

**PACAP signaling to DREAM: A cAMP-dependent pathway that
regulates cortical astrogliogenesis**

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Abstract

Astrocytes constitute a very abundant cell type in the mammalian central nervous system, and play critical roles in brain function. During development, astrocytes are generated from neural progenitor cells only after these cells have generated neurons. This so called gliogenic switch is tightly regulated by intrinsic factors that inhibit the generation of astrocytes during the neurogenic period. Once neural progenitors acquire gliogenic competence, they differentiate into astrocytes in response to specific extracellular signals. Some of these signals are delivered by neurotrophic cytokines via activation of the gp130-JAK-STAT system, whereas others depend on the activity of pituitary adenylate cyclase-activating polypeptide (PACAP) on specific PAC1 receptors that stimulate the production of cAMP. This results in the activation of the small GTPases Rap1 and Ras, and in the cAMP-dependent entry of extracellular calcium into the cell. Calcium, in turn, stimulates the transcription factor DREAM, which is bound to specific sites of the promoter of the glial fibrillary acidic protein gene, stimulating its expression during astrocyte differentiation. Lack of DREAM *in vivo* results in alterations in the number of neurons and astrocytes generated during development. Thus, the PACAP-cAMP-Ca²⁺-DREAM signaling cascade constitutes an important pathway to activate glial-specific gene expression during astrocyte differentiation.

Introduction

Among glial cells, astrocytes constitute the most abundant cell type in the central nervous system (Bass et al., 1971; Nedergaard et al., 2003). In recent years a large body of data has accumulated in favor of the notion that these glial cells participate actively in the physiological processes of the brain by establishing a reciprocal relationship with neurons (Barres, 2008; Theodosis et al., 2008). This relationship is not only important to ensure selectively the availability of nutrients through the blood brain barrier, but also for the performance of important tasks that are essential for the correct function and survival of neurons. Thus, astrocytes are active participants in synaptic transmission and regulate intercellular communication processes (Haydon and Carmignoto, 2006; Perea and Araque, 2007), they prevent neurotoxicity from excitatory neurotransmitters and other compounds (Ashner et al., 1999; Maragakis and Rothstein, 2004), and they produce trophic factors that are essential for neuronal differentiation and survival (Gash et al., 1998; Blondel et al., 2000). Interestingly, in some brain areas astrocytes themselves can give rise to neurons (Seri et al., 2001). In addition, astrocytes appear to be an important element in the development of certain diseases (Araque, 2006; Chen et al., 2007; Lepore et al., 2008), and mutations in the gene encoding glial fibrillary acidic protein (GFAP), an intermediate filament protein characteristic of these cells, are associated with Alexander's disease, a rare but devastating disorder of the central nervous system (Quinlan et al., 2007).

Generation of astrocytes during brain development

The mechanisms by which astrocytes originate from neural precursors during embryonic development have received much attention in recent years. It is now well established that during gestation the generation of astrocytes occurs at a relatively late

stage, only after the generation of neurons has been practically completed, and proceeds postnatally. In rodents, neurogenesis spans approximately from embryonic day 12 (E12) to E18, whereas astrocytogenesis starts at around E17 and peaks a few days after birth. Importantly, both neurons and astrocytes arise in the developing neuroepithelium from the same progenitor cells, which appear to coincide with radial glial cells (Pinto and Gotz, 2007). Therefore, at a specific time during development progenitor cells are instructed to finish with the generation of neurons and to acquire a capacity to generate astrocytes.

This cellular switch from neurogenesis to gliogenesis is tightly regulated at different levels. One level of regulation involves the active inhibition of gliogenesis during the neurogenic period, so that precursors are unable to generate astrocytes even in the presence of astroglial signals. One effective mechanism of inhibition consists on the methylation of astrocyte-specific genes leading to their inactivation in early progenitor cells (Takizawa et al., 2001; Namihira et al., 2004; Fan et al., 2005). In addition, inhibition of glia-specific genes in neuronal progenitors involves epigenetic silencing by chromatin remodeling (Song and Ghosh, 2004), as well as transcriptional repression (Hermanson et al., 2002; Angelastro et al., 2005). Interestingly, it has been found that transcription factors of the basic-helix-loop-helix family that promote the generation of neurons from progenitor cells, actively inhibit the expression of glial-specific genes, thus preventing gliogenesis (Cai et al., 2000; Nieto et al., 2001; Sun et al., 2001).

Neural progenitors gain competence to generate astrocytes due to the activity of growth factors such as basic fibroblast growth factor and epidermal growth factor (Qian et al., 1997; Viti et al., 2003; Song and Ghosh, 2004). This gain of competence allows them to respond to specific gliogenic signals acting at the extracellular level.

Particularly important in this regard are signals provided by neurotrophic cytokines such as ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1) (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Barnabé-Heider et al., 2005). All of these cytokines activate heterodimeric cell surface receptors composed of two subunits named LIFR β and gp130, which in turn activate members of the JAK family of tyrosine kinases, that result in the phosphorylation and nuclear translocation of signal transducer and activator of transcription (STAT) proteins (Schindler et al., 2007). In neural progenitors, two of these proteins, STAT1 and STAT3 act on specific sites in the promoters of the astroglial genes *GFAP* and *SI00 β* to stimulate their transcription during the astrocyte differentiation process. *In vivo* studies carried out using genetically modified mice indicate that the gp130-JAK-STAT system is required for the generation of astrocytes, and that CT-1 provides a key regulatory signal for the activation of this system at the onset of the astrogligenic period (Bugga et al., 1998; Koblar et al., 1998; Nakashima et al., 1999b; Barnabé-Heider et al., 2005).

Neural progenitors also respond to different neurotrophic factors from the bone morphogenetic proteins (BMP) family to generate astrocytes. In this case, BMP2 or BMP4 act on heterotrimeric receptors, which activate Smad transcription factors. These in turn, interact with activated STAT proteins to synergistically stimulate transcription of glial specific genes during astrocyte differentiation (Gross et al., 1996; Mehler et al., 1997; Mabie et al., 1999; Nakashima et al., 1999a).

Identification of PACAP as a cAMP-dependent signal for the generation of astrocytes

Apart from signals generated by diffusible factors acting on kinase-dependent receptors as discussed above, recent studies indicate that seven transmembrane domain

G-protein-coupled receptors activated by pituitary adenylate cyclase-activating polypeptide (PACAP) can trigger the differentiation of astrocytes via a mechanism that requires the production of intracellular cAMP (McManus et al., 1999; Vallejo and Vallejo, 2002; Cebolla et al., 2008).

The studies that led to the identification of PACAP as a neurotrophic factor capable of triggering the differentiation of astrocytes from cortical precursors were initiated with the observation that stimulation of the cAMP-dependent signaling pathway in primary cultures of neuroepithelial progenitors obtained from rat fetal cortex results in the differentiation of astrocytes after exit from the cell cycle (McManus et al., 1999). This response observed after stimulation of the cAMP-dependent pathway in cortical progenitors is specifically instructive, because stimulation of this second messenger pathway does not result in the differentiation of neurons or oligodendrocytes (McManus et al., 1999), although these cells retain the capacity to differentiate into neurons in response to an appropriate stimulus such as BDNF (Lastres-Becker et al., 2008). After this initial observation, a search for an extracellular ligand that could mimic the effect of cAMP was initiated.

As a putative role for monoamine neurotransmitters had been suggested in the developing brain (Levitt et al., 1997; Cameron et al., 1998), and these neurotransmitters act by binding to G-protein-coupled receptors that can activate the synthesis of cAMP, they became obvious candidates for the observed cAMP-dependent astrocytogenic response. Indeed, exposure of primary fetal cortical progenitors to monoamines results in phosphorylation of CREB, indicating that these cells express appropriate functional receptors. However, these experiments showed that these cells do not differentiate into astrocytes when exposed to dopamine, noradrenaline or serotonin, as assessed by changes in morphology and expression of GFAP (Vallejo and Vallejo, 2002).

Having ruled out monoamines, it was considered that certain neuropeptides acting on specific G-protein-coupled receptors could trigger the differentiation of astrocytes from cortical precursors. Because of the known activity of PACAP to stimulate adenylate cyclase in neural cells, this peptide and the closely related vasoactive intestinal polypeptide (VIP) were tested to investigate whether they are able to induce astrocyte differentiation.

Both PACAP and VIP can bind with similar affinities to two specific receptors known as VPAC1 and VPAC2. In addition, PACAP recognizes a third receptor known as PAC1, for which VIP exhibits a comparatively low affinity (Harmar et al., 1998). All of these receptors stimulate adenylate cyclase activity, although PAC1 receptors can also stimulate phospholipase C, and both peptides have been reported to increase intracellular levels of cAMP in a variety of different cell types.

Experiments carried out with primary cultures of rat cortical progenitor cells demonstrated that PACAP, but not VIP, is able to promote their differentiation into astrocytes (Vallejo and Vallejo, 2002). The observed lack of effect of VIP and the specificity of PACAP is due to the circumstance that cortical neural progenitors express relatively high levels of the PAC1 receptor, but not VPAC1 or VPAC2 receptors. However, different isoforms of the PAC1 receptor generated by alternative splicing exist, and each one of these isoforms uses different signal transduction mechanisms. This is due to different configurations that may be adopted by the third intracellular loop of the receptor, which contains the domain that interacts directly with G-proteins to activate adenylate cyclase. Alternative splicing may result in the inclusion in this intracellular loop of one or two small cassettes called Hip and Hop, encoded by two different exons. This favors coupling of the receptor to the polyphosphoinositide-specific phospholipase C-dependent signaling pathway (Spengler et al., 1993; Nicot and

DiCicco-Bloom, 2001). In addition, another PAC1 receptor isoform is able to stimulate calcium entry directly through the activation of an L-type calcium channel (Chatterjee et al., 1996). Therefore, the observation that PACAP is able to specifically mimic the astrocytogenic response of forskolin or 8Br-cAMP in cortical progenitors does not necessarily mean that this neuropeptide is using a cAMP-dependent mechanism to produce this effect.

To clarify this issue, several lines of experimentation were undertaken. First, it was determined that the most abundant isoform of the PAC1 receptor expressed in rat cortical progenitor cells is the short isoform that couples to the G protein-adenylate cyclase system. Relatively small amounts of PAC1 receptor containing the Hop cassette, and even smaller amounts of the isoform containing the Hip cassette were also detected, but interestingly these disappeared upon receptor activation with PACAP or upon treatment of cells with the cAMP analog 8Br-cAMP at the expense of an increase in the expression of the short isoform of the receptor (Vallejo and Vallejo, 2002). Thus, the same stimulus that triggers the production of cAMP response also induces changes at the receptor level to maximize this response.

In a different set of experiments, it was determined that PACAP can stimulate the synthesis of cAMP in rat neural progenitor cells, whereas VIP is practically inactive. Importantly, astrocyte differentiation by PACAP can be inhibited by pretreatment with Rp-cAMPS, a specific antagonist of cAMP, indicating that the production of this second messenger is a critical step to initiate the differentiation response (Vallejo and Vallejo, 2002).

Thus, together these experiments demonstrated that PACAP can specifically induce astrocyte differentiation of primary cortical progenitor cells by acting on the short isoform of PAC1 receptors to stimulate intracellular cAMP production.

Interestingly, only a relative short exposure of cells to PACAP (15-30 minutes) is required to trigger the astrocytogenic response, which can be evident even 5 days after peptide treatment by morphological changes and by the stimulation of GFAP expression. Thus, as it is the case with other neurotrophic factors inducing neuronal or glial differentiation (Gross et al., 1996; Williams et al., 1997; Park et al., 1999), PACAP appears to initiate the response by stimulating cAMP production, but then other intracellular mechanisms take over to promote differentiation.

Watanabe et al. (2006), following studies carried out by the same group (Ohno et al., 2005), confirmed that PACAP is indeed capable to induce astrocyte differentiation by acting on PAC1 receptors. However, they did not find evidence in favor of a role for cAMP in this response, but instead proposed an involvement of phospholipase C and protein kinase C. These authors used relatively early (E14.5) mouse neural progenitor cells, as opposed to the E17 neural progenitors prepared from rat fetuses used in the experiments described above. In addition, relatively low concentrations of PACAP were used, and the differentiation of astrocytes took up to 5 days to be observed (Ohno et al., 2005; Watanabe et al., 2006). Interestingly, it has been found that activation of PAC1 receptors in mouse neural precursors can generate both intracellular cAMP accumulation and activation of phospholipase C-mediated signaling leading to an increase in intracellular calcium, although this latter response appears to be related to proliferation of glial precursors rather than to differentiation (Nishimoto et al., 2007). Thus, differences in the species and experimental conditions may reflect the complex mechanisms activated by PACAP in the developing brain.

Both PACAP and the PAC1 receptor have been described to be present in the brain throughout development and postnatally (Suh et al., 2001; Nishimoto et al., 2007; Watanabe et al., 2007). A role for PACAP in the regulation of astroglialogenesis has been

proposed based on studies carried out by different investigators (Zupan et al., 1998; Vallejo and Vallejo, 2002; Ohno et al., 2005; Cebolla and Vallejo, 2006; Watanabe et al., 2006; Nishimoto et al., 2007; Cebolla et al., 2008; Lastres-Becker et al., 2008). However, it is clear that the role of PACAP during development is complex, because at different times during development it exerts different actions. Thus, independently of its activity as an astrogliogenic signal, PACAP acts as a potent antimitotic factor on relatively early neural progenitors and favors neuronal differentiation, whereas on late progenitors it regulates the generation of oligodendrocytes (Lee et al., 2001; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001; Carey et al., 2002; Cazillis et al., 2004). This variety of responses at different times during development indicates that PACAP is an important neurotrophic factor whose activity is tightly regulated at different levels, including the specific type of receptor isoform expressed by neural progenitors, the different signal transduction pathways used by these receptors, the possible interaction of these pathways with molecules of signaling pathways activated by other neurotrophic factors, the type of transcription factors activated that act on specific target genes, and possibly the degree of responsiveness of these genes which may depend on epigenetic modifications. In turn, each one of these aspects may vary in different cells throughout development, thus providing an explanation for the high degree of versatility with which PACAP exerts different functions depending on the developmental stage and the type of the target cells on which it acts.

Intracellular cAMP-dependent signaling triggered by PACAP in cortical precursors

As the differentiation of astrocytes from progenitor cells is accompanied by stimulation of the expression of the gene encoding GFAP, monitoring the

transcriptional activity of the *GFAP* promoter driving a reporter gene such as luciferase provided a useful approach to investigate the intracellular signaling pathways activated by PACAP during astrocyte differentiation from neural progenitors.

Activation of protein kinase A (PKA) was initially considered to be an obvious mechanism by which PACAP could stimulate *GFAP* gene expression and trigger astrocyte differentiation. This notion was supported by three lines of evidence: First, the astrogligenic effect of PACAP on cortical progenitors can be inhibited by the cAMP antagonist Rp-cAMPS; second, treatment of cortical progenitors with PACAP results in phosphorylation of CREB, a well known substrate for PKA; and third, PACAP-induced CREB phosphorylation can be inhibited by pretreatment with the PKA inhibitor H89 (Lastres-Becker et al., 2008). However, it soon became clear that PKA is not involved, because H89 was not able to inhibit the stimulation of *GFAP* gene expression nor the differentiation of astrocytes induced by PACAP. Thus, although PACAP can activate PKA in cortical progenitors, this activation may be responsible for other effects such as inhibition of proliferation (Lelievre et al., 2002), but not for inducing the differentiation of these progenitors into astrocytes.

Besides PKA, cAMP can stimulate at least two additional intracellular signaling cascades by binding to guanine nucleotide exchange proteins such as Epac and CNrasGEF (Pham et al., 2000; Bos, 2003). In cortical progenitors, PACAP is able to stimulate both pathways (Lastres-Becker et al., 2008). In the first case, exposure of cells to PACAP results in the activation of Rap1, which acts as an effector for cAMP-activated Epac. In the second case, PACAP treatment leads to stimulation of Ras, an effect that can be inhibited by the cAMP-specific antagonist Rp-cAMPS. The activation of Rap1 and Ras by PACAP is not restricted to neural progenitors, since it has been

observed in different types of neurons and astrocytes (Bouschet et al., 2003; Dasgupta et al., 2003; Romano et al., 2003; Obara et al., 2007).

The notion that stimulations of these pathways are involved in triggering astrocyte differentiation by PACAP is supported by the observations that Rap1N17 or RasN17, which act as dominant negative inhibitors of Rap1 and Ras, respectively (Vossler et al., 1997; Matallanas et al., 2003), are able to decrease the stimulation of the *GFAP* gene promoter elicited by PACAP in cortical precursor cells. That Ras is stimulated in a cAMP-dependent manner was demonstrated by the observation that PACAP is unable to activate Ras in the presence of Rp-cAMPS, and that *GFAP* promoter stimulation by the stable cAMP analog 8Br-cAMP is significantly reduced in the presence of the dominant negative inhibitor RasN17 (Lastres-Becker et al., 2008).

Interestingly, stimulation of either the Epac-Rap1 or the cAMP-Ras pathway in isolation by using the selective activator of Epac 8CPT-2-O-Me-cAMP (Enserink et al., 2002), or by expressing RasV12, a constitutively active form of Ras, is not sufficient to stimulate *GFAP* gene expression. For this to occur, both signaling cascades need to be concomitantly stimulated, consistent with the observations that coexpression of both RapN17 and RasN17 completely inhibits *GFAP* promoter stimulation by PACAP, and that combined knock down of Ras and Rap1 isoforms by siRNA prevents expression of *GFAP* after stimulation of cortical progenitors with PACAP (Lastres-Becker et al., 2008).

The data described above, obtained using primary cultures of cortical precursors *in vitro* are in agreement with those observed *in vivo* (Apicelli et al., 2003). These authors generated transgenic mice expressing a constitutively active form of Rap1 under the control of the *GFAP* promoter, so that its expression is restricted to astrocytes from a time coinciding with astrogliogenesis at around E16. In these mice, astrocyte

differentiation was not significantly altered, as the number of astrocytes generated was similar to that of control mice.

Transcriptional stimulation of the *GFAP* promoter by PACAP-cAMP

Studies to identify transcriptional mechanisms that activate and restrict the expression of the *GFAP* gene to astrocytes have been carried out both in cultured cells and in transgenic mice (Barnett et al., 1995; Bonni et al., 1997; Kahn et al., 1997; Rajan and McKay, 1998; Stone et al., 1998; Krohn et al., 1999; Nakashima et al., 1999a; Sun et al., 2001; Rajan et al., 2003; Lee et al., 2006; Lee et al., 2008).

Among the different transcription factors identified to date that regulate GFAP gene expression, some members of the NFI family appear to play a prominent role (Cebolla and Vallejo, 2006; Deneen et al., 2006; Gopalan et al., 2006). The NFI transcription factors comprise a family composed of at least four members with important functions during development (Gronostajski, 2000). Studies carried out in the developing spinal cord indicate that one member of this family, NFIA, is necessary for the gliogenic switch by which neural precursors inhibit their capacity to differentiate into neurons and acquire an astroglial phenotype (Deneen et al., 2006). In addition, *NFI* genes are important for the promotion of astrocyte differentiation, and their deletion results in decreased levels of GFAP expression (Das Neves et al., 1999; Shu et al., 2003; Steele-Perkins et al., 2005; Deneen et al., 2006).

In the brain of rat fetuses NFI occupies specific sites of the *GFAP* promoter *in vivo*, and therefore it is not surprising that the onset of expression of NFI in the developing cortex coincides with the onset of GFAP expression during astroglialogenesis at around E17 (Cebolla and Vallejo, 2006). In the absence of NFI binding to the *GFAP* gene, activation of the promoter by astroglial factors such as PACAP or CNTF

remains functional but is significantly impaired. NFI has been found to regulate transcription by presetting the chromatin for accessibility to other transcription factors (Belikov et al., 2004; Hebbar and Archer, 2007). Therefore, it is possible that NFI could be important in neural cells for maintaining the *GFAP* promoter in a favorable condition for its activation during astrocyte differentiation via a similar mechanism. This remains an important question to be addressed in future studies.

The stimulation of *GFAP* gene transcription by PACAP or by cAMP agonists even in the absence of NFI binding, although reduced, clearly indicates the existence of promoter elements that bind specific transcription factors different from NFI, which are activated by cAMP at the beginning of the astrocyte differentiation process. Analyses of the rat *GFAP* promoter in primary cultures of cortical progenitors led to the characterization of two regulatory elements bound by transcription factor DREAM, and to the demonstration that DREAM is the effector protein that mediates the transcriptional stimulation triggered by PACAP and cAMP at the onset of astrogliogenesis (Cebolla et al., 2008).

In addition, both PACAP and CNTF facilitate astrocytogenesis cooperatively, so that when both factors are present together, cells are generated with longer and more elaborate processes than those exposed to only one of these factors. This synergism does not occur in the absence of NFI binding to the *GFAP* promoter, suggesting that NFI is also important for the integration of different signaling pathways at the transcriptional level (Cebolla and Vallejo, 2006). The mechanism by which this integration resulting in synergism occurs is not known, but it is possible that it involves interactions of NFI with both STAT proteins and DREAM. In the first case, such type of interactions have been described in other cell types (Mukhopadhyay et al., 2001), whereas in the second, the NFI and DREAM binding sites in the rat and mouse *GFAP*

promoter are located in such close proximity (nucleotides -79 to -67 and -62 to -59, respectively, relative to the transcription initiation site) that direct interactions are indeed likely (Fig. 1). An analysis of the promoter sequences of the *GFAP* gene from different species using the UCSC Genome Bioinformatics database (<http://genome.ucsc.edu>) shows that the NFI binding site is well conserved among different species including rat, mouse, dog, cow and human. The adjacent DREAM binding site is conserved in rat and mouse, but is divergent in other species. However, a conserved DREAM binding site is present in the human *GFAP* promoter approximately 160 base pairs upstream from the NFI site.

DREAM transactivates the *GFAP* promoter in response to a cAMP-dependent increase in intracellular calcium

DREAM (Downstream Regulatory Element Antagonist Modulator) was initially identified as a repressor protein bound to regulatory elements located downstream from the transcription initiation site of the prodynorphin and *c-fos* genes (Carrion et al., 1999; Ledo et al., 2000). In cortical progenitor cells, DREAM is bound to at least two sites in the *GFAP* promoter even before they are induced to differentiate by PACAP, and once *GFAP* expression is initiated and astrocytes are generated DREAM remains bound. Indeed, the integrity of these two sites, and therefore binding of DREAM to the *GFAP* gene appears to be essential for promoter activity (Cebolla et al., 2008).

That DREAM is essential for mediating PACAP-induced stimulation of *GFAP* expression and astrocyte differentiation was initially indicated by the observation that cortical progenitor cells derived from DREAM-deficient mice fail to differentiate into astrocytes in response to PACAP, although they can still generate astrocytes in response to CNTF, indicating that the PACAP-DREAM signaling pathway works independently

of the gp130-JAK-STAT signaling pathway used by neurotrophic cytokines (Cebolla et al., 2008).

DREAM can be stimulated by interacting with the transcription factor α CREM after phosphorylation of this factor by PKA following activation by cAMP, or by direct binding of calcium ions to specific domains (Carrion et al., 1999; Ledo et al., 2000). In concert with the previous observation that PKA activation is not involved in the generation of astrocytes induced by PACAP, cAMP-dependent interactions of α CREM and DREAM are not involved in stimulation of GFAP expression by PACAP. On the contrary, PACAP/cAMP-induced GFAP expression in cortical progenitors requires the integrity of calcium-binding domains in DREAM (Cebolla et al., 2008), adding calcium as an additional component of the signaling cascade that links PACAP activation of PAC1 receptors with *GFAP* promoter stimulation.

PACAP can increase intracellular concentrations of calcium by a variety of mechanisms (Przywara et al., 1996; Osipenko et al., 2000; Morita et al., 2002; Liu et al., 2003; Payet et al., 2003; Nishimoto et al., 2007). In rat cortical progenitors, this rise in calcium is due to entry from the extracellular milieu. As observed in other cell types (Przywara et al., 1996; Osipenko et al., 2000), PACAP-induced entry of calcium into the cells is mediated by cAMP, because pretreatment with the cAMP antagonist Rp-cAMPS completely blocks this response (Cebolla et al., 2008).

The mechanism by which DREAM transactivates the *GFAP* promoter in response to calcium is unknown, but it is likely that it involves changes in protein conformation (Carrion et al., 1999; Osawa et al., 2001; Osawa et al., 2005). DREAM binds to DNA as a tetramer and ion-induced conformational changes result in the generation of calcium-bound DREAM dimers that normally do not bind DNA (Osawa et al., 2001; Osawa et al., 2005; Lusin et al., 2008). However, southwestern analysis has

shown that DREAM dimers can bind DNA (Carrion et al., 1999), and in the case of the *GFAP* promoter, this binding could be stabilized by interactions with other proteins bound in close proximity. In this regard, it is important to note that NFI occupies a site in the *GFAP* promoter located adjacent to one of the DREAM binding sites, and that binding of NFI to this site is important for *GFAP* expression during astrocyte differentiation (Cebolla and Vallejo, 2006). Thus, it is possible that DREAM is bound to the *GFAP* promoter as part of a multiprotein complex including NFI, and that calcium binding to DREAM induces conformational changes that favor positive interactions with coactivators or with other transcriptionally active proteins. Indeed, it has been indicated that the mechanism by which DREAM acts on target genes bearing DREAM binding sites upstream, rather than downstream of the TATA box, may be different and may involve interactions with other transcription factors (Rivas et al., 2004; Gomez-Villafuertes et al., 2005; Scsucova et al., 2005).

In this regard, and in view of the observed capacity of DREAM to interact with other transcription factors (Ledo et al., 2000; Rivas et al., 2004; Scsucova et al., 2005), it is important to distinguish between the effects on *GFAP* promoter activation obtained experimentally after mutating either of the two DREAM binding sites *in vitro*, and the effects obtained on endogenous *GFAP* expression after inactivating the expression of DREAM *in vivo*. Thus, whereas the integrity of DREAM binding sites is essential for *GFAP* promoter activity *in vitro*, in cells lacking DREAM the *GFAP* promoter can still be functional (Cebolla et al., 2008). One possible interpretation of these apparently conflicting observations is that DREAM may be engaged in interactions with NFI and/or with other unidentified transcription factors assembled in such a way that promoter activity requires the coordinated binding of the complex, as depicted schematically in figure 1. Mutation of the DREAM binding sites could result in the

sequestration of the entire complex if DREAM is not given access to DNA (Fig. 1B). In contrast, in the absence of DREAM the remaining proteins could still be able to bind to their cognate sites on DNA and activate transcription (Fig. 1C). To validate or reject this model, studies to investigate whether DREAM interacts with NFI, or with STAT proteins or other transcription factors on the GFAP promoter in differentiating astrocytes will be required.

DREAM and the timing of the transition from neurogenesis to gliogenesis

The physiological importance of DREAM in the mechanisms that regulate the generation of astrocytes during development was indicated by the observation that the number of astrocytes present during the early postnatal gliogenic period in mice lacking DREAM is reduced (Cebolla et al., 2008). This reduction is probably a reflection of a defect in the relay of PACAP/cAMP/calcium-dependent signals to the transcription machinery assembled on the *GFAP* gene, and is consistent with previous studies showing reduced number of astrocytes in the offspring of rats treated with a PACAP receptor antagonist during late pregnancy (Zupan et al., 1998). However, this reduction is not permanent. From the seventh day of postnatal life, the number of astrocytes in DREAM-mutant mice rises to even surpass that of control mice (Cebolla et al., 2008). Therefore, lack of DREAM does not affect other astrocyte-generating signals that may act coordinately during development.

The mechanisms that regulate the cellular switch by virtue of which cortical progenitors inhibit their capacity to generate neurons and start generating astrocytes are tightly linked. Therefore, when gliogenesis is inhibited a concomitant increase in neurogenesis may be observed. The reason for this is that signals that activate gliogenesis are also actively involved in the inhibition of neurogenesis (Miller and

Gauthier, 2007). It follows that if DREAM is also involved in repressing neurogenesis when astrogliogenesis is about to start, the initial decrease in the number of astrocytes in DREAM-deficient mice followed by the subsequent rise at a later stage could be interpreted as a delay in the onset of astrogliogenesis at the expense of maintaining neurogenesis for a longer period. Support for this interpretation is given by the observation that cortical progenitors of DREAM-mutant mice generate more neurons than those of control mice both *in vitro* and *in vivo* (Cebolla et al., 2008). But then, why are more astrocytes generated in the end?

The answer to this question may be related to the discovery that neurotrophic cytokines such as cardiotrophin-1 generated from neurons provide a powerful astrogliogenic signal (Barnabé-Heider et al., 2005). Thus, the proposed scenario would be that DREAM may have two different functions: on the one hand, it would inhibit neurogenesis at the end of the neurogenic period, and on the other hand, it would coordinately stimulate the generation of astrocytes from the same progenitor cells. This would explain that when DREAM is not present, neurogenesis would go on for a longer period of time generating an increased number of neurons at the expense of astrocytes. Later, neurotrophic cytokines secreted by these neurons would in turn act on the progenitors to inhibit neurogenesis and stimulate astrogliogenesis, and because of the initial overproduction of neurons these cytokines would be present in higher concentrations, thus resulting in a relative overproduction of astrocytes.

This model has two important implications. One is that lack of DREAM does not alter the gp130-JAK-STAT signaling pathway used by neurotrophic cytokines (Barnabé-Heider et al., 2005; Cebolla et al., 2008). The other is that during normal development, the astrogliogenic instructions delivered by the PACAP-cAMP-calcium-DREAM system precede those delivered by neuron-generated neurotrophic cytokines.

Of course, future studies are needed to confirm or reject these notions. In addition, although lack of DREAM appears to affect astrocyte development throughout the brain (Cebolla et al., 2008), it remains to be determined whether in different regions of the developing central nervous system the stimulation of GFAP promoter by DREAM is activated by PACAP, as it appears to be the case with cortical astrocytes, or by different cAMP-generating factors acting on neural progenitor cells.

Conclusions

Our understanding of the molecular mechanisms by which proliferating neuroepithelial progenitors differentiate into neurons and glial cells during brain development is increasing rapidly. The emerging scenario is one in which different types of processes acting at different levels, including epigenetic changes in chromatin, differential expression of transcription factors and specific interactions among them, as well as coordinated activation of intracellular signaling cascades triggered by factors acting on the cell membrane, are tightly regulated. The studies described in this review support the notion that the PACAP-cAMP-calcium-DREAM cascade constitutes an important signaling pathway that participates in the regulation of the gliogenic switch and the generation of astrocytes in the developing brain (Fig. 2).

Based on these studies emerging questions arise. For example, what is the mechanism by which NFI facilitates CNTF- and PACAP-induced GFAP expression? Does DREAM directly interact with NFI or STAT proteins? How does DREAM participate in the regulation of the number of neurons generated? Is DREAM involved in the stimulation of GFAP expression observed in reactive astrocytes? These questions open new avenues of investigations that will contribute to elucidate the complex mechanisms by which cellular diversity is generated in the mammalian brain.

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FIGURE LEGENDS:

Figure 1. Schematic depiction of a simplified model to illustrate a possible role DREAM in the regulation of *GFAP* gene transcription. A) In a normal situation, DREAM is bound to a site located in close proximity to the site bound by NFI, so that it is possible that DREAM and NFI interact. In addition, it is possible that DREAM and NFI interact with other unidentified transcription factors (UTF) that are required for transcriptional activity (arrow 1). DREAM mediates transcriptional responses to PACAP/cAMP (arrow 2), whereas STAT proteins, which bind to promoter elements located upstream, mediate transcriptional responses to neurotrophic cytokines such as CNTF or cardiotrophin-1 (arrow 3). In addition, NFI binding is required for the synergistic cooperation between CNTF and PACAP, which may involve transcriptional interactions (arrow 4). B) Sequestration of NFI and possibly other UTF by DREAM would explain that mutation of the binding sites for DREAM abolish basal and stimulated activity of the *GFAP* promoter. C) In DREAM deficient mice, PACAP-dependent stimulation of *GFAP* transcription is inhibited, but the binding of other transcriptional transactivators remains unaltered (arrow 1) and gp130-JAK-STAT-induced promoter activity is intact (arrow 3). *In vivo*, this results in delayed astrogliogenesis as a result of the absence of the PACAP-DREAM-dependent signal. D) When the NFI binding site is mutated, stimulation of the gp130-JAK-STAT and PACAP-DREAM signaling pathways are intact (arrows 2 and 3), but synergistic cooperation cannot occur (arrow 4) and overall promoter activity is decreased.

Figure 2. Schematic depiction of the cAMP/calcium-dependent pathway activated by PACAP during astrocyte differentiation. PACAP acts on cortical precursors by activating seven transmembrane spanning domain PAC1 receptors. This results in the

stimulation of intracellular cAMP production by G-protein-mediated stimulation of adenylate cyclase (AC). Cyclic AMP activates PKA, Epac-Rap1 and Ras (probably by acting on CNrasGEF), but PKA does not contribute to stimulation of *GFAP* gene transcription during astrocyte differentiation. Exposure of precursor cells to PACAP results in calcium entry from the extracellular milieu, an effect that requires the activity of cAMP. Finally, calcium binds to DREAM, which occupies specific sites on the *GFAP* promoter, and following this *GFAP* gene transcription is stimulated. Independently of this pathway, *GFAP* transcription can also be stimulated by neurotrophic cytokines such as CNTF or cardiotrophin-1 (CT-1) via activation of the gp130-JAK-STAT pathway. Direct or indirect interactions between STAT proteins and DREAM, as described in Figure 1, could explain synergistic interactions of both pathways at the transcriptional level.

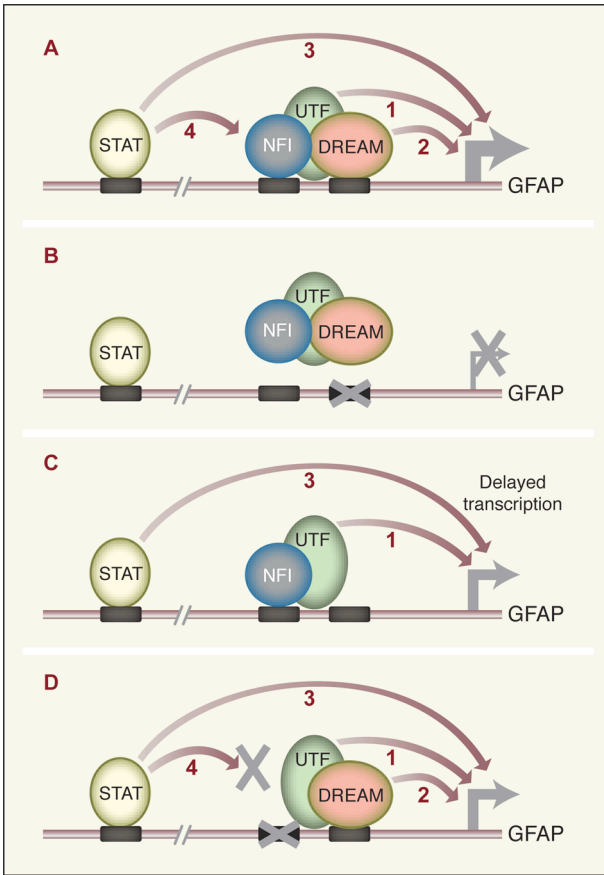


Figure 1

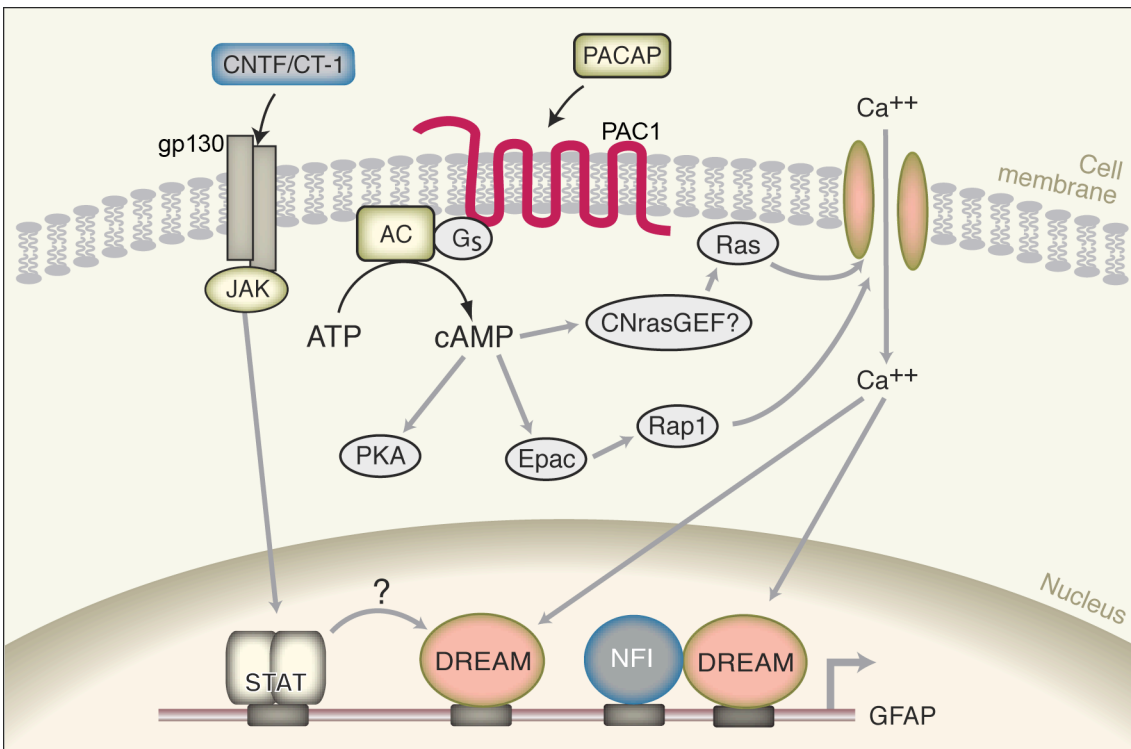


Figure 2