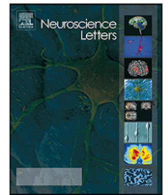
Neuroscience Letters 520 (2012) 26–31

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet



The dynamic expression of Mash1 in the hippocampal subgranular zone after fimbria-fornix transection

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HIGHLIGHTS

- ► Mash1 protein was highly increased in the deafferented hippocampus.
- ► More Mash1 positive cells in the DG differentiated into NeuN positive neurons in deafferented than in the normal side.
- ► After FF transection more newborn cells differentiated into Mash1 positive cells in the deafferented side.
- ► *In vitro*, antibody neutralization showed that Mash1 in the extracts was involved in promoting neuronal differentiation.

ARTICLE INFO

Article history:
Received 17 January 2012
Received in revised form 2 May 2012
Accepted 3 May 2012

Keywords:
Mash1
Hippocampus
Fimbria-fornix transection
Neural progenitor
Differentiation

ABSTRACT

Mash1, a member of the basic helix-loop-helix (bHLH) transcription factor family, has previously been considered essential for neuronal differentiation and specification in the nervous system. In this study, we investigated the expression of Mash1 in the hippocampus after fimbria-fornix (FF) transection. Western blot showed that protein of Mash1 increased significantly and peaked at day 7 after FF transection. Immunofluorescence indicated that after FF transection, more newborn cells differentiated into Mash1 positive cells in the deafferented side than that in the normal side, and we investigated that in the neurogenic area, subgranular zone (SGZ), a part of Mash1 positive cells were NeuN positive, and more Mash1/NeuN double positive neurons were identified in the deafferented side than that in the normal side. Additionally, the number of Mash1/NeuN double positive neurons in SGZ increased significantly and peaked at day 7 after FF transection. *In vitro*, immunofluorescence revealed that extracts of the deafferented hippocampus promoted neuronal differentiation to a greater extent than extracts from normal hippocampus. Deafferented extracts also enhanced Mash1 expression in MAP-2 positive neurons. This study concludes that after FF transection, Mash1 expression in the deafferented hippocampus increased and might play an important role in inducing local progenitors to differentiate into neurons.
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1. Introduction

It is believed that neural stem cells (NSCs) in the central nervous system (CNS) are self-renewing and have multipotent ability to differentiate into cells of either the neuronal or the glial lineage [6,26]. Recent studies demonstrate that basic helix-loop-helix (bHLH) transcription factors play important roles in proliferation, cell lineage determination, and neuronal differentiation of NSCs [3,13]. BHLH transcription factors include Mash1, Math1, neurogenin, and NeuroD. These factors form a heterodimer with ubiquitously expressed bHLH proteins, such as E12 and E47, and activate gene expression by binding to the E box domain, leading to neuronal differentiation [8,16,20].

Mash1 is a bHLH transcription factor restricted to proliferative zones in the developing brain in a spatially specific manner [14,28]. In the adult brain, Mash1 is present in neuronal progenitors in the dentate gyrus (DG) of the hippocampus and the rostral migratory stream in the forebrain [15]. In addition, Mash1 promotes the neuronal fate and inhibits the astrocytic fate of NSCs in the developing forebrain [22]. These findings indicate that Mash1 plays a crucial role in the regulation of nervous system development.

Lesion to the CNS may trigger the increase of some transcription factors to partly or absolutely restore the lost functions. As in the

Abbreviations: NSCs, neural stem cells; DG, dentate gyrus; SVZ, subventricular zone; SGZ, subgranular zone; CNS, central nervous system; BrdU, 5-bromo-2-deoxyuridine; FF, fimbria-fornix; bHLH, basic helix-loop-helix; NGF, nerve growth factor-1; BDNF, brain-derived neurotrophic factor.

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http://dx.doi.org/10.1016/j.neulet.2012.05.016

published reports [7,18] we used the hemisphere fimbria-fornix-damaged animals to model Dementia in order to investigate the development of local progenitors in the hippocampus. We previously reported that NSCs grafted into the adult hippocampus with FF transection showed greater survival, migration and neuronal differentiation than NSCs grafted into normal hippocampus; extracts from deafferented hippocampus effectively promoted NSC differentiation into neurons or AChE positive neurons *in vitro* [29,30]. It was proposed that expression of some neurotrophic or transcription factors are increased in the denervated hippocampus, and participate in the repair of the hippocampus.

In this research, we described the expression of Mash1 in the hippocampus after FF transection. And *in vitro*, we demonstrated that Mash1 levels increase significantly during the differentiation of NSCs stimulated by extracts of the deafferented hippocampus.

2. Materials and methods

2.1. Animals and surgery

Neonatal and adult female Sprague–Dawley ($n = 6$, 220–250 g) rats were purchased from the experimental animal center of Nantong University. All animal experiments were carried out in accordance with the US National Institutes of Health Guide for the care and use of laboratory animals.

Lesion of FF was adapted from Hefti [9]. After transient anesthesia with Chlorpent (2 ml/kg body weight, i.p.), adult SD rats were transferred to the stereotaxic apparatus and a 3-mm length aperture was drilled into the skull between 2 points (AP = 1.4, ML = 1.0 and AP = 1.4, ML = 4.0) according to the atlas of Paxinos and Watson [25]. A wire-knife was lowered to a depth of 5.4 mm ventral to the dura and shifted laterally in the aperture three times. After 1 min, the wire-knife was slowly withdrawn. After suture of skin the animals were caged for tender care. Nissl staining [24] was used to verify the models.

2.2. BrdU labeling

5-Bromo-2-deoxy-uridine (BrdU, Roche, Germany) was administrated twice per day from 2 to 6 days after FF transection. On the 7th day after operation, the brains were perfused for fixation and frozen section. The number of BrdU-labeled cells was measured for assessing neurogenesis after FF transection.

2.3. Immunofluorescence assays

For double immunofluorescence labeling we combined BrdU with Mash1 for assessing the local cell proliferation in DG. The sections were incubated with mouse anti-BrdU antibody (1:1000; Roche, Germany) and rabbit anti-Mash1 (1:60, Abcam, Japan) overnight at 4 °C, respectively, as described previously [32], and then with 568 (1:800, Molecular Probes, USA), and FITC (1:100, Chemicon, USA). To determine the relationship between the expression of Mash1 and NeuN, Sections were incubated with rabbit antiMash1(1:60, Abcam, Japan) and mouse anti-NeuN (1:500, Chemicon, USA) antibodies overnight at 4 °C and then with FITC and 568 for 2 h at RT. Pictures were captured by a fluorescence microscope (Leica, Germany).

2.4. Tissue preparation and Western blotting

Dentate gyrus of normal and deafferented hippocampus were quickly isolated and homogenized in 50 mM Tris–NaCl for 30 min on ice. Protein concentration in the supernatant was determined by Bradford assay [1]. Protein was separated on 10% SDS–PAGE, transferred to nitrocellulose membrane and probed with respective antibodies. The antibodies used were anti-Mash1 and anti-β-actin (Abcam, Japan).

2.5. Extract preparation

On day 7 following unilateral FF transection, deafferented and untreated contralateral hippocampus were quickly dissected and homogenized into precooled Dulbecco's modified Eagle's medium/F12 medium (1:1, DMEM/F12, Gibco, USA) containing 2% B27 (1 ml/100 mg tissue; Gibco, USA) [29]. Homogenates were then centrifuged at $10{,}000 \times g$ at 4 °C for 10 min; supernatants were stored at −70 °C.

2.6. Cell culture and neurosphere formation

Neural progenitors were derived from the hippocampus of E18 rats as described previously [19,29]. Briefly, after anesthesia with Chlorpent (2 ml/kg, i.p.), hippocampus was dissected quickly into single cell suspensions. Single-cell suspensions were then transferred to DMEM/F12 medium with 2% B27, epidermal growth factor (EGF, 10 ng/ml) and basic fibroblast growth factor (bFGF, 10 ng/ml) at a density of 4×10^4 cells/ml and plated into culture flasks and incubated at 37 °C/5% CO_2. After 6–7 d the neurospheres were dissociated into single-cell suspension, and seeded into 96-well plate at 1–2 cells per well. The subclonal neurospheres were digested and passaged again as before. Cells were passaged every 6–7 d to obtain neurospheres originating from a single primary cell.

2.7. Induction of neural progenitors differentiation

Subclonal neurospheres were seeded onto the poly-D-lysine-coated cover-slips in 24-well culture plates containing the extracts from deafferented or normal hippocampus. Five groups with or without Mash1 antibody diluted 1:400 were designed for neutralization experiments: (1) deafferented hippocampal extract; (2) deafferented hippocampal extract added with Mash1 antibody; (3) normal hippocampal extract; (4) normal hippocampal extract added with Mash1 antibody; (5) Control group without any extracts or Mash1 antibody. After 7d culture, cells were fixed with 4% PFA in 0.1 M PB at 4 °C for 30 min and rinsed with 0.01 M PBS. After blocked in 5% goat serum overnight at 4 °C, cells were incubated with rabbit anti-MAP-2 (1:200, Chemicon), mouse anti-Mash1 (1:400, BD Pharmingen) overnight at 4 °C and then with secondary antibodies conjugated to fluorescein 488 and 568 for 2 h at RT. Immunofluorescence signals were visualized by fluorescence microscope. The number, area, and perimeter of MAP-2 positive neurons and the number of Mash1/MAP-2 double-labeled neurons in different groups were analyzed statistically.

2.8. Statistical analysis

To count the immunoreactive cells in the DG, the representative sections through the posterior, middle and anterior part of hippocampus were selected randomly, to ensure their consistence between different animals. All counts were performed using a 200× objective lens on a Leica fluorescence microscope. The optical density and gray scale were analyzed by JD-801morphology software (Nanking, China). Images were processed using Leica Qwin image processing and analysis software (Leica Imaging Systems, Cambridge, UK). Statistical analysis was performed using Stata 8.0 statistical software. Comparison of differences between groups was performed using the Kruskal–Wallis ANOVA on ranks and SNK test, followed by post hoc tests using Dunn's method (SigmaStat; Jandel Scientific, San Rafael, CA, USA). Differences were considered significant when $P < 0.05$.

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