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Gandhervin Kesavamoorthy[1], Sumiko Mikawa[1], Yuya Kusakawa[1,2], Tomonori Sato[3], Michihito Miyagi[4], Satoru Yamagishi[1*], Kohji Sato[1*]

[1)] Department of Anatomy & Neuroscience, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashiku, Hamamatsu, Shizuoka 431-3192, Japan
[2)] AICHI Medical College for Physical and Occupational Therapy, 519 Ichiba, Kiyosu, Aichi 452-0931, Japan
[3)]Department of Physical Therapy, Faculty of Health Science, Tokoha University, 1-30 Mizuochi, Aoi-ku, Shizuoka 420-0831, Japan
[4)]Department of Orthopaedic Surgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashiku, Hamamatsu, Shizuoka 431-3192, Japan

Correspondence should be addressed to Kohji Sato or Satoru Yamagishi.
Department of Anatomy & Neuroscience
Hamamatsu University School of Medicine
1-20-1 Handayama, Higashiku, Hamamatsu,
Shizuoka 431-3192, Japan
Tel & Fax: 81(Japan)-53-435-2582
E-mail: ksato@hama-med.ac.jp or yamagish@hama-med.ac.jp

Abstract

We investigated the distributions of BMP ligands (BMP2, BMP4, BMP5), BMP receptors (BMPR) (BMPRIA, BMPR IB, BMPR II), BMP antagonists (chordin and noggin) in the adult rat cerebellum. They are differentially expressed throughout the cerebellum. Interestingly, they are more abundantly expressed in the molecular cell layer, where neuronal plastic phenomena, such as long-term depression (LTD) and rebound potentiation (RP) are observed, than in the granule cell layer. In addition, neurons in the cerebellar nuclei also express all of them. We also found differential usages of BMPRIA and BMPRIB in the cerebellum. For example, in the molecular layer, BMPRIA is mainly expressed in the processes of Bergmann glia, while BMPRIB is mainly detected in the dendrites of Purkinje cells. In addition, BMPRIB is expressed in both cell bodies and dendrites of Purkinje cells, while BMPRIA is mainly expressed in cell bodies of Purkinje cells, showing that BMPRIA and BMPRIB are differentially targeted in a Purkinje cell. These data strongly indicate that differential BMP signaling should be needed to keep the proper function of the cerebellum in the adult rat cerebellum.

Key words

BMP, BMP receptor, chordin, noggin, immunohistochemistry

Introduction

Bone morphogenetic proteins (BMPs) constitute the large subgroup of the transforming growth factor β (TGF- β) superfamily [1]. Although BMPs were initially identified by their ability to direct ectopic bone formation, they are now shown to play important roles in multiple biological events [1]. The activities of the BMPs are mediated by a heterodimeric complex of type I and type II BMP serine/threonine kinase receptors, consist of a short extracellular domain, a single transmembrane, and the intracellular serine/threonine kinase domain, including bone morphogenetic protein receptor type I (BMPRIA, BMPRIB) and type II (BMPRII) [1]. On BMP binding, the type I BMPRs activate the receptor-activated Smads (R-Smads; Smad1/5/8) which oligomerize with common-mediator Smad (Co-Smad; Smad4). The Smad complex then translocates to the nucleus and acts as a transcription regulator [2]. Although many of the biological effects of BMPs have been related to the Smad-dependent pathways, Smad-independent pathways have been also reported [3]. Functions of BMPs are also regulated in the extracellular space by secreted antagonistic regulators such as chordin, noggin, follistatin, neurogenesin-1, which are reported to bind BMPs extracellularly and prevent their interaction with their receptors [4,5].

BMP signaling has also been reported to be involved in the development of the cerebellum, especially in the development of granule cells [6-8]. In addition, the expressions of some members of BMP signaling-related proteins in the development are also reported [9,10]. However, their detailed expression profiles in the adult cerebellum have not been reported. Thus, we investigated the distributions of BMP2, 4, 5, BMPRIA, IB, II, chordin and noggin in the adult rat cerebellum.

Methods

1. **Animals and section preparation**

Wistar male rats (7 weeks old; Japan SLC Inc., Shizuoka Japan) were used in this study. Under deep diethylether anesthesia, rats were perfused transcardially with saline followed by 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid. The brains were removed rapidly, and then postfixed in the same fixative for 2 h at 4 °C. All brains were immersed in 10% sucrose in PB for overnight and in 25～30% sucrose in PB for 2 overnight at 4 °C. Sections were cut on a cryostat at 20 μm thickness. All experiments conformed to the Guidelines for Animal Experimentation at Hamamatsu University School of Medicine on the ethical use of animals.

2. **Immunohistochemistry**

The following primary antibodies were used: goat anti-BMP2 (200 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-BMP4 antibody, NCL-BMP4 (the initial concentration is not available; Novocastra, Newcastle, United Kingdom); goat anti-BMP5 antibody (100 μg/ml; R & D Systems, Inc., Minneapolis, MN); goat anti-BMPRIA antibody (200 μg/ml; Santa Cruz Biotechnology); goat anti-BMPRIB antibody (200 μg/ml; Santa Cruz Biotechnology); goat anti-BMPRII antibody (200 μg/ml; Santa Cruz Biotechnology); rabbit anti-noggin antibody (200 μg/ml; Santa Cruz Biotechnology); rabbit anti-chordin antibody (100 μg/ml; ABGENT Inc., San Diego, CA).

For immunohistochemical staining of goat primary antibodies, the sections were treated with 10% normal rabbit serum, 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature, and incubated further in goat anti-BMP2 (diluted 1:50), goat anti-BMP5 (diluted 1:100), goat anti-BMPRIA (diluted 1:50), goat

anti-BMPRIB (diluted 1:200) and goat anti-BMPRII (diluted 1:50) for overnight at 4 °C, respectively. After being washed with 0.1 M PB, sections were incubated in rabbit anti-goat IgG with peroxidase complex (no dilution, ready-to-use; Histofine® Simple Stain™ Rat MAX PO (G); Nichirei, Tokyo, Japan) for 2 h at room temperature. For immunohistochemical staining of mouse primary antibody, the sections were treated with 10% normal goat serum, 2% BSA and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature, and incubated further in mouse anti-BMP4 (diluted 1:100) for overnight at 4 °C. After being washed with 0.1 M PB, sections were incubated in goat anti-mouse IgG with peroxidase complex (no dilution, ready-to-use; Histofine® Simple Stain™ Rat MAX PO (M); Nichirei) for 2 h at room temperature. For immunohistochemical staining of rabbit primary antibodies, the sections were treated with 10% normal goat serum, 2% BSA and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature, and incubated further in rabbit anti-noggin (diluted 1:100) and rabbit anti-chordin (diluted 1:50) for overnight at 4 °C, respectively. After being washed with 0.1 M PB, sections were incubated in goat anti-rabbit IgG with peroxidase complex (no dilution, ready-to-use; EnVision ™ System, K4002; DAKO, Tokyo, Japan) for 2 h at room temperature. And then, after being washed with 0.1 M PB, immunoperoxidase of all sections was visualized with 3,3'-diaminobenzidine with nickel (II) sulfate intensification.

Bright-field images were obtained using a microscope (Eclipse 80i; Nikon, Tokyo, Japan), equipped with a CCD camera (Microfire; Optronics). They were further processed in image analysis software (Photoshop; Adobe, Tokyo, Japan).

Results

The specificity of the antibodies used in this study was already confirmed in our previous studies [11-16]. The relative intensities of the BMP signaling-related protein expression in the major areas of the rat cerebellum are shown in Table 1.

BMP2, 4, and 5 expressions in the adult cerebellum

BMP2: Cell bodies of Purkinje neurons were intensely stained (Fig. 1A). The granular cells also exhibited strong BMP2 immunoreactivity (IR) . In the molecular layer, many neurons and the neuropil were moderately labeled. In addition, cell bodies of Bergmann glia seemed to be stained. In the cerebellar nuclei, strong neuronal cell body staining and moderate neuropil staining were observed (Fig. 1B).

BMP4: The molecular layer showed very strong neuropil staining (Fig. 1C). In addition, dendrites of Purkinje neurons were intensely stained. In the Purkinje cell layer, cell bodies of Purkinje neurons showed strong BMP4-IR. In addition, cell bodies of Bergmann glia seemed also stained. The granular layer exhibited a mosaic pattern (Fig. 1C), and the cerebellar nuclei contained intensively labeled neurons (Fig. 1D).

BMP5: We observed very strong staining in the Purkinje cell layer (Fig. 1E). Very strong BMP5-IR was seen in the cell bodies of Purkinje neurons as well as BMP2/4. In addition, small Bergmann glia around the cell bodies of Purkinje cells also seemed to show very strong BMP5-IR. The molecular cell layer contained many strongly-positive neurons, and strong neuropil staining was also observed. Moderate BMP5-IR was also seen in granular cells. Strong neuronal cell body and moderate neuropil staining were observed in the cerebellar nuclei (Fig. 1F).

BMP receptor IA, B and II expression in the cerebellum

BMPRIA: BMPARIA-IR was moderately observed in Purkinje neurons (Fig. 2A). In the molecular layer, BMPRIA-IR was strongly seen in vertical line-like structures, suggesting that it is expressed in the processes of Bergmann glia. In addition, moderate neuropil staining was also observed. Granule cells also expressed BMPRIA moderately (Fig. 2A). In the cerebellar nuclei, BMPARIA-IR was strongly detected in neural cell bodies (Fig. 2B). In addition, moderate dot-like BMPRIA-IR was observed in neropil.

BMPRIB: Cell bodies and dendrites of Purkinje neurons were intensively stained with the anti-BMPRIB antibody (Fig. 2C). Granular cells exhibited weak BMPRIB-IR. In the cerebellar nuclei, BMPARIB-IR was strongly detected in neural cell bodies (Fig. 2D). In addition, BMPRIB-IR was also seen in dendrites of neurons.

BMPRII: Strong BMPRII-IR was observed in the Purkinje cell layer and molecular layer (Fig. 2E). Granular cells also expressed BMPRII moderately. In the cerebellar nuclei, BMPARII-IR was strongly detected in neural cell bodies (Fig. 2F). In addition, moderate dot-like BMPRII-IR was observed in neropil.

Noggin and chordin expressions in the adult cerebellum

Noggin: Very strong neuropil staining was observed in the molecular layer (Fig. 3A). Cell bodies of Purkinje neurons were weakly stained (Fig. 3A). The granular cells also exhibited weak noggin-IR. In the cerebellar nuclei, strong neural cell body staining and moderate neuropil staining were observed (Fig. 3B).

Chordin: Intensive staining was observed in the Purkinje cell layer (Fig. 3C). Cell bodies of Purkinje neurons were very strongly stained. In addition, small Bergmann glial-like cells also showed chordin-IR (Fig. 3C). The molecular cell layer contained many chordin-positive neurons, in addition, strong neuropil staining was also observed (Fig. 3C). Granular cells also exhibited strong chordin-IR (Fig. 3C). In the cerebellar nuclei, strong neural cell body staining and moderate neuropil staining were observed (Fig. 3D).

Discussion

In the present study, we found that BMP signaling-related proteins are differentially expressed throughout the adult rat cerebellum. In general, they are abundantly expressed in the molecular cell layer, where neuronal plastic phenomena, such as long-term depression (LTD) [17] and rebound potentiation (RP) are observed [17,18]. As BMP signaling is reported to be deeply involved in neural plasticity [19,20], BMP signaling-related proteins expressed in the molecular cell layer may play pivotal roles in these phenomena. In addition, Purkinje cells also abundantly express most of BMP signaling-related proteins. Especially BMP4, BMP5, BMPRIB are expressed very strongly in dendrites of Purkinje cells. Such characteristic expression patterns in the dendrites suggest that BMP signaling is needed to keep the complicated morphology of the dendrites of Purkinje cells.

Interestingly, differential usage of BMPRIA and B is observed. For example, in the molecular layer, BMPRIA is mainly expressed in the processes of Bergmann glia, while BMPRIB is mainly detected in the dendrites of Purkinje cells. In addition, even in a given Purkinje cell, BMPRIB is expressed in both the cell bodies and dendrites of Purkinje cells, while BMPRIA is mainly expressed in the cell bodies of Purkinje cells, showing that BMPRIA and BMPRIB are differentially targeted in a Purkinje cell. In the other parts of the CNS, we also found very strong BMPRIB in dendrites of many neurons, while BMPRIA was mainly expressed in cell bodies [13]. Although the functional differences between BMPRIA and BMPRIB have not been elucidated so far, we speculate that to keep the complicated structure of Purkinje cells, different kinds of BMP signaling should be needed in the different parts of the neuron.

Finally, noggin shows also very characteristic expression patterns in the cerebellum, i.e. strong neuropil expression in the molecular layer (Fig. 3A). Noggin is an extracellular BMP antagonist that binds BMP-2/4 with high affinity and thus interferes their binding to BMP receptors. Interestingly, Lim et al. have reported that, in the adult subventricular zone, BMP2 and 4

expressed in the type B/C cells potently inhibits neurogenesis, and its antagonist noggin secreted from the ependymal cells makes a niche for adult neurogenesis [21]. In addition, we reported that noggin is expressed in the subgranular zone of the hippocampus, where adult neural stem cells also exist [14]. Furthermore, mossy fibers originated from granule cells also express abundant noggin. Therefore, noggin in the molecular layer might also play important roles in neural plasticity and morphogenesis.

Conclusion

We show here that BMP signaling-related proteins are differentially expressed throughout the adult rat cerebellum. Further investigations using various techniques are needed to fully delineate the function of BMP signaling in the adult cerebellum.

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Figure legends

Fig. 1. BMP2, 4 and 5 expressions in the rat cerebellum and cerebellar nucrei. CN, cerebellar nucrei; Gr, granular layer; Mol, molecular layer; Pur, Purkinje cell layer. Scale bar = 20 μm for A-F

Fig. 2. BMPRIA, BMPRIB and BMPRII expressions in the rat cerebellum and cerebellar nucrei CN, cerebellar nucrei; Gr, granular layer; Mol, molecular layer; Pur, Purkinje cell layer. Scale bar = 20 μm for A-F

Fig. 3. Noggin and chordin expressions in the rat cerebellum and cerebellar nucrei. CN, cerebellar nucrei; Gr, granular layer; Mol, molecular layer; Pur, Purkinje cell layer. Scale bar = 20 μm for A-F

Table1. Distribution and Intensity of BMPs, BMP receptors and BMP Antagonists in the Adult Rat Cerebellum

	Molecular layer	Purkinje cell layer	Guranule cell layer
BMP2	++	++++	+++
BMP4	++++	++++	+
BMP5	+++	++++	++
BMPRIA	+++	++	++
BMPRIB	+++	++++	+
BMPRII	+++	+++	++
Noggin	++++	+	+
Chordin	+++	++++	+++

Relative intensities were estimated by visual comparison of immunostained slides: +, low; ++, moderate; +++, strong; ++++, very strong.

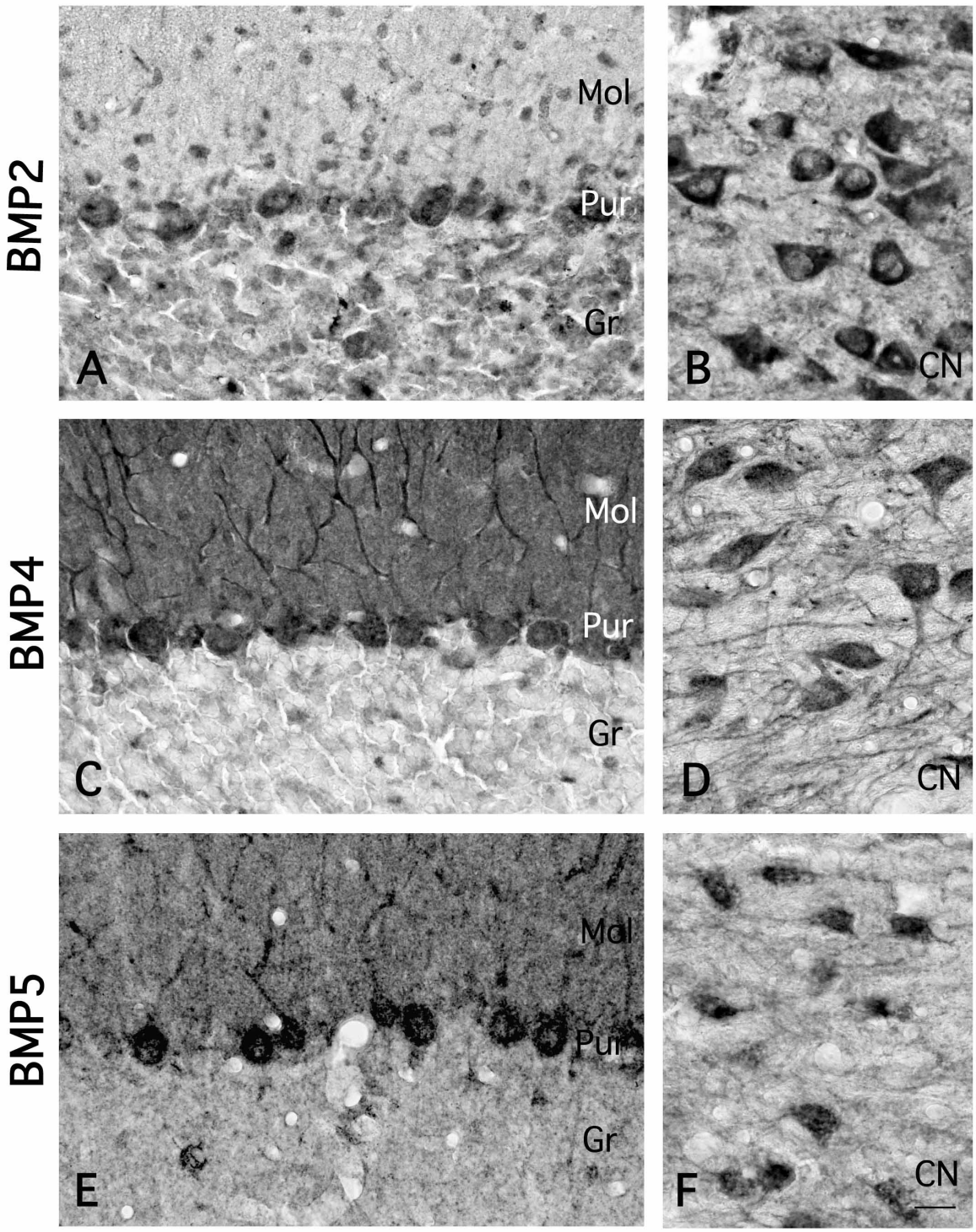

Fig. 1

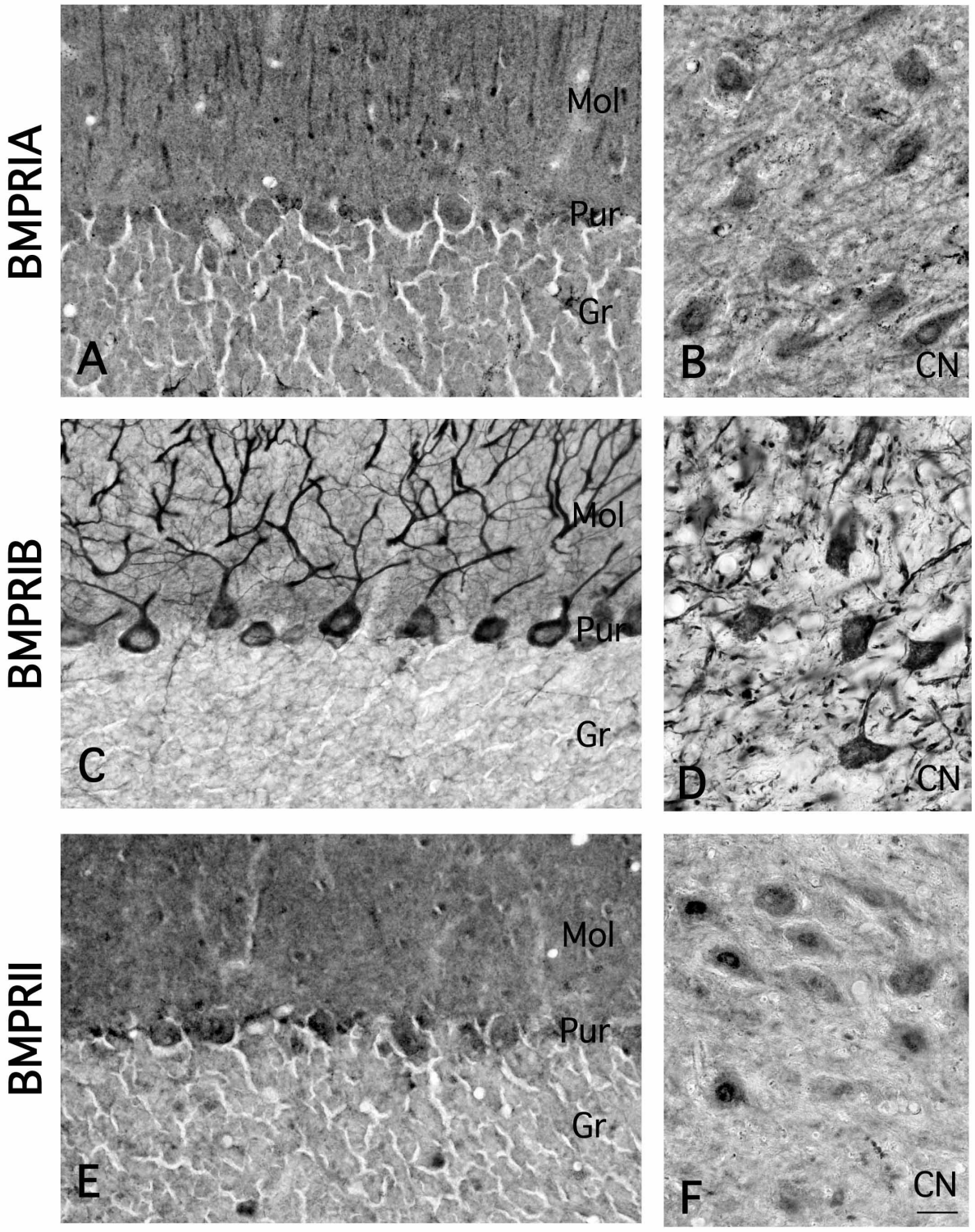

Fig. 2

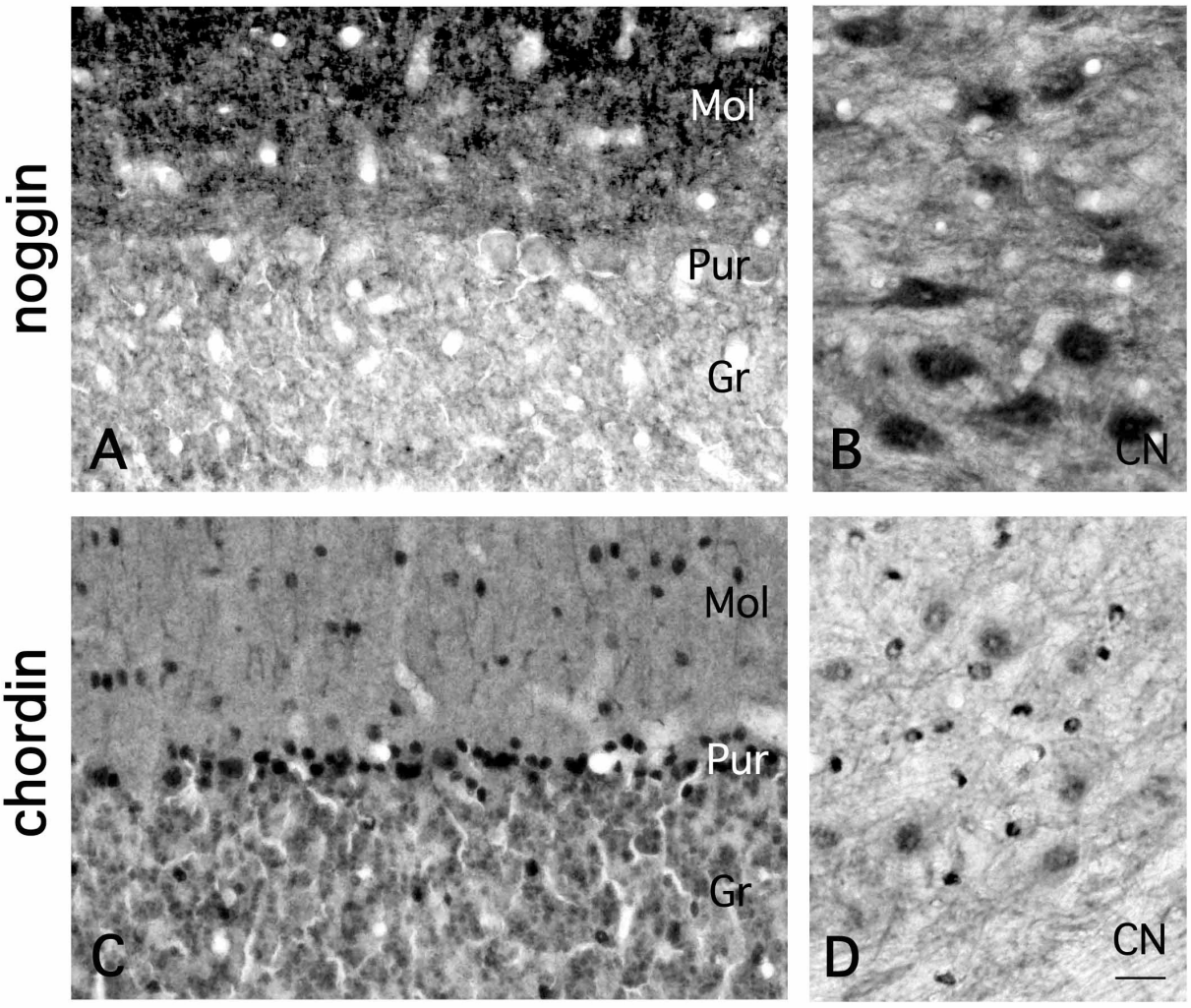

Fig. 3