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Monoamine oxidase A regulates neural differentiation of murine embryonic stem cells

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Abstract

Monoamine oxidase (MAO) A is the major metabolizing enzyme of serotonin (5-hydroxytryptamine, 5-HT) which regulates early brain development. In this study, wild-type (WT) and MAO A^{neo} embryonic stem (ES) cell lines were established from the inner cell mass of murine blastocysts and their characteristics during ES and differentiating stages were studied. Our results show that the differentiation to neural cells in MAO A^{neo} ES cells was reduced compared to WT, suggesting MAO A played a regulatory role in stem cells neural differentiation.

Keywords

Embryonic stem cells; Neural differentiation; Neurogenesis; Monoamine oxidase (MAO) A

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) regulates neural as well as non-neural development. Monoamine oxidase (MAO) A, is the major metabolic enzyme of 5-HT (Bach et al. 1988; Bortolato et al. 2008; Shih et al. 1999), which controls 5-HT homeostasis and is involved in

cell apoptosis and the development of the central nervous system (CNS) (Cheng et al. 2010; Ou et al. 2006; Vitalis and Parnavelas 2003).

MAO A KO mice show increased 5-HT levels and aggressive behavior (Cases et al. 1995; Scott et al. 2008) and they also lack the clustering of layer IV granular neurons around thalamocortical afferents (TCA), characteristic of the barrel fields (Cases et al. 1996). They exhibited other abnormalities in addition to primary somatosensory cortex (S1), such as abnormal segregation of contralateral and ipsilateral retinogeniculate projections (Upton et al. 1999), and aberrant maturation of the brainstem respiratory network (Burnet et al. 2001). These abnormalities were believed to be due to increased 5-HT levels resulting from the lack of MAO A. The MAO A^{neo} is a novel hypomorphic line of MAO A: there is no MAO A activity in all brain regions except prefrontal cortex and amygdala in adults and no detectable MAO A activity in embryonic stem (ES) cells (unpublished data). Thus, ES cells from the MAO A^{neo} mice were used to study the role of MAO A in stem cell differentiation.

ES cells differentiate into different types of cells including all the neural lineages. In vitro ES cells can differentiate into neural cells. This can be used as a model to mimic some of the processes associated with neural development (Bibel et al. 2004; Keller 2005). In this study, MAO A^{neo} and WT ES cells were established and the characteristics of ES cells were examined. Embryoid body (EB) and monolayer culture-induced neural differentiation model were used initially to explore the effects of MAO A on the neural development of ES cells. Our results have revealed that MAO A has important regulatory effects on ES cell neural differentiation.

Materials and methods

Isolation and establishment of MAO A^{neo} and wild type (WT) murine embryonic stem cells

ES cells were isolated and amplified from MAO A^{neo} and wild type (WT) mice embryos (Martin 1981). Briefly, flush blastocyst stage embryos (d 3.5 post coitus) were obtained from uterine horns of plugged females. The embryos were transferred into Tyrode's solution and incubated for 1–2 min to remove the zona pellucida and to isolate the inner cell mass (ICM). The ICM was washed with medium and transferred into a 4-well dish with a layer of irradiated mouse embryonic fibroblasts (MEFs) in Glasgow Minimum Essential Medium (GMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamate, 0.1 μM β-mercaptoethanol, 1× EME nonessential amino acids and 10⁶ U/L leukemia factor (LIF). After amplification, the ES cells were routinely cultured without feeder cells. ES cell identification was performed by immunocytochemistry and RT-PCR using the ES cell marker OCT3/4 and Nanog (Chambers et al. 2003; Mitsui et al. 2003; Nichols et al. 1998).

Neural differentiation of ES cells by monolayer culture

For monoculture differentiation (Ying et al. 2003), undifferentiated ES cells were dissociated and plated onto 0.1% gelatin-coated tissue culture plates at a density of 0.5–1.5 × 10⁴/cm² in N2B27 medium. Medium was renewed every 2 days. After several days, some of the cells differentiated into nestin-positive neural stem cells. Continuous culture could induce the cells to differentiate into mature neural cells.

Neural differentiation of ES cells by the embryoid body (EB) induced method

ES cells hanging-drop culture (500 cells/20 μl/drop) was performed in LIF-free medium to generate EBs according to Kuo's protocol (Kuo et al. 2003). EBs were then suspension-cultured for 3 days and plated onto 0.2% gelatin-coated dishes in the defined medium (N2B27: F12/DMEM/ NEUROBASAL medium 1:1:2, 19 N2 supplement, 1× B27 supplement, bovine serum albumin fraction V 5 ug/ml) to induce the ectodermal lineages

differentiation. Some of the cells were differentiated into neural cells which could be immunostained with the neuron-specific marker β -tubulin III.

Cell morphology and immunocytochemistry—For immunocytochemistry, cells were fixed with methanol for 15 min, washed with PBS, and incubated with 10% goat serum and 0.1% Triton X-100 for 1 h at room temperature. The cells were then incubated with primary antibodies at 4°C overnight or at room temperature for 2 h. After several washes, the secondary antibody was added for 30 min. The cells were finally stained with DAPI to identify the cell nucleus.

The following primary antibodies were used: mouse monoclonal antibody against OCT3/4 (1:200, Santa Cruz Biotechnology), rabbit polyclonal anti-nestin (1:200, Chemicon), mouse monoclonal anti- β -tubulin III (1:500, Sigma-Aldrich, Ltd). Secondary antibodies were goat anti-rabbit fluorescein isothiocyanate (FITC) and goat anti-mouse rhodamine (all at 1:400, Chemicon).

MTT conversion assay—To determine cell proliferation and viability, the MTT conversion assay was used. Cells were cultured in two different culture conditions. Medium of GMEM + 10% FBS + 10^5 U/L LIF was used to maintain the cells in an undifferentiated state. N2B27 medium was used to induce differentiation. After culturing, 30 μ l of MTT dye (5 mg/ml) was added into medium in each well and incubated for 4 h. Then, the supernatant was discarded carefully and the formazan crystal was dissolved by addition of 1 ml of DMSO per well. Optical density of each well at 570 nm was determined by a spectrophotometric analyzer (Shimadzu, USA).

RNA isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Gibicol BRL) or QIAGEN RNeasy kit (Qiagen Inc., Valencia, CA). First-strand cDNA synthesis and PCR were performed according to standard protocols. Primer sequences and their PCR conditions are shown as follows: MAO A (genotyping) primers (S: 5'-CCTCTCTTCCAAGTATTAGG-3', A: 5'-GGAAAAGAGGGAGGAGTAAG-3', 58°C, 35 cycles) OCT3/4 (S: 5'-AGGGATGGCATACTGTGGAC-3', A: 5'-CCTGGGAAAGGTGTCCTGTA-3', 60°C, 32 cycles) Nanog (S: 5'-AGATGCCTCACACGGAGACT-3', A: 5'-AAGTGGGTTGTTTGCCTTTG-3', 60°C, 35 cycles), β -actin (S: 5'-TGACGGGGTCACCCACTGTGCCCATCTA-3', A: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' 60°C, 25 cycles). The PCR products were resolved by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining.

Results

WT and MAO A^{neo} murine embryonic stem cells display similar characteristics in ES stage

Figure 1a shows the constructs of a partial MAO A gene from WT and MAO A^{neo} mice. ES cells were isolated and amplified from each mouse and genotyped by RT-PCR. MAO A^{neo} displayed a 500 bp band and WT displayed a 220 bp band (Fig. 1b). Both ES cell lines grow as island-shaped colonies on the feeder cells (data not shown) which is characteristic of ES cells. The RNA levels of ES cell marker OCT3/4 and Nanog determined by RT-PCR was also similar in WT and MAO A^{neo} ES cells (Fig. 1c), suggesting that MAO A^{neo} ES cells have the same properties as that of WT.

Decreased neural differentiation in MAO A^{neo} murine ES cell compared to WT

Using monolayer-induced differentiation, ES cells were allowed to differentiate into neural cells. The expression of the neural stem cell marker nestin was determined at the early stage of ES neural differentiation. OCT was used as a marker for undifferentiated cells. Our results showed that at day 3, most cells were still undifferentiated. However, there were more neural stem cells expressing nestin in WT culture compared to MAO^{neo} (Fig. 2a). The difference in the nestin expression between WT and MAO^{neo} was further demonstrated at day 4 (Fig. 2b, c), and day 6 (Fig. 2d, e). These results indicated that the differentiation of MAO A^{neo} ES cell to neural stem cells was delayed compared to WT.

Embryonic body (EB)-induced neuronal differentiation also revealed a similar pattern. Moreover, the morphological analysis revealed obvious differences between MAO A^{neo} and WT cultures. On days 8 to 10, there were fewer neuron-like cells in MAO A^{neo} cultures than in WT cultures (Fig. 2f). Western blot revealed that there was less β -tubulin III expression in MAO A^{neo} cultures than in WT cultures (Fig. 2g, h), indicating there were fewer mature neural cells in MAO A^{neo} cultures.

To explore why there were fewer neural cells in the MAO A^{neo} differentiated culture, cell proliferation was examined using a MTT conversion assay. Interestingly, there was no significant difference in the proliferation characteristics of MAO A^{neo} ES cells compared with WT ES cells in the undifferentiated stage in LIF-containing medium (Fig. 2i). However, during differentiation a significant reduction of cell number was found in MAO A^{neo} culture compared to WT, this effect being particularly evident at 48 h of culture (Fig. 2j).

Discussion

There is increasing evidence showing that the neurotransmitter 5-HT modulates a number of developmental events, including cell division, neuronal migration, cell differentiation and synaptogenesis (Levitt et al. 1997; Vitalis and Parnavelas 2003). Disturbances of 5-HT homeostasis induced by MAO A deletion, the key enzyme responsible for 5-HT degradation, induces many developmental disorders (Bou-Flores et al. 2000). Recently, we have shown that 5-HT can regulate telencephalic neural progenitors in late embryonic and early postnatal development (Cheng et al. 2010). Using the ES differentiation model, we now show for the first time that MAO A also participates in neural differentiation from ES cells, thus implicating a potential 5-HT-dependent mechanism. We also show that MAO A^{neo} ES differentiated cultures produced fewer neural stem cells and mature neuronal cells. This may be due to a delay in the differentiation to a neuronal pheno-type in MAO A^{neo} ES cells. Development is a systematic process comprising aspects of cell proliferation, differentiation and apoptosis. The involvement of 5-HT in development has been shown by the fact that 5-HT has a trophic autocrine effect and increases axon outgrowth of raphe 5-HT neurons (De Vitry et al. 1986). However, 5-HT can also inhibit the differentiation of neural precursors into serotonergic neurons (Branchereau et al. 2002). In this study, we showed that MAO A^{neo} mice induced significant reduction in neural cells during ES cell differentiation suggesting that the excess 5-HT inhibits neural differentiation during development. Perhaps this could explain the smaller size of MAO A KO neonates (Shih and Chen 1999). Although MAO A has been reported to be closely associated with cell apoptosis (Ou et al. 2006), the role of MAO in apoptosis during differentiation and neural development remains to be further explored.

Taken together, this study has provided evidence to demonstrate for the first time that MAO A exerts important regulatory effects on the neural differentiation of murine embryonic stem cells.

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Fig. 1. Genotyping and characteristics of ES cells from WT and MAO A^{neo} mice. **a** Schematic representation of constructs of MAO A partial gene from ES cells from WT and MAO A^{neo} mice, showing the location of the PCR primers. WT: primer S; In11F2, A; in11R1, the PCR product size 220 bp. MAO A^{neo} mice: primer S; In11F2, A; In11R1, PCR product size 500 bp. **b** Representative PCR products of ES cells from WT and MAO A^{neo} mice shown on an 1.5% agar gel. **c** The expression of ES cell markers OCT and Nanog in WT and MAO A^{neo} ES cells. Same amount of PCR product of OCT (702 bp), or Nanog (105 bp) was shown for WT and MAO A^{neo} ES cells

Fig. 2. Reduced neural differentiation from ES cells in MAO A-neo mice compared to WT. **a–e** Less nestin expression in the cultures from MAO A^{neo} ES cells during differentiation by monolayer differentiation culture. Nestin immunostaining was conducted on day 3 (**a**), day 4 (**b, c**) and day 6 (**d, e**) to investigate the neural stem cells differentiated from ES cells. OCT was stained as undifferentiated stem cell marker in **a** to distinguish from differentiated cells and DAPI was used to stain the nuclei. All images were taken at a magnification of $\times 40$. **c** and **e** depict the quantification of the Nestin fluorescence density at Day 4 and Day 6, respectively. The fluorescence density of the images was quantified by using image-pro 5.0

software. The relative value for WT fluorescence intensity was set at '1'. **f** MAO A^{neo} differentiated cultures produced fewer neural cells versus WT cultures by EB-induced differentiation method. *Upper panel:* morphology of WT (*left*) and MAO A^{neo} (*right*) in lower magnification ($\times 10$); *Lower panel:* morphology of WT (*left*) and MAO A^{neo} (*right*) in higher magnification ($\times 40$). **g** Western blot showed less β -tubulin III expression in MAO A^{neo} differentiated cultures compared to WT. Actin was used as the loading control. **h** shows the quantitative data from **g** WT was taken as 1. **i** MAO A^{neo} and WT ES cells showed similar growth pattern in un-differentiated state in LIF + medium. MTT conversion assay was conducted at 0, 48, 72 h after plating. **j** MAO A^{neo} ES-cell differentiated cultures showed more cell loss during monolayer differentiation compared to WT cultures. MTT conversion assay was conducted on 0, 48, 72 h to evaluate the cell viabilities in the differentiated stage. The relative cell viability compared with control was presented. * $P < 0.01$, ** $P < 0.01 (n = 3)$