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Localization of *bcl-2* in the mouse brain: An anatomical study by immunohistochemistry

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SUMMARY

The presence of *bcl-2* has been demonstrated in neurons of the developing adult brain of different species, including humans, the monkey, rat, rabbit and mice. Although *bcl-2* expression in mouse embryo and its distribution in the central nervous system during development have been described in the cerebellar cortex and hippocampus, its distribution in the entire adult mouse brain has not yet been described. In the present work we describe *bcl-2* expression in the mouse brain by immunohistochemistry and we compare it with other species showing similar patterns. We found that limbic areas have the highest bcl-2 density, suggesting a protective role of this protein in these regions.

Key words: Bcl-2 – Limbic areas – Mouse brain – Neuroprotection – Oxidative stress

INTRODUCTION

The distribution of *bcl-2* protein or bcl-2 mRNA in the adult brain has been described in some species such as humans, the monkey, rabbit, rat and in certain regions of the mouse brain (Castren et al., 1994; Hara et al., 1996; Merry et al., 1994; Vinet et al., 2002). Although, there is a general common pattern of *bcl-2* expression in the brain, regional and cellular differentiation may differ among different species (Castren et al., 1994; Hara et al., 1996; Merry et al., 1994; Shin et al., 2000). For example, *bcl-2*$^+$cells are present in the rat and rabbit hippocampus (Castren et al., 1994) but not in the human counterpart (Hara et al., 1996). In the rabbit, the Purkinje cells of the cerebellum show a *bcl-2* signal (Shin et al., 2000) but this is not seen in human or rat cerebellum (Castren et al., 1994). The reason for the diverse distribution remains unknown, but during the development of the rat brain *bcl-2* has been suggested to function as a controller of programmed cell death (Castren et al., 1994) and in the adult primate brain it has been considered a protector preferentially of limbic areas (Bernier and Parent, 1998). *bcl-2* inhibits programmed cell death in response to different stimuli, including oxidative stress (Bernier and Parent, 1998 a, 1998 b; Castren et al., 1994; Jacobson and Raff, 1995), and it may play a role in the survival/differentiation of newborn neurons (Abe-Dohmae et al., 1993; Berard et al., 2002; Kuhn et al., 2005).

bcl-2 prevents apoptosis through antioxidant pathways, being considered as a free radical scavenger (Zhong et al., 1993). One proposal for the action of bcl-2 is that it would protect the integrity of mitochondrial oxida-

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tive phosphorylation and thus limits the mitochondrial dysfunction induced by several apoptotic stimuli (Reed et al., 1998; Shimizu et al., 1998; Green et Reed, 1998; Kroemer, 1997).

In the present work we study the distribution of *bcl-2* in the adult mouse brain and compare the results with those obtained in other species in order to know whether there is any relationship between the content of *bcl-2* and neuronal vulnerability.

Material and Methods

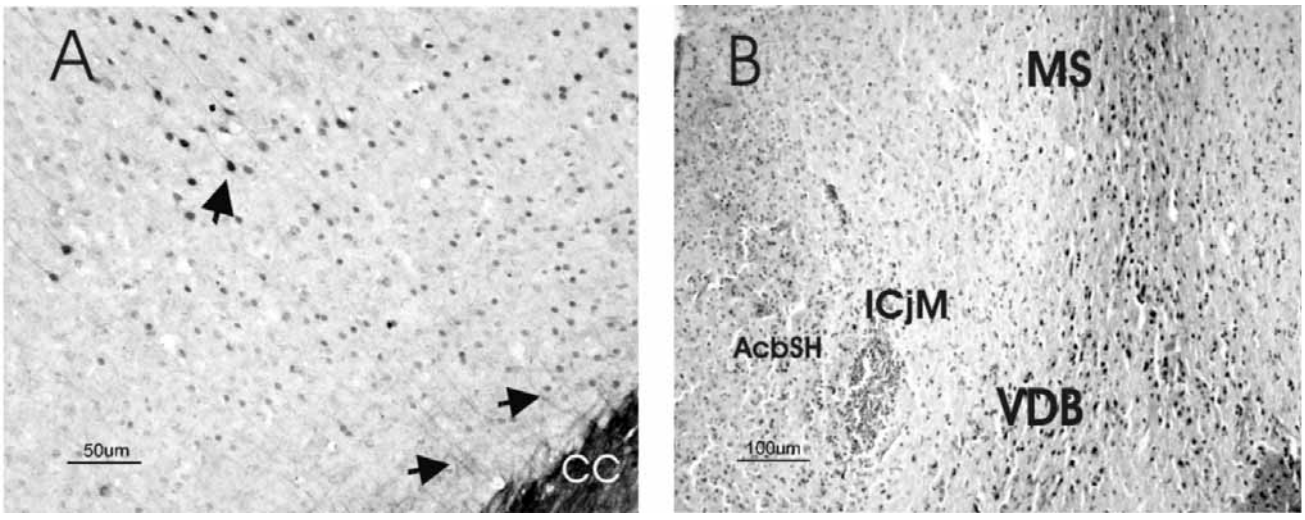
Adult ICR mice (Harlan) were housed in a single group with water and food *ad libitum* and with controlled temperature, humidity and photoperiod. The animals were treated in accordance with the guide for the care and use of laboratory animals (NIH Publications No. 80-23). Mice were sacrificed by an overdose of sodium pentobarbital and then perfused with saline followed by 4% para-formaldehyde in 0.1M PB, pH 7.4. Brains were post-fixed in paraformaldehyde solution and placed in a 30% sucrose solution for 24 h. 40 micrometers thick sections were obtained using a cryostat. Sections were rinsed and pre-treated with citrate buffer for antigen retrieval. Briefly, glass tubes containing 10 mM citrate buffer pH 6 were heated in a water bath up to 95-100°C. Then, brain sections were immersed for 20 minutes. The tubes were then removed from the water bath and the slides were kept at room temperature for 20 minutes. Following this, sections were rinsed with 0.02M PBS, and blocked with 30% H_2O_2 and methanol for 20 minutes followed by incubation overnight with primary rabbit anti *bcl-2* (Santa Cruz, sc-783) (dilution 1:500 in PBS 0.08% Triton X-100 and 5% normal goat serum (NGS) and the sections were rinsed in PBS 0.02M and incubated at room temperature shaking for 1 hour in 0.4% biotinylated anti-rabbit IgG (Vector Labs, Burlingane, CA, USA). Finally, they were rinsed and re-incubated with avidin-biotin complex (ABC, Vector) for 1 hour. The reaction was developed with DAB (Vector). Sections were mounted and covered with DPX for microscope analysis.

Results

In the mouse brain, the *bcl-2* proto-oncogene was located preferentially in the cytoplasm, but also in processes and cell nuclei. Nuclear *bcl-2*$^+$ staining was found in pyramidal cells of the cerebral cortex, Purkinje cells of the cerebellum, cells of the *locus coeruleus* and cells of the motor trigeminal nucleus or olivary nuclei.

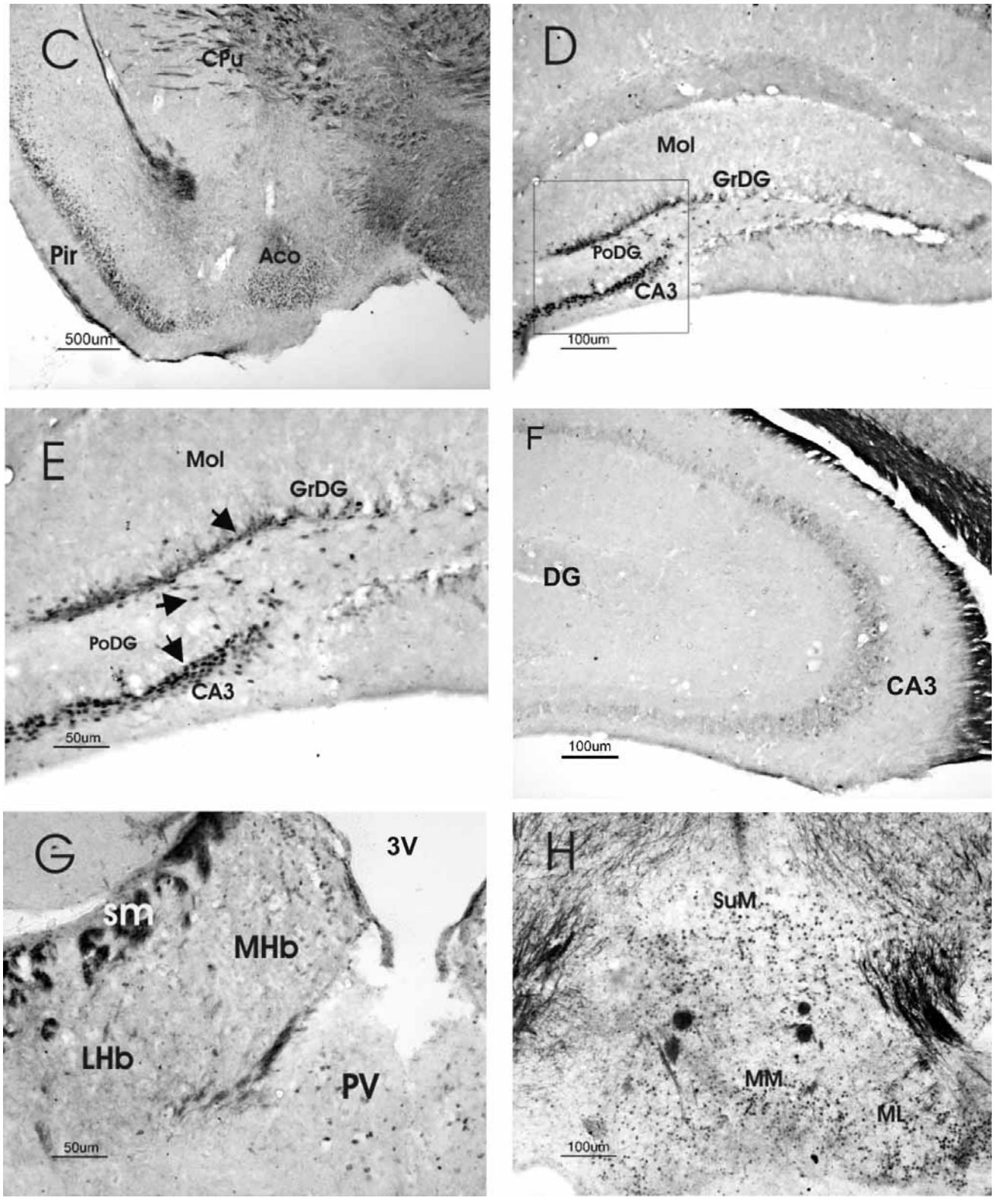
The cerebral cortex displayed a generalised *bcl-2* staining in several cortical areas. Thus, the motor cortex, including M2 and M1, showed *bcl-2*$^+$ cells in several cortical layers; No apparent staining was found in the first cortical layer, levels III and IV showed sparse

Fig. 1. *bcl-2* expression in telencephalic and diencephalic areas: (A) arrowheads indicate *bcl-2* expression in pyramidal neurons of the motor cortex (M1) and fibers of the corpus callosum (cc); (B) medial septum (MS), vertical limb of the diagonal band (VDB), shell of the accumbens (AcbSH) and major Islands of Calleja (ICjM); (C) shows piriform cortex (Pir), caudate-putamen (CPu) and anterior amygdaloid area (ACo); (D) dentate gyrus of hippocampus granular layer (GrDG), polymorphic layer (PoDG), molecular layer (Mol) and field CA3 of hippocampus (CA3) and (E) magnification (20X) of the boxed area in D arrowheads show *Bcl-2* staining in PoDG, GrDG CA3 and Mol. (F) CA3 of hippocampus; (G) Epithalamus: medial habenular nucleus (MHb), lateral habenular nucleus (LHb), stria medularis (sm) and paraventricular thalamic nuleus (PV); (H) Supramammillary nucleus (SuM), medial mammillary (MM) and lateral mammillary nucleus (ML).



bcl-2$^+$ labeling and layers II and V of motor cortex presented some populations of *bcl-2*$^+$ neurons. The pyramidal neurons of layer V showed *bcl-2*$^+$ immunostaining in somata, nuclei and fibers. Some fibers projected from the corpus callosum to several cortical, such as areas as the motor cortex (fig. 1.A). Extensive bcl-2 staining was found in the prelimbic cortex (PrL), the infralimbic cortex (IL) and the dorsal peduncular cortex (DP). No apparent staining was found in Cg1. A dense *bcl-2*$^+$ population was found in the basal forebrain, medial septum, VDB and HDB (fig. 1.B). *bcl-2* positive cells were also found in the latero-septal regions, the Islands of Calleja or the shell of the nucleus accumbens (AcbSH) (see figure 1.B).

Low *bcl-2* reactivity was found in the CPu and a dense population was found in the piri-



form cortex (Pir) with apparent nuclear staining in several neurons (fig. 1.C).

The amygdalar formation and its boundaries displayed sparse *bcl-2*$^+$ cells, although no labeling was found in the lateral amygdala (LA) or basolateral amygdala (BLA) nuclei. The highest concentration of bcl-2 cells in the temporal area was located in the nucleus of the lateral olfactory tract (LOT), medial amygdaloid nucleus (MeA), anterior cortical amygdaloid nucleus (ACo), basomedial amygdalar nuclei, central amygdaloid nucleus (CeA) and BSTIA (fig. 1.C).

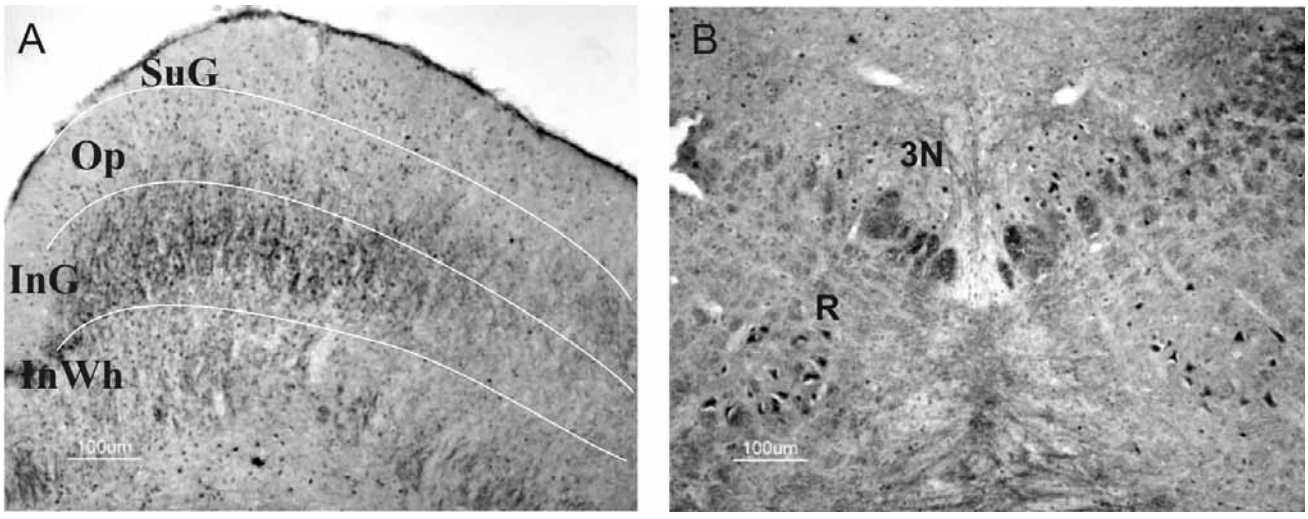
Immunoreactivity for *bcl-2* in the hippocampus seemed to be strictly confined to discrete zones, CA1, CA3 and DG, although some dispersed cells were found in the stratum radiatum (Rad) and the oriens layer of the hippocampus (Or). The CA1 zone had a dense *bcl-2*$^+$ cells whose somata, nuclei and fibers were labeled. These fibers projected to the Rad. The CA3 located close to the dentate gyrus (DG), showed a small group of *bcl-2*$^+$ cells stained in their somata and nuclei (fig. 1.F). No labeling was found in CA2. The dentate gyrus showed *bcl-2*$^+$ cell populations located mainly in the granular layer (GrDG) and less frequently in the polymorphic layer (PoDG) (fig. 1.D). Some *bcl-2* fibers of the upper part of the DG projected to the molecular layer of the DG (Mol) (fig. 1.E). No apparent staining was found in the mol, DG or stratum lucidum (SLu).

The medial and lateral habenular nuclei in the epithalamus, had a small population of *bcl-2*$^+$ cells and fibers (fig. 1.G). The *Stria medularis* of thalamus and the *fasciculus retroflexus* also showed immunoreactivity for *bcl-2*. Thalamic nuclei, such as the paraventricular thalamic, intermediodorsal, reuniens or ventrolateral thalamic nuclei also showed *bcl-2* immunoreactivity. Some hypothalamic nuclei, such as the anterior hypothalamic area and dorsomedial hypothalamic nucleus, showed *bcl-2* immunoreactivity, and this immunoreactivity was also found in the caudal-most diencephalic and first mesencephalic levels located in the MM and SuM nuclei (fig. 1.H).

In addition, we found *bcl-2* immunoreactivity in the different layers of the superior colliculus: in the superficial superior colliculus gray layer (SuG) and a large amount of fibers in the intermediate superior colliculus gray and white matter (InG, InWh resp.). We also found some bcl-2 positive cells in the superior colliculus commissure (csc) (fig. 2.A), medial geniculate nucleus (MG) and pretectal areas.

bcl-2 labeling in the PAG was reduced to some discrete cells and fibers around the aqueduct of Silvius. The area of the oculomotor nucleus (3N) had a very low number of *bcl-2*$^+$cells; some of them were also seen in the red nucleus with a discrete group of cells also showing nuclear staining (R) (fig. 2.B). The DR nucleus had a small population of *bcl-2*$^+$cells in the caudal-most levels of the aqueduct. A densely stained population was present in the interpeduncular nucleus mainly located in the rostral part of the nucleus (IPR)

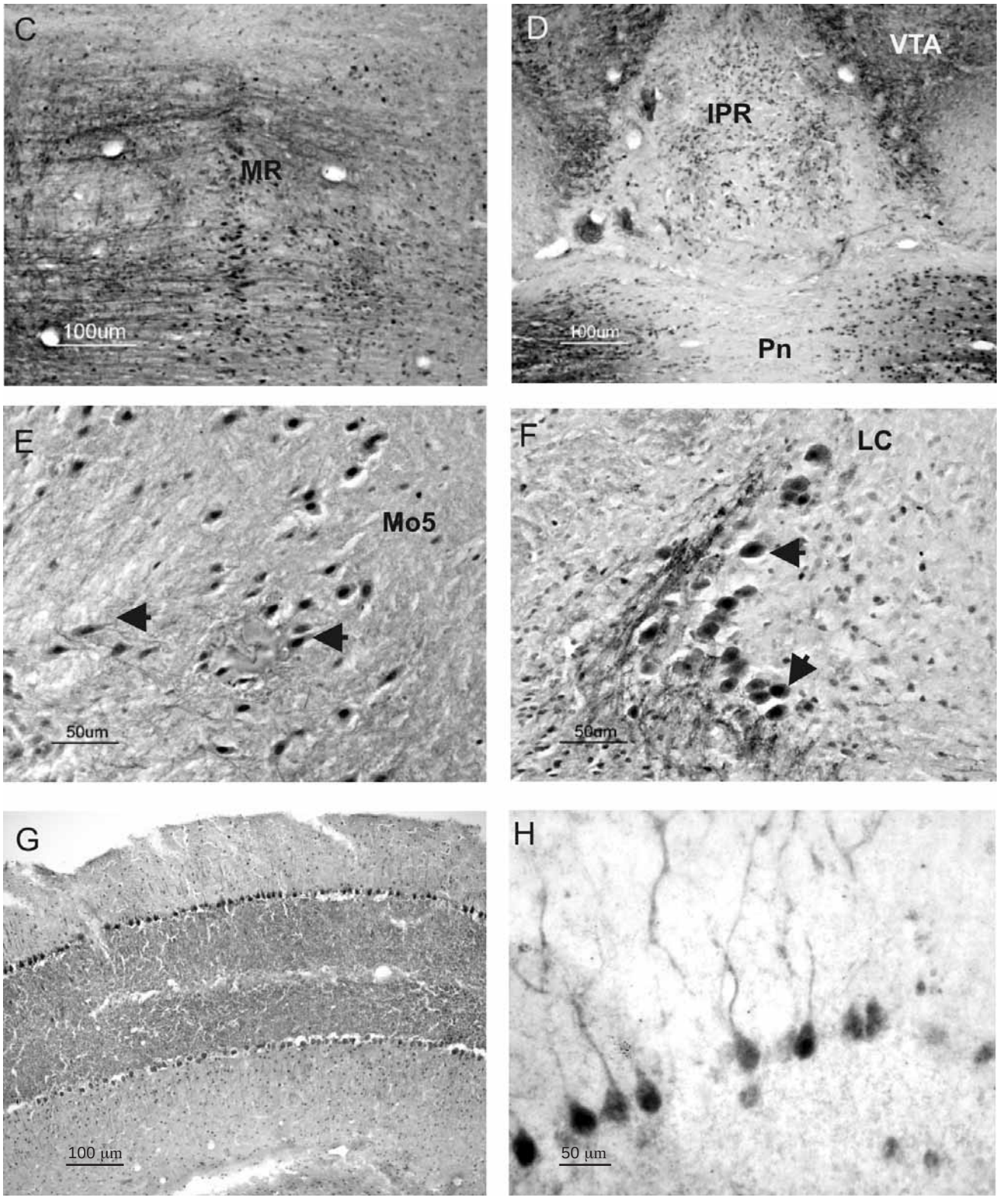
Fig. 2. *bcl-2* expression in hindbrain structures: (A) Superior colliculus with cells and fibers labelled in SuG, Op InG, InWh; (B) Periaqueductal grey (PAG), oculomotor nucleus (3N), red nucleus (R); (C) Interprduncular rostral (IPR), ventral tegmental area (VTA), pons (pn). (D) median raphe nucleus (MR); (E) Arrowheads show nuclear staining of motor trigeminal nucleus cells (Mo5) and (F) in the locus coeruleus; (G) Purkinje (PC) and molecular cells in the molecular layer (ML) of the cerebellum. Note also the absence of labelling in granular layer (GL); (H) higher magnification of Purkinje cells in the cerebellum showing nuclear staining.



and in the ventral tegmental area (VTA) (fig. 2.C). Fibers and cells were also stained in the MR (fig. 2.D).

The substantia nigra *pars reticulata* (SNR) had a *bcl-2*$^+$ population of large cells with strong nuclear labeling, whereas in the *pars compacta* (SNC) we found smaller cells with staining in cytoplasm.

At the level of the pons and medulla oblongata there were many well-defined *bcl-2*$^+$ cells in the *locus coeruleus*, the motor trigeminal nucleus and the olivary nuclei. Some of these cells showed nuclear immunoreactivity, such as in the *locus ceruleus*, Mo5 (fig. 2.E and F, respectively), olivary nuclei, and reticulotegmental nucleus of the pons (RtTg). The tegmental region of the fourth ventricle displayed dense *bcl-2*$^+$ cell labeling in nuclei such as the LDTg, dorsal tegmental nuclei (DT) and Barrington's nucleus (Bar). At caudal lev-



els of the mesencephalon and pons, we found many cells and fibers along the pons and medulla oblongata showing *bcl-2*$^+$ immunoreactivity.

The Purkinje layer of the cerebellum showed *bcl-2*$^+$ cells (fig. 2.G) with intense labeling in their somata, fibers and nuclei (fig. 2.H). However, the molecular layer had few *bcl-2*$^+$ cells. No labeling was found in the granular layer (fig. 2.G).

Discussion

Bcl-2 is widely expressed in mouse brain in several nuclei and fibers. Previous publications measuring mRNA *bcl-2* expression (Castren et al., 1994; Inoue et al., 2002; Shin et al., 2000) or bcl-2 protein levels (Bernier and Parent 1998, Hara et al., 1996; Merry et al., 1994) have reported some differences and similarities among species, in agreement with our results. On comparing the pattern of *bcl-2* expression in the rodent brain, we found low variability between the mouse, rat and rabbit. In general, these species showed a substantial expression of the proto-oncogene, ranging from telencephalic areas to structures of the brain stem, most of the expression being related to hippocampal formation. Rabbit, mice and rat express *bcl-2* in CA1, CA3 and DG. Cortical areas also showed intense staining in the rabbit, rat and mouse. However, in the cerebellum there were some differences: While the rabbit (Shin et al., 2000) and mouse show *bcl-2* reactivity in the Purkinje cells, this labelling is not present in rat (Castren et al., 1994). Moreover, cells of the granular layer in the rat cerebellum showed strong bcl-2 reactivity (Castren et al., 1994) while only occasional labelling is present in the rabbit (Shimizu et al., 1995) or mouse. More differences were sseen between the rat and mouse on comparing septal areas. The mouse showed profuse signals in several septal areas such as the ICj, MS or the vertical and horizontal parts of the diagonal band while the rat showed low *bcl-2* signals. The results of previous studies in the primate brain are also in agreement with our findings, and it has been suggested that basal *bcl-2* expression located in limbic areas might be related to the protection of these areas. (Bernier and Parent, 1998 a; 1998 b; 2002). *bcl-2* may act not only as a signal of protection against different injuries leading to apoptosis (Jacobson and Raff, 1995; Shimizu et al., 1995; Zhong et al., 1993) but also in neuronal remodeling (Najbauer et al., 1995) or neurogenesis (Abe-Dohmae et al., 1993; Berard et al., 2002; Kuhn et al., 2005). This protective role seems to be related to the mitochondrial oxidative phosphorylation and mitochondrial dysfunction induced by apoptosis (Reed et al., 1998; Shimizu et al., 1998; Green et Reed, 1998; Kroemer, 1997). In this context, and with focus on the hippocampus, all rodents species showed *bcl-2* expression throughout the hippocampal structures. Moreover, an increase has been found in DG neurogenesis due to *bcl-2* overexperession in mice (Kuhn et al., 2005) and the newly developed neurons of the amygdala, piriform cortex, and inferior temporal cortex in primate brain also express *bcl-2* (Bernier et al., 2002)

Our group has previously demonstrated that chronic ethanol consumption in the rat blocks neurogenesis in the DG, but not in the subventricular zone (Herrera et al., 2003). Moreover, this inhibition can be reversed by the antioxidant Ebselen, a peroxynitrite scavenger (Herrera et al., 2003). Thus, we suggest that *bcl-2* could be expressed in vulnerable or important areas to protect these against the mitochondrial oxidative-nitrosative steps related to apoptosis. Further research on these issues should focus on studying the effects of the antioxidant therapy in the prevention or treatment of neurodegenerative disorders.

Abbreviations

3N	oculomotor nucleus
3V	third ventricle
4V	fourth ventricle
AcbSH	shell of nucleus accumbens
ACo	anterior cortical amygdaloid nucleus
Aq	aqueductus (Sylvius)
Bar	Barrington's nucleus
BSTIA	bed nucleus of stria terminalis, amygdaloid division
CA1	field CA1 of hippocampus
CA2	field CA2 of hippocampus
CA3	field CA3 of hippocampus
cc	corpus callosum
Cg1	cingular cortex, area 1
CNS	central nervous system
CPu	caudate putamen nucleus
cs	commissure of superior colliculus
csc	superior colliculus
DG	dentate gyrus
DP	dorsal peduncular cortex
DR	dorsal raphe nucleus

DT	dorsal tegmental nucleus
fr	fasciculus retroflexus
GC	granular layer of cerebellum
GrDG	granular layer of dentate gyrus
HDB	horizontal limb of diagonal band
ICjM	Islands of Calleja, major island
IL	infralimbic cortex
InG	intermediate grey layer of the superior colliculus
InW	intermediate white layer of the superior colliculus
IPN	interpeduncular nucleus
IPR	interpeduncular nucleus, rostral
LA	lateral amygdaloid nucleus
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LM	lateral mammillary nucleus
LOT	nucleus of lateral olfactory tract
M1	primary motor cortex
M2	secondary motor cortex
MeA	medial amygdaloid nucleus
MG	medial geniculate nucleus
MHb	medial habenular nucleus
ML	medial mammillary nucleus, lateral
MM	medial mammillary nucleus, medial
Mo5	motor trigeminal nucleus
MolC	molecular layer of cerebellum
Mol	molecular layer of dentate gyrus
MR	median raphe
MS	medial septum
Op	optic nerve layer of superior colliculus
Or	oriens layer of hippocampus
PAG	periaqueductal gray
PC	Purkinje cell
Pir	piriform cortex
Pn	pons
PoDG	polymorphic layer of dentate gyrus
PV	paraventricular thalamic nucleus
R	red nucleus
Rad	stratum radiatum of hippocampus
RtTg	reticulotegmental nucleus of pons
SLu	stratum lucidum of hippocampus
sm	stria medullaris of thalamus
SNC	substantia nigra, compact part
SNR	substantia nigra, reticular part
SuG	superficial grey layer of superior colliculus
SuM	suprammmillary nucleus
VDB	vertical limb of diagonal band
VTA	ventral tegmental area

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