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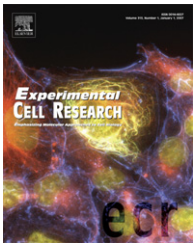

Research Article

# Contrasting and brain region-specific roles of neurogenin2 and mash1 in GABAergic neuron differentiation *in vitro*

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ABSTRACT

We have cultivated highly uniform populations of neural precursor cells, which retain their region-specific identities, from various rat embryonic brain regions. The roles of the proneural basic–helix–loop–helix (bHLH) factors neurogenin2 (Ngn2) and Mash1 in γ-aminobutyric acid (GABA) neuron differentiation were explored in the region-specific cultures. Consistent with previous *in vivo* studies, forced expression of Mash1 promoted GABA neuron formation from the precursors derived from the developing forebrains, whereas Ngn2 displayed an inhibitory role in forebrain GABA neuron differentiation. Functional analyses of mutant bHLH proteins indicated that the helix–loop–helix domains of Mash1 and Ngn2, known as the structures for protein–protein interactions, impart the distinct activities. Intriguingly, the regulatory activities of Mash1 and Ngn2 in GABA neuron differentiation from the hindbrain- and spinal cord-derived precursor cells were completely opposite of those observed in the forebrain-derived cultures: increased GABA neuron yield by Ngn2 and decreased yield by Mash1 were shown in the precursors of those posterior brain regions. No clear difference that depended on dorsal–ventral brain regions was observed in the bHLH-mediated activities. Finally, we demonstrated that Otx2, the expression of which is developmentally confined to the regions anterior to the isthmus, is a factor responsible for the anterior–posterior region-dependent opposite effects of the bHLH proteins.



## Introduction

A central issue in developmental neurobiology is how brain region-specific neuronal subtypes arise at precise positions in the developing brain along the anterior–posterior and dorsal–ventral axes. Despite extensive study, these developmental processes have been only partly understood. The major problem comes from the fact that the developmental processes can not be achieved simply by the individual actions of single factors, but must be accomplished in the molecular

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context of the developing brain [1]. Specifically, the repertories of region-specific gene expression, established in uncommitted neural precursor cells during early brain development, critically influence the generation of the region-specific types of cells [2,3].

*In vivo* functional analyses have the benefit that one can appropriately evaluate the normal physiology of the cell fate determination phenomenon resulting from the summation of the interactions between cells, between cells and the surrounding environments, and the existing extrinsic molecules. However, such complicated interactions in *in vivo* systems often hinder an adequate identification of the contribution by each individual single component, which may be essential for a clear understanding of the developmental processes. In this regard, there is intense interest in studying central nervous system (CNS) stem/precursor cells in culture models, in which each molecular component can be managed, to understand the fundamental cellular, biochemical, and physiological bases of the *in situ* regulation of cell fate determination and differentiation. Obviously, *in vivo* cellular characteristics cannot be predicted *a priori* from culture studies, yet cultures provide a powerful means to test or make hypotheses on the *in vivo* properties and behavior of cells. In addition, information obtained in the culture system could be applied directly to guide differentiation of neural precursor cells towards specific neuronal subtypes for experimental and transplantation purposes.

Neurons secreting γ-aminobutyric acid (GABA) neurotransmitter (GABAergic neurons; GABA neurons), which reside in almost all of the brain regions, are the most common inhibitory neurons in the CNS. The molecular machinery governing GABA subtype specification largely remains to be determined, but recent studies have defined several transcription factors that play essential roles in GABA neuron productions of various brain regions, such as Dlx1, 2 in the forebrain [4,5], Heslike/Megane in the midbrain [6,7], Ptf1a in the cerebellum [8], and Lbx1/Tlx3 in the spinal cord [9]. Proneural basic helix–loop–helix (bHLH) transcription factors are well-acknowledged pan-neurogenic molecules that specify neural precursor cell fates towards neuronal lineages and neuronal differentiation [10,11]. Two families of proneural bHLHs, such as the achaete–scute homolog Mash1 and the atonal-related Neurogenin (Ngn) genes, have been identified in the developing mammalian nervous system [12,13]. A conventional explanation for bHLH's role was that it simply activates generic neuronal differentiation of precursor cells, neurotransmitter subtypes of which had been committed by the actions of regionally-specified proteins. Recent studies, however, have reported that the bHLH proteins are more actively and specifically involved in the processes of neurotransmitter subtype determination [10]. Specifically, *in vivo* knock-out mouse analyses revealed a critical role for the bHLH factors in forebrain GABA neuron formation [14,15].

In this study, we performed cultures for highly homogenous populations of neural precursor cells from dorsal and ventral parts of rat embryonic brain regions throughout the forebrain to the spinal cord. The precursor cells derived from the different regions of the developing brain shared common properties in their *in vitro* proliferation and generic differentiation towards neurons and glia, but retained unique region-specific neuronal subtype differentiation and gene expression patterns inherited from the regions of the developing brain. The cultures of the region-specific precursors were utilized to obtain further insights into the roles of the proneural bHLH proteins neurogenin 2 (Ngn2) and Mash1 in GABA neuron differentiation. Consistent with previous *in vivo* findings [14,15], over-expression of Mash1 bHLH factor enhanced GABAergic neuronal yield from precursor cells derived from the developing forebrains [cortex (Ctx) and lateral ganglionic eminence (LGE)], whereas Ngn2 repressed forebrain precursor-derived GABA neuron differentiation. Functional analyses of Ngn2 and Mash1 mutants identified the structures of Ngn2 and Mash1 responsible for the activities of GABA subtype differentiation. The GABA differentiation control activities of the bHLH factors, in contrast, were completely opposite to those of the forebrain cultures in the precursors derived from the regions posterior to the isthmus (midbrain–hindbrain junction) such as the hindbrain and spinal cord. We further demonstrate data suggesting that Otx2, expression of which is restricted in the embryonic brain anterior to the isthmus [16–18], is, at least in part, responsible for the contrasting roles of the bHLH in the anterior and posterior embryonic brain.

## Materials and methods

### *Cultures for neural precursor cells derived from rat embryonic brains*

Timed-pregnant Sprague Dawley (SD) rats were purchased from Koatech (Seoul; Korea). Embryonic brain tissues were dissected from dorsal and ventral parts of the forebrain, midbrain, hindbrain and spinal cord at embryonic day 13 (E13; day of conception was called day 0). Dissected tissues were mechanically triturated in $Ca^{2+}$/$Mg^{2+}$ free Hank's balanced salt solution (HBSS; Invitrogen, Grand Island, NY) and seeded at 19,000 cells/$cm^2$ on 10-cm culture dishes (Corning, Corning, NY) pre-coated with polyornithine/fibronectin [poly-L-ornithine (15 μg/mL; Sigma-Aldrich, St Louis, MO) in a 37 °C incubator overnight followed by incubation with fibronectin (1 μg/mL, Sigma-Aldrich) for at least 2 h]. Neural precursor cells were allowed to proliferate in serum-free N2 media with basic fibroblast growth factor (bFGF; 20 ng/mL; R&D system, Minneapolis, MN) for 4–5 days [19]. Clusters of proliferated cells were then dissociated by trypsin–EDTA (Invitrogen) and plated on freshly coated 12-mm glass coverslips (45,000 cells/$cm^2$; Carolina Biological Supply Company, Burlington, NC) or 6-cm culture dishes, and grown in N2+bFGF for an additional 1–5 days. All the experiments were performed in the passaged (P1) cultures. Differentiation of the precursor cells was induced by withdrawal of bFGF from the medium for 3–6 days. The medium was changed every other day, and bFGF was supplemented daily. Cell cultures were maintained at 37 °C in a 5% $CO_2$ incubator.

### *Construction of the mutants of Ngn2 and Mash1*

The DNA-binding mutants, Ngn2/AQ [20] and Mash1/AQ (modeled after Ngn2AQ), were generated by changing two