

Neuroanatomical distribution of galectin-3 in the adult rat brain

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Received: 10 January 2017 / Accepted: 15 February 2017 / Published online: 2 March 2017
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Abstract Galectin-3 is a member of the lectin subfamily that enables the specific binding of β -galactosides. It is expressed in a broad spectrum of species and organs, and is known to have various functions related to cell adhesion, signal transduction, and proinflammatory responses. Although, expression of galectin-3 in some activated neuroglia under neuroinflammation has been well documented in the central nervous system, little is known about the neuronal expression and distribution of galectin-3 in normal brain. To describe the cellular and neuroanatomical expression map of galectin-3, we performed galectin-3 immunohistochemistry on the entire normal rat brain and subsequently analyzed the neuronal distribution. Galectin-3 expression was observed not only in some neuroglia but also in neurons. Neuronal expression of galectin-3 was observed in many functional parts of the cerebral cortex and various other subcortical nuclei in the hypothalamus and brainstem. Neuroanatomical analysis revealed that robust galectin-3 immuno-signals were present in many hypothalamic nuclei related to a variety of physiological

functions responsible for mediating anxiety responses, energy balance, and neuroendocrine regulation. In addition, the regions highly connected with these hypothalamic nuclei also showed intense galectin-3 expression. Moreover, multiple key regions involved in regulating autonomic functions exhibited high levels of galectin-3 expression. In contrast, the subcortical nuclei responsible for the control of voluntary motor functions and limbic system exhibited no galectin-3 immunoreactivity. These observations suggest that galectin-3 expression in the rat brain seems to be regulated by developmental cascades, and that functionally and neuroanatomically related brain nuclei constitutively express galectin-3 in adulthood.

Keywords Galectin-3 · Immunohistochemistry · Neurons · Rat brain

Introduction

Galectins comprise a ubiquitous, ancient family of β -galactoside-binding animal lectins sharing a common structural fold and at least one conserved carbohydrate recognition domain (CRD) of approximately 135 amino acids that mediate carbohydrate binding. Galectins are widely expressed in a variety of tissues among different species of vertebrates, invertebrates, protochordates, mushrooms, and viruses (Rabinovich et al. 2002). Thus far, 15 members of the galectin family have been identified in vertebrates. On the basis of their molecular architecture, galectins have been classified into three main types: (a) “proto-type” galectins (galectin-1, -2, -5, -10, -11, -13, -14, and -15), which contain a single CRD capable of forming homodimers; (b) “tandem repeat-type” galectins (galectin-4, -6, -8, -9, and -12), which harbor two distinct CRDs connected by a linker peptide and

Dae-Yong Song and Tai-Kyoung Baik have contributed equally to the work of this manuscript.

Electronic supplementary material The online version of this article (doi:10.1007/s10735-017-9712-9) contains supplementary material, which is available to authorized users.

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oligomerize via CRD–CRD interactions; and (c) “chimera-type” galectin-3, which consists of one C-terminal CRD linked to an N-terminal linker that can pentamerize upon binding to glycan ligands on cells or matrix (Hirabayashi and Kasai 1993). These multivalent members of the galectin family have been suggested to play different roles in various biological responses (Barondes et al. 1994).

Galectin-3/Mac-2 is found in the cytoplasm and nucleus, on the cell surface, and in the extracellular space (Yoshii et al. 2002; Krzeslak and Lipinska 2004). Galectin-3 on the cell surface plays important roles in cell-to-cell or cell-to-extracellular matrix adhesion (Ochieng et al. 2004), tumor growth, angiogenesis, and metastasis (Krzeslak and Lipinska 2004; Takenaka et al. 2004; Dumic et al. 2006). Cytoplasmic galectin-3 takes part in cell proliferation and differentiation and regulates apoptosis signaling cascades (Matarrese et al. 2000; Liu et al. 2002; Yu et al. 2002; Shalom-Feuerstein et al. 2005). Meanwhile, extracellular galectin-3 mediates the adhesion of immune cells to the endothelium during inflammation and is involved in the recruitment and activation of neutrophils (Almkvist and Karlsson 2004).

Galectin-3 is widely distributed among tissues and is highly expressed in a variety of inflammatory and epithelial cells (for review, Yang et al. 2008). With regard to the central nervous system (CNS), it is expressed in a specific subset of activated microglia that phagocytose myelin following induction of experimental allergic encephalomyelitis, ischemia, and sciatic nerve transection (Reichert et al. 1994; Reichert and Rotshenker 1999; Walther et al. 2000). Galectin-3 is also observed in Müller cells during retinal degeneration in rat and bovine (Uehara et al. 2001) and in neoplastic astrocytes in human tumor tissue (Camby et al. 2001). Meanwhile, galectin-3 is not expressed in any types of cells in the intact CNS of mice, with the exception of ependymal cells, subventricular zone (SVZ) astrocytes (Comte et al. 2011), and developing oligodendrocytes (Pasquini et al. 2011). Thus far, the distribution of galectin-3 in the rat brain has not been analyzed in detail. Therefore, in the present study, we conducted galectin-3 immunohistochemistry of the entire normal rat brain and presented a comprehensive neuroanatomical map of galectin-3-expressing neurons in the adult rat brain. Our findings suggest that galectin-3 is expressed in several CNS nuclei in the normal rat brain.

Materials and methods

Animals

Adult male Wistar rats ($n=10$, 8 weeks old, Charles River Laboratories, Wilmington, MA, USA) weighing 250–300 g

were purchased from SamTako Bio Korea (Osan, Korea). They were housed in a room maintained at constant room temperature (20–22 °C) with a 12-h light–dark cycle, with lights on at 7:00 AM. Food and water were available *ad libitum*. All experimental procedures performed on the animals were approved by the Animal Review Board of Eulji University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 1996).

Tissue preparation

Rats were anesthetized via intraperitoneal injection of ketamine (70 mg/kg) and xylazine (8 mg/kg) and transcardially perfused with physiological saline, followed by 400 mL of 4% paraformaldehyde in phosphate–buffered saline (PBS). The brains were removed immediately, post-fixed in the same fixative for 2 h, and infiltrated with 30% sucrose solution for 24 h at 4 °C until they sank. The whole brains were rapidly frozen in 2-methylbutane chilled on dry ice and mounted in Tissue-Tek OCT compound (Sakura Fine-technical Co., Tokyo, Japan). Serial coronal sections of 40 μm -thickness were obtained on a Cryostat Microtome (Leica Microsystems Inc., Wetzlar, Germany).

Immunohistochemistry

The list of primary antibodies used in this study is summarized in Table 1. Free-floating brain sections were pre-treated for 20 min with 0.3% hydrogen peroxide in PBS to quench any endogenous peroxidase activity. They were then incubated for 1 h at room temperature in 0.1 M PBS containing 0.1% Triton X-100 and 5% normal rabbit serum to reduce non-specific staining. Then, sections were incubated for 16 h at 4 °C with goat polyclonal anti-galectin-3 antibody (R&D Systems, Minneapolis, MN, USA), at a final dilution of 1:500. The specificity of the anti-galectin-3 antibody has previously been confirmed by Western blot analysis and immunohistochemistry (Choy et al. 2015). In order to verify the specificity of labelling, additional sections were incubated in the absence of primary antibody. Sections were washed extensively with PBS and incubated for 2 h with biotinylated rabbit anti-goat IgG (Vector Labs, Burlingame, CA, USA), at a final dilution of 1:200. After rinsing three times for 5 min in PBS, sections were exposed to avidin–biotin peroxidase complex (Vector Labs), which was applied for 1 h prior to incubation for 3–5 min for peroxidase detection using 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) containing 0.01% hydrogen peroxide. Sections were mounted on gelatin-coated slides, counterstained with hematoxylin, dehydrated through a graded ethanol series,

Table 1 Antibodies used

Name	Immunogen	Manufacturer, catalog number, RRID, host species	Concentration
Galectin-3	<i>E. coli</i> -derived recombinant mouse Galectin-3	R&D Systems, Cat# AF1197, RRID: AB2234687, goat polyclonal	IHC: 1:500 IF: 1:100
NeuN	Purified cell nuclei from mouse brain	Millipore, Cat# MAB377, RRID: AB2298772, mouse monoclonal	IF: 1:100
GFAP	Purified GFAP from porcine spinal cord	Millipore, Cat# MAB360, RRID: AB2109815, mouse monoclonal	IF: 1:200
Iba1	Synthetic peptide corresponding to C-terminus of Iba1	Wako Pure Chemical Industries, Cat# 27030, RRID: AB2314667, rabbit polyclonal	IF: 1:50
Rip	Rat olfactory bulb	DSHB, Cat# Rip, RRID: AB531796, mouse polyclonal	IF: 1:100

cleared in xylene, and covered with coverslips using Permount (Fisher Scientific, Pittsburgh, PA, USA).

Data analysis for immunohistochemistry

Light photomicrographic images were acquired using a Nikon Optiphot microscope (Nikon Inc., Tokyo, Japan) fitted with a Nikon digital camera (DXM1200; Nikon Inc.) and Nikon ACT-1 image capture software (version 2.2; Nikon Inc.). Images were optimized for image resolution, contrast, evenness of illumination, and background using Adobe Photoshop 7.0.1 (Adobe Systems, San Jose, CA, USA). Anatomical structures (nuclei) were identified according to an adult rat brain atlas (Paxinos and Watson 1998). The relative intensity of galectin-3 was scored as negative (–; no reactivity), scarce (+/–; faint reactivity of one or two cells), moderate (+; reactivity <10% of cells), intense (++; reactivity in 10–30% of cells), and very intense (+++; reactivity >30% of cells).

Double immunofluorescence

Double immunofluorescence labeling for galectin-3/NeuN (Neuronal nuclear antigen), galectin-3/GFAP (glial fibrillary acidic protein), galectin-3/Iba1 (ionized calcium-binding adapter molecule 1), and galectin-3/Rip was conducted to examine which cell types express galectin-3 in the normal rat brain. Following incubation in PBS containing 10% normal horse serum for 30 min, sections were incubated for 16 h at 4 °C with galectin-3 antibody diluted to 1:100 in PBS. After rinsing in PBS, a Cy3-conjugated horse anti-goat secondary antibody (Jackson ImmunoResearch Labs., West Grove, PA, USA) diluted to 1:200 in PBS was applied for 2 h at room temperature. The incubation and rinse steps were repeated using NeuN (mouse anti-NeuN monoclonal antibody, 1:100, Millipore, Temecula, CA, USA), GFAP (mouse anti-GFAP monoclonal antibody, 1:200, Millipore), Iba1 (rabbit anti-Iba1 polyclonal antibody, 1:50, Wako Pure Chemical Industries, Osaka, Japan), and Rip (mouse anti-oligodendrocyte polyclonal antibody, 1:100, DSHB, Iowa

City, IA, USA) antibodies and either FITC-conjugated horse anti-mouse secondary antibody (for NeuN, GFAP, and Rip, Jackson ImmunoResearch Labs.) or FITC-conjugated horse anti-rabbit secondary antibody (for Iba1, Jackson ImmunoResearch Labs.). Sections were mounted on gelatin-coated slides and cover-slipped with Vector-shield medium (Vector). All procedures were conducted in dark conditions.

Results

Cell types that express galectin-3 in the rat brain

Galectin-3 immunoreactivity in the normal rat brain was assessed via immunohistochemistry. As presented in Fig. 1a–f, galectin-3-immunoreactive cells were observed in various brain areas. In the gray matter (cerebral cortex), many morphologically typical pyramidal neurons (with large vesicular nuclei and prominent apical dendrites directed toward the surface of the cortex) were immunopositive for galectin-3. In the white matter (corpus callosum and internal capsule), many morphologically typical neuroglia (with small cell bodies and many long and slender radiating processes) were also immunopositive for galectin-3. Consistent with the findings of a previous report (Comte et al. 2011), ependymal cells lining the ventricles (especially the third ventricle) and the SVZ were also densely immunostained with galectin-3. No immunostaining was observed in sections incubated without primary antibodies (data not shown).

Next, double immunofluorescence experiments were performed to verify that the labeling with galectin-3 was indeed specific for neurons or neuroglia. As presented in Fig. 1g, most of the galectin-3-immunoreactive cells in the gray matter were co-localized with NeuN. In contrast, in the white matter, the majority of the galectin-3-immunoreactive cells were not co-localized with any neuroglia markers used in the present study (Fig. 1h–j, GFAP for astrocytes; Iba1 for microglia; Rip for oligodendrocytes).

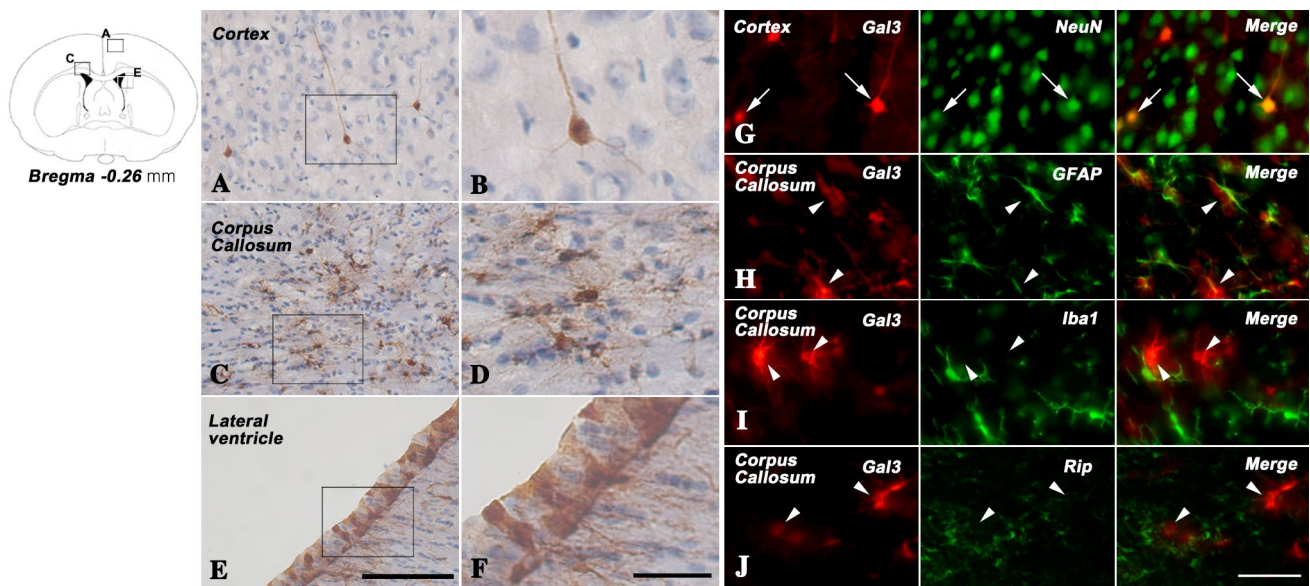


Fig. 1 Identification of cell types that express galectin-3 in the adult normal rat brain. Regions corresponding to pictures are depicted in coronal diagrams taken from the adult rat brain atlas (Paxinos and Watson 1998). **a** In the cerebral cortex, many pyramidal neurons are stained with galectin-3. **b** A higher-power image from *panel a* shows a large vesicular nucleus and prominent apical dendrites, which are typical features of pyramidal neurons. **c** In the corpus callosum, many neuroglial cells exhibited immunoreactivity for galectin-3. **d** A higher-power image from *c* showing a relatively small cell body and many slender radiating processes, which are typical features of

neuroglial morphology. **e** In the ventricular wall, ependymal cells are strongly stained with galectin-3. **f** A higher-power image from *e*. **g–j** Double immunofluorescence images of galectin-3/NeuN (**g**), galectin-3/GFAP (**h**), galectin-3/Iba1 (**i**), and galectin-3/Rip (**j**) in the corpus callosum. Most of galectin-3-immunoreactive cells in the cerebral cortex are co-localized with NeuN (*arrows in g*). However, in the *white matter* (corpus callosum), galectin-3-immunoreactive cells scarcely co-localized with neuroglia markers used in the present study (*arrowheads in h–j*). *Scale bars in e, f, and j* represent 100, 30, and 50 μm , respectively

Occasionally, only a few cells expressing GFAP or Iba1 were co-localized with galectin-3 (Supplementary Figure 1).

Regional distribution of neuronal galectin-3 immunoreactivity

Telencephalon

The distribution of galectin-3 immunoreactivity in the telencephalon is presented in Table 2. The cerebral cortex exhibited strong immunoreactivity for galectin-3 with variations in the laminar distribution. In the primary and secondary motor cortex, galectin-3 immunoreactivity was mainly distributed in cell bodies and apical dendrites in layers III–VI (Fig. 2a, b, i, j). A similar pattern of galectin-3 expression was observed in the somatosensory (Fig. 2e, f, Supplementary Figure 2E, F), insular (Fig. 2c, d), visual (Supplementary Figure 2C, D), temporal association (Supplementary Figure 2A, B), parietal association (Supplementary Figure 2G, H), and auditory cortices (Fig. 2g, h). However, in the cingulate, frontal, orbital, entorhinal, and rhinal cortices as well as the claustrum, galectin-3 immunostaining was barely detectable (data not shown).

The olfactory region, the anterior olfactory area (especially the lateral region) (Fig. 2m, n), and the rostral migratory stream (RMS; Fig. 2o, p) exhibited strong galectin-3 immunoreactivity. However, the main bulb, olfactory tubercle, and piriform cortex did not (data not shown). The hippocampal formation (CA1, CA2, CA3, dentate gyrus, and subiculum) also showed negative reaction against galectin-3 anti-sera.

The medial amygdaloid nucleus (Fig. 2k, l), central amygdaloid nucleus (Supplementary Figure 2K, L), and bed nucleus of the stria terminalis (Fig. 2q, r) contained neurons with prominent galectin-3 expression, but no significant expression was observed in the anteroventral, basolateral, or intercalated amygdaloid nuclei (data not shown). Scattered cells with moderate to sparse labeling of galectin-3 were observed in the basal forebrain regions, including the substantia innominata (Supplementary Figure 2I, J), the nucleus of the horizontal limb of the diagonal band of Broca (Fig. 2s, t), and the lateral limb of the diagonal band of Broca (data not shown). Other septal nuclei (lateral/medial septal nucleus, ventral limb of the diagonal band of Broca, and island of Calleja) and the basal ganglia (caudoputamen, globus pallidus, and nucleus accumbens) were

Table 2 Distribution of galectin-3-immunoreactive neuronal cells in the telencephalon of the rat brain

Subdivision	Abb.	Region	Immunore-activity	Figures
Cortex	M1	Primary motor cortex	+	Figure 2a, b
	M2	Secondary motor cortex	+	Figure 2i, j
	S1	Primary somatosensory cortex	+	Supplementary Figure 2E, F
	S2	Secondary somatosensory cortex	+	Figure 2e, f
	Au	Auditory cortex	+	Figure 2g, h
	Ins	Insular cortex	++	Figure 2c, d
	TmAC	Temporal association cortex	++	Supplementary Figure 2A, B
	V1	Primary visual cortex	+	Supplementary Figure 2C, D
	V2	Secondary visual cortex	+	Data not shown
	PaAC	Parietal association cortex	+	Supplementary Figure 2G, H
	Rh	Rhinal cortex	+/-	Data not shown
	Orb	Orbital cortex	-	Data not shown
	Fr	Frontal cortex	-	Data not shown
	Cl	Clastrum	-	Data not shown
	Cin	Cingulate cortex	-	Data not shown
	EnRh	Entorhinal cortex	-	Data not shown
	Olfactory	MOI	Main bulb	-
Tu		Olfactory tubercle	-	Data not shown
AOL		Anterior olfactory area, lateral	++	Figure 2m, n
RMS		Rostral migratory stream	++	Figure 2o, p
Pir		Piriform cortex	-	Data not shown
Amygdala and extended amygdala	AmyAv	Anteroventral amygdaloid area	-	Data not shown
	AmyMe	Medial amygdaloid nucleus	++	Figure 2k, l
	AmyIc	Intercalated nucleus of amygdala	-	Data not shown
	AmyBl	Basolateral amygdaloid nucleus	-	Data not shown
	AmyCe	Central amygdaloid nucleus	++	Supplementary Figure 2K, L
Hippocampal formation	STL	Bed nucleus of the stria terminalis	++	Figure 2q, r
	CA1-CA3	CA1-CA3	-	Data not shown
	DG	Dentate gyrus	-	Data not shown
Basal ganglia and septum	Sub	Subiculum	-	Data not shown
	Cpu	Caudate putamen	-	Data not shown
	GP	Globus pallidus	-	Data not shown
	Accb	Nucleus accumbens	-	Data not shown
	Sep	Lateral/medial septal nucleus	-	Data not shown
	HDB	Horizontal limb of diagonal band of Broca	++	Figure 2s, t
	VDB	Ventral limb of diagonal band of Broca	-	Data not shown
	LDB	Lateral limb of diagonal band of Broca	+	Data not shown
	ACj	Island of Calleja	-	Data not shown
	SIB	Substantia innominata	+	Supplementary Figure 2I, J
VOLT	Vascular organ of the lamina terminalis	+++	Supplementary Figure 2M, N	

not immunolabeled with galectin-3 (data not shown). Meanwhile, the vascular organ of the lamina terminalis (VOLT) exhibited very strong galectin-3 immunoreactivity (Supplementary Figure 2M, N).

Diencephalon

The distribution of galectin-3 immunoreactivity in the diencephalon is presented in Table 3. In the thalamus,

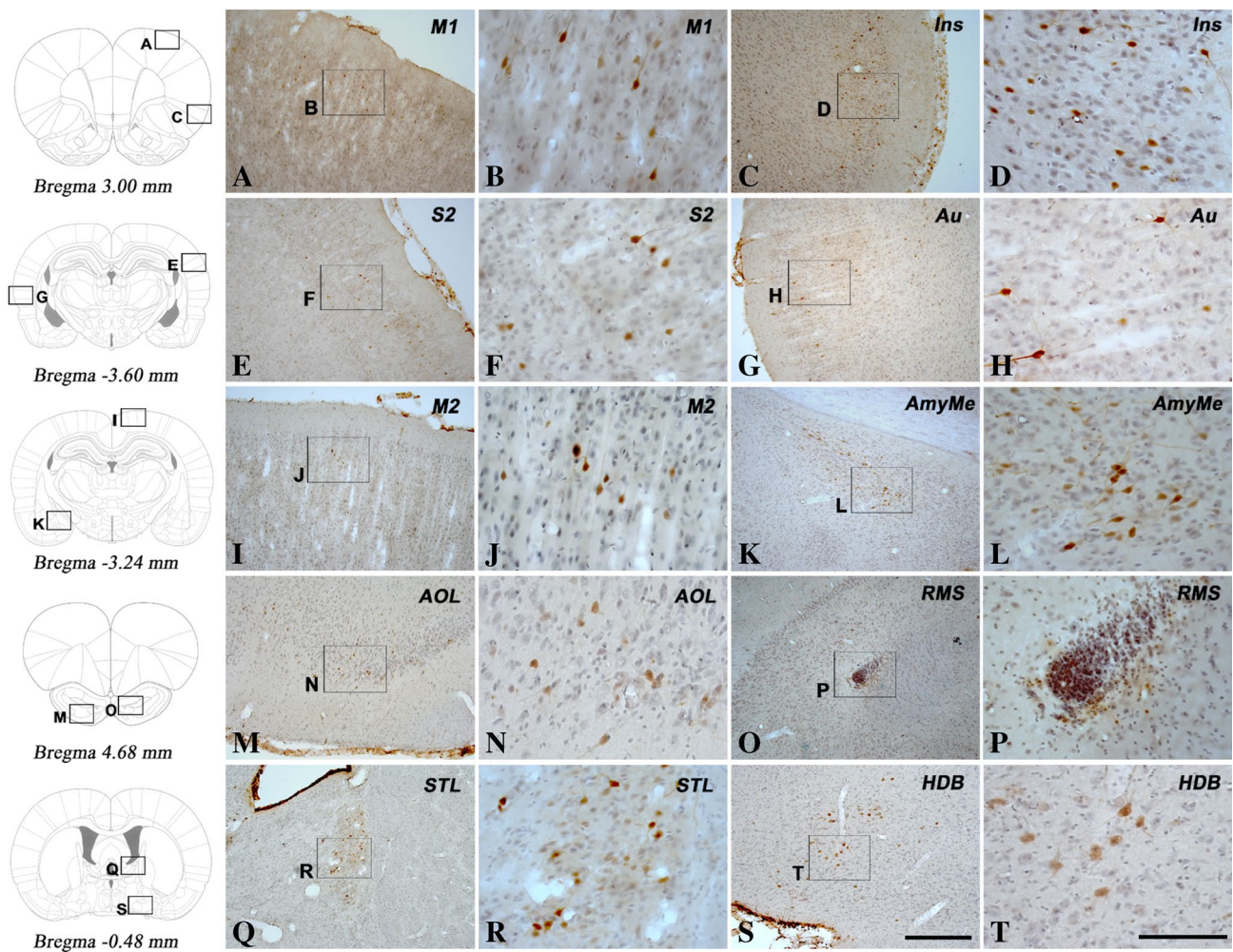


Fig. 2 Neuroanatomical distribution of galectin-3 immunoreactivity in the telencephalon. Regions corresponding to pictures are depicted in coronal diagrams taken from the adult rat brain atlas (Paxinos and Watson 1998). In the cerebral cortex, galectin-3 immunoreactivity is mainly distributed in neurons within layers III–IV of M1 (a and b), M2 (i and j), Ins (c and d), S2 (e and f), and Au (g and h). In the olfactory region, the AOL (m and n) and RMS (o and p) exhibit significant expression of galectin-3. Furthermore, AmyMe (k and l),

STL (q and r), and HDB (s and t) also exhibit moderate immunoreactivity for galectin-3. Scale bars in s and t represent 300 and 100 μm , respectively. M1 primary motor cortex; Ins insular cortex; S2 secondary somatosensory cortex; Au auditory cortex; M2 secondary motor cortex; AmyMe medial amygdaloid nucleus; AOL anterior olfactory area; RMS rostral migratory stream; STL bed nucleus of stria terminalis; HDB horizontal limb of the diagonal band of Broca

galectin-3 immunostaining was highly concentrated in the paraventricular thalamic nucleus (Supplementary Figure 3I, J). However, most parts of neurons in other thalamic nuclei showed no galectin-3 immunoreactivity, except for only a few neurons in the anteroventral thalamic nucleus (Supplementary Figure 3A, B), rhomboid thalamic nucleus (Fig. 3c, d), medial geniculate nucleus (Fig. 4g, h), reticular thalamic nucleus (data not shown), and zona incerta (Fig. 3a, b).

In the hypothalamus, many galectin-3-immunoreactive neurons were found in multiple hypothalamic midline neurons, including the dorsomedial hypothalamic nucleus (Fig. 3e, f), arcuate hypothalamic nucleus (Fig. 3g, h), ventral hypothalamic nucleus (Supplementary Figure 3E,

F), paraventricular hypothalamic nucleus (Supplementary Figure 3C, D), and supraoptic nucleus (Fig. 3k, l). Moderately-labeled neuronal cells were also detected in the medial preoptic area (Fig. 3i, j), suprachiasmatic nucleus (Supplementary Figure 3K, L), ventral tuberomammillary hypothalamic nucleus (Supplementary Figure 3M, N), medial tuberal nucleus (Fig. 3m, n), tuberal region of the lateral hypothalamic nucleus (Supplementary Figure 3G, H), and perifornal part of the lateral hypothalamic nucleus (Fig. 3o, p). Other hypothalamic nuclei, including those in the anterior hypothalamic area, lateral preoptic area, paraventricular hypothalamic nucleus, lateral hypothalamic area, posterior hypothalamic area, and mammillary nucleus were not immunostained with galectin-3 (data not shown).

Table 3 Distribution of galectin-3-immunoreactive neuronal cells in the diencephalon of rat brain

Subdivision	Abb.	Region	Immunoreac-tivity	Figures	
Thalamus	AM	Anteroventral thalamic nucleus	+	Supplementary Figure 3A, B	
	AD	Anterodorsal thalamic nucleus	–	Data not shown	
	PV	Paraventricular thalamic nucleus	+++	Supplementary Figure 3I, J	
	Re	Reuniens thalamic nucleus	–	Data not shown	
	Rhom	Rhomboïd thalamic nucleus	+	Figure 3c, d	
	CL	Centrolateral thalamic nucleus	–	Data not shown	
	CM	Centromedial thalamic nucleus	–	Data not shown	
	PC	Paracentral thalamic nucleus	–	Data not shown	
	RetTh	Reticular thalamic nucleus	+/-	Data not shown	
	VP	Ventral posterior thalamic nucleus	–	Data not shown	
	VA	Ventral anterior thalamic nucleus	–	Data not shown	
	MGN	Medial geniculate nucleus	+	Figure 4g, h	
	LGN	Lateral geniculate nucleus	–	Data not shown	
	Hb	Habenular nucleus	–	Data not shown	
	ZI	Zona incerta	+	Figure 3a, b	
	STN	Subthalamic nucleus	–	Data not shown	
	Hypothalamus	AH	Anterior hypothalamic area	–	Data not shown
		MPO	Medial preoptic area	+	Figure 3i, j
		LPO	Lateral preoptic area	–	Data not shown
		SCH	Suprachiasmatic nucleus	+	Supplementary Figure 3K, L
SO		Supraoptic nucleus	++	Figure 3k, l	
PaH		Paraventricular hypothalamic nucleus	++	Supplementary Figure 3C, D	
PeH		Periventricular hypothalamic nucleus	–	Data not shown	
Arc		Arcuate hypothalamic nucleus	+++	Figure 3g, h	
DMH		Dorsomedial hypothalamic nucleus	+++	Figure 3e, f	
VMH		Ventromedial hypothalamic nucleus	++	Supplementary Figure 3E, F	
VTMH		Ventral tuberomammillary hypothalamic nucleus	+	Supplementary Figure 3M, N	
MTu		Medial tuberal nucleus	+	Figure 3m, n	
TuLH		Tuberal region of the lateral hypothalamic nucleus	+	Supplementary Figure 3G, H	
PeFLH		Perifrontal part of the lateral hypothalamic nucleus	+	Figure 3o, p	
LH		Lateral hypothalamic area	–	Data not shown	
PH	Posterior hypothalamic area	–	Data not shown		
Ma	Mammillary nucleus	–	Data not shown		

Brain stem and cerebellum

The distribution of galectin-3 immunoreactivity in the brainstem and cerebellum is presented in Table 4. In the mesencephalon, the central nucleus of the inferior colliculus (Fig. 4a, b) and the lateral parabrachial nucleus (Fig. 4c, d) exhibited intense galectin-3 immunoreactivity. A few weakly labeled perikarya were apparent in the subbrachial nucleus (Fig. 4e, f), Edinger–Westphal nucleus (Supplementary Figure 4K, L), and mesencephalic trigeminal nucleus (Supplementary Figure 4G, H). However, neurons of the substantia nigra (pars compacta, reticulata, and lateralis), ventral tegmental area, red nucleus, superior colliculus, deep mesencephalic nucleus, oculomotor nucleus, and

trochlear nucleus were not immunolabeled with galectin-3 (data not shown).

In the rhombencephalon, high levels of galectin-3 immunoreactivity were observed in the neurons of the pontine nucleus (Fig. 4i, j) and cochlear nucleus (Fig. 4m, n). Moderately-labeled neuronal cells were also detected in the ventral tegmental nucleus (Fig. 4k, l), NA5 norepinephrine cells (Fig. 4o, p), motor trigeminal nucleus (Supplementary Figure 4I, J), and reticulotegmental nucleus (Supplementary Figure 4M, N). No expression of galectin-3 was detected in the locus coeruleus, A7 norepinephrine cells, pontine raphe nucleus, vestibular nucleus, principal sensory trigeminal nucleus, or facial motor nucleus (data not shown). The cerebellum, including the neurons composing

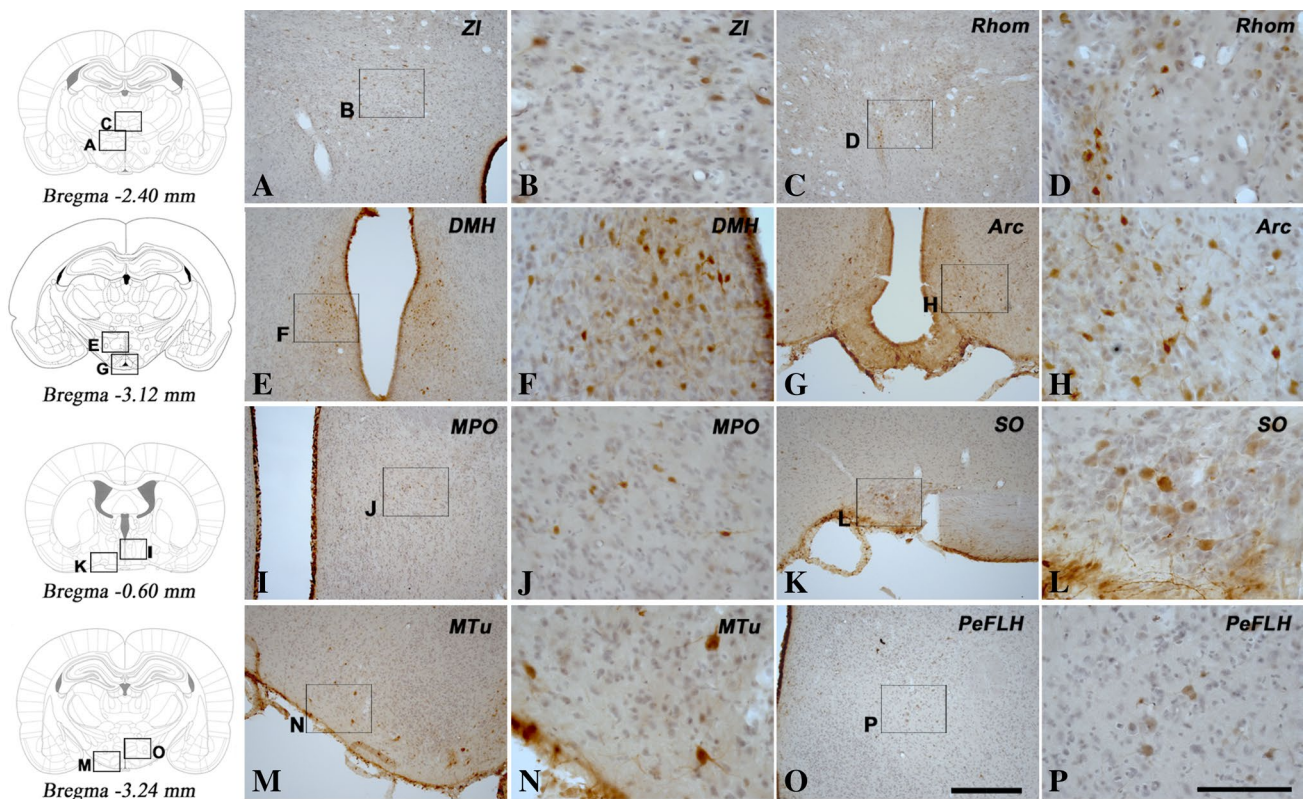


Fig. 3 Neuroanatomical distribution of galectin-3 immunoreactivity in the diencephalon. Regions corresponding to pictures are depicted in coronal diagrams taken from the adult rat brain atlas (Paxinos and Watson 1998). In the thalamus, ZI (a and b) and Rhom (c and d) exhibit weak galectin-3 immunoreactivity. In contrast, many hypothalamic nuclei, including the DMH (e and f), Arc (g and h) and SO (k and l), exhibit moderate-to-strong galectin-3 immunoreactivity.

Moreover, the MPO (i and j), MTu (m and n), and PeFLH (o and p) exhibit weak-to-moderate expression of galectin-3. Scale bars in s and t represent 300 and 100 μm , respectively. ZI zona incerta; Rhom rhomboid thalamic nucleus; DMH dorsomedial hypothalamic nucleus; Arc arcuate hypothalamic nucleus; MPO medial preoptic area; SO supraoptic nucleus; MTu medial tuberal nucleus; PeFLH perifornical part of the lateral hypothalamic nucleus

the cerebellar cortex and deep cerebellar nuclei, also exhibited no expression of galectin-3 (data not shown). However, the fibers composing cerebellar peduncles showed high levels of galectin-3 immunoreactivity (data not shown).

In the myelencephalon, neurons in the solitary nucleus (Fig. 4q, r), superior salivatory nucleus (Fig. 4s, t), vagal motor nucleus (Supplementary Figure 4A, B), hypoglossal nucleus (Supplementary Figure 4E, F), spinal trigeminal caudal nucleus (Supplementary Figure 4C, D), and cuneate nucleus (Supplementary Figure 4O, P) expressed high levels of galectin-3 in their perikarya. Areas of the lateral reticular nucleus, matrix region of the medulla, and area postrema contained scattered cells with low levels of galectin-3 expression (data not shown).

Discussion

Galectin-3 was originally identified as a macrophage-associated molecule, referred to as Mac-2 (Ho and

Springer 1982). Although discovered first in macrophages, this protein has been suggested to modulate a wide variety of biological processes, such as cell adhesion, tumor growth, cell cycling, cell signaling, and immune surveillance (Dumic et al. 2006; Yang et al. 2008). The general expression of galectin-3 in normal tissues is relatively well described. Briefly, galectin-3 is constitutively expressed under normal conditions in a variety of cell types in the kidneys, lungs, intestines, and the male and female reproductive organs (Kim et al. 2007), as well as the skin (Larsen et al. 2011). However, the expression of galectin-3 in the normal brain was underestimated because it was detected less strongly when compared with other organs using Western blotting (Kim et al. 2007). In the present study, we investigated the neurochemical profiles of galectin-3-synthesizing cells in the CNS and provided a detailed map of galectin-3 expression throughout the adult normal rat brain, which revealed that galectin-3 is expressed in a variety of cell types throughout the rat brain.

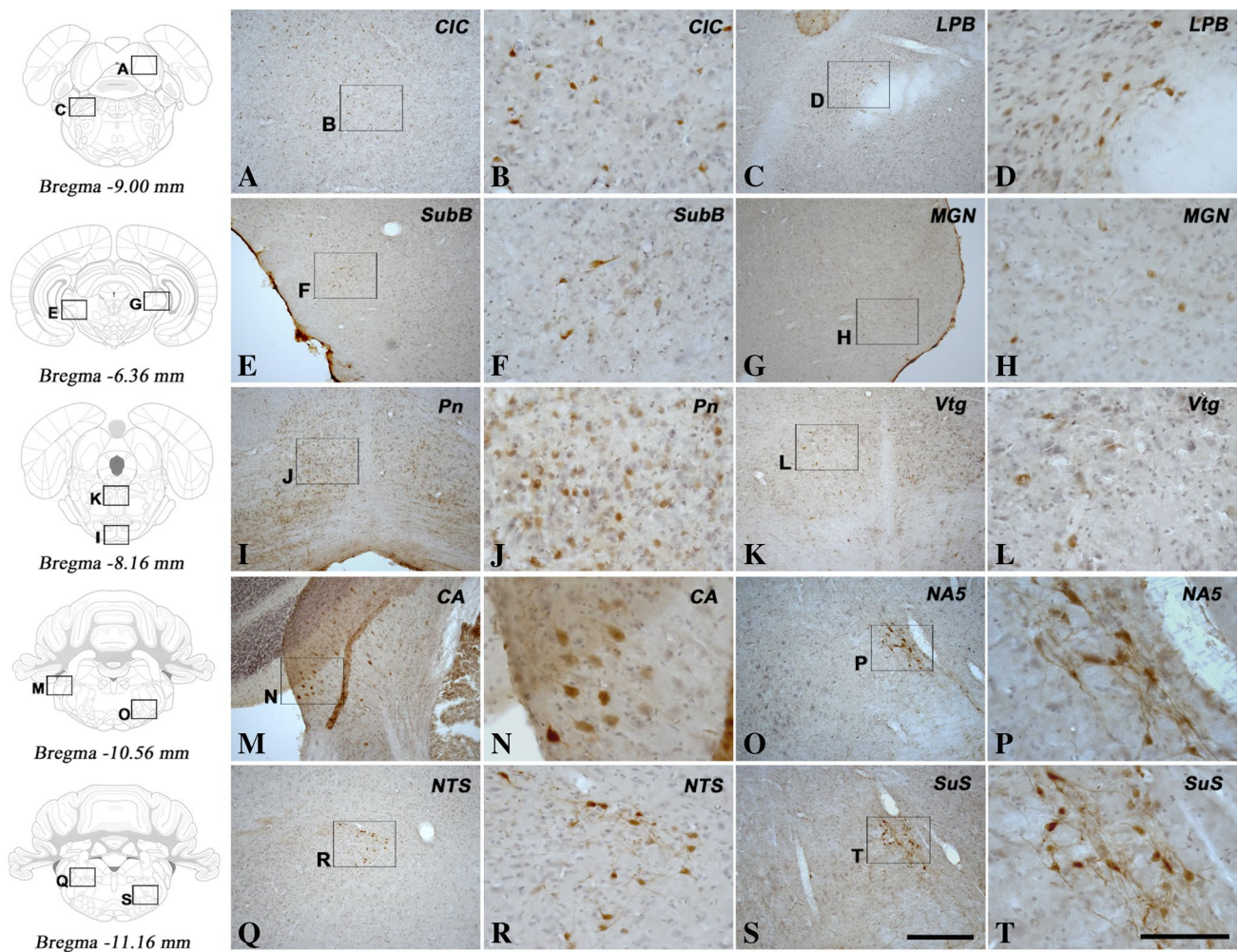


Fig. 4 Neuroanatomical distribution of galectin-3 immunoreactivity in the brainstem. Regions corresponding to pictures are depicted in coronal diagrams taken from the adult rat brain atlas (Paxinos and Watson 1998). In the mesencephalon, the CIC (a and b) and LPB (c and d) exhibit moderate to intense galectin-3 immunoreactivity, while, in the SubB (e and f) and MGN of the thalamus (g and h), only a few galectin-3-immunoreactive neurons are found. In the rhombencephalon, high levels of galectin-3-immunoreactive neurons are present in the Pn (i and j) and CA (m and n), while weak-to-moderate

galectin-3-immunoreactivity is observed in the Vtg (k and l) and NA5 (o and p). In the myelencephalon, neurons in the NTS (q and r) and SuS (s and t) express galectin-3. Scale bars in s and t represent 300 and 100 μ m, respectively. CIC central nucleus of inferior colliculus; LPB lateral parabrachial nucleus; SubB subbrachial nucleus; MGN medial geniculate nucleus; Pn pontine nucleus; Vtg ventral tegmental nucleus; CA cochlear nucleus; NA5 A5 norepinephrine cells; NTS nucleus of the solitary tract; SuS superior salivatory nucleus

As presented in Fig. 1, most of the galectin-3-immunoreactive cells in the gray matter were co-localized with NeuN. This indicates that, in the gray matter, galectin-3 is expressed mainly in neurons. This finding of galectin-3 expression in neurons is somewhat puzzling since previous studies have reported that galectin-3 expression did not occur in neurons (for a review, Shin 2013), except for one study that reported the expression of galectin-3 in subsets of dorsal root ganglion (DRG) neurons (Pesheva et al. 2000). This disparity is not readily understood, but may be related to differences in experimental conditions and antibodies used. Moreover, the study of galectin-3 in neurons under normal conditions has largely been neglected

because previous studies placed emphasis on elucidating the role of galectin-3 in the neuroglia (especially microglia) under pathological conditions (Wesley et al. 2013; Hisamatsu et al. 2016).

In the white matter, galectin-3-immunoreactive neuroglial cells and their fibers were also detected in various regions, including the internal capsule, corpus callosum, optic tract, alveus, and cerebellar peduncle. The morphological characteristics (small cell body and many long and slender radiating processes) of galectin-3-immunoreactive cells in the white matter strongly suggest that they are neuroglia. However, double labeling of galectin-3 with various glial markers (GFAP for astrocytes, Iba1 for microglia,

Table 4 Distribution of galectin-3-immunoreactive neuronal cells in the brainstem and cerebellum of the rat brain

Subdivision	Abb.	Region	Immunoreactivity	Figures	
Mesencephalon	SN	Substantia nigra	–	Data not shown	
	VTA	Ventral tegmental area	–	Data not shown	
	R	Red nucleus	–	Data not shown	
	RRF	Retrorubral field	–	Data not shown	
	SC	Superior colliculus	–	Data not shown	
	CSC	Commissure of superior colliculus	–	Data not shown	
	ECIC	External cortex of inferior colliculus	–	Data not shown	
	CIC	Central nucleus of inferior colliculus	+++	Figure 4a, b	
	LPB	Lateral parabrachial nucleus	++	Figure 4c, d	
	SubB	Subbrachial nucleus	+	Figure 4e, f	
	EW	Edinger–Westphal nucleus	+	Supplementary Figure 4K, L	
	Me5	Mesencephalic trigeminal nucleus	+	Supplementary Figure 4G, H	
	3 N	Oculomotor nucleus	–	Data not shown	
	4 N	Trochlear nucleus	–	Data not shown	
Rhombencephalon	LC	Locus coeruleus	–	Data not shown	
	A7	A7 norepinephrine cells	–	Data not shown	
	RtTg	Reticulotegmental nucleus	+	Supplementary Figure 4M, N	
	PnR	Pontine raphe nucleus	–	Data not shown	
	Vtg	Ventral tegmental nucleus	+	Figure 4k, l	
	Pn	Pontine nucleus	+++	Figure 4i, j	
	NA5	A5 norepinephrine cells	++	Figure 4o, p	
	Ve	Vestibular nucleus	–	Data not shown	
	CA	Cochlear nucleus	+++	Figure 4m, n	
	5MT	Motor trigeminal nucleus	+/-	Supplementary Figure 4I, J	
	Pr5	Principal sensory trigeminal nucleus	–	Data not shown	
	7 N	Facial motor nucleus	–	Data not shown	
	Myelencephalon	NTS	Solitary nucleus	+++	Figure 4q, r
		SuS	Superior salivary nucleus	++	Figure 4s, t
Sp5c		Spinal trigeminal nucleus, caudalis	++	Supplementary Figure 4C, D	
Cu		Cuneate nucleus	++	Supplementary Figure 4O, P	
Gr		Gracile nucleus	–	Data not shown	
LRt		Lateral reticular nucleus	+/-	Data not shown	
10 N		Vagal motor nucleus	++	Supplementary Figure 4A, B	
12 N		Hypoglossal nucleus	++	Supplementary Figure 4E, F	
Mx		Matrix region of the medulla	+/-	Data not shown	
AP		Area postrema	+/-	Data not shown	
Cerebellum	Cbll	Cerebellar cortex	–	Data not shown	
	CbllDp	Deep cerebellar nuclei	–	Data not shown	

and Rip for oligodendrocytes) was barely matched (Fig. 1), except in a small number of GFAP or Iba-1 immunopositive cells (Supplementary Figure 1). Since morphology is an unreliable way of classifying cells, neuroglia have come to be defined by their high content of cytoplasmic intermediate filaments, such as GFAP or Iba1 (Kessaris et al. 2008). Though these markers have been most widely used to distinguish specific neuroglia from other glial cells in the CNS, they are not able to discern all types of neuroglial cells definitively (Kessaris et al. 2008). For example, there

are cells with a protoplasmic astrocyte-like morphology that do not normally express GFAP (Scotti 2003). Therefore, the galectin-3 expressing cells not matched with glial markers used in the present study may represent other types of neuroglia that cannot be detected with routine commercial neuroglial markers. Although this is an interesting research topic to examine, we focused on the anatomical distribution of galectin-3-immunoreactive neurons.

We observed extensive galectin-3 immunoreactivity in many functional parts of the cerebral cortex. In particular,

pyramidal and granular neurons in layers III–VI of the cerebral cortex exhibited considerable galectin-3 immunoreactivity (Fig. 2, Supplementary Figure 2). However, the functional limbic areas, including the rhinal, entorhinal, and cingulate cortices, were negative for galectin-3. In accordance with this finding, the neurons comprising limbic system, major parts of the olfactory areas (main bulb and olfactory tubercle), and the hippocampal formation (CA1, CA2, CA3, and dentate gyrus), also exhibited no expression of galectin-3. Meanwhile, the RMS (Fig. 2o, p) and anterior olfactory area (Fig. 2m, n) in the olfactory region exhibited strong galectin-3 immunoreactivity. The RMS is a specialized migratory channel for neuroblasts originating from the SVZ to travel to the olfactory bulb, where they differentiate into granular and periglomerular interneurons (Luskin 1993). As presented in Figs. 1e, f and 2o, p, strong galectin-3 immunoreactivity was observed in the ependyma, SVZ, and RMS. These results are in line with previously published findings, which have suggested a potential role for galectin-3 in the modulation of neuroblast motility via the RMS (Storan et al. 2004; Comte et al. 2011). However, it remains unclear why only the neurons of the anterior area of the olfactory region express galectin-3.

The reactivity of galectin-3 in the amygdala, an important limbic structure, was different depending on nuclei. The medial amygdaloid nuclei (Fig. 2k, l), central amygdaloid nuclei (Supplementary Figure 2O, P), and bed nuclei of the stria terminalis (Fig. 2q, r) exhibited intense galectin-3 immunoreactivity, though other amygdaloid nuclei did not. The central and medial amygdaloid nuclei and the bed nuclei of the stria terminalis project their fibers primarily through the stria terminalis to many parts of the hypothalamus and brain stem, where they act to influence hormonal and somatomotor aspects of behavior (Shimogawa et al. 2015). As discussed later, many nuclei of the hypothalamus and brain stem were also positive for galectin-3, suggesting a correlation between the nuclei that interconnect the amygdaloid nuclei with the hypothalamus (or brain stem) and galectin-3 expression. This hypothesis is further supported by the observation that the anterolateral and basolateral amygdaloid nuclei, which have closed triangular connectivity with the medial prefrontal cortex and mediodorsal thalamic nuclei in rats (Matyas et al. 2014), are all galectin-3-negative. Taken together, these findings indicate that regions of the amygdala connected with hypothalamus (galectin-3 positive area) may exhibit galectin-3 immunoreactivity, while regions connected with the prefrontal cortex and thalamus (galectin-3 negative area) may not.

The subcortical nuclei responsible for the control of voluntary motor functions (caudoputamen, globus pallidus, and nucleus accumbens) and their interconnected brain areas (substantia nigra and subthalamic nuclei) exhibited no galectin-3 immunoreactivity. However, dense galectin-3

staining was observed in some septal nuclei, including the horizontal limb of the diagonal band of Broca (Fig. 2s, t), lateral limb of the diagonal band of Broca (data not shown), and substantia innominata (Supplementary Figure 2I, J). These nuclei are basal forebrain cholinergic neurons that mainly send their signals to broad cortical areas to promote cortical arousal and activation (Rieck et al. 1995). They also play an important role in both cortical development and adult neurogenesis (Bruehl-Jungerman et al. 2011). If the neural connections are actually associated with galectin-3 immunoreactivity, this may explain the broad presence of galectin-3-immunoreactive neurons in the cortical areas.

Our galectin-3 immunohistochemistry analysis revealed that galectin-3 immunoreactivity was widely distributed in many nuclei of the diencephalon, especially the hypothalamus, and the brain stem. The hypothalamus is the predominant brain center for regulating the activity of the autonomic nervous system (ANS) and neuroendocrine system. High levels of galectin-3 expression have been observed in the dorsomedial hypothalamic nucleus (DMH; Fig. 3e, f) and the ventromedial hypothalamic nucleus (VMH; Supplementary Figure 3E, F), which are the main centers for regulating feeding behavior. Lesion studies have shown that destruction of the DMH (Bernardis and Bellinger 1998) or VMH (Shimizu et al. 1987) leads to hyperphagia, demonstrating that these regions may act as “satiety” centers. In addition, significant expression of galectin-3 was detected in the arcuate hypothalamic nucleus (Fig. 3g, h), supraoptic nucleus (Fig. 3k, l), and paraventricular hypothalamic nucleus in the present study (Supplementary Figure 3C, D). These nuclei are the main components of the hypothalamic–pituitary–adrenal (HPA) axis, which involves a wide array of ANS and neuroendocrine systems (Heinrichs and Koob 2004). Moreover, many brain regions associated with the HPA axis also exhibited intense galectin-3 immunoreactivity. For example, dense galectin-3 immunostaining was observed in the neurons of the nucleus of the solitary tract (Nucleus tractus solitarius, NTS; Fig. 4q, r) and VOLT (Supplementary Figure 2M–N). The NTS relays sensory information to the paraventricular hypothalamic nucleus from the cranial nerves that innervate large parts of visceral organs (King et al. 2012). The VOLT is one of the circumventricular organs of the brain and is strongly interconnected with the medial preoptic nucleus (Camacho and Phillips 1981). As discussed earlier, the medial and central amygdaloid nuclei, which are thought to activate the HPA axis (Petrovich and Swanson 1997; Dong et al. 2001), also exhibited strong galectin-3 expression. The lateral parabrachial nucleus (Fig. 4c, d), which is densely interconnected with the central amygdaloid nucleus and the paraventricular thalamic nucleus (Supplementary Figure 3I, J), which in turn is interconnected with collateral projections from the

lateral parabrachial nucleus (Liang et al. 2016), also exhibited galectin-3 immunoreactivity. Therefore, galectin-3 expression seems to be correlated with neuroanatomically interconnected areas that regulate functions of the ANS and neuroendocrine system.

Many other brain regions associated with autonomic functions also exhibited galectin-3 immunoreactivity: Edinger–Westphal nucleus (Supplementary Figure 4K, L), A5 norepinephrine cells (Fig. 4o, p), superior salivatory nucleus (Fig. 4s, t), and vagal motor nucleus (Supplementary Figure 4A, B). The Edinger–Westphal nucleus is composed of two types of neuronal groups: parasympathetic preganglionic oculomotor neurons, which innervate the iris sphincter muscles and the ciliary muscles and non-preganglionic neuropeptide-containing neurons, which project to the spinal cord, hypothalamus, and central amygdaloid nucleus (Dos Santos Junior et al. 2015). Although it remains to be elucidated which types of neurons are immunoreactive for galectin-3, the observed neuroanatomical connections suggest that non-preganglionic neurons seem to express galectin-3. A5 norepinephrine cells act as sympathetic premotor neurons by innervating the intermediolateral cell column in the thoracic spinal cord and activating the sympatho-adrenal system (Mravec et al. 2012). The superior salivatory nucleus contains preganglionic parasympathetic neurons whose fibers project to the submandibular ganglion and pterygopalatine ganglion. The vagal motor nucleus is a cranial nerve nucleus that serves parasympathetic vagal functions in the thoracic and abdominal visceral organs.

It is worth noting that the major nuclei accepting information from the sensory receptors also exhibited galectin-3 immunoreactivity. Galectin-3 expression was observed in the nuclei of relaying auditory pathways [cochlear nucleus (Fig. 4m, n), central nucleus of inferior colliculus (Fig. 4a, b), medial geniculate nucleus (Fig. 4g, h)], and auditory cortex (Fig. 2g, h). These findings suggest a correlation between galectin-3 expression and auditory circuit formation. The observation of galectin-3 expression in the mesencephalic trigeminal nucleus (Supplementary Figure 4G, H) appears to diverge from the data published by Pesheva and colleagues, who observed galectin-3 expression in subsets of DRG neurons (Pesheva et al. 2000). Because mesencephalic trigeminal nucleus is the only structure in the CNS to contain the neurons of a primary afferent, which are usually contained within ganglion (trigeminal ganglion), galectin-3 expression in the spinal trigeminal nucleus shows regional differences. Galectin-3 was expressed in the pars caudalis but not in the pars oralis and pars interpolaris (Supplementary Figure 4C, D). The pars oralis is associated with the transmission of discriminative touch sensation, while the pars interpolaris is associated with the transmission of tactile sense. The pars caudalis is associated

with the transmission of nociception and thermal sensation. Therefore, galectin-3 expression seems to be associated with nociceptive transmission. This hypothesis is supported by the findings of a previous report, which demonstrated the expression of galectin-3 in DRG neurons projecting into laminae I and II of the dorsal horn in the spinal cord, where pain modulation occurs (Dodd and Jessell 1986). The cuneate nucleus and gracile nucleus are the main sensory relaying nuclei composing part of the posterior column-medial lemniscus pathway, carrying discriminative touch and conscious proprioceptive information from the upper (cuneate nucleus) and lower body (gracile nucleus). It is interesting that galectin-3 immunolabeling was observed in the cuneate nucleus (Supplementary Figure 4O, P) but not in the gracile nucleus. Some motor nuclei in the brain stem [faintly in the trigeminal motor nucleus (Supplementary Figure 4I, J) and intensely in the hypoglossal nucleus (Supplementary Figure 4E, F)] also expressed galectin-3.

Neurons in the cerebellar cortex and deep cerebellar nuclei showed complete negative immunoreactivity for galectin-3, while the fibers composing the cerebellar peduncles exhibited high levels of galectin-3 immunoreactivity (data not shown). This finding seems to be correlated with the pattern of intense galectin-3 expression observed in the pontine nucleus (Fig. 4i, j) and reticulotegmental nucleus (Supplementary Figure 4M, N). The pontine nucleus is the main motor relay center that receives information from a variety of cortical areas via corticopontine fibers, projecting to the contralateral cerebellum via the middle cerebellar peduncle. The reticulotegmental nucleus in the pons is known to be topographically related to the pontine nucleus, and so projects and receives reciprocal fibers with the cerebellum. Therefore, galectin-3 molecules expressed in the soma of neurons comprising the pontine nucleus and reticulotegmental nucleus can be transferred along their axons to the cerebellum, resulting in high levels of galectin-3 immunoreactivity in the cerebellar peduncles.

In conclusion, the aim of the present work was to provide substantial evidence of the cellular and neuroanatomical distribution of galectin-3 immunoreactivity in the rat brain. To our knowledge, the present study is the first to provide *in vivo* evidence that galectin-3 is constitutively expressed not only in neuroglial cells but also in many kinds of neurons. Various hypothalamic areas are involved in the regulation of a variety of physiological functions, such as anxiety, energy balance, and neuroendocrine regulation, express galectin-3. Furthermore, brain regions functionally and neuroanatomically related to these hypothalamic areas, such as the central and medial amygdaloid nuclei, VOLT, and NTS, also express galectin-3. Extensive galectin-3 expression was also observed in regions associated with ANS function (hypothalamic, sympathetic, and parasympathetic nuclei in the brain stem). The main

regions of the sensory centers also exhibited galectin-3 immunoreactivity. Although the functional consequences of these observations are unclear at present, they suggest that galectin-3 expression seems to be regulated by developmental cascades, and that functionally and neuroanatomically related nuclei seem to constitutively express galectin-3 in adulthood. Thus, further studies would be needed to elucidate why galectin-3 is expressed in several hypothalamic nuclei, how they interconnect with other nuclei, and what mechanisms act on these galectin-3 immunopositive neurons.

Acknowledgements Grant sponsor: Institute for Information & Communications Technology Promotion (IITP) Grant funded by the Korean government (MSIP) (No. B01321510010003003, Next Imaging System XIS) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (NRF-2015R1D1A3A03020164). We would like to thank Editage (<http://www.editage.co.kr>) for English language editing.

Authors contribution All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: HIY, TKB and DYS. Acquisition of data: EGK, EJJ, SYH, and SJP. Analysis and interpretation of data: HIY and RSW. Drafting of the manuscript: MJH, CSY, and DYS. Critical revision of the manuscript for important intellectual content: TKB and RSW. Obtained funding: TKB and DYS. Study supervision: TKB and DYS.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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