WT) mice (Grismby et al, 1997). The MAOB KO mice did not show altered levels of dopamine, norepinephrine and serotonin in the cerebral cortex, substantia nigra and hippocampus, compared to that of the WT, whereas the OlfB levels of the monoamines are unknown and the brain PEA level was increased in the KO mice. However, detailed neurochemical mechanisms await clarification underlying the behavior of the KO mice.

Our previous study found that the KO mice had a lower level of OlfB phosphorylated CREB (cAMP response element binding protein), a key neuronal transcription factor, than WT, as detected by immunoblotting (Mayr and Montminy, 2001; Yin et al, 2006). This study further found that in response to the repeated Amph administration, the KO mice displayed a lower level of behavioral hyperactivity, including a weaker sniffing behavior, than the WT mice. Amph is known to be an indirect catecholamine agonist, and Amph-administered animals are utilized as models for examining addiction and Amph psychosis. The Amph-induced abnormal behavior is associated with the release of dopamine and/or other monoamines in distinct brain areas, including OlfB (Seiden et al, 1993; Karler et al, 1997; Featherstone et al, 2007; Mesfiou et al, 1998; Deng et al, 2007). There was also greater down-regulation of the OlfB pCREB in KO mice produced by the Amph exposure than that of the WT mice. Furthermore, baclofen, a $GABA_B$ receptor agonist blocked the Amph-evoked changes in behavior and the pCREB levels (Yin et al, 2006).

Thus, relationships are proposed to exist among the activity of MAOB, expression of GABA system and activation of CREB. In order to elucidate the significance of MAOB activity in the regulation of OlfB dopaminergic, GABAergic and pCREB expression, the present work examined the morphological expression of GAD_{67}, a GABA synthesizing enzyme, tyrosine hydroxylase (TH), a L-dopa synthesizing enzyme, and pCREB in distinct OlfB layers of MAOB KO and WT mice after saline or Amph administration by employing immunocytochemistry.

Materials and Methods

Drug administration

Adult male WT and MAOBKO mice (Grimsby et al, 1997) were housed in plastic cages with free access to water and food at 12 h light/12 dark cycle (lights on between 07:00 and 19:00 h) at 20°C. The mice received repetitive intraperitoneal (i.p.) injections of saline or d-Amph (Sigma), 5 mg/kg, twice daily at 9:00 and 17:00 h for three consecutive days and once on the 4th day at 9:00 for a total of 7 injections. At 4 h after the last injection, each mouse was anaesthetized with an i.p. injection of chloral hydrate, 0.32 g/kg, followed by transcardial perfusion with Bouin's fixative. Serial coronal paraffin OB sections were prepared and subjected to immunocytochemistry. All animal protocols were performed in accordance with guidelines of an animal research review committee of National Taiwan University.

Immunoblotting

The immunoblotting was conducted primarily following a previous protocol (Choe et al., 2002). The olfactory bulbs of a number of anaesthetized mice were dissected on ice and lysed in the SDS buffer (sodium dodecyl sulfate, 0.1%, and phenylmethyl-sulfonyl fluoride, 1.25 mM, in Trizma Base, 25 mM, pH 7.6) for western blot analysis. The lysed tissue samples were sonicated for 30 s on ice and centrifuged at 14,000 rpm for 10 min at 4°C using a table top centrifuge. The concentration of proteins was determined in the supernatant of each sample by using the bicinchoninic acid assay kit (Amersham). Samples with equal amounts of proteins were subjected to 10% polyacrylamide gel electrophoresis. Afterward, protein bands on the gels were electrically transferred onto nitrocellulose membranes (Scheiller and Schuell). The membranes were blocked with 2.5% nonfat milk in the PBST buffer (NaCl, 137 mM; KCl, 0.27 mM; Na2HPO4, 10.14 mM; KH2HPO4, 1.76 mM; and Tween-20, 0.1%) for 1 h at RT and incubated with an anti-GAD_{67} antiserum (Chemicon), 1:2000 diluted in the buffer, for 17 h at 48°C. β-actin was used as an internal control. Thereafter, the membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h at room temperature (RT). The immunoreactive bands were visualized with the chemiluminescence method and X-ray films (Amersham). The intensities (ODs) of the immunoreactive bands were quantified with a computer program (Gel Probe, Media Cybernetics).

Immunocytochemistry

The immunostaining was carried out according to our previous procedures (Yin et al, 2004). Briefly, coronal paraffin OlfB sections (7 μm thick) were deparaffinized with xylene, rehydrated and incubated with 1% H_2O_2 in PBS for 30 min. The samples were then blocked with 2.5% nonfat milk in PBS for 30 min at RT and then incubated with the anti-tyrosine hydroxylase (TH) (DiaSorin), anti-GAD_{67} (Chemicon), anti-CREB, anti-phosphorylated CREB (Cell signaling) or anti-NeuN (Chemicon) antibody diluted 1:200~2000 in PBS at 4°C for 20 h. Thereafter, the sections were incubated with a biotinylated secondary antibody for 1 h at RT and the avidin–biotin–peroxidase complex for 45 min (Vector). The immunoreaction products were visualized by incubating the samples with 0.05% 3–3'-diaminobenzidine tetrachloride (DAB). Subsequently the samples were dehydrated and coverslipped with Permount. For immunostaining of CREB and pCREB, the deparaffinized sections were microwave-treated in 0.1 mM citrate buffer, pH 6 for antigen retrieval before incubation with 1% H_2O_2 in PBS (Vector). Absence of positive immunoreactivity was detected by omission of primary antibody or secondary antibody during the staining procedure to define non-specific staining. The histological organization of mouse OlfB was shown by staining certain deparaffinized sections with the Nissl method (Fig. 1). A number of OlfB sections were doubly immunostained first with the anti-GAD_{67} antibody and color-developed with DAB as described above, and then stained with the anti-pCREB antibody and SG (blue color, Vector).

Image analysis

To reduce variability among the data, the procedures of immunostaining were performed as regularly and consistently as possible. Quantification of the TH or GAD_{67} immunoreactivity in the somata, punctates and processes was conducted using a computerized image analysis system (Image-Pro Plus, Media Cybernetics) including a Zeiss microscope and Kodak digital camera, as described previously (Yin et al, 2007). In brief, under a fixed level of illumination and a 40X objective, the immunostained OlfB layers were photographed and the color images were converted to gray-leveled. The TH or GAD_{67} immunopositive profiles with areas ≧0.2 μm^2, and optical densities (ODs) ≧0.12, were measured in the layers of OlfB. The OD 0.12 was determined after using a gray-level thresholding procedure. The levels of staining intensity all fell below the maximum, equivalent to 256 pixels, and thus excluded the presence of saturated staining. The numbers of CREB or pCREB positive and dually-labeled somata were also counted using the image analysis system. For each protein, six 40X fields under the microscope in each of 10 OlfB sections, from every third of consecutive sections at Bregma 3.9~4.7 mm, were quantified for each animal (n=6) of respective experimental groups.

The levels (densities) of the TH or GAD_{67} immunoreactive profiles were presented by ratios, which were derived from dividing the cumulative sums of immunopositive profile areas by the selected total areas. The data are presented as means ± SDs. One-way ANOVA and Scheffe's post-hoc comparison were employed to analyze the data. P-Values less than 0.05 were considered as statistically significant.

Results

Western blotting analysis revealed significant differences in the levels of GAD_{67} proteins in the entire OlfBs among the saline or Amph administered WT and MAOB KO mice ($F(3, 27) = 28.52$, $p<0.05$) (Fig, 2). The level of GAD_{67} protein was approximate 12% lower in the OlfB of the saline-treated KO mice than that of the WT ($p<0.05$). The Amph exposure down-regulated the levels of OlfB GAD_{67} by 13% and 14%, compared with that of saline-control WT and KO mice respectively ($p<0.05$).

As shown from counting the neurons on OlfB sections immunostained with an anti-NeuN antibody, the numbers were 170 ± 16~197 ± 48/0.1 mm^2 in the glomerular layer (GloL), 48 ± 4~55 ± 6 external plexiform layer (EPL), 125 ± 7~130 ± 12 mitral cell layer (ML), and 650 ± 29~669 ± 42 granule cell layer (GraL, including internal plexiform layer) of the saline or Amph-treated WT and MAOB KO mice. For each layer, insignificant differences were found among the values of the four experimental groups, indicating that MAOB gene deficiency and the Amph exposure did not induce significant changes in the numbers of neurons.

Because dopaminergic neurons are present in the GloL of OlfB, the immunostaining of the L-dopa synthesizing enzyme, tyrosine hydroxylase (TH), was conducted to examine the distribution of the dopaminergic neurons. The TH-immunoreactivity (ir) was seen in the perikarya, processes and punctates of the GloL, and almost absent in other layers. There were significant differences in the levels of the GloL TH-ir among the repeated saline or Amph administered WT and KO groups ($F(3, 16)=14.27$, $p<0.05$). The level of TH in the saline-treated KO was 0.22 ± 0.03, around 39% lower than 0.36 ± 0.04 of the WT. However, the Amph exposure did not significantly alter the levels of TH of WT and KO (Fig. 3).

The GAD_{67}-ir was present in the perikarya and as punctates surrounding the somata or in the neuropil in all OlfB layers of WT and KO mice. In each of the four OlfB layers observed, the densities of GAD_{67} were significantly different among the four experimental groups, saline or Amph treated WT and KO mice ($F(3, 16)=32.25$, $p<0.001$ for GloL; $F(3,16)=17.25$, $p<0.05$, EPL; $F(3, 16)=12.82$, $p<0.05$ ML; $F(3, 16)=18.75$, $p<0.05$ GraL).

The GloL GAD_{67} level of saline-administered KO mice was approximate 25% lower than that of the saline-treated WT. The repeated Amph exposure significantly reduced the level of GloL GAD_{67} of WT mice by 46%, and that of KO by 71%, compared with the values of respective saline-treated animals. The saline-treated WT and KO mice had similar levels of EPL GAD_{67}. However, in the EPL of Amph-administered WT or KO, the value of GAD_{67} was 52% or 69% lower than that of saline-WT or KO.

The level of ML GAD_{67} was around 38% lower in the saline-treated KO mice than that of WT. The ML GAD_{67} level of Amph-treated WT mice was 44% lower than that of the saline-treated WT, whereas the saline-treated and Amph-treated KO mice had similar ML GAD_{67} levels. The saline-treated WT and KO mice had similar values of GraL GAD_{67}. The repeated Amph exposure decreased the WT and KO values of GraL GAD_{67} by 47% and 65% (Fig. 3).

The CREB and pCREB-immunoreactivity was seen primarily in nuclei of neurons and non-neuronal cells in the OlfB, as verified by double staining using the anti-CREB or pCREB and anti-NeuN antibodies. The numbers of CREB-positive neurons were similar in corresponding OlfB layers of WT and KO mice. The Amph exposure did not alter the numbers of CREB-neurons in respective OlfB layers of WT or KO mice. By contrast, significant differences were detected in the numbers of pCREB-positive neurons of the GraLs among saline or Amph treated WT and KO mice ($F(3, 16)=27.56$, $p<0.05$), and were undetected in other layers. The saline-treated KO mouse had a 38% lower number of pCREB-neurons than that of saline-WT. The number of pCREB-neurons was decreased by 60% or 45% in the GraL of Amph-treated WT or KO, compared with that of saline-treated control (Fig. 4).

Double immunostaining revealed the coexistence of pCREB and GAD_{67} in a number of granule cell somata. In the GraL, the percent of pCREB positive cells that expressed GAD_{67} was 14.6 ± 2.6% in the saline-treated WT mice, which was similar to 10.6 ± 2.8% of saline-KO. In the GraL of WT mice after repeated Amph exposure, the percent of the number of pCREB-positive cells that expressed GAD_{67} was 8.1 ± 1.94%, which was lower than that of the saline-treated WT ($p<0.05$). Similarly, 4.6 ± 1.3 % of pCREB-cells that expressed GAD_{67} in the Amph-treated KO was less than 10.6 ± 2.8% of saline-KO ($p<0.05$) (Fig. 5).

Discussion

The present study provides mainly morphological evidence for the participation of MAOB activity in maintaining the regular GABAergic expression and activation of CREB in the main olfactory bulb (OlfB) layers and in responses of the OlfB to the perturbation of Amph. The characteristic MAOB activity in the OlfB is signified by lower levels of GAD_{67} and pCREB seen in distinct OlfB layers of the MAOB KO than that of WT mice, and more profound decreases in the GABAergic expression in the KO than WT following the repetitive Amph administration. These changes may be associated with the sniffing behavior of the mice induced by Amph (Yin et al, 2006). .

The deficiency in the MAOB activity resulted in a lower level of TH immunostaining in the glomerular cell layer (GloL) of the saline-treated KO mice than that of saline-WT, probably implicating different dopamine content and/or other differences in the OlfB between the WT and KO mice (Ennis et al, 2001; Davila et al, 2003). However, in other brain regions, such as cerebral cortex and substantia nigra, the content of dopamine was alike between the WT and KO mice, as disclosed by HPLC methods (Grimsby et al, 1997; Fornai, et al, 2001). Although the OlfB dopamine content is unknown, it is suggested that dopamine in the GloL may be catabolized slower in the KO than WT, leading to down-regulated TH. Nevertheless, after the repeated Amph exposure, the level of TH was unaltered in GloLs of both WT and KO mice. By contrast, an earlier work showed greater reductions in dopamine levels of the KO striatum

and substantia nigra than that of WT, at 7 days post-MDMA (3,4-methylene-dioxy–methamphetamine) injection (Fornai et al, 2001).

Similar to the response of GloL TH, the levels of GAD_{67} were lower in the GloL and mitral cell layer (ML) of saline-treated KO mice than that of WT. This may be due to, at least in part, different GABA content in the GloLs or MLs between the WT and KO mice. Previous studies have found that dopamine interacted with GABA and that the administration of phenelzine, a nonselective MAO inhibitor, produced increased brain levels of GABA in the rat (Paslawski et al., 1995; Parent et al, 2000). The OlfB layers of mice lacking MAOB probably possess higher levels of GABA, and thereby contribute to the decreased level of the GABA synthesizing enzyme, GAD_{67}. In addition, the KO mouse brain contains a high level of PEA, which has been found to depress the $GABA_B$ activity (Federici et al, 2005). Further investigation is required to ascertain the changes in the OlfB GABA content.

The repeated Amph injections differentially down-regulated the levels of GAD_{67} in the 4 OlfB layers of both WT and KO mice, except the ML of KO. This down-regulated GAD_{67} expression likewise could be compensation to presumably altered levels of GABA induced by Amph, as it was shown that the level of GABA release was increased or decreased in the nucleus accumbens and striatum of the rat after receiving acute or repeated Amph administration (Lindefors et al, 1992; Del Arco et al, 1998; Bartolletti et al, 2004). Moreover, Amph may induce alterations in dopamine, and/or other monoamine content in the layers to affect the GABA level and GAD_{67} expression (Mesfiou et al, 1998; Deng et al, 2007). By contrast, our previous study found up-regulation of GAD_{67} after repeated Amph treatment in the rat neocortical areas, nucleus accumbens and hippocampus (Yin et al, 2004). The response of OlfB GAD_{67} protein apparently reflects the unique structure and connectivity of the OlfB.

Whether GAD_{65}, the other isoform of GAD, is associated with the reduced expression of OlfB GAD_{67} in the KO mice and after Amph exposure is unknown, but it has been shown that protein levels of both isoforms were differentially changed in the rat frontal cortex by chronic treatment of psychotropic drugs, such as fluoxetine and olanzapine (Fetami et al, 2009). Nevertheless, reduced levels of mRNA and protein of GAD_{67} were seen in postmortem prefrontal and temporal regions of schizophrenics (Guidotti et al, 2000; Akbarian and Huang, 2007).

After the Amph exposure, the KO mice manifested greater decreases in GAD_{67} levels of GloL and GraL than the WT mice, indicating an association between MAOB and the action of Amph. Higher GABA levels are possibly generated in the KO OlfB layers than that of WT by Amph, for the whole brain level of GABA was increased by phenelzine and the striatal GABA altered after Amph administration (Parent et al, 2000; Tunnicliff and Malatynska, 2003; Federici et al, 2005). Furthermore, in response to the MDMA treatment, the MAOB KO mice indeed showed a greater depletion of striatal dopamine than WT, whereas a lower depletion of striatal 5-hydroxytryptamine (Fornai et al, 2001).

A relation is suggested between the MAOB activity and activation of CREB in the GraL, because the number of pCREB-positive neurons was lower in the saline-treated KO mice than WT. Moreover, the Amph-elicited decreased pCREB expression of both WT and KO mice match our previous study, which by using immunoblotting exhibited down-regulated levels of pCREB in the entire OlfBs of both mice after the Amph exposure (Yin et al, 2006). The dopamine receptor 1 (D1) and 2 (D2) mRNAs were seen in the OlfB granule cells (Coronas et al, 1997). Activation of the D1 or D2 receptor could lead to elevated or reduced phosphorylation of CREB via regulating cAMP levels (Berke and Hyman, 2000). Thus, the decreases in the pCREB levels are probably related to the altered activity of dopamine receptors (Deng et al, 2007). That the altered pCREB was seen only in the GraL rather than other layers could be

relevant with that the granule cells are abundant and predominantly participate in modulating the activity of mitral and tufted cells (Shipley and Ennis, 1996).

Variable GraL GABA content may likewise account for differential pCREB levels in GraLs between WT and KO mice after saline or Amph administration. Many granule cells are GABAergic, and a $GABA_B$ receptor agonist, baclofen, blocked the decreased OlfB pCREB expression induced by Amph in both mice, as detected by immunoblotting (Kosaka et al, 1987; Yin et al, 2006). In addition, the repeated Amph exposure decreased the ratio of pCREB-somata that expressed GAD_{67} in the GraL of WT or KO mice, implying an association of the pCREB with GAD_{67}. A previous study indeed proposed a relationship between the GABA–induced BDNF expression and activation of CREB (Obrietan et al, 2003).

Our present findings demonstrate that the MAOB activity is associated with maintaining the regular OlfB dopaminergic, GABAergic and pCREB expression. After the Amph exposure, compared with the response of WT mice, the MAOB KO mice manifested different magnitude of reductions in the levels of GAD_{67} and pCREB in OlfB layers, which may participate in mechanisms underlying the weaker sniffing behavior of the KO mice evoked by Amph than that of WT (Yin et al, 2006). The data support interactions among the activity of MAOB, GABAergic expression and action of Amph, as well as contribute to understanding the pathogenesis of addiction and Amph psychosis.

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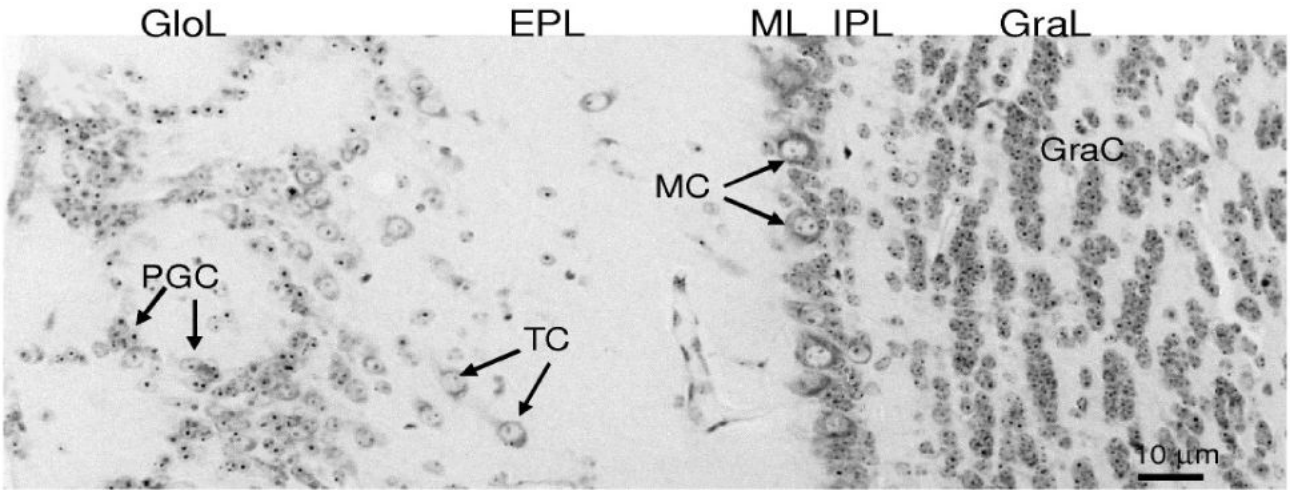

Fig. 1.

A representative micrograph illustrates cellular organization of the layers of mouse main mouse olfactory bulb (OlfB). Coronal paraffin OlfB sections were prepared from wild type (WT) mice after perfusion with fixative and then stained with the Nissl method. Note the periglomerular cells (PGC, arrows) in the glomerular layer (GloL), tufted cells (TC, arrows) in the external plexiform layer (EPL), mitral cells (MC, arrows) in the mitral cell layer (ML) and granule cells (GraC) in the granule cell layer (GraL). IPL: internal plexiform layer.

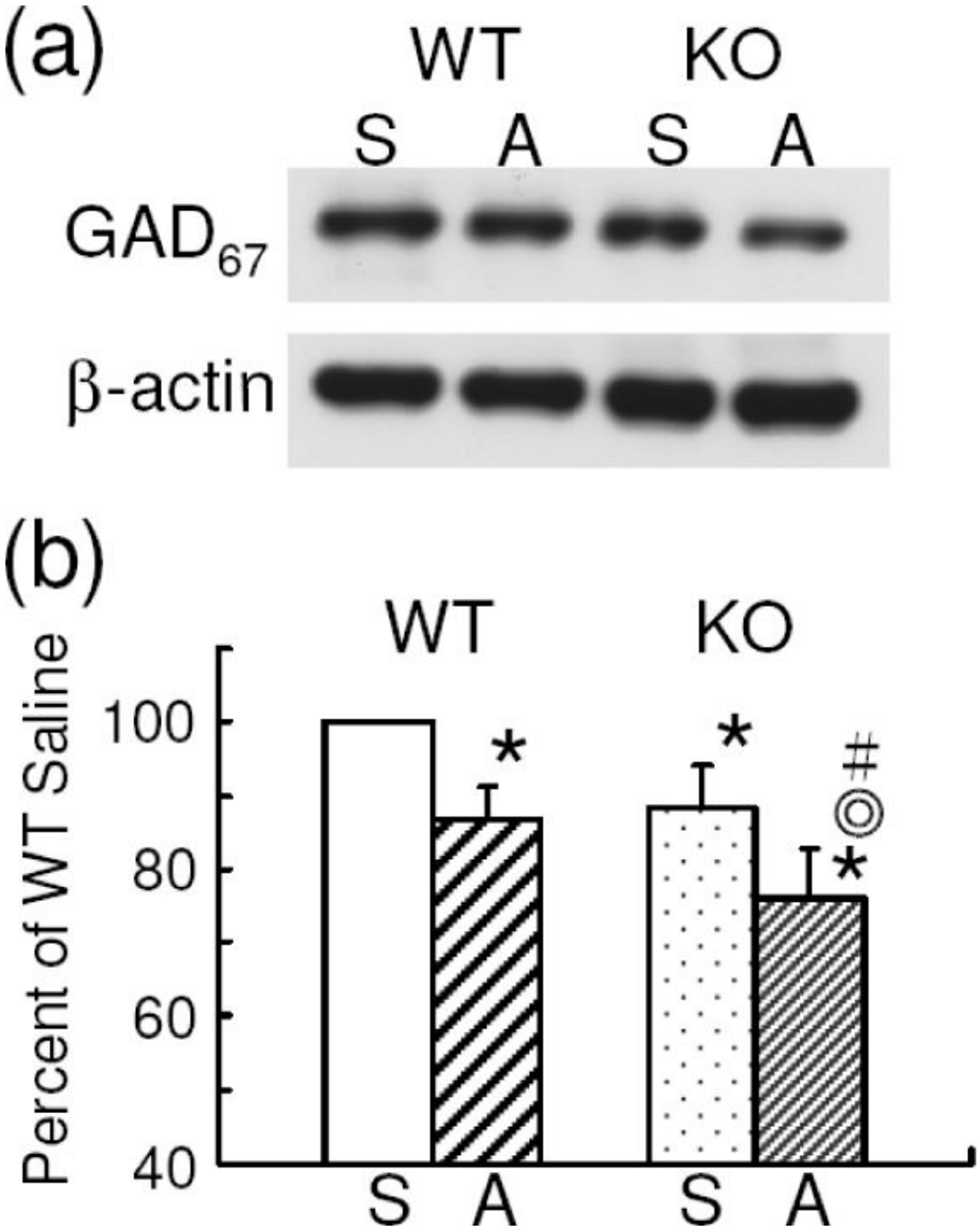

Fig. 2.

The level of the olfactory bulb GAD_{67} is lower in the MAOB KO mouse than WT and is decreased in either animal after the Amph treatment. The mice received i.p. injections of saline (S) or Amph (A) for 3 days, and after the final injection on day 4, were subjected to brain dissection for removal of OlfBs. Gel electrophoresis and immunoblotting were conducted on the homogenates of the OlfBs by using an anti-GAD_{67} antibody (a), as described in the Methods. Levels of GAD_{67} are presented as percentages of that of the saline-treated WT (b). *: significantly different from the value of saline-treated WT mice, $p<0.05$; ⊙: significantly

different from Amph-treated WT; #: significantly different from saline-treated MAOB KO, $p<0.05$.

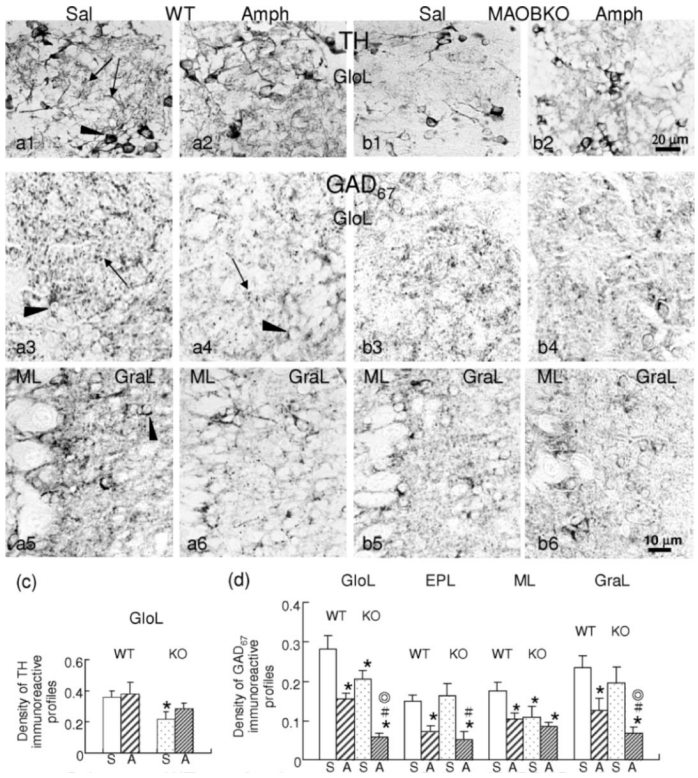

Fig. 3.

Amph induces differential changes in the expression of tyrosine hydroxylase (TH) or GAD_{67} in the distinct layers of main olfactory bulb (OlfB) of WT and MAOB KO mice. Representative micrographs (a–b) and bar graphs (c, d) illustrate that the level of TH is higher in the OlfB glomerular layer (GloL) of the WT mouse (a1, a2) than that of the KO (b1, b2) after repeated saline injections (S), and that the levels of GAD_{67} are reduced to different extents in respective OlfB layers of WT (a3–a6) and KO (b3–b6) mice by repeated Amph injections. The mice received multiple injections of Amph or saline (Sal), and then were perfused with fixative 4 h later, followed by preparation of coronal paraffin OlfB sections and immunocytochemical analysis using an anti-TH or GAD_{67} antibody, as described in the Methods. The TH and GAD_{67} immunoreactivity is seen in the perikarya (arrowheads), processes and punctates (arrows). Levels of the immunoreactive profiles were quantified by using an image analysis computer system. The densities of TH and GAD_{67} are presented as means ± SDs (c, d). *: significantly different from the value of saline-treated WT mice, $p<0.05$; ⊙: significantly different from Amph-treated WT; #: significantly different from saline-treated MAOB KO, $p<0.05$. GloL: glomerular layer; EPL: external plexiform layer; ML: mitral cell layer; GraL: granule cell layer.

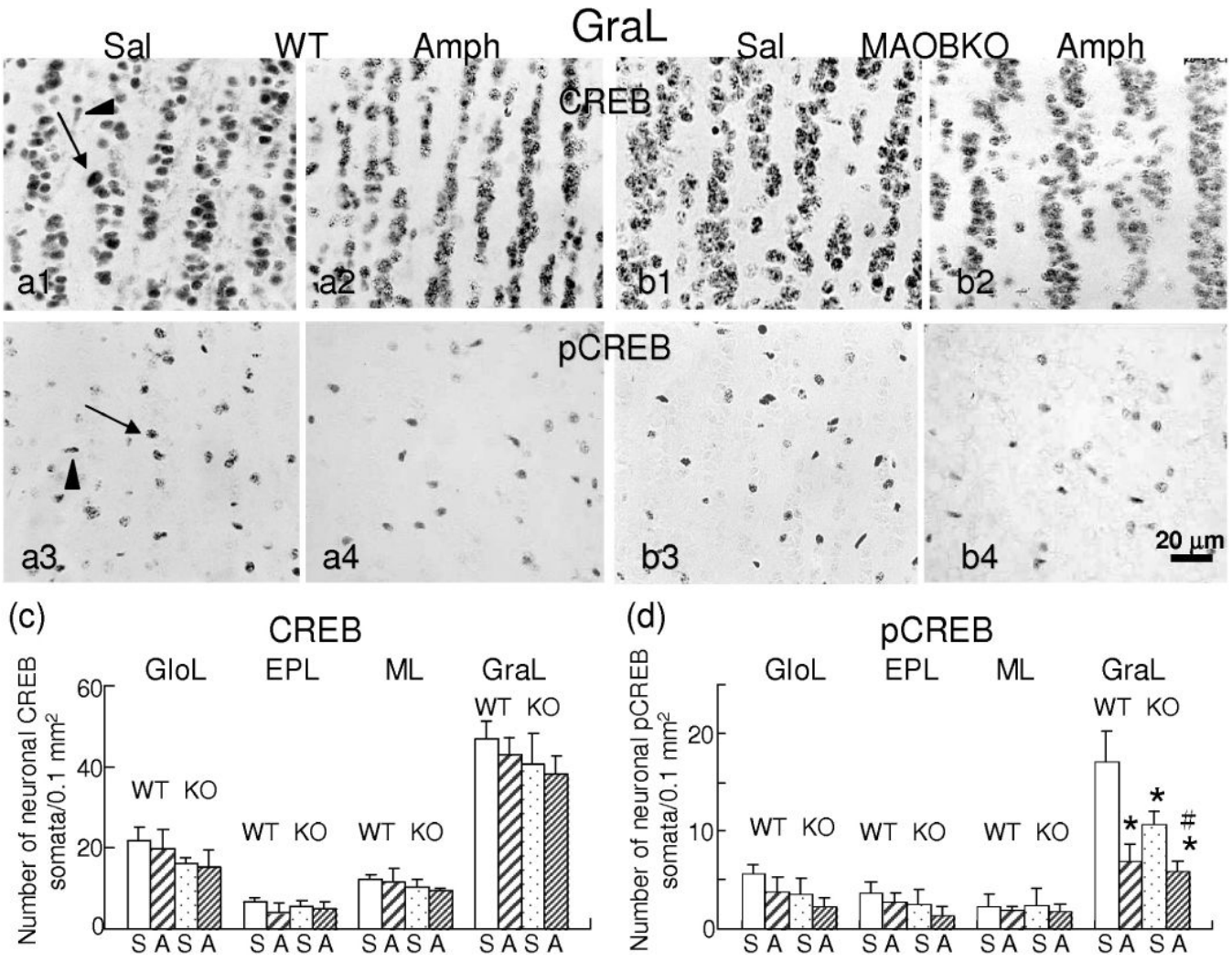

Fig. 4.

Differential expression of CREB and pCREB in OlfB layers of Amph-treated WT and MAOB KO mice. Immunostaing with an anti-CREB or pCREB antibody was performed on coronal OlfB sections of mice at 4 h post-repeated saline or Amph administration. Micrographs of the GraL illustrate that the CREB or pCREB immunoreactivity is seen in the nuclei of neurons (arrows) and glia (arrowheads) of WT (a1–a4) and MAOB KO (b1–b4) mice. *: significantly different from the value of saline-treated WT; $p<0.05$. #: significantly different from saline-treated MAOB KO. GloL: glomerular layer; EPL: external plexiform layer, ML: mitral cell layer; GraL: granule cell layer.

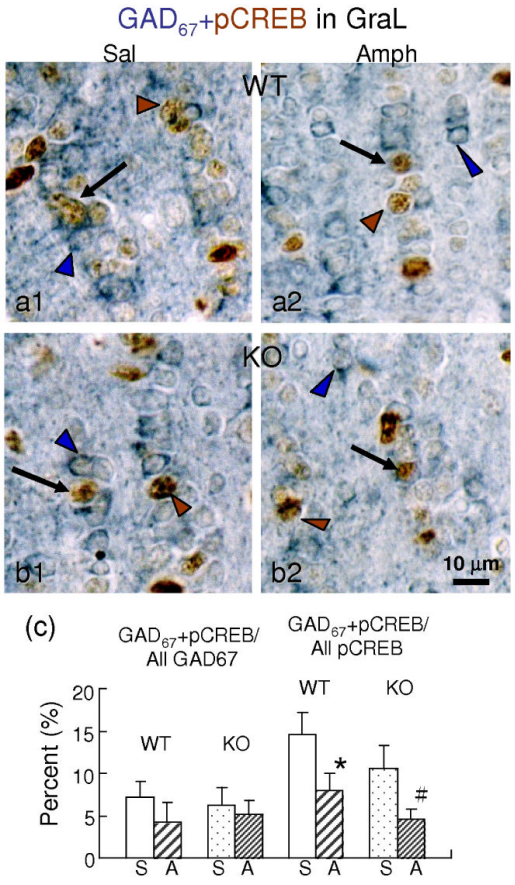

Fig 5.

Representative micrographs of GraL of WT (a1, a2) and MAOB KO (b1,b2) mice and bar graphs (c) illustrate that Amph decreased the numbers of somata that show coexistence of GAD_{67} and pCREB in the granule cell layer (GraL). Double immunostaining of pCREB and GAD_{67} was conducted on the OlfB sections of WT and KO mice at 4 h post-repeated saline or Amph treatment, as described in the Methods. The numbers were counted of positively single-immunostained somata (arrowheads) and dually stained somata (arrows). The percents were determined by dividing the numbers of dually stained somata with the total numbers of somata containing either of the proteins. *: significantly different from the value of saline-treated WT mice, $p<0.05$. #: significantly different from saline-treated MAOB KO.