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Expression and Localization of Brain Glutamate Dehydrogenase with Its Monoclonal Antibody

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Glutamate dehydrogenase (GDH) is one of the main enzymes involved in the formation and metabolism of the neurotransmitter glutamate. In the present study, we investigated the distribution of the GDH-immunoreactive cells in the rat brain using monoclonal antibodies against bovine brain GDH isoprotein. GDH-immunoreactive cell were distributed in the basal ganglia, thalamus and the nuclei belong to substantia innominata, and its connecting area, subthalamic nucleus, zona incerta, and substantia nigra. We could see GDH-immunoreactive cells in the hippocampus, septal nuclei associated with the limbic system, the anterior thalamic nuclei connecting between the hypothalamus and limbic system, and its associated structures, amygdaloid nuclear complex, the dorsal raphe and median raphe nuclei and the reticular formation of the midbrain. The GDH-immunoreactive cells were shown in the pyramidal neurons of the cerebral cortex, the Purkinje cells of the cerebella cortex, their associated structures, ventral thalamic nuclei and the reticular thalamic nuclei that seem to function as neural conduction in the thalamus.

Glutamate is a major excitatory neurotransmitter (Fonnum, 1984) and is also known to be the immediate precursor in the biosynthesis of γ-aminobutyric acid, a widely distributed inhibitory neurotransmitter. Due to its neurotoxic potentials, glutamate may be involved in the pathogenesis of human degenerative disorders (McGeer and McGeer, 1976; Plaitakis et al., 1982). One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH) (EC 1.4.1.3). GDHs are a family of enzymes which catalyze the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+, $NADP^+$ or both as coenzymes (Stillman et al., 1993). They are hexameric enzymes with a subunit molecular mass of 56,500 Da.

The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest. Four different forms of GDH isoproteins were detected from the human cerebellum of normal subjects and patients with neurodegenerative disorders (Duvoisin et al., 1983; Plaitakis et al., 1984; Hussain et al., 1989). The isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (Plaitakis et al., 1993). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed a marked reduction of one of the GDH isoproteins (Hussain et al., 1989). The origin of the GDH polymorphism is not known. It was reported that the presence of four differently sized mRNAs and multiple gene copies for GDH occur in the human brain (Plaitakis et al., 1993). A novel cDNA encoded by an X chromosome-linked intronless gene also has been isolated from human retina (Shashidharan et al., 1994).

Recently, we have isolated two types of glutamate dehydrogenase isoproteins (designated GDH I and GDH II) from bovine brain (Cho et al., 1995) and the regulatory properties of the GDH isoproteins have been reported (Cho et al., 1996; Cho and Lee, 1996; Kim et al., 1997; Lee et al., 1997). Our work led to the finding that GDH is present in bovine brain in "heat-labile (GDH I)" and "heat-stable (GDH II)" forms (Cho et al., 1995). Very recently, it has been reported that nerve tissue-specific human GDH is thermolabile and highly regulated by ADP (Shashidharan et al., 1997). It also has been reported that two the of GDH activities in rat brain differ in their relative resistance to thermal inactivation, detergent extractability, and allosteric regulation characteristics (Colon et al., 1986). To our knowledge, comparison of the detailed structure and functions of any GDH isoproteins rarely has been reported.

It would appear that we have just begun to unravel the mystery of GDH proteins and their role in neurobiology. It is, therefore, essential to have a detailed structural and functional description of the various types of brain GDH to elucidate the pathophysiological

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nature of GDH-deficient neurological disorders. In the present work, we investigated the distribution of GDH-immunoreactive cells in the rat brain using monoclonal antibodies against bovine brain GDH II.

Materials and Methods

Materials

The glutamate dehydrogenase isoprotein II (GDH II) was purified from bovine brain as described previously (Cho et al., 1995). Fetal bovine sera were purchased from Hyclone Lab. Other reagents, media, and antibiotics used for production of monoclonal antibodies were purchased from GIBCO-BRL. Goat anti-rabbit gamma globulin (GAR) and peroxidase- antiperoxidase (PAP) conjugates were used by Chemicon International Inc.. Diaminobenzidine (DAB) were purchased from Sigma and all other chemicals and solvents were reagent grade.

Production of monoclonal antibodies against GDH isoprotein

Monoclonal antibodies against GDH II were made according to the method of Choi et al. (1995). Purified GDH II was denatured by adding a final concentration of 0.1% SDS and by heat treatment for 1 min at 100 ℃. The denatured protein solution was mixed with an equal volume of complete Freund's adjuvant by sonication and injected into BALB/c mice (6-8 wk old). The first injection was followed by three booster injections at 3- to 4-wk intervals.

Three or four days after final injection, the spleen was obtained from the animal and cut into small pieces. Prepared spleen and Sp2/o-Ag-14 cell suspensions were combined and 1 ml of 50% polyethylene glycol 1500 in incomplete Dulbecco's modified Eagle's medium (DME) was added slowly. The fusion process was stopped by adding incomplete DME after 90 s and 20 ml of incomplete DME was then added slowly for a period of 10 min. Cells were collected by centrifugation for 1 min at 650 g, suspended carefully in 20 ml of HAT medium by swirling, and centrifuged for 1 min at 650 g. The cells were resuspended in 120 ml of HAT medium and the cell suspension was transferred into each well of 96-well plates. Two weeks after the fusion, cultured supernatants were collected and first screened by immuno dot blot analysis with purified enzyme, and then by Western blot analysis. Positive clones, selected by the screening methods, were grown in tissue culture flasks (75 cm^2) and frozen in a liquid nitrogen tank. All positive clones were frozen first and cloned by limiting dilution after thawing.

Tissue preparation for light microscope observation

Sprague-Dawley rats (150-180 gm) were anesthetized by ether and then perfused through the left ventricle with saline followed by 4% paraformaldehyde (0.1 M phosphate buffered saline, pH 7.4). The brain was removed from the skull and placed in a fresh fixative for 4 to 24 h at 4℃. Tissues were trimmed according to the atlas of Paxinos and Watson (1986). After being dehydrated and cleared, they were embedded in paraplast. Tissues were sectioned at 6 μm thickness, and placed on slides treated with gelatin. One tissue section was immunohistochemically stained for GDH by using the peroxidase-antiperoxidase (PAP) method of Sternberger (1986), and the other section was Nissl stained for counter staining.

Immunoperoxidase staining

All tissue sections were immunohistochemically stained with the GDH monoclonal antibody by using the peroxidase-antiperoxidase (PAP) method of Sternberger (1986). Before staining, tissues were pretreated in 3%

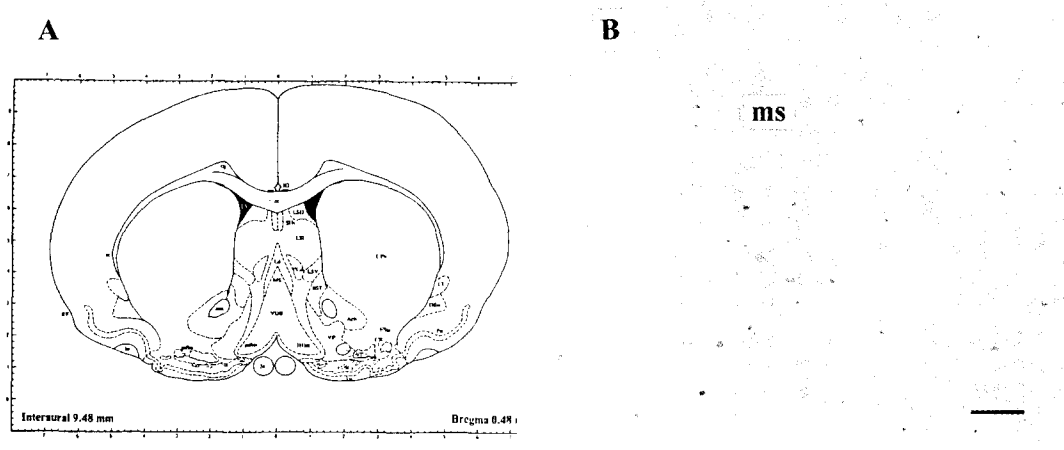

Fig. 1. Distribution of GDH in the rat brain. The section through 0.48 mm from the bregma and 9.48 mm from the interaural line. a, Schematic figure of the same section. b, We can see GDH-immunoreactive cells in the medial septal nucleus (ms). Scale bar=5 mm.

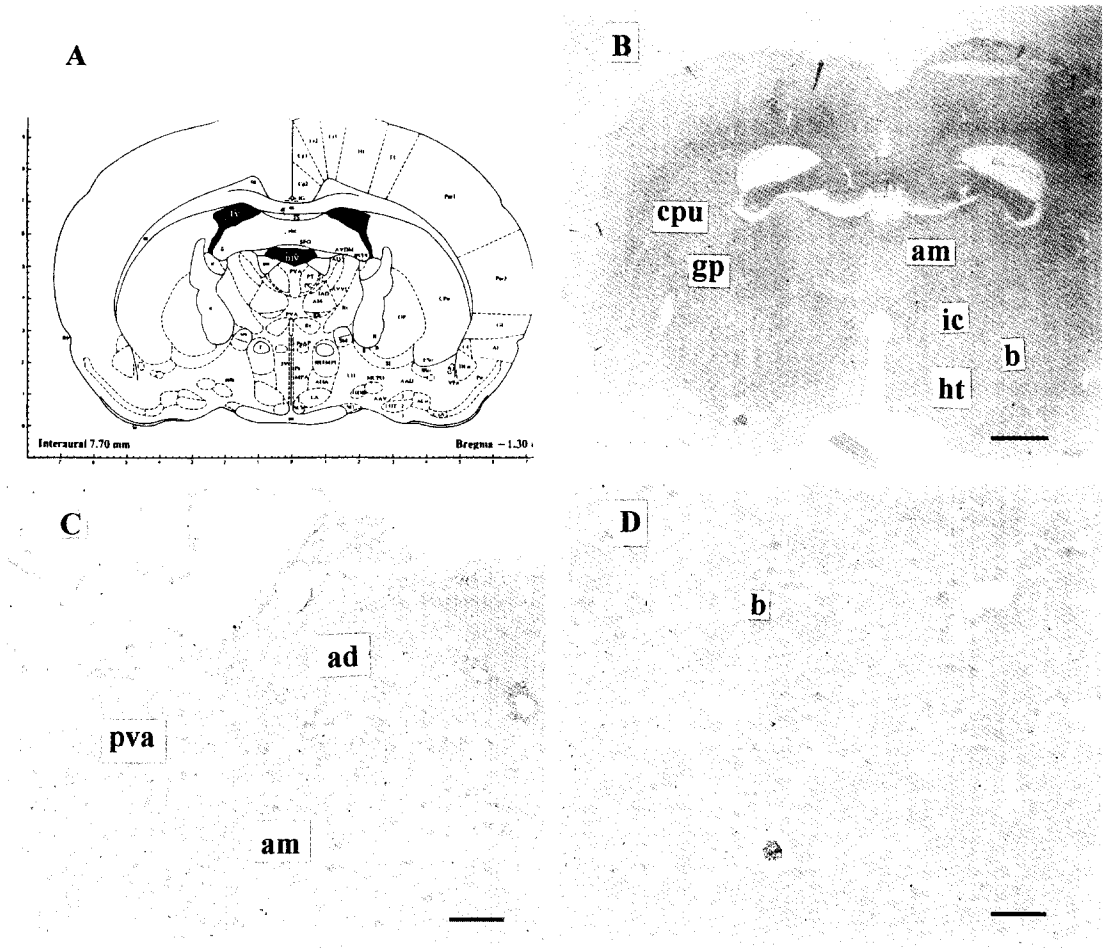

Fig. 2. The section through -1.30 mm from the bregma and 7.70 mm from the interaural line. A, Schematic figure of the same section. B, GDH-immunoreactive cells were observed in the caudateputamen (cpu), globus pallidus (gp), internal capsule (ic), hypothalamus (ht), basal nucleus of Mynert (b). C, Higher magnification micrographs show the GDH-immunoreactive cells in the paraventricular thalamic nucleus (pva), anterolateral thalamic nucleus (ad), anteromedial thalamic nucleus (am). D, In the basal nucleus of Mynert (b). Scale bars=0.1 mm (B) and 10 μm (C, D).

hydrogen peroxide solution for 10 min and several rinses in phosphate buffer(pH 7.4, containing 0.1% Triton X-100). Sections were placed in 20% normal goat serum (NGS; GIBCO-BRL) for 1 h. They were incubated in the same 0.1% Triton-PBS solution containing GDH monoclonal antibody at a dilution of 1:100 for 48 h at 4℃. Following several rinses in phosphate buffer(pH 7.4, containing 0.1% Triton X-100), the tissue was incubated with goat anti-rabbit gamma globulin (GAR, USA; Chemicon Inc.) at a dilution of 1:50 for 1 h at room temperature. The sections were rinsed several times with the same phosphate buffer and then placed in peroxidase-antiperoxidase (PAP) conjugates (Chemicon Inc.) at a dilution of 1:100 for 1 h at room temperature. Following several rinses in phosphate buffer, the sections were placed in 0.05% diaminobenzidine solution containing 0.01% hydrogen peroxide for 10 minutes. Controls were prepared using incubating solution without primary antibodies. All procedures were performed in a moisture chamber.

Results and Discussion

Glutamate dehydrogenase (GDH) is expressed at high levels in the mammalian brain (Smith et al., 1975). The exact function of GDH in the brain is not fully understood. Although thermodynamically the GDH reaction favors glutamate synthesis, aspartate aminotransferase may be more important than GDH in forming glutamate from α-ketoglutarate *in vivo* (Palaiologos et al., 1988; Christensen et al., 1991). On the other hand, Yu et al. (1982) suggested that GDH may play a role in glutamate oxidation in astrocytes.

The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest. Previous studies have shown that GDH activity is reduced in leukocytes and fibroblasts of patients with multisystemic neurological disorders (Aubby et al., 1988; Hussain et al., 1989; Abe et al., 1992). Molecular biological studies revealed that multiple GDH-specific genes are present in the

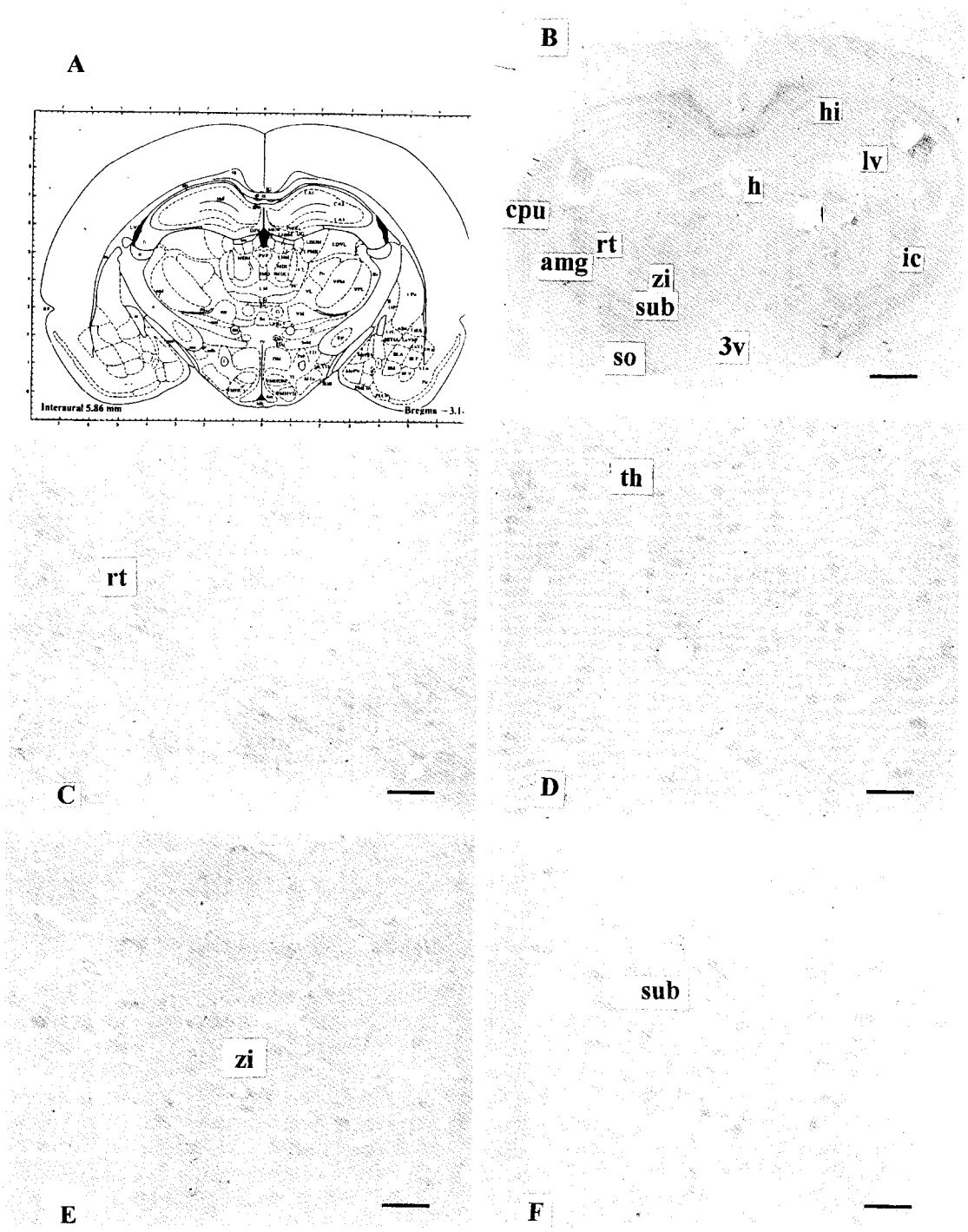

Fig. 3. The section through -3.14 mm from the bregma and 5.86 mm from interaural line. A, Schematic figure as above. B, GDH-immunoreactive cells were found in the hippocampal formation (hi), habenular nuclei (hi), thalamus (th), caudate putamen (cpu), reticular thalamic nuclei (rt), amygdaloid complex (amg), zona incerta (zl) and subthalamic nuclei (sub). C, Higher magnification micrograph of the reticular thalamic nucleus (rt). D, Higher magnification micrograph of the thalamus (th). E, The zona incerta (zl), F, The subthalamic nucleus (sub). Scale bars=5 μm (C-F) and 0.1 μm (B).

mammalian tissues. At least two of these genes are functional. The first one is an intron-containing gene and the mRNA of this gene is expressed in all tissues (Marvrothalassitis et al., 1988; Anagnou et al., 1993; Michaelidis et al., 1993). The second one is encoded by an X-linked intronless gene, of which the mRNA is

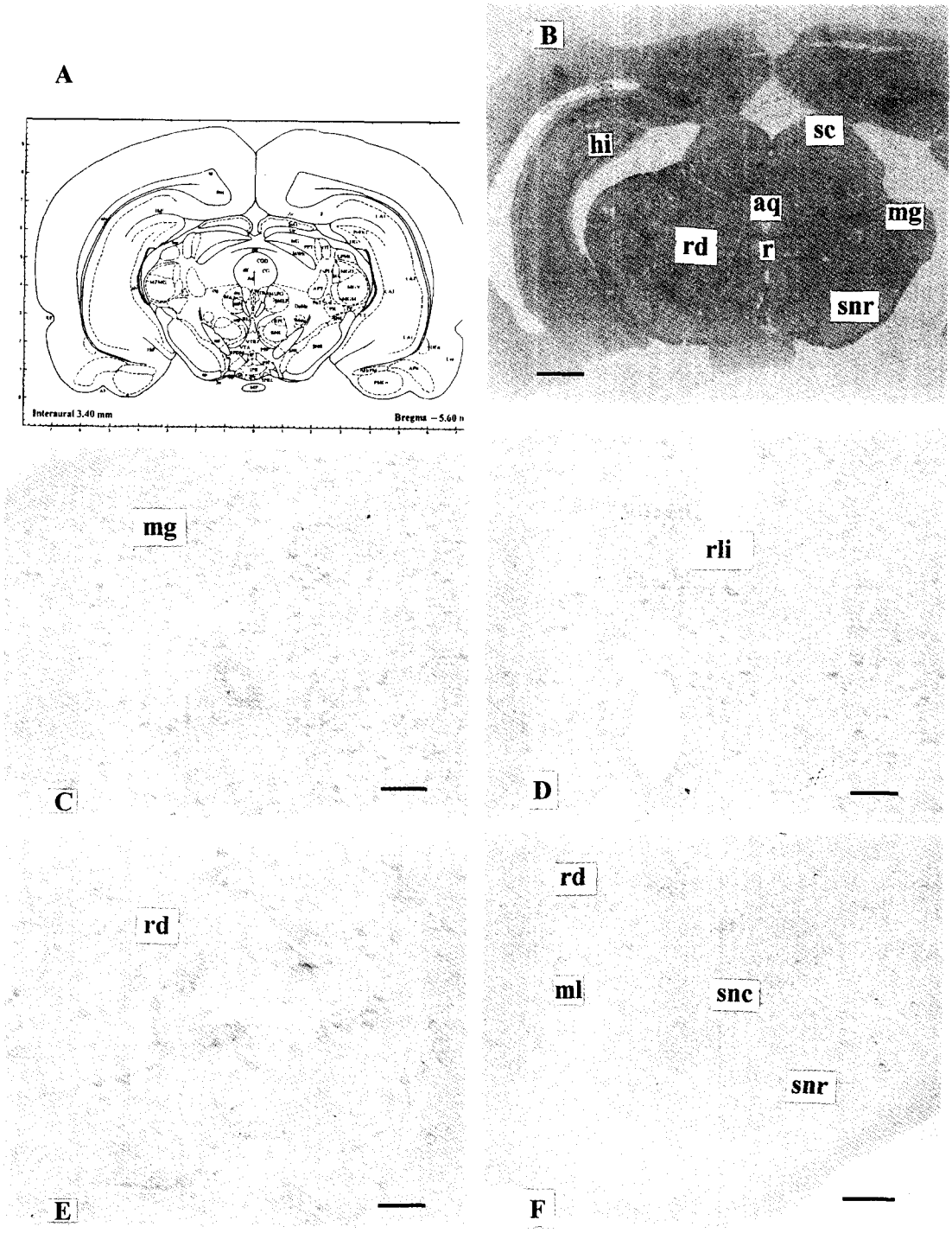

Fig. 4. The section through -5.60 mm from bregma and 3.40 mm from interaural line of midbrain level. A, Schematic figure as above. B, We can see the hippocampus (hi), superior colliculus (sc), cerebral aqueduct (aq), medial geniculate body (mg), rostral linear raphe nucleus (r), red nucleus (rd), substantia nigra (snr) in the same section. The GDH-immunoreactive cells were observed in the medial geniculate body (C, mg), the rostral linear raphe nucleus (D, rli), the red nucleus (E, rd) and the substantia nigra, pars reticularis (F, snr). Scale bars=0.1 mm (B), 5 μm (C, D, E), and 10 μm (F).

specifically expressed in neural and testicular tissues (Shashidharan et al., 1994). Recent studies from our laboratory have shown that two different types of GDH isoproteins (GDH I and GDH II) are also present in bovine brain (Cho et al., 1995). At present, the functional significance of GDH in nerve tissue remains

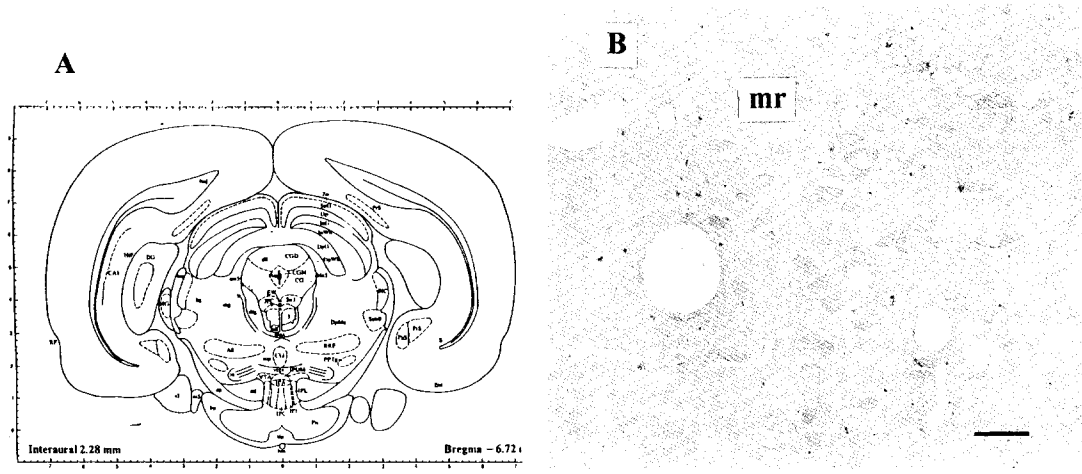

Fig. 5. The section through -6.72 mm from bregma and 2.28 mm from interaural line. A, Schematic figure. B, GDH-immunoreactive cells distributed in the median raphe nuclei (mr). Scale bar=5 μm.

uncertain. In the present study, we performed immunohistochemical staining with monoclonal antibodies against bovine brain GDH II in the rat brain for a first step of localization of the GDH isoproteins.

The stained tissue slices of rat brain were examined with reference to the atlas of Paxinos and Watson (1986). Immunoreactive cells were found to be present in all tissues. In the section through 9.48 mm from the interaural line and 0.48 mm from the bregma (Fig. 1a), GDH-immunoreactive cells were mainly observed in the septal nucleus (Fig. 1a and b), caudate putamen, and cerebral cortex. In the section through 7.70 mm from the interaural line and -1.30 mm from the bregma (Fig. 2a), GDH-immunoreactive cells were distributed evenly and were in the caudate putamen (Fig. 2b), anteromedial and anterodorsal thalamic nucleus of the thalamus, paraventricular thalamic nucleus (Fig. 2c), and basal nucleus of Mynert (Fig. 2d) of the substantia innominata. In the section through 5.86 mm from the interaural line and -3.14 mm from the bregma (Fig. 3a), immunoreactive cells were mainly found in the thalamus and hypothalamus (Fig. 3b), especially the reticular thalamic nucleus which is associated with sleep (Fig. 3c), ventrolateral and ventroposteral thalamic nucleus including periventricular thalamic nucleus (Fig. 3d), zona incerta (Fig. 3e) and subthalamic nucleus (Fig. 3f).

On the level of the superior colliculus, section through 3.40 mm from the interaural line and -5.60 mm from the bregma (Fig. 4a and b), we can see the GDH-immunoreactive cells in the hippocampus (Fig. 4b), medial geniculate body (Fig. 4c), rostral linear raphe nucleus (Fig. 4d), red nucleus (Fig. 4e), and substantia nigra (Fig. 4f). In the section through 2.28 mm from the interaural line and -6.72 mm from the bregma (Fig. 5a), the immunoreactive cells were observed in the dorsal and median raphe nuclei (Fig. 5b). In the junction of midbrain and pons, section through 0.20 mm from the interaural line and -8.80 mm from the bregma (Fig. 6a and b), cells showed strong GDH-immunoreactivity in the dorsal raphe nucleus (Fig. 6c) and traphezoid body nucleus (Fig. 6d). On the medulla oblongata level, sections through 1.04 mm from the interaural line and -10.04 mm from the bregma (Fig. 7a and b), the GDH-immunoreactive cells were distributed in the locus ceoluleus (Fig. 7c), mesencephalic trigeminal nucleus (Fig. 7d), superior olivary nucleus (Fig. 7e) and periolivary nucleus (Fig. 7f). Some immunoreactive cells were observed in the paraabducens nucleus, raphe pallidus nucleus, and Purkinje cell of the cerebellum.

Filla et al. (1986) investigated GDH activity in the 17 regions of six human brains, and found that GDH activity was highest in the inferior olivary complex and hypothalamus and lowest in the cerebellum and lenticular nucleus. GDH activity has been reported in the rat brain (Filla et al., 1984; Leong and Clark, 1984) and cat brain (Johnson, 1972). In these studies, GDH activity was detected mainly in the inferior olive, hypothalamus, base of pons, medulla oblongata, thalamus, caudate nucleus, hippocampus, and precentral gyrus, etc. These biochemical data agree with our immunohistochemical staining results. Our results show that GDH-immunoreactive cells are distributed in the basal ganglia, thalamus and the nuclei belong to sustantia innominata, and its connecting area, subthalamic nucleus, zona incerta and substantia nigra. We could see the GDH-immunoreactive cells in the hippocampus, septal nuclei associated with limbic system and the anterior thalamic nuclei connected between the hypothalamus and limbic system, and its associated structure, amygdaloid nuclear complex, the dorsal and median raphe nuclei and the reticular formation of the midbrain. The GDH-immunoreactive cells were distributed in the pyramidal neurons of the cerebral cortex, the Purkinje cells of the cerebella cortex, their associated structure, ventral thalamic

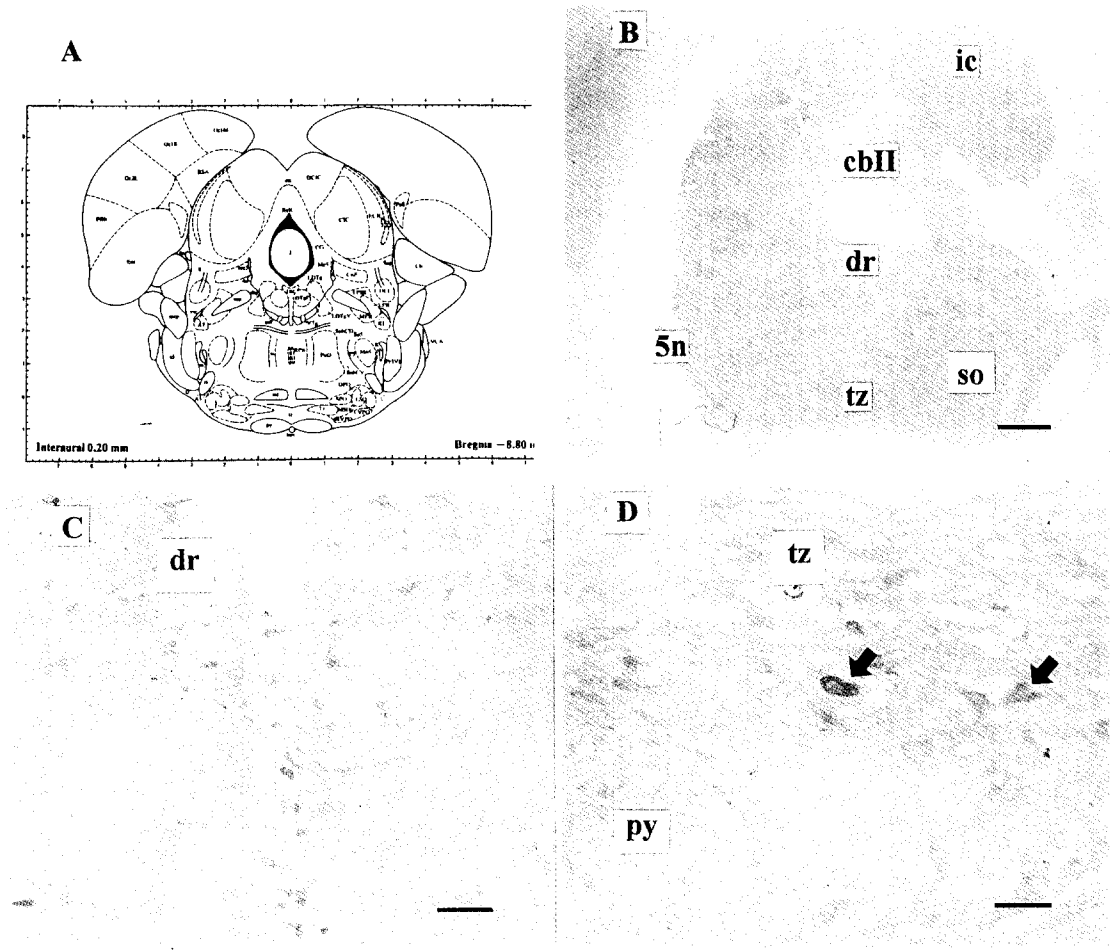

Fig. 6. The section through -8.80 mm from the bregma and 0.20 mm from the interaural line of the lower midbrain level. A, Schematic figure. B, We can see the inferior colliculus (ic), cerebellum (cbll), dorsal raphe nucleus (dr), traphezoid body nucleus (tz), superior olivary nucleus (so) and trigeminal nerve (5n) in this section. C, Higher magnification micrograph of the dorsal raphe nuclei (dr). d, GDH-immunoreactive cells with strong immunoreactivity were fond in the traphezoid body nucleus (tz). Scale bars=0.1 mm (B) and 5 μm (C, D).

nuclei and the reticular thalamic nuclei that seem to function as neural conduction in the thalamus that are the nuclei known to contain high densities of receptors for glutamate or aspartate (Halpain et al., 1984: Monaghan and Cotman, 1985). A local accumulation of this amino acid could lead to neuronal degeneration because of the well-known neurotoxic effect of glutamate and its analogs when present in excess (Olney, 1978).

Since the antibody was monoclonal against bovine brain GDH II, it is possible that it does not necessarily detect all GDH in rat brain. Indeed the cloning of genes encoding GDH suggests the presence of a gene family. The studies on the distribution of GDH in brain using monoclonal antibodies against different GDH isoprotein (GDH I) is in progress in our laboratory. It remains to be further studied whether the different types of GDH play a role in protecting neurons against excitotoxicity under conditions of enhanced glutamate release and energy failure.

Abbreviations of Figure Legends

cerebellar lobules, 2n, optic nerve, 3V, 3rd ventricle, 3n, oculomotor nerve, 4n, trochlear nerve, 4V, 4th ventricle, 7n, facial nerve, 8vn, vestibulocochlear nerve, aca, anterior commissure, AcbC, accumbens nucleus core, AD, anterodorsal thalamic nucleus, AF, amygdaloid fissure, AH, anterior hypothalamic area, AM, anteromedial thalamic nucleus, Amg, amygdaloid complex area, APT, anterior pretectal nucleus, Aq, cerebral aqueduct, Arc, arcuate nucleus, ATg, anterior tegmental nucleus, AV, anteroventral thalamic nucleus, B, basal nucleus of Mynert, bas, basilar artery, bic, brachium of inf. colliculus, BIC, nucleus of inf. colliculus brachium, CC, corpus callosum, cg, cingulum, CG, central gray, CL, centrolateral thalamic nucleus, CM, central medial thalamic nucleus, CPu, caudate putamen, ctg, central tegmental tract, DA, dorsal hypothalamic area, D3V, dorsal 3rd ventricle, DB, nucleus of diagonal band, DEn, dorsal entopiriform nucleus, DG, dentate gyrus,

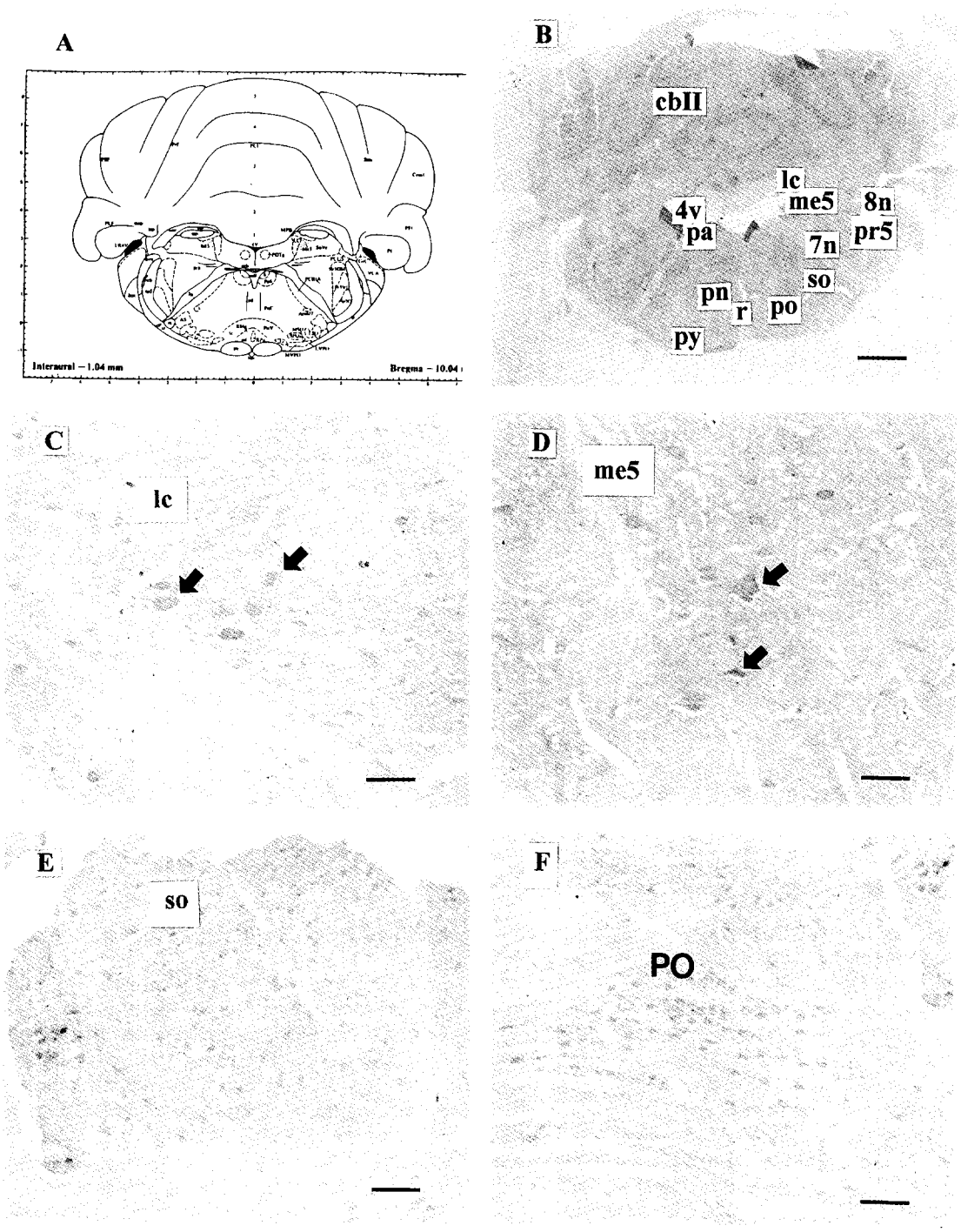

Fig. 7. The section through -10.04 mm from the bregma and -1.04 mm from the interaural line of the medulla oblongata level. A, Schematic figure. B, Cerebellum (cbll), the fourth ventricle (4v), paraabducens nucleus (pa), locus ceoluleus (lc), mesencephalic trigeminal nucleus (me5), superior olivary nucleus (so), periolivary nucleus (po), raphe pallidus nucleus (r), facial nerve (7n) and vestibulocochlear nerve (8n) were observed in this section. bar=0.1 mm. GDH-immunoreactive cells were located in the locus ceoluleus (C, lc) and mesencephalic trigeminal nucleus (D, me5), the superior olivary nucleus (E, so), periolivary nucleus (F, po). Scale bars=0.1 mm (B), 5 μm (C, D), and 10 μm (E, F).

DMH, dorsomedial hypothalamic nucleus, DpMe, deep mesencephalic nucleus, DR, dorsal raphe nucleus, En, entopiriform nucleus, Ent, enrorhinal cortex, EW, Edinger-Westphal nucleus, f, fornix, Fl, flocculus, Fr, frontal cortex, GP, globus pallidus, HC, hippocampus, HDB, nucleus of horizontal limb of diagonal band, ic,

internal capsule, IC, inferior colliculus, ICj, islands of Calleja, IMD, intermediodorsal thalamic nucleus, IMLF, interstitial nucleus of mlf, IP, interpeduncular nucleus, LC, locus coeruleus, LDDM, laterodorsal thalamic nucleus, dorsomedial, LDVL, laterodorsal thalamic nucleus, ventrolateral, LH, lateral hypothalamic nucleus, ll, lateral lemniscus, LP, lateral posterior thalamic nucleus, LPMR, lateral posterior thalamic nucleus, mediorostral, LSD, lateral septal nucleus, dorsal, LSI, lateral septal nucleus, intermediate, LSV, lateral septal nucleus, ventral, LV, lateral ventricle, m5, motor root of trigeminal nerve, MA3, medial accessory oculomotor nucleus, mcp, middle cerebellar peduncle, MDC, mediodorsal thalamic nucleus, central, MDM, mediodorsal thalamic nucleus, medial, MDL, mediodorsal thalamic nucleus, lateral, ME, median eminence, Me5, mesencephalic trigeminal nucleus, mfbb, medial forebrain bundle, MG, medial geniculate body, ml, medial lemniscus, mlf, medial longitudinal fasciculus, MnR, median raphe nucleus, Mo5, motor trigeminal nucleus, MPA, medial preoptic area, MS, medial septal nucleus, mt, mammillothalamic tract, MTu, medial tuberal nucleus, NIC, nucleus of inf. colliculus, OC, opt, optic tract, OT, nucleus of optic tract, ox, optic chiasm, P5, peritrigeminal zone, PaAP, paraventricular hypothalamic nucleus, Par, parietal cortex, PC, paracentral thalamic nucleus, PFl, paraflocculus, Pir, piriform nucleus, PLF, posterolateral fissure, Pn, pontine reticular nucleus, PnO, pontine reticular nucleus, Po, posterior thalamic nuclear group, PO, paraolivary nucleus, PPTg, pedunculopotine tegmental nucleus, Pr5, priciple sensory trigeminal nucleus, PrF, primary fissure, PRh, perirhinal cortex, PrS, presubiculum, PT, paratenial thalamic nucleus, PVA, paraventricular thalamic nucleus, PVP, paraventricular thalamic nucleus, posterior, py, pyramidal tract, R, red nucleus, Re, reuniens thalamic nucleus, RF, rhinal fissure, RLi, rostral linear raphe nucleus, Rmg, raphe magnus nucleus, Rt, reticular thalamic nucleus, RtTg, reticulotegmental nucleus, s5, sensory root of trigeminal nerve, SCh, suprachiasmatic nucleus, SC, sup. colliculus, scp, superior cerebellar peduncle, SHi, septohippocampal nucleus, SI, substantia innominata, sm, stria medullaris thalami, SNC, substantia nigra compacta, SNL, substantia nigra lateral, SNR, substantia nigra reticular, SO, supraoptic nucleus, Subl, subincertal nucleus, Te, temporal cortex, Tg, tegmental nucleus, tz, trapezoid body, TZ, nucleus of trapezoid body, VC, ventral cochlear nucleus, VEn, ventral entopiriform nucleus, VM, ventromedian thalamic nucleus, VMH, ventromedian hypothalamic nucleus, VP, ventral poster ior thalamic nucleus, VPM, ventral posteromedial thalamic nucleus, VPL, ventral posterolateral thalamic nucleus, xscp, decussation of sup. cerebellar peduncle, ZI, zona incerta.

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