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Mechanisms of Development 108 (2001) 207–211

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Gene expression pattern

Expression pattern of *Zac1* mouse gene, a new zinc-finger protein that regulates apoptosis and cellular cycle arrest, in both adult brain and along development

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Received 5 June 2001; received in revised form 26 June 2001; accepted 11 July 2001

Abstract

Using in situ hybridization, we analyzed the expression pattern of the *Zac1* gene in mouse brain during the embryonic and postnatal development. *Zac1* is a new gene that regulates extensive apoptosis and cell cycle arrest through unrelated pathways. At embryonic stages, strong expression was observed in brain areas with active proliferation (ventricular zone and numerous neuroepithelius) and in nervous system (neural retina and neural tube). In addition, some areas with differentiation activity were noticeably labeled such as arcuate nucleus and amygdaloid region of the brain together with other embryonic sites (hindlimb, forelimb and somites). From P0 onwards, the expression appeared in some proliferative areas, such as subventricular zone and cerebellum (external granular layer and Purkinje cells) and in some synaptic plasticity areas, such as the dorso and ventromedial hypothalamic nuclei, arcuate nucleus, ventral thalamic nucleus.

Keywords: Zac1; Brain development; Proliferation; Hypothalamus; Mouse embryo and postnatal; Eye; PACAP; Differentiation; Apoptosis; Cell cycle; Tumor

Recently (Spengler et al., 1997), a new zinc finger protein, *Zac1*, has been described which regulates extensive apoptosis and cell cycle arrest (in the G1 phase) through unrelated pathways. Studies in vitro revealed that *Zac1* inhibits tumor cell growth, therefore the *Zac1* gene could be a candidate for tumor suppression (Pagotto et al., 1999, 2000). Northern Blot methods showed that *Zac1* was highly expressed in the anterior pituitary gland and at lower levels in other brain areas (Spengler et al., 1997; Piras et al., 2000).

1. Results

High levels of *Zac1* gene expression were determined early in brain development. Thus, between E9.5 and E12.5 noticeable labeling was detected in brain regions with high active cellular proliferation, such as telencephalic vesicles and the infundibular recess of the third ventricle (irIIIv) destined to form the neurohypophysis (pituitary gland), where *Zac1* was intensely expressed in adult (Spengler et al., 1997). In addition, *Zac1* expression was detected in other proliferative areas of central nervous system, such as neural tube at E9.5 and neural retina at E10.5 (Fig. 1 and Table 1). Other embryonic sites revealed also strong label, such as the second branchial arch, liver primordium, the somites and body wall of the umbilical region (Fig. 1 and Table 1). Moreover, from E10.5 strong expression appeared

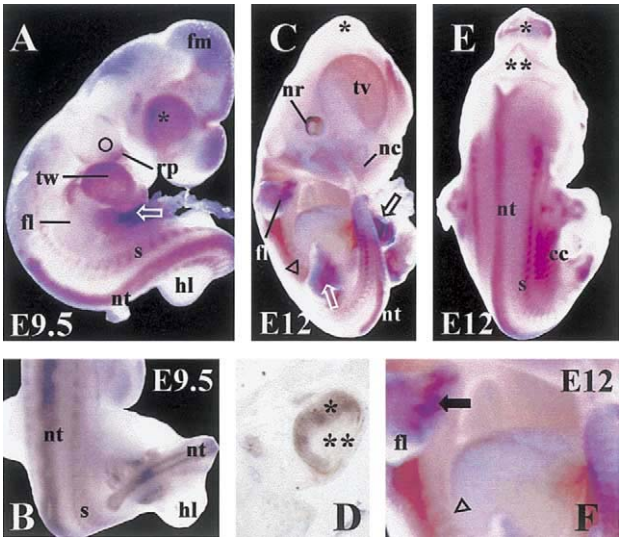

Fig. 1.

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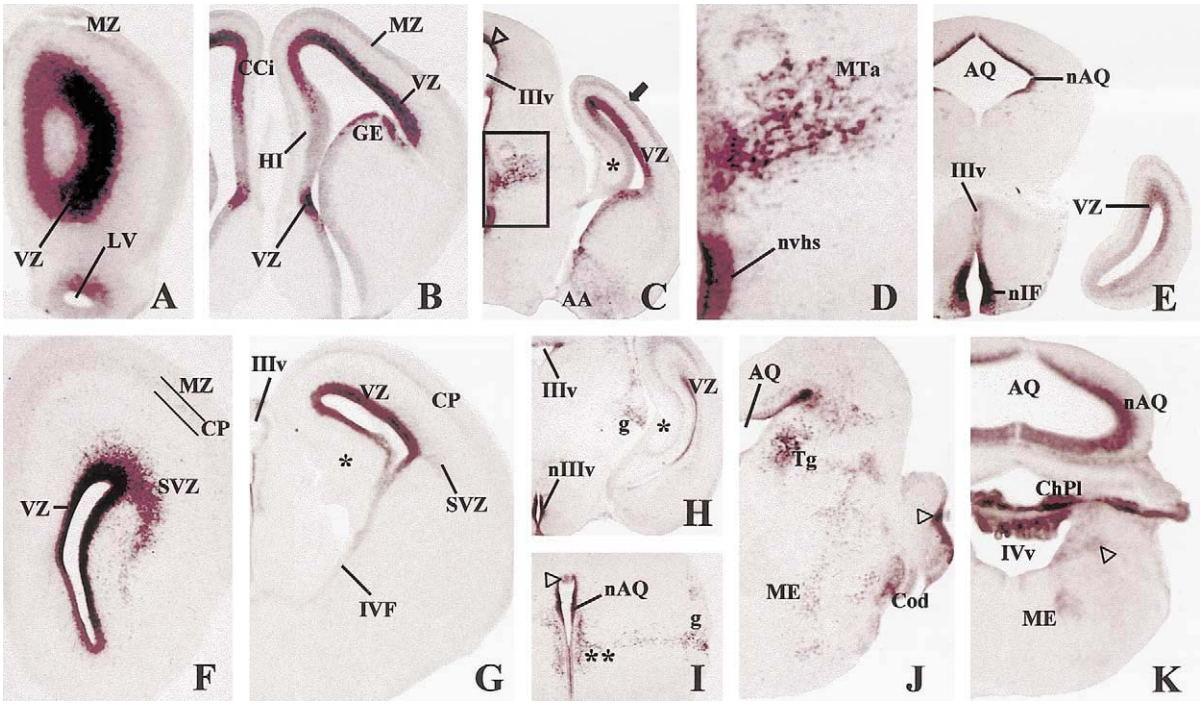

Fig. 2. Expression pattern of *Zac1* mRNAs in brain coronal sections of E14 (A–E) and E18 (F–K) embryonic stages. The expression is detected in areas where active cellular proliferation occurs. Thus, at E14 (A–E) intense label is observed in the ventricular zone (VZ) of the lateral ventricles (A,B) and in many neuroepithelia (C–E), such as in ventral hypothalamic sulcus (nvhs), infundibulum (nIF), aqueduct of Sylvius (nAQ) and third ventricle (IIIv). The neuroepithelium of the lateral ventricle (LV), the ventricular zone of the cingulate cortex (CCi) and the mammillothalamic tract area (MTa) are strongly labeled (A,B and C,D, respectively). In amygdaloid area (AA) and the ventricular zone of the ganglionic eminence (GE), the label is weaker (C and B, respectively). At this time, the ventricular zone of the hippocampal area (Hi) is unstained (B). By E18 (F–K), intense expression is still found in areas with cellular proliferation, such as the ventricular zone (VZ) of the lateral ventricles (F,G), the neuroepithelia (H,I,K) of third ventricle (nIIIv), the aqueduct of Sylvius (nAQ), and the choroid plexus (ChPl) of the fourth ventricle (K). At this stage, in anterior anatomic levels (F,G), the expression of *Zac1* disappears in the marginal zone (MZ) while it appears in the cortical plate (CP) and subventricular zone (SVZ). Moreover, the label is very weak in the interventricular foramen of Monroe (IVF) and the neuroepithelium of the hippocampal formation (asterisk). At posterior levels (H–K), strong label appears in external granular layer of the cerebellum (arrowhead in J) and moderate signals of expression are detected in dorsal cochlear nucleus (cod) and tegmentum (Tg). Expression is weaker in the medulla (ME), the subcommissural organ (arrowhead in I), the geniculate body (g) and the medial vestibular nucleus (arrowhead in K). (I) *Zac1*-positive cells are observed in the dorsomedial thalamic nucleus (double asterisk), which are probably in the process of migration (arrows) to the geniculate body (g). Magnification: 24× (A), 17.8× (B), 15.7× (C,E), 46.8× (D), 20.7× (F), 18.6× (G), 11.7× (H), 15.2 (I), 17.6× (J) and 22.8× (K).

in the hindlimb and forelimb (Fig. 1C–E and Table 1) which were in differentiation process, starting the demarcation of the digital interzones and the development of the precartilage primordium (Kaufman, 1999). All these findings suggest that *Zac1* have an important role in cellular growth control that occurs during embryogenesis, concretely in the digits and precartilage (forelimb and hindlimb), in neural retina, in the pituitary gland (irIIIv) and in the neural tube and telencephalic vesicles).

From E14 to E18, intense *Zac1* expression was detected in many brain areas with a high cellular proliferation activity, such as the ventricular zone, the external granular layer of the cerebellum (EGL) and different neuroepithelia (Fig. 2). Specifically, the label was found in neuroepithelium of the aqueduct of Sylvius, ventral hypothalamic sulcus, third ventricle, preoptic recess, infundibulum and fourth ventricle (Fig. 2 and Table 1). Moreover, strong expression was detected in brain regions with intense cellular differentiation, like arcuate nucleus, different hypothalamic and thalamic nuclei (Fig. 2 and Table 1), and in regions that regulate

Fig. 1. Expression patterns of *Zac1* in the mouse embryos. (A,B) At embryonic day 9.5 (E9.5), high expression of *Zac1* is found in telencephalic vesicles (asterisk), neural tube (nt) which is not yet joined, future midbrain (fm) and pericardial region (arrow). Moreover, expression is weaker in the entrance to Ranthke's pouch (rp), on the thoracic wall (tw) and in somites (s), while the mandibular components of the first branchial arch (circle), hindlimb (hl) and forelimb (fl) are unstained. (C–F) At E12, the expression is more specific and many areas are well delimited. Thus, intense expression is observed on the body wall of the umbilical region (black arrow in C), forelimb (fl) and hindlimb (white arrow in C). In these two last structures, the label is located in the precartilage primordium of the hands, feet (white arrow in C) and digits (arrow in F). Strong expression is detected in the neural layer of the retina (nr in C and asterisk in D). Weaker label is observed in the costal cartilage (arrowhead in C and cc in E), neural tube (nt), telencephalic vesicles (tv), midbrain (asterisk in E) and somites (s). The lens vesicle (double asterisk in D) and the hindbrain (double asterisk in E) are unstained, while the nasal cavity (nc) is very weak labeled. Image (E) was from slices of 12-day embryos. Magnification: 7× (A), 6× (B), 3× (C,E), 18× (D) and 14× (F).

Table 1
Expression pattern of *Zac1* gene in both adult brain and along development[a]

Anatomic structure	E9.5	E10.5	E12	E14	E16	E18	P0−P9	>P12
	Whole embryos			Brain slices				
Body wall of the umbilical region	++	++	+++ +					
Costal cartilage (cc)	−	−	++					
Forelimb (fl)	−	+/−	+++ +					
Hindlimb (hl)	−	+/−	+++ +					
Liver primordium (l)	+++ +	+++ +	++					
Midbrain (fm)	++	+	+					
Neural tube (nt)	+++	+++	++					
Optic eminence (oe) or neural retina (nr)	−	+/−	+++					
Pericardial region or Heart (h)	+++	+++ +	+					
Second branchial arch	−	+	++					
Somite (s)	+/−	+	++					
Telencephalic vesicle (tv)	++	++	++					
Dorsal diencephalic sulcus				+++	++	++		
Dorsal thalamus				+	−	−		
Entopeduncular nucleus				+	−	−		
Epithalamus area				+++	−	−		
Geniculate body, dorsal lateral and medial (g)				−	+/−	+		
Interventricular foramen of Monroe (IVF)				+	+/−	+/−		
Mammillothalamic tract (MTa)				++	−	−		
Medial forebrain bundle, posterior				+/−	+/−	+/−		
Medial vestibular nucleus				+/−	+/−	+/−		
Neuroepithelium of aqueduct of Sylvius (nAQ)				+++	+++	+++		
Neuroepithelium of fourth ventricle				+/−	+/−	+/−		
Neuroepithelium of infundibulum n(IF)				+++ +	+++	+++		
Neuroepithelium of pineal recess				++	++	−		
Neuroepitheium of periventricular hypothalamic nucleus				+	+	+		
Neuroepithelium of preoptic recess				+/−	+	++		
Neuroepithelium of third ventricle				+++	+++	+++		
Neuroepithelium of ventral hypothalamic sulcus (nvhs)				+++	+++	+++		
Reticular thalamic nucleus				+/−	+	+		
Subcommissural organ (SCO)				−	+/−	+		
Tegmentum (Tg)				++	++	++		
Ventricular zone (VZ)				+++ +	+++ +	+++ +	++	+/−
Subventricular zone (SVZ)				+/−	+	++	+	+/−
Marginal zone (MZ)				+	+/−	+/−	−	−
Cortical plate (CP)				−	+/−	+	+/−	−
Arcuate nucleus (arc)				++	++	++	++	+++ +
Dorsomedial hypothalamic nucleus (dmh)				++	++	++	+++	++
Ventromedial hypothalamic nucleus (vmh)				++	++	++	++	++
Ventral posterior thalamic nucleus (vpm)				+	++	++	+/−	−
Dorsomedial thalamic nucleus (dmt)				−	+/−	+	+	+
Amygdaloid area (AA)				+	+	+	+	++
CA3 field of the hippocampus, posterior levels (Py)				−	−	−	+++	+++ +
Pyramidal cells of the hippocampal formation				−	−	−	+/−	+/−
Granular cells of the dentate gyrus				−	−	−	+/−	+/−
Septal area				−	−	−	−	+/−
Piriform area				−	−	−	−	+/−
Choroid plexus (ChPl)				+++	+++	+++	++	+
Medulla (Me)				−	−	+/−	+/−	+
Dorsal cochlear nucleus (cod)				−	−	+	+/−	−
External granular layer of the cerebellum (EGL)						+++	++	−
Internal granular layer of the cerebellum (IGL)						−	+	−
Differentiating field of the olfactory bulb				++	++	++	+	+
Accessory olfactory bulb (differentiating field)							++	++
Nerve of olfactory bulb							+	+/−
Mitral cell layer of the olfactory bulb (Mi)							+/−	+
Anterior hypothalamic area (AH)							+	++
Posterior hypothalamic nucleus (PH)							+	++
Lateral hypothalamic nucleus (LH)							+++	++
Purkinje cells (Pj)							++	+

[a] +++ +, intense; +++, strong; ++, moderate; +, weak; +/−, very weak.

chemical stability of the cerebrospinal fluid, such as the choroid plexus in ventricles.

During brain postnatal development and until adulthood, *Zac1* was strongly expressed in the arcuate nucleus, different hypothalamic and thalamic nuclei, amygdaloid nuclei and CA3 field of the posterior hippocampus (Fig. 3D–L

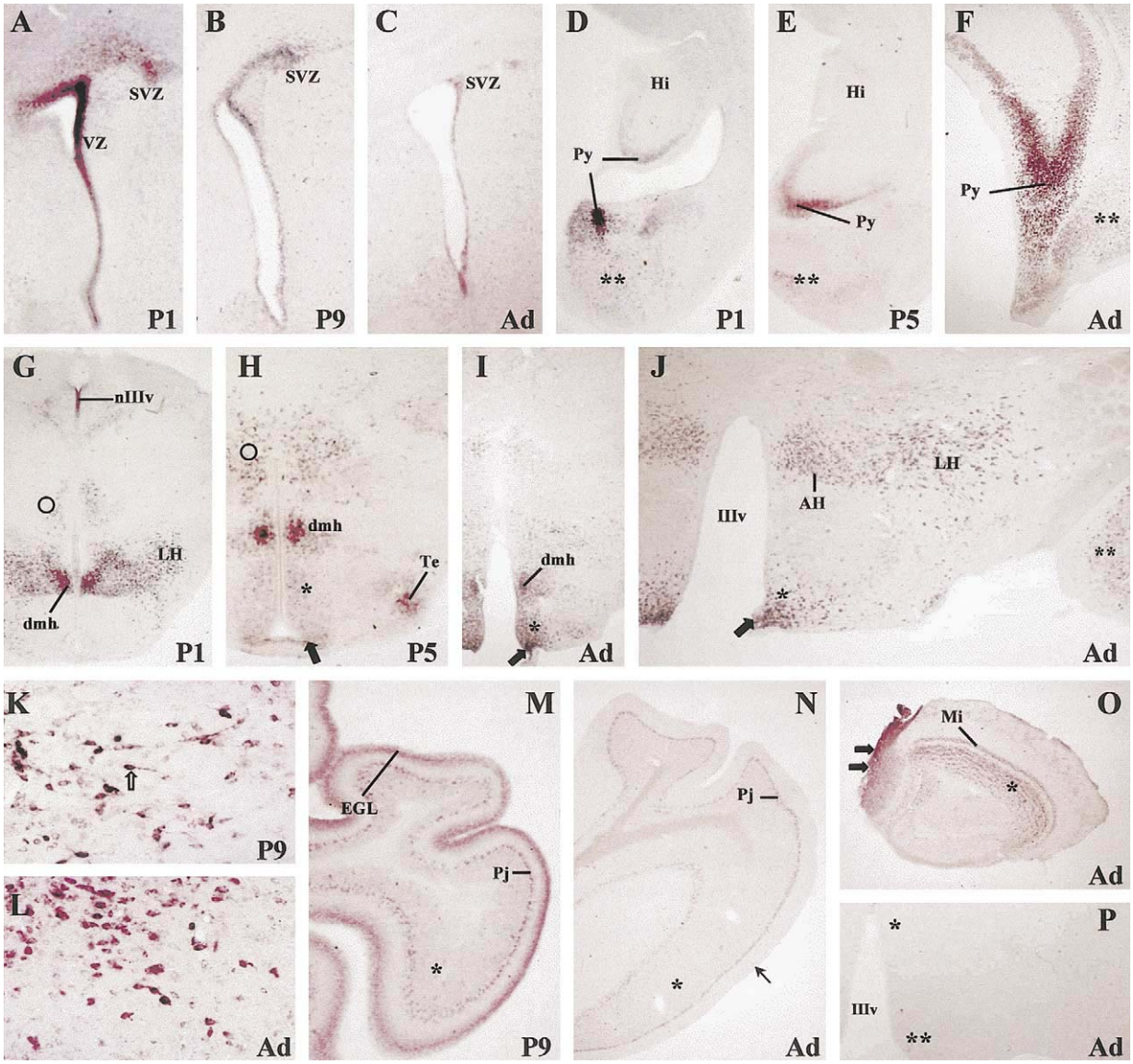

Fig. 3. Expression pattern of *Zac1* gene from postnatal day 1 (P1) until adult. (A–F) During this period, the expression of *Zac1* decreases within ventricular and subventricular zones (VZ and SVZ, respectively) while it increases in regions of posterior brain such as in pyramidal cells (Py) of the CA3 field of the hippocampus (Hi) and the amygdaloid area (double asterisk). Moreover during these postnatal stages, the label is found in some hypothalamic nuclei, begin well-defined in adult (G–J). Thus, as development proceeds *Zac1* expression is increasing in the arcuate nucleus (solid arrow), anterior hypothalamic area (AH) and posterior hypothalamic nucleus (circle) while a decrease is revealed in lateral (LH) and dorsomedial hypothalamic (dmh) nuclei. However, in the ventromedial hypothalamic nucleus (asterisk) the expression is maintained as in embryonic stages, while a rapid decrease is seen in the neuroepithelium of the third ventricle (nIIIv in G). By P5 (H) moderate staining is found in terete hypothalamic nucleus (Te). (K,L) These microphotographs shows the morphological features of *Zac1* positive cells which are located in the lateral hypothalamic nucleus (LH). (K) At P9 the cells within LH reveal typical features of migrating cells (arrow). However, at later stages of development until adult (L) these cells in LH acquire the morphology of mature cells. At P9, strong label is observed in the cerebellum (M), especially in the external granular layer (EGL) and Purkinje cells (Pj). However, a weaker label than before is found in the internal granular layer (asterisk). (N) In the adult cerebellum, the levels of *Zac1* mRNAs are maintained in Purkinje cells (Pj), however a decrease is seen in the internal granular layer (asterisk), disappearing in EGL (arrow) (O) During postnatal development and in the adult, the expression pattern in the olfactory bulb is constant. Thus, a moderate label is found in mitral cell layer (Mi) and in differentiating field of the olfactory bulb (asterisk), while label is stronger in other differentiating field of the olfactory bulb, such as accessory (double arrow). (P) The sense probe of *Zac1* gene was used in adult mouse and no positive cells were detected in anterior hypothalamic area (asterisk) or in the ventromedial hypothalamic nucleus (double asterisk). Magnification: 22.5× (A,F), 31× (B), 27× (C), 17.4× (D,I), 14.6× (E), 15.7× (G,N), 18.9× (H,O), 29× (J), 79.5× (K), 76× (L), 38× (M) and 23.8× (P).

and Table 1). In the cerebellum, moderate expression was detected in the external granular layer (EGL) and Purkinje cells up to P9. However, from P9 onwards, the label in the EGL layer quickly decreased, whereas in Purkinje cells it was maintained (Fig. 3M,N). A lesser label was found in the subventricular zone.

The expression pattern of *Zac1* in the subventricular zone, cerebellum and hypothalamic-pituitary axis suggests

that the *Zac1* gene could be implicated in the cellular growth control, both in adult brain and along development. Besides, *Zac1* induces expression of the *PACAP$_1$-R* gene in pituitary gland (Spengler et al., 1997) and since this gene was highly expressed during early embryogenesis (Basille et al., 2000), like *Zac1*, the neurotrophic role of both genes during brain development is reinforced.

2. Methods

2.1. Subcloned

To prepare digoxigenin (DIG)-labeled RNA probes for in situ hybridization (in vitro transcription), a cDNA fragment (2790 bp long) of the *Zac1* gene (GenBank accession No. X95503) was subcloned into the *Bam*HI and *Hind*III sites of pBluescript SK+. Sequencing of the cloned fragments of the *Zac1* gene was performed with an ABI PRISM™ dye terminator cycle sequencer (PERKIN ELMER).

2.2. In situ hybridization

Fetal and postnatal animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed immediately and together with whole embryos were immersed in the same fixative solution for 24–48 h at 4°C. In situ hybridizations were performed on free-floating tissue sections and on whole embryos. Briefly, were rinsed in buffer solutions and treated with acid to increase the tissue permeability. After this treatment, the hybridizations were performed at 60–65°C. The hybridization solution containing formamide (50%), sodium saline citrate (SSC), sodium dodecyl sulfate (SDS), sheared salmon sperm DNA, yeast tRNA and *Zac1* riboprobes. After hybridizations, sections and embryos were washed in stringency solutions (with formamide, SSC and SDS) at 60–65°C and incubated with RNase A. Subsequently, they were rinsed in buffer solutions, blocked with normal goat serum, incubated with an alkaline phosphatase-labeled antidigoxigenin antibody (Roche) and revealed with NBT and BCIP solution.

Acknowledgements

We thank Drs Dietmar Spengler and Laurent Journot for providing the *Zac1* probe and for research support, Dr Casto Ramos for contribution to the in situ analysis, Fèlix Junyent for technical support and Robin Rycroft for linguistic advice. We would also like to thank Dr Rafael Romero for research support. This work was supported by grants from the Ministry of Education of Spain (Spanish Agency for International Cooperation: AECI99).

References

Basille, M., Vaudry, D., Coulouarn, Y., Jegou, S., Lihrmann, I., Fournier, A., Vaudry, H., Gonzalez, B., 2000. Comparative distribution of pituitary adenylate cyclase-activating polypeptide (PACAP) binding sites and PACAP receptor mRNAs in the rat brain during development. J. Comp. Neurol. 425, 495–509.

Kaufman, M.H., 1999. The Atlas of Mouse Development, 3rd revised edition, Academic Press Ltd, London.

Pagotto, U., Arzberger, T., Ciani, E., Lezoualc'h, F., Pilon, C., Journot, L., Spengler, D., Stalla, G.K., 1999. Inhibition of Zac1, a new gene differentially expressed in the anterior pituitary, increases cell proliferation. Endocrinology 140 (2), 987–996.

Pagotto, U., Arzberger, T., Theodoropoulou, M., Grubler, Y., Pantaloni, C., Saeger, W., Losa, M., Journot, L., Stalla, G.K., Spengler, D., 2000. The expression of the antiproliferative gene ZAC is lost or highly reduced in non-functioning pituitary adenomas. Cancer Res. 60 (24), 6794–6799.

Piras, G., El Kharroubi, A., Kozlov, S., Escalante-Alcalde, D., Hernandez, L., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Stewart, C.L., 2000. Zac1 (Lot1), a potential tumor suppressor gene, and the gene for epsilon-sarcoglycan are maternally imprinted genes: identification by a subtractive screen of novel uniparental fibroblast lines. Mol. Cell. Biol. 20 (9), 3308–3315.

Spengler, M., Villalba, M., Hoffmann, A., Pantaloni, C., Houssami, S., Bockaert, J., Journot, L., 1997. Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J. 16 (10), 2814–2825.

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DEVELOPMENTAL DYNAMICS 233:000–000, 2005

PATTERNS & PHENOTYPES

Zac1 Is Expressed in Progenitor/Stem Cells of the Neuroectoderm and Mesoderm During Embryogenesis: Differential Phenotype of the Zac1-Expressing Cells During Development

Tony Valente,* Fèlix Junyent, and Carme Auladell

Zac1, a new zinc-finger protein that regulates both apoptosis and cell cycle arrest, is abundantly expressed in many neuroepithelia during early brain development. In the present work, we study the expression of *Zac1* during early embryogenesis and we determine the cellular phenotype of the Zac1-expressing cells throughout development. Our results show that *Zac1* is expressed in the progenitor/stem cells of several tissues (nervous system, skeleton, and skeletal muscle), because they colocalize with several progenitor/stem markers (Nestin, glial fibrillary acidic protein, FORSE-1, proliferating cell nuclear antigen, and bromodeoxyuridine). In postnatal development, Zac1 is expressed in all phases of the life cycle of the chondrocytes (from proliferation to apoptosis), in some limbic γ-aminobutyric acid-ergic neuronal subpopulations, and during developmental myofibers. Therefore, the intense expression of *Zac1* in the progenitor/stem cells of different cellular lineages during the proliferative cycle, before differentiation into postmitotic cells, suggests that Zac1 plays an important role in the control of cell fate during neurogenesis, chondrogenesis, and myogenesis. *Developmental Dynamics 233:000–000, 2005.* © 2005 Wiley-Liss, Inc.

Key words: zinc-finger protein; apoptosis; neurogenesis; progenitor/stem cells; chondrogenesis; myogenesis; cell cycle arrest; GABAergic system; cell fate

Received 5 January 2005; Revised 19 January 2005; Accepted 22 January 2005

INTRODUCTION

Fu

Neurogenesis, the processes by which neural cells are generated, occurs through the production of postmitotic neurons and glial-like cells (oligodendrocytes and astrocytes) from neuroepithelial stem cells localized in the ventricular zone of the neural tube by progressive steps of cell division, cell cycle arrest, differentiation, and migration, as well as the natural developmental death of the neural precursors (Roth and D'Sa, 2001; Moskowitz and Lo, 2003). Growing evidence indicates that cell cycle arrest and neurogenesis are highly coordinated and interactive processes, governed by cell cycle genes and neural transcription factors, which control the correct positional identity of the neural cells from the stem/progenitor cells (Johnson et al., 1990; Akazawa et al., 1995; Ben-Arie et al., 1996; Ma et al., 1996; Edenfeld et al., 2002). Several transcription factor expressions contribute to a series of genetic and cellular processes that eventually produce a fully differentiated brain. Zac1, a new zinc-finger protein, is known to be capable of inducing G1 cell cycle arrest and apoptosis, and Zac1 inhibits tumor cell proliferation in vitro and in vivo (Spengler et al., 1997). Thus, Zac1, together with p53, has the singular capacity to simultaneously control these two fundamental cellular mechanisms (Bates and Vousden, 1996; Spengler et al., 1997). The gene encoding Zac1 is located in human and mouse chromosomal regions that are maternally imprinted (Piras et al., 2000; Kamiya et al., 2000; Smith et al., 2002). Zac1

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DOI 10.1002/dvdy.20373
Published online 00 Month 2005 in Wiley InterScience (www.interscience.wiley.com).

was found to induce expression of the PACAP type 1 receptor in multiple transfected cell lines (Hoffmann et al., 1998). This receptor is the most potent known insulin secretagog and an important mediator of autocrine control of insulin secretion in the pancreatic islet (Yada et al, 1998; Filipsson et al., 1999). *Zac1* is mainly expressed in the brain and pituitary gland (Spengler et al., 1997; Valente and Auladell, 2001). In early mouse brain development, *Zac1* is strongly expressed in several neuroepithelia, and in adulthood, *Zac1* is moderately expressed in some limbic structures, such as hypothalamus, amygdala, hippocampus, and olfactory bulb (Valente and Auladell, 2001). Nevertheless, their biological role in neural development and in mature brain remains uncertain. Furthermore, *Zac1* and *hZAC* are highly expressed in somites and limbs during early embryonic stages (Piras et al., 2000; Valente and Auladell, 2001; Tsuda et al., 2004), as well as in cartilage primordium sites (Valente and Auladell, 2001; Tsuda et al., 2004) and skeletal muscle (Arima et al., 2000; Ma et al., 2004). In adult tissues, *hZAC* (human homolog of *Zac1* mouse gene) and *Zac1* are expressed to a low extent in skeletal muscle and bone marrow (Varrault et al., 1998; Piras et al., 2000). The development of skeleton (bone and cartilage) and skeletal muscle in vertebrates begins during early embryonic stages, and their mesodermal origin is interrelated with the ectodermal origin of the nervous system (Bailey et al., 2001; Yang and Karsenty, 2002). Some transcription factors are predominantly expressed in the early phases of the skeletal and skeletal muscle formation, and their functions are restricted to controlling their cell proliferation and differentiation (Yang and Karsenty, 2002). The cartilage and bone cells and the majority of skeletal muscle cells arise from the somites, which are situated adjacent to the neural tube and notochord and respond to signals from the notochord (Bailey et al., 2001). In the present study, our aim was to report a detailed analysis of *Zac1* expression during early embryonic stages of development and to characterize Zac1-expressing cell populations during development, by immunohistochemistry and in situ hybridization techniques.

RESULTS AND DISCUSSION

Zac1 Expression in Neural Cells

Embryonic stages.

At E12–E14, strong *Zac1* expression was detected within the germinal matrices and weak detectable signals were found in the differentiating fields (Figs. 1, 2; Table 1). In the nervous system, strong expression was observed within active proliferating fields, such as in the neural tube and neural layer of retina, as well as in several neuroepithelia (telencephalic vesicles, third and fourth ventricles, and optic recess). Between E12 and E16, in the innermost cell layer surrounding the lumen of the neural tube and in the ventricular zone of telencephalic vesicles, some *Zac1*-expressing cells were colocalized with Nestin (Fig. 3B), which recognized an intermediate filament protein (class type VI) expressed in some stem/progenitor cells in the most primitive neuroepithelium, or with glial fibrillary acidic protein (GFAP), which reacted with the class III intermediate filament in both undifferentiating and differentiating astroglia, as well as in stem/progenitor neural cells (Doetsch et al., 1999; Laywell et al., 2000; Alvarez-Buylla et al., 2001; Fig. 3I; Table 2). In addition, many ventricular and subventricular cells that expressed *Zac1* gene were colocalized with the proliferative marker proliferating cell nuclear antigen (PCNA), which begins to accumulate during the G1 phase of the cell cycle, becomes most abundant during the S phase, and declines during the G2/M phase (Kurki et al., 1988; Fig. 3F; Table 2). Moreover, some *Zac1*-expressing cells were colo-

Fig. 1. Expression of *Zac1* gene in whole mouse embryos at embryonic day (E) 12 (A–E, in toto), E12.5 (F–M, in sections), and E14 (N–U, in sections) by in situ hybridizations. **A–E:** At E12, intense expression is observed in telencephalic vesicles (tv in A), neural retina (nr in A), the vascular system of the hindbrain (black arrows in A), lateral dermomyotomes (white arrowheads in B), the ventral part of the somites and sclerotome (white arrows in B,C), the cartilage primordium of humerus (hmc in D), and the cartilage condensation of the digital zone of the forelimb (cc in E), as well as the vascular cells that surround the cartilaginous digits of the interdigital zone (open arrows in E). **F–M:** At E12.5, intense expression is detected in the inner (neural) layer of retina (nlr in F), extrinsic ocular muscle (eom in F), optic recess (or in F), neopallial cortex in the region of the future olfactory lobe (nc in F), ventricular zone of the telencephalic vesicles (vz in G), vascular cells of the interdigital zone (idiz, open arrows in H), and humeral (hmc in H,K and double asterisk in J), radial (rmc in H), phalangeal (double asterisk in H), metacarpal (mc in H) and carpal (single asterisk in H) mesenchymal condensations, as well as in the dorsal premuscle mesenchymal condensation (dmc in H, solid arrows in K and single asterisk in J), cartilage primordium of body vertebrae (cpbv in L), and mantle region of spinal cord in lumbosacral region (mrsc in M). In addition, moderate expression was observed in the neural tube (nt in I,K), in the notochord (n in K) and in the entrance of esophagus (eop in L). **N–U:** At E14, intense expression is observed in the neural tube (nt in N,P,Q,S,U), notochord (n in N,S), proliferating muscle cells (mpc in N,Q,R,S), muscle cells surrounding the ribs (mr in N), muscle cells that surround the femur (asterisk in N), radial nerve (rn in N), cartilage primordium of femur (cpf in N), cartilage primordium of ribs (cpr in N,P,U), cartilage primordium of neural arch (cpa in N), masseter muscle (mm in O), Meckel's cartilage (mc in O), cartilage primordium of acromion of left scapula (cpas in O), primordium of lower molar tooth (pmt in O), dorsal surface of tongue primordium (dst in O), tooth primordium (tp in O,T), primordium of shoulder joint (psj in Q and R), primordium of joint between tubercle of rib and neural arch of its own vertebra (pj in Q), muscle cells of the limb (mcl in R), cartilage primordium of humerus (cph in R), as well as in the hypothalamus (h in O,T), mamillothalamic tract (mta in O,T), ventricular zone of the telencephalic vesicles (vztv in O,T), neuroepithelium of the third ventricle (nIIIv in O,T), and the plexus choroids (pch in P). In addition, moderate expression is detected in the cartilage condensation primordium of sacral vertebral body (centrum, ccs in U) and aorta area (aa in N), whereas weak expression is observed in the esophagus (o in N), metanephros (definitive kidney, mn in S), and the muscle cells that surround the ribs (mcr in U).

Fig. 2. Immunolocalization of Zac1 protein in the development of the nervous system. **A,C:** Some migrating Zac1-expressing cells (black cells) are leaving neural tube (nt) region at embryonic day (E) 12. **B,D:** Zac1-expressing cells in the germinative layers (ventricular, vz, and subventricular zone, svz) of the telencephalic vesicles (B) and in the hypothalamic area (h in D) at embryonic E14. **E–G:** At E16, many Zac1-expressing cells are found in the ventricular (vz) and subventricular (svz) zones of the lateral ventricles (LV in E) and third ventricle (IIIv in F). G: In addition, in the spinal cord (sc), there is a clear layer with Zac1-expressing cells. Zac1-expressing cells are in black in A–D, and in brown in E–G.

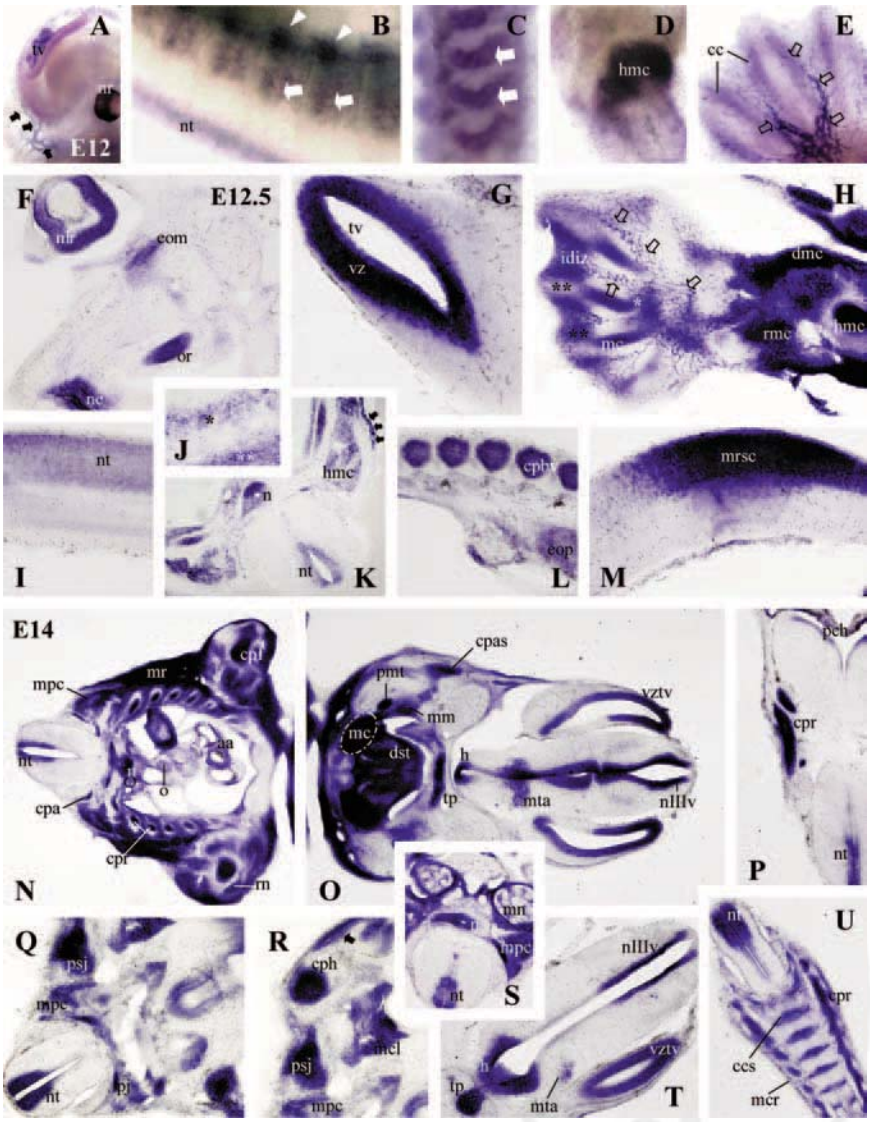

Fig. 1.

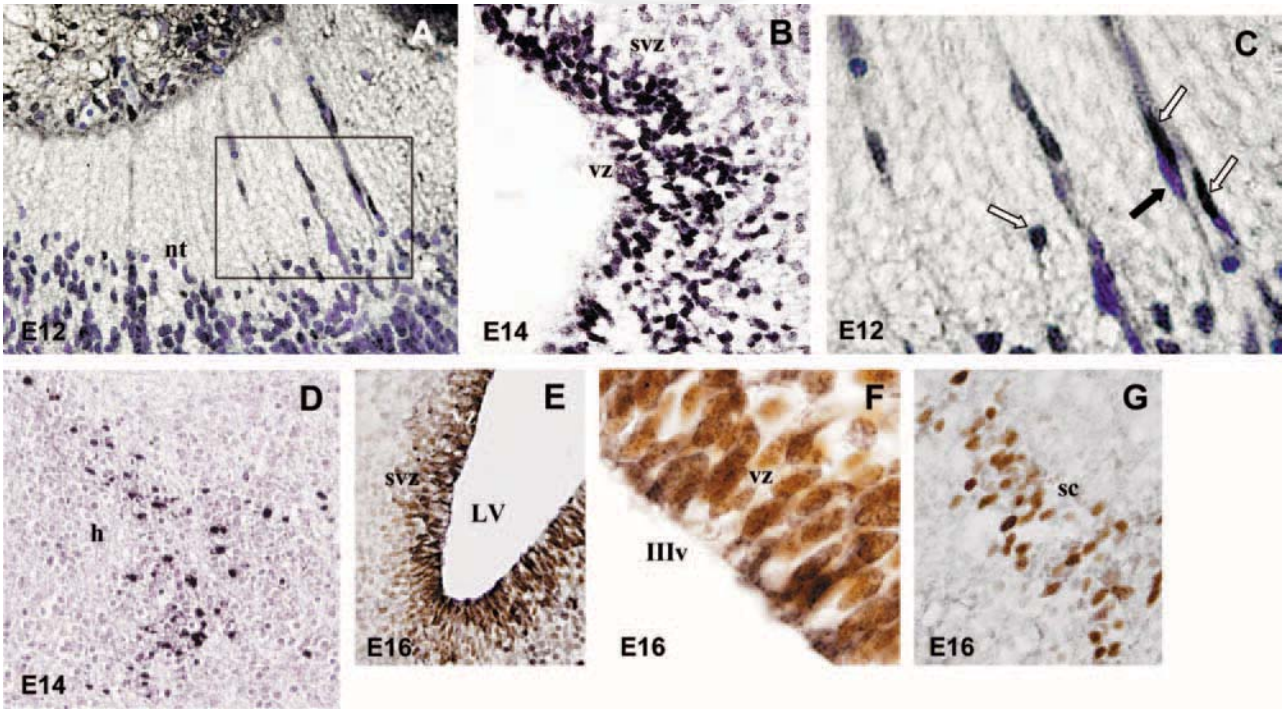

Fig. 2.

calized with FORSE-1 within of the ventricular zone and neural tube (Table 2). FORSE-1, an antibody that labels regionally restricted subpopulations of progenitor cells in the embryonic central nervous system and that recognizes the Lewis-X (LeX) carbohydrate epitope, shares expression boundaries with neural regulatory genes and may be involved in patterning of the neural tube by creating domains of differential cell adhesion (Allendoerfer et al., 1999).

With prosencephalon and mesencephalon development, variable levels of *Zac1* gene expression were found in the presumptive olfactory bulb, thalamus, hypothalamus, and amygdaloid area (limbic areas), where a few of these cells were colocalized with β-tubulin (C-terminus of the beta-III isoform of tubulin, which is known to be a specific marker of immature neurons; Tables 1, 2). Furthermore, some *Zac1*-expressing cells that leave the third ventricle present a migratory cellular route toward the future mamillothalamic tract and dorsomedial and lateral hypothalamic nuclei (Valente and Auladell, 2001). Colocalization studies (Table 2) have demonstrated that these *Zac1*-expressing cells are positively labeled to the calcium-binding protein calbindin D-28k or calretinin, as well as to Netrin-1 (Fig. 4A), which is a protein impli-

COLOR

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TABLE 1. *Zac1* Expression in Early Embryonic Tissues

Anatomic area	E10	E12.5	E14
Aorta region		+/−	+
Body wall (thoracic) overlying pericardial cavity	+	+	+/−
Cartilage condensation being primordium of sacral vertebral body		+	+
Cartilage primordium of acromion of left scapula		+	++
Cartilage primordium of body vertebras		+	++
Cartilage primordium of femur		+++	+++
Cartilage primordium of humerus		+++	+++
Cartilage primordium of neural arch		+	+
Cartilage primordium of phalangeal bone and metacarpal bone		+	++
Cartilage primordium of pubic bone		+	++
Cartilage primordium of radius		+	++
Cartilage primordium of ribs		+	+++
Cartilage primordium of thoracic vertebral body		++	+++
Caudal part of medulla oblongata		++	++
Conective tissue of the spinal cord		+/−	+
Choroid plexus		+	++
Diencephalon (dorsal and ventral thalamus)		++	++
Diencephalon (hypothalamus)		+++	+++
Digital interzone			+/−
Dorsal surface of tongue		++	+++
Ependimal layer			++
Epithalamus		++	+++
Epithelium of the Rathke's pouch/lumen of anterior lobe of pituitary	+	++	+++
Extrinsic Ocular Muscle		+/−	+
Forelimb bud	+	+	
Infundibular recess/Infundibulum (future pars nervosa)	+	++	+++
Inner (neural) layer of retina		+	++
Mantle region of spinal cord in lumbosacral region	+++	+++	++
Masseter muscle		+	++
Maxillary bone		+/−	+
Meckel's cartilage		++	+++
Metanephros (definitive kidney)			+/−
Middle region of clavicle, with early evidence of ossification			++
Nasal cartilage		+/−	+
Neopallial cortex in the region of the future olfactory lobe		+	++
Neural tube	+++	++	++
Neuroepithelium of forebrain region (telencephalic vesicles)	++	+++	+++
Neuroepithelium of hindbrain (aqueduct/fourth ventricle)	+/−	+	+
Neuroepithelium of midbrain region (third ventricle)	+	++	++
Notochord	+	++	++
Oesophageal region of foregut	+/−	+	
Oesophagus			+/−
Olfactory epithelium		+	++
Olfactory placode	+/−		
Optic nerve			+
Optic recess		+	+
Optic vesicle/optic stalk	+/−		
Ossification within cartilage primordium of rib			+++
Ossification within cartilage primordium of the humerus			+++
Primordium of follicle of vibrissa			+
Primordium of lower molar tooth			+++
Primordium of pancreas			+
Radial nerve			+
Region of optic chiasma		+	++
Segmental inter-zone, future localization of intervertebral disc			+
Skeletal muscle of the limbs		++	+++
Somite	++	++	
Tongue		++	+++
Tooth primordium			+++
Trachea	+/−	+/−	+
Umbilical vein	++	++	+

+/−, weak; +, moderate; ++, strong; +++, intense.

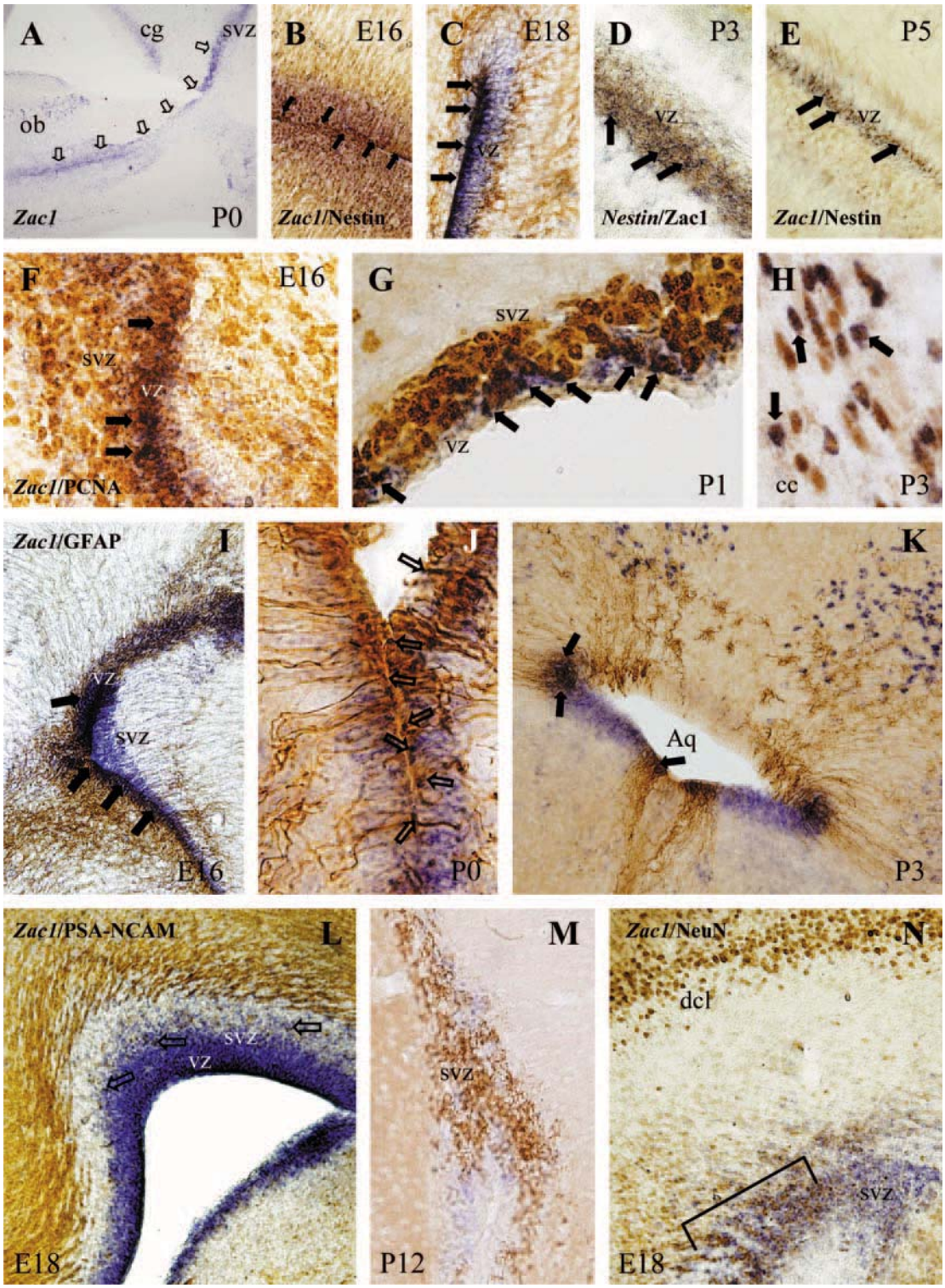

Fig. 3. Expression of Zac1 in the neural progenitors of the germinative layers during mouse embryonic and early postnatal development. **A:** In newborn mice, as in embryonic mice, the *Zac1* gene is expressed in the olfactory bulb (ob) and along the rostral migratory stream pathway (open arrows) from the subventricular zone (svz). In addition, some *Zac1*-expressing cells are found transitorily in the cingular cortex (cg). **B–E:** Some progenitor/stem cells in the ventricular zone (vz) coexpress the *Zac1* gene (in blue) and Nestin protein (in brown; solid arrows in B,C,E), as well as the *Nestin* gene (in blue) and Zac1 protein (in brown; solid arrows in D), during high mitotic phase of this germinative layer. **F–H:** In the embryonic and postnatal stages, *Zac1*-expressing cells (in blue) are colocalized with PCNA (in brown) in the vz and svz of the lateral ventricles, as well as (solid arrows in F,G) in many cells that leave the vz and start a migratory route along the corpus callosum (cc; solid arrows in H). **I–K:** Progenitor cells express GFAP immunolabel (in brown) in the neural germinative layers. Thus, in embryo (E16) and newborn mice (postnatal day [P] 0) the *Zac1*-expressing cells (in blue) in the vz are colocalized with GFAP (arrows in I and K). K: In the subsequent postnatal stages (P3), a noticeable decrease in the colocalization between *Zac1* and GFAP is observed (see the arrows in the neuroepithelium of the aqueduct of Sylvius, Aq). **L,M:** In the last stages of embryonic development, some *Zac1*-expressing cells (in blue color) among the subventricular cells differentiate and start to express PSA-NCAM (in brown; L). M: However, this coexpression is transitory, and at P12, no detectable colocalization is found in vz. **N:** At E18, the majority of *Zac1*-expressing cells (in blue) are in the vz and only some of them are in the svz, where they express neuronal markers such as NeuN (in brown). However, when these *Zac1*-NeuN–expressing cells leave the svz and migrate to the deeper cortical layer (dcl), the expression of *Zac1* is completely down-regulated.

COLOR

TABLE 2. Cellular Phenotype of Zac1-expressing Cells During Development

		Developmental Colocalization							
First Marker	Second Marker	E12	E14	E16	E18	P0-P3	P5-P9	P12	Anatomic areas
Zac1	Nestin	+	+	+	+	+	+/−	+/−	VZ of several ventricular neuroepithelia; skeletal muscle; follicle of vibrissa
Nestin	Zac1			+	+	+/−	+/−	+/−	VZ of several ventricular neuroepithelia; skeletal muscle; follicle of vibrissa
EGFR1	Zac1			+	+	+	+/−	+/−	VZ of several ventricular neuroepithelia; callosum corpus
Zac1	FORSE-1	+	+	+	+/−	+/−	−		VZ of several ventricular neuroepithelia; skeletal muscle
PCNA	Zac1	+	++	++	++	+	+	+/−	VZ of several ventricular neuroepithelia; chondrocytes; perichondrium; follicle of vibrissa; vascular bone cells; neural layer of retina; skeletal muscle; nasal epithelium; callosum corpus
BrdU	Zac1		++	++	++	+	+	+/−	VZ of several ventricular neuroepithelia; chondrocytes; perichondrium; follicle of vibrissa; vascular bone cells; neural layer of retina; skeletal muscle; nasal epithelium; callosum corpus
ssDNA-Apostain	Zac1	+/−	+	+	+	+/−	−	−	VZ of lateral ventricles; vascular cells of interdigital zone; postmitotic chondrocytes
DNA fragment	Zac1	+/−	+	+	+	+/−	−	−	VZ of lateral ventricles; vascular cells of interdigital zone; postmitotic chondrocytes
Zac1	GFAP	+	++	++	+	+	+/−	+/−	VZ of several ventricular neuroepithelia
Zac1	PSA-NCAM		−	+/−	+	+	+/−	−	SVZ of several ventricles; RMS; thalamus
Zac1	Vimentin	+	+	+/−	+/−	+/−	−		SVZ of several ventricles
Zac1	B-tubulin	−	+/−	+/−	+	+	−	−	SVZ of several ventricles; RMS; hypothalamic and amygdaloid nuclei; olfactory bulb
Zac1	Netrin-1	−	+/−	+	+/−	−			hypothalamus; thalamus; skeletal muscle
Zac1	Ng2				−	−	−	−	—
Zac1	NeuN		−	+/−	+	+	++	+++	in all Zac1 positive areas, excepted in VZ
GAD65	Zac1		−	+/−	+	+	++	++	in all Zac1 positive areas, excepted in VZ, SVZ and brain stem
Zac1	Calretinin	−	−	+	+	+	+	+/−	olfactory bulb; marginal cortical layer; enthorinal cortex; hypothalamus
Zac1	Calbindin	−	−	+	+	++	+++	+++	in all Zac1 positive areas, excepted in VZ
Zac1	Parvalbumin	−	−	+	+	+	+	+	amygdala; CA3 posterior hippocampal regio; hypothalamus
Zac1	Somatostatin		−	−	+/−	+/−	+	+	hypothalamus; amygdala; CA3 posterior hippocampal regio
NPY	Zac1		−	+/−	+	++	++	++	hypothalamus; amygdala; CA3 posterior hippocampal regio
Zac1	TH				+/−	+	+	+/−	hypothalamus; thalamus; Zona incerta; brain stem; spinal cord
Zac1	LHRH					+/−	+	+/−	thalamus; olfactory tract; medial eminence
Zac1	CFR					−	+	+/−	medial eminence; amygdala
Zac1	GluR1					−	−	−	—
Zac1	GluR2/3					+/−	+	+/−	deeper cortical layers; CA3 posterior
Zac1	GluR4					−	−	−	—
Zac1	GluR5/6					−	−	−	—

−, none; +/−, few; +, some; ++, many; and +++, all Zac1-expressing cells are colocalized.

cated in the chemotropic and outgrowth-promoting activities of plate cells to guide commissural axons toward the ventral midline and regulate axon pathway formation and neuronal position during hypothalamic development (Deiner and Sretavan, 1999). At the same time, only very few *Zac1*-expressing cells are colocalized with F4

NeuN (nuclear neuron-specific protein, which is present in mature neuronal cells of the central nervous system [CNS] and peripheral nervous system; Table 2). Therefore, the *Zac1*-expressing cells that begin to express neuronal markers (calbindin and calretinin) are not completely differentiated and for this reason they display a much lower reactivity for NeuN.

In subsequent stages (embryonic day [E] 16–E18), there is an increase in *Zac1* gene expression in the subventricular zone and, consequently, the number of *Zac1*-expressing cells that leave this area and begin their differentiating process, especially in the third ventricle. Thus, in the outer cell layer of the subventricular zone of the neuroepithelia, we observed some *Zac1*-expressing cells that colocalized with differentiating neuronal markers (Table 2), such as PSA-NCAM (polysialic acid neural cell adhesion molecule recognizes the adhesion of neuronal cells; Fig. 3L) and β-tubulin. However, the number of *Zac1*-expressing cells that colocalized with NeuN was still low (Fig. 4N; Table 2) and was restricted to the amygdaloid and hypothalamic nuclei. At E16, in the limbic areas, some *Zac1*-expressing cells immunoreacted with several calcium-binding protein markers, such as calretinin, parvalbumin, and calbindin (Figs. 4B,E–H; Table 2), and with the interneuron markers (Table 2), such as the glutamic acid decarboxylase (GAD65), responsible for the conversion of glutamic acid to gamma-aminobutyric acid (GABA), and the neuropeptide Y (NPY). In addition, some cells of the spinal cord expressed GAD65 and tyrosine hydroxylase (TH), which is a catecholinergic marker, and as determined by double-stain techniques, these cells are colocalized with Zac1 protein.

At E18, very few *Zac1*-expressing cells are colocalized with somatostatin (interneuron subpopulations) in the hypothalamic and amygdaloid nuclei and with TH in the brainstem and spinal cord (Table 2). Nevertheless, the coexpression of *Zac1* with TH or somatostatin was more evident during postnatal development (Fig. 4K; Table 2).

Therefore, in the embryonic stages, many cells with high mitotic activity express the *Zac1* gene before entering into a differentiation process, which suggests that Zac1 could regulate the proliferation/mitotic phase of the neurogenesis process, probably by the regulation of cell cycle arrest (Spengler et al., 1997). Zac1 is a transcription factor capable of inducing cell cycle arrest in the G1 phase and, by an independent pathway, of inducing apoptosis (Spengler et al., 1997). Recent studies have shown high rates of apoptosis in the neural progenitors during embryogenesis (D'Sa-Eipper and Roth, 2000; D'Sa-Eipper et al., 2001; Zaidi et al., 2001). In this way, we tried to colocalize the Zac1-ventricular cells with apoptotic markers (like ssDNA-F7-26-Apostain or DNA fragmentation), and we observed that the number of Zac1-ventricular cells that die by apoptosis during the proliferation of the neural progenitors is very low (data not shown). Curiously, these apoptotic Zac1-ventricular cells only appear in the lateral ventricles, whereas they are nonexistent in the third and fourth ventricles. Consequently, Zac1 might also be implicated in the apoptotic mechanisms that occur in restricted neural progenitor subpopulations. However, the high expression of *Zac1* gene in non-apoptotic neural progenitors of the ventricular zone and their down-expression in differentiated neural cells reinforces the hypothesis that Zac1 might be more involved in the cell cycle arrest of the neural progenitors than in the apoptotic process of these progenitors.

Proliferative areas in postnatal stages.

In newborn animals (at postnatal day [P] 0/P1), a general decrease in the *Zac1*-expressing progenitor cells was observed, although a restricted *Zac1*-expressing progenitor pool is maintained within of the ventricular zone and in the rostral migratory stream (RMS) of the olfactory system (Fig. 3A; Table 2). Thus, a very fine line of ventricular cells expressed *Zac1* gene and colocalized with *EGFR-1*, *Nestin*, GFAP, 5′-bromo-2′-deoxyuridine (BrdU), or PCNA (Fig. 3G,J; Table 2). However, when these progenitor cells begin a differentiation process along the RMS, the *Zac1* gene is progressively down-regulated and the *Zac1*-expressing cells are colocalized with PSA-NCAM and β-tubulin. In the olfactory bulb, The *Zac1*-expressing cells are completely differentiated and located in the several olfactory layers, mainly in the mitral and granular layers. The expression pattern of the *Zac1* gene in the RMS and olfactory system was maintained in the adult brain, although at lower levels (data not shown).

Postnatal Zac1-interneuron subpopulations.

The calcium-binding proteins parvalbumin, calretinin, and calbindin D-28k are markers of different classes of GABAergic interneurons and display different functions (Yan et al., 1995). However, calbindin is not an exclusive marker of interneurons, because it is also in certain glutamatergic neuron populations, such as in the granular cells of the dentate gyrus (Baimbridge, 1992). At P0–P3, many *Zac1*-expressing cells leave the third ventricle and acquire a differentiation character, since they are labeled positively for neuronal markers (β-tubulin or NeuN) and negatively for macroglial markers (GFAP or Ng2, which is an immature oligodendrocyte marker). Many of these cells in the limbic areas (hypothalamic, amygdaloid, olfactory bulb, and hippocampus) were labeled for calbindin (Fig. 4C; Table 2), whereas some of them in the olfactory bulb were labeled for calretinin (Table 2). In addition, some *Zac1*-expressing cells in the hypothalamus and amygdala were labeled for parvalbumin (Table 2). In the subsequent stages, we observed a decline in the number of *Zac1*/calretinin-expressing cells; whereas a noticeable increase in the number of the *Zac1*/calbindin-expressing cells is found in the limbic areas. In the adult, all *Zac1*-expressing cells also expressed calbindin protein. In addition, some cortical cells in the cingular area expressed the *Zac1* gene transiently during the first postnatal week. These cells also displayed immunoreactivity to the calbindin protein. Moreover, the *Zac1*/parvalbumin-expressing cells seemed to be more stable throughout postnatal development (Table 2).

In neonates, some Zac1-expressing cells are colocalized with NPY, GAD65, and somatostatin, concretely in the limbic areas (Fig. 4I,J; Table 2);

however, it is during postnatal stages that these cell subpopulations represent the majority of Zac1-expressing cells. GAD65 are present in almost all GABAergic neurons (Soghomonian and Martin, 1998). GAD is the biosynthetic enzyme for GABA, the major inhibitory neurotransmitter in the CNS and is strongly expressed in brain development (Lauder et al., 1986; Van Eden et al., 1989). The high cellular levels of both GAD and GABA during early embryogenesis suggests a signalling role during development (Katarova et al., 2000), whereas in mature neural circuits they have a predominant neuronal inhibitory role (Fonnum and Storm-Mathisen, 1969). During embryogenesis, *Zac1* gene is expressed in ventricular progenitor/stem cells. With the maturation of the nervous system, these Zac1-expressing cells begin a differentiating process, in which they start to express GAD65 until adulthood. Therefore, all these data together show that Zac1-expressing cells are mainly GABAergic neurons and suggest that Zac1 may be involved in the neuronal differentiation of these GABAergic neuron subpopulations within of the limbic system.

In addition, a few *Zac1*-expressing cells in the hypothalamic and brainstem areas coexpress TH (Table 2). Colocalization studies confirm the heterogeneity of the *Zac1*-expressing interneurons, which are nonoverlapping: GABAergic neurons colocalize with somatostatin, NPY, or calcium-binding proteins in the limbic areas; catecholinergic neurons express TH in the brainstem and zona incerta, as well as in the spinal cord. These catecholinergic neurons are probably dopaminergic neurons, because the *Zac1* gene is dynamically regulated after in vivo induction of D1 and D2 dopaminergic receptors by the administration of selective agonists and antagonists (Valente and Auladell, unpublished results). In this way, catecholinergic neurons are implicated in the regulation of hormone release in the pituitary gland (Gonzalez et al., 1989; Dorton, 2000). The *Zac1* gene is intensely expressed in arcuate nucleus (Valente and Auladell, 2001), which is responsible for the production and release of many hormonal factors in the pituitary gland by the axonal pathway that crosses the eminence media (Szentagothai, 1969). Previous reports (Pagotto et al., 1999, 2000) showed a coexpression of *ZAC* gene (human homolog of *Zac1*) and hormonal factors in the pituitary gland. In this way, many *Zac1*-expressing projections in the arcuate nucleus and eminence media are colocalized with some release hormonal factors, such as LHRH and CFR, during postnatal development (Fig. 4L–O; Table 2). All together, these findings suggest that Zac1 is implicated in the maturation of the neuronal endocrine hypothalamic system.

Postnatal Zac1-glutamatergic cells.

During postnatal developmental stages, the *Zac1*-expressing cells are not colocalized with glutamatergic receptors, except for a few cells in the deeper developmental cortical layer that are transitorily immunoreacted for GluR2/3. However, in adult mice, very few *Zac1*-expressing cells are colocalized with GluR2/3 receptors in the hippocampal formation (Table 2). Additionally, *Zac1* is up-regulated in the granular layer of the dentate gyrus of the hippocampus after seizures induced by KA (Valente et al., 2004). These granular cells are essentially glutamatergic, although recent studies also show the presence of GAD67 in some granular cells (Schwarzer and Sperk, 1995; Sloviter et al., 1996). Thus, some

Fig. 4. Characterization of *Zac1*-expressing cells in the hypothalamic area. **A:** At embryonic day (E) 14, the *Zac1*-expressing cells (in blue) surround the third ventricle starting a migratory route toward hypothalamic region and during this early process they coexpress Netrin-1 (in brown). **B,C:** All the *Zac1*-expressing cells that leave the third ventricle are colocalized with calbindin (in brown; B), and this coexpression is maintained throughout adulthood in the hypothalamic (h) and amygdaloid areas (C). **D–H:**In contrast, only a few *Zac1*-expressing cells (in blue) of this migratory route in the hypothalamic area (h) are colocalized with calretinin (solid arrows in D–F) and parvalbumin (solid arrows in G,H). I,J: During the establishment of the hypothalamic nuclei between E18 and P5, the neuropeptide Y (NPY) -expressing cells (in blue; I) and GAD65-expressing cells (in blue; J) are colocalized with most of the Zac1 protein cells (in brown). **K:** In the postnatal stages, some *Zac1*-expressing cell subpopulations (in blue) are colocalized with Somatostatin (in brown), concretely in the CA posterior region of hippocampus, hypothalamus, and amygdala. **L:** At postnatal day (P) 9, Zac1 protein is detected in many neuronal cells and axonal projections of the arcuate nucleus and eminence media, respectively. **M–O:** Many *Zac1*-expressing cells (in blue) in arcuate nuclei (arc) send their terminations to the eminence media (em) at P9, where they are colocalized with CFR (solid arrows in N). At P3, the Zac1 projections are not actively mature, and the colocalization with CFR (M) and LHRH (O) is much lower.

Fig. 5. Immunolabeling for Zac1 in chondrogenic sites of the bone during development. **A:** At embryonic day (E) 14, in the ribs, Zac1 protein (in black) is found in high levels in the resting (CMMA, chondrocytes with moderate mitotic activity) and proliferating (CHMA, chondrocytes with high mitotic activity) chondrocytes and in low levels in the postmitotic chondrocytes (PMC). No detectable immunoreaction is found in bone (Bn). In addition, some Zac1-expressing cells are found in the perichondrium (solid arrows) as well as in the muscle cells (open arrowheads) that surround the cartilage anlage. **B–G:** At E16, Zac1 protein is found in all developing cartilaginous sites, especially in the limbs (B) and the ribs (D–G). All Zac1-expressing chondrocytes (in brown) in the CHMA are in proliferation, because they are colocalized with proliferating cell nuclear antigen (PCNA; in black; solid black arrows in D), whereas some of the Zac1-expressing chondrocytes in CMMA are not in proliferation (only labeled for Zac1, solid white arrows in D). E,F: Colocalization studies with Zac1 (in brown) and ssDNA-F7-26-Apostain (in black) show that some chondrocytes are double labeled in the postmitotic zone surrounding the bone site (solid white arrows in E), whereas no detectable colocalization is found in the CHMA and CMMA areas or in the perichondrium (solid black arrows in F) and muscle connective cells (arrowheads in F). G: The expression of Zac1 (in black) in chondrogenic sites is maintained in postnatal stages included the craniofacial cartilages. The sections in A–C and G are counterstained with methyl green–pyronin.

Fig. 6. *Zac1* expression in muscle cells during embryonic development. **A–C:** Some *Zac1*-expressing skeletal muscle cells (in blue) are coexpressed with the progenitor/stem cell marker Nestin (in brown) in the tail (A), limbs (B), and craniofacial areas (C). **D,E:** Some *Zac1*-expressing skeletal muscle cells (in blue) are colocalized with the proliferating marker proliferating cell nuclear antigen (PCNA; solid arrows in D) and with migrating marker Netrin-1 (solid arrows in E), in the same areas described above. **F:** The immunolabeling for Zac1 protein is found in the same areas described for the *Zac1* gene. **G,H:** As the development proceeds, the *Zac1* expression is maintained in the skeletal muscle cells (in blue) and these cells colocalize with progenitor markers such as FORSE-1 (solid arrows). E, embryonic day.

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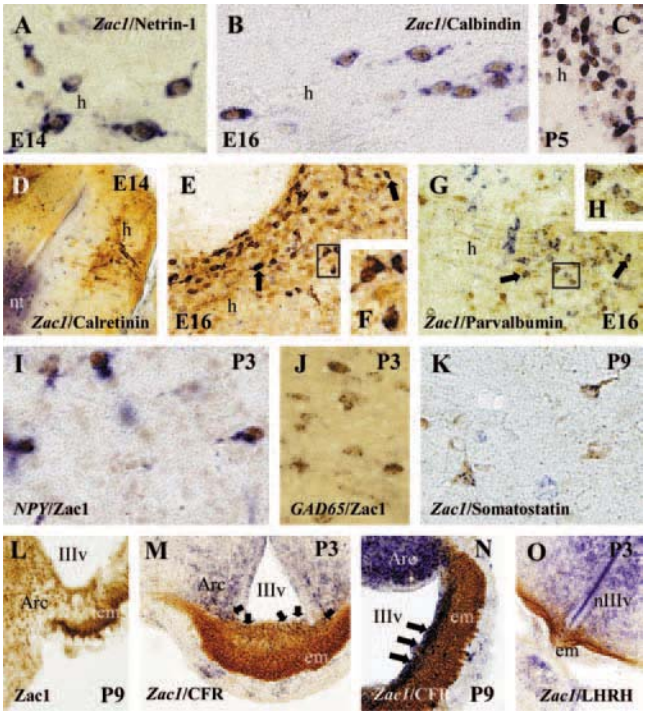
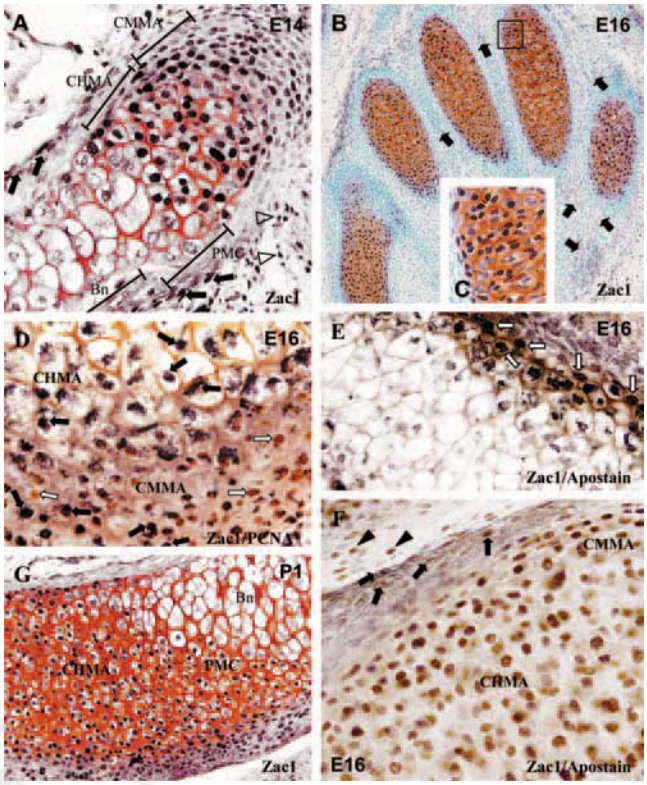


Fig. 4.



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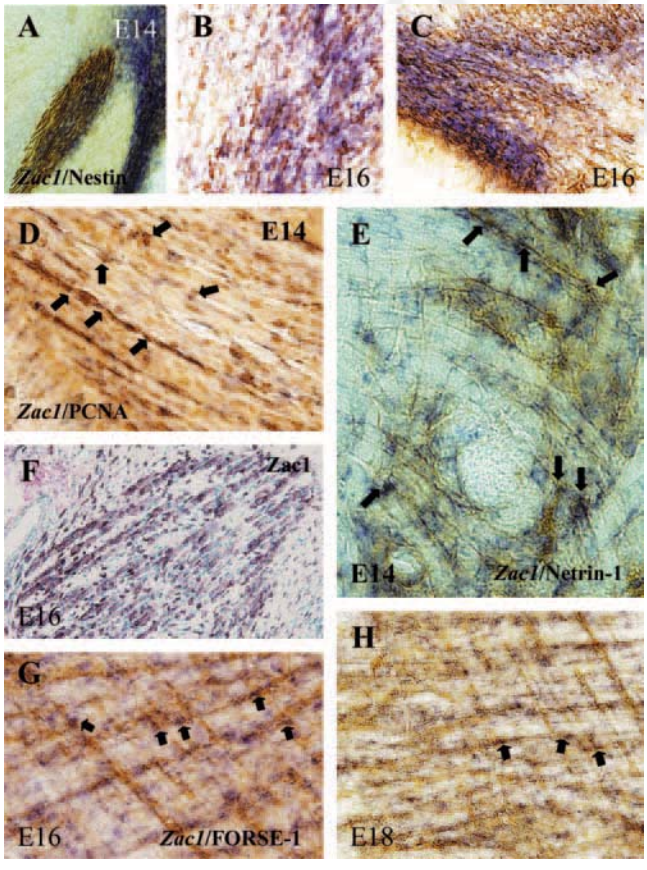

Fig. 6.

glutamatergic cell subpopulations of the hippocampal formation express *Zac1* during development and after injury, suggesting the involvement of Zac1 in the early plasticity processes related with excitatory neurons.

Taken together, these developmental results suggest that, in the CNS, there are several subpopulations of neural progenitor cells that express *Zac1*. Thus, in the lateral ventricles, Zac1 is expressed in the progenitor/stem cells of the ventricular and subventricular zones. However, if the new neural cells leave the ventricular zone and migrate to the cortical layers, the expression of *Zac1* gene in these cells is completely down-regulated, whereas if the new neural cells follow the RMS, the expression of *Zac1* is weakly down-regulated and persists in all of the olfactory system, even in adult stages. Moreover, Zac1 regulates the expression of the PACAP type 1 receptor gene (Hoffmann et al., 1998), and PACAP is implicated in the regulation of the development of the neuronal and glial precursors, because PACAP guides the transition from cell proliferation to cell cycle exit (for review, see Waschek, 2002). Therefore, the ex-

pression of *Zac1* gene in the lateral neuroepithelia suggests an important coordination of the cell fate by Zac1 and PACAP, probably by the regulation of both proliferation and determination of the neural progenitor cells, as well as by the induction of the apoptotic process in restricted progenitor subpopulations. On the other hand, in the third and fourth ventricles, the expression of the *Zac1* gene appears in the neural progenitor cells as in the neural cells (essentially GABAergic interneurons) that leave the subventricular zone and migrate toward their final position in the hypothalamic and brainstem area, respectively, where *Zac1* gene is strongly expressed in the adult brain (Valente and Auladell, 2001). Therefore, Zac1 may play an important role in the differentiation of the several GABAergic subpopulatons.

Zac1 Expression in the Development of Vertebrate Skeleton

Positioning and patterning of the limb involves cellular interactions between the ectoderm surrounding the limb bud and the mesenchymal cells that form the core of the limb bud (Christ and Brand-Saberi, 2002). At E10–E12, *Zac1* gene is expressed in the dorsal region of the neural tube (neural crest cells; Fig. 1B,K), in the notochord (Fig. 1K), in the somites (Fig. 1B,C), and in the apical ectodermal ridge of the limb bud (Table 1).

At E12.5, intense *Zac1* gene expression is found within several cartilaginous sites of bones: in the craniofacial skeleton (such as Meckel's cartilage, tooth primordium, nasal cartilage, and maxillary bone), in the limb skeleton (such as radius, humerus, phalangeal, and metacarpal), and in the axial skeleton (such as ribs, body vertebrae, and thoracic vertebral body), as well as in many vascular cells of the limbs (Fig. 1C–E,H,L; Table 1). Immunohistological studies with Zac1 protein confirm the same expression pattern obtained with *Zac1* gene (data not shown). At E14.5, intense expression of *Zac1* gene is observed in cartilaginous and ossification bone sites (Fig. 1N–U; Table 1). However, at this embryonic stage, the immunolabeled for Zac1 protein is only detected in the chondrocytes and perichondrium cells, as well as in the vascular cells that surround the limb bones (Fig. 5A). At E16, the expression of *Zac1* gene decreases strongly in the ossification sites and no detectable expression is found at E18, whereas the expression in chondrocytes is maintained throughout postnatal stages (Fig. 5B–F) and in adulthood. Therefore, the *Zac1* gene is transitorily expressed in ossification bone sites, whereas its expression is permanent within cartilaginous sites.

Three embryonic lineages are responsible in the formation and development of vertebrate skeleton: neural crest cells give rise to the craniofacial skeleton; paraxial mesoderm cells (somites) form the axial skeleton (vertebrae column and ribs), the dermis of dorsal skin, and the skeletal muscle of the body wall and the limbs; and lateral plate mesoderm cells form the limb skeleton (Erlebacher et al., 1995; Olsen et al., 2000). Chondrocytes arise from these three lineages (for review see Shum and Nuckolls, 2002), migrate toward specific embryonic locations where the skeleton will develop, form a dense nuclei of condensations, and then the chondrocytes or osteoblasts differentiate (Hall and Miyake, 2000; DeLise et al., 2000). Thus, at early embryonic stages, in the clavicles and in the majority of facial bones, the cells of mesenchymal condensations differentiate directly into osteoblasts (membranous bone development), whereas in the rest of bones (limb skeleton, ribs, vertebrae, and so on), the cells in mesenchymal condensations differentiate into chondrocytes to create a cartilaginous anlage of the future bone (endochondral skeletal development; for review, see Shum and Nuckolls, 2002; Horton, 2003). Chondrocytes show a life cycle of proliferation, differentiation, maturation, and apoptosis. In accordance with the present results, *Zac1* is observed mainly in the endochondral skeletal development, in which they are detected in all cartilage primordia between E12 and E14, and where the expression of Zac1 in chondrocytes is differential, depending upon the cell cycle phase. Thus, four different classes of Zac1-chondrocytes are detected (Fig. 5A,E; Table 2): (1) the resting chondrocytes (quiescent) located within of the most remote region of the ossification zone, which express high levels of Zac1 and, consequently, show lower colocalization with PCNA or BrdU; (2) the proliferating chondrocytes located in the middle part of the chondrogenic site, which express high levels of Zac1, PCNA, and BrdU; (3) the differentiated chondrocytes in the most internal chondrogenic area (prehypertrophic chondrocytes), which express low-to-moderate levels of Zac1; and (4) the apoptotic hypertrophic chondrocytes located in the border of the ossification site, which express low levels of Zac1 and high levels of Apostain (Fig. 5E; Table 2). Therefore, as occurs in the embryonic nervous system, Zac1 expression is intense in proliferating chondrocytes and its expression is down-regulated when the progenitor cells leave the cell cycle.

In addition, hypertrophic chondrocytes induce sprouting angiogenesis from the perichondrium (Gerber et al., 1999). Vascular invasion from the perichondrium or bone collar brings osteoblast progenitors that will from ossification centers (Yang and Karsenty, 2002). Thus, with the formation of primary ossification centers, the cartilage matrix is degraded and the mature chondrocytes undergo apoptosis. Zac1 is expressed in the perichondrium and vascular cells that surround the limb bones sites, which are positively PCNA-labeled (Table 2), whereas their expression in the vascular interdigital zone overlaps with Apostain (Table 2).

Therefore, Zac1, like collagen type II, is expressed in all steps of the chondrogenesis (including mesenchymal condensations that occur between E10 and E12), which suggests a regulatory Zac1 role in the development of the skeleton, in which the proliferating chondrocytes differentiate into hypertrophic chondrocytes and facilitate the ossification spread with the degradation and replacement of the cartilage by apoptosis (Shum and Nuckolls, 2002). The present results suggest that Zac1 participates in all cellular steps of the chondrocyte life cycle, from proliferation to apoptosis, and they reinforce the previous reports of the expression of *Zac1* gene in the primordium of the cartilage sites (Valente and Auladell, 2001; Tsuda et al., 2004) and of the *ZAC* gene (human

homolog of *Zac1*) in adult bone marrow (Varrault et al., 1998).

Development of the Skeletal Muscle

At E12–E14, many migrating dermomyotomal cells of the limbs express the *Zac1* gene (Fig. 1K,Q,R; Table 1), as well as some differentiated muscle cells surrounding the axial primordium cartilage skeleton (vertebrae column and ribs). In addition, many facial and tongue muscle cells express the *Zac1* gene at E14 and during all embryonic stages. Many of these *Zac1*-expressing cells are positively labeled to Nestin, PCNA, BrdU, or Forse-1, which confirms their mitotic character, as well as to Netrin-1, which is implicated in the migratory process (Fig. 6; Table 2). At E16, the muscle *Zac1*-expressing cells are colocalized with Vimentin (intermediate filament proteins within cells of mesenchymal derivation; Table 2). Furthermore, at E15, it is possible to detect the *Zac1* gene expression in the primordium of the nails. The epidermis is a derivate of the surface ectoderm that forms a protective barrier and specific appendages, including hair, nails, and different eccrine glands. The surface ectoderm also forms the epithelium of the oral cavity and tongue (Jonker et al., 2004). Moreover, *Zac1* gene is expressed in the muscle cells that surround the follicle and in the primordium of follicle of vibrissae (Table 2), where some *Zac1*-expressing cells are colocalized with Nestin or PCNA. The follicle contains a distinct population of presumptive follicular stem cells that express Nestin (Amoh et al., 2004). Zac1 could be implicated in the regulation of mitotic activity of the stem cells of the follicle of vibrissae.

All muscle cells produced by the somites take their origin from the dermomyotome located in the dorsal part of the somite, which receives signals from the notochord and the floor plate of the neural tube, initiating a de-epithelialization; the individual mesenchymal muscle precursor cells migrate, proliferate, and differentiate to form individual muscles (Christ and Ordahl, 1995; Schmidt et al., 1998; Borycki and Emerson, 2000). At E9–E10, the *Zac1* gene is strongly expressed during somite formation (Piras et al., 2000; Valente and Auladell, 2001; Tsuda et al., 2004) and at E14–E18 in the migrating and proliferative mesenchymal muscle precursor cells (Fig. 6; Table 2), as well as in several waves of muscle fiber formation (myofibers) during development. However, in the postnatal stages and with the differentiating process of the muscle cells, expression of the *Zac1* gene decreases and persists at low levels in some differentiated myofibers of the craniofacial, limb, and tail muscle cells. The present data reinforce the previous results obtained for *ZAC* and *Zac1* genes in adult and fetal tissues (Varrault et al., 1998; Piras et al., 2000; Arima et al., 2000; Ma et al., 2004).

Therefore, during early embryonic stages, *Zac1* gene is strongly expressed in progenitor cells of the skeleton and in the skeletal muscle tissues, as well as in apoptotic cells of the bone development. The ventral part of the somite (sclerotome) gives rise to the cartilage and bone of the vertebral column and ribs, whereas the dorsal part of somite (dermomyotome) produces the dermis of the back and the skeletal muscle of the body and limbs and the muscular connective tissue, except in the head muscles, which proceed from prechordal and paraxial mesoderm (Buckingham et al., 2003). The wide expression of the *Zac1* gene in most of these tissues, as well as in most, if not in all, cellular phases, suggests that Zac1 could be a regulating control factor, like other transcription factors (Pax, bHLH, Sox, and so on) in chondrogenesis and myogenesis during embryonic development (Stockdale et al., 2000; Olsen et al., 2000; Moran et al., 2002; Yang and Karsenty, 2002).

EXPERIMENTAL PROCEDURES

Animals

We used embryo, postnatal, and adult NMRI (Iffa Credo, Lyon, France) mice. The day on which a vaginal plug was detected was considered E0. E1 began 24 hr later. The day of birth was designated P0. The fetal animals were removed from the mother under anesthesia by intraperitoneal injection of ketamine (100 mg/kg) and Xilacine (10 mg/kg). All animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer and processed for in situ hybridization (ISH) and immunohistochemistry (IHC). Alternatively, six NMRI pregnant mice were injected i.p. at E14, E16, and E18 (two mice for each age) with 50 mg/kg of BrdU (Sigma, in Tris-buffered saline, pH 7.6). Two hours after injection, the mice were killed and the embryos were collected, perfused, and fixed in 4% paraformaldehyde overnight. The animals were kept under controlled temperature, humidity, and light conditions, and they were treated according to European Community Council Directive 86/609/EEC and the procedure was registered at the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering.

ISH

Antisense and sense riboprobes were labeled with digoxigenin-d-UTP (Boehringer-Mannheim). ISH was performed on free-floating tissue sections as described by Valente and Auladell (2001). Briefly, sections were pretreated with H_2O_2 and HCl and hybridized overnight at 61°C with antisense or sense digoxigenin–d-UTP-labeled riboprobes (mouse *Zac1* cDNA used in Valente and Auladell, 2001; rat NPY cDNA; rat Nestin cDNA; mouse GAD65 cDNA; mouse EGFR1 cDNA). After ISH, sections were stringently washed in 50% formamide solutions at 61°C and incubated with 100 μg/ml RNase A (at 37°C). After that, the sections were blocked with 10% normal goat serum (NGS) and incubated overnight at 4°C with an alkaline phosphatase-labeled anti-digoxigenin antibody (1:2,000; Boehringer-Mannheim). To view alkaline phosphatase activity, sections were incubated with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP). The anatomic analysis of the embryonic areas was made in accordance with "The Atlas of Mouse Development" by Kaufman (1999).

Double ISH-IHC Procedure

After obtaining an intense labeling for *Zac1*, *Nestin*, *EGFR1*, *NPY*, or

GAD65 transcripts in the ISH, free-floating sections were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-Zac1 (1:1,000, L. Journot), anti-TH (1:2,000, Chemicon), anti-GFAP (1:2,000, Dako), anti-Ng2 (1:1,000, W.B. Stallcup), anti-calbindin (1:4,000, Swant), anti-calretinin (1:2,500, Swant), anti-parvalbumin (1:5,000, Swant), anti-LHRH (1:5,500, Chemicon), anti-CRF (1:3,500, Chemicon), anti-GluR1 (1:1,000, Chemicon), anti-GluR2/3 (1:1,000, Chemicon), anti-GluR4 (1:1,000, Chemicon), anti-GluR5/6 (1:800, Chemicon), anti-GAD65 (1:700, Chemicon), anti-somatostatin (1:1,000, Dakkopats), and mouse anti-PCNA (1:700, Chemicon), anti–Nestin-Rat-401 (1:200, hybridoma bank), anti-FORSE-1 (1:500, hybridoma bank), anti-BrdU (1:300, Roche), anti–β-tubulin (1:100, Chemicon), anti–Netrin-1 (1:300, Oncogene), anti–PSA-NCAM (1:7,500, G. Rougon), anti-vimentin (1:400, Dako), anti-NeuN (1:300, Chemicon), and mouse anti-ssDNA (F7-26) Apostain (1:150, Alexis). After that, sections were sequentially incubated with biotinylated goat anti-rabbit or horse anti-mouse antibodies (1:200), and then with the avidin-biotin-peroxidase complex (ABC, 1:200). Peroxidase was developed with 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2.

IHC Techniques

IHC was performed on free-floating tissue sections as described by Valente and Auladell (2004). Briefly, free-floating sections were treated with 0.5% H_2O_2, blocked with 10% of NGS and incubated overnight with the Zac1 antibody and processed as described above until developed with 0.05% DAB–0.01% H_2O_2, for single immunohistochemistry, or with 0.05% DAB–0.01% H_2O_2–0.2% $NiNH_4SO_4$, for double immunohistochemistry. In this latter case, the immunolabeled sections were washed and incubated again with a second primary antibody and developed with 0.05% DAB–0.01% H_2O_2. Alternatively, some sections were counterstained with hematoxylin and methyl green–pyronin.

Detection of the In Situ DNA Fragmentation

Free-floating sections were treated following the protocol supplied by Klenow-FragEl DNA Fragmentation Detection Kit (Oncogene).

ACKNOWLEDGMENTS

We thank Drs. Steven Sabol, Toshiyuki Takeuchi, Kunihiko Obata, and Harley Kornblum for providing us with NPY, Nestin, GAD65, and EGFR1 probes, respectively. We would also thank Drs. W.B. Stallcup, G. Rougon, and L. Journot for providing us with Ng2, PSA-NCAM, and Zac1 antibodies, respectively. We thank Tom Yohannan and Jordi Correas for editorial and technical assistance, respectively.

REFERENCES

Akazawa C, Ishibashi M, Shimizu C, Nakanishi S, Kageyama R. 1995. A mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. J Biol Chem 270:8730–8738.

Allendoerfer KL, Durairaj A, Matthews GA, Patterson PH. 1999. Morphological domains of Lewis-X/FORSE-1 immunolabeling in the embryonic neural tube are due to developmental regulation of cell surface carbohydrate expression. Dev Biol 211:208–219.

Alvarez-Buylla A, Garcia-Verdugo JM, Tramontin AD. 2001. A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci 2:287–293.

Amoh Y, Li L, Yang M, Moossa AR, Katsuoka K, Penman S, Hoffman RM. 2004. Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. Proc Natl Acad Sci U S A 101:13291–13295.

Arima T, Drewell RA, Oshimura M, Wake N, Surani MA. 2000. A novel imprinted gene, HYMAI, is located within an imprinted domain on human chromosome 6 containing *ZAC*. Genomics 67:248–255.

Bailey P, Holowacz T, Lassar AB. 2001. The origin of skeletal muscle stem cells in the embryo and the adult. Curr Opin Cell Biol 13:679–689.

Baimbridge KG. 1992. Calcium-binding proteins in the dentate gyrus. Epilepsy Res Suppl 7:211–220.

Bates S, Vousden KH. 1996. p53 in signaling checkpoint arrest or apoptosis. Curr Opin Genet Dev 6:12–18.

Ben-Arie N, McCall AE, Berkman S, Eichele G, Bellen HJ, Zoghbi HY. 1996. Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. Hum Mol Genet 5:1207–1216.

Borycki AG, Emerson CP Jr. 2000. Multiple tissue interactions and signal transduction pathways control somite myogenesis. Curr Top Dev Biol 48:165–224.

Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D, Relaix F. 2003. The formation of skeletal muscle: from somite to limb. J Anat 202:59–68.

Christ B, Ordahl CP. 1995. Early stages of chick somite development. Anat Embryol (Berl) 191:381–396.

Deiner MS, Sretavan DW. 1999. Altered midline axon pathways and ectopic neurons in the developing hypothalamus of netrin-1- and DCC-deficient mice. J Neurosci 19:9900–9912.

DeLise AM, Fischer L, Tuan RS. 2000. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage 8:309–334.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.

Dorton AM. 2000. The pituitary gland: embryology, physiology, and pathophysiology. Neonatal Netw 19:9–17.

D'Sa-Eipper C, Roth KA. 2000. Caspase regulation of neuronal progenitor cell apoptosis. Dev Neurosci 22:116–124.

D'Sa-Eipper C, Leonard JR, Putcha G, Zheng TS, Flavell RA, Rakic P, Kuida K, Roth KA. 2001. DNA damage-induced neural precursor cell apoptosis requires p53 and caspase 9 but neither Bax nor caspase 3. Development 128:137–146.

Edenfeld G, Pielage J, Klambt C. 2002. Cell lineage specification in the nervous system. Curr Opin Genet Dev 12:473–477.

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R. 1995. Toward a molecular understanding of skeletal development. Cell 80:371–378.

Filipsson K, Sundler F, Ahren B. 1999. PACAP is an islet neuropeptide which contributes to glucose-stimulated insulin secretion. Biochem Biophys Res Commun 256:664–667.

Fonnum F, Storm-Mathisen J. 1969. GABA synthesis in rat hippocampus correlated to the distribution of inhibitory neurons. Acta Physiol Scand 76:35A–36A.

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623–628.

Gonzalez HA, Kedzierski W, Aguila-Mansilla N, Porter JC. 1989. Hormonal control of tyrosine hydroxylase in the median eminence: demonstration of a central role for the pituitary gland. Endocrinology 124:2122–2127.

Hall BK, Miyake T. 2000. All for one and one for all: condensations and the initiation of skeletal development. Bioessays 22:138–147.

Hoffmann A, Ciani E, Houssami S, Brabet P, Journot L, Spengler D. 1998. Induc-

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tion of type I PACAP receptor expression by the new zinc finger protein Zac1 and p53. Ann N Y Acad Sci 865:49–58.

Horton WA. 2003. Skeletal development: insights from targeting the mouse genome. Lancet 362:560–569.

Johnson JE, Birren SJ, Anderson DJ. 1990. Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. Nature 346:858–861.

Jonker L, Kist R, Aw A, Wappler I, Peters H. 2004. Pax9 is required for filiform papilla development and suppresses skin-specific differentiation of the mammalian tongue epithelium. Mech Dev 121: 1313–1322.

Kamiya M, Judson H, Okazaki Y, Kusakabe M, Muramatsu M, Takada S, Takagi N, Arima T, Wake N, Kamimura K, Satomura K, Hermann R, Bonthron DT, Hayashizaki Y. 2000. The cell cycle control gene ZAC/PLAGL1 is imprinted-a strong candidate gene for transient neonatal diabetes. Hum Mol Genet 9:453–460.

Katarova Z, Sekerkova G, Prodan S, Mugnaini E, Szabo G. 2000. Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. J Comp Neurol 424:607–627.

Kaufman MH. 1999. The atlas of mouse development. 3rd ed. London: Academic Press Ltd.

Kurki P, Ogata K, Tan EM. 1988. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 109:49–59.

Lauder JM, Han VK, Henderson P, Verdoorn T, Towle AC. 1986. Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study. Neuroscience 19:465–493.

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA. 2000. Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc Natl Acad Sci U S A 97:13883–13888.

Ma Q, Kintner C, Anderson DJ. 1996. Identification of neurogenin, a vertebrate neuronal determination gene. Cell 87:43–52.

Ma D, Shield PHJ, Dean W, Leclerc I, Knauf C, Burcelin R, Rutter GA, Kelsey G. 2004. Impaired glucose homeostasis in transgenic mice expressing the human transient neonatal diabetes mellitus locus, *TNDM*. J Clin Invest 114:339–348.

Moran JL, Li Y, Hill AA, Mounts WM, Miller CP. 2002. Gene expression changes during mouse skeletal myoblast differentiation revealed by transcriptional profiling. Physiol Genomics 10:103–111.

Moskowitz MA, Lo EH. 2003. Neurogenesis and apoptotic cell death. Stroke 34: 324–326.

Olsen BR, Reginato AM, Wang W. 2000. Bone development. Annu Rev Cell Dev Biol 16:191–220.

Pagotto U, Arzberger T, Ciani E, Lezoualc'h F, Pilon C, Journot L, Spengler D, Stalla GK. 1999. Inhibition of Zac1, a new gene differentially expressed in the anterior pituitary, increases cell proliferation. Endocrinology 140:987–996.

Pagotto U, Arzberger T, Theodoropoulou M, Grubler Y, Pantaloni C, Saeger W, Losa M, Journot L, Stalla GK, Spengler D. 2000. The expression of the antiproliferative gene ZAC is lost or highly reduced in non-functioning pituitary adenomas. Cancer Res 60:6794–6799.

Piras G, El Kharroubi A, Kozlov S, Escalante-Alcalde D, Hernandez L, Copeland NG, Gilbert DJ, Jenkins NA, Stewart CL. 2000. Zac1 (Lot1), a potential tumor suppressor gene, and the gene for epsilon-sarcoglycan are maternally imprinted genes: identification by a subtractive screen of novel uniparental fibroblast lines. Mol Cell Biol 20:3308–3315.

Roth KA, D'Sa C. 2001. Apoptosis and brain development. Ment Retard Dev Disabil Res Rev 7:261–266.

Schmidt C, Christ B, Patel K, Brand-Saberi B. 1998. Experimental induction of BMP-4 expression leads to apoptosis in the paraxial and lateral plate mesoderm. Dev Biol 202:253–263.

Schwarzer C, Sperk G. 1995. Hippocampal granule cells express glutamic acid decarboxylase-67 after limbic seizures in the rat. Neuroscience 69:705–709.

Shum L, Nuckolls G. 2002. The life cycle of chondrocytes in the developing skeleton. Arthritis Res 4:94–106.

Sloviter RS, Dichter MA, Rachinsky TL, Dean E, Goodman JH, Sollas AL, Martin DL. 1996. Basal expression and induction of glutamate decarboxylase and GABA in excitatory granule cells of the rat and monkey hippocampal dentate gyrus. J Comp Neurol 373:593–618.

Smith RJ, Arnaud P, Konfortova G, Dean WL, Beechey CV, Kelsey G. 2002. The mouse Zac1 locus: basis for imprinting and comparison with human ZAC. Gene 292:101–112.

Soghomonian JJ, Martin DL. 1998. Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci 19:500–555.

Spengler D, Villalba M, Hoffmann A, Pantaloni C, Houssami S, Bockaert J, Journot L. 1997. Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J 16:2814–2825.

Stockdale FE, Nikovits W Jr, Christ B. 2000. Molecular and cellular biology of avian somite development. Dev Dyn 219: 304–321.

Szentagothai J. 1969. The synaptic architecture of the hypothalamo-hypophyseal neuron systems. Acta Neurol Psychiatr Belg 69:453–468.

Tsuda T, Markova D, Wang H, Evangelisti L, Pan TC, Chu ML. 2004. Zinc finger protein Zac1 is expressed in chondrogenic sites of the mouse. Dev Dyn 229: 340–348.

Valente T, Auladell C. 2001. Expression pattern of Zac1 mouse gene, a new zinc-finger protein that regulates apoptosis and cellular cycle arrest, in both adult brain and along development. Mech Dev 108:207–211.

Valente T, Dominguez MI, Bellmann A, Journot L, Ferrer I, Auladell C. 2004. Zac1 is up-regulated in neural cells of the limbic system of mouse brain following seizures that provoke strong cell activation. Neuroscience 128:323–336.

Van Eden CG, Mrzljak L, Voorn P, Uylings HB. 1989. Prenatal development of GABA-ergic neurons in the neocortex of the rat. J Comp Neurol 289:213–227.

Varrault A, Ciani E, Apiou F, Bilanges B, Hoffmann A, Pantaloni C, Bockaert J, Spengler D, Journot L. 1998. hZAC encodes a zinc finger protein with antiproliferative properties and maps to a chromosomal region frequently lost in cancer. Proc Natl Acad Sci U S A 95:8835–8840.

Waschek JA. 2002. Multiple actions of pituitary adenylyl cyclase activating peptide in nervous system development and regeneration. Dev Neurosci 24:14–23.

Yada T, Sakurada M, Nakata M, Shioda S, Yaekura K, Kikuchi M. 1998. Autocrine action of PACAP in islets augments glucose-induced insulin secretion. Ann N Y Acad Sci 865:451–457.

Yan YH, Van Brederode JF, Hendrickson AE. 1995. Transient co-localization of calretinin, parvalbumin, and calbindin-D28K in developing visual cortex of monkey. J Neurocytol 24:825–837.

Yang X, Karsenty G. 2002. Transcription factors in bone: developmental and pathological aspects. Trends Mol Med 8: 340–345.

Zaidi AU, D'Sa-Eipper C, Brenner J, Kuida K, Zheng TS, Flavell RA, Rakic P, Roth KA. 2001. Bcl-X(L)-caspase-9 interactions in the developing nervous system: evidence for multiple death pathways. J Neurosci 21:169–175.

Neuroscience 128 (2004) 323–336

ZAC1 IS UP-REGULATED IN NEURAL CELLS OF THE LIMBIC SYSTEM OF MOUSE BRAIN FOLLOWING SEIZURES THAT PROVOKE STRONG CELL ACTIVATION

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Abstract—Zac1, a new zinc-finger protein that regulates both apoptosis and cell cycle arrest, is abundantly expressed in many proliferative/differentiation areas during brain development. In the present work, we studied *Zac1* gene expression and protein in experimental seizure models following i.p. injection of pentylenetetrazole (PTZ) or kainic acid (KA). Following KA treatment, an early and intense up-regulation of *Zac1* is detected in the limbic areas, such as the hippocampus, cortex and amygdaloid and hypothalamic nuclei. Pretreatment with MK-801, an antagonist of the NMDA receptors, fully blocks the effect of KA in the hippocampus, whereas it only attenuates KA-induced *Zac1* up-regulation in the other areas of the limbic system. A reduced induction is obtained with PTZ-treated animals, specifically in the entorhinal and piriform cortices as well as in amygdaloid and hypothalamic nuclei. Thus, *Zac1* is highly induced in the seizure models that generate strong neuronal stimulation and/or extensive cell damage (cell death), reinforcing its putative role in the control of the cell cycle and/or apoptosis. Moreover, strong induction is observed in the granular cells of the dentate gyrus (which are resistant to neurodegeneration) and in some glial cells of the dentate gyrus and subventricular zone, suggesting that *Zac1* may be implicated in the mechanisms of neural plasticity following injury.

Key words: zinc-finger protein, seizures, apoptosis, neuronal damage, neural plasticity, gene expression.

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Abbreviations: ABC, avidin–biotin–peroxidase complex; DEDTC, sodium diethyldithiocarbamate; IHC, immunohistochemistry; ISH, *in situ* hybridization; KA, kainic acid; NGS, normal goat serum; $PACAP_1$-R, pituitary adenylate cyclase-activating polypeptide receptor; PBS, phosphate buffer saline; PBST, phosphate buffer saline with 0.5% of Triton X-100; PFA, paraformaldehyde in phosphate buffer; PTZ, pentylenetetrazole; SDS, sodium dodecyl sulfate; SSC, sodium saline citrate; SVZ, subventricular zone; TBST, 135 mM NaCl, 3 mM KCl, 0.1% Tween-20, 2 mM levamisole and 25 mM Tris–HCl; VZ, ventricular zone.

Experimental models of epilepsy do not offer the entire complex etiologies and variety of syndromes that have been identified in humans. However, since there are basic features in common, experimental models have allowed for the determination of the basic molecular and cellular mechanisms of epileptogenesis and their relation to brain damage (Ben-Ari and Cossart, 2000). A careful analysis of the genomic responses may provide the means for uncovering the molecular events leading to such long-lasting phenomena as neural plasticity and neurodegeneration (Zagulska-Szymczak et al., 2001).

Kainic acid (KA), an analog of glutamate, has been the most commonly used agent for the generation of seizures *in vivo*, and consequently for creating an animal model of epilepsy (for review see Sperk, 1994; Ben-Ari and Cossart, 2000). Systemic doses of KA result in sustained depolarisation of neurons manifested by a complex seizure behavior starting with mild head nodding and culminating in severe limbic seizures (Lothman et al., 1981). These seizures can be associated with cell death, both apoptosis and necrosis (Filipkowski et al., 1994; Pollard et al., 1994a,b), and, consequently, with multiple brain genome responses (Kaminska et al., 1997; Goodenough et al., 1997; Djebaïli et al., 2001). The mechanisms affecting these alterations involve transcriptional changes in a vast number of genes in several regions of the brain. Thus, many of these genes have been implicated in the early mechanisms associated with cell death, such as the zinc-finger immediate early genes (c-fos and c-jun) or genes that encode proteins with zinc-finger DNA binding motif (Zif268, NGFI-B, egr-2, egr-3 and Nurr 1). However, many other genes seem to encode proteins involved in transduction of extracellular signal and structural proteins, related with synaptic remodelling (e.g. Furin, Syndecan and SNAP 25a; Becker et al., 1999; Zagulska-Szymczak et al., 2001).

Gene alterations may occur during the regulation of the brain developmental mechanisms, occasioning brain injuries that consequently converge in several neuropathologies. Therefore, neurodegenerative models are useful tools for gene expression studies, especially for the genes involved in CNS development.

During the development of the mouse brain most areas undergo programmed cell death, or apoptosis (Oppenheim, 1991), and this process is much more complex than the classic cell death mechanisms. This apoptotic neuronal death could contribute to the brain damage following seizures, especially in the hippocampus and other limbic areas (Goodenough et al., 1997; Honkaniemi and Sharp, 1999; Ben-Ari and Cossart, 2000; Zagulska-Szymczak et

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doi:10.1016/j.neuroscience.2004.06.056

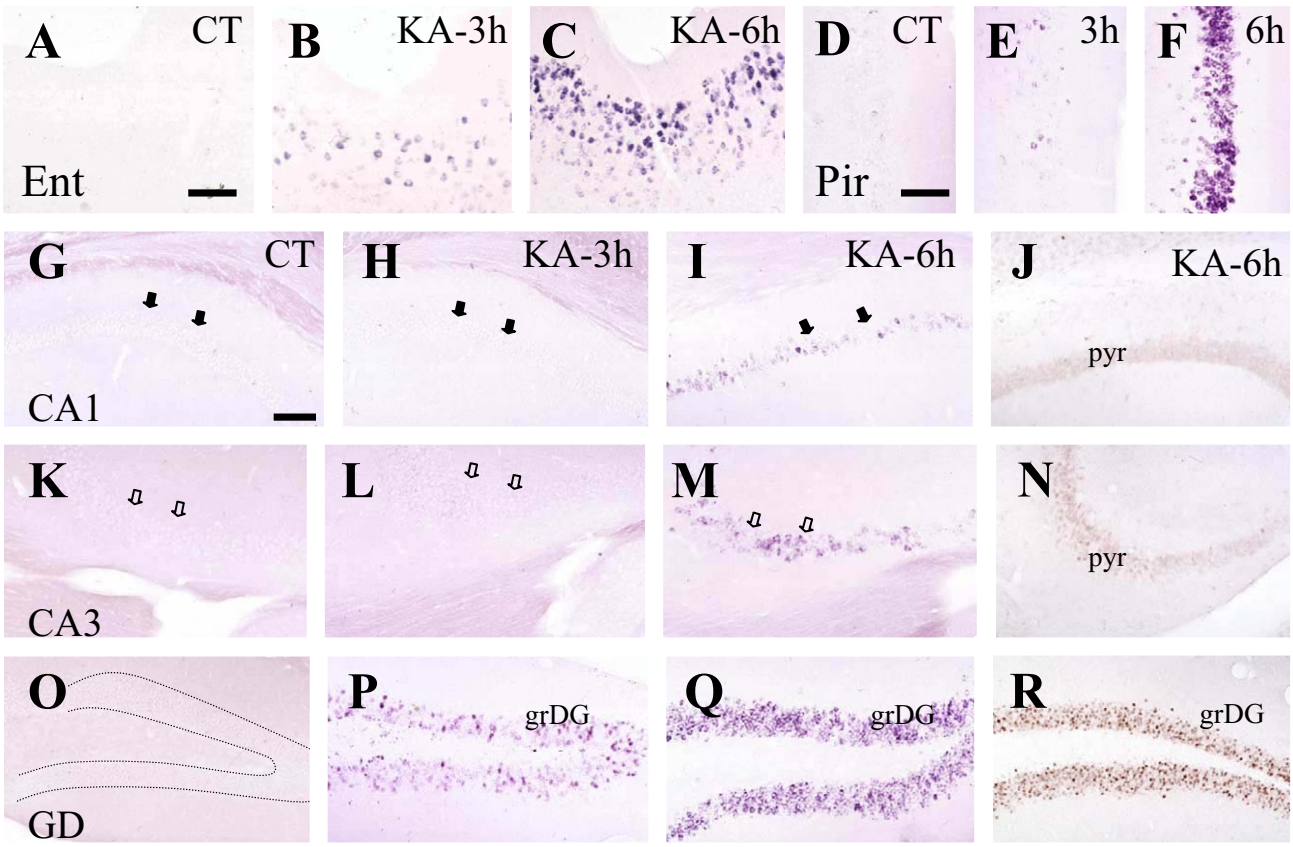

Fig. 1. ISH to Zac1 mRNA in control (A, D, G, K, O) and KA-treated mice at 3 h (B, E, H, L, P) and 6 h (C, F, I, M, Q) following administration. Immunohistochemical for Zac1 protein at 6 h (J, N, R) following KA administration. In the entorhinal cortex, Ent (A–C), the induction of Zac1 mRNA is detected in a few pyramidal cells at 3 h following KA-treatment and in many pyramidal cells at 6 h. A similar pattern of zac1 transcripts is found in the piriform cortex, Pir (D–F); however, the induction is more discreet at 3 h after KA-treatment, whereas it is more intense at 6 h, compared with the Ent. In the pyramidal cell layer of the CA1 and CA3 hippocampal regions (G–L), the induction of Zac1 mRNA is detected at 3 h for CA3 region (only in a very few cells) and at 6 h for CA1 region after KA injection. The intensity of the label in these cells is weak in the CA1 region while it is weak to moderate in the CA3 region. In the dentate gyrus (M–O), a moderate induction is observed in many granular cells at 3 h after KA treatment. This induction increases subsequently (6 h) in almost all granular cells, which appear strongly labeled. Some immunolabeled Zac1 cells were observed in the pyramidal cell layer of the CA1 (J) and CA3 (N) regions at 6 h. In the dentate gyrus (R), a large number of immunolabeled Zac1 cells were observed at 6 h after the treatment. Scale bar=70 μm (shown in A), A–C; (shown in D): D–F=100 μm; (shown in G): G–R=100 μm.

al., 2001; Bengzon et al., 2002). Consequently, apoptotic processes could be the result of alterations in the expression and activity of cell-death regulatory proteins (Estus et al., 1994; Liu et al., 1999; Becker et al., 1999; Zagulska-Szymczak et al., 2001; Bengzon et al., 2002; Verdaguer et al., 2002). Recently, Spengler and colleagues (1997) cloned a new zinc-finger protein called Zac1, which regulates apoptosis and cell cycle, like the p53 protein. High levels of Zac1 mRNA were found in some cell populations with intense proliferative activity, cellular differentiation, and active synaptic plasticity during mouse development (Valente and Auladell, 2001). The Zac1 gene induces and regulates expression of the pituitary adenylate cyclase-activating polypeptide receptor ($PACAP_1$-R) gene (Hoffmann et al., 1998; Rodríguez-Henche et al., 2002). This receptor is the target of the pituitary adenylate cyclase-activating polypeptide, and the two of them seem to promote mitogenesis, survival and differentiation in some cell subpopulations during fetal brain development (Vaudry et al., 1998, 1999; Sherwood et al., 2000). Curiously, Zac1 and $PACAP_1$-R genes show parallel expression patterns during early brain development (Basille et al., 2000; Valente and Auladell, 2001). Both genes are highly expressed in transient focal cerebral ischemia (Gillardon et al., 1998). Moreover, $PACAP_1$-R is also up-regulated following excitotoxic damage by KA treatment (Boschert et al., 1997), as is the p53 gene, which, like Zac1, induces $PACAP_1$-R expression (Spengler et al., 1997; Liu et al., 1999). Therefore, our aim was to elucidate the role of the Zac1 gene and protein in a mouse seizure model induced by KA and to determine whether its role is related to the cell death process (using another convulsing agent that does not induce neuronal death: pentylenetetrazole, PTZ).

EXPERIMENTAL PROCEDURES

Animals

We used 51 ICR adult male mice (Iffa Credo, Lyon, France), each weighing approximately 35 g. The animals were kept under controlled temperature, humidity, and light conditions, and they were

treated according to European Community Council Directive 86/609/EEC and the procedure registered at the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Seizure models (drug treatment)

A first group of ICR animals (12) was i.p. injected with 15 mg/kg of KA and, after 15 min, the animals were injected again with 150 mg/kg of sodium diethyldithiocarbamate, DEDTC (a chelatable agent of endogenous Zn^{2+}). A second group of animals (10) was i.p. injected with 1 mg/kg of MK-801 (an antagonist of the NMDA receptors) and, after 1 h, these were injected with 15 mg/kg of KA and, 15 min later, with 150 mg/kg of DEDTC. A third group of animals (10) was i.p. injected with 60 mg/kg of PTZ (another convulsing agent which is a potent antagonist of the $GABA_A$ receptors). A fourth group of animals (seven) was injected with a saline solution and, 15 min later, they received an additional injection of 150 mg/kg of DEDTC. These animals were used as KA treatment controls. A fifth group of animals (two) was injected with saline solution and, 15 min later, with 1000 mg/kg of DEDTC. They were used as DEDTC treatment controls. A sixth group of animals (five) was injected with 1 mg/kg of MK-801, 1 h later with saline solution, and 15 min after this with DEDTC, preparing them for use as MK-801/KA treatment controls. A final group of animals (five) was injected with saline solution for use as controls for PTZ and general treatment. All the animals used were injected with the same total volume of 0.5 ml/animal.

One, 3, 6, 8 and 18 h after the treatment, the animals were deeply anesthetized by i.p. injection of ketamine (100 mg/kg) and Xylocaine (10 mg/kg), and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA). The brains were removed immediately and processed for *in situ* hybridization (ISH) and immunohistochemistry (IHC). Briefly, the brains were postfixed in PFA for 48 h at 4 °C, cryoprotected with 30% sucrose in PFA for 48 h at 4 °C, and frozen in dry ice. Coronal sections 30 μm thick were processed free-floating for ISH and IHC.

ISH

Antisense and sense riboprobes were labeled with digoxigenin-*d*-UTP (Boehringer-Mannheim, Roche, Spain) by *in vitro* transcription of mouse cDNA encoding Zac1, and ISH was performed on free-floating tissue sections as described by Valente and Auladell (2001). Briefly, sections were rinsed in phosphate buffer saline 0.1 M, pH 7.2 (PBS) and treated with H_2O_2 and HCl. Subsequently, the sections were hybridized overnight at 61 °C with antisense or sense Zac1 digoxigenin-*d*-UTP-labeled riboprobes in a solution containing 50% formamide, sodium saline citrate (SSC), sodium dodecyl sulfate (SDS), sheared salmon sperm DNA and yeast tRNA. After ISH, sections were washed in mixed solution with 50% formamide, 5× SSC, and 1% SDS at 61 °C. Then the sections were incubated with 100 μg/ml RNase A (at 37 °C) for 30 min and washed in mixed solution with 50% formamide and 2× SSC, at 56 °C. After stringent washes, sections were rinsed in TBST solution (135 mM NaCl, 3 mM KCl, 0.1% Tween-20, 2 mM levamisole and 25 mM Tris–HCl, pH 7.5), blocked with 10% normal goat serum (NGS) in TBST and incubated overnight at 4 °C with an alkaline phosphatase-labeled antidigoxygenin antibody (1:2000; Boehringer-Mannheim). To view alkaline phosphatase activity, sections were incubated with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl-phosphate toluidinium salt (BCIP) diluted in an NTMT solution (100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, 2 mM levamisole and 100 mM Tris–HCl, pH 9.5). Alkaline phosphatase activity was then stopped in PBS and the sections were mounted on gelatinized slides. Control of hybridization with sense strand-labeled riboprobes showed no hybridization signal.

Zac1 IHC

Free-floating sections were rinsed in 0.1 M PBS, pH 7.2, and then treated with 0.5% H_2O_2 and 10% methanol in PBS. After that, they were pre-incubated first in a blocked solution (10% of NGS, 0.25% of BSA and 0.2 M of glycine in PBST, PBS with 0.5% of Triton X-100) and then with rabbit anti-Zac1 (1:1000; L. Journot, Montpellier) overnight at 4 °C. Some sections were incubated with rabbit serum as an immunohistochemical control technique. After this, sections were sequentially incubated with biotinylated goat anti-rabbit antibody (1:200) and with the avidin–biotin–peroxidase complex (ABC, 1:200, Vector, USA). Peroxidase was developed with 0.05% diaminobenzidine in 0.1 M PB and 0.01% H_2O_2, and immunoreacted sections were mounted onto gelatinized slides. Alternatively, some sections were counterstained with hematoxylin.

Double IHC technique

After development of the Zac1 immunolabeling, the sections were again incubated in the primary antibody solution, but this time with GFAP antibody (Dako, Spain), as described above. All the subsequent steps were followed up to peroxidase development. At that point, the sections were pre-washed in a PB solution, 0.01 M and pH=6.0, and then incubated in the same PB solution with 0.01% benzydamine hydrochloride, 0.025% sodium nitroferricyanide and 0.01% H_2O_2, for peroxidase development. Immunolabeled sections were mounted onto gelatinized slides. Alternatively, some sections were counterstained with hematoxylin.

Double ISH-IHC procedure

After obtaining an intense labeling for Zac1 transcripts in the ISH, some free-floating sections were incubated overnight at 4 °C with 8% NGS or normal horse serum and with one of the following primary antibodies in PBST: rabbit anti-GFAP (1:2500; DAKO, USA), rabbit anti-cFos (1:2000; Santa Cruz, USA), and rabbit anti-Zac1 (L. Journot). Thereafter, sections were sequentially incubated with biotinylated goat anti-rabbit or horse anti-mouse antibodies (1:200) and with the ABC (1:200). Peroxidase was developed with 0.05% diaminobenzidine in 0.1 M PB and 0.01% H_2O_2, and immunoreacted sections were mounted onto gelatinized slides.

Preparation of brain extracts and Western blotting

The forebrain and cerebellum of control adult mice were homogenized in 1 ml of lysis buffer (125 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM EDTA, 1 μg/ml leupeptin, 5 μg/ml aprotinin and 0.125 mg/ml PMSF) at 4 °C, and the homogenate was centrifuged in order to obtain the cytosolic and nuclear fractions. Total extracts (30 μg) were resolved on 8% SDS-PAGE gels and electrotransferred to nitrocellulose membrane using a semi-dry blotting system. The membranes were blocked with 4% BSA in Tris-buffered saline (140 mM NaCl, 10 mM Tris–HCl, pH 7.4, with 0.1% Tween 20) at room temperature for 1 h and incubated with purified rabbit polyclonal against Zac1 (1:5000) overnight at 4 °C. After that, the membranes were incubated with the second antibody conjugated to horseradish peroxidase. After washing, bound IgG-HRPs were visualized using the ECL chemiluminescence system (Amersham Biosciences, UK).

Detection of the *in situ* DNA fragmentation

Free-floating sections were rinsed in 0.1 M TBS (Tris–HCl/NaCl) and then mounted onto gelatinized slides and air-dried. After that, the sections were treated following the protocol supplied by Klenow-FragEl DNA Fragmentation Detection Kit (Oncogene, USA).

Fluoro-Jade technique

For the Fluoro-Jade histofluorescent staining (Schmued et al., 1997; Schmued and Hopkins, 2000) we used the Tom Hallam

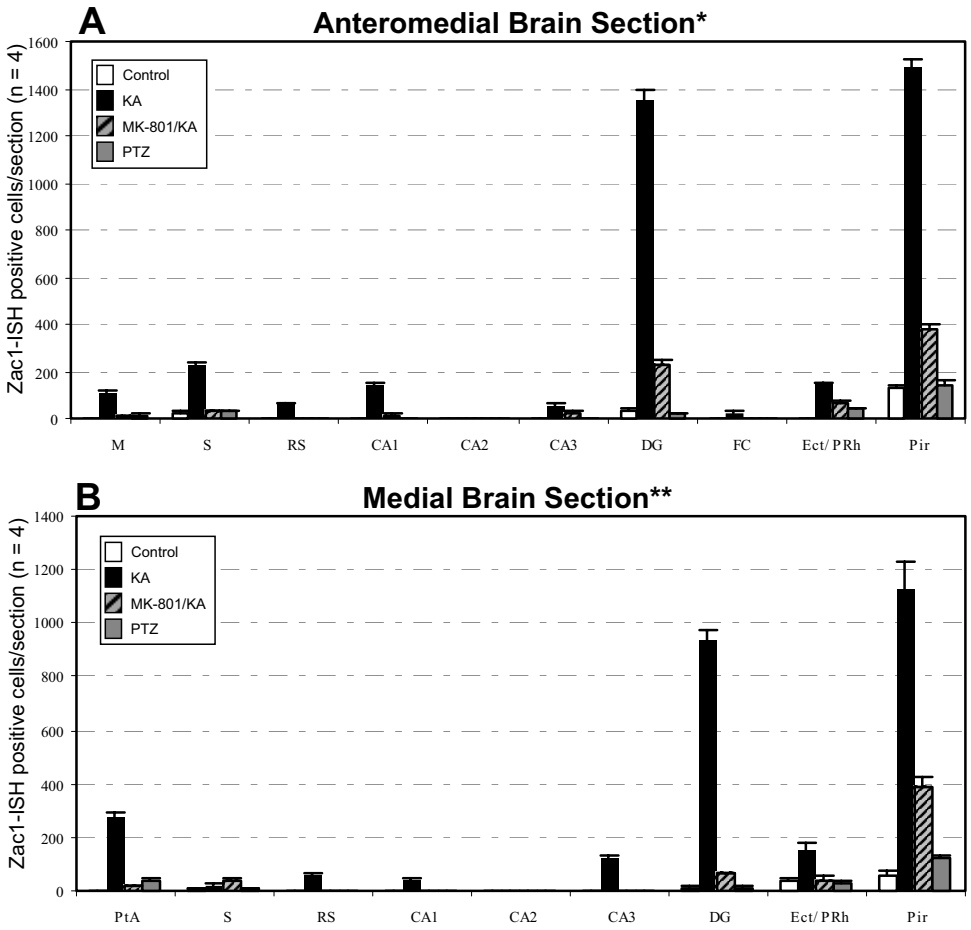

Fig. 2. Number of Zac1 positive cells (mRNA) induced by several seizure models following 6 h of treatment in the anterior (A) and medial (B) brain areas. (A) In the rostral brain sections, an evident cellular induction of the Zac1 mRNA is observed in the KA-treated mice, while a somewhat weak induction is detected in the MK-801/KA-treated mice and very weak one in the PTZ-treated mice. Anterior limbic brain areas have a large number of cells with up-regulated Zac1 transcripts, especially in the cells of the DG and Pir. Moderate cell numbers are induced in some cortical areas, such as the somatosensory, motor and entorhinal cortices, as well as in some pyramidal cells of the CA1 region. Less cellular induction is detected in the cells of the RS, CA3 region and the hippocampal commissure. In contrast, no cell induction at all is found in the CA2 region throughout the course of the seizure treatments. (B) In caudal brain sections, a similar pattern of induction is observed. Thus, in the DG and in the retrosplenial, Ect/PRh, a large number of induced Zac1 cells are detected. However, the induction in the CA1 region is not as strong as in the rostral sections, whereas in the CA3 region and the PtA, the induction of Zac1 mRNA is stronger in the caudal sections than in the rostral brain levels. In addition, the induction is barely appreciable in the S, where there is an inversion of the tendency and the MK-801/KA-treated mice are more sensitive than KA-treated mice. CA1, CA2 and CA3, hippocampal regions; DG, dentate gyrus; Ect/PRh, entorhinal and perirhinal cortices; FC, hippocampal fissure; M, motor cortex; Pir, piriform cortex; PtA, parietal association cortex; RS, retrosplenial cortex; S, somatosensory cortex; *, interaural 2.46 mm and bregma −1.34 mm; **, interaural 1.50 mm and bregma −2.30 mm.

protocol. Briefly, Zac1 IHC and ISH sections were mounted onto gelatinized slides and then dehydrated and re-hydrated in alcohol solutions. The slides were washed in distilled water and incubated in a 0.06% potassium permanganate solution for 15 min. After that, they were incubated in a 0.001% Fluoro-Jade staining solution for 30 min. Following the fluorescent staining, the slides were washed in distilled water and dried at 50 °C. Then they were immersed in xylene and coverslipped with DPX, and microscopically analyzed under fluorescent/FITC filter.

Counting analysis

For each case, the cells positively labeling for Zac1 mRNA and protein of 4 sections were counted in the induced brain areas between interaural 2.46 mm and bregma −1.34 mm, in the anterior brain areas (Figs. 2A and 5), and between interaural 1.50 mm and bregma −2.30 mm, in the medial brain areas (Fig. 2B). The counts comprised all those cells positively labeling for Zac1 mRNA and/or protein that could be clearly distinguished from the background. Means and standard deviations were calculated using the Student's *t*-test.

RESULTS

Seizure models

In the KA-treated mice, we observed numerous convulsions during the first 2 h. In subsequent hours, the animals had moderate continuous convulsions up to 8 h following KA administration. After this time, there were practically no convulsions detected. Only the animals that presented continued convulsions were used. The percentage of dead animals with this treatment was

Table 1. Up-expression of Zac1 mRNAs in brain areas following i.p. KA administration[a]

	3 h KA	6 h KA	8 h KA	18 h KA	Controls
Granular cells of the dentate gyrus	2	4	4	2	1
CA1	0	1	1	0	0
CA2	0	0	0	0	0
CA3	0	1	1	0	0
CA3 posterior	4	5	5	4	4
Cingular and retrosplenial cortices	0	1	1	0	0
Piriform cortex	2	5	5	1	1
Primary somatosensorial cortex	0	2	3	0	0
Parietal association cortex	2	3	3	0	0
Entorhinal cortex	1	4	4	1	0
Hypothalamus nuclei	4	5	5	4	4
Purkinje cells	2	2	2	2	2
Amygdaloid nuclei	4	5	5	4	3
Olfactory bulb area	3	5	5	2	2

[a] 0, no expression; 1, very weak; 2, weak; 3, moderate; 4, strong; 5, intense.

around 25%. The PTZ-treated mice displayed many convulsions during the first 4-h period after treatment. However, from this time until perfusion the animals remained still and convulsions were not detected. In the MK-801-treated mice, we observed hyperactivity, jumps, and an absence of co-ordination with resulting falls. In addition, these physiological symptoms remained unchanged after KA injection, except in one of the mice which seemed to show a slow increase in the jumps, probably as a consequence of the lessened convulsions, within the first 2 h following KA administration. The animals treated with DEDTC presented the same behavioral effects previously described in rats (Danscher et al., 1973) and mice (Domínguez et al., 2003a,b). Thus, in the present work no convulsions were detected in the control animals, which were treated with 150 mg/kg of DEDTC in saline solution. In addition, the DEDTC injection following 15 min of systemic administration of non-convulsant doses of KA (15 mg/kg) provoked epileptic seizures and neural damage in the hippocampus, as occurred with the high dose of KA, but it also increased the survival rate of the convulsing mice.

Zac1 gene expression in KA treatment

At only 3 h after KA treatment we detected moderate Zac1 positive cells among the granular cells of the dentate gyrus of the hippocampus and among the pyramidal cells of the entorhinal and piriform cortices (Fig. 1B, E and P, and Table 1). In addition, some neuronal cells of the amygdaloid nuclei were moderately stained (Table 1). At 6–8 h following KA treatment, we found an increase in the amounts of Zac1 transcripts as well as an increase in the number of Zac1 positive cells in these vulnerable areas (Figs. 1C, 1F, 1Q, 2 and 3A–F). The induction extended throughout the hippocampus. Thus, we found a few labeled pyramidal cells in the CA3 hippocampal region (Fig. 1M), while a few positive cells were visualized in the CA1 hippocampal region (Fig. 1I). In contrast, all these limbic brains areas were unstained in the control animals (Figs. 1A, D, G, K, O and 2, and Table 1), except in the amygdaloid and hypothalamic areas. The induction of Zac1 transcripts was maintained very intensely until 8 h after KA injection within these limbic areas (Table 1). Moreover, the up-regulation was also found in some thalamic and hypothalamic nuclei (Table 1), as well as in many cells of the olfactory bulb within the proliferative and/or differentiation layer (Table 1). At 18 h following KA injection, we observed a recovery of the normal levels of the Zac1 transcripts (Fig. 4A versus B), though we still found some induced cells in the hippocampus (Table 1).

Zac1 gene expression in combined MK-801 and KA treatment

Weak induction of Zac1 mRNA was also observed at only 6–8 h following MK-801/KA treatment (Figs. 2 and 3G–I). Small amounts of Zac1 transcripts were detected in the entorhinal and piriform cortices (Figs. 2 and 3H, I), as well as in the olfactory bulb (data not shown). Very weak induction was also detected in the pyramidal cells of the CA1 and CA3 hippocampal regions and in the granular cells of the dentate gyrus, as well as in the somatosensorial and parietal cortices (Fig. 2). Zac1 induction was not observed in the animals injected only with a single MK-801 injection.

Zac1 gene expression in PTZ treatment

Induction of Zac1 mRNA in the animals injected only with PTZ was observed at 6–8 h following treatment, similar to results obtained for MK-801/KA-treated mice (Figs. 2 and 3J–L). Nevertheless, in the PTZ-treated mice the weak induction was much lower in the piriform cortex and in the granular cells of the dentate gyrus, whereas it was higher in the amygdaloid (Figs. 2 and 3J–L), hypothalamic and thalamic nuclei (data not shown).

Zac1 gene expression in control animals

In all the saline-injected or saline-DEDTC-injected mice, the expression of the Zac1 gene was absent in many limbic areas, such as the hippocampus and the somatosensorial,

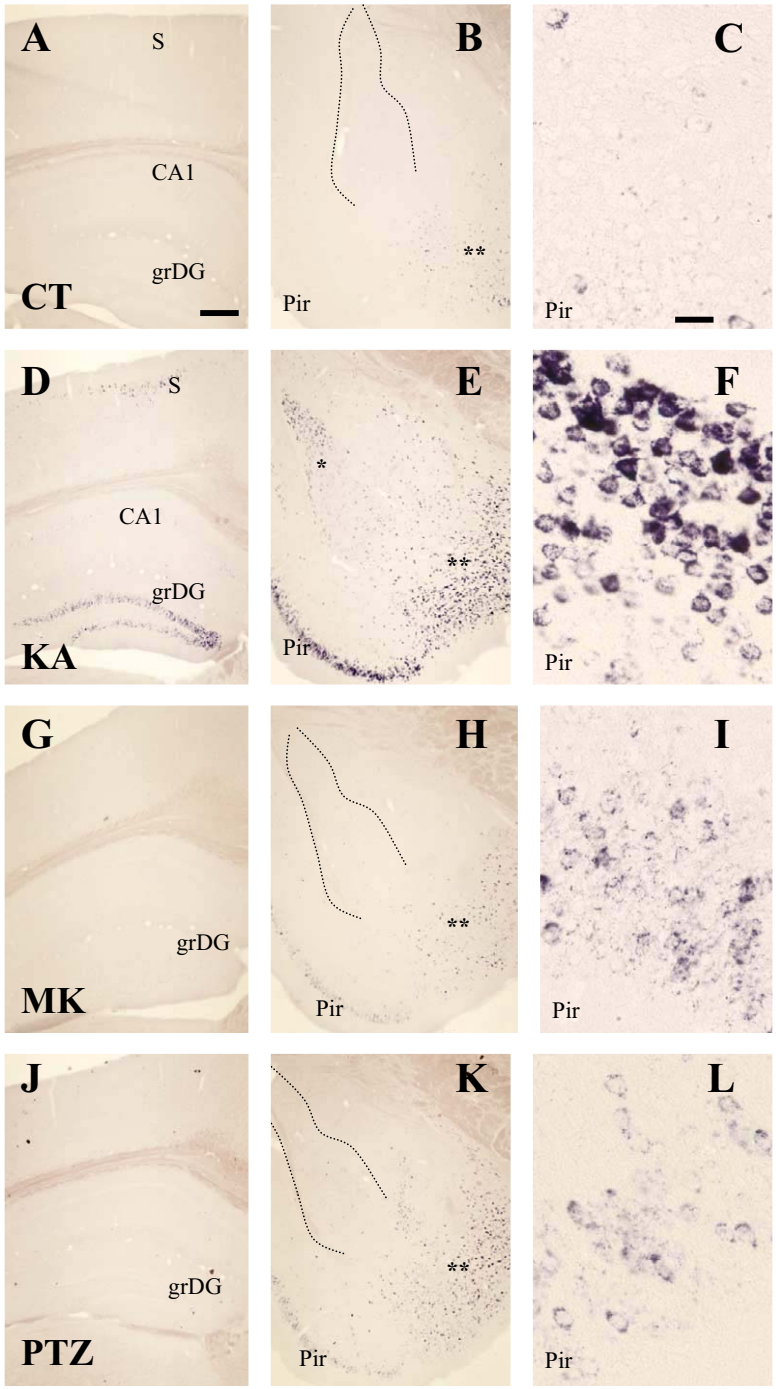

Fig. 3. ISH to Zac1 mRNA in control (A–C), KA-treated (D–F), MK-801/KA-treated (G–I) and PTZ-treated mice (J–L) at 8 h following injection. A moderate-strong induction of Zac1 transcripts is observed in the KA-treated mice (D–F), specifically in the granular cells of the dentate gyrus (grDG), in the pyramidal layer of the entorhinal and piriform cortices (Pir in E and in F, high magnification) and in the cortical amygdaloid and amygdalohippocampal areas (double asterisks in E). In addition, some cells are labeled in layers II–III of the somatosensorial cortex (S in E) and in the lateral amygdaloid nuclei (single asterisk in E). In the animals injected with MK-801 1 h before KA administration (G–I), a weak induction is observed in the entorhinal cortex and Pir (Pir in H and I) while very weak induction is detected in the cortical amygdaloid and amygdalohippocampal areas (double asterisks in H). In the PTZ-treated mice (J–L), weak induction is observed in the cortical amygdaloid and amygdalohippocampal areas (double asterisks in K), whereas very weak induction is detected in the entorhinal cortex and Pir (K, L). Scale bar=250 μm (shown in A): A, B, D, E, G, H, J, K; (shown in C) C, F, I, L=50 μm.

cingular, retrosplenial, entorhinal and piriform cortices. However, the posterior CA3 hippocampal region, amygdaloid complex and hypothalamic areas were strongly labeled in the control animals (Figs. 3 A, B and 2, and Table

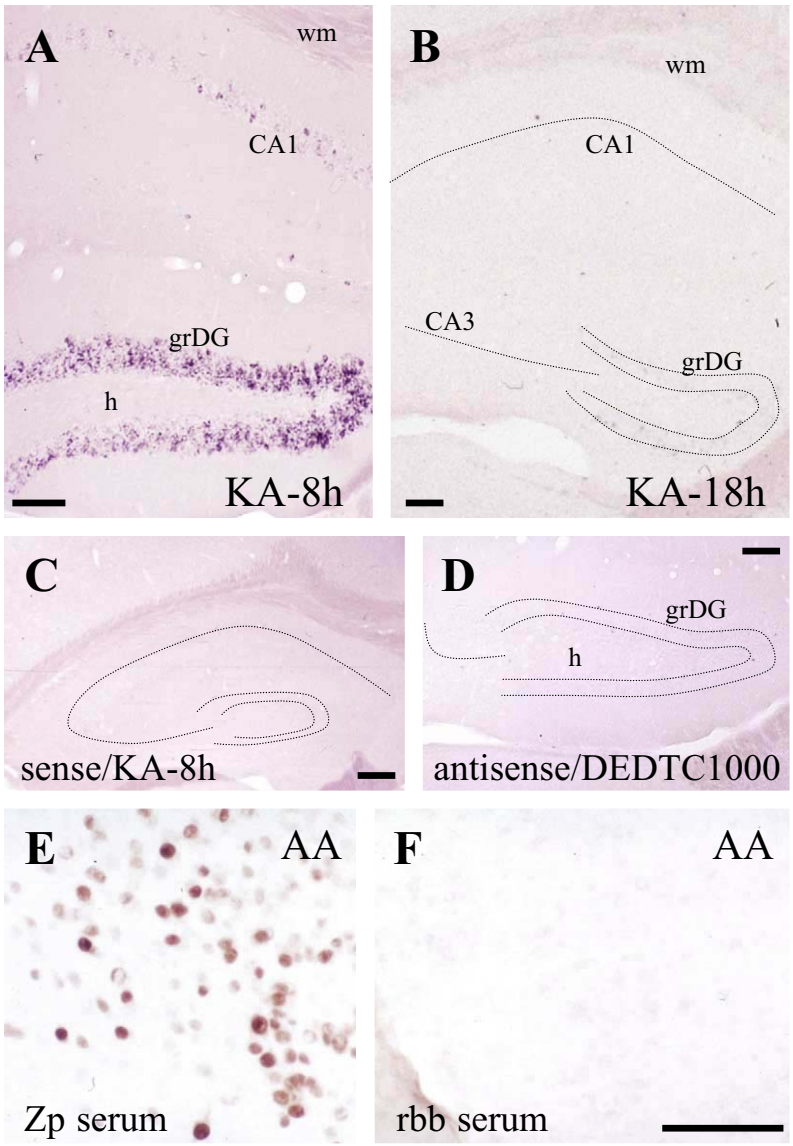

Fig. 4. Control mice for ISH and immunohistochemical techniques. (A) This microphotograph shows strong Zac1 mRNA up-expression in the hippocampal area with a single KA injection. (B) This strong induction was almost completely reversed 18 h after KA treatment. (C) No cell labeling is found in the brain sections that were incubated with the Zac1 sense probe (mRNA). (D) The administration of several concentrations of DEDTC failed to provoke an induction of Zac1 gene. (E) Immunopositive Zac1 cells in the sections incubated with the rabbit anti-Zac1 protein serum, while no labeled cells are found when the rabbit anti-Zac1 protein serum is substituted for the rabbit serum in the incubation, as a negative control for the immunohistochemical technique. CA1 and CA3, hippocampal regions; grDG, granular cells of dentate gyrus; h, hilus of dentate gyrus; rbb serum, rabbit serum; Zp serum, rabbit anti-Zac1 protein serum. Scale bar=100 μm (A); B, 150 μm; C, 280 μm; D, 100 μm; (shown in F): E, F, 50 μm.

1), as well as in the untreated animals previously described by Valente and Auladell (2001).

Zac1 IHC

In all animals, the distribution pattern of Zac1 protein correlated well with the up-regulation of *Zac1* transcripts, as much in terms of the areas induced as in the duration of this induction. Thus, a noticeable induction of Zac1 protein was found with the treatments that occasioned more cell damage. In KA-treated mice we detected moderate Zac1 protein levels at 3 h and high levels at 6 h in the granular cells of the dentate gyrus (Figs. 1R and 5) and the pyramidal cells of the entorhinal and piriform cortices (Fig. 5). A slight increase was observed in the CA1 and CA3 of hippocampal regions (Fig. 1N and R). Furthermore, in the co-localization studies with both the Zac1 gene and protein, we found a total correspondence in the distribution of the double-positive cells (Fig. 6B and E). And in the KA-treated mice the Zac1 protein was clearly found within the dendrites and soma of the amygdaloid neurons (Fig. 6I), as well as in the glial cells of the dentate gyrus (Fig. 6H) and ventricular zone (Fig. 6J–L). Alternatively, a Zac1 immunoblot was performed in untreated mice (data not shown), in

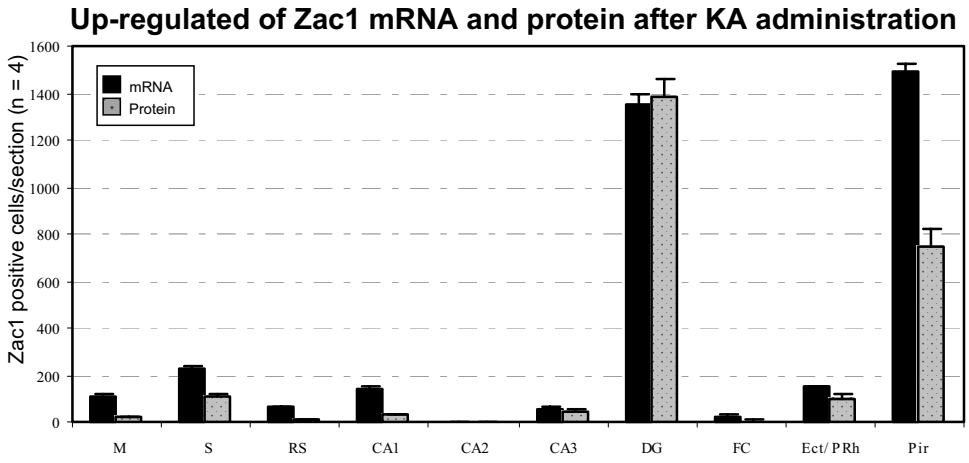

Fig. 5. Comparison between Zac1 mRNA and protein cell number induction following KA-treatment in mice. Induction is 1.5-2-fold higher in the Zac1 mRNA than in the Zac1 protein, except in the dentate gyrus and CA3 region. In these hippocampal areas, the number of Zac1 positive cells is very similar in both mRNA and protein. CA1, CA2 and CA3, hippocampal regions; DG, dentate gyrus; Ect/PRh, entorhinal and perirhinal cortices; FC, hippocampal fissure; M, motor cortex; Pir, piriform cortex; RS, retrosplenial cortex; S, somatosensory cortex; interaural 2.46 mm and bregma −1.34 mm.

order to corroborate the Zac1 immunohistochemical analysis and the specificity of the Zac1 antibody.

Immunoreactivity for c-fos and GFAP and co-localizations with Zac1 positive cells

In the animals treated with convulsing agents we observed an early up-regulation of c-fos, concretely in the first 3 h. This up-regulation appeared in all vulnerable cells, as much inside the limbic system as in many other brain areas. As a result, all the *Zac1* expressing-cells were immunolabeled for c-fos (Fig. 6C and F). Thus, both Zac1 transcripts and c-fos protein were up-regulated in the KA-treated mice and down-regulated when the mice were pre-treated with MK-801 before the KA treatment. However, in the PTZ-treated mice, we detected weak-to-moderate c-fos immunolabeling, whereas the levels of *Zac1* mRNA were very weak (data not shown).

The antibody against GFAP was co-localized with the Zac1 transcripts only in the animals treated with KA. We found co-localization in a very few glial cells distributed in the subgranular layer of the dentate gyrus (Fig. 6G), as well as in some glial cells of the ventricular and subventricular zone (SVZ; data not shown). These data are further supported by the existence of double-immunolabeling between GFAP and Zac1 proteins. Therefore, we observed a noticeable co-localization with GFAP and Zac1 protein in some glial cells of the SVZ and ventricular zone (Fig. 6J and K).

Lesion pattern

In order to measure the degree of neural lesion, histological (hematoxylin), histofluorescent (Fluoro-Jade) and immunohistological (Klenow-FragEl DNA Fragmentation Detection Kit) techniques were performed in the various treatments. Six hours following KA injection, we observed evident neuronal damage, specifically a shrunken appearance and small size, in a few mossy cells of the hilus as well as in some pyramidal cells of the CA3 with hematoxylin (Fig. 7A–E) and Fluoro-Jade, Fig. 7G–I). However, lesser co-localization was found between Zac1 immunolabeled cells and hematoxylin (Fig. 7B, D, E, and Table 2) or Fluoro-Jade stain (Fig. 7H and I) at only 6 h following KA treatment. Moreover, some Zac1 ISH cells co-localized with Fluoro-Jade stain (Fig. 7F and G). Concretely, with hematoxylin stain very few Zac1 immunopositive damaged cells (11%, number of Zac1 immunohistochemical-damaged cells/number of damaged cells in percentage counted in six hemispheres of the interaural 2.46 mm and bregma −1.34 mm, Table 2) were detected, especially in the CA3 hippocampal area at 6 h following KA treatment, whereas at 18 h the number of these cells noticeably decreased (5%, Table 2). In contrast, we found very few apoptotic cells in these brain areas with Klenow-FragEl DNA Fragmentation Detection Kit (Fig. 7J and M). However, at 18 h following KA treatment, a large number of apoptotic cells was detected within the CA3 pyramidal cell layer (Fig. 7N and P), while only a few apoptotic cells in the mossy cells of the hilus (Fig. 7K and L) were observed, in addition to a moderate number of apoptotic cells in the CA1 pyramidal cell layer (Fig. 7O). Conversely, with hematoxylin and Fluoro-Jade stains very few damaged cells were observed, only in the MK-801/KA-treated mice at 18 h after treatment, whereas in the PTZ animals no cell damage was detected at the several time points studied (data not shown).

DISCUSSION

Controls of the experimental seizure models

In the control-treated mice, both the saline and saline–DEDTC injected as well as the MK-801–saline–DEDTC injected, the expression pattern of Zac1 mRNA was

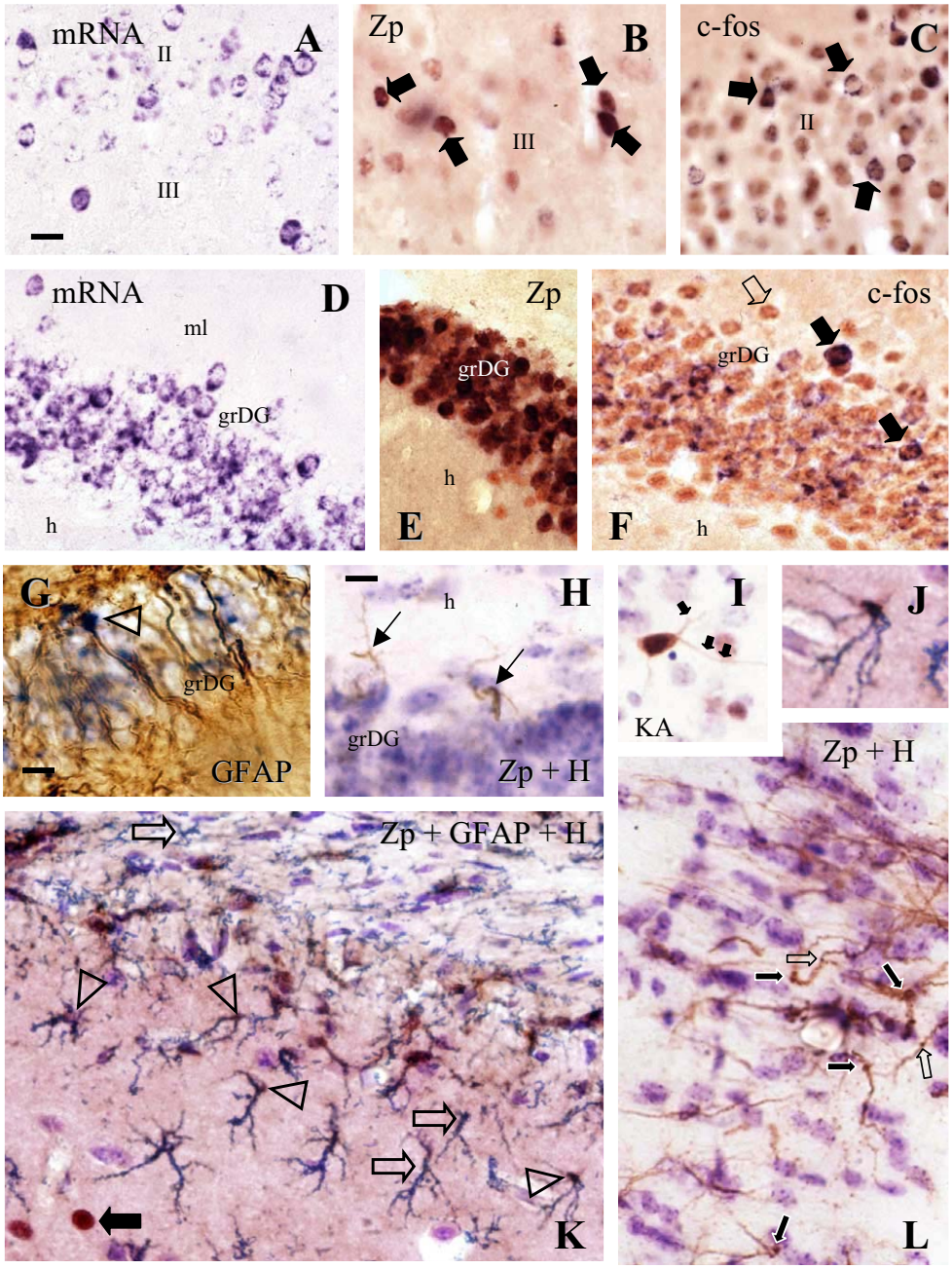

Fig. 6. Characterization of the Zac1 cells induced in some limbic areas in KA-treated and control mice with double ISH-immunohistochemical technique to Zac1 mRNA and Zac1, c-fos, GFAP antibodies, and double-immunohistochemical techniques for Zac1 and GFAP antibodies. In the somatosensory cortex (A–C), only the cells that expressed Zac1 mRNA presented Zac1 protein (solid arrows in B). The c-fos protein is detected in almost all neuronal cells of the somatosensory cortex following KA-treatment, whereas Zac1 mRNA is up-regulated only in the cells of the deep layers, which are positively labeled for c-fos (solid arrows in C). Similar findings are observed in the granular cell dentate gyrus (D–G), where a total co-localization of both Zac1 protein and mRNA (E) is detected along with a partial co-localization with c-fos protein (double-labeled cells are shown by solid arrows in F), since all the cells that expressed Zac1 mRNA are c-fos positive; the reverse case does not occur, however (open arrows in F). A few positive GFAP cells are co-localized with Zac1 mRNA in the dentate gyrus (open arrowhead in G), concretely in the granular cells. In addition, some glial cells of the subgranular layer of the dentate gyrus (arrows in H) and SVZ (solid arrows in K, L) are positively labeled to Zac1 antibody. In some of these Zac1 glial cells the dendrite spines are positively marked (see open arrows in L). These Zac1 immunolabeled cells are co-localized with some GFAP subpopulation cells (J, K), especially in the SVZ (double-labeled cells are shown by open arrowheads, GFAP-labeled cells by open arrows and Zac1 labeled cells by solid arrows in K). Morphological changes can be observed in some neuronal cells of the amygdaloid nuclei where the Zac1 immunolabeling appears either in the soma or in the dendrites (see solid arrows in I). Positive ISH cells are shown with a blue–violet color and positive IHC cells with a brown color, except in figure J and K, where positive GFAP IHC cells are blue–black in color. grDG, granular cells of dentate gyrus; Zp, IHC to Zac1 protein; II–III, layers II and III of somatosensory cortex. Scale bar = 12 μm (shown in A): A–F, K, L; (shown in H): H, I, 7 μm; (shown in G): G, J, 4 μm.

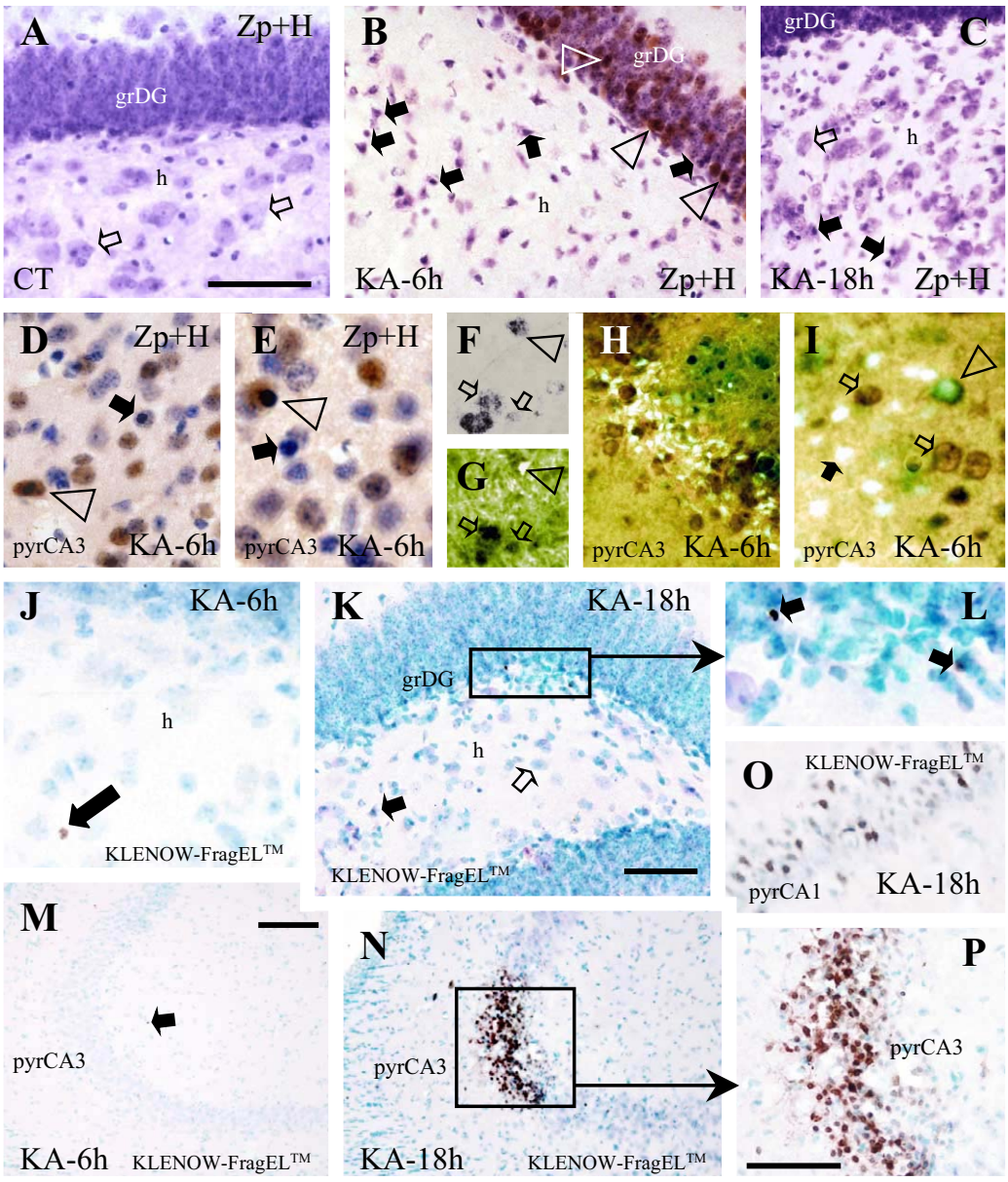

Fig. 7. Cell damage induced by KA in the hippocampus at 6 h and 18 h following i.p. injection. In the control mice (A), no damaged cells (open solid arrows) are found in the hilus of the hippocampus with hematoxylin stain, nor is immunolabeling to Zac1 found in the granular cells of dentate gyrus. In the KA-treated mice, damage cell morphology (shrunken appearance and small size; see the solid filled arrows) was found in the mossy cells of the hilus at 6 h (B) and 18 h following KA administration (C) with hematoxylin staining. Similar observations are obtained with Fluoro-Jade technique and Zac1 immunopositive cells (H, I), as well as with Zac1 positive ISH cells (F, G). In the subgranular layer of the dentate gyrus and CA3 area, low co-localization (arrowheads) is observed between Zac1 immunopositive cells (in brown) or Zac1 positive ISH cells (in dark blue) and damage cells (solid filled arrows) at 6 h after KA treatment, by hematoxylin (A–E) and Fluoro-Jade stains (F–I). Alternatively, very few apoptotic cells (solid filled arrows) are observed in the hippocampus at 6 h following KA treatment (J, M) by apoptotic technique (Klenow-FragEl DNA Fragmentation Detection Kit). However, at 18 h following KA treatment a few apoptotic cells are found in the subgranular layer of the dentate gyrus (K, L), as well as a moderate and large number of these cells in the pyramidal layer of the CA1 and CA3 region (N–P), respectively. The microphotographs F (under light field) and G (under fluorescent/FITC filter) are of exactly the same brain area, the CA3 region. grDG, granular cells of the dentate gyrus; h, hilus of dentate gyrus; pyrCA1, pyramidal cells of the CA1 hippocampal region; pyrCA3, pyramidal cells of the CA3 hippocampal region. Scale bar=50 μm (shown in A): A–D, F–H, J–L, O, P; (shown in E): E, I, 40 μm; (shown in M): M, N, 150 μm.

similar to that observed in untreated adult mice (Valente and Auladell, 2001; Spengler et al., 1997). This expression pattern did not change even when the concentrations of DEDTC increased to 1000 mg/kg or when the animals were severely behaviorally affected by a single MK-801 injection (1 mg/kg). The present experimental seizure model using DEDTC to prevent the over-excitation provoked by KA injection has been described previously in rats (Mitchell et al., 1990), and more recently in mice (Domínguez et al., 2003a,b). The administration

Table 2. Number of damage cells (hematoxylin stain) following 6 and 18 h of KA treatment

	Zac1 IHC cells with picnotic nuclei (a)		Picnotic nuclei (b)	
Hippocampal area	6 h	18 h	6 h	18 h
pyrCA1	2±1	2±1	35±3	67±4
pyrCA3	11±2	10±1	51±5	110±12
Hilus	0	0	29±3	87±7
Total cell number	13±3	12±2	115±11	264±23
(a)/(b) in %			11.3	4.55

of DEDTC after non-convulsant doses of KA induced epileptic seizures and neural damage in the hippocampus, as previously described with the high dose of KA (Strain and Tasker, 1991; Kim et al., 2001), but it also increased the survival rates of the convulsing-mice.

Zac1 mRNA and protein induction following seizures

The induction of Zac1 transcripts was prominent in the seizure model that occasioned strong cell activation and/or extensive neuronal death, as in the KA-treated mice. Thus, Zac1 is up-regulated following an intense activation of the excitatory neurons. This up-regulation of Zac1 mRNA was found in many areas of the limbic system (hippocampus, amygdala, hypothalamus, olfactory bulb and neocortex) that are extremely vulnerable to neuronal damage (Lothman et al., 1981; Filipkowski et al., 1994; Pollard et al., 1994a,b; Goodenough et al., 1997; Djebaïli et al., 2001). These limbic areas are well joined by a complex network of connections (Price, 2003; Stoop and Pralong, 2000; Calderazzo et al., 1996; Witter and Amaral, 1991; Wilson et al., 1990), through which the KA induction could extend. The up-regulation of *Zac1* transcripts was detectable starting 3 h after KA injection and persisted until 18 h. In the KA-treated mice previously injected with MK-801, which acts as a blocker of the NMDA receptors (Lee et al., 2002; Planas et al., 1995; Clifford et al., 1990), we found a noticeable attenuation of the induction of *Zac1*, as has been observed with other genes in diverse studies (Hughes et al., 1998; Bendotti et al., 1997; McNamara and Routtenberg, 1995), but only at 6 h following KA injection. *Zac1* induction seems to require activation of the NMDA receptors. Zac1 protein expression is identical with the pattern of Zac1 mRNA, both in the untreated and the variously treated mice. All induced cells positively labeling to Zac1 protein also expressed Zac1 transcripts with co-localization techniques. Moreover, the number of Zac1 transcript cells is almost twice the number of Zac1 protein cells, except in the dentate gyrus. The discrepancy in the dentate gyrus could be explained by the high density of granular cells and the diffuse ISH signal presented in some of them judged to be negative labeling. On the other hand, Zac1 was expressed starting at 3 h following KA treatment in cells of the dentate gyrus and neocortex which did not normally express it, as is the case for many other transcription factors, such as the early genes c-fos and c-Jun, which are implicated in cell rescue and cell survival (Ressler et al., 2002; Zagulska-Szymczak et al., 2001; Becker et al., 1999). These early genes encode transcription factor proteins that have been implicated in cell proliferation, gene transcription, stress responses, regeneration, and cell death (Zhang et al., 2002; Salvat et al., 1999; Goodenough et al., 1997; Pandey and Wang, 1995; Rinaudo and Zelenka, 1992). Moreover, c-fos is also up-regulated following KA treatment as a consequence of a massive entry of Ca^{2+} into vulnerable cells (Morgan and Curran, 1986, 1989; Greenberg et al., 1992). Our co-localization studies of Zac1 transcripts and c-fos protein have shown that all *Zac1*-positive cells are c-fos immunolabeled. Additionally, noticeable Zac1 up-regulation is found in the granular cells of the dentate gyrus, which are resistant to seizure-nduced injury (Bugra et al., 1994; Zagulska-Szymczak et al., 2001).

PTZ-treated mice showed the same lessened induction of the Zac1 transcripts in entorhinal and piriform cortices at only 6 h following treatment, such as that with MK-801/KA. PTZ is a convulsing drug that does not provoke neuronal death (Klioueva et al., 2001; da Silva et al., 1998; Planas et al., 1994) while MK-801 administration prevents the cellular damage provoked by KA (Lee et al., 2002; Clifford et al., 1990). Taken together, these results suggest that *Zac1*, like the early genes, may be implicated in the early cellular mechanisms that control neural survival following seizure-induced strong neuronal stimulation.

Zac1 protein expression in glial cells following KA treatment

In the KA-treated mice we also found immunolabeling to Zac1 protein within glial cells, especially in many cells of the SVZ and ventricular zone (VZ), which co-localized with GFAP protein as well as Zac1 transcripts. The SVZ and VZ are extensively involved in early cellular processes, such as proliferation and differentiation (Frederiksen and McKay, 1988; Lois and Alvarez-Buylla, 1993; Cremisi et al., 2003), in which Zac1 mRNA is strongly expressed throughout embryonic and early postnatal development (Valente and Auladell, 2001). Moreover, Zac1 has an anti-proliferative activity, as it induces extensive apoptosis and G1 arrest (Spengler et al., 1997; Pagotto et al., 1999, 2000). Several studies have described an extensive process of glial proliferation (gliosis) in the hippocampus as

well as in the VZ following KA administration (Altar and Baudry, 1990; Niquet et al., 1994a,b). Thus, Zac1 could be implicated in the control of the glial plasticity process that occurs following injury.

Zac1 positive damaged cells are a very limited subpopulation of damaged cells

In previous reports, the Zac1 gene has been related with apoptosis and arrested cell cycle (Spengler et al., 1997; Rozenfeld-Granot at al., 2002). It has been noted that apoptosis, like necrosis, contributes to degeneration following seizures in rats (Pollard et al., 1994; Weiss et al., 1996; Portera-Cailliau et al., 1997). In the present work, the lesion pattern obtained is very similar to that previously described for the same experimental seizure model (Domínguez et al., 2003) and other seizure models in mice (Strain and Tasker, 1991) and rats (Ben-Ari, 1985). Thus, with hematoxylin and Fluoro-Jade stains we found some damaged cells in the hippocampus at 6 h following KA treatment, but, curiously, not within the hippocampal areas where Zac1 up-regulation is more evident. Moreover, the number of damaged cells increases rapidly at 18 h in the hippocampus, at the same time during which Zac1 expression disappears within the hippocampus, except for a few cells of CA3. Consequently, the low number of Zac1 positive damaged cells at 6 h decreases sharply at 18 h following KA treatment. Alternatively, the histological studies to detect apoptotic cells with Klenow-FragEl DNA Fragmentation Detection Kit showed a distribution pattern similar to that obtained for hematoxylin and Fluoro-Jade stains, but in fewer cells. Thus, a very few apoptotic labeled cells are found in the hilus (where Zac1 is not induced) and in the CA3 region at 6 h following KA treatment (where Zac1 is induced in few cells), whereas there are many apoptotic labeled cells in the CA3 region at 18 h (where Zac1 is induced in very few cells). The cellular processes that lead to programmed cell death are lengthy (Mikati et al., 2003; Araki et al., 2002; Tooyama et al., 2002; Djebaïli et al., 2001) and have been shown to occur exactly when the induction of Zac1 decreases. It is possible, then, that some cells up-regulated by Zac1 in the early hours could die in the subsequent days by apoptosis, even when there is no evidence of co-localization at this time, thereby reinforcing to some degree their putative role in the cell death process (Spengler et al., 1997).

CONCLUSIONS

Tumor suppressor genes encode proteins involved in growth regulation in differentiating and proliferating cells (for review see Macleod, 2000; Teh et al., 1999). The Zac1 gene is highly expressed in differentiating and proliferating brain areas during embryonic and early postnatal development (Valente and Auladell, 2001). In the present seizure convulsing model, Zac1 is induced in the limbic system and appears to be produced by both glia and neurons, which suggests a novel role for the candidate tumor suppressor gene Zac1 in growth regulatory pathways involved in cellular plasticity in response to injury. Recently, some studies have reported that the Zac1 gene induces and regulates the expression of the $PACAP_1$-R gene, which has been implicated in neurotrophic processes during normal brain development (Rodriguez-Henche et al., 2002). In this work, the induction of the Zac1 gene in the hippocampus of KA-treated mice has shown many analogies with $PACAP_1$-R gene expression in another KA model created by Boschert and colleagues (1997). Furthermore, the Zac1 and $PACAP_1$-R genes show co-induction under stressful conditions after ischemic injury (Gillardon et al., 1998). Taking together this co-induction with the present co-relation in seizure models, we suggest the possible involvement of both the Zac1 and $PACAP_1$-R genes in diverse biological responses following strong neural activation, concretely in the neural plasticity processes.

Acknowledgments—We wish to thank Tom Yohannan for editorial assistance. The study was supported by grants from the Ministry of Education (AECI01) and the Ministry of Health (FIS 01/0096–02) of Spain, and from the University of Barcelona.

REFERENCES

Altar CA, Baudry M (1990) Systemic injection of kainic acid: gliosis in olfactory and limbic brain regions quantified with [^{3}H]PK11195 binding autoradiography. Exp. Neurol 109:333–341.

Araki T, Simon RP, Taki W, Lan J-Q, Henshall DC (2002) Characterization of neuronal death induced by focally evoked limbic seizures in the C57BL/6 mouse. J. Neurosci. Res 69:614–621.

Basille M, Vaudry D, Coulouarn Y, Jegou S, Lihrmann I, Fournier A, Vaudry H, Gonzalez B (2000) Comparative distribution of pituitary adenylate cyclase-activating polypeptide (PACAP) binding sites and PACAP receptor mRNAs in the rat brain during development. J Comp Neurol 425:495–509.

Becker AJ, Gillardon F, Blumcke I, Langendorfer D, Beck H, Wiestler OD (1999) Differential regulation of apoptosis-related genes in resistant and vulnerable subfields of the rat epileptic hippocampus. Mol Brain Res 67:172–176.

Ben-Ari Y, Cossart R (2000) Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci 23:580–587.

Ben-Ari Y (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14:375–403.

Bendotti C, Baldessari S, Pende M, Southgate T, Guglielmetti F, Samanin R (1997) Relationship between GAP-43 expression in the dentate gyrus and synaptic reorganization of hippocampal mossy fibres in rats treated with kainic acid. Eur J Neurosci 9:93–101.

Bengzon J, Mohapel P, Ekdahl CT, Lindvall O (2002) Neuronal apoptosis after brief and prolonged seizures. Prog Brain Res 135:111–119.

Boschert U, Muda M, Camps M, Dickinson R, Arkinstall S (1997) Induction of the dual specificity phosphatase PAC1 in rat brain following seizure activity. Neuroreport 8:3077–3080.

Bugra K, Pollard H, Charton G, Moreau J, Ben-Ari Y, Khrestchatisky M (1994) aFGF, bFGF and flg mRNAs show distinct patterns of induction in the hippocampus following kainate-induced seizures. Eur J Neurosci 6:58–66.

Calderazzo L, Cavalheiro EA, Macchi G, Molinari M, Bentivoglio M (1996) Branched connections to the septum and to the entorhinal cortex from the hippocampus, amygdala, and diencephalon in the rat. Brain Res Bull 40:245–251.

Clifford DB, Olney JW, Benz AM, Fuller TA, Zorumski CF (1990) Ketamine, phencyclidine, and MK-801 protect against kainic acid-induced seizure-related brain damage. Epilepsia 31:382–390.

Cremisi F, Philpott A, Ohnuma S (2003) Cell cycle and cell fate interactions in neural development. Curr Opin Neurobiol 13:26–33.

Danscher G, Haug FM, Fredens K (1973) Effect of diethyldithiocarbamate (DEDTC) on sulphide silver stained boutons. Reversible blocking of Timm's sulphide silver stain for "heavy" metals in DEDTC treated rats (light microscopy). Exp Brain Res 16:521–532.

da Silva LF, Pereira P, Elisabetsky E (1998) A neuropharmacological analysis of PTZ-induced kindling in mice. Gen Pharmacol 31:47–50.

Djebaïli M, Lerner-Natoli M, Pascale M, Baille V, Bockaert J, Rondouin G (2001) Molecular events involved in neuronal death induced in the mouse hippocampus by in-vivo injection of kainic acid. Mol Brain Res 93:190–198.

Domínguez MI, Blasco-Ibáñez JM, Crespo C, Marqués-Marí AI, Martínez-Guijarro FJ (2003a) Zinc chelation during non-lesioning overexcitation results in neuronal death in the mouse hippocampus. Neuroscience 116:791–806.

Domínguez MI, Blasco-Ibáñez JM, Crespo C, Marqués-Marí AI, Martínez-Guijarro FJ (2003b) Calretinin/PSA-NCAM immunoreactive granule cells after hippocampal damage produced by kainic acid and DEDTC treatment in mouse. Brain Res 966:206–217.

Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J Cell Biol 127(6 Pt 1):1717–1727.

Gillardon F, Hata R, Hossmann KA (1998) Delayed up-regulation of Zac1 and PACAP type I receptor after transient focal cerebral ischemia in mice. Mol Brain Res 61:207–210.

Goodenough S, Davidson M, Chen W, Beckmann A, Pujic Z, Otsuki M, Matsumoto I, Wilce P (1997) Immediate early gene expression and delayed cell death in limbic areas of the rat brain after kainic acid treatment and recovery in the cold. Exp Neurol 145(2 Pt 1):451–461.

Greenberg ME, Thompson MA, Sheng M (1992) Calcium regulation of immediate early gene transcription. J Physiol Paris 86:99–108.

Filipkowski RK, Hetman M, Kaminska B, Kaczmarek L (1994) DNA fragmentation in rat brain after intraperitoneal administration of kainate. Neuroreport 5:1538–1540.

Frederiksen K, McKay RD (1988) Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. J Neurosci 8:1144–1151.

Hoffmann A, Ciani E, Houssami S, Brabet P, Journot L, Spengler D (1998) Induction of type I PACAP receptor expression by the new zinc finger protein Zac1 and p53. Ann NY Acad Sci 865:49–58.

Honkaniemi J, Sharp FR (1999) Prolonged expression of zinc finger immediate-early gene mRNAs and decreased protein synthesis following kainic acid induced seizures. Eur J Neurosci 11:10–17.

Hughes PE, Young D, Preston KM, Yan Q, Dragunow M (1998) Differential regulation by MK801 of immediate-early genes, brain-derived neurotrophic factor and trk receptor mRNA induced by a kindling alter-discharge. Mol Brain Res 53:138–151.

Kaminska B, Filipkowski RK, Biedermann IW, Konopka D, Nowicka D, Hetman M, Dabrowski M, Gorecki DC, Lukasiuk K, Szklarczyk AW, Kaczmarek L (1997) Kainate-evoked modulation of gene expression in rat brain. Acta Biochim Pol 44:781–789.

Kim HJ, Moon KD, Oh SY, Kim SP, Lee SR (2001) Ether fraction of methanol extracts of *Gastrodia elata*, a traditional medicinal herb, protects against kainic acid-induced neuronal damage in the mouse hippocampus. Neurosci Lett 314:65–68.

Kliouéva IA, van Luijtelaar EL, Chepurnova NE, Chepurnov SA (2001) PTZ-induced seizures in rats: effects of age and strain. Physiol. Behav 72:421–426.

Lee JK, Choi SS, Lee HK, Han KJ, Han EJ, Suh HW (2002) Effects of MK-801 and CNQX on various neurotoxic responses induced by kainic acid in mice. Mol Cells 14:339–347.

Liu W, Rong Y, Baudry M, Schreiber SS (1999) Status epilepticus induces p53 sequence-specific DNA binding in mature rat brain. Mol Brain Res 63:248–253.

Lois C, Alvarez-Buylla A (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci USA 90:2074–2077.

Lothman EW, Collins RC, Ferrendelli JA (1981) Kainic acid-induced limbic seizures: electrophysiologic studies. Neurology 31: 806–812.

Macleod K (2000) Tumor suppressor genes. Curr Opin Genet Dev 10:81–93.

McNamara RK, Routtenberg A (1995) NMDA receptor blockade prevents kainite induction of protein F1/GAP-43 mRNA in the hippocampal granule cells and subsequent mossy fiber sprouting in the rat. Mol Brain Res 33:22–28.

Mikati MA, Abi-Habib RJ, El Sabban ME, Dbaibo GS, Kurdi RM, Kobeissi M, Farhat F, Asaad W (2003) Hippocampal programmed cell death after status epilepticus: evidence for NMDA-receptor and ceramide-mediated mechanisms. Epilepsia 44:282–291.

Mitchell CL, Barnes MI, Grimes LM (1990) Diethyldithiocarbamate and dithizone augment the toxicity of kainic acid. Brain Res 506:327–330.

Morgan JI, Curran T (1986) Role of ion flux in the control of c-fos expression. Nature 322:552–555.

Morgan JI, Curran T (1989) Calcium and proto-oncogene involvement in the immediate-early response in the nervous system. Ann NY Acad Sci 568:283–290.

Niquet J, Ben-Ari Y, Represa A (1994a) Glial reaction after seizure induced hippocampal lesion: immunohistochemical characterization of proliferating glial cells. J Neurocytol 23:641–656.

Niquet J, Jorquera I, Ben-Ari Y, Represa A (1994b) Proliferative astrocytes may express fibronectin-like protein in the hippocampus of epileptic rats. Neurosci Lett 180:13–16.

Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453–501.

Pagotto U, Arzberger T, Ciani E, Lezoualc'h F, Pilon C, Journot L, Spengler D, Stalla GK (1999) Inhibition of Zac1, a new gene differentially expressed in the anterior pituitary, increases cell proliferation. Endocrinology 140:987–996.

Pagotto U, Arzberger T, Theodoropoulou M, Grübler Y, Pantaloni C, Saeger W, Losa M, Journot L, Stalla GK, Spengler D (2000) The expression of the antiproliferative gene ZAC is lost or highly reduced in non-functioning pituitary adenomas. Cancer Res 60:6794–6799.

Pandey S, Wang E (1995) Cells en route to apoptosis are characterized by the upregulation of c-fos, c-myc, c-jun, cdc2, and RB phosphorylation, resembling events of early cell-cycle traverse. J Cell Biochem 58:135–150.

Planas AM, Soriano MA, Ferrer I, Rodriguez F (1994) Regional expression of inducible heat shock protein-70 mRNA in the rat brain following administration of convulsant drugs. Mol Brain Res 27:127–137.

Planas AM, Soriano MA, Ferrer I, Rodriguez F (1995) Kainic acid-induced heat shock protein-70, mRNA and protein expression is inhibited by MK-801 in certain rat brain regions. Eur J Neurosci 7:293–304.

Pollard H, Cantagrel S, Charriaut-Marlangue C, Moreau J, Ben-Ari Y (1994a) Apoptosis associated DNA fragmentation in epileptic brain damage. Neuroreport 5:1053–1055.

Pollard H, Charriaut-Marlangue C, Cantagrel S, Represa A, Robain O, Moreau J, Ben-Ari Y (1994b) Kainate-induced apoptotic cell death in hippocampal neurons. Neuroscience 63:7–18.

Portera-Cailliau C, Price DL, Martin LJ (1997) Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in the adult brain are morphologically distinct: further evidence for an apoptosis-necrosis continuum. J Comp Neurol 378:88–104.

Price JL (2003) Comparative aspects of amygdala connectivity. Ann NY Acad Sci 985:50–58.

Ressler KJ, Paschall G, Zhou X, Davis M (2002) Regulation of synaptic plasticity genes during consolidation of fear conditioning. J Neurosci 22:7892–7902.

Rinaudo JA, Zelenka PS (1992) Expression of c-fos and c-jun mRNA in the developing chicken lens: relationship to cell proliferation, quiescence, and differentiation. Exp Cell Res 199:147–153.

Rodríguez-Henche N, Jamen F, Leroy C, Bockaert J, Brabet P (2002) Transcription of the mouse PAC1 receptor gene: cell-specific expression and regulation by Zac1. Biochim Biophys Acta 1576:157–162.

Rozenfeld-Granot G, Krishnamurthy J, Kannan K, Toren A, Amariglio N, Givol D, Rechavi G (2002) A positive feedback mechanism in the transcriptional activation of Apaf-1 by p53 and the coactivator Zac-1. Oncogene 21:1469–1476.

Salvat C, Aquaviva C, Jariel-Encontre I, Ferrara P, Pariat M, Steff AM, Carillo S, Piechaczyk M (1999) Are there multiple proteolytic pathways contributing to c-fos, c-jun and p53 protein degradation in vivo? Mol Biol Rep 26:45–51.

Schmued LC, Albertson C, Slikker W Jr (1997) Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 751:37–46.

Schmued LC, Hopkins KJ (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 874:123–130.

Sherwood NM, Krueckl SL, McRory JE (2000) The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocr Rev 21:619–670.

Spengler D, Villalba M, Hoffmann A, Pantaloni C, Houssami S, Bockaert J, Journot L (1997) Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J 16:2814–2825.

Sperk G (1994) Kainic acid seizures in the rat. Prog Neurobiol 42:1–32.

Stoop R, Pralong E (2000) Functional connections and epileptic spread between hippocampus, entorhinal cortex and amygdala in a modified horizontal slice preparation of the rat brain. Eur J Neurosci 12:3651–3663.

Strain SM, Tasker RA (1991) Hippocampal damage produced by systemic injections of domoic acid in mice. Neuroscience 44:343–352.

Teh BT, Larsson C, Nordenskjold M (1999) Tumor suppressor genes (TSG). Anticancer Res 19:4715–4728.

Tooyama I, Bellier JP, Park M, Minnasch P, Uemura S, Hisano T, Iwami M, Aimi Y, Yasuhara O, Kimura H (2002) Morphologic study of neuronal death, glial activation, and progenitor cell division in the hippocampus of rat models of epilepsy. Epilepsia 43:39–43.

Valente T, Auladell C (2001) Expression pattern of Zac1 mouse gene, a new zinc-finger protein that regulates apoptosis and cellular cycle arrest, in both adult brain and along development. Mech Dev 108:207–211.

Vaudry D, Basille M, Anouar Y, Fournier A, Vaudry H, Gonzalez BJ (1998) The neurotrophic activity of PACAP on rat cerebellar granule cells is associated with activation of the protein kinase A pathway and c-fos gene expression. Ann NY Acad Sci 865:92–99.

Vaudry D, Gonzalez BJ, Basille M, Fournier A, Vaudry H (1999) Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development. Proc Natl Acad Sci USA 96:9415–9420.

Verdaguer E, Garcia-Jorda E, Canudas AM, Domínguez E, Jimenez A, Pubill D, Escubedo E, Pallas JC, Camins A (2002) Kainic acid-induced apoptosis in cerebellar granule neurons: an attempt at cell cycle re-entry. Neuroreport 13:413–416.

Weiss S, Cataltepe O, Cole AJ (1996) Anatomical studies of DNA fragmentation in rat brain after systemic kainate administration. Neuroscience 74:541–551.

Wilson CL, Isokawa M, Babb TL, Crandall PH (1990) Functional connections in the human temporal lobe: I. Analysis of limbic system pathways using neuronal responses evoked by electrical stimulation. Exp Brain Res 82:279–292.

Witter MP, Amaral DG (1991) Entorhinal cortex of the monkey: V. Projections to the dentate gyrus, hippocampus, and subicular complex. J Comp Neurol 307:437–459.

Zagulska-Szymczak S, Filipkowski RK, Kaczmarek L (2001) Kainate-induced genes in the hippocampus: lessons from expression patterns. Neurochem Int 38:485–501.

Zhang J, Zhang D, McQuade JS, Behbehani M, Tsien JZ, Xu M (2002) c-fos regulates neuronal excitability and survival. Nat Genet 30:416–420.

(Accepted 26 June 2004)
(Available online 21 August 2004)

Title:

Zac1, a new anti-proliferative zinc finger protein, is required for neural progenitors differentiation towards restrict interneurons and glial cells.

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Running title:

Zac1 is implicated in neural differentiation

Keywords: Zinc-finger protein, apoptosis, neurogenesis, progenitor/stem cells, cell cycle arrest; oligodendrocytes; GABAergic interneurons; cell fate; cellular differentiation

ABSTRACT

Zac1 is expressed in the progenitor/stem cells during the embryogenesis and in the germinative niches of the adult brain mice. In seizures models, Zac1 is induced in the glial and neuronal cells of the limbic system. Nevertheless, its role in the central nervous system isn't known. In the present work, we tried to enrich the actual information of Zac1 gene in the CNS through the study of the brain phenotype of the Zac1 mutant mice and its implication in the damage processes following seizures. The obtained results showed that the mutant mice had a high incidence of hydrocephalia, displayed an important reduction in the brain size and a noticeable increase in the proliferation rate of progenitor cells. Moreover, important changes in the glial and neuronal populations were determined. Thus, in the mutant mice an evident decrease in the immature and mature oligodendrocytes, as well as, in the microglial cells was detected. In contrast, an important increase in the immature and mature GFAP-cell subpopulations was found. On the other hand, except for calretinin interneurons, which were down-regulated in the Zac1 mutant mice, a general increase in several GABAergic interneurons subpopulations was determined. Further, a reduction in the catecholinergic neurons was observed. Altogether, our study reinforces the anti-proliferative role of Zac1 and shows that Zac1 is important in the neural differentiation of several progenitor cells of the central nervous system.

INTRODUCTION

The gene Zac1 encode a zinc-finger protein which regulates both the cell cycle arrest (in phase G1) and the apoptosis, by independent routes (Spengler et al., 1997). Zac1 is a maternally imprinted gene and also implied in the transitory neonatal diabetes mellitus, which is a rare inherited diabetic syndrome apparent in the first weeks of life and again during early adulthood (Gardner et al., 2000; Arima et al., 2001; Varrault et al., 2001; El Kharroubi et al., 2001; Abdollahi et al., 2003; Ma et al., 2004). Zac1 is a transcriptional co-activator and repressor for nuclear receptors (Huang and Stallcup, 2000), as well as is a co-activator of p53/Apaf-1 (Huang et al., 2001; Rozenfeld-Granot et al., 2002). In the adult mice, Zac1 is expressed mainly in the pituitary gland, brain (hypothalamus, amygdala, olfactory bulb and hippocampus) and chondrogenic sites (Spengler et al., 1997; Valente and Auladell, 2001; Valente et al, 2005). Zac1 is expressed in the progenitor/stem cells during the embryogenesis and in the proliferative niches of the adult brain mice (Valente et al., 2005; Valente and Auladell, 2001). In kainic acid seizures models, Zac1 is induced in the glial and neuronal cells of the limbic system, possibly being implied in the neuronal plasticity processes that are triggered after seizures that caused an extensive cell activation (Valente et al., 2004). Nevertheless, Zac1 functions in the central nervous system are not yet known. Thus, the study of the mutant mice for Zac1 bring new lights into the role of Zac1 along embryonic development and, in particular, in the development of the central nervous system. In this work, we studied the brain phenotype of the Zac1 mutant adult mice by immunohistochemical and western blot techniques, under normal conditions as following seizures induced by kainic acid.

RESULTS

Histological analysis of the Zac1 mutant mouse brain

A macroscopic analysis of the mutant and wild type mice brain sections showed a clear reduction in the brain size of the mutant mice. In addition, one of each three mutant mice displayed hydrocephalia, whereas any case of hydrocephalia was observed in the wild type mice brain.

By histochemical techniques (Nissl and Methyl Green-Pyronin) a noticeable increase in the cell number of the subventricular zone and in the granular layer of the olfactory bulb was observed in the mutant mice (Figures 1A-B). However, the position and the limits of the brain nuclei and areas seemed similar in mutant and wild type mice.

Immunohistological phenotype of the Zac1 mutant mouse brain

Proliferative phenotype

The immunohistochemical techniques detecting the PCNA protein and the incorporation of BrdU showed the same cellular pattern of distribution in the adult proliferative areas. Thus, in the mutant mice we observed a noticeable increase of the number of proliferative cells in the subventricular zone of the lateral and third ventricles, in the RMS of the olfactory system and in the subgranular zone of dentate gyrus (Figures 1C-H and Table I). However, no differences were detected with Nestin-positive cells in the subventricular zone of the lateral and third ventricles and in the subgranular zone of the dentate gyrus (Table I).

Neuronal phenotype

In the mutant mice, we observed a decrease of PSA-NCAM-positive cells within proliferating/differentiating fields of the RMS (Figures 1I-L), whereas no evident changes were observed for β-tubulin-positive cells (Table I). The NeuN-positive cells

were very similar in both mutant and wild type mice, except in the septal area of the mutant mice, where a weak reduction of the neuronal cell number was detected (Figures 1M-N).

The immunodetection for MAP2a&b (neuronal and dendrite marker) showed a general decrease all over the limbic brain areas of the mutant mice, except for upper layers of the cingular and retrosplenial cortices, in which an increase of this immunostaining was determined (Figures 1O-T).

The expression of the calcium-binding proteins was altered in the mutant mice (Figure 2 and Table I). Thus, in the mutant mice, we observed a noticeable increase in the number of calbindin-positive cells in the habenular thalamic nuclei, amygdaloid region and hypothalamic area (Figures 2A-F). In addition, these mutant mice presented a cellular increase of parvalbumin-positive cells in the cingular and retrosplenial cortices, reticular thalamic nucleus and in the dentate gyrus of hippocampus (Figures 2G-L). On the other hand, we observed a noticeable decrease of calretinin-positive cells in all brain areas (Figures 2M-P).

By ISH we detected a general down-regulation of the *GAD65*mRNA levels in all brain areas of the mutant mice (Table I). However, the number of *GAD65*-positive cells is comparable to wild type, except for the olfactory bulb, which showed a cellular diminution of this neuronal subpopulations (Figures 3A-D). In contrast, in the mutant mice, an increase of the cellular subpopulations that expressed the *NPY* gene in the limbic areas could be observed, especially in the hippocampus and hypothalamus (Figures 3E-H).

The TH-positive cells (catecholaminergic cells) were clearly reduced in the hypothalamic and brainstem nuclei of the mutant mice, as well as their projections (Figures 4A-D).

Axonal phenotype

In the white matter of the mutant mice, the number of MBP-positive fibers (myelin-binding protein) was decreased weakly and moderately in the rostral and caudal brain areas, respectively (Figures 4E-H).

Glial phenotype

We detected a significant increase of the GFAP-positive cells in the germinative fields of the adult mutant mice brain (Table I). Thus, many immature GFAP-positive cells were found in the subventricular zone of the lateral and third ventricle, and in the subgranular layer of the dentate gyrus (Figures 5B-E and 5G-J). Co-localization studies confirmed the proliferative character of many of these glial cells (data not shown). In addition, we observed a clear increase of the mature GFAP-positive cells within of the olfactory bulb (Figures 5A and 5F).

Contrarily, we detected an evident decrease in the immature (Ng2-positive cells) and mature (CAII-positive cells) oligodendrocytes mainly within the white matter of the mutant mice brain (Figures 5K-V). In addition, we found a general reduction of the microglial cells (CD11-positive cells) in all of the brain areas of the mutant mice (Figures 5W-Z).

No difference in the immunostaining against vimentin (radial glia marker) could be found between mutant and wild type mice brain (Table I).

Zac1 mutant mice behaviour following KA administration

The mutant and wild-type mice developed comparable convulsions following KA administration. Nevertheless the survival rate of the mutant (6 of each 10 mutant mice) was lower compared to the wild type (8 of each 10 wild type mice).

All mice acquire a position of corporal contraction after KA injection, since in the following fifteen minutes the convulsions begin.

Immunohistological phenotype of the Zac1 mutant mouse brain following KA administration

Neuronal loss and Cellular activation

At 3 hours followed KA treatment, the presence of neurodegenerative cells could be observed only in the hippocampus of the wild type mice (Figure 6 and Table II), in particular in many mossy cells of hilus and in some pyramidal cells of CA3 region. At 8 hours following KA-treatment, the number of neurodegenerative cells of the wild type mice clearly increases in the hilus and in the CA3 hippocampal region. At this time (8 hours), some neurodegenerative cells appeared for the first time in the hilus and in the CA3 region of the treated-mutant mice. At 24 and 48h, in the treated-wild type mice many neurodegenerative cells were found within hilus and CA3 hippocampal region, as well as in the CA1 hippocampal region (Figure 6 and Table II). At the same time, we didn't observe any neurodegenerative cells in the treated-mutant mice. In the treated-wild type mice, the number of neurodegenerative cells decreased progressively after 48 hours in the hippocampal areas and disappeared completely at 7 days following KA treatment, except in the CA1 hippocampal region (Figure 6 and Table II).

Similar results were found with the apoptotic markers in treated-mutant and treated-wild type mice (Figure 7 and Table II). Thus, a complete absence of apoptotic cells were observed in the treated-mutant mice in the several times used, whereas in the treated-wild type mice many apoptotic cells were detected between 24h and 48h in the CA3 hippocampal area. In addition, 48h following KA-injection some apoptotic cells were found in the CA1 hippocampal region of the treated-wild type mice, which were maintained for 7th day (data not shown).

Between 1 and 8 hours following KA injection, we found an intense c-fos immunolabelling (which is an indicative marker of the cellular calcium entrance) in the hippocampal area of the treated-wild type mice, whereas, in the treated-mutant mice, a very weak c-fos labelling could be found only at 8 hours following KA-treatment (Figure 7 and Table II).

Neural Proliferation

Between 24 and 48 hours following KA administration, many cellular alterations in the hippocampus and septum of the treated-wild type mice were found, whereas few alterations were observed in the treated-mutant mice. Thus, in the treated-mutant mice, we observed a faint increase of the cell proliferation in the septum whereas in the dentate gyrus compared with the mutant untreated mice a clear decrease of cell proliferation could be detected (Figures 8A-B). In contrast, treated-wild type mice showed a clear increase in the number of proliferative cells (BrdU-positive cells) in the subgranular layer of the dentate gyrus (Figure 8) and in the septum (Figure 9). These proliferative cells co-localized differentially with glial cells. Therefore, in the dentate gyrus, the major part of proliferative cells (BrdU-positive cells) co-localized with GFAP (Figures 8E-H), whereas only a minor groups co-localized with Ng2 (a marker for immature oligodendrocytes; Figures 8I-J) or with CD11 (a marker for microglia; Figures 8K-L). In contrast, in the septum of the treated-mutant mice, the major groups of the proliferative cells are expressed Ng2 (Figures 9E-H) and CD11 (Figure 9I), and a minor group expressed GFAP (Figure 9J).

As well in the treated-wild type mice as in the treated-mutant ones, no proliferative cell co-localized with undifferentiating neuronal markers (β-tubulin-positive cells). On the other hand, we observed a clear decrease of the NeuN immunostaining in the mutant mice mainly in the septum (Figures 9C-D).

DISCUSSION

Histological analysis

The high incidence of hydrocephalia in the mutant mice and the strong expression of Zac1 in the choroids plexus, which regulates the cerebrospinal fluid, suggest that Zac1 is an important factor in the brain homeostasis.

Our results confirm the anti-proliferative character of the Zac1 protein in adult brain mice (Spengler et al., 1997), since the mutant animals display a remarkable increase in the proliferative rate of the germinative niches, in the subventricular and subgranular zones (Alvarez-Buylla and Lim, 2004; Doestch, 2003; Kintner, 2002). Moreover, the absence of alterations in the nestin-positive cells of the mutant mice, suggests that Zac1 is involved in the control of the proliferative rate of the progenitor cells but not of the stem cells.

Immunohistological analysis

Neuronal populations in the RMS

The expression of Zac1 seems to be important in the RMS, since in the mutant mice showed a clear reduction of the number of undifferentiated neurons, PSA-NCAM-positive cells, that arrive to olfactory bulb, as well as, a noticeable decrease in the calretinin neurons of the glomerular layer of the olfactory bulb. Moreover, there was a reduction in the intracellular levels of *GAD65* within of the granular layer, as well as in the number of neurons and dendrite projections (MAP2a&b-positive neurons) located within the glomerular layer of the olfactory bulb. Nevertheless, a significant increase in the number of proliferative cells was found in the RMS, which were co-localised with GFAP. In addition, we found a general increase of differentiated GFAP-positive cells in the glomerular layer of the olfactory bulb. Thus, the precursor cells of the mutant mice

adopt a glial fate causing an over production of astrocytes and a down production of calretin-positive cells (a restricted GABAergic interneurons subpopulation). Zac1 was strongly expressed in the neuroepithelial cells (Valente and Auladell, 2001; Valente et al., 2005). These cells are the progenitors of all neurons and macroglial cells of the mammalian central nervous system during development. Nevertheless, in the adult brain the proliferation persists at least in two concrete regions, in the subventricular zone and in the subgranular zone (for revision see Doetsch, 2003, and Alvarez-Buylla and Lim, 2004). Taken together all the data, we suggests that Zac1 protein is determinant in the specification of restricted neuronal progenitors (calretinin-positive interneurons) in the olfactory bulb, and their absence favours the production of glial cells (astrocytes).

Neural populations in the limbic system

A general increase of some neuronal populations (calbindin-positive cells, parvalbumin-positive cells and *NPY*-positive cells) in the limbic areas of the mutant mice was observed. In contrast, a noticeable reduction of the immature and mature oligodendrocytes was found in all parts of the limbic system. Moreover, the axonal myelin fibers of these areas were clearly reduced. Zac1 was expressed in the progenitor cells of the lateral and medial ganglionic eminence (Valente and Auladell, 2001; Valente et al., 2005), which were implied in the generation of early GABAergic interneuron and oligodendrocyte progenitors cells (Olsson et al., 1998; Wilson and Rubenstein, 2000; Rakic and Zecevic, 2003). Therefore, the inactivation of Zac1 gene reduce the oligodendrocyte differentiation, thereby favouring production of GABAergic interneurons, in the limbic system. This way, Zac1 can play an important role in the control of the neural differentiation balance towards interneurons or towards oligodendrocytes.

Catecholinergic neurons

In the brainstem of mutant mice, the subpopulations of catecholinergic neurons were strongly reduced, as well as their axonal projections. Since Zac1 was expressed in catecholinergic cells during development and their expression was down-regulated in the adult (Valente et al., 2005), possibly Zac1 expression was necessary for the differentiation of the neuronal progenitors towards these catecholinergic subpopulations.

Microglial cells

In mutant mice we observed a decrease of the microglial cells, in which Zac1 was not expressed during the embryonic development. In the brain, the microglial cells were considered to be the only cell population of mesodermal origin, since they arise from a hematopoietic lineage (Ling and Wong, 1993; Corti et al., 2002). The microglial cells expressed markers for oligodendrocyte precursor cells, and in vitro studies suggested a novel role of microglia as multipotential stem cells to give rise to neurons, astrocytes, or oligodendrocytes (Yokoyama et al., 2004). Taking together these data and the present results, we suggests that Zac1 may be play an important role in the microgliogenesis process.

Zac1 in the neuronal loss following seizures

The mutant mice develop intense convulsions like wild type mice following KA treatment. Nevertheless, in these mice some neuronal damage cells were found only a short time following KA injection. Therefore, the mutant mice do not get to activate the apoptotic mechanisms that lead to the cellular death, which would be observed 24 hours after the treatment with KA. These results reinforce the Zac1 role in the induction of the apoptosis and suggests that in the KA-seizure models, the early induction of *Zac1* gene is indispensable for the activation of the apoptotic processes that will be evident in the

following days. On the other hand, the rate of mortality of the mutant mice KA-treated is significantly greater than the rate obtained for wild type mice. It has been determined that only between the 2 to 4% of the injected concentration of KA arrives at the brain (Ben-Ari, 1985), which means that the majority would follow the detoxifying metabolic routes. This date is supported if we considered that Zac1 is expressed in different peripheral tissues during development and in adult mice, and in this way a genetic failure in the development of these organs can diminish the metabolic tolerance of the mutant mice to KA administration.

Moreover, some changes in the neuronal and glial populations were observed between mutant and wild type mice 48h following KA injection. Thus, in the treated-mutant mice: (a) the proliferative rate in the dentate gyrus was noticeable reduced; (b) some proliferative cells were found in septum, which were mainly oligodendrocytes; (c) a clear reduction of the number of differentiated neurons was determined in the septum. Moreover, in the septum of the mutant mice the number of proliferative oligodendrocytes found is much smaller than in the wild type mice. Therefore, in the treated-mutant mice the proliferation was strongly reduced at the same time that the apoptosis was blocked, which makes suppose that the expression of Zac1, on the one hand was determinant to induce the apoptotic cell death after brain injury and on the other hand it was important to induce the proliferation of the neural progenitors of the dentate gyrus and of the subventricular zone.

Therefore, Zac1, the new imprinting gene that regulates the cell cycle arrest and the apoptosis by independent pathways, plays an important role in the differentiating processes of the neural progenitors during the development of the central nervous system, as well as in the early processes that activate the apoptotic pathways which are associated with the seizures.

EXPERIMENTAL PROCEDURES

Animals

We used forty-six BL6C57 adult Zac1 +/+ and forty-eight Zac1 BL6C57 adult Zac1 +/- (mutant) mice produced in the CNRS (Montpellier). The animals were kept under controlled temperature, humidity, and light conditions, and they were treated according to European Community Council Directive 86/609/EEC. Every effort was made to minimise animal suffering.

Animals treatment

A first group of BL6C57 animals (eighteen mutant and eighteen wild type mice) were intraperitoneally (IP) injected with 15 mg/Kg of KA and following 15 minutes they were injected again with 150 mg/Kg of sodium diethyldithiocarbamate, DEDTC (a chelatable agent of endogenous Zn^{2+}). A second group of animals (eighteen mutant and eighteen wild type mice) was injected with a saline solution and 15 minutes after they received an additional injection with 150 mg/kg of the DEDTC for using as KA treatment controls.

Three, six, eight and twenty-fourth and forty-eight hours after the treatment, the animals were deeply anaesthetised by IP injection of ketamine (100 mg/Kg) and Xilacine (10 mg/Kg) and: (a) for Western blot technique, the brains are quickly remove, dissectionated in specific brain areas (olfactory bulb, hippocampus, piriform cortex, somatosensory cortex, hipothalamus, and cerebellum) and processed for obtained the protein extracts (see below); (b) perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA), removed immediately and processed for *in situ* hybridisation (ISH).

All mice used for histological studies (KA-treated and untreated mice) were injected i.p. with 50 mg/kg of 5'-bromo-2'-deoxyuridine (BrdU, Sigma, in Tris-buffered saline, pH 7.6) 2 hours before perfusion.

***In Situ* hybridisation**

Antisense and sense riboprobes were labelled with digoxigenin-d-UTP (Boehringer-Mannheim). *In situ* hybridisation (ISH) was performed on free-floating tissue sections as described by Valente and Auladell (2001). Briefly, sections were pre-treated with H_2O_2 and HCl and hybridised overnight at 61 ºC with antisense or sense digoxigenin-d-UTP-labelled riboprobes (mouse *Zac1* cDNA and GAD65 cDNA used in Valente et al., 2005; rat NPY cDNA; mouse GAD65 cDNA). After ISH, sections were stringently washed in 50% formamide solutions at 61 ºC and incubated with 100 µg/ml RNase A (at 37 ºC). After that, the sections were blocked with 10% normal goat serum (NGS) and incubated overnight at 4 ºC with an alkaline phosphatase-labelled antidigoxygenin antibody (1:2000; Boehringer-Mannheim). To view alkaline phosphatase activity, sections were incubated with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate toluidinium salt (BCIP). The anatomic analysis of the embryonic areas was made in accordance with "The Atlas of Mouse Development" by Kaufman (1999).

Double ISH-immunohistochemistry procedure

After obtaining an intense labelling for *Zac1*, *NPY* or *GAD65* transcripts in the ISH, free-floating sections were incubated overnight at 4 ºC with one of the following primary antibodies: rabbit anti-Zac1 (1:1000, L. Journot), anti-Tirosine hidroxylase (1:2000, Chemicon), anti-GFAP (1:2000, Dako), anti-Ng2 (1:1000, W.B. Stallcup), anti-Calbindin (1:4000, Swant), anti-Calretinin (1:2500, Swant), anti-Parvalbumin (1:5000, Swant), anti-Gad65 (1:700, Chemicon) and anti-somatostatin (1:1000,

Dakkopats), and mouse anti-PCNA (1:700, Chemicon), anti-Nestin-Rat-401 (1:200, hybridoma bank), anti-BrdU (1:300, Roche), anti-β tubulin (1:100, Chemicon), anti-Netrin-1 (1:300, Oncogene), anti-PSA-NCAM (1:7500, G. Rougon), anti-Vimentin (1:400, Dako), anti-NeuN (1:300, Chemicon) and mouse anti-ssDNA (F7-26) Apostain (1:150, Alexis). After that, sections were sequentially incubated with biotinylated goat anti-rabbit or horse anti-mouse antibodies (1:200), and then with the avidin-biotin-peroxidase complex (ABC, 1:200). Peroxidase was developed with 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2.

Immunohistochemistry (IHC) techniques

IHC was performed on free-floating tissue sections as described by Valente and Auladell (2004). Briefly, free-floating sections were treated with 0.5 % H_2O_2, blocked with 10 % of NGS and incubated overnight with the Zac1 antibody and processed as described above until developed with 0.05% DAB- 0.01% H_2O_2, for single immunohistochemistry, or with 0.05% DAB- 0.01% H_2O_2- 0.2% $NiNH_4SO_4$, for double immunohistochemistry. In this latter case, the immunolabeled sections were washed and incubated again with a second primary antibody and developed with 0.05% DAB-0.01% H_2O_2. Alternatively, some sections were counterstained with hematoxylin and methyl green-pyronin.

Detection of the *in situ* DNA fragmentation

Free-floating sections were treated following the protocol supplied by Klenow-FragEl^{TM} DNA Fragmentation Detection Kit (Oncogene).

Preparation of brain extracts and Western blotting

The forebrain and cerebellum of treated and untreated adult Zac1 +/+ and +/- mice were homogenized in 1 ml of lysis buffer (125 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM EDTA, 1 μg/ml leupeptin, 5 μg/ml aprotinin and 0,125 mg/ml PMSF) at 4ºC, and the homogenate was centrifuged in order to obtain the cytosolic and nuclear fractions. Total extracts (30 μg) were

resolved on 8% SDS-PAGE gels and electrotransferred to nitrocellulose membrane using a semi-dry blotting system. The membranes were blocked with 4% BSA in Tris-buffered saline (140 mM NaCl, 10 mM Tris-HCl, pH 7.4, with 0.1% Tween 20) at room temperature for 1 hour and incubated overnight at 4ºC with one of the primary antibody described above. After that, the membranes were incubated with the second antibody conjugated to horseradish peroxidase. After washing, bound Igs were visualized using the ECL chemiluminescence system (Amersham).

Acknowledgements

We would like to thank Drs. Steven Sabol and Kunihimutant Obata for providing us with NPY and GAD65 probes, respectively. We would also like to thank Drs. W.B. Stallcup and G. Rougon for providing us with Ng2 and PSA-NCAM antibodies, respectively. We wish to thank Ondrej Krsicka for editorial assistance, respectively. The study was supported by grants from the University of Barcelona.

References

Abdollahi A, Gruver BN, Patriotis C, Hamilton TC. Identification of epidermal growth factor-responsive genes in normal rat ovarian surface epithelial cells. Biochem Biophys Res Commun. 2003 Jul 18;307(1):188-97.

Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. Neuron. 2004 Mar 4;41(5):683-6.

Arima T, Drewell RA, Arney KL, Inoue J, Makita Y, Hata A, Oshimura M, Wake N, Surani MA. A conserved imprinting control region at the HYMAI/ZAC domain is implicated in transient neonatal diabetes mellitus. Hum Mol Genet. 2001 Jul 1;10(14):1475-83.

Ben-Ari Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neurosci. 1985, 14, 375-403.

Corti S, Locatelli F, Donadoni C, Strazzer S, Salani S, Del Bo R, Caccialanza M, Bresolin N, Scarlato G, Comi GP. Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sensory ganglia. J Neurosci Res. 2002 Dec 15;70(6):721-33.

Doetsch F. The glial identity of neural stem cells. Nat Neurosci. 2003 Nov;6(11):1127-34. Epub 2003 Oct 28.

El Kharroubi A, Piras G, Stewart CL. DNA demethylation reactivates a subset of imprinted genes in uniparental mouse embryonic fibroblasts. J Biol Chem. 2001 Mar 23;276(12):8674-80. Epub 2000 Dec 21.

Gardner RJ, Mackay DJ, Mungall AJ, Polychronakos C, Siebert R, Shield JP, Temple IK, Robinson DO. An imprinted locus associated with transient neonatal diabetes mellitus. Hum Mol Genet. 2000 Mar 1;9(4):589-96.

Huang SM, Schonthal AH, Stallcup MR. Enhancement of p53-dependent gene activation by the transcriptional coactivator Zac1. Oncogene. 2001 Apr 19;20(17):2134-43.

Huang SM, Stallcup MR. Mouse Zac1, a transcriptional coactivator and repressor for nuclear receptors. Mol Cell Biol. 2000 Mar;20(5):1855-67.

Kintner C. Neurogenesis in embryos and in adult neural stem cells. J Neurosci. 2002 Feb 1;22(3):639-43.

Ling EA, Wong WC. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia. 1993 Jan;7(1):9-18.

Ma D, Shield JP, Dean W, Leclerc I, Knauf C, Burcelin R R, Rutter GA, Kelsey G. Impaired glucose homeostasis in transgenic mice expressing the human transient neonatal diabetes mellitus locus, TNDM. J Clin Invest. 2004 Aug;114(3):339-48.

Olsson M, Bjorklund A, Campbell K. Early specification of striatal projection neurons and interneuronal subtypes in the lateral and medial ganglionic eminence. Neuroscience. 1998 Jun;84(3):867-76.

Rakic S, Zecevic N. Early oligodendrocyte progenitor cells in the human fetal telencephalon. Glia. 2003 Jan 15;41(2):117-27.

Rozenfeld-Granot G, Krishnamurthy J, Kannan K, Toren A, Amariglio N, Givol D, Rechavi G. A positive feedback mechanism in the transcriptional activation of Apaf-1 by p53 and the coactivator Zac-1. Oncogene. 2002 Feb 28;21(10):1469-76.

Spengler D, Villalba M, Hoffmann A, Pantaloni C, Houssami S, Bockaert J, Journot L. Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J. 1997 May 15;16(10):2814-25.

Valente T, Junyent F, Auladell C. Zac1 is expressed in progenitor/stem cells of the neuroectoderm and mesoderm during embryogenesis. Differential phenotype of the Zac1-expressing cells during development. Dev. Dyn. 2005; XX: xxx-xxx. (in press)

Valente T, Dominguez MI, Bellmann A, Journot L, Ferrer I, Auladell C. Zac1 is up-regulated in neural cells of the limbic system of mouse brain following seizures that provoke strong cell activation. Neuroscience. 2004;128(2):323-36.

Valente T, Auladell C. Expression pattern of Zac1 mouse gene, a new zinc-finger protein that regulates apoptosis and cellular cycle arrest, in both adult brain and along development. Mech Dev. 2001 Oct;108(1-2):207-11.

Varrault A, Bilanges B, Mackay DJ, Basyuk E, Ahr B, Fernandez C, Robinson DO, Bockaert J, Journot L. Characterization of the methylation-sensitive promoter of the imprinted ZAC gene supports its role in transient neonatal diabetes mellitus. J Biol Chem. 2001 Jun 1;276(22):18653-6. Epub 2001 Apr 10.

Wilson SW, Rubenstein JL. Induction and dorsoventral patterning of the telencephalon. Neuron. 2000 Dec;28(3):641-51.

Yokoyama A, Yang L, Itoh S, Mori K, Tanaka J. Microglia, a potential source of neurons, astrocytes, and oligodendrocytes. Glia. 2004 Jan 1;45(1):96-104.

FIGURE 1

Nissl staining (A-B), PCNA (C-H), PSA-NCAM (I-N) and MAP2a&b (O-T) immunostains in wild type (WT) and Zac1 mutant (KO) mice. An increase in the subventricular cells is detect in mutant mice (solid arrows in B), which are proliferative cells, since they are positive for PCNA (solid arrows in F-H). A decrease in the undifferentiating (PSA-NCAM (K and L) and MAP2a&b (R-T)) and mature (NeuN (N)) neurons is observed. SVZ, subventricular zone; pdr, proliferative/differentiate olfactory region; OB, olfactory bulb; grDG, granular cells of dentate gyrus; ab, accessory of olfactory bulb; g, granular layer; gl, glomerular layer; gab, granular layer of accessory of olfactory bulb; cc, corpus callosum; RCx, retrosplenial cortex; arrows, upper layers of RCx; dm, dorsomedial hypothalamic nucleus.

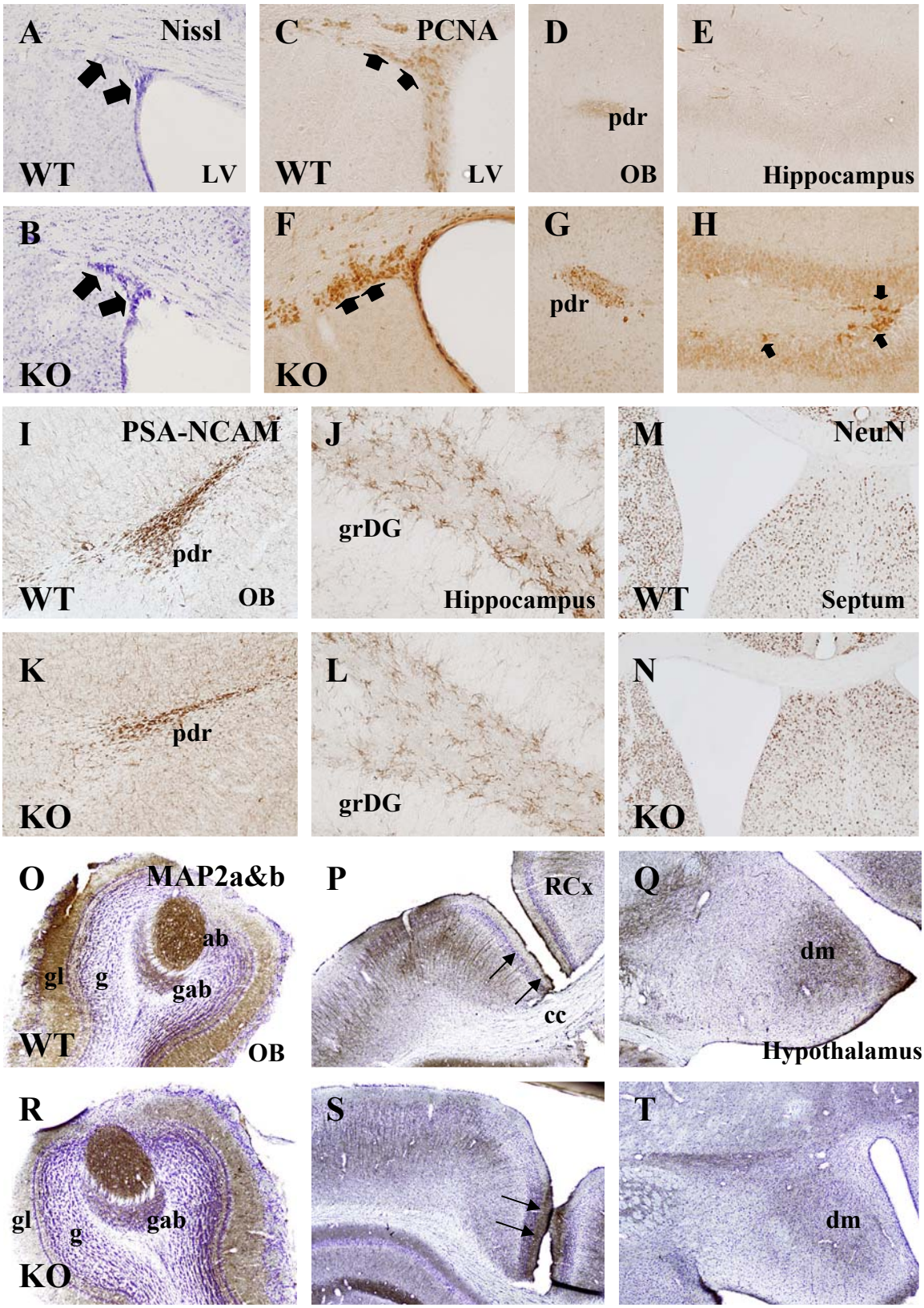

A
Nissl
WT
LV
C
PCNA
WT
LV
D
pdr
OB
E
Hippocampus
B
KO
F
KO
G
pdr
H
I
PSA-NCAM
pdr
WT
OB
J
grDG
Hippocampus
M
NeuN
WT
Septum
K
pdr
KO
L
grDG
N
KO
O
MAP2a&b
ab
gl
g
gab
WT
OB
P
RCx
cc
Q
dm
Hypothalamus
R
gl
g
gab
KO
S
T
dm

FIGURE 2

Immunohistochemical detection of the calcium-binding proteins, calbindin (A-F), parvalbumin (G-L) and calretinin (M-P), in wild type (WT) and Zac1 mutant (KO) mice. The calbindin-positive and parvalbumin-positive cells noticeable increase in the mutant mice in all the limbic brain areas. In contrast, in the Zac1 mutant mice the calretinin-positive cells are strongly decreased in all of the brain. hb, habenular thalamic nucleus; ac, anterior commissure; RCx, retrosplenial cortex; rt, reticular thalamic nucleus; vt, ventral thalamic nucleus; grDG, granular cells of dentate gyrus; g, granular layer; gl, glomerular layer; OB, olfactory bulb.

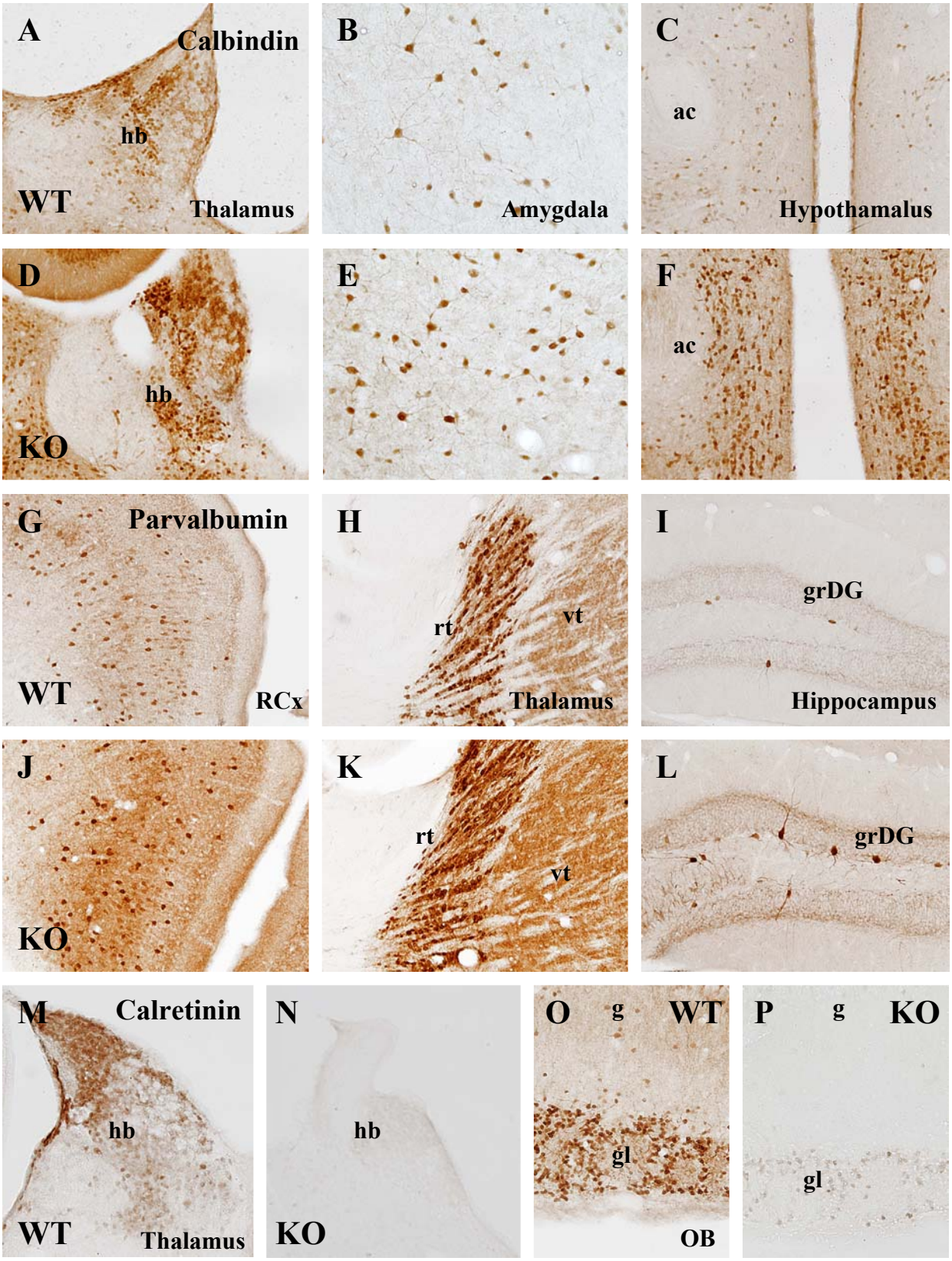

A
Calbindin
hb
WT
Thalamus
B
Amygdala
C
ac
Hypothamalus
D
hb
KO
E
F
ac
G
Parvalbumin
WT
RCx
H
rt
vt
Thalamus
I
grDG
Hippocampus
J
KO
K
rt
vt
L
grDG
M
Calretinin
hb
WT
Thalamus
N
hb
KO
O
g
WT
gl
OB
P
g
KO
gl

FIGURE 3

GAD65 (A-D) and NPY (E-H) gene expression in wild type (WT) and Zac1 mutant (KO) mice. A general down-regulation of *GAD65* gene is observe in all the brain areas. In contrast, a clear up-regulation of *NPY* gene is detect in the hippocampus and in the hypothalamus of the mutant mice. grDG, granular cells of dentate gyrus; arc, arcuate nucleus; g, granular layer; gl, glomerular layer; OB, olfactory bulb.

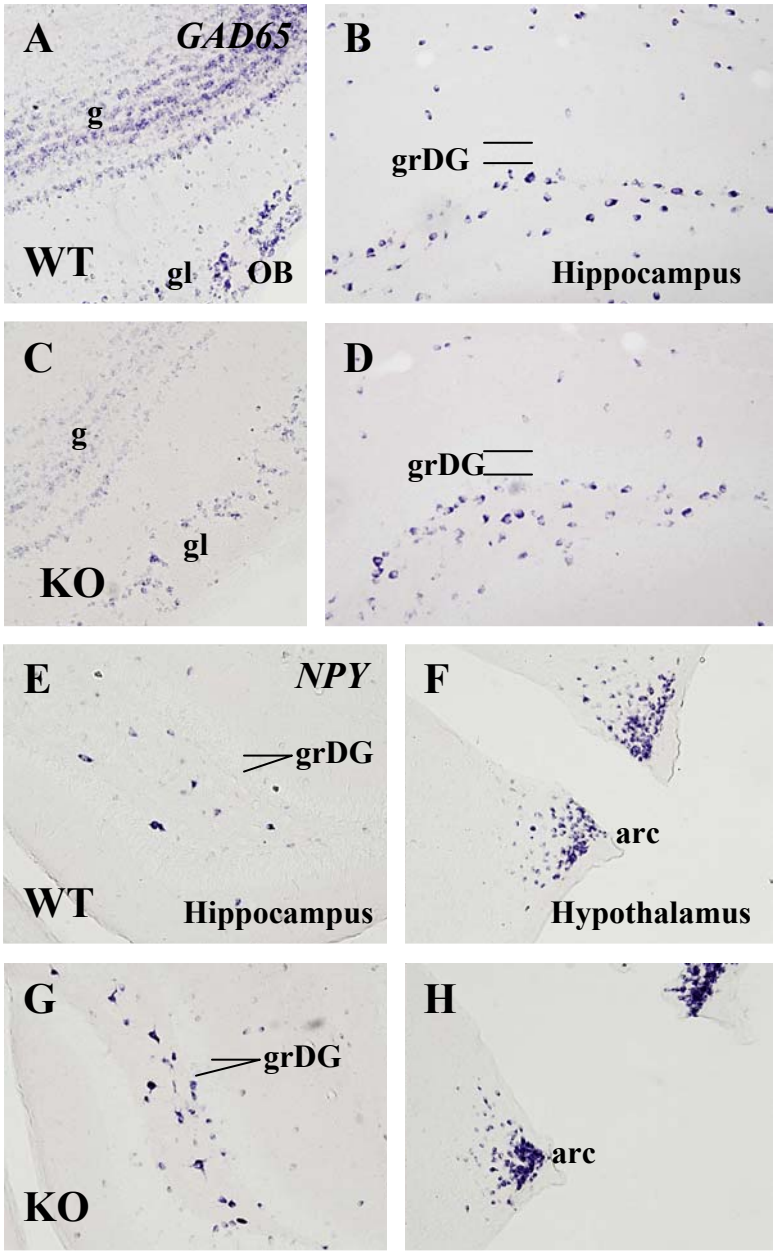

A
GAD65
g
WT
gl
OB
B
grDG
Hippocampus
C
g
gl
KO
D
grDG
E
NPY
grDG
WT
Hippocampus
F
arc
Hypothalamus
G
grDG
KO
H
arc

FIGURE 4

TH (A-D) and MBP (E-H) immunostains in the wild type (WT) and Zac1 mutant (KO) mice. A noticeable reduction of the TH-positive cells is observed in the hypothalamus (A and B) and the brain steam of the mutant mice, as well as a strong reduction of their projections within the brain steam is detect (B and D). In addition, a general reduction of MBP is detected in all the brain areas of the mutant mice, such as in the septum (E and G) and in the brain steam (F and H). sn, substancia nigra; vta, ventral tegmental area; lh, lateral hypothalamic nucleus; cc, corpus callosum; wm, white matter; st, striatum; spt, septum; ac, anterior commissure; aq, aqueduct of Sylviu's; xscp, decuss superior of cerebellar peduncle.

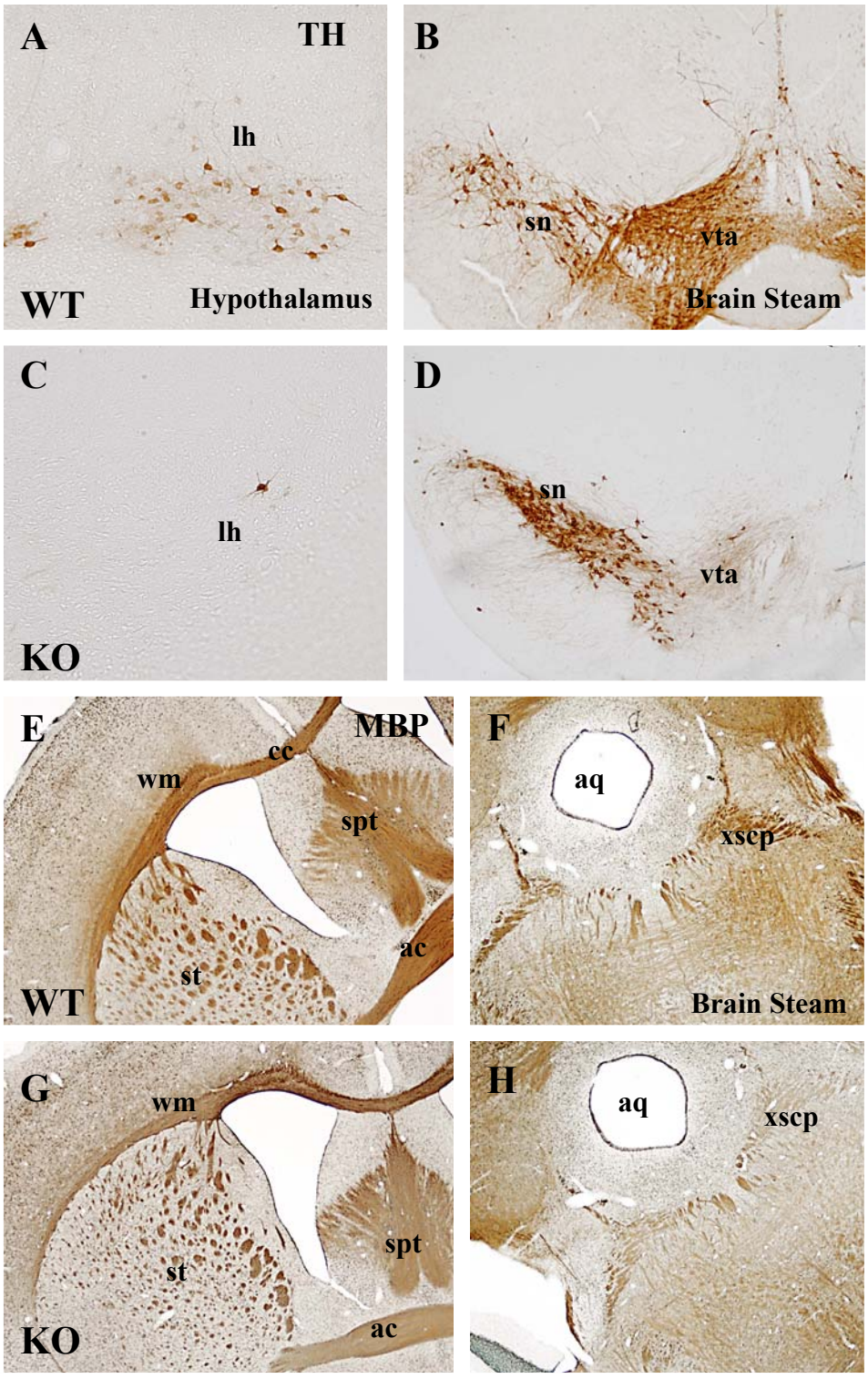

A
TH
lh
WT
Hypothalamus
B
sn
vta
Brain Steam
C
lh
KO
D
sn
vta
E
MBP
cc
wm
spt
ac
st
WT
F
aq
xscp
Brain Steam
G
wm
spt
st
ac
KO
H
aq
xscp

FIGURE 5

Differential glial subpopulation alterations, GFAP-positive cells (A-J), Ng2-positive cells (K-R), CAII-positive cells (S-V) and CD11-positive cells (W-Z), in wild type (WT) and Zac1 mutant (KO) mice. The mutant mice present an increase in the mature astrocytes populations of the olfactory bulb (A and F). Moreover, a noticeable increase of GFAP immunolabelling is observed in the subventricular zone of the lateral (B-C and G-H) and third ventricle (E and J), as well as in the subgranular zone of the dentate gyrus (D and I). In contrast, a clear reduction of the immature Ng2-oligodendrocytes (K-R) and mature CAII-oligodendrocytes (S-V) is detected in many brain areas of the mutant mice (O-R and U-V). The immunostain for microglial cells shows a down-regulation in the mutant mice, mainly in the hippocampus (W and Y) and in the septum (X and Z). gl, glomerular layer; m, mitral layer; LV, lateral ventricle; SVZ, subventricular zone; grDG, granular cells of dentate gyrus; 3V, third ventricle; RCx, retrosplenial cortex; wm, white matter; dm, dorsomedial hypothalamic nucleus; hb, habenular thalamic nucleus; AC, anterior commissure.

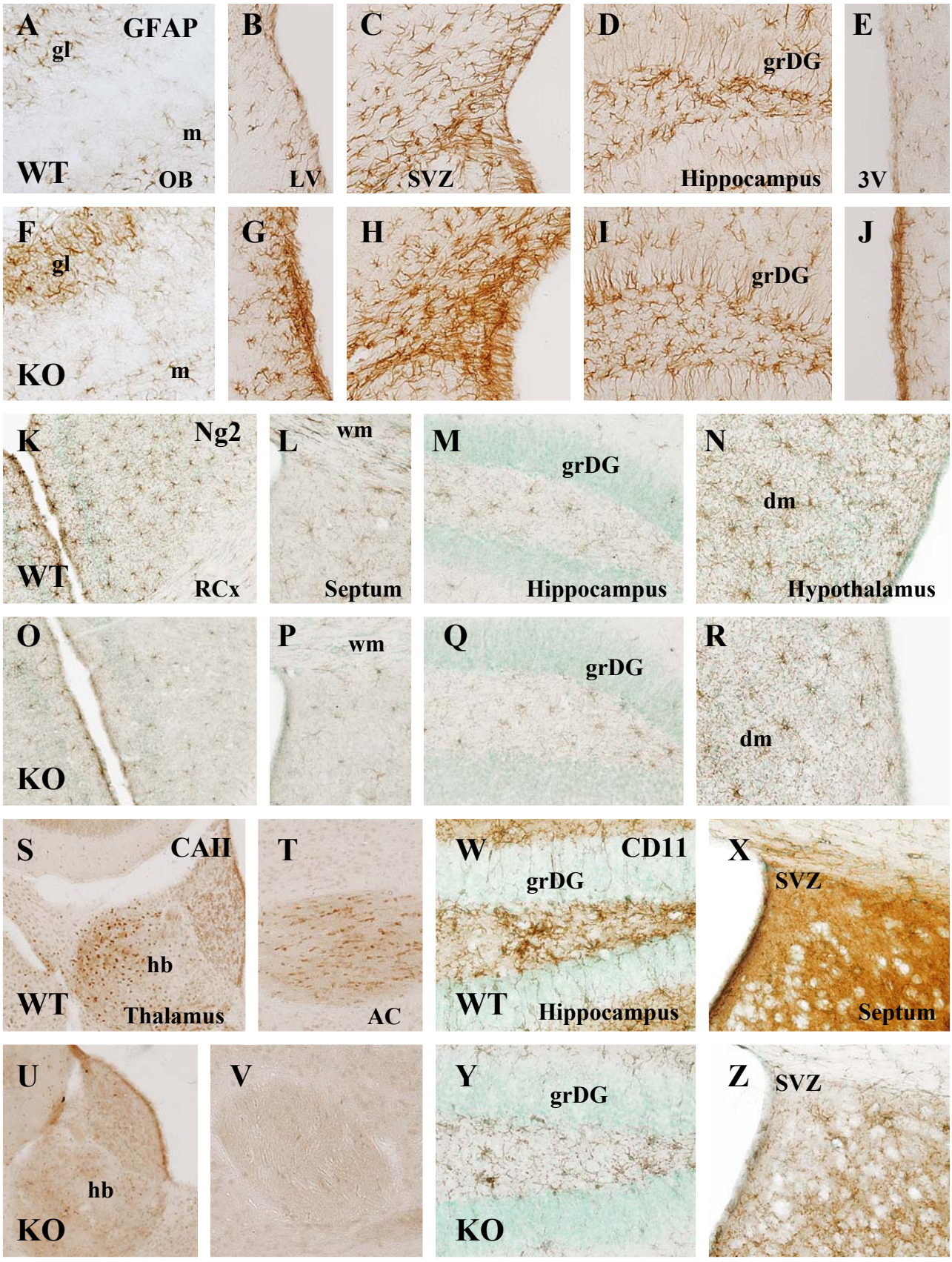

A
GFAP
gl
m
WT
OB
B
LV
C
SVZ
D
grDG
Hippocampus
E
3V
F
gl
KO
m
G
H
I
grDG
J
K
Ng2
WT
RCx
L
wm
Septum
M
grDG
Hippocampus
N
dm
Hypothalamus
O
KO
P
wm
Q
grDG
R
dm
S
CAII
hb
WT
Thalamus
T
AC
W
CD11
grDG
WT
Hippocampus
X
SVZ
Septum
U
hb
KO
V
Y
grDG
KO
Z
SVZ

FIGURE 6

FluoroJade labelling of hippocampal areas at several times following KA administration in the wild type (WT) and Zac1 mutant (KO) mice. At 3 hours after KA treatment, few and very few neurodegenerative neurons are observed in the hilus (A) and in the CA3 region (B) of the wild type, respectively. At the same time, not one neurodegenerative cell is detected in the mutant mice (I-J). At 8 hours following KA injection, the neurodegenerative cells in the hilus (C) and CA3 region (D) of wild type mice increase and few neurodegenerative cells appear in the same hippocampal areas of the mutant mice (K-L). At 24 hours after KA injection, the neurodegenerative cells persists in the wild type mice (E-F) whereas disappears in the mutant mice (M-P). In the wild type, the labelling for neurodegenerative cells is not detected 7 days following KA treatment in the hilus and CA3 region, however persists in the CA1 region (R-S). Moreover, the mutant mice never show neurodegenerative-positive cells in the CA1 region (Q). h, hilus; DG, dentate gyrus; py, pyramidal cells.

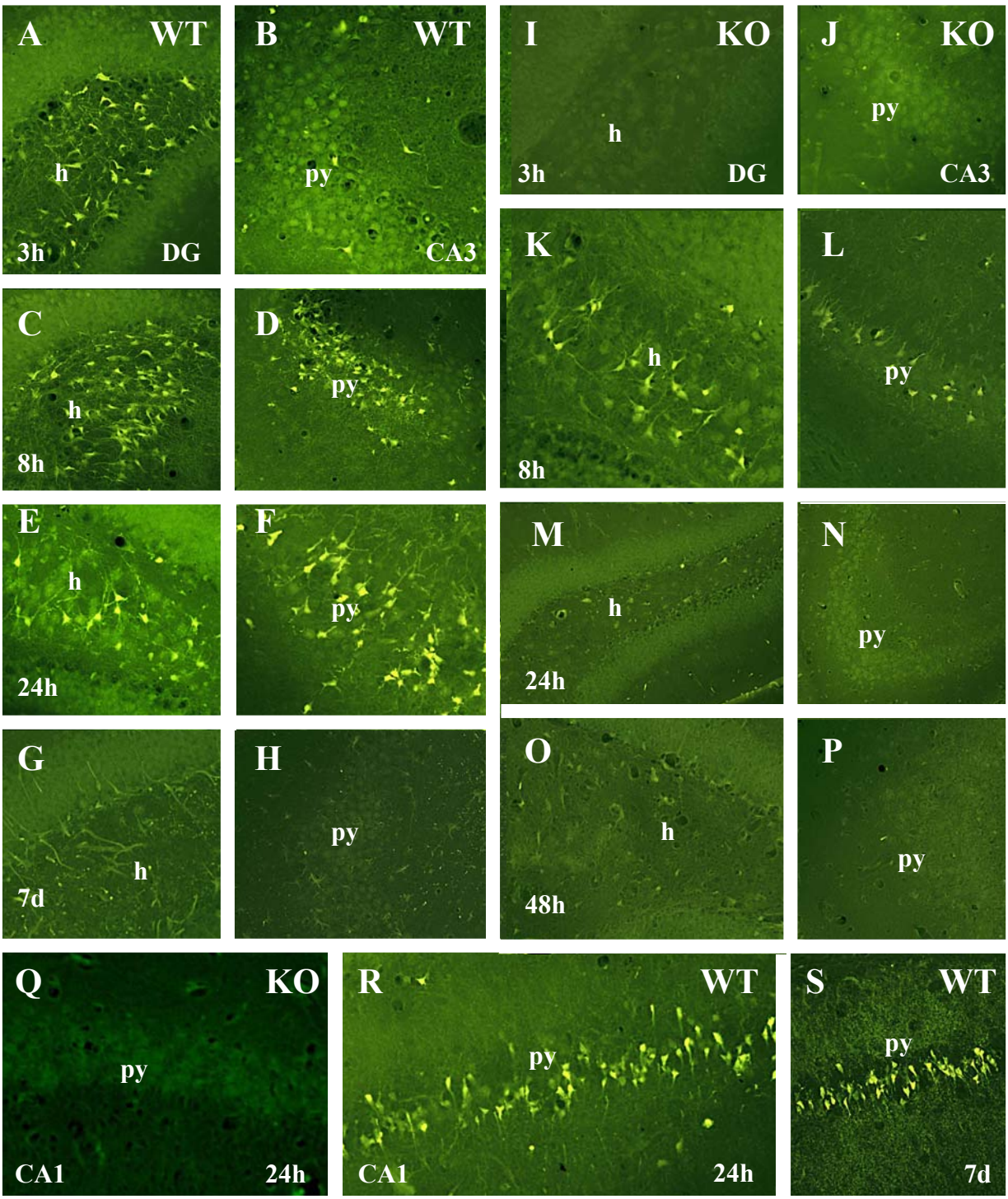

A
WT
h
3h
DG
B
WT
py
CA3
I
KO
h
3h
DG
J
KO
py
CA3
C
h
8h
D
py
K
h
8h
L
py
E
h
24h
F
py
M
h
24h
N
py
G
h
7d
H
py
O
h
48h
P
py
Q
KO
py
CA1
24h
R
WT
py
CA1
24h
S
WT
py
7d

FIGURE 7

Apostain (A-D) and c-fos (E-H) immunolabelling in wild type (WT) and Zac1 mutant (KO) mice following KA administration. The Apostain-positive cells are found only in the hippocampus of the wild type mice after 24 hours following KA treatment (A-D). In addition, the cellular activation (c-fos positive cells) is detected only in the wild type at 3 hours following KA injection and strongly increases at 8 hours (E-F). However, only few cells are observed in the mutant mice at 8 hours following KA administration (G-H). pyrCA3, pyramidal cells of CA3 hippocampal region.

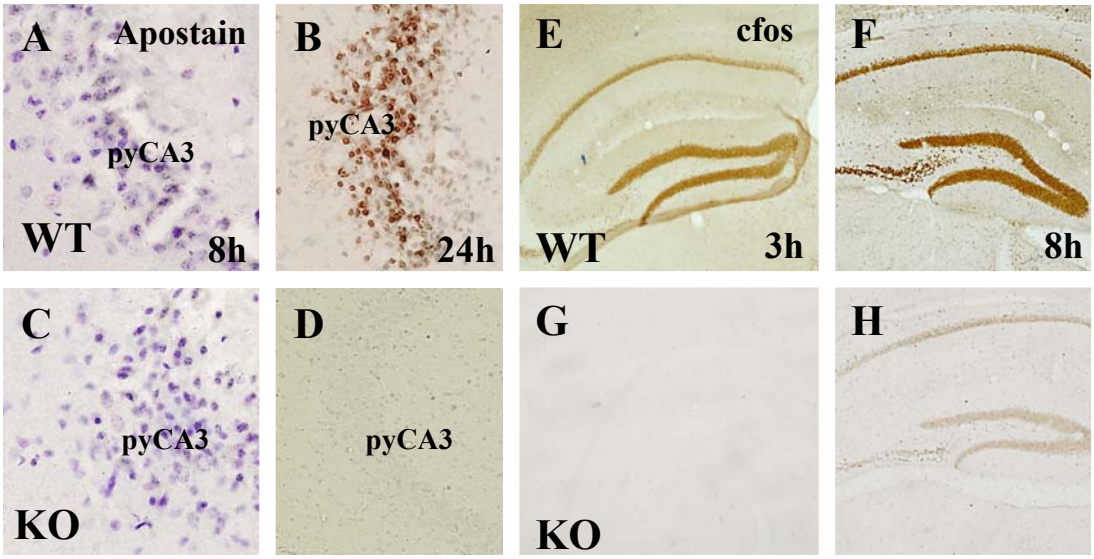

A
Apostain
pyCA3
WT
8h
B
pyCA3
24h
E
cfos
WT
3h
F
8h
C
pyCA3
KO
D
pyCA3
G
KO
H

FIGURE 8

Co-localization studies of the proliferative cells (BrdU-positive cells) in the dentate gyrus of the wild type, WT (A, C, E, G, I, and K) and Zac1 mutant, KO (B, D, F, H, J and L) mice 48 hours following KA treatment. None BrdU-positive cell is observed in the subgranular zone of the dentate gyrus of the mutant mice (arrows in A-B and in I-L). No co-localization is detect with NeuN (C-D)**,** whereas a high co-localization is observed with GFAP (E-H). Thus, many BrdU-positive cells are co-localized with GFAP mainly in the wild type mice (arrows in G-H). Moreover, few co-localizations are detected with immature oligodendrocytes (Ng2-positive cells) and microglial cells (CD11-positive cells) in the wild type mice (I-L). grDG, dentate gyrus; h, hilus

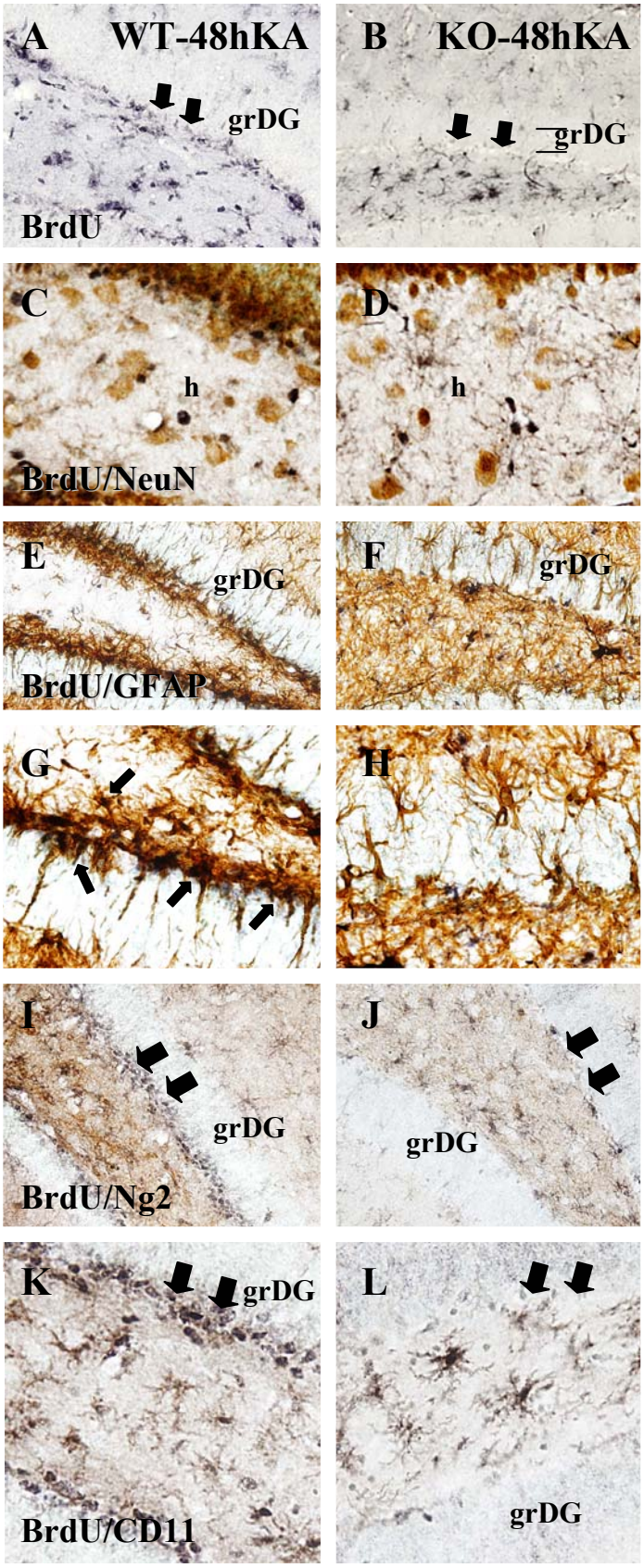
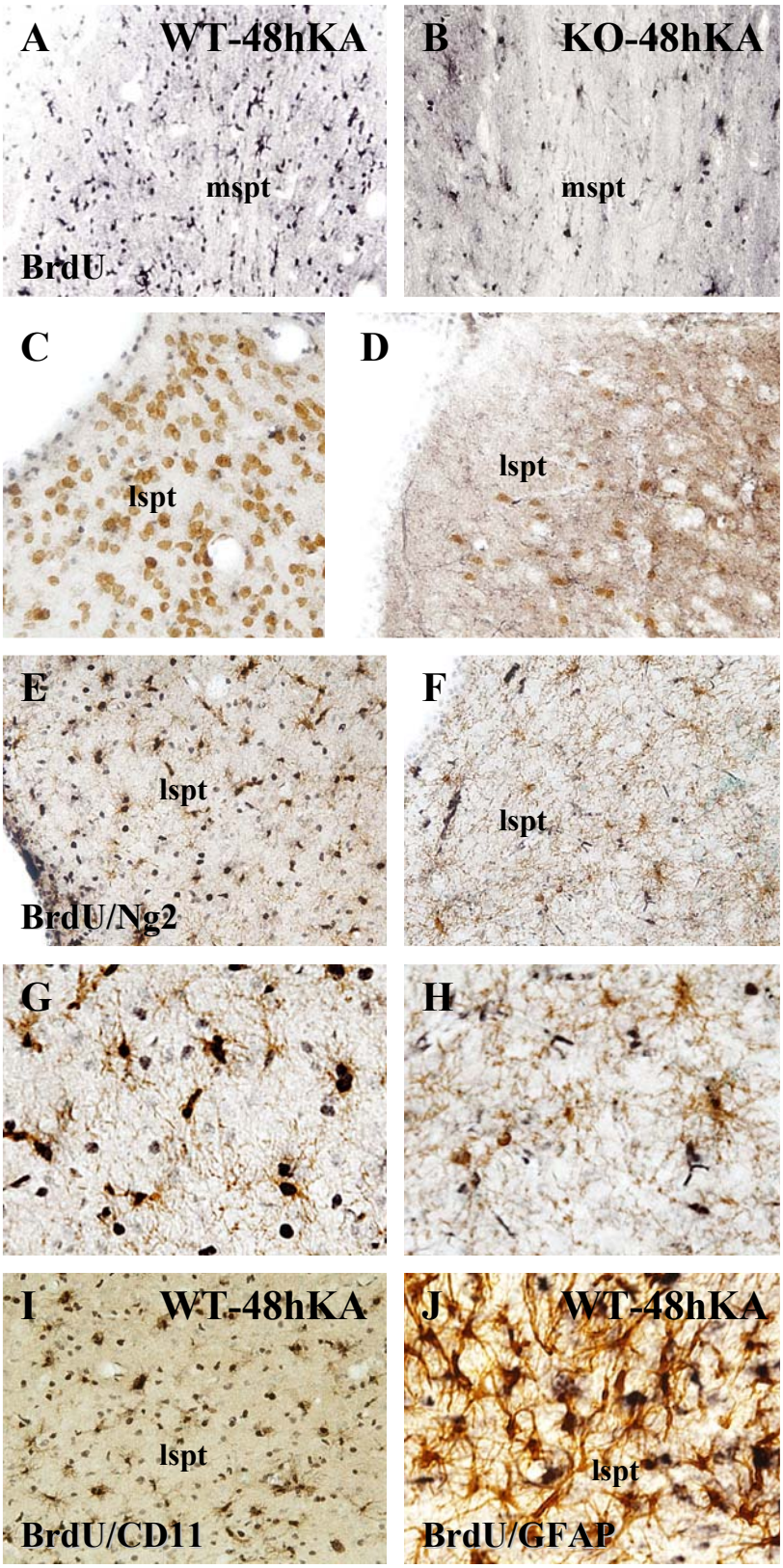

A
WT-48hKA
grDG
BrdU
B
KO-48hKA
grDG
C
h
BrdU/NeuN
D
h
E
grDG
BrdU/GFAP
F
grDG
G
H
I
grDG
BrdU/Ng2
J
grDG
K
grDG
BrdU/CD11
L
grDG

FIGURE 9

Co-localization studies of the proliferative cells (BrdU-positive cells) in the septum of the wild type, WT (A, C, E, G, I, and J) and Zac1 mutant, KO (B, D, F and H) mice 48 hours following KA treatment. Few BrdU-positive cells are observed in the septal area of the mutant mice (A-B). Few NeuN-positive cells are detected in the mutant mice (C-D), and in both wild type and mutant mice no co-localization is found between NeuN and BrdU. In the wild type, many BrdU-positive cells are co-localized with Ng2 (E-H) and few are observe in mutant mice. Moreover, many BrdU-positive cells co-localize with microglial cells (CD11-positive cells) in the wild type mice (I). Some BrdU-positive cells co-localize with GFAP (J). lspt, lateral septal area; mspt, medial septal area.


A
WT-48hKA
mspt
BrdU
B
KO-48hKA
mspt
C
lspt
D
lspt
E
lspt
BrdU/Ng2
F
lspt
G
H
I
WT-48hKA
lspt
BrdU/CD11
J
WT-48hKA
lspt
BrdU/GFAP