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REGULATION OF TRANSCRIPTION FACTOR *Lot1* EXPRESSION DURING PROLIFERATION/DIFFERENTIATION OF NEURONAL CELLS

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1. AIM OF THE STUDY

The balance between cell proliferation, differentiation and death during development is a key factor in the formation of the CNS. The identification of the genes that regulate developmental processes provides the opportunity to identify the molecular interactions that co-ordinate cell-cycle regulation with neural proliferation/differentiation and pattern formation in the CNS.

Lost-on-transformation 1 (*LOT1* also named *ZAC1/PLAGL1*) is a potential tumor suppressor gene with antiproliferative activity. *LOT1* expression is lost in many types of human cancers and its forced expression in tumor derived cell lines leads to cell cycle arrest and induction of apoptosis.

It has recently been shown that Lot1 is highly expressed during brain development. Notably, Lot1 expression is high in rat cerebellum in the early postnatal period, when neurogenesis of cerebellar granule cells (CGC) does take place, while its expression undergoes progressive decrease at later stages. Similarly, *Lot1* is expressed in freshly plated proliferating CGC in culture with a progressive decrease of activity occurring in parallel with differentiation towards mature neuronal phenotype. While the available data suggest that Lot1 may play important an important role in reducing tumor progression, its physiological role in the processes of brain development still remains to be elucidated. By analogy, it is coneceivable that a role of Lot1 during periods at which neurogenesis is particularly prominent is to limit cell proliferation.

Evidence from both in vivo and in culture studies indicates that neuronal precursor proliferation is negatively regulated by an increase in intracellular cAMP. The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is a potent stimulator of cAMP production. In addition, it has been shown that PACAP regulates neurogenesis by exerting antimitogenic effect on neuronal precursors. The molecular

mechanisms by which the PACAP-cAMP system exerts its effects on neurogenesis are still largely unknown. The antiproliferative properties of *Lot1* in tumor cells and its expression in proliferating neuronal precursors may indicate that *Lot1* is one of the genes that are triggered by the cAMP cascade and that mediate the antiproliferative effects exerted by cAMP. The presence of a high density of PACAP receptors coupled to adenylate cyclase in the external granule cell layer of the rat cerebellum during early postnatal development, in a time period closely matching *Lot1* expression strongly suggests that *Lot1* is regulated by the PACAP/cAMP system.

Aim of the present study was to ascertain whether the expression of *Lot1* occurring during proliferation of the cerebellar granule cells is induced by cAMP and to identify the molecular mechanisms that underlie *Lot1* transcription.

2. INTRODUCTION

2.1. *LOT1/ZAC1/PLAGL1*

LOT1 (for lost-on-transformation and also named *ZAC1* or *PLAGL1*) is a member of the a family of zinc-finger transcription factors, designated as PLAG family. Other members in this group include *PLAG1* and *PLAGL2*, which share high homology with each other and with *LOT1/ZAC1/PLAGL1*. PLAG family members are structurally similar but appear to be functionally different. *LOT1/ZAC1/PLAGL1* is a candidate tumor suppressor gene localized on human chromosome 6q24-25, a chromosomal region that is frequently deleted in many types of human cancers. *PLAG1* is a protooncogene that is localized on chromosome 8q12 and was found to be a target of several types of chromosomal rearrangement in many tumors.

2.1.1. *PLAG* genes cloning and nomenclature

Lot1 was originally identified using a rat tissue culture model of ovarian cancer. In this model, a series of malignant rat ovarian surface epithelial (ROSE) cell lines were developed by spontaneous transformation from long term culture of progenitor cells. The malignant cells were examined for differentially expressed genes as compared to their corresponding normal progenitor cells. A cDNA, that was expressed in the normal cell line but not in its malignant counterpart, was cloned and named *Lot1* (accession number U72620) (Abdollahi et al., 1997a). Simultaneously, the human *LOT1* sequence was cloned (accession number U72621) (Abdollahi et al., 1997b).

Zac1 (for Zinc-finger protein which regulates apoptosis and cell-cycle arrest; accession number X95503), which is the mouse orthologue of *LOT1* and *Lot1*, was isolated by a functional expression cloning technique (Spengler et al., 1997).

PLAG1 (for pleomorphic adenoma gene; GenBank accession number NM_002655) was discovered as a novel developmentally regulated gene at chromosome 8q12 and was found to be a main target of pleomorphic adenomas of the salivary gland. In these adenomas, frequent occurrence of t(3;8)(p21;q12) chromosomal rearrangement results in promoter swapping between *PLAG1* and β-catenin and thus *PLAG1* constitutive expression (Kas et al., 1997). *PLAGL2* (PLAG Like 2; another *PLAG* family member; accession number AF006005) was identified by database homology search (Kas et al., 1998). Recently the zebrafish orthologue of *PLAG1* (AY864859), *PLAGL2* (AF186476), and a novel member, *PLAGX* (AY864858) have been cloned (Pendeville et al., 2006).

Gene symbol for the family member *LOT1/ZAC1/PLAGL1* has been designated as *PLAGL1* or *Plagl1* by the Genome Nomenclature Committees based on the homology with the other members of PLAG family. Nevertheless in the past years different authors have named the human gene as *LOT1*, *ZAC1* or *PLAGL1*, while the mouse and rat orthologues have been historically named *Zac1* and *Lot1* respectively. In this dissertation the symbols *PLAGL1*, *LOT1*, and *ZAC1* all refer to the same human gene, while *Lot1* and *Zac1* refer to the rat and mouse orthologues respectively.

2.1.2. LOT1/ZAC1 protein structure

The human *LOT1/ZAC1* gene spans at least 70 kb of the human genome on chromosome 6q24-25. The gene does not display a TATA box element (Abdollahi et al., 2003b). The LOT1/ZAC1 protein is encoded by exons 5 and 6, resulting in a large 5’ UTR. The translation initiation codon (ATG) is mapped at the 326-bp downstream

from the beginning (5' end) of exon 5, and the stop codon (TAA) is located at 1,238-bp downstream from the 5' end of exon 6, followed by about 1.2 kb of nucleotides including the poly(A) tail (Abdollahi et al., 2003b). Unlike the human *ZAC1*, which consists of 9 exons, the mouse *Zac1* transcript is spliced from 10 exons (Varrault et al., 2001).

The human *LOT1/ZAC1* and rat/mouse orthologues share about 76.4% identity at the nucleotide level and 67.7% at the amino acid level. *LOT1/ZAC1* encodes a 463 amino acid protein while rat and mouse orthologues encode a 583 and 704 amino acid protein respectively. Four domains common to human, mouse and rat can be identified: a seven C2H2-type zinc-finger motifs at the amino-terminal, a linker domain, a central region largely comprising repeats of proline, glutamic acid, and glutamine residues and a carboxyl terminal domain (Figure 2.1). These domains are rather well conserved (85.5%, 54%, 58%, and 63% identity, respectively) between human and rodent proteins. On the other hand rat and mouse orthologues display a clusters of glutamic acid residues within the carboxyl region that are absent in the human protein. Besides the mouse protein displays a central region containing 34 proline-rich tripeptide repeats (PLE, PMQ, or PML), possibly important in protein–protein interaction (Abdollahi et al., 1997a; Spengler et al., 1997; Varrault et al., 1998). Despite these structural differences, the mouse and human LOT1/ZAC1 proteins appear to have identical functional roles in apoptosis and growth inhibition (Varrault et al., 1998). An alternative splicing in the coding region of the human gene has been shown to result in the translation of a protein with five zinc fingers (instead of seven) named ZACΔ2 (Bilanges et al., 2001).

2.1.3. Expression of *LOT1/ZAC1*

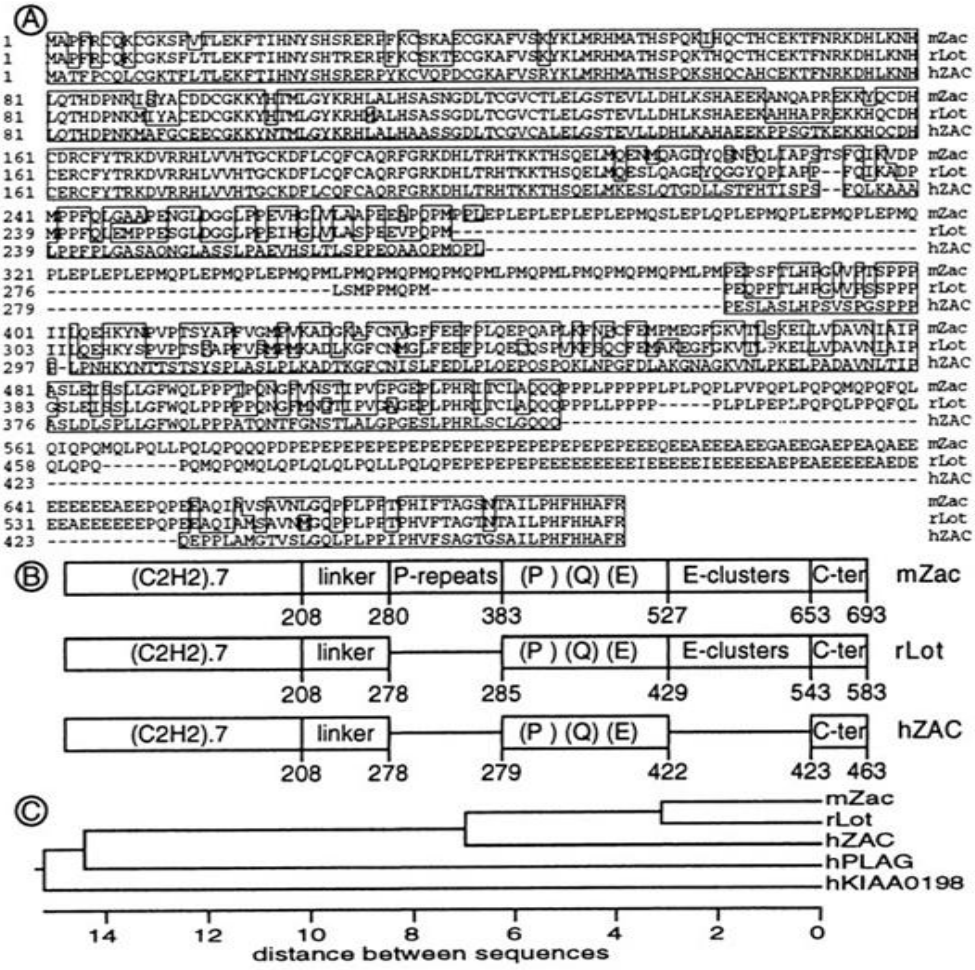

Figure 2.1. Sequences of the LOT1/ZAC1 proteins. *A*) Sequence alignments of the LOT1/ZAC1 proteins. Human ZAC1 (hZAC), mouse Zac1 (mZac), and rat Lot1 (rLot) were aligned according to a Clustal method. Residues that matched hZAC with one distance unit are boxed. *B*) Schematic representation of the LOT1/ZAC1 proteins structures. Each domain is indicated as a box: the seven zinc-finger of C2H2 type, the linker region, the proline repeats present in mZac only, the proline, glutamine and glutamic acid rich region, the glutamic acid clusters absent in hZAC, and the C terminus. The numbering of the amino acid residues is indicated below the boxes. *C*) Phylogenetic tree for LOT1/ZAC1 family. Only the zinc-finger domains were taken into account for the analysis.

(Varrault et al., 1998)

Expression during development. Zac1 transcripts appear to be abundantly expressed in many proliferative/differentiative areas during mouse brain development. At embryonic stages *Zac1* is expressed in the neural tube from E9.5 (Tsuda et al., 2004), at later stages strong expression is observed in brain areas with active proliferation such as the ventricular zone and several neuroepithelius (Valente and Auladell, 2001). In addition, some differentiating areas, such as arcuate nucleus and amygdaloid region of the brain, are noticeably labeled. From P0 onwards, the expression is evident in some proliferative areas, such as the subventricular zone (SVZ) and the external granular layer (EGL) of the cerebellum, as well as in Purkinje cells and in some synaptic areas characterized by high plasticity, such as the dorso and ventromedial hypothalamic nuclei, arcuate nucleus, ventral thalamic nucleus (Table 2.1.) (Valente and Auladell, 2001; Valente et al., 2005).

At the protein level Lot1-specific immunostaining has been observed at postnatal days 2–7 in the external granule layer (EGL) of rat cerebellum, which is composed primarily of proliferating neuronal precursor cells (Ciani et al., 2003). Purkinje cells showed distinct nuclear labelling at postnatal day 7, and at lower level, in adult cerebellum. It was also shown that primary cultures of cerebellar granule cells exhibit a temporal pattern of *Lot1* expression resembling the one observed during in vivo development, with mRNA and protein levels progressively decreasing with differentiation (Figure 2.2.) (Ciani et al., 2003).

Zac1 is broadly expressed in non neuronal embryonic tissues, being highly expressed in the liver primordium and the umbilical region (Piras et al., 2000). *Zac1* is also expressed transiently in the myocardium and basal portion of the stratified embryonic epithelia (Tsuda et al., 2004) together with other embryonic sites (hindlimb, forelimb and somites) (Valente and Auladell, 2001).

Analysis of cell types expressing *Zac1* during mouse development revealed that the gene is expressed by progenitors of several tissues (brain, skeleton, and skeletal muscle). The intense expression of *Zac1* in the progenitors of different cellular lineages during proliferation, before differentiation into postmitotic cells, suggests

that *Zac1* plays an important role in the control of cell fate during neurogenesis and myogenesis (Valente et al., 2005).

During postnatal development, *Zac1* appears to be expressed in developing chondrogenic tissue (Tsuda et al., 2004; Valente et al., 2005). In this regard *Zac1* was shown to be a potential regulatory gene involved in chondrogenic differentiation. During cartilage development, the pattern of *Zac1* expression is associated with proliferating and pre-differentiating chondrocytes, while in mouse ATDC5 cells undergoing in vitro chondrogenesis, *Zac1* mRNA appeared to be upregulated in parallel with genes expressed in the pre-cartilage stage but was low when type II collagen mRNA was markedly increased in differentiated cells (Tsuda et al., 2004).

Expression in adult tissues. LOT1/ZAC1 is ubiquitously expressed in normal human tissues. The gene was shown to be variably transcribed in all the tissues examined: spleen, thymus, prostate, testis, ovary, intestine, colon, pituitary gland, kidney, placenta, adrenal gland, uterus, mammary gland, lung, lymphoid tissues, skeletal muscle, peripheral leucocytes, liver, whole brain, and spinal cord (Abdollahi et al., 1997b; Bilanges et al., 1999; Kas et al., 1998; Varrault et al., 1998). In adult brain, the strongest signals were observed in the occipital lobe, cerebral cortex, and thalamus (Varrault et al., 1998). RNA in situ hybridization experiments have shown that *LOT1/ZAC1* is expressed in normal mammary epithelial cells, most abundantly in the most luminal cells (Bilanges et al., 1999). *LOT1/ZAC1* was also found to be strongly expressed in the human ovarian surface epithelium (which is the origin of most ovarian cancers) and in the follicle epithelial cells (Kamikihara et al., 2005).

In adult mice the anterior pituitary gland showed the highest *Zac1* expression, while lower level was observed in other brain areas such as olfactory bulb, cortex, hippocampus, hypothalamus-thalamus, brain stem and cerebellum, and faintly in stomach, kidney, adrenal gland, heart, and lung (Spengler et al., 1997). In the pituitary *Zac1* mRNA was more abundantly expressed in the anterior lobe compared to the intermediate and posterior lobes. *Zac1* transcripts were found in all hormone-

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)				–	+/–	+		

Table 2.1. Expression pattern of *Lot1/Zac1* during mouse embryonic and postnatal development.

(Valente and Auladell, 2001)

Anatomic structure	E9.5	E10.5	E12	E14	E16	E18	P0–P9	>P12
	Whole embryos				Brain slices			
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)							++	+

Table 2.1 (continued). Expression pattern of *Lot1/Zac1* during mouse embryonic and postnatal development.

(Valente and Auladell, 2001)

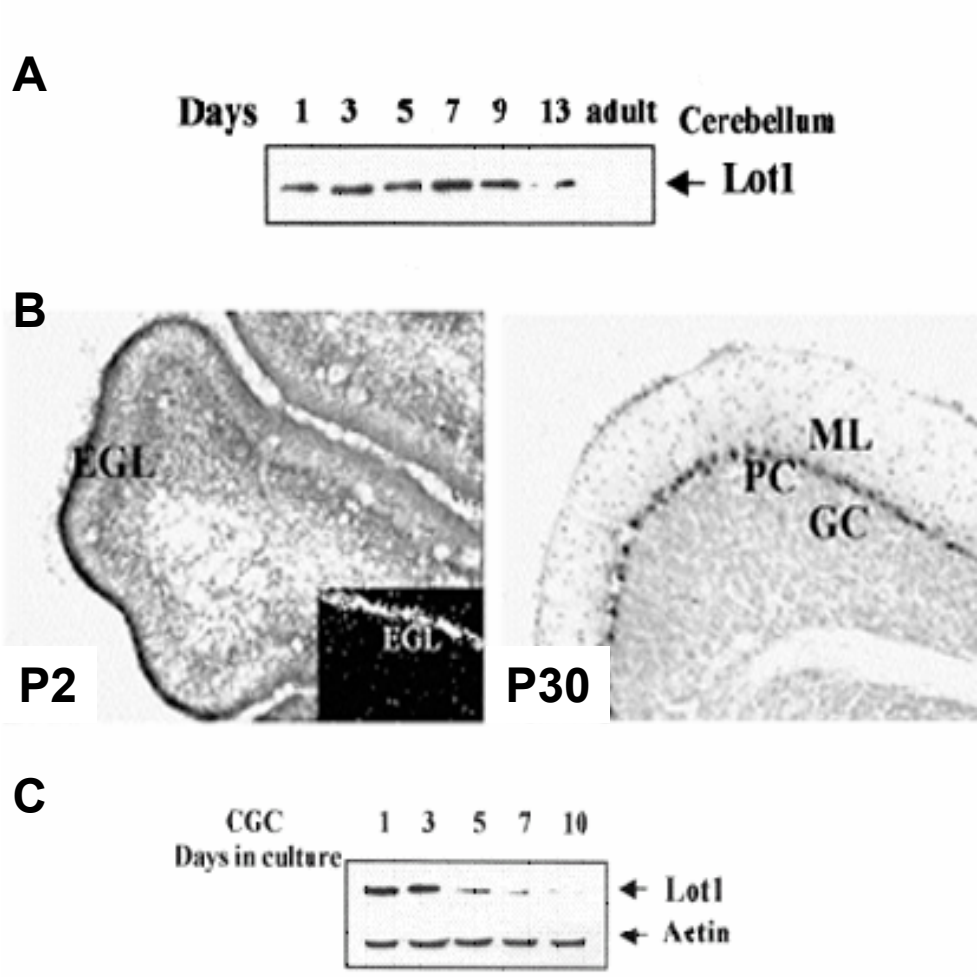

Figure 2.2. Expression of Lot1 during rat cerebellum development. *A*) Western blot analysis of Lot1 expression at different stages of cerebellum development. *B*) Immunostaining for Lot1 on rat cerebellum at postnatal day 2 and 30 (P2, P30) and in situ hybridisation for *Lot1* mRNA (insert) at P2. EGL external granule layer; ML molecular layer; PC Purkinje cell layer; GC granule cell layer. *C*) Western blot analysis of Lot1 expression during in vitro differentiation of cerebellar granule cells (CGC) culture.

(Ciani et al., 2003)

secreting cell types; with the highest levels in growth hormone (GH)- and prolactin (PRL)-producing cells (Pagotto et al., 1999).

Expression in tumors and cancer cell lines. Reduction of the *LOT1/ZAC1* gene expression was found to be a potential genetic event silencing the gene in cancers such as in breast and ovarian primary tumors and tumor-derived cell lines (Abdollahi et al., 1997a; Abdollahi et al., 1997b; Bilanges et al., 1999). The majority of human ovarian carcinoma cell lines examined showed decreased or undetectable *LOT1/ZAC1* expression (Abdollahi et al., 1997b). In agreement with the in vitro data, examination of ovarian carcinomas reduction of LOT1/ZAC1 expression that in more than 80% of the tumor tissues tested was of sizable extent, while in about 39% of ovarian cancer cases brought to no detectable expression (Cvetkovic et al., 2004). A recent study has confirmed the previous results showing a significant reduction in the mRNA expression level of this gene in 24 out of 28 primary ovarian cancer tissues, compared to the non-cancerous counterparts (Kamikihara et al., 2005). Primary breast tumors also showed reduced expression of this gene. Breast cancer cell lines compared to the normal mammary epithelial cells exhibited reduced *LOT1/ZAC1* expression and more then 60% of the breast cancer cells examined showed complete loss of the gene expression (Bilanges et al., 1999). Also head and neck squamous cell carcinoma (HNSCC) cell lines exhibited downregulation of *LOT1/ZAC1* expression compared to normal oral mucosa (Koy et al., 2004). It has also been suggested that *LOT1/ZAC1* gene downregulation in chondrocytes may be a significant contributing factor in the development of extraskeletal myxoid chondrosarcoma (EMC) tumors since *LOT1/ZAC1* mRNA was found to be downregulated in EMC tumors (Poulin and Labelle, 2005). Besides, recent data suggest that *LOT1/ZAC1* plays a role at an early stage of keratinocyte differentiation and in carcinogenesis (Basyuk et al., 2005). In normal skin, a high expression level of *LOT1/ZAC1* was observed in basal keratinocytes and a lower expression in the first suprabasal differentiating layers of epidermis. Accordingly, in vitro, *LOT1/ZAC1* was shown to be upregulated upon

induction of keratinocyte differentiation and was capable of triggering keratinocyte differentiation (Basyuk et al., 2005). *LOT1/ZAC1* expression is dramatically lost in basal cell carcinoma, a neoplasm characterized by a relatively undifferentiated morphology; whereas, in squamous cell carcinoma that exhibits differentiated phenotype, *LOT1/ZAC1* expression is maintained (Basyuk et al., 2005). *LOT1/ZAC1* may also play a role both in growth regulation and differentiation of pituitary gland (Pagotto et al., 2000). Indeed a strong reduction or absence of *LOT1/ZAC1* mRNA and protein expression was detected in non-functioning pituitary adenomas, while in clinically active pituitary neoplasias, the decrease in *LOT1/ZAC1* expression was variable (Pagotto et al., 2000). Taken together, the absence or reduced expression of *LOT1/ZAC1* in majority of the tumor cells or tissues suggested the *LOT1/ZAC1* downregulation has a role in cancer. Accordingly, forced expression of *LOT1/ZAC1* into cancer cell lines, such as ovarian cancer cell lines (Abdollahi et al., 1999), the osteosarcoma cell line SaOs-2, and the porcine epithelial cell line LLC-PK1, both in vitro (culture) and in vivo (nude mice), inhibited cellular proliferation and induction of morphological transformation of tumor cells (Spengler et al., 1997). The anti-proliferative activity of *Zac1* was found to be due to induction of extensive apoptosis and of G_1 arrest, which proceeded independently of retinoblastoma protein (Rb) and of expression of the cyclin dependent kinase inhibitors (CDKIs) $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, and $p16^{INK4a}$ (Spengler et al., 1997). It was also shown that *Zac1*-mediated apoptosis was unrelated to cell-cycle phase and the G_1 arrest observed was independent of apoptosis, which suggested separate mechanism controlling apoptosis and cell-cycle arrest by *Zac1* (Spengler et al., 1997). Furthermore, recent data have suggested that the *LOT1/ZAC1*-mediated apoptotic death in human ovarian cancer cells is p53-independent and can be blocked by caspases inhibitors (Kamikihara et al., 2005). Downregulation of endogenous *Zac1* expression by antisense treatment in two murine tumoral pituitary cell types, AtT-20 (anterior pituitary corticotrophin cell line) and TtT/GF (anterior pituitary folliculostellate cell line), that are representative of

granular and agranular cell lineages, respectively, increased cell proliferation in a dose-dependent manner (Pagotto et al., 1999).

ZAC1 and its alternatively spliced variant, *ZACΔ2*, which lacks two zinc-finger domains, were both found to be nuclear proteins and equally efficient in preventing cell proliferation. However, the anti-proliferative activity of these variants is believed to result from a differential regulation of apoptosis versus cell cycle progression since *ZACΔ2* was found to be more efficient in inducing cell cycle arrest than *ZAC1*, whereas the reverse was true for apoptosis induction (Bilanges et al., 2001).

2.1.4. *LOT1/ZAC1* gene regulation

Chromosomal deletion in cancer. LOT1/ZAC1 is localized at human chromosome 6q24-25, a chromosomal region known to harbor a tumor suppressor gene for several types of neoplasia (Wan et al., 1999). Analysis of allelic deletion in the tumor tissues provided additional evidence supporting a potential role for LOT1/ZAC1 in the pathogenesis of ovarian and breast cancer and possibly in other types of cancer. Ovarian and breast tumors showed about 36.4 and 40% loss of heterozygosity (LOH), respectively, of the *LOT1/ZAC1* gene, based on allelic loss of one or more single nucleotide polymorphic sites within its genomic sequences (Cvetkovic et al., 2004). In another study, using primary squamous cell carcinomas of the head and neck (HNSCC) and microsatellite markers flanking *LOT1/ZAC1*, an average LOH rate of 31.4% in the region was detected (Koy et al., 2004). Additionally, a recent study revealed a high frequency of 6q23-25 LOH in pheochromocytomas (PCCs) (Lemeta et al., 2006). Similarly, another report has concluded that in human hepatocellular carcinomas *LOT1/ZAC1* is often silenced by allelic loss (Midorikawa et al., 2006). Together with its functional properties and chromosomal localization, these findings

substantiated *LOT1/ZAC1* as a suitable candidate for the tumor suppressor gene localized at 6q24-q25.

Epigenetic regulation in cancer. Alterations in the expression of *LOT1/ZAC1* can occur also as a consequence of changes in epigenetic events. Analysis of *LOT1/ZAC1* genomic region revealed the presence of a CpG island in exon 1 that extends into the flanking promoter region of the gene (Abdollahi et al., 2003b; Arima et al., 2001; Varrault et al., 2001). This observation suggested that genomic DNA methylation is a mechanism of silencing the *LOT1/ZAC1* gene expression. The hypothesis was tested in an in vitro reporter assay by modifying the *LOT1/ZAC1* promoter region by DNA methylation, which resulted in a significant loss in the promoter ability to drive the reporter gene transcription in transfected cell lines (Abdollahi et al., 2003b; Arima et al., 2001; Varrault et al., 2001). Also, bisulfite sequencing of the genomic DNA showed that the *LOT1/ZAC1* CpG island is a differentially methylated region (DMR) in ovarian and breast cancer cell lines or tumors samples (Abdollahi et al., 2003b). In these cell lines, the ratio of methylated to unmethylated CpG nucleotides in the DMR ranged from 31 to 99% and the ovarian tumors had relatively higher CpG methylation than in normal tissues. In similar studies, LOT1/ZAC1 mRNA expression was shown to be reduced in the majority of human ovarian cancer samples, which correlated with hypermethylation of the DMR (Kamikihara et al., 2005). It has also been observed that treatment with the methylation-interfering agent 5-azacytidine induced *LOT1/ZAC1* re-expression in breast cancer cell lines (Bilanges et al., 1999). In addition to DNA methylation, epigenetic modification involving histone deacetylation may play an important role in transcriptional silencing and loss of gene function of *LOT1/ZAC1*. Trichostatin-A, a specific inhibitor of histone deacetylase (HDAC), was shown to relieve transcriptional silencing of *LOT1/ZAC1* mRNA in malignantly transformed cells (Abdollahi et al., 2003b). Taken together, these data suggest that expression of *LOT1/ZAC1* is under the control of two epigenetic modifications, CpG methylation and histone deacetylation and that, in the absence of

loss of heterozygosity, the maximal silencing of *LOT1/ZAC1* may require both processes (Abdollahi et al., 2003b).

Maternal imprinting regulation. In 1995, it was reported that transient neonatal diabetes mellitus (TNDM), a rare condition characterized by intrauterine growth retardation, dehydration, reduced postnatal development, and hyperglycemia due to a lack of normal insulin secretion, is associated with paternal uniparental disomy of chromosome 6 (UPD6), suggesting the involvement of an imprinted gene in this disease (Gardner et al., 1999; Temple et al., 1996; Temple et al., 1995). Genomic analysis of TNDM critical region for the presence of methylation differences between maternal and paternal alleles, using DNA from TNDM patients with paternal UPD6 and normal controls, suggested *LOT1/ZAC1* as a candidate imprinted gene for this disease (Gardner et al., 2000). Simultaneously, another report, identified *LOT1/ZAC1* as an imprinted gene and confirmed that this gene is expressed only from the paternal allele in a variety of tissues (Kamiya et al., 2000). Another study, using monochromosomal hybrid cells with a human chromosome 6 of defined parental origin, identified a novel maternally imprinted non-coding (untranslated) gene, *HYMAI*, in addition to *LOT1/ZAC1*. The *HYMAI* gene was found to be in close proximity of and partially overlapping with *LOT1/ZAC1*. This report also showed maternal imprinting of *Zac1* in mice (Arima et al., 2000). This observation was confirmed by another report which identified *Zac1* as an imprinted gene in mice that was expressed predominantly from the paternal allele in all adult mouse tissues tested (pituitary, ovary, lung, brain, and heart tissue) (Piras et al., 2000). In TNDM fibroblasts, the monoallelic expression of both *LOT1/ZAC1* and *HYMAI* was found to be relaxed, which provided strong supportive evidence that the presence of two unmethylated alleles of this locus was indeed associated with the inappropriate gene expression of neighboring genes (Mackay et al., 2002). The DMR that partially overlaps mouse *Zac1* and *Hymai* at the syntenic mouse locus is a likely imprinting control region (ICR) which is unmethylated in sperm but methylated in oocytes, a

difference that persists between parental alleles throughout pre- and post-implantation development (Arima et al., 2001). Within the ICR, there is a region that exhibits a high degree of homology between mouse and human. In the majority of TNDM patients with normal karyotype, there was a loss of methylation (LOM) within this highly homologous region, which suggested that epigenetic or genetic mutations of this region probably result in TNDM, possibly by affecting expression of *LOT1/ZAC1* in the pancreas and/or the pituitary (Arima et al., 2001). Recently, a high-copy transgenic mouse line carrying the human TNDM locus has been developed, which displayed hyperglycemia in newborns and impaired glucose tolerance in adults mice (Ma et al., 2004). Expression of human *LOT1/ZAC1* and *HYMAI* in these transgenic mice recapitulated key features of TNDM, implicating impaired development of the endocrine pancreas and beta cell function in pathogenesis of the disease (Ma et al., 2004).

A recent report has confirmed the maternal imprinting of *Zac1* using knockout mice. Expression of Zac1 protein was lost in both $Zac1^{-/-}$ homozygous mice and in heterozygous mice in which only the paternal allele was deleted ($Zac1^{+/-pat}$) indicating the paternal origin of *Zac1* active allele and the silencing of the maternal allele (Varrault et al., 2006). Main phenotypic features of $Zac1^{-/-}$ and $Zac1^{+/-pat}$ mice were intrauterine growth restriction, reduction of body weight, altered bone formation, and neonatal lethality, while $Zac1^{+/-mat}$ heterozygotes were indistinguishable from wild-type pups. Interestingly while liver, hindlimb and lung were reduced in $Zac1^{+/-pat}$ mice, no absolute weight change was observed in brain (Varrault et al., 2006). The embryonic growth restriction phenotype observed in *Zac1*-deficient mice is counterintuitive. Indeed, it is generally observed that inactivation of genes promoting cell proliferation (e.g. oncogenes) leads to growth restriction, and inactivation of genes reducing cell proliferation (e.g., tumor suppressor genes) leads to overgrowth. It was therefore unexpected that the inactivation of *Zac1,* a putative tumor suppressor gene with proapoptotic and cell-cycle-blocking activities, resulted in embryonic growth reduction. On the other hand, accepted theory on imprinted genes, predicts

that paternally expressed imprinted genes are growth promoting, and maternally expressed genes are growth restraining (Moore and Haig, 1991; Wilkins and Haig, 2003). The growth restriction phenotype of *Zac1*-deficient embryos shows that *Zac1* complies with this theory and accounts for the paternal expression of this antiproliferative gene (Varrault et al., 2006).

Growth factor-dependent expression. LOT1/ZAC1 potential role in cancer and development was further supported by the evidence showing that this gene is a novel target of growth factor signaling pathways (Abdollahi et al., 1999). In normal ROSE cells, the *Lot1* expression was strongly downregulated by epidermal growth factor (EGF) receptor ligands (Abdollahi et al., 1999; Abdollahi et al., 2003a). However, blocking the ligand-activated EGF receptor signal transduction pathway by a specific EGF receptor inhibitor (AG1478) or a mitogen-activated protein kinase (MEK) inhibitor (PD098059) restored the normal level of *Lot1* expression, indicating that the signaling from the EGF receptor to *Lot1* expression is relies upon MEK/ERK (extracellular-regulated protein kinase) pathway. The EGF effect was also confirmed in human ovarian epithelial cells. In addition, the growth promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) was able to downregulate *Lot1* expression (Abdollahi et al., 1999). Furthermore, a recent report showed that in rat pituitary tumor cells line GH3, the somatostatin analogue octreotide produces its antiproliferative action by inducing expression of *Lot1* (Theodoropoulou et al., 2006). Octreotide inhibits cell proliferation by binding to a family of receptors (SSTR1-5), which belong to G-protein coupled receptors (GPCR). Knocking down *Lot1* by RNA interference abolished the antiproliferative effect of octreotide in pituitary tumor cells, indicating that *Lot1* is necessary for the action of octreotide. In this study *Lot1* expression appeared to be a target of the phosphatidylinositol 3-kinase (PI3-kinase) pathway. Octreotide treatment decreased the tyrosine phosphorylation levels of the PI3-kinase regulatory subunit p85, induced dephosphorylation of phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt), and activated glycogen

synthase kinase 3β (GSK3β). Therefore, in pituitary tumor cells, somatostatin analogues produce their antiproliferative action by acting on the PI3K/Akt/GSKβ signaling pathway and increasing *Lot1* gene expression (Theodoropoulou et al., 2006).

2.1.5. Role of *LOT1/ZAC1* as nuclear transcription factor

The deduced amino acid sequences of the cDNAs for rat, mouse, and human LOT1/ZAC1, which contain C2H2-type zinc-finger domains at the N-terminal domain, suggested that they act as transcription factors (Abdollahi et al., 1997a; Abdollahi et al., 1997b; Kas et al., 1997; Spengler et al., 1997). Subsequent studies showed that the carboxyl terminal (the non-zinc-finger region) of these proteins can serve as an activator domain (Abdollahi et al., 1999; Kas et al., 1998). Lot1/Zac1 and p53 were found to be capable of inducing transcription of the PACAP type 1 receptor (PAC1-R) in different cell lines (Ciani et al., 1999; Hoffmann et al., 1998). In addition to both being capable of inducing PAC1-R expression, functional interaction between Zac1 and p53 was determined by investigating possible effects of Zac1 on the transcriptional activator function of p53 (Huang et al., 2001). Zac1 specifically enhanced the activity of p53-responsive promoters in cells expressing wild-type p53 but not in cells lacking functional p53. The Zac1 effect was restored by coexpression of p53 in p53-null cells. The results suggested that Zac1 served as a transcriptional coactivator for p53 by directly binding to p53 (Huang et al., 2001). In addition, using luciferase-promoter construct, it has been shown that p53 is capable of activating Apaf-1, which can be enhanced by Zac1 (Rozenfeld-Granot et al., 2002). It is also believed that a sequence upstream to the first coding exon of Zac1 may contain a p53 recognition site that is activated by p53 (Rozenfeld-Granot et al., 2002). Zac1 was also found to bind GRIP1, CREB-binding protein (CBP), and p300, and act as a

potent coactivator or repressor of nuclear receptors, including estrogen, androgen, glucocorticoid, and thyroid hormone receptor activity (Huang and Stallcup, 2000). In a more recent study, it has been shown that p300/CBP increases Zac1 transactivation in a strictly histone acetyltransferase (HAT)-dependent manner. The HAT activity is controlled by the coordinated binding of Zac1 zinc-fingers and carboxyl terminal region to p300, increasing the affinity for histone and acetyl-coenzyme-A (Hoffmann et al., 2006). The consensus DNA-binding site for LOT1/ZAC1 was identified as GGGGCCCC (Varrault et al., 1998). An alternatively spliced variant of ZAC1 (designated as ZACΔ2), which lacks the sequence encoding the two N-terminal zinc-finger domains, has been identified (Bilanges et al., 2001). Both ZAC1 and ZACΔ2 were shown to localize in the nucleus, however, ZACΔ2 displayed an improved transactivation activity and an enhanced affinity for a ZAC1-binding site, which suggested that the two N-terminal zinc fingers negatively regulate ZAC binding to its target DNA sequences (Bilanges et al., 2001).

2.2. CEREBELLUM DEVELOPMENT

2.2.1. Overview of adult cerebellar cortex

The cortex of the cerebellum is the region of the brain in which the most precise correlation between structure and function has been established. This brain region involved in the control of motor coordination, is composed of only eight different neuronal populations: six classical ones, already described by Cajal (1911), Purkinje cells, Golgi cells, Lugaro cells, granule cells, basket cells, stellate cells, and two more

recently reported ones, unipolar brush cells and candelabrum cells (Mugnaini and Floris, 1994). With the exception of unipolar brush cells that are much more numerous in ventral than dorsal lobules, the others repeat their specific pattern all along the numerous folia composing the cortex. This characteristic neuronal arrangement consists of a strict positioning of neurons and afferent fibers, conferring to the cortex a stereotyped three-dimensional geometry (Sotelo, 2004).

In adult mammals and birds, the cortex of the cerebellum contains three main layers. The most superficial one is the molecular layer (ML), a region of low cellular density but of high synaptic density. The deepest layer exhibits, on the contrary, the highest cellular density of any central structure; it contains the granule cell neurons and is called the inner granular layer (IGL). At the interface between these two layers, a thin middle zone in which the cell bodies of the Purkinje cells are aligned into a single row can be found; this zone is the Purkinje cell layer (PCL). From the eight classes of neurons encountered in the cerebellar cortex only one, the Purkinje cell, has an axon which projects outside the cortex. The other seven populations are local circuit neurons (granule and unipolar brush cells are glutamatergic, and the five others GABAergic), and their axons never reach extracortical structures (Cajal, 1911; Mugnaini and Floris, 1994). Inhibitory interneurons are localized either in the ML (stellate, basket cells), in the PCL (candelabrum cells) or in the IGL (Lugaro, and Golgi cells) (Sotelo, 2004).

2.2.2. Overview of cerebellar development.

The cerebellum is derived from the dorsal plate of the neural tube and is part of the metencephalon. The cerebellum is one of the few exceptional structures in the brain where neuronal populations arise not from a single germinal zone but from at least two different germinal zones. The initial germinal matrix of the cerebellum is

bounded by the isthmus anteriorly and the choroid plexus posteriorly. The germinal matrix initially consists of a typical neuroepithelial ventricular zone and a more caudal germinal trigone region, also known as the rhombic lip. The first neurons to leave the ventricular zone are the nuclear neurons (embryonic days E10–12 in the mouse) that will settle deep to the cerebellar cortex. The birth and exit of Purkinje cells (E11–13 in the mouse) from the ventricular zone to form a temporary plate-like structure occur soon after the birth of the first nuclear neurons. The formation of a secondary germinal matrix from the rhombic lip, the external granular layer (EGL), occurs at about the time when nuclear and Purkinje cells have stopped dividing. The formation of the EGL is characterized by the migration of granule cells precursors (GCP) over the cerebellar surface in a subpial position. Postnatally, GCP proliferate extensively in the EGL and then migrate and differentiate into the developing IGL. Also generated at this time are the stellate and basket cells that colonize the ML. Granule cell migrate from the EGL to the IGL in an inward, radial manner helped in their journey by radial glial fibers (more properly known as the Bergmann fibers), through the molecular layer and past the developing Purkinje cells. It is during this time that the cerebellum is transformed from a curved structure into one with deep fissures and large finger-like appendages called folia. The EGL ceases to exist by the end of the second postnatal week in the mouse and the rat, and this period also sees the maturation of the two major extrinsic inputs to the cerebellar cortex: the inferior olive's climbing fibers to the Purkinje cells and the spinal and reticular mossy fibers to the granule cells (Goldowitz and Hamre, 1998).

2.2.3. Genetic dissection of the origins of the cerebellum.

Traditionally, the cerebellum was believed to arise exclusively from the metencephalic territory of the developing neural tube (a region that extends

posteriorily from the choroid plexus to the constriction between the mesencephalon and metencephalon anteriorily, termed the isthmus). However, experimental studies using the chick–quail chimera system provided compelling evidence for a dual origin of the cerebellum. These studies demonstrated that the region of the neural tube that gives rise to the cerebellum not only includes the metencephalon, but extends anteriorily past the isthmus and into the caudal mesencephalon (Goldowitz and Hamre, 1998). The junction between the mesencephalon and metencephalon, the isthmus, has been shown to have an organizing potential on surrounding neural tissue. The special nature of the isthmic region is inherent in the number of important secreted and regulatory genes that are expressed in this region: *Fgf8*, *Wnt1*, *En1*, *En2*, *Pax2*, *Pax5*, *Pax6*, *Pax8*, *Otx1*, *Otx2*, *Gbx2*, *Wnt5a* and *Nkx2.2*. The isthmus is a transition region for the expression of many of these molecules (Joyner, 1996). For example, *Fgf8* is expressed in the caudal portion of the isthmus, whereas *Wnt1* is expressed rostrally, in a nonoverlapping, complementary fashion (Crossley and Martin, 1995). To attest the importance of these molecules, mutations or ectopic expression of several of these genes have marked impact upon cerebellar development. The best documented examples are *Wnt1* and *En1*. Knockouts of these genes largely or totally eliminate the cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994). In mouse, as in fruit fly, it is believed that *En1* expression is regulated by the secreted glycoprotein Wnt1 (McMahon et al., 1992). The expression of these molecules might, in turn, be regulated by pairedbox-containing genes of the *Pax* family (Song et al., 1996). Indeed *Pax2* is found to precede *En1* and *Wnt1* expression in the presomitic embryo. Inactivation of *Pax2* leads to a frequent loss of the cerebellum and posterior midbrain (Favor et al., 1996). Another *Pax* gene that is expressed in the midbrain–hindbrain region is *Pax5*. Although deletions of *Pax5* have little effect on cerebellar development, when a *Pax5* knockout is complemented with a hemizygous deletion of *Pax2* there is complete agenesis of the cerebellum in addition to the inferior colliculus (Urbanek et al., 1997). Also Pax6 appear an important player in cerebellum

morphogenesis as is mutation (*Sey* mouse) leads to cerebellum defects and perturbation of granule cells differentiation (Swanson et al., 2005). Other molecules that are expressed in the neighborhood of the isthmic region, namely Fgf8 and the homeodomain proteins Gbx2 and Islet3, are known to be important players in cerebellar development, as their elimination results in large deletions of the cerebellum (Kikuchi et al., 1997; Meyers et al., 1998; Wassarman et al., 1997). These three molecules are believed to act upstream to *Wnt1* and *En1*. These results allow to start constructing a molecular framework of the initial development of the cerebellar anlagen.

The contribution of the ventricular zone to neuronal populations in the cerebellum. The cells that colonize the cerebellum arise from either the ventricular neuroepithelium or the rhombic lip. The ventricular neuroepithelium is responsible for the generation of most of the neuronal populations within the cerebellum, including the nuclear, molecular layer, Purkinje and Golgi neurons. Fundamental questions are just being answered about the lineage relationships of these various neurons, although little is known about the cellular interactions and molecules that are crucial to commitment to each lineage. A recent report has found that all cerebellar neurons, with the exception of the granule cells, are clonally related. From this work it was concluded that Purkinje cells and nuclear cells arise from a common progenitor (Mathis and al., 1997). A further issue related to the ventricular epithelium concerns the generation of inhibitory interneurons (Golgi, stellate and basket cells). Until recently, the prevailing dogma was that GABAergic interneurons were generated in the secondary germinal zone (the EGL). This belief was based on the fact that the generation of both of these neuronal populations occurs postnatally and that the only substantive germinal zone at this time is the EGL. Recently, several lines of evidence make it clear that the formation of the GABAergic lineage occurs independently of the formation of the EGL. Using retroviral infections either superficially into the EGL or deep into the white matter of cerebellar cortex, it was found that injections into the

EGL generated only granule cells. Alternatively, injections into the white matter generated both astrocytes and GABAergic interneurons (Golgi, stellate and basket cells). Because the retrovirus is taken up only by cells undergoing mitosis, the molecular layer interneurons must undergo a round of mitosis within the white matter (Zhang and Goldman, 1996a; Zhang and Goldman, 1996b). This led to the hypothesis that the generation of inhibitory interneurons is a two-step process, with the initial generation occurring in the ventricular layer and subsequent migration into the white matter of the cerebellar cortex, where they undergo further cell division before translocation to their final destination. These findings were confirmed by a more recent report using mice expressing green fluorescent protein (GFP) from the *Pax2* locus (Weisheit et al., 2006).

The contribution of the secondary germinal zone to the cerebellum. Granule cells have been recognized to arise from the secondary germinal zone since the time of Cajal (1911). Besides, he also reported that the EGL was composed of two distinct zones: a superficial one (the outer EGL), where granule cells precursors actively proliferate, and a deep zone (the inner EGL) containing undifferentiated postmitotic granule cells. The origin of granule cells has been confirmed by both retroviral labelling studies (Miyake et al., 1993; Ryder and Cepko, 1994a) and cell culture studies in which only granule cells were generated from cultured EGL cells (Gao and Hatten, 1994). Granule cell lineage is very clear: the cells all arise from the EGL, and the EGL appears to originate exclusively from the rombic lip (Goldowitz and Hamre, 1998). A major finding that clarified the early development of the granule cell was the discovery that the helix–loop–helix (bHLH) transcription factor *Math1* is essential for the establishment of the granule cell lineage. Mice with a knockout in the *Math1* gene lack all granule cells due to the absence of the formation of the EGL (Ben-Arie et al., 1997). The importance of *Math1* in granule cell genesis was confirmed by overexpression of the gene in transgenic mice that resulted in perturbation of granule cell differentiation (Helms et al., 2001). Mechanisms regulating proliferation and

differentiation of GCP will be discussed in detail in a following section of this dissertation.

2.2.4. Migration of cerebellar neurons

The developing cerebellum can well serve as a model for most of the recognized ways by which neurons migrate in the CNS. There is an outward radial (Purkinje and nuclear neurons migrating from the ventricular zone) and inward radial migration (granule cells migrating from the inner part of the EGL to the IGL) (Altman and Bayer, 1997), as well as a circumferential (formation of the EGL) and tangential migration of cells (postmitotic granule cells in the inner part of the EGL) (Ryder and Cepko, 1994a). The initial migration in the cerebellum is the departure of the first cohorts of cells from the ventricular zone and germinal rhombic lip, which occurs at about E13 in the mouse. These migrating cells appear to define the future boundaries of the cerebellum. In *Unc5h3* mutant mice (*rostral cerebellar malformation, rcm* mice) there is an exuberant migration of granule and Purkinje cells beyond the normal boundaries of the cerebellum into the inferior colliculus and hindbrain. *Unc5h3* (the *Caenorhabditis elegans unc-5* homologue) is a receptor for mouse *Netrin* (Ackerman et al., 1997; Leonardo et al., 1997). *Netrin* is found to be expressed in regions surrounding the cerebellum at E13 (Przyborski et al., 1998). Thus, there seem to be molecules that establish the normal boundaries of the cerebellum, with the proteins Netrin and its receptor, Unc5h3, as key players. On the other hand, *Netrin* signaling did not appear to be crucial for EGL formation (Bloch-Gallego et al., 1999).

Two recently identified genes (*reelin* and *disabled*) that are known to be critical to neuronal migration in the cortex are also important for Purkinje cells migration. In mutant *reeler* and *scrambler* mice, caused by mutations in the extracellular matrix molecule Reelin (D'Arcangelo et al., 1995), and the tyrosine kinase signaling adaptor

molecule Disabled (encoded by *Dab1*) (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997), respectively, Purkinje cells fail to migrate and largely remain in several ectopic clusters deep in the cerebellar cortex, while the granule cells appear to migrate normally but are greatly reduced in number (Goffinet et al., 1984; Goldowitz et al., 1997). While in neocortex there is a single population of Reelin-positive cells (the Cajal–Retzius cells) that are spatially juxtaposed to Dab1-positive cortical cells (Howell et al., 1997), in the cerebellum two populations of Reelin-positive cells have been identified: a subpopulation of cerebellar nuclear neurons and cells of the external granular layer. The responding cells in the cerebellum appear to be the Purkinje cells in that they both bind Reelin and express the Dab1 protein (Miyata et al., 1996). These two molecules are believed to be in the same molecular pathway with Dab1 downstream of Reelin (Goldowitz et al., 1997; Sheldon et al., 1997).

Granule cells exhibit a two-stage migrating behavior in reaching their final destination. A tangential migration in the inner part of the EGL and a glial-guided radial migration across the ML to the IGL. The use of retrovirus in chick embryos has revealed that the unipolar and bipolar cells with "horizontal polarity" reported by Cajal (1911) in the inner EGL are tangentially migrating granule cells, that can move over a long distance, medially and laterally (Ryder and Cepko, 1994b). Tangential migration in the inner EGL has also been confirmed in slice preparations of P10 mouse cerebellum using DiI labelling and time-lapse imaging (Komuro et al., 2001). Once tangential migration is completed, neurons in the deeper region of the EGL start to acquire a "vertical polarity" by emitting a new protoplasmic expansion, provided with a growth cone, which descends vertically from the basal pole of the cell body towards the white matter. The nucleus begins to descend within the vertical expansion once this third process penetrates into the forming ML (Cajal, 1911; Terrazas, 1897) (Komuro and Yacubova, 2003). Detailed electron microscopic study of granule cell migration across the molecular layer of the monkey cerebellum, reported that each descending expansion (the leading process) maintains an intimate contact with the vertically oriented Bergmann fibers (Rakic, 1971). Bergmann fibers are characterized

by the expression of a number of molecules, including nestin, brain-lipid-binding protein (BLBP), the antigens RC1 and RC2 and D4 (Soriano et al., 1997) that, together with other specific molecules, are required by the radial glial for supporting neuronal migration. The expression of these molecules is transient and, after the migration of granule cells is completed, Bergmann fibers lose their early features and become the radially oriented processes of mature astrocytes (the Golgi-Bergmann glia) (Sotelo, 2004).

To date, large numbers of molecules and genes have been discovered as potential regulators or modulators of neuronal cell migration. Accordingly also granule cell migration is modulated by the action of many genes. One of such modulators appear to be brain-derived neurotrophic factor (*Bdnf*). Cerebellar granule cells express *Bdnf* and its high-affinity receptor (*TrkB*) (Wetmore et al., 1990). In vitro, $Bdnf^{-/-}$ granule cells initiate migration poorly and exogenous Bdnf induces both wild-type and $Bdnf^{-/-}$ cells to migrate. In vivo, a lack of Bdnf results in impaired migration of granule cells (Borghesani et al., 2002).

9-O-acetyl-GD3 is a ganglioside expressed in the developing nervous system during periods of neuronal migration. Electron microscopic analysis reveals that 9-O-acetyl-GD3 is localized at the contact sites between migrating granule cells and Bergmann fibers in the EGL and ML. Furthermore, the application of an antibody against 9-O-acetyl-GD3, blocks the migration of cerebellar granule cells in a dose-dependent manner (Santiago et al., 2001). These results suggest that 9-O-acetyl-GD3 is involved in glia-associated migration of cerebellar granule cells in the developing ML.

In the developing cerebellum, granule cells turn on the gene for tissue plasminogen activator (*tPA*) as they begin their migration into the ML (Friedman and Seeds, 1995). Granule cells both secrete tPA (an extracellular serine protease that converts the proenzyme plasminogen into the active protease plasmin) and bind tPA to their cell surface. Interestingly, mice lacking the *tPA* gene ($tPA^{-/-}$) have greater than twofold more migrating granule cells in the ML during a period of active cell migration. Granule cells in $tPA^{-/-}$ mice migrate through the ML at about half the rate

measured for migration of granule cells in *tPA*$^{+/+}$ mice (Seeds et al., 1999). These findings suggest that *tPA* gene expression is required for maintaining the maximal rate of granule cell migration during cerebellar development.

The human neuronal migration disorder Miller-Dieker lissencephaly, has been ascribed to a defect in *Platelet-activating-factor* (*PAF*) signaling (Jellinger and Rett, 1976; Stewart et al., 1975). In this disorder, the brain has a smooth cortical surface (lissencephaly) caused by a lack of outer cortex complexity and a disruption of the migration of cerebellar granule cells (Jellinger and Rett, 1976; Miller, 1963; Stewart et al., 1975). This brain malformation results from a haploinsufficiency of the *LIS-1* gene (Ledbetter et al., 1992; Mizuguchi et al., 1995; Reiner et al., 1995), which encodes a 45-kDa subunit of a brain PAF acetylhydrolase, an enzyme that converts PAF to the inactive lyso-PAF (Hattori et al., 1994). It was found that the application of the non hydrolyzable PAF receptor agonist methyl-carbamyl-PAF (mc-PAF) yields a dose dependent decrease in cerebellar granule cell migration. This effect can be blocked by PAF receptor antagonists (Bix and Clark, 1998). Taken together, these results suggest that the stimulation of neuronal PAF receptors could be one critical step for the regulation of cerebellar granule cells as well as immature cortical neurons.

Cyclin-dependent kinase 5 (*Cdk5*) is one unique member of the cyclin-dependent kinases (Meyerson et al., 1992). Unlike other cyclin-dependent kinases, which are known to control cell cycle in eukaryotes, *Cdk5* expression and kinase activity are not associated to cell division. The appearance of active Cdk5 is correlated with the termination of neurogenesis and the beginning of differentiation of neuronal cells in the developing brain (Tsai et al., 1993). To elucidate the role of *Cdk5* during postnatal cerebellar development, a series of *Cdk5*$^{-/-}$-*Cdk5*$^{+/+}$ chimeric mice have been generated, since *Cdk5*$^{-/-}$ mice die around birth and much cerebellar development occurs postnatally (Ohshima et al., 1999; Ohshima et al., 1996). In the *Cdk5*$^{-/-}$-*Cdk5*$^{+/+}$ chimeric cerebella of 2- to 3-month old mice, significant numbers of granule cells are located in the ML, suggesting a failure to complete migration from the EGL

to the IGL. In fact, the granule cells found within the ML of chimeric cerebella are nearly all *Cdk5* deficient. In contrast, the granule cells within the IGL are a mixture of $Cdk5^{-/-}$ and $Cdk5^{+/+}$ cells (Ohshima et al., 1999). These results indicate that the block in cell migration that leaves many granule cells within the ML is intrinsic to $Cdk5^{-/-}$ cells, suggesting that *Cdk5* may play crucial roles in cerebellar granule cell migration. Monitoring intracellular calcium levels using Ca^{2+} indicator dyes demonstrated that migrating granule cells exhibit dynamic changes inside the cell body (Komuro and Rakic, 1998). Importantly, the reduction of Ca^{2+} influx by lowering extracellular Ca^{2+} concentrations or by blocking N-type voltage-gated Ca^{2+} channels results in a decrease in the amplitude and frequency of spontaneous Ca^{2+} elevations (Komuro and Rakic, 1996). This reduction is linearly related to a reduction in the rate of cell movement. Taken together, these results indicate that intracellular Ca^{2+} fluctuations provide an intracellular signal controlling granule cell migration.

It has been also demonstrated an important role for N-methyl-D-aspartate (NMDA) receptor activation in the migration of granule cells from the EGL. The presence of spontaneous activity of the NMDA receptors on the surface of migrating cerebellar granule cells has been confirmed by patch-clamp analysis. The frequency of spontaneous NMDA receptor-coupled channel activity is low in the middle and the bottom of the EGL, but high in the ML (Rossi and N., 1993). Most importantly, blocking NMDA receptor activity with its antagonists significantly decreases the rate of granule cell movement in the ML. In contrast, the rate of cell migration is not substantially altered by blocking the activity of non-NMDA receptors (kainate and AMPA receptors), $GABA_A$ and $GABA_B$ receptors (Komuro and Rakic, 1993; Komuro and Rakic, 1996; Komuro and Rakic, 1998).

The *weaver* (*wv*) mutation, a single base pair substitution in the G-protein-coupled inward-rectifying K^+ channel protein Girk2 (Patil et al., 1995), appears to specifically affect the preparation of cells to migrate (Smeyne and Goldowitz, 1989). In this mutation, postmitotic and premigratory cells experience massive death. It is believed that the inactive Girk2 does not offer the compensatory hyperpolarization following

the predominant depolarizing current evoked by NMDA receptor activation (Surmeier et al., 1996). The result is sustained depolarization, excessive Ca^{2+} entry (Tucker et al., 1996) and cell death. Accordingly, abolishing of functional NMDA receptors in the *weaver* cerebellum rescues granule cells from death (Jensen et al., 1999).

At the cellular level it has been shown that granule cell survival is compromised and migration does not occur when radial glia are disordered and 'atrophic' (Rakic and Sidman, 1973; Ross et al., 1990). Additionally, it has been determined that, if glia are experimentally eliminated from the developmental cerebellum, GCP suffer large scale cell death (Delaney et al., 1996). On the other hand, using co-cultures of premigratory neurons and glial cells, it has been shown that the induction of the radial glial phenotype is in fact mediated by neuron-glial interactions (Hunter and Hatten, 1995). The molecular underpinnings of this two-way interaction appear to be, on one hand, a neuron–glial trophic signal and, on the other, a glial–neuron buffering of the surrounding milieu. Astrotactin and Neuregulin are two molecules that are expressed in migrating cerebellar granule cells and provide a trophic support for glial development (Rio et al., 1997; Zheng et al., 1996). The EGF receptor tyrosine kinase family ErbB4 is known to be activated following Neuregulin stimulation. Neuregulin and ErbB4 are localized to granule cells and glia, respectively, this signaling pathway appears to be important to granule cell migration as well as the induction of radial glia morphology (Rio et al., 1997). Besides *Astrotactin* null mutation results in a decrease of the rate of granule cell migration, with increased apoptosis and secondary abnormalities in Purkinje cell development (Adams et al., 2002).

2.3. PROLIFERATION OF CEREBELLAR GRANULE CELLS

2.3.1. Overview of neuron number regulation in the cerebellum

The cerebellum is a highly ordered structure with a tight regulation of the ratio of Purkinje cells to granule cells (Wetts and Herrup, 1983). It appears that the number of Purkinje cells determines to a large extent the size of the granule cell population. Numerous mutations, as well as experimental perturbations, that eliminate Purkinje cells demonstrate that there is a concomitant reduction in the number of the granule cells (Goldowitz and Hamre, 1998). Transgenic mice in which subpopulations of Purkinje cells were ablated at various developmental times have been generated. In the EGL overlying areas of deleted Purkinje cells, the mitotic activity of EGL cells was significantly diminished relative to regions in which Purkinje cells were still present (Smeyne et al., 1995). This demonstrated that Purkinje cells can control the mitotic activity of GCP within the EGL. Support for this premise comes from a comparison of *meander-tail*/wild-type and *weaver*/wild-type chimeric mice (Goldowitz, 1989; Goldowitz and Mullen, 1982; Hamre and Goldowitz, 1997). In *meander-tail* mutant mice (gene symbol *mea*), cerebellar granule cells are largely depleted from the anterior cerebellum and somewhat compromised in the posterior lobe. In *wv* mutant mice, the generation of granule cells is normal and granule cells die after they become postmitotic. When the mutant gene acts early, as in *mea*, the chimeric cerebellum contains a near-normal number of granule cells in the anterior lobe as a result of repopulation by wild-type granule cells. In contrast, when the mutant gene affects postmitotic granule cells, as in *wv*, the chimeric cerebellum has a depletion in the granule cell population. These studies indicate a feedback mechanism

by which the EGL informs the Purkinje cells of the size of the population engaged in proliferation.

2.3.2. Regulation of granule cells precursor proliferation

The fact that Purkinje cells stimulate granule cell proliferation implies that some kind of mitotic signal must be generated from Purkinje cells. A good candidate for this role appeared to be the secreted glycoprotein Sonic hedgehog (Shh) which is expressed by Purkinje cells (Traiffort et al., 1999). *Shh* was known to be involved in limb skeletal development as well as cell proliferation and cell fate determination in many brain areas during development (Jensen and Wallace, 1997; Roelink et al., 1995) (Ingham and McMahon, 2001; Litingtung et al., 2002; Palma, 2004; Wang et al., 2000). Besides granule cells in the EGL express Shh receptors and downstream effectors of *Shh* pathway (Corrales et al., 2004; Traiffort et al., 1999). This hypothesis received strong support by the demonstration that antibody-blocking of Shh action resulted in a marked reduction of granule cell production in vivo. In contrast, treatment with recombinant Shh prevented the GCP in culture to exit from the cell cycle and prolonged their proliferation (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). This findings were confirmed later on by *Shh* conditional allele (*L7-Cre* and *Pax2-Cre*) knockout mice ($Shh^{-/-}$ mice die at birth) in which granule cell proliferation and cerebellum foliation are compromised (Lewis et al., 2004). Additionally transgenic mice overexpressing *Shh* display increased size of the cerebellum and prolonged proliferation of granule cells (Corrales et al., 2004).
Shh receptor system is composed of two different proteins: Patched1 (*Ptc1*), a 12-trasmembrane receptor for Shh and Smoothened (*Smo*) a 7-trasmembrane protein that mediates Shh downstream signals. In the absence of Shh, Ptc1 inhibits Smo activity but when Shh binds Pct1 the inhibition is released allowing activation of Smo (Fuse

et al., 1999; Kalderon, 2000). Through an unknown mechanism, activated Smo initiates a series of downstream events culminating in the activation of the Gli family of transcription factors (Gli1, Gli2, and Gli3) that control expression of *Shh* target genes. Shh signaling induces transcription, among other genes, of *Ptc1* and *Gli1*, thus creating both negative and positive feedback effects (Goodrich et al., 1996; Marigo et al., 1996). Furthermore *Gli1* transcription in response to Shh appear to be dependent on Gli2 or Gli3 with respect to the tissue analyzed. In the spinal cord Gli2 is the primary activator of Shh signaling, whereas Gli3 functions mainly (but not exclusively) as a repressor (Bai et al., 2002; Bai and Joyner, 2001; Bai et al., 2004; Persson et al., 2002). By contrast, in the limb Shh signaling inhibits Gli3 repressor and only *Gli3* is required for digit patterning and for achieving normal level of proliferation (Litingtung et al., 2002; te Welscher et al., 2002; Wang et al., 2000). The three *Gli* genes, which display different patterns of expression in the cerebellum, appear to differently contribute to cerebellum development. *Gli1*$^{-/-}$ mice are viable, have a normal development and display altered Shh signaling only in combination with *Gli2* heterozygosis (Park et al., 2000). In contrast *Gli2*$^{-/-}$ and *Gli3*$^{-/-}$ mice die at birth thus precluding evaluation of postnatal cerebellum development. Unlike *Gli3* mutants, *Gli2*$^{-/-}$ mice display a reduced thickness of the EGL at E18.5 (just before birth) and impaired transcription of *Gli1* indicating a major role for *Gli2* in transducing Shh signaling in the developing cerebellum (Corrales et al., 2004). Accordingly *Gli2* conditional allele (*En1-Cre*) mutants, in which the target gene is deleted from E9 in the cells that will give rise to the cerebellar cortex are viable and display reduced size of the cerebellum and a premature cessation of proliferation in the EGL (Corrales et al., 2006). Importantly, elevation of intracellular cyclic adenosine monophosphate (cAMP) levels and subsequent protein kinase A (PKA) activation inhibits Shh signaling (Concordet et al., 1996; Dahmane and Ruiz-i-Altaba, 1999; Kenney and Rowitch, 2000; Wallace, 1999; Wechsler-Reya and Scott, 1999) possibly by enhancing Gli3 repressor activity (Dai et al., 1999) and/or promoting

cAMP responsive element-binding protein (CREB) phosphorylation (Pons et al., 2001).

The major target gene involved in proliferation that is directly upregulated in granule cells in response to Shh signaling is the protooncogene *Nmyc* (Kenney et al., 2003; Oliver et al., 2003). Nmyc in turns enhances D-type cyclins (*CcnD1* and *CcnD2*) expression, promotes GCP proliferation and prevents their differentiation (Kenney et al., 2003; Kenney and Rowitch, 2000; Knoepfler et al., 2006; Oliver et al., 2003). Accordingly, the cerebellum is reduced in *Nmyc* conditional allele (*Nestin-Cre*) knockout mice (*Nmyc* mutant dye during gestation) and granule cells proliferation in response to Shh signaling is inhibited in cultures obtained from this mutant (Hatton et al., 2006; Knoepfler et al., 2006). This results clearly indicates *Nmyc* as a downstream effector of Shh signaling in the cerebellum. Besides other pathways may give important contribution in supporting GCP proliferation by synergize with Nmyc. For example it has been shown that PI3-kinase pathway activation enhances granule cell precursor proliferation by stabilizing Nmyc protein via activation of Akt and subsequent inhibition of GSK3β-dependent Nmyc phosphorylation and degradation. The effects of PI3-kinase activity on Nmyc stabilization are mimicked by insulin-like growth factor (IGF), an activator of PI3-kinase cascade which have been shown to have a role in central nervous system precursor proliferation (Kenney et al., 2003b). These findings indicate that Shh and PI3-kinase signaling pathways converge on *Nmyc* to regulate neuronal precursor cell cycle progression.

2.3.3. Regulation of granule cells precursor differentiation

As stated above GCP proliferate in the outer EGL under the influence of mitogenic Shh secreted by Purkinje cells and then translocate in the inner EGL where they cease proliferation and begin differentiation. An obvious question arise from this behavior:

what are the mechanisms that drive the exit from cell cycle of granule cell precursor and initiate their differentiation program? Several explanations have been proposed to answer this question involving the action of extracellular matrix (ECM) components, cell-cell interactions or diffusible signals.

A first explanation comes from the observation that, in the prenatal and early postnatal cerebellum, stromal cell-derived factor 1α (*SDF-1α*) is expressed by cells in the pia membrane, while GCP and immature granule cells express its $G_{\alpha i}$-coupled receptor *CXCR4* (Zou et al., 1998). Importantly, the deletion of *SDF-1α* or *CXCR4* leads to the premature migration of GCP away from the proliferative zone of the EGL and small numbers of GCP are found ectopically outside the EGL of *SDF-1α*- or *CXCR4*-deficient mice (Ma et al., 1998; Zou et al., 1998). Accordingly, SDF-1α has been found to induce chemotactic responses in GCP (Klein et al., 2001). These results suggest that *SDF-1α* and *CXCR4* play a crucial role in retaining GCP in the EGL. Besides SDF-1α signaling has also been found to enhance Shh mediated proliferation of GCP, probably by inhibiting, through $G_{\alpha i}$, cAMP production by adenylate cyclase and therefore reducing PKA activity, which is known to repress Shh mediated proliferation (Klein et al., 2001). Although the expression of *SDF-1α* or *CXCR4* persists during the early postnatal cerebellum, postmitotic granule cells initiate their migration toward the IGL. A possible molecular mechanisms that explains this behavior have been proposed. *Ephrin-B2* and its receptor *EphB2* are expressed in the developing EGL. The chemoattractant effect of SDF-1α to the granule cells is selectively inhibited by soluble EphB2 receptor through reverse signaling of Ephrin-B2 (Lu et al., 2001). These results suggest that when granule cells are ready to migrate, they would lose responsiveness to SDF-1α. Such changes in responsiveness to SDF-1α could be mediated at least in part by *EphB2* receptor and *Ephrin-B2*.

A second explanation to the fact that GCP exit the cell cycle in the inner part of the EGL, just as they approach Purkinje cells, the putatively primary source of mitogenic Shh, has been ascribed to pituitary adenylate cyclase-activating polypeptide (PACAP)

signaling. The neuropeptide PACAP is expressed by Purkinje cells, deep cerebellar nuclei while PACAP immunoreactivity has been detected in nerve fibers in the EGL, during cerebellum development. On the other hand granule cells express PACAP receptor 1 (PAC1-R) in the EGL with an increased abundance in its inner part (Nicot et al., 2002; Nielsen et al., 1998; Skoglosa et al., 1999). In contrast *Gli1* appears to be expressed mainly in the outer EGL (Nicot et al., 2002). PACAP signaling, through PAC1-R coupled to protein G_s, stimulates cAMP production by adenylate cyclase thus inhibiting Shh mediated proliferation of granule cell (Nicot et al., 2002). By contrast SDF-1α secreted by the pia inhibits cAMP production and enhances Shh mediated proliferation (Klein et al., 2001). In this scenario it has been proposed that PACAP, through cAMP elevation and PKA activation, could antagonize Shh and SDF-1α signaling in the inner EGL, leading to exit from the cell cycle and initiation of differentiation of granule cells (Nicot et al., 2002). The observation that *Gli1*, a marker of Shh signaling, is expressed in the outer EGL, while PAC1-R, that mediate PACAP antiproliferative effects, is mainly found in the inner EGL together with CREB phosphorylation (Pons et al., 2001), is consistent with this theory (Figure 2.3).
An alternative explanation has been provided by the observation that some ECM components are differentially represented in the inner and outer EGL. Laminin and its receptors (α6 integrins) are highly expressed in mitotically active GCP in the outer EGL, whereas vitronectin and its major receptors (αv integrins) are expressed in postmitotic cells in the inner EGL and in the IGL. Besides this pattern of expression appears to have a functional role in granule cell proliferation: cells cultured on laminin responded much more strongly to Shh-induced proliferation than cells cultured on vitronectin, which, in addition, expresses differentiation markers. Apparently vitronectin, initiates an intracellular signaling cascade that changes the response of granule cells to Shh. The pathway in GCP, responsible for the conversion from a proliferative Shh-mediated response to a differentiation signal, appear to depend on CREB. Vitronectin stimulates phosphorylation of CREB, and over-

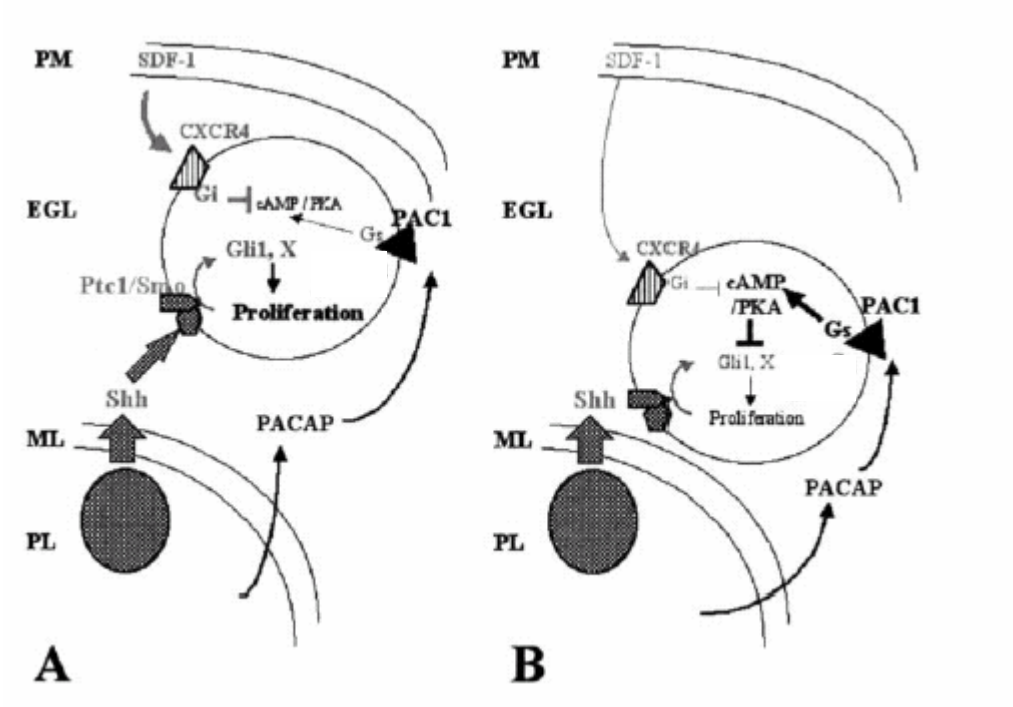

Figure 2.3. Model of PACAP regulation of granule precursor proliferation. Granule precursors are depicted in the outer EGL (*A*) and inner EGL (*B*), with the complex of soluble, extracellular signals and their receptors, including Shh/Ptc-Smo, SDF-1/CXCR4, and PACAP/PAC1-R. *A*) Near the pia matter during the first postnatal week, granule cell proliferation is high. Prominent expression of SDF-1/CXCR4 blocks adenylate cyclase activity and cAMP via Gαi coupling, thereby promoting Shh-induced proliferation. *B*) In the inner EGL as development progresses, SDF-1 and CXCR4 levels decrease. In turn, the proliferative action of Shh is now blocked by PACAP via Gs activation of adenylate cyclase through PAC1 receptors. PACAP may be derived from nerve fibers emerging from the Purkinje layer or brainstem nuclei. *ML*, Molecular layer; *PL*, Purkinje cell layer; *PM*, pia matter. *(Nicot et al., 2002)*

expression of CREB appears to be sufficient to induce granule cell differentiation in the presence of Shh. Taken together, these data suggest that granule neuron differentiation is regulated by the vitronectin-induced phosphorylation of CREB, a critical event that terminates Shh-mediated proliferation and permits the differentiation program to proceed in these cells (Pons et al., 2001).

While mitogenic signaling by Shh appears to be the main source driving granule cell proliferation, the mechanisms regulating exit from cell cycle and differentiation of GCP appear to be intricate and not yet fully understood. It is also possible that different mechanisms may contribute in parallel in regulating this process possibly converging on PKA activation and CREB phosphorylation.

2.4. PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamic extracts on the basis of its ability to stimulate cyclic adenosine monophosphate (cAMP) formation in anterior pituitary cells (Miyata et al., 1989). PACAP is produced by both hypotalamic neurons and other neurons in the CNS as well as by many peripheral tissues. PACAP has been found to exert pleiotropic effects including modulation of neurotransmitter release, vasodilation, bronchodilation, activation of intestinal motility, increase of insulin and histamine secretion, as well as stimulation of cell multiplication and/or differentiation (Vaudry et al., 2000a).

2.4.1. PACAP peptides and regulation

PACAP exists in two forms, a longer 38- and a shorter 27-amino acid peptides (PACAP-38 and PACAP-27) (Miyata et al., 1990) whose sequences appear to be highly conserved along evolution (Vaudry et al., 2000a). The human PACAP cDNA encodes a 176-amino acid prepro-protein with a N-terminal 24-amino acid signal peptide (Hosoya et al., 1992). In mammals (human, sheep, rat and mouse) PACAP precursor comprises both PACAP and a second peptide termed PACAP-related peptide (PRP) located upstream (Vaudry et al., 2000a). By the action of different enzymes (prohormone convertases, carboxypeptidases and peptidyl glycine α-amidating monooxygenase) PRP, PACAP-38 and PACAP-27 are generated from the precursor (Li et al., 1999; Okazaki et al., 1992; Vaudry et al., 2000a). The sequence of human PACAP-27 is 68% identical to vasoactive intestinal polypeptide (VIP), identifying PACAP as a member of the VIP-growth hormone releasing factor (GRF)-glucagon-secretin superfamily (Campbell and Scanes, 1992).

The human PACAP gene is composed of five exons with a promoter sequence comprising two regions, termed P1 and P2, which correspond, respectively, to an initiator-like sequence and a CT-rich domain with GC boxes. Besides, the PACAP promoter possesses two cAMP-response elements (CRE), a 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) and a pair of sequences homologous to the consensus sequence for pituitary-specific growth hormone factor 1 (GHF-1) binding sites, which is known to play a role in the tissue specific expression of growth hormone (GH) (Vaudry et al., 2000a). Investigation of the promoter activity has revealed that PACAP is constitutively expressed and that transcription of the *PACAP* gene can be enhanced by cAMP, TPA and even by PACAP itself (Suzuki et al., 1994).

2.4.2. PACAP expression in the CNS

During rat development PACAP mRNA is expressed from E13 in distinct regions of rodent brain: developing cortex, hippocampus, amygdala and hypothalamus as well as in spinal cord and dorsal root ganglia (Skoglosa et al., 1999). In adult rat brain PACAP appears to be highly expressed in the hypothalamus in particular in the magnocellular neurons of paraventricular (PVN) and supraoptic (SON) nuclei (Arimura et al., 1991; Hannibal et al., 1995a; Hannibal et al., 1995b; Koves et al., 1991; Skoglosa et al., 1999). Dense accumulation of PACAP-immunoreactive fibers is found in the internal zone of the median eminence and in the vicinity of the capillaries of the hypothalamo-hypophysial portal system (Hannibal et al., 1995a). Significant amounts of PACAP-38 are also found in extrahypothalamic regions, including the substantia nigra, nucleus accumbens, septum, globus pallidus, cerebral piriform cortex, and pons (Ghatei et al., 1993; Masuo et al., 1993). In the limbic system, PACAP-immunoreactive fibers are detected in the amygdaloid complex and in the mediodorsal and paraventricular nuclei of the thalamus (Koves et al., 1991; Masuo et al., 1993). In the lateral septum area, a dense network of immunoreactive fibers innervates blood vessels (Koves et al., 1991). PACAP and its mRNA have also been detected in the cerebellum (Purkinje cells and deep cerebellar nuclei) (Ghatei et al., 1993; Hannibal et al., 1995a; Nielsen et al., 1998; Skoglosa et al., 1999). Specifically, PACAP immunoreactivity is localized in the soma and dendrites of Purkinje cells, whose axons directly contact granule cells precursors proliferating in the EGL (Nielsen et al., 1998).

2.4.3. PACAP receptors

Two classes of PACAP receptors have been identified so far in mammals on the basis of their different affinity for PACAP and VIP. Type I PACAP receptors (PAC1-R) have high affinity for PACAP ($K_d \sim 0.5$ nM) and low affinity for VIP ($K_d \sim 500$ nM), whereas type II (VPAC1-R and VPAC2-R) have similar affinity for both PACAP and VIP ($K_d \sim 1$ nM)(Vaudry et al., 2000a).

PAC1-R are G protein-coupled receptor that can activate either phospholipase C (PLC) or adenylate cyclase (AC). Adenylate cyclase activation leads to cAMP production whereas PLC activation leads to inositol triphosphate (IP3) and diacylglycerol (DG) production and subsequent Ca^{2+} mobilization and protein kinase C (PKC) activation. The coupling of PAC1-R to distinct second messenger appear to depend upon different splice variants of the receptor. The presence of alternative cassettes in the third intracellular loop of the receptor (Hip and Hop variats) confers AC or PLC responsiveness (Journot et al., 1994; Spengler et al., 1993). On the other hand VPAC receptors have been found to activate only adenylate cyclase to produce cAMP (Vaudry et al., 2000a).

In the brain the expression of PAC1-R is generally higher than VPAC receptors. PAC1-R transcripts appear to be particularly abundant in the olfactory bulb, the dentate gyrus of the hyppocampus, the supraoptic nuclei of the hypothalamus, the cerebellar cortex, and the area postrema. High levels of PAC1-R mRNA have been also found in the cingulate, entorhinal and piriform cortex, pyramidal and nonpyramidal cells of the hippocampal formation, the amygdaloid nuclei, the centromedial, mediodorsal, and ventromedial nuclei of the thalamus, the hypothalamus, the central gray, the raphe nuclei, and the superior colliculus (Vaudry et al., 2000a). VPAC1-R are expressed mainly in the cerebral cortex, hippocampus and olfactory bulb while VPAC2-R are found in the thalamus, suprachiasmatic nucleus, central nucleus of the amygdala, pontine nucleus hippocampus and olfactory bulb (Vaudry et al., 2000a). During rodent brain development PAC1-R are expressed from E9.5 in the neural tube and at later stages in all proliferating neuroephitelia (Vaudry et al., 2000a). During postnatal rat cerebellum development PAC1-R are

found initially in the proliferating EGL and then in the ML and IGL (Basille et al., 1994). A similar distribution during brain development has been found for VPAC1-R while VPAC2-R transcripts appear to be more abundant in the thalamus and hypothalamus (Vaudry et al., 2000a).

2.4.4. Effects of PACAP in the CNS

Due to its broad distribution in the CNS, it is not surprising that PACAP has been shown to exert multiple function in the different areas. In the hypothalamus, where the highest expression of both PACAP and its receptors has been detected, the peptide appears to activate and increase firing of magnocellular neurons of the PVN and SON. PACAP also modulates the activity of other hypothalamic population enhancing the expression of gonadotropin-releasing hormone (GnRH), somatostatin, prolactin (PRL) and corticotropin-releasing factor (CRF) (Vaudry et al., 2000a). PACAP has also been involved in circadian clock regulation in the suprachiasmatic and supraoptic nuclei as well as rhythmic melatonin release in the pineal gland. This latter action appears to depend upon Ca^{2+} influx and CREB phosphorylation (Vaudry et al., 2000a). In the pituitary neural lobe PACAP appears to stimulate the release of vasopressin and oxytocin through cAMP/PKA signalling. PACAP has also been shown to stimulate the release from the anterior pituitary of growth hormone (GH), adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL) but not of thyroid-stimulating hormone (TSH). Besides it has also been shown that PACAP increases secretion of melanotropin α-melanocyte-stimulating hormone (α-MSH) from melanotrope cells of the intermediate lobe of the pituitary. The action of PACAP on secreting cells in the pituitary is mediated via cAMP production and/or Ca^{2+} mobilization (Vaudry et al., 2000a).

PACAP has also been found to exert nurotrophic effects on neurons. The peptide appears to protect cerebellar granule cells and cortical neurons from programmed cell death when cultured in apoptosis promoting conditions (Gonzalez et al., 1997; Morio et al., 1996). The protective effects of PACAP on cerebellar granule neurons appears to depend on cAMP production from adenylate cyclase and subsequent PKA activation (Gonzalez et al., 1997; Villalba et al., 1997). Besides, it has been suggested that the anti-apoptotic action of PACAP via cAMP/PKA transduction pathway, involves inhibition of delayed outward rectifier K^+ current and long-term depolarization of the resting membrane potential (Mei et al., 2004). The neuroprotective action of PACAP has also been demonstrated in vivo where is able to prevent ischemic death of hippocampal neurons (Uchida et al., 1996).

The presence of high levels of PACAP and its receptors in germinative areas during brain development suggests that the peptide might be involved in the regulation of CNS morphogenesis. PACAP has been found to reduce proliferation and promote differentiation of cortical neuron precursors (Lu and DiCicco-Bloom, 1997), to antagonize Shh mitogenic signalling on cerebellar granule precursors and stimulate neurite outgrowth of cerebellar granule neurons (Gonzalez et al., 1997; Nicot et al., 2002). On the contrary PACAP appears to stimulate proliferation of sympathetic neuroblasts (Lu and DiCicco-Bloom, 1997). Opposite effects on neuroblasts proliferation appear to depend upon differential coupling of PACAP receptors to PLC or adenylate cyclase second messenger cascade. PLC coupled PAC1-R Hop variant expression appears to stimulate proliferation whereas PAC1-R coupled to AC correlates with reduced proliferation of cortical precursors (Nicot and DiCicco-Bloom, 2001).

2.5. cAMP SIGNALING IN NEURONS

Cyclic adenosine monophosphate (cAMP) is a widely used intracellular second messenger synthesized from ATP by the action of adenylate cyclase (AC). Adenylate cyclase is an enzyme bound to the inner face of the plasma membrane that trasduce a variety of extracellular signals acting through G protein-coupled receptors. Many substances utilize cAMP as second messenger, by binding to receptors coupled to AC through G proteins: hormones (e.g. glucagon), neurotransmitters (e.g. adrenalin), peptides (e.g. PACAP) and prostaglandins. Main target of cAMP in all cells is protein kinase A (PKA). PKA is a tetramer composed of two regulatory and two catalytic subunits. Binding of cAMP to PKA regulatory subunit releases the catalytic subunit that can then phosphorylate target proteins in the cytoplasm and nucleus. In eukaryotes gene transcription in response to cAMP elevation is mediated by transcription factors of the leucine-zipper family. Genes under the control of cAMP contains in their promoter region a cAMP response element (CRE), a palindrome sequence of 8 bp (TGACGTCA) that is recognized by cAMP responsive element-binding protein (CREB) and related proteins. CREB is a 43 kD leucine-zipper transcription factor that is activated by phosphorylation on a serine residue (Ser133). CREB can be phosphorylated by PKA, thus linking cAMP signaling to gene transcription. Besides CREB can also be phosphorylated by other kinase including calcium/calmoduline-dipendent kinase (CaMK) and PKC.

In neurons and neuronal-like cell line (e.g. the neuroendocrine cell line PC12) cAMP signaling appears also to activate the extracellular-regulated kinase 1 and 2 (ERK1/2) cascade. Canonical ERK1/2 activation in response to growth factors (e.g. EGF or NGF) is achieved through activation of the small G protein Ras (retrovirus-associated DNA sequence) that in turn activates the kinase c-Raf that then phosphorylates the mitogen-activated kinasae 1 and 2 (MEK1/2) which finally phosphorylates ERK1/2.

In neurons and PC12 cells the MEK-ERK pathway is also activated by the action of cAMP. This pathway involves the PKA-dependent activation of the Ras-related small G protein Rap1 and the subsequent activation of B-Raf (a c-Raf isoform). Given that B-Raf is highly expressed in neuronal and neuroendocrine cells, this Rap1-dependent pathway may be unique to these types of cells (Grewal et al., 1999). This signalling pathway has been implicated in ERK1/2 activation by PACAP/cAMP signaling in cultured cerebellar granule cells (Obara et al., 2007). A key mediator of MEK-ERK cascade on gene transcription has been identified in the transcription factor Elk-1 (a member of the Ets family of transcription factors). Elk-1 is a substrate for ERK1/2 and mediates the expression of immediate early genes involved in MEK-ERK cascade response (Marais et al., 1993; Vossler et al., 1997). Besides in cerebellar granule cells cAMP/PKA-dependent ERK1/2 activation leads to the expression of the inducible transcription factor c-Fos (Figure 2.4) (Vaudry et al., 1998). c-Fos expression can be induced by the action of a variety of transcription factors acting on regulatory region of the gene, including CREB and Elk-1 (Seternes et al., 1998). c-Fos is part of a family of inducible transcription factors that also comprise FosB, Fra1 and Fra2. This proteins are characterized by a leucin-zipper motif that mediates the heterodimerization with members of the Jun family (c-Jun, JunB e JunD) of transcription factors to form the AP1 (activator protein 1) complex. The AP1 complex mediates the transcription of target genes by binding the palindromic sequence TGA(C/G)TCA. AP1 complex binding on this sequence can either activate or repress the expression of target gene depending on the subunit composition of the complex. It has been reported that AP1 complexes composed of Fos/Jun are mainly activator of transcription while Fos/JunB heterodimer are mainly repressor of transcription (Kovacs, 1998).

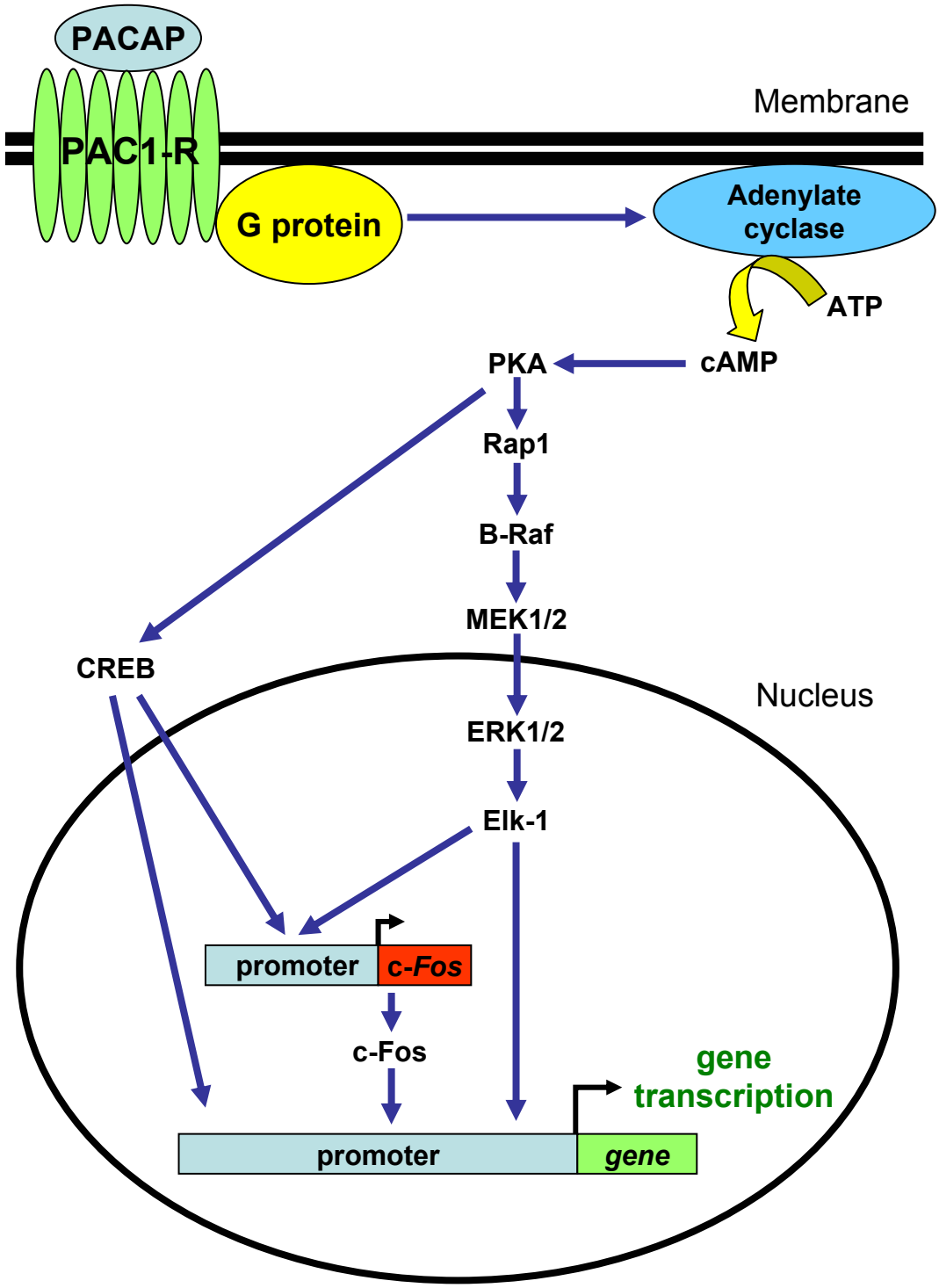

Figure 2.4. Schematic representation of pathway activated by cAMP signaling in neurons.

3. EXPERIMENTAL PROCEDURES

Plasmids. The following reporter plasmids were used for this study: pRL-TK (Promega, Madison, WI), pTK-βgal, pTK-Luc (Ciani et al., 2002) in which the tymidine kinase (TK) promoter drives the expression of *Renilla* luciferase, β-galactosidase and *Firefly* luciferase respectively; AP1-Luc, CRE-Luc, and NFκB-Luc (Ciani et al., 2002) and Lot1-Luc (Ciani et al., 2003) in which the expression of *Firefly* luciferase is driven by tandem repeats of consensus-binding sequence for AP1, CREB, NFκB and Lot1 transcription factors, respectively.
The following expression plasmids bearing the cDNA of the indicated proteins were also used: pSV40-cFos, pCMV-cJun, and pCMV-Fra1 (these plasmids were a kind gift of Dr. G. Perini, University of Bologna, Bologna, Italy); pCI-JunB (a kind gift of Dr. D. Chalbos, INSERM U540, Montpellier, France); pCMV-A-Fos and pCMV-A-CREB (a kind gift of Dr. C. Vinson, National Institutes of Health, Bethesda, MD).

Cloning of the Rat Lot1 Promoter and Construction of the Luciferase Reporter Vectors. For luciferase reporter vector construction, the rat Lot1 promoter region from 2099 bases upstream of the *Lot1* transcription start site (GenBank™ accession number U72620) to 2 bases downstream (from base 8682293 to base 8684394 of contig NW_043337) was cloned. by nested PCR. The target rat genomic region was amplified with primers Lot-F1 (5'-caaggaaagaaaaccacccc-3') and Lot-R1 (5'-aactcccgagcgttctcc-3') for the first reaction and primers Lot-F1nex (5'-cggatcctgggattacagatgctcataaa- 3') and Lot-R1nex (5'-cgggatccgcgagtgaggctggagaa-3') for the second (the BamHI sites used for cloning is underlined). The product was digested and cloned into the BAmHI site of BlueScript SK- plasmid (Stratagene, La Jolla, CA). The ~2.1-kb insert representing the putative *Lot1* promoter was sub-cloned upstream of the firefly luciferase gene into the pGL2-Basic vector (Promega)

with an EcoRV/SacI cut to generate the pGLot-2099 vector. Plasmids pGLot-1373, pGLot-515, and pGLot-327 were also generated by restriction endonuclease digestion of pGLot-2099. Mutation of the AP1 binding site at -268 bases from the transcription start site was introduced by PCR. The region upstream of the AP1 site (from -2099 to -269 bases) was amplified by PCR with primers Lot-F1nex and LotAPm (5'-cggaattcttcggctgtctacgcacagc-3'), whereas the region downstream of the AP1 site (from -262 to +2 bases) was amplified by PCR with primers Lot-R1nex and LotAPv (5'-cggaattccgcagcgggtgcgtgg- 3', the EcoRI sites used for cloning are underlined). The EcoRI site overlapping the AP1 site at -268 bp was used for cloning and to introduce the mutation. The two segments (digested with BamHI and EcoRI) were cloned into pBlueScript SK- to generate the full-length promoter bearing the mutation of the AP1 site at -268 bp (TGACTCA→GGAATTC). The mutated promoter was then subcloned into pGL2-Basic to generate vectors pGLot-2099APmut and pGLot-327APmut. Finally, the region downstream of the AP1 site was sub-cloned in pGL2-Basic to generate the pGLot-262 vector. All plasmids were sequenced with an ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and run on an ABI PRISM 3730 DNA analyzer (Applied Biosystems).

Cell Culture. All cells were kept in a 5% CO_2 humidified atmosphere at 37 °C. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Sigma, St. Louise, MO), 2 mM glutamine, 100 μg/ml penicillin, and 50 μg/ml streptomycin (Sigma). Primary cultures of CGC were prepared from the cerebella of 7-day old Wistar rat pups as described previously (Gallo et al., 1982). Cells were plated on poly-D-lysine (20 μM; Sigma) coated dishes at a density of $2x10^3$ cells/mm^2 and maintained in basal modified Eagle's (BME) medium (Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (Invitrogen), 25 mM KCl, 2 mM glutamine, and 0.05 mg/ml gentamycin (Sigma).

Treatments. Cells were stimulated with 10 μM forskolin (Sigma), 10 nM PACAP-38 (Sigma) or 1 mM dibutyryl cAMP (Sigma) for the indicated times. The protein synthesis inhibitor cycloheximide (Sigma) was used at 10 μg/ml. The PI3-kinase inhibitor wortmannin (Calbiochem, La Jolla, CA) was used at 100 nM. The MEK1/2 inhibitor U0126 (Calbiochem) was used at 10 μM. The ERK1/2 inhibitor KT5720 (Calbiochem) was used at 10 μM (Davies et al., 2000). The specific PKA inhibitor H89 was used at 10 μM (Calbiochem) (Vaudry et al., 1998b). Cells were preincubated with inhibitors for 1 h before stimulation.

Real-time Reverse Transcription-PCR. For quantification of Lot1 expression, total RNA was extracted from CGC with TriReagent (Sigma) according to the manufacturer's instructions. Approximately 5 μg of RNA were digested with 10 units of RNase-free DNase (Promega) for 30 min at 37 °C. After DNase inactivation, RNA was retrotranscribed with an oligo$(dT)_{12–18}$ primer (0.5 μM) and 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. For real-time PCR, the following primers pairs were used: *Lot1* (5'-ttagctcgtagttgcgtgtta-3' sense and 5'-cgggtccctgaaaagaacaca-3' antisense); glyceraldehyde-3-phosphate dehydrogenase (GADPH) (5'-gaacatcatccctgcatcca-3' sense and 5'-ccagtgagcttcccgttca-3' antisense); c-*Fos* (5'-tggacctgtctggttccttc-3' sense and 5'-atgcaccagctcagtcagtg-3' antisense). Real-time PCR was performed using a SYBR Premix Ex Taq kit (Takara, Shiga, Japan) according to the manufacturer's instructions in an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). Fluorescence was determined at each cycle. Real-time PCR was done under the following universal conditions: 10 min at 95 °C, 40 cycles of denaturation for 15 s at 95 °C, and annealing/extension for 30 s at 60 °C. Relative quantification of target gene expression towards the housekeeping

gene GAPDH was performed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) .

Northern Blotting. The expression of *Lot1* in CGC was determined by Northern blotting. Approximately 30 μg of total RNA was loaded per lane and fractionated on a formaldehyde-containing 1% agarose gel. Following transfer to Hybond-N membrane (Amersham Biosciences, Upsala, Sweden), *Lot1* mRNA was detected using an α-^{32}P-labeled full-length cDNA probe. Cyclophilin probes were used for quantification as described previously (Taubenfeld et al., 2001). Membranes were washed at a maximum stringency of 0.1X SSC and 0.1% SDS at 65 °C.

Transfection. All cells were transfected with polyethyleneimine (25 kDa; Sigma) as described previously (Boussif et al., 1995; Pennuto et al., 2002). For luciferase assays, HEK293 cells were plated 24 h before transfection in 24-well plates (10^5 cells/well). Cells were transfected with 1–2 μg of plasmid DNA as indicated for 3 h in DMEM. The pTK-βgal reporter plasmid was cotransfected together with the *Lot1*-luciferase reporter vectors to normalize for transfection efficiency. For luciferase assays, CGC were plated in poly-D-lysine-coated 24-well plates ($5x10^5$ cells/well) and transfected after 24 h with 1.05–1.55 μg of plasmid DNA as indicated for 2 h in complete medium. CGC were cotransfected with the pRL-TK reporter plasmid together with *Lot1*-luciferase reporter vectors at 1:20 ratio to normalize for transfection efficiency.

Luciferase Assay. Luciferase activity in CGC was measured 48 h after transfection using a Dual-Luciferase assay kit (Promega) according to the manufacturer's instructions on a TD-20/20 luminometer (Promega). *Firefly* luciferase activity was normalized for each sample by dividing by the *Renilla* luciferase activity in the same sample. Luciferase activity in HEK293 cells was measured as described previously

(de Wet et al., 1987) and normalized for β-galactosidase activity in the same sample (see below).

β-galactosidase Assay. β-galactosidase activity was measured as described previously (Ciani and Paulsen, 1995). In brief, 20 μl of HEK293 lysate was added to 100 μl of β-galactosidase assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, and 0.34% β-mercaptoethanol, pH 7.5) containing 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) and incubated for 1 h at 37 °C. The reaction was stopped with 50 μl of 1 M Na_2CO_3, and the absorbance at 420 nm was measured using a Benchmark multiplate reader (Bio-Rad).

Western Blotting. The following antibodies were used: anti-phosphorylated ERK1/2 and anti-phosphorylated MEK1/2 polyclonal antibodies (1:1000 dilution; Cell Signaling Technology, Beverly, MA); anti-phosphorylated cAMP-responsive element-binding protein (CREB) polyclonal antibody (1:2000 dilution; Upstate Biotechnology, Lake Placid, NY); anti-Lot1 polyclonal antibody (1:1000 dilution) (Ciani et al., 2003); anti-c-Fos and anti-c-Jun polyclonal antibodies (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-β-actin polyclonal antibody (1:2000 dilution; Sigma). For the preparation of total cell extracts, cells were lysed in lysis buffer (2% SDS, 10 mM dithiothreitol, 10 mM Tris-HCl, pH 8 and 1% protease and phosphatase inhibitors mixture (Sigma)). For the preparation of nuclear extracts, cells were lysed in low salt buffer (10 mM Hepes, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 8) for 10 min at 4 °C. After centrifugation, nuclei were extracted with hypertonic salt buffer (20 mM Hepes, 420mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride, pH 8). The protein concentration of samples was estimated by the Lowry method (Lowry et al., 1951). Equivalent amounts (50 μg) of protein were subjected to electrophoresis on an SDS-10%

polyacrylamide gel. The gel was then blotted onto a nitrocellulose membrane (Amersham Biosciences), and equal loading of protein in each lane was assessed by brief staining of the blot with 0.1% Ponceau S. Blotted membranes were blocked for 1 h in 5% milk in Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl, pH 8.0) and 0.1% Tween-20 and incubated overnight at 4 °C with primary antibodies. Membranes were washed and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit antibody (1:1000 dilution; Amersham Biosciences). Specific reactions were revealed with the ECL Western blotting detection reagent (Amersham Biosciences).

Immunofluorescence. For immunofluorescence studies, the following antibodies were used: monoclonal anti-NeuN antibody (Chemicon), anti-neural cell adhesion molecule (NCAM) and anti-glial fibrillary acidic protein (GFAP) antibodies (Sigma), anti-bromodeoxyuridine (BrdU) and FITC-conjugated anti-BrdU antibodies (Roche Applied Science, Mannheim, Germany), and anti-Lot1 polyclonal antibody (Ciani et al., 2003). Cells (plated on poly-D-lysine-coated coverslips) were fixed for 30 min in 4% paraformaldehyde in 120 mM sodium phosphate buffer, pH 7.4, and then rinsed three times with phosphate-buffered saline (PBS). For BrdU immunofluorescence, coverslips were treated with 2 N HCl for 30 min and extensively washed with PBS. Coverslips were then incubated overnight at 4 °C with appropriate dilutions of the primary antibody in 1.5% goat serum and 0.1% Triton X-100 in PBS, pH 7.4. Cells were then incubated with Cy3-conjugated goat anti-mouse secondary antibody or with FITC-conjugated goat anti-rabbit secondary antibody (1:200 dilution; Sigma) for 1–2 h at room temperature. After all incubations, specimens were extensively washed with PBS containing 0.1% Triton X-100. Coverslips were mounted on glass slides in PBS containing 70% glycerol and Hoechst 33342 (2 μg/ml). Confocal images were taken using a Leica TCS confocal microscope. Phase-contrast and fluorescence

images were taken using a Nikon Eclipse TE 2000-S microscope equipped with a Zeiss AxioCam MRm digital camera.

Determination of the Labeling Index. CGC (plated on poly-D-lysine coated coverslips) were cultured for 18 h, treated with 10 μM bromodeoxyuridine (BrdU; a thymidine analogue that is incorporated in the DNA of proliferating cells during the S phase of the cell cycle) for an additional 6 h, fixed, and processed for BrdU immunofluorescence as described above. The corresponding phase-contrast and fluorescence images (taken from random microscopic fields, 10–12 for each coverslip) were superimposed and used to determine the labeling index), defined as the percent of BrdU-positive cells out of the total number of cells in three independent experiments performed in duplicate. Glial cells, which represent ~3-5% of the total cell number in CGC cultures (Ciani and Paulsen, 1995), were recognized on the basis of phase-contrast morphology and were excluded from scoring.

Bioinformatics. Transcription factors binding site search on the rat *Lot1* promoter was performed with MatInspector software (Quandt et al., 1994). Alignment of mouse and rat *Lot1/Zac1* promoter sequences was performed with Clustal W software (Thompson et al., 1994).

Statistics. Data are expressed as the means ± S.E., and statistical significance was assessed by Student t-test or one-way analysis of variance (ANOVA), followed by Bonferroni's or Dunnett's *post hoc* test, where appropriate. Differences were considered to be significant starting from $p<0.05$. Statistical analysis was performed using Prism 4.0 (GraphPad Software, San Diego, CA).

4. RESULTS

4.1. PACAP AND FORSKOLIN UP-REGULATE *Lot1* EXPRESSION THROUGH cAMP

As shown in Fig. 4.1, PACAP-38 treatment induced a large increase in *Lot1* mRNA in differentiated CGC kept for 7 days *in vitro* (DIV). The effect of PACAP on *Lot1* induction was mimicked by the stable cAMP analogue dibutyryl-cAMP and by forskolin, a potent activator of adenylate cyclase. This clearly indicates the involvement of cAMP signaling cascade in the induction of *Lot1* expression in response to PACAP.

As it has been reported that *Lot1* overexpression correlates with apoptotic cell death in other systems (Spengler et al., 1997), we tested whether *Lot1* expression was affected by changing culture conditions to a well established paradigm of apoptosis induction in CGC: serum and potassium withdrawal (Contestabile, 2002). Shifting culture conditions to low potassium (5 mM) and serum-free medium did not alter *Lot1* expression (Fig. 4.1 A), indicating that it is not involved in induction of apoptosis in CGC.

To study the time course of cAMP-mediated *Lot1* up-regulation in CGC, we used forskolin to maximally increase intracellular cAMP and examined the expression of *Lot1* at the mRNA level. Northern blot analysis of CGC culture extracts demonstrated that induction of *Lot1* mRNA was detectable by 30 min after exposure to forskolin, reached its maximum between 2.5 and 6 h, and remained elevated for at least 12 h (Fig. 4.1 *B*). By Western blot analysis using a specific anti-Lot1 antibody (Ciani et al., 2003), we found that induction of Lot1 expression by either PACAP or forskolin at the protein level reflected that observed at the mRNA level (Fig. 4.1, *C* and *D*).

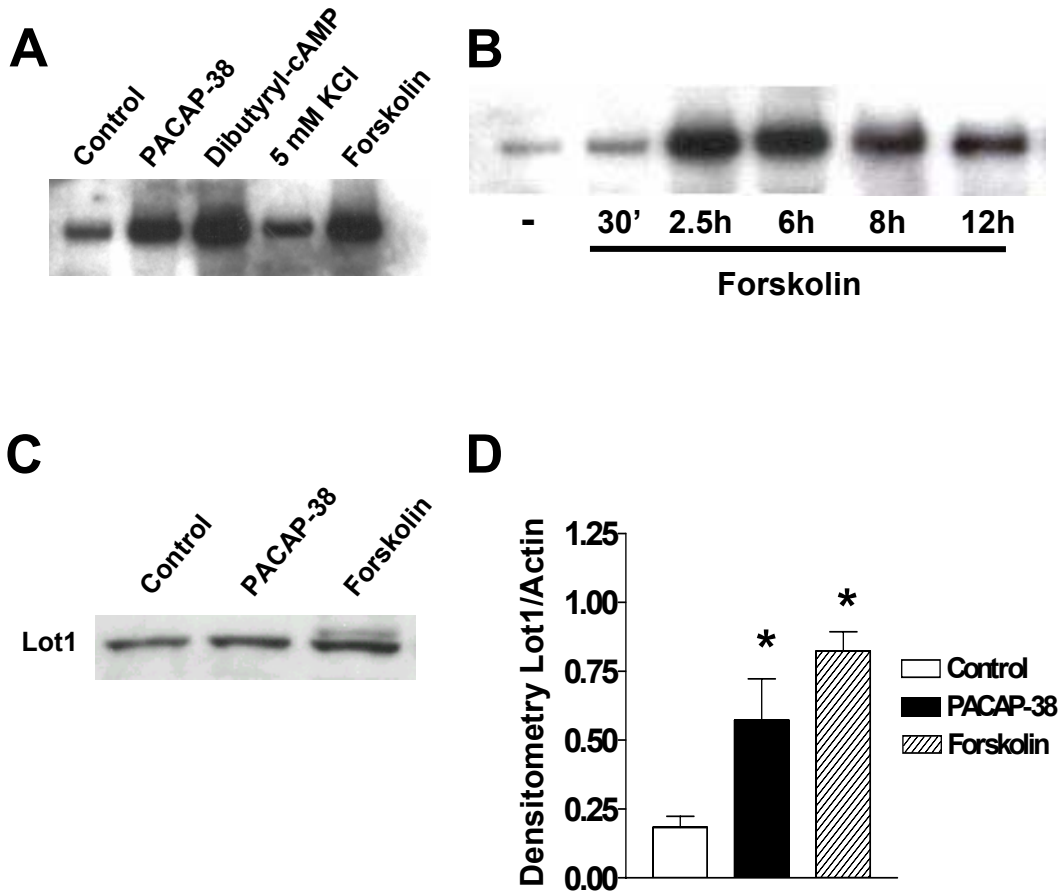

FIGURE 4.1. **Effect of PACAP-38 and forskolin on *Lot1* mRNA and protein expression in CGC.**
A) CGC at 7 DIV were stimulated with PACAP-38 (10 nM), dibutyryl-cAMP (1mM), or forskolin (10 μM) for 12 h or shifted to 5mM KCl serum-free medium for 6 h, and total RNA (20 μg) was analyzed by Northern blotting using a *Lot1* cDNA probe. A representative Northern blot is shown. *B*) CGC at 7 DIV were stimulated with forskolin (10 μM) for 0.5, 2.5, 6, 8, or 12 h, and total RNA (20 μg) was analyzed by Northern blotting using a *Lot1* cDNA probe. A representative Northern blot is shown. *C*) expression of Lot1 protein was assayed by Western blotting of nuclear extracts from CGC at 7 DIV that had been treated with PACAP-38 (10 nM) or forskolin (10 μM) for 24 h. *D*) Lot1 protein expression was quantified and normalized for β-actin. *Error bars* are the means ± S.E. of four experiments. *, $p<0.01$ compared with the control (Bonferroni's test after ANOVA).

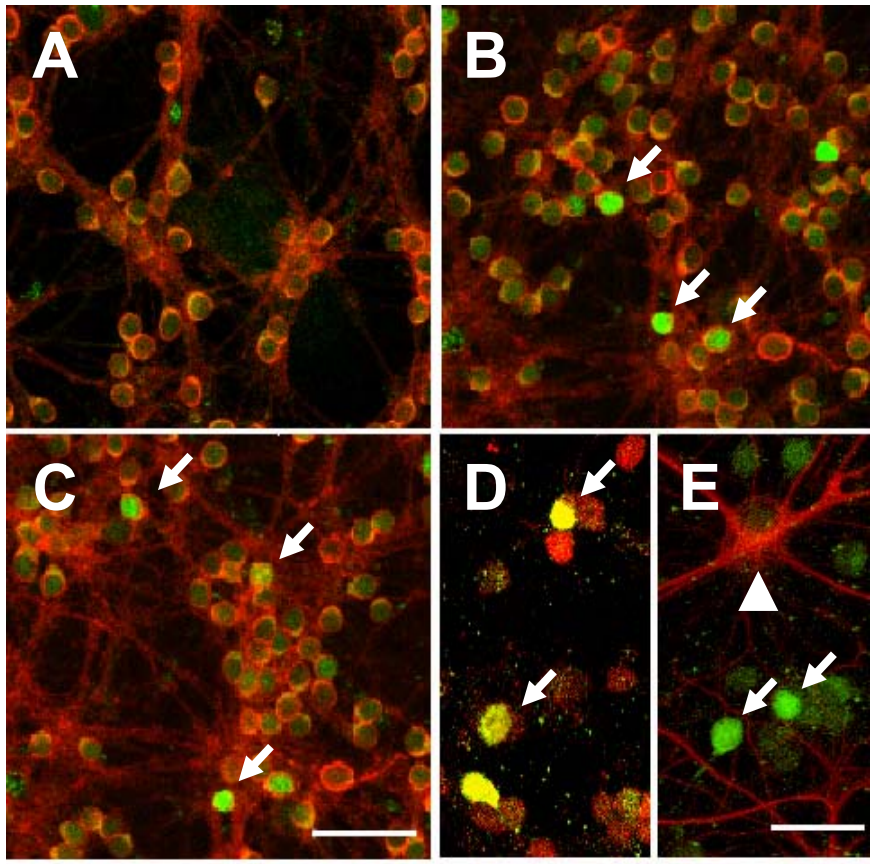

FIGURE 4.2. **Cytological evaluation of Lot1 immunoreactivity in CGC.**
CGC cultures at 7 DIV were treated for 24 h, after which time cells were processed for immunocytochemistry. *A–C*) confocal images of doubly immunostained CGC for Lot1 (*green*) and NCAM (*red*). *A*) control; *B*) forskolin (10 μM); *C*) PACAP-38 (10 nM). Arrows indicate CGC nuclei strongly stained for Lot1. *Bar* 40 μm. *D*) confocal image of CGC treated with forskolin and doubly immunostained for Lot1 (*green*) and NeuN (*red*), arrows indicate double labeled cells (yellow). *E*) confocal image of CGC culture stimulated with forskolin and doubly immunostained for Lot1 (*green*) and GFAP (*red*). Arrows indicate Lot1-positive GFAP-negative cells; arrowhead points to a GFAP positive glial cell not expressing Lot1. *Bar* 20 μm.

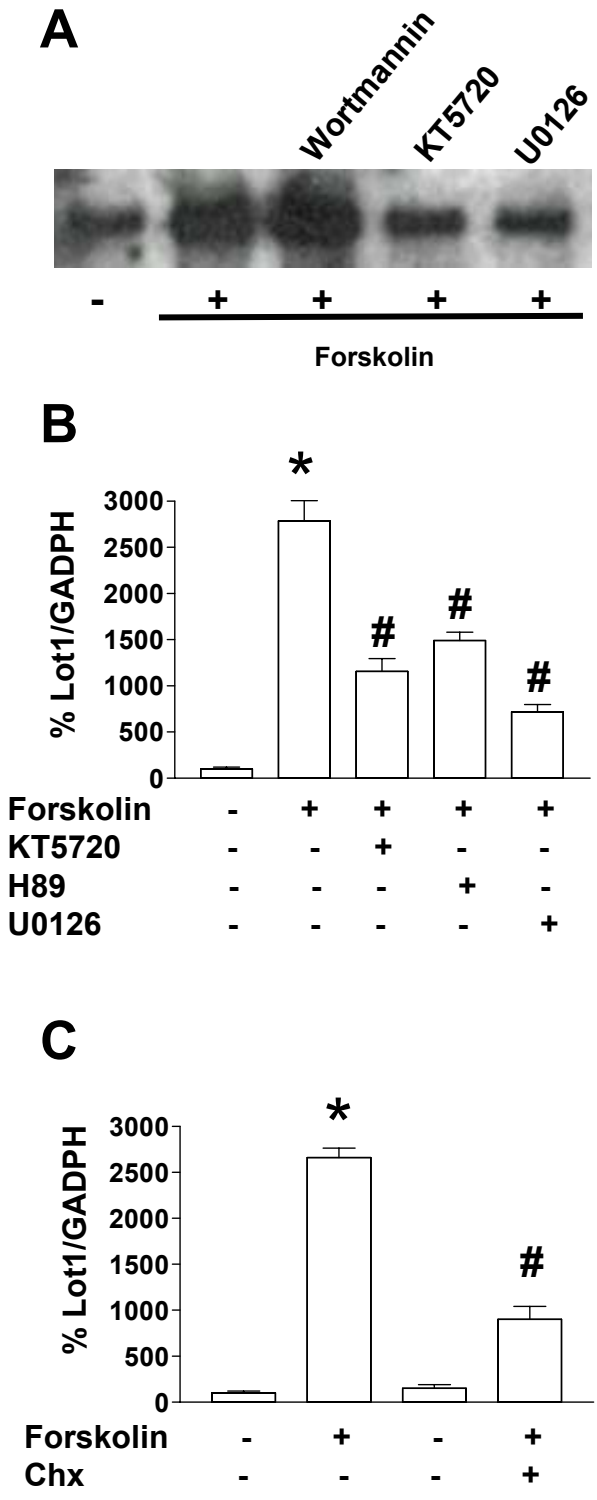

FIGURE 4.3. **Pharmacological dissection of pathways involved in *Lot1* expression.**
A) CGC were treated with forskolin alone or in combination with wortmannin (100 nM), KT5720 (100 nM), or U0126 (10 μM) for 12 h, and total RNA (20 μg) was analyzed by Northern blotting using a *Lot1* cDNA probe. A representative Northern blot is shown. *B*) *Lot1* mRNA expression was quantitatively measured by real-time reverse transcription-PCR of CGC that had been treated for 6 h with forskolin (10 μM) alone or in combination with KT5720 (100 nM), H89 (10 μM), or U0126 (10 μM). Data are expressed as the means ± S.E. of three independent experiments. *, $p<0.001$ compared with the control; #, $p<0.001$ compared with forskolin-stimulated samples (Bonferroni's test after ANOVA). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *C*) the dependence of forskolin-induced *Lot1* mRNA expression on protein synthesis was evaluated by realtime reverse transcription-PCR from CGC that had been treated for 6 h with forskolin (10 μM) alone or in combination with the protein synthesis inhibitor cycloheximide (Chx; 10 μg/ml). Data are expressed as the means ± S.E. of three independent experiments. *, $p<0.001$ compared with the control; #, $p<0.001$ compared with forskolin-stimulated samples (Bonferroni's test after ANOVA).

As it was previously demonstrated that Lot1 is a nuclear protein (Spengler et al., 1997), we used immunocytochemistry to verify its subcellular localization in CGC. Cultures at 7 DIV were treated with forskolin or PACAP-38 for 24 h; and thereafter, cells were fixed and processed for immunocytochemistry for Lot1 and the neuronal marker NCAM (neuronal cell adhesion molecule) as described under "Experimental Procedures". The results showed that Lot1 was barely visible in the nuclei of untreated cells (Fig. 4.2 *A*), whereas forskolin (Fig. 4.2 *B*) and PACAP (Fig. 4.2 *C*) induced a generalized increase in Lot1 expression in the nuclei of granule cells. Notably the nuclei of some CGC exhibited a particularly strong up-regulation of nuclear protein expression (Fig. 4.2, *B* and *C*, *arrows*).

CGC cultures are highly enriched in neurons but contain a 3-5% of glial cells (Ciani and Paulsen, 1995); in order to clearly identify cell type expressing *Lot1* we performed a double immunostaining for Lot1 and a cell-specific marker for neuronal or glial cells (NeuN or GFAP respectively).

Confocal microscopic observation of doubly immunostained cultures showed that Lot1 immunoreactivity was localized in nuclei of granule cells that were also positive for the antibody against the neuronal marker NeuN (Fig. 4.2 *D, arrows*), but not in nuclei of astrocytes labeled by the glial marker GFAP (Fig. 4.2 *E*, *arrowhead*).

To explore the signaling cascades involved in *Lot1* gene expression, we examined the effect of inhibitors of various protein kinases known to be activated in response to the forskolin-induced cAMP increase. We found that induction of *Lot1* mRNA caused by exposure to forskolin was strongly counteracted by co-treatment with the PKA inhibitor H89, the MEK1/2 inhibitor U0126, or ERK1/2 inhibitor KT5720 (Fig. 4.3, *A* and *B*), but not by exposure to the PI3-kinase inhibitor wortmannin (Fig. 4.3 *A*), which is know to block, at the concentration used, Akt phosphorylation in response to insulin activation in CGC cultures (van Weeren et al., 1998). These finding indicates that cAMP-dependent *Lot1* expression depends upon activation of PKA and MEK-ERK signaling cascade.

In the presence of the protein synthesis inhibitor cycloheximide (Chx), forskolin-dependent *Lot1* induction was largely counteracted, implying the requirement of *de novo* protein synthesis for *Lot1* mRNA induction in response to cAMP elevation (Fig. 4.3. *C*).

4.2. PACAP AND FORSKOLIN ACT AS ANTI-MITOGENIC STIMULI IN CGC

To define the effects of cAMP elevation on CGC proliferation, we analyzed the incorporation of BrdU (a thymidine analogue which is incorporated in the DNA of proliferating cells during the S phase of the cell cycle) in cells exposed to PACAP or forskolin for 24 h after cell plating.

We first performed a double labeling immunocytochemistry experiments for BrdU and the neuronal marker NCAM (Fig. 4.4 *A*) or BrdU and the glial marker GFAP (Fig. 4.4 *B*). This experiment demonstrated that most BrdU-positive cells were also labeled by NCAM, indicating that the dividing cells were, at this stage, mainly neuronal precursors.

We next investigated the proliferative activity of cells expressing Lot1 in culture, by double immunocytochemistry experiments with anti-BrdU and anti-Lot1 antibodies. Microscopic observation of doubly immunostained cultures showed that Lot1 immunoreactivity did not colocalize with proliferating BrdU-positive cells after forskolin treatment while cells showing strong Lot1 expression were found to be BrdU-negative (Fig. 4.4, *C–E*) indicating that the expression of this putative antiproliferative transcription factor negatively correlates with CGC mitotic activity.

We verified the effect of intracellular cAMP increase on CGC proliferation by treating freshly plated cells with either PACAP or forskolin. Cell proloferation was evaluated by labeling cycling cells with BrdU. Calculating the labelling index (BrdU-

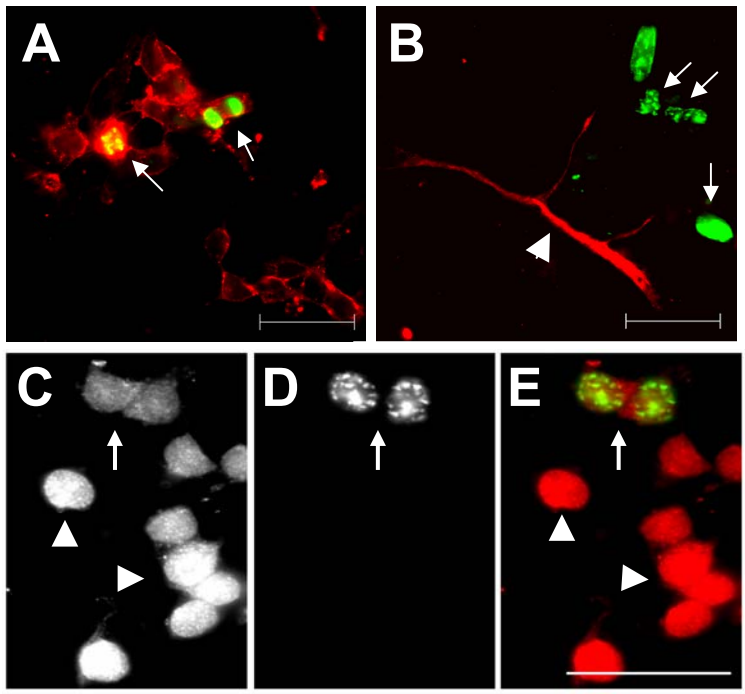

FIGURE 4.4. **Relationship between *Lot1* expression and CGC proliferation.**
A) double immunofluorescence for NCAM (*red*) and BrdU (*green*). Arrow points to a BrdU/NCAM doubly labeled neuronal cells. *B*) double immunofluorescence for GFAP (*red*) and BrdU (*green*). Arrows indicate BrdU-positive GFAP-negative cells; arrowhead points to a GFAP-positive BrdU-negative glial cell. *Bars* 20 μm. *C–E*) double immunofluorescence for Lot1 (*red*) and BrdU (*green*) of CGC at 1 DIV treated with forskolin (10 μM). *C*) Lot1 immunoreactivity; *D*) BrdU immunoreactivity; *E*) merged color image. Arrows indicate BrdU positive cells showing faint Lot1 expression; arrowheads point to BrdU-negative cells strongly labeled for Lot1. *Bar* 25 μm.

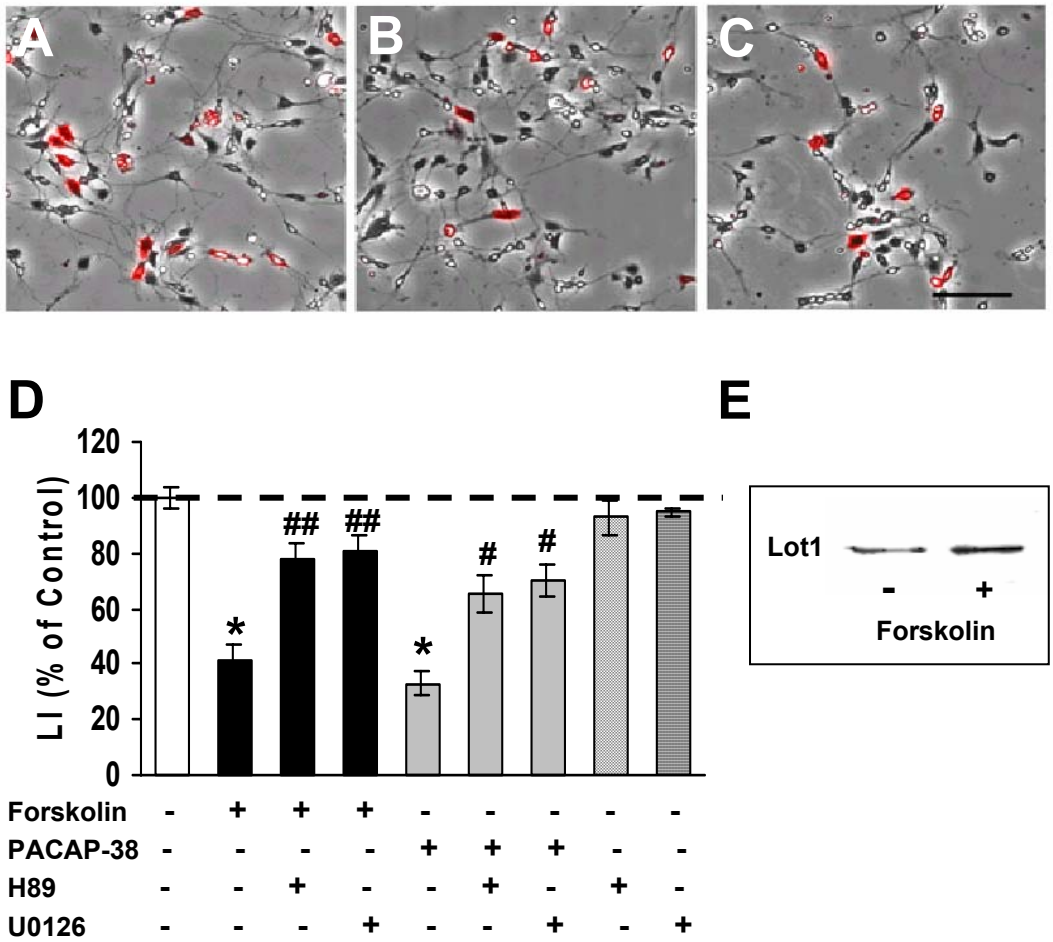

Figure 4.5. **Effect of PACAP-38 and forskolin on proliferation of CGC in culture.** *A-C*) freshly plated CGC cultures were stimulated (as indicated below) for 20 h. BrdU (10 μM) was added for the last 6 h, after which time cells were processed for immunofluorescence with anti-BrdU antibody (*red*). Final pictures were obtained by merging phase-contrast images with fluorescence images. *A*) control; *B*) forskolin (10 μM); *C*) PACAP-38 (10 nM). *Bar* 50 μm. *D*) labeling index (*LI*) determination for CGC in culture. Four hours after plating, CGC were treated for 20 h with forskolin (10 μM), PACAP-38 (10 nM), H89 (10 μM), or U0126 (10 μM) as indicated, and exposed to BrdU (10 μM) for the last 6 h. Data are expressed as the means ± S.E. of three independent experiments in duplicate. *, $p<0.01$ compared with the control; #, $p<0.05$ compared with PACAP-treated samples; ##, $p<0.01$ compared with forskolin treated samples (Bonferroni's test after ANOVA). *E*) Western blot analysis of Lot1 expression on nuclear extracts obtained from CGC at 1 DIV treated with forskolin (10 μM) for 24 h prior to analysis.

labeled neurons/total neurons) based on direct counting of neurons identified by their morphology observed by phase-contrast microscopy, we found that exposure to forskolin or PACAP reduced the CGC proliferation rate, which was decreased by ~60 or 70%, respectively, compared with untreated cells (Fig. 4.5, *A-D*). Moreover, we verified whether the PKA or MEK1/2 inhibitors, that blocked forskolin- or PACAP-induced *Lot1* up-regulation, also counteracted the proliferation rate reduction of CGC precursors. We found that the reduced CGC proliferation rate caused by exposure to forskolin or PACAP was significantly counteracted by co-treatment with H89 or U0126 (Fig. 4.5 *D*), whereas these two inhibitors had, by themselves, no effect on cell proliferation. Taken together, these results clearly correlate the anti-mitotic effect of cAMP signaling on CGC proliferation with *Lot1* expression.

4.3. FUNCTIONAL ANALYSIS OF THE *Lot1* PROMOTER

We cloned the 5'-flanking region of the Lot1 gene into the pGL2-Basic vector upstream of the *Firefly* luciferase gene to determine whether this region possesses transcriptional activity. The cloned rat *Lot1* promoter region appears to be highly similar to the mouse *Lot1* promoter (GenBank™ accession number AF314094) as observed by aligning the two sequences (Fig 4.6). To identify, within the *Lot1* promoter, the binding region responsible for the cAMP-induced *Lot1* activation, serial deletions of the promoter were made. Complete or deleted constructs were transfected in CGC as described under "Experimental Procedures", and cells were stimulated with forskolin for 24 h before luciferase measurements. The structures of the different constructs are schematically shown in Fig. 4.7 together with the results of experiments performed using the different plasmids. Insertion of the ~2.1-kb rat *Lot1* genomic fragment (nucleotides -2099 to +2 with respect to the transcription start site of the *Lot1* sequence in GenBank™ accession number U72620) into pGL2-Basic

vector (designated pGLot-2099) resulted in a high increase (up to ~30-fold) in luciferase activity compared to the empty pGL2-Basic vector in basal culture conditions. Strong increase in pGLot-2099 activity (up to~160-fold) was obtained after forskolin stimulation, confirming cAMP responsiveness of the promoter (Fig. 4.7). Results from transfection studies based on different truncated constructs of *Lot1* promoter, indicated that the cAMP-dependent activation domain in the *Lot1* promoter is localized in proximal region. Indeed, the transcriptional activity stimulated by forskolin was abolished following the 5'-truncation of the promoter at nucleotide -262, as shown for the pGLot-262 plasmid, whereas other truncated constructs, in which the promoter was progressively deleted up to nucleotide -327, only marginally affected forskolin-induced transcriptional activity (Fig. 4.7). This indicates the requirement of the promoter region between -327 and -262 bp from the transcription start site, for cAMP-dependent *Lot1* transcription. Software analysis with MatInspector (Quandt et al., 1994) was used to identify putative binding sites for transcription factors on *Lot1* promoter. Within the region between -327 and -262 bp, that confers cAMP responsiveness to the promoter, an AP1 site (located at -268 bp and conserved in the mouse *Lot1/Zac1* promoter; Fig. 4.6) was found. Accordingly, a reporter vector bearing the minimal promoter responsive to cAMP with a mutated form of this site (pGLot-327AP1mut) did not exhibit any significant response to forskolin stimulation (Fig. 4.7). A similar mutation in the complete construct (pGLot-2099AP1mut) also dramatically decreased the effect of forskolin stimulation (compare with pGLot-2099 in figure 4.7). Besides, software analysis also identified additional potential binding site with partial homology to canonical binding sequences: two AP1-like binding site (at -1887 and -891) and a cAMP response element (CRE)-like binding site (at -1411). However this binding sites did not appear to be essential for cAMP-dependent *Lot1* expression as their deletion in the truncated construct did not significantly affect *Lot1* promoter activity. Despite this fact we can not exclude a minor role for this potential binding site in mediating *Lot1* transcriptional activation. Indeed a minor stimulating effect of forskolin over basal

FIGURE 4.6. **Rat and mouse *Lot1* promoters are highly conserved.** Sequence alignment of *Lot1/Zac1* proximal promoter region for mouse (mLot1; AF314094) and rat (rLot1; NW_043337) according to Clustal method. The figure shows the alignment of the proximal portions of the promoters (~420 bp upstream of the transcription start site). Results indicate an high degree of similarity between the two sequence (89.5% identity in the region shown). Asterisks indicate conserved nucleotide, the arrow indicates the transcription start site, while in red is outlined the AP1 binding site, located at -268 bp from the transcription start site (rLot1 numeration), which is conserved in rat and mouse sequences.

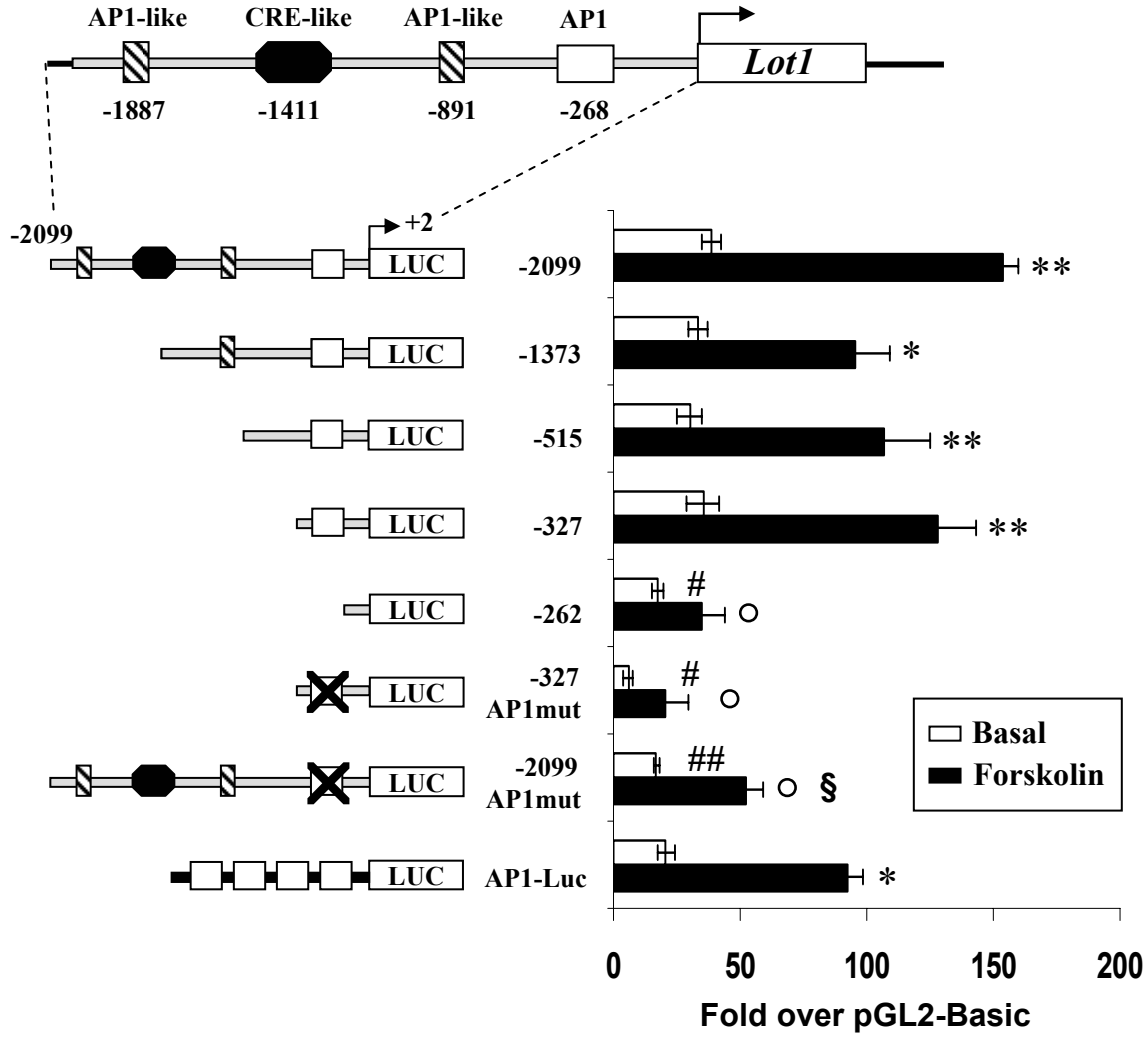

FIGURE 4.7. **The *Lot1* promoter is activated by forskolin through an AP1-responsive element.** *Upper*, schematic representation of the cloned *Lot1* promoter region (not drawn to scale). The AP1 response elements and CRE found by software analysis are indicated. *Lower left*, schematic representation of the reporter plasmids used in these experiments. The plasmids contained a progressively shorter fragment of the *Lot1* promoter or *Lot1* promoter site mutants driving the expression of the luciferase gene. *Lower right*, luciferase reporter analysis of the *Lot1* promoter. One day after plating, CGC were transfected with 1 μg of the indicated *Lot1*-luciferase (*Luc*) reporter plasmids and 0.05 μg of *Renilla* luciferase reporter plasmid (pRL-TK) to normalize for transfection efficiency. Twenty-four hours after transfection, cells were incubated with (*black bars*) or without (*white bars*) 10 μM forskolin for an additional 24 h. The results are given as fold increase over pGL2-Basic activity and are the means ± S.E. of four to five experiments done in triplicate. Data were analyzed with Bonferroni's test after ANOVA. **, $p<0.001$, and *, $p<0.01$, difference in the forskolin-stimulated *versus* basal conditions for the same vector; ##, $p<0.01$, and #, $p<0.05$, difference in the basal *versus* pGLot-2099 basal conditions; °, $p<0.05$, difference in the forskolin-stimulated *versus* pGLot-2099 stimulated conditions. A *post hoc t*-test confirmed the results from Bonferroni's test and additionally revealed a difference between pGLot-2099AP1mut-stimulated *versus* basal conditions. §, $p<0.05$ (*t*-test).

conditions was noticed with the pGLot-2099AP1mut construct, possibly due to the presence in the long upstream genomic fragment of forskolin-responsive site(s) with lesser effect on transcription. Nevertheless our results clearly demonstrate that full activation of *Lot1* expression by forskolin requires the intact AP1 site at -268 bp. Accordingly, a construct in which luciferase expression was driven by tandem repeats of AP1 consensus motifs (AP1-Luc) (Fig. 4.7), used as control for the AP1-dependent transcriptional activity induced by forskolin, exhibited strong activation when transfected in CGC. Moreover, these experiments revealed that the promoter activity of the constructs deleted or mutated in the AP1 site (pGLot-262, pGLot-2099APmut, and pGLot-327APmut) caused a decrease in basal transcription, suggesting that this site is also important for unstimulated basal transcription (Fig. 4.7).

4.4. INDUCTION OF *Lot1* TRANSCRIPTIONAL ACTIVITY AND *c-Fos* EXPRESSION BY FORSKOLIN IN CGC.

To study in further detail the mechanism of cAMP mediated induction of Lot1 transcriptional activity, reporter gene assays were performed using plasmids containing a tandem repeat of Lot1, CREB, AP1, or NF-κB consensus response elements placed upstream of a *Firefly* luciferase cDNA (Lot1-Luc, CRE-Luc, AP1-Luc, and NFκB-Luc) or a control plasmid in which the expression of the *Firefly* luciferase is driven by the thymidine kinase promoter (TK-Luc). Twelve hours after transfection with the reporter plasmids, cells were cultured for an additional 24 h in the presence or absence of forskolin. Although no changes in luciferase activity were observed upon forskolin treatment in NFκB-Luc- or TK-Luc-transfected cells, this treatment remarkably induced the transcriptional activity of Lot1-Luc (~5-fold), CRE-Luc (~25- fold), and AP1-Luc (~3.5-fold) (Fig. 4.8), thus showing that, besides CREB and AP1 transcriptional activity, also Lot1 transcriptional activity was induced

by forskolin in CGC. As shown in Fig. 4.3 *B*, inhibition of the MEK-ERK cascade by the MEK inhibitor U0126 or of the PKA cascade by the PKA inhibitor H89 abrogated Lot1 induction by forskolin, implying that forskolin induces *Lot1* expression through activation of these pathways. In agreement with this conclusion, exposure of CGC to forskolin caused a rapid (30 min) and long lasting (3 h) phosphorylation of MEK1/2 and ERK1/2 (Fig. 4.9 *A*). The MEK-ERK signaling pathway regulates the activity of AP1 sites by increasing both the synthesis and activation of the ligands of this binding site, the immediate-early genes c-*Fos* and c-*Jun* (Raingeaud et al., 1996). The protooncogene c-*Fos* is known to play a key role in the control of cell proliferation and differentiation and programmed cell death (Didier et al., 1992; Holt et al., 1986; Muller and Wagner, 1984; Ruther et al., 1987) and to be induced by PACAP in CGC (Vaudry et al., 1998b). We therefore examined the kinetics of expression of c-Fos as well as c-Jun in CGC after forskolin treatment. As shown in Fig. 4.9 *A*, forskolin induced c-Fos protein expression, which peaked 3 h after forskolin stimulation, while c-Jun appeared to be constitutively expressed. Inhibition of PKA by H89 prevented forskolin-induced MEK1/2 phosphorylation, with a consequent decrease in ERK1/2 and CREB phosphorylation (Fig. 4.9 *A*), and c-Fos induction (Fig. 4.9, *A*-*C*). These results demonstrate that PKA is required for forskolin-induced transcription of c-Fos in CGC. Forskolin-induced *c-Fos* expression at both the protein and mRNA levels was also significantly decreased by directly interfering with the MEK-ERK pathway through the specific MEK1/2 inhibitor U0126 (Fig. 4.9, *A*–*C*) indicating the requirement of this pathway activation for full achievement of *c-Fos* expression.

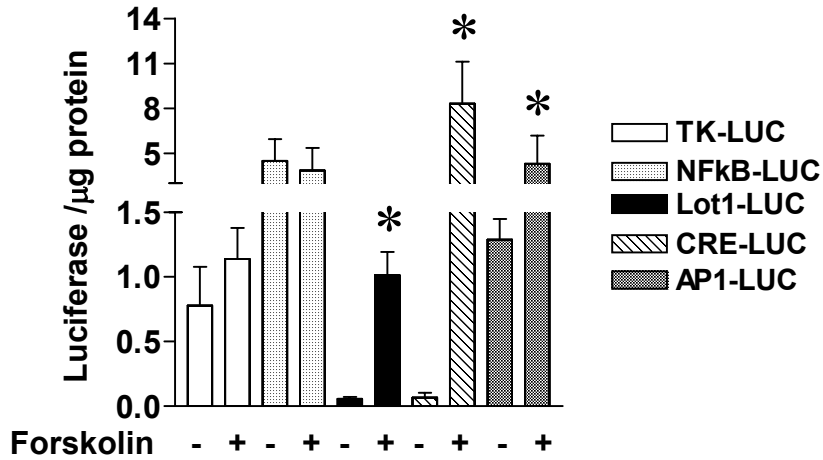

FIGURE 4.8. **Analysis of pathways activated by cAMP in CGC.** CGC were transfected with 1 μg of reporter plasmids containing tandem repeats of Lot1, CREB, AP1, or NF-κB consensus response elements as indicated. CGC were cultured for 24 h and treated with forskolin (10 μM) for an additional 24 h, and then luciferase activity was measured. *Error bars* are the means ± S.E. of three experiments. *, $p<0.01$ *versus* unstimulated conditions (Bonferroni's test after ANOVA).

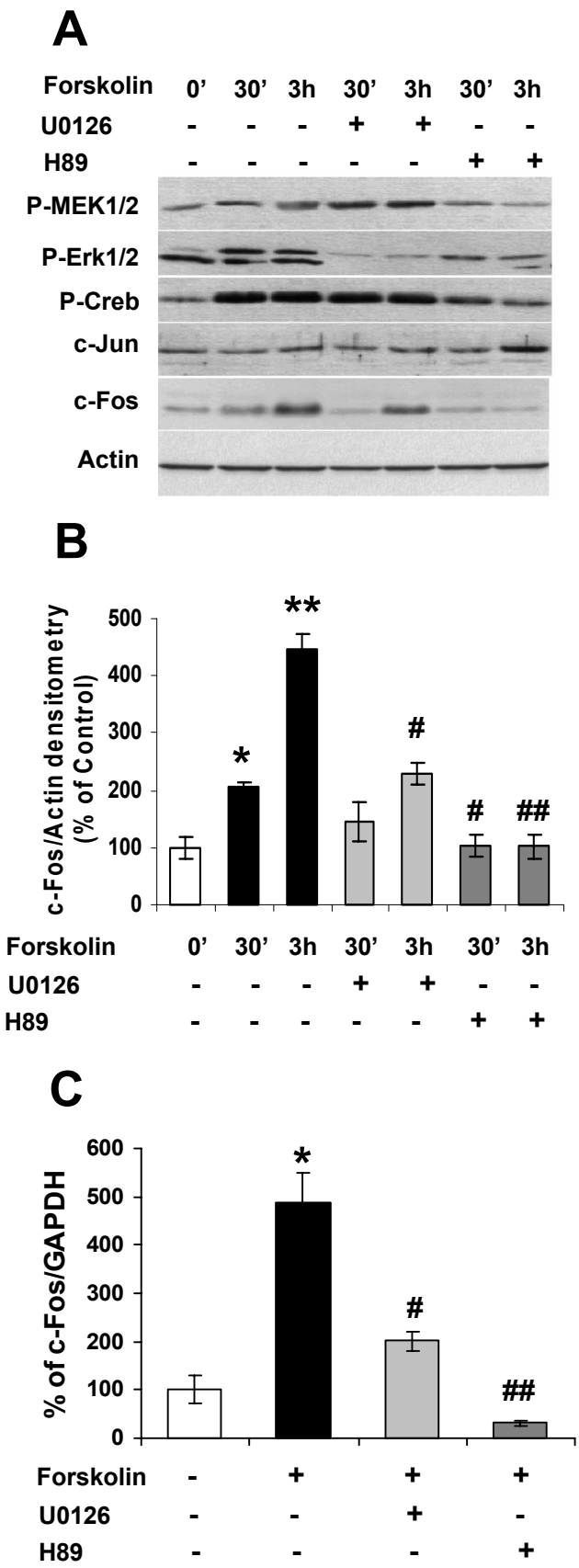

FIGURE 4. 9. **Analysis of pathways involved in cAMP-dependent *Lot1* expression**
A) shown are the results of Western blot analysis of MEK, ERK, and CREB phosphorylation (*P*) and c-Fos and c-Jun expression in CGC. Cells were treated for the indicated times with forskolin alone or with the MEK inhibitor U0126 (10 μM) or the PKA inhibitor H89 (10 μM). *B*) c-Fos protein expression was quantified and normalized for β-actin. *Error bars* are the means ± S.E. of three experiments. *, $p<0.05$ compared with the control; **, $p<0.01$ compared with the control; #, $p<0.05$ compared with the corresponding forskolin-treated samples; ##, $p<0.01$ compared with the corresponding forskolin-treated samples (Bonferroni's test after ANOVA). *C*) c-*Fos* mRNA expression was quantitatively measured by real-time reverse transcription-PCR in CGC treated with forskolin (10 μM), U0126 (10 μM), or H89 (10 μM). Data are expressed as the means ± S.E. of two independent experiments. *, $p<0.001$ compared with the control; #, $p<0.05$ compared with forskolin-stimulated conditions; ##, $p<0.001$ compared with forskolin-stimulated conditions (Bonferroni's test after ANOVA). *GAPDH*, glyceraldehyde-3- phosphate dehydrogenase.

4.5. *Lot1* EXPRESSION DOES NOT DEPEND ON cAMP-INDUCED AP1 TRANSCRIPTION IN HEK293 CELLS

We examined the promoter activity of Lot1 in a non-neuronal cell model, HEK293 cell line. Transfection of these cells with the *Lot1* reporter vector pGLot-2099 resulted in remarkable levels of luciferase activity. However, the transcriptional activity was slightly decreased by forskolin stimulation in these cells. Besides, deletion of the 5' distal most region of the promoter at nucleotide -1373 from the transcription start site resulted in a strong reduction of *Lot1* promoter activity. This indicates that the AP1 site located at -268 bp is not essential for *Lot1* transcription in this cells that in turns appear to depend upon the presence of the region upstream of nucleotide -1373 (Fig. 4.10 *A*). Accordingly Western blot analysis of Lot1 protein expression on HEK293 nuclear extracts showed a very low level of expression that was not increased by forskolin treatment (Fig. 4.10 *insert*). Furthermore, c-Fos expression in HEK293 cells was not stimulated but was actually depressed by forskolin, whereas the MEK-ERK cascade was only slowly and moderately activated (Fig. 4.10 *B*). To study in further detail Lot1 transcriptional activity in HEK293 cells, reporter gene assays were performed using the AP1-Luc or CRE-Luc reporter plasmid. Although forskolin treatment remarkably induced the transcriptional activity of CRE-Luc, no change in luciferase activity was observed in AP1-Luc transfected cells, thus confirming that AP1 transcriptional activity was not induced by forskolin in HEK293 cells (Fig. 4.10 *C*). Taken together, these results converge in demonstrating the lack of effect of cAMP on *Lot1* expression in HEK293 cells.

4.6. c-Fos/c-Jun FAMILY PROTEINS CONTROL *Lot1* PROMOTER ACTIVITY

To determine the mechanisms leading to AP1 site activation on the Lot1 promoter in CGC, we examined the transcriptional effects of c-Fos and c-Jun family members in transient transfection assays. We transfected CGC with wild type expression plasmid for c-Fos, Fra1, c-Jun, JunB proteins or a dominant-negative mutant of c-Fos (named A-Fos) (Olive et al., 1997) together with the reporter vector bearing the minimal *Lot1* promoter region responsive to cAMP elevation (pGLot-327), and cells were then stimulated or not with forskolin (Fig. 4.11 *A*). We found that only concomitant c-Fos/c-Jun overexpression significantly increased pGLot-327 promoter activity under basal and forskolin-stimulated conditions (Fig. 4.11 *A*). All other conditions tested (overexpression of c-Fos, Fra1, c-Jun, JunB, c-Fos/JunB, or c-Jun/JunB) failed to induce any significant increase in pGLot-327 activity. On the other hand, overexpression of dominant-negative A-Fos significantly decreased pGLot-327 activity upon forskolin treatment. We were next interested in determining whether enforced overexpression of c-Fos and c-Jun would activate the *Lot1* promoter at the level of the AP1 site. To perform these experiments we cotransfected *Lot1*-luciferase reporter plasmids (pGLot-2099, pGLot-2099AP1mut, pGLot-327, pGLot-327AP1mut, or pGLot-262) together with the c-Fos and c-Jun expression plasmids in CGC. Fig. 4.11 *B* shows that overexpression of c-Fos and c-Jun approximately doubled *Lot1* transcription only when the *Lot1*-luciferase reporter plasmids contained the intact AP1 site (pGLot-2099 and pGLot-327). Furthermore, c-Fos/c-Jun overexpression no longer stimulated reporter gene activity when the AP1 site was selectively mutated (Fig. 4.11 *B*). These results indicate that *Lot1* up-regulation elicited by cAMP elevation relies upon AP1-mediated transcriptional activity and that the composition of the heterodimer that binds at the AP1 site on the *Lot1* promoter is likely c-Fos/c-Jun. We also tested whether c-Fos/c-Jun overexpression activates *Lot1*

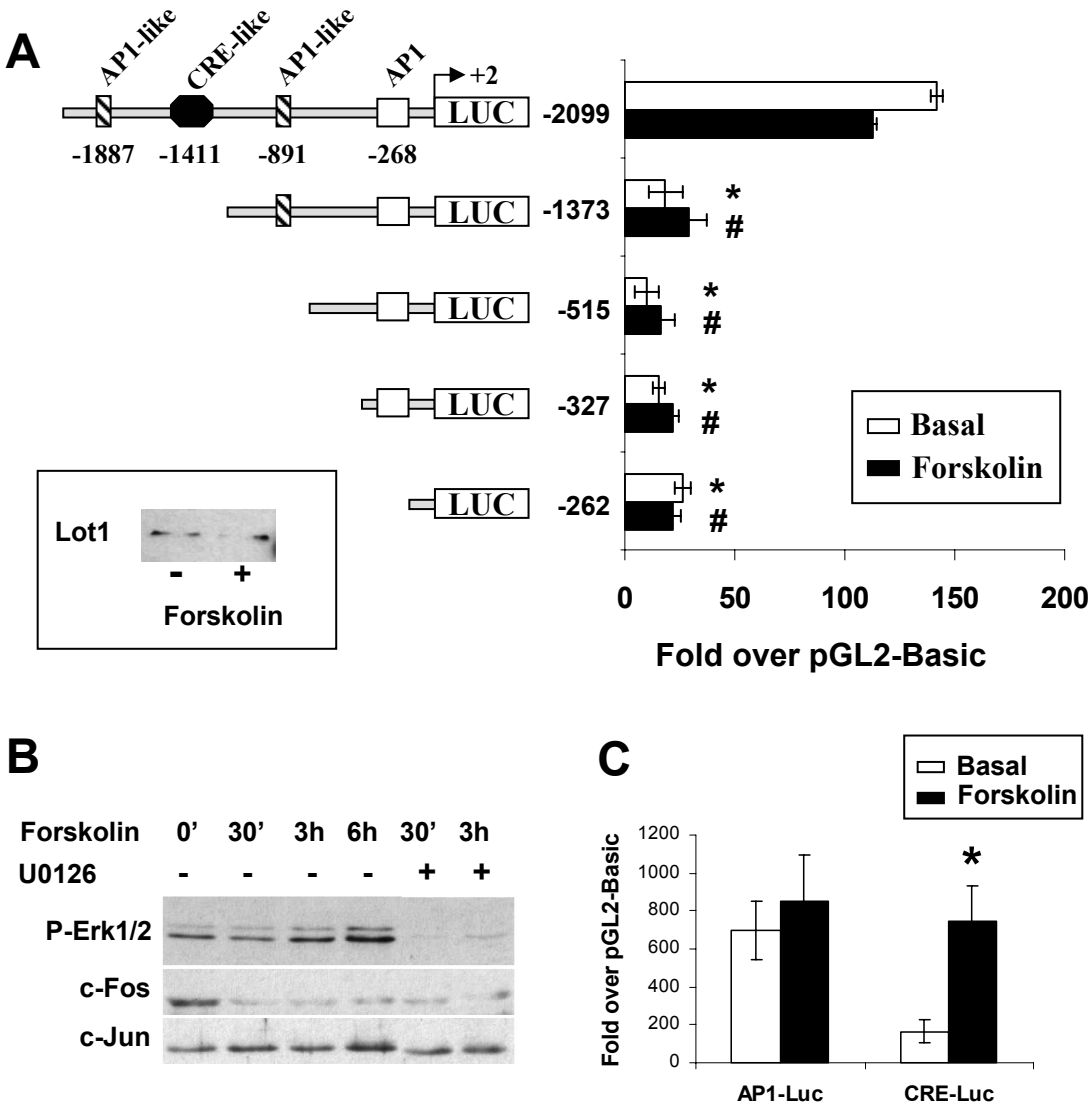

FIGURE 4.10. ***Lot1* promoter activity in HEK293 cells.**
A) shown are the results from luciferase reporter analysis of the *Lot1* promoter. HEK293 cells were transfected with 0.5 μg of the indicated *Lot1*-luciferase reporter plasmids and 0.5 μg of the β-galactosidase reporter plasmid (pTK-βgal) to normalize for transfection efficiency. After transfection, cells were incubated with (*black bars*) or without (*white bars*) 10 μM forskolin for 24 h prior to luciferase measurement. *Error bars* are the means ± S.E. of three experiments. *, $p<0.01$ *versus* pGLot-2099 under basal conditions; #, $p< 0.01$ *versus* pGLot-2099 under forskolin-stimulated conditions (Bonferroni's test after ANOVA). Insert show Lot1 protein expression in HEK293 nuclear extracts. Expression of Lot1 appear to be extremely low and not influenced by forskolin treatment in this cells. *B*) Western blot analysis of ERK phosphorylation (*P*) and c-Fos and c-Jun expression in HEK293 cells. Cells were treated for the indicated times with forskolin (10 μM) alone or with the MEK inhibitor U0126 (10 μM). *C*) HEK293 cells were transfected with 0.5 μg of reporter plasmids containing tandem repeats of CREB (CRE-Luc) or AP (AP1-Luc) consensus response elements as indicated and 0.5 μg of the β-galactosidase reporter plasmid (pTK-βgal) to normalize for transfection efficiency. After transfection, cells were incubated with (*black bars*) or without (*white bars*) forskolin (10 μM) for 24 h prior to luciferase measurement. *Error bars* are the means ± S.E. of three experiments. *, $p<0.01$ *versus* unstimulated conditions (Bonferroni's test after ANOVA).

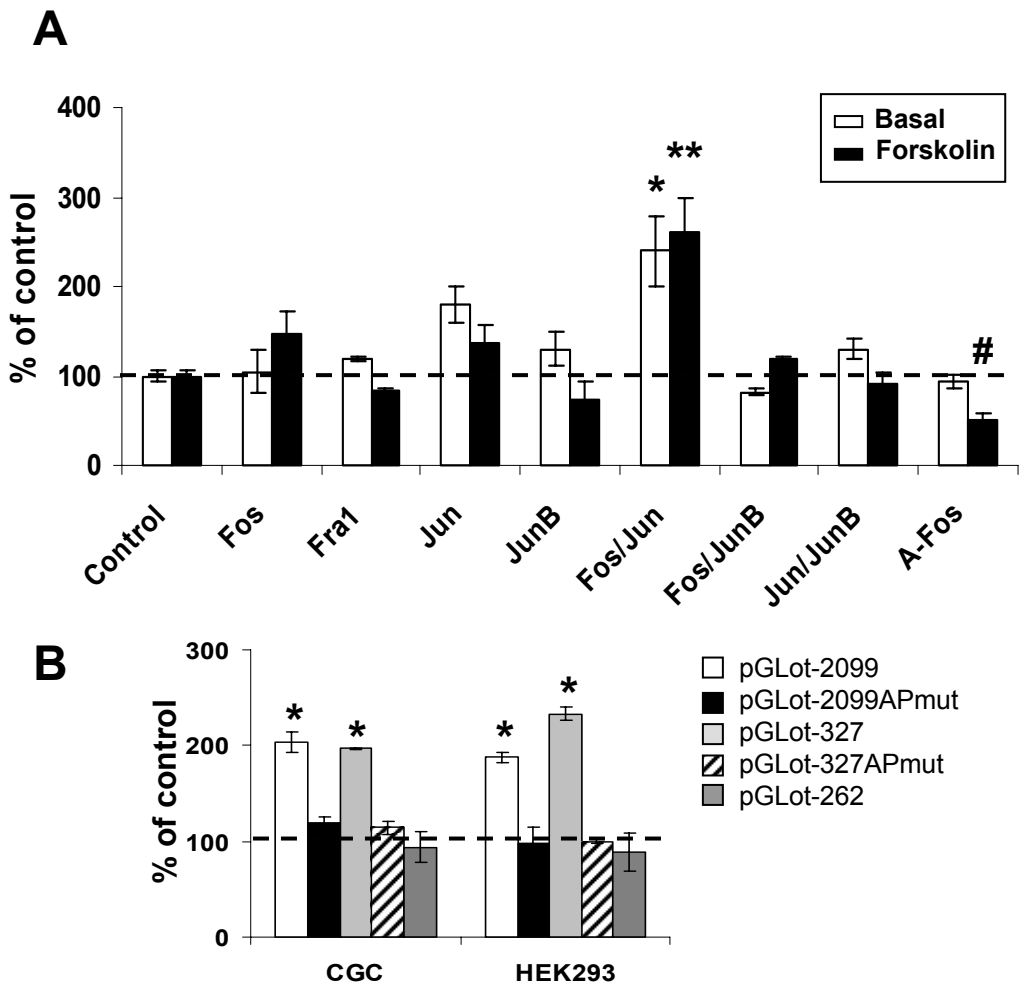

FIGURE 4.11. **The c-Fos/c-Jun heterodimer is responsible for cAMP-mediated *Lot1* transcriptional activity.**

A) CGC were cotransfected with 0.5 μg of the pGLot-327 reporter plasmid without (*Control*) or with 0.5 μg of the indicated expression vector for AP1 family proteins in the presence of 0.05 μg of pRL-TK. DNA quantity for each transfection was equalized with empty vector pcDNA3. Twenty-four hours after transfection, cells were incubated with (*black bars*) or without (*white bars*) 10 μM forskolin for an additional 24 h. The results are expressed as percent induction over control conditions and are the means ± S.E. of three to four experiments done in triplicate. *, $p< 0.01$ *versus* control conditions; **, $p<0.001$ *versus* control conditions; #, $p<0.05$ *versus* control conditions (Dunnett's test after ANOVA). *B*) shown is the specificity of c-Fos and c-Jun binding to the AP1 element involved in *Lot1* promoter activation. CGC were cotransfected with 0.5 μg of the indicated *Lot1*-luciferase reporter plasmids without (control) or with 0.5 μg of c-Fos and c-Jun expression plasmids in the presence of 0.05 μg of pRL-TK. DNA quantity for each transfection was equalized with empty vector pcDNA3. HEK293 cells were cotransfected with 0.5 μg the indicated *Lot1*-luciferase reporter plasmids without (control) or with 0.5 μg of c-Fos and c-Jun expression plasmids in the presence of 0.5 μg of pTK-βgal. DNA quantity for each transfection was equalized with empty vector pcDNA3. The results are given as percent induction over control conditions and are the means ± S.E. of three to four experiments done in triplicate. *, $p<0.01$ *versus* pGLot-2099APmut, pGLot-327APmut, and pGLot-262 (Dunnett's test after ANOVA).

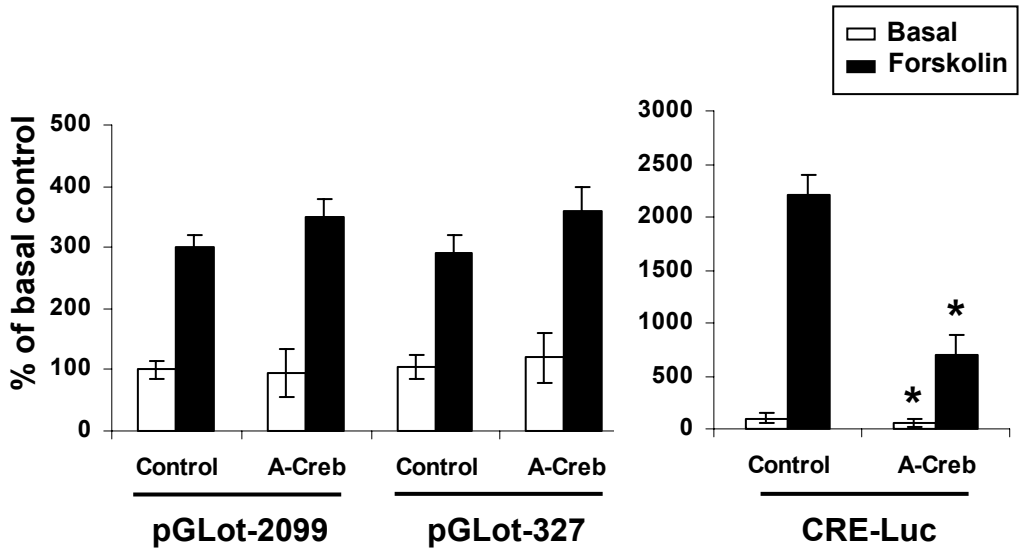

FIGURE 4.12. **CREB is not involved in cAMP-dependent activation of *Lot1* promoter.**

CGC were cotransfected with 0.75 μg of pGLot-2099, pGLot-327 reporter plasmids or a vector containing tandem repeats of CREB (CRE-Luc) consensus response elements, without (*Control*) or with 0.75 μg of the expression vector for dominant-negative A-CREB in the presence of 0.05 μg of pRL-TK to normalize for transfection efficency. DNA quantity for each transfection was equalized with empty vector pcDNA3. Twenty-four hours after transfection, cells were incubated 10 μM forskolin for an additional 24 h. The results are given as the percent over basal control conditions and are the means ± S.E. of three experiments done in triplicate. *, $p<0.01$ *versus* control conditions (Dunnett's test after ANOVA).

in HEK293 cells, in which cAMP does not induce c-Fos and *Lot1* expression. These experiments revealed that the effect of overexpression of c-Fos/c-Jun on *Lot1* transcription in HEK293 cells was similar to that observed in CGC (Fig. 4.11 *B*). We have shown before that, in CGC, treatments that increased cAMP levels promoted CREB phosphorylation (Fig. 4.9 *A*). To test whether CREB may be a primary factor in controlling cAMP-dependent *Lot1* transcriptional activation, we performed cotransfections with the full-length *Lot1*-luciferase reporter plasmid pGLot-2099 or the truncated pGLot-327, which lacks any obvious cAMP response element (CRE) binding site, and the dominant-negative mutant A-CREB (Ahn et al., 1998). Results indicate that, first of all, the *Lot1* promoter constructs possessing (pGLot-2099) or not (pGLot-327) a CRE binding site were stimulated by forskolin in a similar manner, thus suggesting that CREB is not necessary for *Lot1* activation (Fig. 4.12). Under our experimental conditions and in the presence of very strong activation of endogenous CREB by forskolin (see Fig. 4.9 *A*), the dominant-negative mutant A-CREB did not significantly inhibit forskolin-induced *Lot1* transcription in both constructs (Fig. 4.12). As a positive control, a plasmid containing tandem repeats of CREB (CRE-Luc) consensus response elements was used. The results shown in Fig. 4.12 demonstrate that, in this control plasmid, A-CREB was able to strongly inhibit both basal and forskolin-induced CRE-Luc activation.

5. DISCUSSION

In this study, we have shown, at both the mRNA and protein levels, that expression of the growth suppressor gene *Lot1* is up-regulated in CGC culture after elevation of cAMP intracellular concentration by either PACAP-38, forskolin or dibutyryl-cAMP treatments. Stimulation of the PACAP/cAMP signaling cascade was in parallel able to reduce proliferation of CGC in culture. Physiologically, cAMP-dependent *Lot1* induction parallels the negative regulation of CGC precursor proliferation mediated by cAMP, as we never observed its induction in dividing (BrdU-positive) cells. Furthermore, *Lot1* induction was not related to apoptotic elimination of mature CGC whose survival was challenged by shifting them to non-depolarizing conditions and serum deprivation. These results implicate *Lot1* as one of the elements in the cascade of molecular events triggered by PACAP/cAMP and suggest a role for *Lot1* as mediator of the antiproliferative activity of PACAP.

We have also analyzed the intracellular mechanisms involved in cAMP-dependent *Lot1* expression by pharmacological dissection of the pathway with specific protein kinases and protein synthesis inhibitors. We have found that, in CGC, cAMP-dependent *Lot1* stimulation requires activation of PKA and MEK-ERK pathways activation as well as *de novo* protein synthesis of the immediate early gene c-*Fos*. We have identified a putative promoter region within 2.1 kb 5'-upstream of the *Lot1* transcription start site, and we have characterized a regulatory element that is involved in neuronal expression of the rat *Lot1* gene after cAMP stimulation. We have provided evidence that the transcription of this gene depends upon an AP1 binding site in the proximal promoter regulatory region (268 bp 5'-upstream of the transcription start site) through the binding of the c-Fos/c-Jun heterodimer.

The cAMP-dependent effects elicited by the exogenous adenylate cyclase activator forskolin were also replicated by the physiological neurotrophic factor PACAP, which we have demonstrated here to down-regulate neuronal precursor proliferation in parallel with induction of *Lot1* expression. PACAP and its receptors are present in the developing nervous system (Basille et al., 1993; Gonzalez et al., 1997; Lioudyno et al., 1998; Masuo et al., 1992). PACAP belongs to a peptide family that includes secretin, glucagon, growth hormone releasing factor, and vasoactive intestinal peptide and interacts with target cells via G-protein-coupled receptors (Vaudry et al., 2000a). Cerebellar granule neurons express PACAP and its receptors during early neurogenesis *in vivo* while the peptide elicits survival and differentiation response in culture (Basille et al., 1995; Basille et al., 1993; Favit et al., 1995; Gonzalez et al., 1997; Tabuchi et al., 2001). Notably, a high density of PACAP receptors linked to adenylate cyclase activation was found in the external granular layer of the developing cerebellum (Basille et al., 1993; Masuo et al., 1994), with a time window temporally matching *Lot1* expression in the same layer (Ciani et al., 2003). Activation of the PAC1 receptors by PACAP has been shown to stimulate adenylate cyclase and phospholipase C activities in CGC, leading to activation of PKA and PKC (Basille et al., 1995; Favit et al., 1995). The signal transduction pathways that mediate the anti-apoptotic effect of PACAP in cerebellar granule neurons have been investigated in several experimental models. While the cAMP-PKA pathway has been clearly implicated in PACAP survival response (Chang et al., 1996; Kienlen Campard et al., 1997; Vaudry et al., 1999; Vaudry et al., 1998), the activation of phospolipase C and PKC does not seems to be involved (Vaudry et al., 1998; Vaudry et al., 2000b). The MEK-ERK pathway is a major signaling pathway in neural cells that is activated by PKA in CGC (Obara et al., 2007). Besides a it has been shown that inhibition of activation of the PKA-dependent MEK-ERK pathway abolishes the protective effect of PACAP on rat CGC (Villalba et al., 1997). Therefore, in addition to neurogenesis, PACAP signaling through PKA-MEK-ERK pathway is important for neuroprotection. Here, we have demonstrated that PACAP increases *Lot1* gene

expression in cultured rat cerebellar neurons, thus suggesting the involvement of *Lot1* in the PACAP-mediated survival- and differentiation-promoting action in neurodevelopment. This relationship is supported by studies demonstrating neurotrophic interactions between *Lot1*/*Zac1* and PACAP (Rodriguez-Henche et al., 2002) as well as by available reports on *Lot1*/*Zac1* expression in some brain areas during development (Ciani et al., 2003; Valente and Auladell, 2001).

It is also important to note that, in this study, increased *Lot1* expression induced by PACAP as well as by forskolin clearly did not compromise cell viability, as no signs of apoptosis could be detected in Lot1-positive neurons. Recent findings in animal models of excitotoxic injuries go in the same direction by linking *Lot1* expression to neuroprotection and brain plasticity (Gillardon et al., 1998; Valente and Auladell, 2001; Valente et al., 2004). Interestingly, in these models, a relationship between *Lot1* expression and PACAP (Gillardon et al., 1998) or c-Fos (Valente et al., 2004) was suggested, similar to what we have demonstrated in this study. This functional relationship is likely essential for the physiological role of Lot1.

The role of cAMP in the maturation of newborn neurons is well accepted (Fujioka et al., 2004; Nicot et al., 2002), and both PKA-dependent and PKA-independent pathways have been implicated in cAMP-mediated effects. Our study has shown that, in CGC, cAMP activation of *Lot1* is PKA- and MEK-ERK-dependent, as it was counteracted by specific pathway inhibitors. Notably, the PKA-dependent mechanism implicated in *Lot1* activation did not act through the CRE binding site present in the gene promoter. Indeed, deletion of the CRE-binding site on the cloned promoter did not abrogate on *Lot1* activation after forskolin stimulation. Besides CREB, previous studies have shown that the cAMP-PKA pathway also activates the MEK-ERK signaling cascade in CGC trough the recruitment of Rap1 and subsequent B-Raf activation (Obara et al., 2007). Our data imply that forskolin-induced *Lot1* transcription in CGC is mediated by the PKA and MEK-ERK pathways. In many neuronal cells, increased cAMP can induce neuronal differentiation through activation of the mitogen-activated protein kinase cascade (Dugan et al., 1999;

Vossler et al., 1997). We have shown that inhibition of PKA completely blocked MEK1/2 first and consequently ERK1/2 activation by forskolin, demonstrating dependence of ERK1/2 activation by PKA. Moreover, blockade of forskolin-induced MEK1/2 activity with a selective inhibitor (U0126) not only reduced the magnitude and duration of ERK1/2 phosphorylation, but also blocked transcriptional activation of the *Lot1* gene. These results provide clear evidence that ERK activation is absolutely required for transcriptional activation of the *Lot1* gene. Interestingly, prolonged ERK1/2 activation in some cell types leads to the expression of a differentiated phenotype such as neurite outgrowth in PC12 cells (Marshall, 1995; Traverse et al., 1992). One of the targets of activated MEK-ERK pathway in mammalian cells is the transcription factor AP1, which is composed of members of the c-Fos and c-Jun family of DNA-binding proteins (Angel and Karin, 1991; Karin, 1995). The MEK-ERK signaling pathways regulate AP1 activity in several ways, including increased synthesis and/or activation of c-Fos (Raingeaud et al., 1996). Our present results clearly suggest a link between the PKA-MEK-ERK pathways and c-Fos activity in the regulation of *Lot1* expression through the critical promoter AP1 binding site disclosed by the reported experiments. We isolated, mapped, and functionally characterized the 5'-flanking region of the rat *Lot1* gene. By both 5'-deletion analysis and site mutagenesis of the native *Lot1* promoter, we directly demonstrated that the AP1 binding site at -268 bp from the transcription start site is necessary to achieve a full transcriptional response to cAMP elevation by forskolin stimulation. Moreover, our results show that cAMP elevation by forskolin up-regulated the expression of c-Fos, implicating this immediate-early gene as the transcriptional mediator linking ERK1/2 phosphorylation to *Lot1* promoter activation. Our data show, however, that overexpression of c-Fos alone was not sufficient to activate the *Lot1* promoter, but cotransfection of c-Jun and c-Fos together resulted in significant induction of *Lot1* promoter activity. Overexpression of other heterodimers such as c-Fos/JunB and c-Jun/JunB was not effective. The unique role of the c-Fos/c-Jun heterodimer in activation of the AP1 complex on the *Lot1* promoter is supported

by A-Fos overexpression studies. Putative knockout of the AP1-binding activity by overexpression of A-Fos was effective in blocking activation of *Lot1* promoter activity. This dominant interfering mutant has been shown previously to disrupt c-Fos/c-Jun heterodimer binding to DNA in a remarkably specific manner (Olive et al., 1997).

Our results provide novel information on the functional significance of the up-regulation of *Lot1* expression by cAMP stimulation in CGC. First, we have shown that *Lot1* is induced in CGC by the anti-mitogenic and differentiative stimulus exerted by PACAP and cAMP on these neuronal cells. Second, our analysis revealed that the cellular mechanisms regulating *Lot1* activation, *i.e.* the PKA-MEK-ERK cascade and c-Fos induction, are the same that regulate crucial steps of the neurogenetic process (Moody et al., 2003). Third, the characterization of these mechanisms, together with the previous data on *Lot1* expression in brain development, enforces the notion that the gene may play an essential role in neurogenesis, with particular reference to cerebellar development (Ciani et al., 2003). Fourth, regulation of the *Lot1* gene, described here for neuronal cells, may be different in other cell types. It has been indeed noted in the Introduction that *Lot1* acts as a tumor suppressor gene in cancer cells (Pagotto et al., 1999; Spengler et al., 1997). With the present observations, we have shown that the epithelial cell line HEK293 responds to cAMP elevation in a very different way compared with CGC and that c-Fos induction is not part of the signaling elicited in these cells. Thus, depending upon the availability of AP1 protein factors, the dimerization among different members of the AP1 family might lead to different regulatory effects on *Lot1* expression. These studies clearly indicate a possible role for *Lot1* gene expression controlled by different stimuli in regulating proliferation of neuronal precursors and brain development.

6. CITED LITERATURE

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