Role of EPAC in axon determination
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Chapter 1:
General Introduction

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General introduction

Neurons are highly polarized cells involved in the generation of functional networks. These networks are formed based on the molecular, structural, morphological and functional properties of axons and dendrites. This process begins early during development with the establishment of neuronal polarity. In the past 40 years, the breaking of neuronal symmetry and maintenance of polarity had been a fundamental question in neurobiology.

In vivo Neuronal polarization

The neuron as the basic unit of brain function, as well as neuronal polarization were first described by Santiago Ramón y Cajal as in his law of Dynamic polarization (Ramón y Cajal, 1914, 1954). This law establishes that each neuron has domains specifically involved in the reception and emission of signals allowing a vectorial flux of information between neurons. Since this first description about nerve cells, several works has been done in embryonic rodent brains to study the formation of the central nervous system (CNS); in particular, studying the neuronal migration. In this context, radial glia fibers play a key role in providing a temporary scaffold and enforcing restrictions in the developing CNS that facilitate neuronal migration and axon growth (McDermott et al., 2005).

In vivo polarization of mammalian neocortical pyramidal neurons starts when migrating neurons travel long distances from the germinal ventricular zone (VZ) toward the margin of the cerebral wall to form the primordial layer or preplate (PP), which is further split into the superficial marginal zone (MZ) and the deeper subplate (SP). Preplate splitting defines the margins of cortical plate (CP) formation, which develops in an ‘inside-out’ pattern: newly-arriving neurons
bypass the subplate, causing the newest neurons to migrate radially past their preceding neurons before stopping at the top of the CP (Berry and Rogers, 1965; Rakic, 1972; Gleeson and Walsh, 2000) (**Fig 1A**). This process arises at the cell-cycle exit from embryonic day (E) 11 to E18 in the mouse cortex (Lewis et al., 2013). Migrating neurons adopt a bipolar morphology with a leading process and exhibit a trailing process that will further develop as dendrites and axon, respectively (Calderon de Anda et al., 2008) (**Fig 1A**). Later on, axons extend rapidly to their final destination, guided by extracellular cues. Upon arrival to their targets, axons evolve into more complex morphologies due to axonal branching and are allowed to establish a presynaptic domain (**Fig 1B**). Thus, the sequence of events described accounts for the development of cortical and hippocampal pyramidal neurons, two of the best-studied models for neuronal polarization (Barnes and Polleux, 2009; Lewis et al., 2013). Axon specification and elongation is indeed determined by a combination of external signals from the extracellular environment and internal signaling pathways during active cell migration.

**In vitro neuronal polarization**

The study of in vitro neuronal polarity started with the establishment of cultured primary neurons derived from rodent hippocampi at the end of the 70’s by Banker and Cowan (Banker and Cowan, 1977). Later on, Dotti and colleagues described the morphological and morphometric events involved in the recapitulation of neuronal polarity, leading to a polarized cell displaying axonal and dendritic domains (Dotti et al., 1988).

Hippocampal culture is a well-suited model in physiological studies, as it allows for direct observation and manipulation of living neurons in a low-density
culture, which is ideal for the study of subcellular localization and trafficking of proteins coupled to fluorescence imaging, manipulation of DNA expression, and pharmacological treatments. (Banker and Cowan, 1977; Kaech and Banker, 2006).

A key advantage of cultured hippocampal neurons is that most of the cells are homogeneous, corresponding to pyramidal neurons, with little presence of glial cells. In addition, cultured pyramidal neurons phenotypically develop as in vivo neurons, showing defined axo-dendritic compartments and synapse formation (Banker and Cowan, 1977; Kaech and Banker, 2006).

Ex vivo cultured hippocampal neurons undergo dramatic morphological changes during their polarization, following a highly stereotyped sequence of developmental events that can be divided into five stages, as described by Dotti and Banker (Dotti et al., 1988): Stage I is defined soon after cells are plated on substrate (Banker and Cowan, 1977; Dotti et al., 1988; Arimura and Kaibuchi, 2007), where neurons form a continuous actin-based structure all along the cell perimeter. This actin-dependent structure is composed of a lamellipodia (thin sheets of cytoplasm containing networks of actin filaments that have their fast growing bordering the membrane) (Koestler et al., 2008) and extends around the cell body and several filopodia (thin finger-like structures projecting from the plasma-membrane, which are composed of parallel bundles of filamentous [F]-actin) (Mattila and Lappalainen, 2008) (Fig. 2). After 6-18 hours, this lamella coalesces at distinct spots around the cell periphery, leading to the protrusion of several “minor neurites” of 20-30 μm in length. These minor neurites undergo intermittent growth and retraction over short distances, corresponding to Stage 2
of neuronal development (Dotti et al., 1988; Barnes and Polleux, 2009; Polleux and Snider, 2010). At this stage, neurite tips are decorated with large growth cones that are important for motility. Cells remain in Stage 2 up to 36 h with subtle net growth of neurites, leading to a symmetric appearance, where all neurites possess the capacity to become axons or dendrites (Dotti et al., 1988; Kaech and Banker, 2006; Polleux and Snider, 2010) (Fig 2). Stage 3 starts during the second day after plating when one of the minor neurites begins to extend more rapidly and continuously than its siblings, becoming two to three times longer than other neurites. This fast-growing neurite is the axon; the other neurites undergo brief bursts of slow growth and retraction and will further acquire a dendritic identity (Dotti et al., 1988; Kaech and Banker, 2006; Barnes and Polleux, 2009; Polleux and Snider, 2010). The transition between Stage 2 and Stage 3 is a critical hallmark for neuronal polarization, since it is the initial break in symmetry during neuronal development (Craig and Banker, 1994; Bradke and Dotti, 2000). The transition from Stage 2 to Stage 3 does not occur synchronously across the cell population; half of the neurons in culture under optimal conditions reach Stage 3, 24 h after plating, with 80% of all cells reaching this stage after 36-48h. (Kaech and Banker, 2006). Interestingly, such a transition is the focus for most drug treatments or neuron manipulation to assess whether these experimental variations affect development of neuronal polarity (Fig 2). After 3–4 days in culture, neurons can be classified as Stage 4, where remaining neurites grow and branch acquiring the morphological characteristic of dendrites (shorter and thicker than axons) (Polleux and Snider, 2010; Caceres et al., 2012). At this stage, axonal and dendritic proteins are segregated, indicating molecular
polarization in axons and dendrites (Dotti et al., 1988; Kaech and Banker, 2006; Barnes and Polleux, 2009; Polleux and Snider, 2010) (Fig 2). Molecular changes distinctive of somatodendritic morphology in Stage 3 and Stage 4 are more easily observed by immunostaining with antibodies against microtubule-associated proteins (MAPs), such as MAP2 (Kosik and Finch, 1987); while axonal identity is observed using antibodies against posttranslational modification on Tau-1 (Mandell and Banker, 1996) and MAP1B (Johnstone et al., 1997). Stage 5 is reached by 7 days in culture (Kaech and Banker, 2006). During this process, dendrites become highly branched and establish dendritic components to construct premature dendritic spines, which lead neurons to form an extensive network of synaptic contact (Dotti et al., 1988; Polleux and Snider, 2010; Caceres et al., 2012). In addition, at Stage 5, the assembly of the axonal initial segment, a region where action potentials originate (Kole and Stuart, 2012), indicate the neuronal maturation required to allow the transmission of electrical activity (Segal, 1983; Bartlett and Banker, 1984) (Fig 2).

The development of neuronal polarity in cultured cells is very consistent from laboratory to laboratory. Moreover, primary culture of neurons has been used over the last 30 years to study several important processes in neurobiology. The use of continuous (clonal) cell lines, derived from the central nervous system, to study axonal determination is not advisable, since these cells do not form bona fide axons and dendrites, which are further required for proper synapse formation.
Extracellular signaling during neuronal polarization

Over the last years, several extracellular and environmental cues have been identified, improving the knowledge on the signaling pathways involved in neuronal polarity both in vitro and in vivo. In this section, we will present an overview of the main mechanisms regulated by mechanotrophic environmental signals and particular extracellular ligands as regulators of neuronal polarity. Some of the studies that address the generation of neuronal polarity have been done in the nematode Caenorhabditis elegans (C. elegans), due to the stereotyped nature of their neuronal morphology and its facility for genetic manipulation and lineage studies (Quinn and Wadsworth, 2008). This is exemplified by studies assessing the role of the diffusible signal, UNC-6 (mammalian ortholog, netrin) and its receptor UNC-40 (mammalian ortholog, DCC). UNC-6 induced neuronal polarization gives spatial information for axonal formation as an attracting-guidance signal during cell migration in developmental brain (Adler et al., 2006; Killeen and Sybingco, 2008). Concomitantly, another diffusible guidance signal termed Semaphorin 3A (Sema-3A) and its receptor plexin regulate asymmetric growth of cortical neurons, acting as a repellent for axons and an attractant for apical dendrites (Whitford et al., 2002; Dent et al., 2004). In addition, the identification of another diffusible signal, termed Lin-44 (mammalian ortholog, Wnt) and its receptor Lin-17 (mammalian ortholog, Frizzled) have been reported to determine neuronal polarity and axon outgrowth, independent of their role in the planar cell polarity (PCP) response (Hilliard and Bargmann, 2006; Prasad and Clark, 2006; Arimura and Kaibuchi, 2007) (Fig 3B).
Development of neuronal polarity in mammals is also linked to discrete sources of extracellular cues, such as the family of neurotrophins that are involved in many different functions in the nervous system, such as survival, neural development, and neuronal functions in both the central (CNS) and peripheral nervous systems (PNS) (Huang and Reichardt, 2001, 2003). For instance, cultured neurons secrete neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) through autocrine and paracrine mechanisms. Local application of these factors on immature neurites induces axon specification through tropomyosin receptor kinases (Trks) (Nakamuta et al., 2011a). A similar approach was performed by Shelly and colleagues (Shelly et al., 2007) in neurites from cultured neurons growing on patterned substrate or in contact with coated-beads with BDNF, become axons. In these experiments, when two neurites had contact to a BDNF-source, both become axons, leading to neurons bearing multiples axons. (Shelly et al., 2007). The mechanism by which BDNF signaling generated these changes in neurons may be related to a self-amplifying autocrine response, triggered by two nested positive-feedback mechanisms: First, BDNF elevates cytoplasmic cAMP and protein kinase A activity, which triggers further secretion of BDNF and membrane insertion of its receptor, TrkB. Second, BDNF/TrkB signaling activates PI3-kinase, which promotes anterograde transport of TrkB in the putative axon, further enhancing local BDNF/TrkB signaling (Cheng et al., 2011b). In cultured neurons, BDNF and other extracellular signals such as Wnts, the insulin-like growth factor-1 (IGF-1) or the transforming growth factor beta (TGF- β), may act in a paracrine mode as well (Nakamuta et al., 2011a; Cheng and Poo, 2012). This can induce, for
example, activation of the PI3K signaling in neighbouring neurons, suggesting that these cues likely work collectively in determining neuronal polarity. Furthermore, genetic deletion or chemical inhibition in any single factor is unlikely to be significant in neuronal development alone, further supporting the idea that a culmination of factors is ultimately responsible for the end determination of neurons (Cheng and Poo, 2012). These results suggest that the break in symmetry likely involves a specific amplification signal, one that requires contribution from multiple factors. It is noteworthy that local contact of a neurite with any of these signals is enough to generate an axon and impair the development of this structure in the remaining neurites (Nakamuta et al., 2011a; Cheng and Poo, 2012) (Fig 3B).

In addition to secreted molecules, cultured neurons are influenced by the extracellular matrix and cell adhesion molecules, which induce mechanical action from the surrounding environment in the neurons. Contact of immature neurites with extracellular matrix proteins, such as laminin or neuron-glia cell adhesion molecule (NgCAM) (Esch et al., 2000; Barnes and Polleux, 2009) promote axonal specification or enhance neurite outgrowth, both in vitro and in vivo, suggesting that neurons detect changes in the composition of extracellular substrate (Esch et al., 1999; Menager et al., 2004).

However, cultured neurons are preferentially incubated on substrate such as poly-L or poly-D-lysine, which promote cell adhesion through their ionic interactions (Kaech and Banker, 2006) with neurons, allowing for similar actions of an in vivo environment, such as sensing, transduction, and cellular and molecular responses. Therefore, the extracellular information is translated into
biological responses that produce intracellular signals, such as the second messenger cascade, leading to changes in protein synthesis, gene expression, or cytoskeleton dynamics; thereby regulating the establishment of neuronal polarity.

**Intracellular signaling of neuronal polarization**

The establishment of a polarized morphology requires two active and complementary phenomena: a positive feedback that permits the selective elongation of a single neurite that will later develop as the axon, and a negative signal that prevents the growth of the remaining neurites. This has been proposed as the principle of self-organization in neurons (Turing, 1990; Cheng and Poo, 2012). This principle predicts that a cell can initiate internal molecular changes that trigger the appearance of polarity by using a combination of an enhanced local-activator that acts through positive feedback inside the cell, and a global long-range-inhibitor (Arimura and Kaibuchi, 2007; Hutchins, 2010; Toriyama et al., 2010; Cheng and Poo, 2012) (Fig 3A). Moreover this signal should involve at least four main steps to induce neurite outgrowth or axon specification; First, it may modify the amount of plasma membrane recruited by vesicle fusion. Second, it may alter the local concentration and activation of signalling molecules. Third, it should trigger an increase of actin dynamics and microtubule polymerization, (Andersen and Bi, 2000; Arimura and Kaibuchi, 2007). And forth, there may be an opposite reaction induced by a global inhibitor, which precludes the growth of other minor neurites (Naoki et al., 2011) (Fig 3A).

The fact that neurons can spontaneously polarize in the absence of an asymmetric signal suggests that there exists an intrinsic principle of self-
organization, which is central to the establishment of neuronal polarity. In addition, it may reflect preservation of an asymmetry determinant, which is conserved after tissue dissociation to promote axon regrowth in plated cells (Menchon et al., 2011; Pollarolo et al., 2011; Caceres et al., 2012).

Amongst the several intracellular signaling cascades involved in the generation of neuronal asymmetry, the PI3K-Akt-GSK3\(\beta\) axis is a key player in promoting neuronal polarity.

Phosphatidylinositol-3 kinase (PI3K) regulates multiple biological functions, including gene expression, survival, establishment of cell polarity, and axonal specification (Shi et al., 2003; Manning and Cantley, 2007; Barnes and Polleux, 2009). PI3K is activated by upstream regulator proteins, such as Ras (Huang and Reichardt, 2003; Yoshimura et al., 2006a; Yoshimura et al., 2006b), or the insulin receptor, substrate-1 (IRS-1) (Yamada et al., 1997) in response to neurotrophic factors such as BDNF or NT3. Active PI3K triggers phosphorylation of phosphatidylinositol 4,5- bisphosphate (PIP2), producing phospholipid phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) and inositol 1,4,5-trisphosphate (InsP3). Next, PIP3 is concentrated in the plasma membrane of the tip of the axon during Stage 3, promoting neurite outgrowth and axon specification (Menager et al., 2004). PIP3 activates the phosphoinositide-dependent kinase (PDK), which phosphorylates and activates Akt [also known as protein kinase B (PKB)] (Burgering and Coffer, 1995; Downes et al., 1997). Consequently, activated Akt phosphorylates and inactivates the glycogen synthase kinase-3b (GSK-3b). Inactivation of GSK-3b promotes dephosphorylation and activation of the microtubule assembly–promoting proteins, such as collapsin response
mediator protein-2 (CRMP-2) and Tau (Kim et al., 2006), enhancing axon specification. On the other hand, the deletion of the phosphatase and tensin homologue (PTEN) antagonize PI3K signaling by decreasing PIP3 levels at the tip of the neurites, thus disrupting development of polarity (Shi et al., 2003). PIP3 promotes interaction with proteins containing pleckstrin homology (PH) domains with a high affinity (Hyvonen et al., 1995), favoring the recruitment of such proteins to the plasma membrane. Interestingly, both the expression of constitutively active forms of PI3K or Akt induces the formation of multiple axons in cultured hippocampal neurons (Yoshimura et al., 2006a; Yoshimura et al., 2006b) (Fig 3B).

In addition to the PI3K-Akt- GSK-3b axis, there are many other signaling proteins that are important in promoting axon specification and elongation. In this context, the small GTPases have key roles. This family of proteins cycle between an active GTP-bound and an inactive GDP-bound state (Nobes and Hall, 1995), and its activity is mainly controlled by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide exchange inhibitors (GDIs) (Gonzalez-Billault et al., 2012).

The most studied small GTPases are the family of Rho GTPases and their effectors, which are involved in functions such as cytoskeletal and membrane dynamics, gene transcription, cell polarity, and cell cycle progression. More than 20 members have been identified, with RhoA (ras homolog gene family, member A), Cdc42 (cell division cycle 42) and Rac1 (ras-related C3 botulinum toxin substrate 1) being the most characterized members. Activation of Rac1 and Cdc42 induces neurite elongation, whereas activation of RhoA is associated with
inhibiting the formation of neurites (Sebok et al., 1999; Gonzalez-Billault et al., 2012). Rac1 and Cdc42 induce extensive protrusive activities that include the formation of lamellipodia and filopodia, respectively (Ridley et al., 1992; Nobes and Hall, 1995), while RhoA regulates stress fiber formation and cell contraction (Ridley et al., 1992; Nobes and Hall, 1995). More recent studies have revealed that small Rho GTPases, in association with factors that control their expression, activity, lifespan, or subcellular localization, act as “spatiotemporal signaling modules,” (Pertz, 2010) modifying microtubule organization, dynamics, plus-end capture, and cross talk with actin-based structures, such as growth cone actin ribs and the subcortical cytoskeleton (Paglini et al., 1998; Li and Gundersen, 2008; Lowery and Van Vactor, 2009). Although it has been proposed that there are mutually exclusive and opposite roles for Rho and Rac/Cdc42 family members, this vision has started to change with the development of biosensors to monitor spatio/temporal changes in GTPase activity (Gonzalez-Billault et al., 2012) (Fig 3B).

Aside from the Rho GTPases, there is another family of GTPases involved in neuronal polarity. The Ras proteins (H-Ras, K-Ras, N-Ras and R-Ras), which are small GTPases that regulate cell growth and differentiation (Hancock, 2003), are also reported to be activators of PI3K (Yoshimura et al., 2006b; Oinuma et al., 2007) during neuronal polarization. Active Ras interacts with several effector proteins; the best characterized are PI3K and Raf (Vojtek et al., 1993). Overexpression of wild-type Ras induced multiple axons in cultured hippocampal neurons, whereas ectopically expressed dominant-negative Ras inhibited
axon formation (Yoshimura et al., 2006a; Yoshimura et al., 2006b; Oinuma et al., 2007) (Fig 3B).

A member of the Ras subfamily of GTPases, Ras-related protein 1B (Rap1B) in hippocampal neurons localize to a single neurite during development, promoting the specification of the future axon. Rap1B is initially present in all neurites of unpolarized neurons, but becomes accumulated to a single neurite during neuronal polarization (Schwamborn and Puschel, 2004). Interestingly, Rap1B function depends on PIP3 and seems to act upstream of Cdc42 and the Par complex, presumably via activating a Cdc42-GEF (Schwamborn et al., 2007). At present, the identity of the GEF protein involved in Rap1B activation remains elusive (Fig 3B).

The tripartite complex, Par6/Par3/aPKC, which is conserved by many species, from worms to vertebrates, is essential for the determination of axons. The complex is formed by two scaffold proteins, Par6 and Par3, which interact with several proteins involved in cell polarization, such as atypical forms of protein kinase C (aPKC: PKCλ and ζ) (Lin et al., 2000; Qiu et al., 2000; Etienne-Manneville and Hall, 2001); the kinesin motor protein KIF3A (Nishimura et al., 2004); the guanine exchange factor Tiam1 (Chen and Macara, 2005; Nishimura et al., 2005); the lipid and protein phosphatase PTEN (von Stein et al., 2005); the tumor suppressor lethal giant larvae (lgl) (Plant et al., 2003); the ubiquitin ligases Smurf1 (Ozdamar et al., 2005a; Cheng et al., 2011a) and Smurf2 (Schwamborn et al., 2007); the transforming growth factor receptor 1 (TGF β R1) (Ozdamar et al., 2005a); and the active version of Cdc42 (Schwamborn and Puschel, 2004; Warner et al., 2010). Each of these proteins
has been implicated in controlling neuronal and cell polarity as being part of the Par3/Par6 complex (Fig 3B).

Several studies show that in hippocampal neurons, Par proteins and aPKC are concentrated at the nascent tip of the extending axon in Stage 3. Moreover, inhibition of aPKC activity prevents axon formation (Shi et al., 2003) whereas phosphorylated (active) aPKC decorates the tips of growing axons (Schwamborn and Puschel, 2004). Furthermore, the Par complex is regulated downstream of PI3K, since inhibitors of PI3K prevent polarization and cause mislocalization of Par3 and Par6. This indicates that the correct localization and activity of the Par complex is necessary for a normal polarization (Shi et al., 2003).

The second messenger cAMP and its role in neuronal polarity

Cyclic adenosine monophosphate (cAMP) is a second messenger that is produced by activation of membrane-bound or soluble adenylyl ciclases (ACs). Its production is generally initiated upon the binding of extracellular ligands to Gs protein-coupled receptors (Simonds, 1999; Beavo and Brunton, 2002) (Fig 4). cAMP regulates fundamental physiological processes, including metabolism, secretion, calcium homeostasis, muscle contraction, cell fate, gene transcription, development in posmitotic neurons, and neuronal regeneration (Schmidt et al., 2013) (Fig 4). Shelly et al (2010) showed that local changes in cAMP signaling promotes axonal growth concomitantly with a long distance decrease in cAMP concentration on minor neurites (Fig. 5) (Hutchins, 2010). A mechanism involving cAMP-dependent regulation had been proposed in hippocampal neurons. Elevated local levels of cAMP can activate PKA, which can modify two different
and complementary molecular events. PKA-dependent phosphorylation stabilizes and allows the accumulation of LKB1, an early event involved in axonal differentiation (Shelly and Poo, 2011). Moreover, PKA-dependent phosphorylation of Smurf1 directs the selective degradation of Par6 or RhoA in neurons (Cheng et al., 2011a).

During neuronal differentiation, neurons must extend their axons to distant regions inside nervous system, in a very well controlled, topographic manner. cAMP-PKA signaling is very important since it is involved in axonal guidance and neuronal migration processes during nervous system development (Murray and Shewan, 2008; Murray et al., 2009). Most of the actions triggered by changes in the concentration of cAMP inside cells had been historically linked to changes in the activity of PKA, its main effector protein. However, this notion began to change with the discovery of a family of novel cAMP effector proteins, exchange proteins directly activated by cAMP (EPAC) (de Rooij et al., 1998). The EPAC protein family is comprised of EPAC1 (cAMP-GEF-I) and EPAC2 (cAMP-GEF-II), which are guanine nucleotide exchange factors for the monomeric G proteins, Rap1 and Rap2 (de Rooij et al., 1998; Kawasaki et al., 1998a; Kawasaki et al., 1998c). These proteins have a cAMP-affinity similar to the PKA holoenzyme, suggesting that both effectors may respond to similarly to physiological concentrations of this second messenger (Dao et al., 2006).

EPAC and PKA may not be the only effector molecules acting downstream of cAMP signaling. A member of the A-kinase anchoring protein (AKAP) family expressed in the brain, namely AKAP150, may be important in the fine-tuning of the local concentration of cAMP in neurons (Moita et al., 2002). AKAP150 levels
are also regulated during the development of the nervous system, being lower during embryogenesis, but increasing simultaneously with dendritic spine formation and maturation (Robertson et al., 2009). Interestingly, AKAP150 may provide a platform to integrate cAMP signaling with other signaling cascades, such as the PKB/Akt signaling (Nijholt et al., 2008) (Fig 4).

In addition, cAMP microdomains inside the cell are facilitated by local phosphodiesterase (PDE) pools, this cAMP-specific PDEs (PDE4, PDE7, and PDE8) regulate cAMP and limite its diffusion into the cell by degradation (Xu et al., 2011). The PDEs, together with the scaffold protein AKAP, modify the cAMP signalling in time and space, localizing and facilitating crosstalks between its effectors (Fig 4).

**EPAC: a new mediator of cAMP-signaling dependent.**

The discovery of EPAC1 and EPAC2 has profoundly altered the prevailing idea on cAMP signaling, which had historically been associated only with PKA. The analysis of molecular mechanisms related to EPAC signaling, has shown that the EPAC family regulates many physiological processes (Grandoch et al., 2010b; Schmidt et al., 2013), such as apoptosis, cell adhesion, control of insulin secretion (Schmidt et al., 2013), neurotransmitter release (Gekel and Neher, 2008; Ster et al., 2009), axonal guidance (Murray et al., 2009) and growth of neurites in dorsal ganglion neurons (Murray and Shewan, 2008). EPAC1 and EPAC2 are proteins containing multiple domains, which account for an N-terminal regulatory region and a C-terminal catalytic region. The regulatory domain presents in the cAMP-binding site, which autoinhibits its catalytic activity in absence of cAMP (Bos, 2003, 2006) (Fig 6A, B). The catalytic region is
conserved between both EPAC isoforms and contains the GEF activity that specifically activates Rap1 (Fig 6A). Nevertheless, EPAC2 contains a second cAMP-binding domain (domain A) in its amino terminal area (Bos, 2003, 2006). EPAC1 and EPAC2 proteins contain a Dishevelled/Egl-10/Pleckstrin (DEP) domain, which would explain the binding of EPAC to the plasma membrane, and a Ras exchanger motif (REM) that stabilizes the CDC25 domain acting as an intramolecular bridge between the catalytic and regulatory region (Bos, 2003, 2006) (Fig 6B). In addition, EPAC has a Ras association domain (RA), which is present in several proteins and interacts with active Ras (Fig 6B).

Although both EPAC1 and 2 present a RA domain, to date, only EPAC2 shows association with Ras, which results in a different subcellular location of EPAC2 (de Rooij et al., 2000; Bos, 2003) (Fig 6B). EPAC1 and EPAC2 exhibit a distinct expression pattern in mature and developing tissues (Schmidt et al., 2013). EPAC1 is expressed ubiquitously (thyroid, kidney, ovary, skeletal muscle and specific brain regions such as the septum and thalamus) (Kawasaki et al., 1998a; Kawasaki et al., 1998c; Bos, 2003, 2006) and to a greater extent, in embryos (Murray and Shewan, 2008). Meanwhile; EPAC2 is expressed predominantly in the adult nervous system, mainly in the brain cortex, hippocampus (specially CA3 and dentate gyrus), habenula, and cerebellum, as well in the adrenal gland (Kawasaki et al., 1998a; Kawasaki et al., 1998c; Gekel and Neher, 2008; Murray and Shewan, 2008; Niimura et al., 2009; Ster et al., 2009). However spatial and temporal differences in the expression of EPAC1 and 2 suggest functional redundancy between these two proteins (Murray and Shewan, 2008; Schmidt et al., 2013).
Since changes in the concentration of cAMP will ultimately modify the functions of PKA and EPAC proteins, many efforts had been made to generate pharmacological tools to discriminate and specifically target PKA or EPAC functions. These tools have been based on cell-permeable cAMP analogues, such as N6-benzyladenosine-3’, 5’-cyclic monophosphate (6-Bnz-cAMP, 6-Bnz) for PKA or 8-(4-chlorophenylthio)-2’-O-methyl-cAMP (8-pCPT-2'-O-Me-cAMP, 8-pCPT) (Fig 7B), for EPAC (Holz et al., 2008). Similarly, structural analogues of cAMP, such as Rp-8-CPT-cAMPS and PKI-(Myr-14-22)-amide (PKI) were designed to act as antagonists for PKA. Rp-8-CPT-cAMP inhibits the dissociation of PKA regulatory subunits upon binding to cAMP. In contrast, PKI-(Myr-14-22)-amide (PKI) binds to the free catalytic subunit of PKA, preventing phosphorylation of PKA substrates (Dalton and Dewey, 2006). EPAC specific antagonists have just recently been developed (Tsalkova et al., 2012a; Tsalkova et al., 2012b; Chen et al., 2013). ESI-05 is a specific inhibitor for EPAC2 while ESI-09 can inhibit both EPAC1 and EPAC2 (Tsalkova et al., 2012a; Tsalkova et al., 2012b; Almahariq et al., 2013) (Fig 7C, D).

**EPAC as mediator of neuronal polarity**

Whether or not EPAC1 or EPAC2 has a role in the regulation of axon specification, the underlying molecular mechanisms should be dependent on their molecular target, Rap1, particularly Rap1B (Rehmann et al., 2008), since the localization of the active Rap1B at the distal end of a single neurite is a crucial step in determining which neurite becomes an axon. It was previously shown that the ubiquitin ligase Smurf2 mediates Rap1B degradation in its inactive GDP-form but activation of Rap1B at the tip of nascent axon would
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protect it against proteasomal degradation (Schwamborn et al., 2007). This evidence suggests that a necessary initial event would be the local activation of a Rap1B-GEF in the distal end of the neurite that will ultimately become the axon. EPAC proteins may serve this function.

Accordingly, Murray et al. (2009) evaluated the role of PKA and EPAC in growth cone responses (attraction and/or repulsion) mediated by axonal guidance signals (e.g Netrin-1 and MAG) and suggested that the response of attraction and repulsion are conducted by the differential activation of PKA or EPAC. One likely mechanism would involve differential activity for these proteins in events where cAMP levels are oscillating (Murray et al., 2009).

One of the earliest events contributing to axon specification is the localization and local activation of PI3K, which triggers accumulation of PIP3 at the membrane, leading to Akt kinase plasma membrane recruitment. Akt activation in the axon, in turn, induces inactivation of GSK3, providing a differential activity of GSK3 in axons and dendrites (Tahirovic and Bradke, 2009). A possible molecular link between PI3K and cAMP signaling is associated with differential effects on PKA and EPAC activation of Akt, since it has been shown that PKA and EPAC can modulate phosphorylation of Akt Ser-473. This occurs with opposite effects on the levels of phosphorylation in this residue, through mechanisms that are still unclear (Nijholt et al., 2008).

Molecular changes involved in neuronal polarization will ultimately modify cytoskeleton dynamics to provide the structural frame needed to support proper establishment of neuronal polarity. For instance, EPAC1 may interact with microtubule-associated proteins, particularly, the light chain (LC2) of MAP1A and
light chain (LC1) of MAP1B. LC1 interactions can increase the association between EPAC1 and cAMP and thus, the ability to activate Rap1 (Gupta and Yarwood, 2005; Borland et al., 2006). A similar interaction between LC1 and PKA has not been demonstrated. Moreover, the interaction between EPAC and MAP1B is interesting, as it has been demonstrated that MAP1B has a crucial role in the formation of the axon (Gonzalez-Billault et al., 2001; Gonzalez-Billault et al., 2005; Riederer, 2007). Furthermore, MAP1B interacts with Tiam1, a GEF for Rac1, contributing to axonal elongation (Montenegro-Venegas et al., 2010; Henriquez et al., 2012). Interestingly, EPAC1 activates Rac through the interaction between Rap1 and Rac GEFs, STEF, in CHO and Cos-1 cells and Tiam1 in pulmonary endothelial cells (HPAEC) (Maillet et al., 2003; Zaldua et al., 2007; Birukova et al., 2008). These antecedents suggest that a molecular interaction between MAP1B and EPAC would contribute to efficiently activating Rap1B.

In addition, EPAC has been studied in cell differentiation, using the neuroblastoma and neuro-endocrine cell lines, which are cellular Paradigms for neuronal differentiation. For instance, PC12 cells are robustly differentiated after EPAC activation by EPAC-selective agonist 8-pCPT, leading to a high and sustained activation of ERK1/2 (Kiermayer et al., 2005). In addition, EPAC1 is involved in SH-SY5Y (Birkeland et al., 2009) and PC6 cell differentiation, triggered by neurotrophic actions of the pituitary adenylate cyclase-activating polypeptide (PACAP) signaling. PACAP-dependent mechanism involves activation of ERK, p38 MAP kinase, and Rit signaling pathways (Shi et al., 2006; Monaghan et al., 2008).
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With all these antecedents, the aim of this work is to explore the role of EPAC proteins as a key player downstream of cAMP signaling during neuronal polarization.
Figure legends

Figure 1: In vivo polarization of cortical neurons; A) Establishment of mammalian neurons in vivo from E11-E18: axon-dendrite polarity of pyramidal neurons (blue cell) is derived from the polarized emergence of the trailing (TP) and leading processes (LP). B) At postnatal stages (P1-P21), pyramidal neurons acquire mature features such as the axon initial segment (AIS, yellow cartridge) and dendritic spines (gray protrusions) which are key for synaptic function. Ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), marginal zone (MZ), cortical plate (CP), White matter (WM). Numbers in B represent cortical layers, WM (Modified from (Barnes and Polleux, 2009))

Figure 2: Neuronal polarization in cultured hippocampal neurons: (A) Hippocampal neurons change from round cells bearing lamellipodia (Stage 1) into multipolar cells (Stage 2). One neurite extends rapidly to become the axon (Stage 3). The remaining shorter neurites will become dendrites (Stage 4). This is followed by formation of dendritic spines, synapses, and functional maturation and polarization (Stage 5). (B) Phase contrast images of hippocampal neurons in culture during stages of development; 1, 2, 3, 4 and 5. Scale bar in stages 1-4, 25 µm. (Modified from (Polleux and Snider, 2010) and (Kaecch and Banker, 2006))

Figure 3: A tentative model of the principle of self-organization and the coordination of extracellular and intracellular signaling in axon formation. (A) Local amplification mechanisms for axon formation have random fluctuations within cytoplasmic axon determinants and growth-promoting activities. In Stage 2, the initial fluctuation of a local activator (Positives regulation) in one of the neurites could be stabilized and amplified by a local autocatalytic process that
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generates the activator as well as by a long-range diffusible inhibitor (Negative regulation) that amplifies the local asymmetry, when this balance is upset (In Stage 3) by extracellular signals, auto-activation of receptors or adhesion molecules and by the recruitment of signaling molecules lead to spontaneous axon formation. (B) Overview of selected signaling pathways that may initiate neuronal polarization and axon specification. (Adapted from Arimura and Kaibuchi, 2007; Cheng and Poo, 2012; Lalli, 2012).

**Figure 4: Global cAMP signaling and its biological response:**

Generation of the second messenger cyclic AMP (cAMP) is initiated upon stimulation of G protein-coupled receptors through binding of appropriate ligands and subsequent activation of membrane-bound AC family members. Next to G protein-coupled receptors and ACs, cAMP-specific PDEs shape the cAMP gradient throughout the cell to maintain the spatiotemporal nature of cAMP signaling. EPAC may, act either alone or in concert with PKA regulating diverse biologic responses through Rap GTPases or other effectors. In addition, A-Kinase anchoring proteins (AKAPs) are signal-organizing molecules providing a molecular framework that orients kinase such as PKC and PKA towards selected substrates. Finally, cAMP-PKA signaling may amplify the biological response through a crosstalk with calcium signaling through the inositol 1,4,5-trisphosphate receptor (IP3R), releasing calcium from intracellular stores.
Figure 5: cAMP signals promote axonal growth.

(A) Postmigratory neuron with several undifferentiated neurites. One of these stochastically exhibits higher cAMP concentrations (at the top). (B) The neurite with high cAMP concentrations reinforces its own cAMP signaling through positive feedback and reduces cAMP concentrations in the other neurites. This higher cAMP concentration stimulates growth of the top neurite (which becomes the axon) leading a reduction in the other neurites (Adapted from (Hutchins, 2010).

Figura 6: EPAC Overview

The hypothetical model predicts equilibrium between active and inactive states of EPAC, both in the cAMP-bound state and nonbound state. Depicted in the model is the cAMP-B domain of EPAC1. (B) Multidomain structure of EPAC. Interaction Partners that determine both intracellular localization and activity of EPAC1 and EPAC2 are indicated such as small GTPase Ran and Ras. cAMP-A, low-affinity cAMP-binding site; PA, phosphatidic acid; RA, Ras association domain; REM, Ras exchange motif; ERM, (Ezrin, Radixin, Moesin); RANBP2, RAN-binding protein-2. (Adapted from (Schmidt et al., 2013))

Figure 7: EPAC-selective agonist and antagonist.

(A) Structure of the cAMP. (B) Structure of the superagonist 8-pCPT-2′-O-Me-cAMP for EPAC1 and EPAC2, which is cleaved into the active form by the action of esterases. (C) Structure of the selective antagonist for EPAC1 and EPAC2 which displays an at least 100-fold selectivity for EPAC proteins compared to PKA type II and I in in vitro assays. (D) Structure of EPAC2 antagonist, which displays an at least 100-fold selectivity for EPAC2 over EPAC1 and PKA type II
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and I in in vitro assays. (Adapted from (Bos, 2003; Tsalkova et al., 2012b; Almahariq et al., 2013; Schmidt et al., 2013)
Figures

Figure 1
Figure 2

A

<table>
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<tr>
<th>Days in culture</th>
<th>0.25</th>
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<th>1.5</th>
<th>4</th>
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<td>Stage 5</td>
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- Lamellipodial and filopodial protrusion
- Multiple immature neurite extension
- Breaking of symmetry: axon specification
- Axon and dendrite outgrowth, branching
- Spine morphogenesis, synapse formation

B

Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | Stage 5

Scale bars: 10 μm
Figure 3

A

Negative regulation
- Membrane elimination
- Degradation of proteins
- Decrease in dynamics of F-actin
- Microtubule catastrophe

Retraction
- Phosphatase
- Rho GAP

Extension
- Rho GTPases and GEF
- PI3K
- Centrosome

Stage 2
- Negative feedback signals

Stage 3
- Negative feedback signals

Positive regulation
- Membrane recruitment
- Protein transport
- Increase in dynamics of F-actin
- Microtubule assembly

Extracellular signals, receptors, adhesion molecules, transport of key regulators

B

Inhibition

Activation

Pathway details:
- GPCR
- Wnt
- Adhesion
- Wnt
- Ret
- PI3K
- AKT
- MAPK
- RhoA
- RhoB
- RhoC
- ROCK
- MRTF
- TnC
- Myosin

Membrane dynamics
- Actin dynamics
- F-actin dynamics
- Membrane delivery
- Cytoskeleton
- Cytoplasm
- Cell membrane

Cellular processes
- Differentiation
- Migration
- Growth
- Apoptosis
- Invasion
Figure 5
Figure 7

A: cAMP

B: 8-pCPT-2'-O-Me-cAMP
   EPAC1 and EPAC2 superagonist

C: ESI-09
   EPAC1 and EPAC2 antagonist

D: ESI-05
   EPAC2 antagonist
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Scope of the thesis

The establishment of a polarized morphology and functional specialization of different cellular compartments are essential for the differentiation of neurons. The second messenger cyclic adenosine monophosphate (cAMP) plays a central role in neuronal differentiation. EPAC is a cAMP effector, which suggests a possible role during neuronal polarization. In this thesis, we aim to study the specific role of EPAC and its mechanism downstream during neuronal polarity in hippocampal neurons.

In chapter 2, we present a review that focuses on the regulation of microtubule and actin cytoskeleton during neuronal morphogenesis by small GTPases, in particular the small RhoGTPase family members, which have emerged as crucial regulators of cytoskeletal dynamics. In this review, we will comprehensively analyse findings that support the participation of RhoA, Rac, Cdc42, and TC10 in different neuronal morphogenetic events.

In chapter 3, we focus on EPAC and the polarity complex (Par3/Par6/aPKC). First, we determined the expression and subcellular localization of both EPAC in culture of neuronal cells from mouse hippocampal tissue. Second, we measured the expression and subcellular distribution of the proteins that are involved in regulation of the polarity complex and the signalling upstream and downstream Rap1B. Third, we measured the effect on neuronal polarity with pharmacological treatments and investigated the role of EPAC, PKA, PI3K, and atypical PKC (a member of the polarity complex). Furthermore, we performed genetic modifications with a Par6 construct to relationship with the EPAC-Rap1B pathway. The last step was to conduct experiments in live cell
imaging, to analyse the dynamics of actin and the subcellular activation of the effector of EPAC, Rap1B.

In chapter 4, we discuss how both isoforms of EPAC (EPAC1 and EPAC2) are differentially expressed during the development of axons in primary neuronal cell culture from rats and mice. Furthermore, neurons expressing a constitutively active EPAC1 or treated with the EPAC-specific agonist, 8-pCPT were characterized by the presence of supernumerary axons and 8-pCPT treated-neurons show positive stain for mature axonal markers, which are required for the identity of the axon initial segment and trafficking of synaptic proteins along the axon. Moreover, the knockdown and knockout of EPAC1 induced a reduction in neuronal polarization. We also observed that the axonal regulation by EPAC during neuronal polarity is related with its effector downstream, Rap1B. Finally, we determined that cAMP-EPAC signaling alters the neuronal polarity PKA independent, thereby providing alternative and complementary mechanism for PKA in the development of the axon.

In chapter 5, we describe a possible role of cAMP compartmentalization by AKAPs in neuronal processes, such as learning and memory; and the physiological events that could involve AKAP in pathological neurodegenerative diseases.

In chapter 6, we study neuronal differentiation in the N1E-115 neuroblastoma cell line under cAMP analog treatment such as Dybuturyl-cAMP. Our focus was to study some neuronal cytoskeleton associated proteins and the cAMP players: EPAC, PKA, and AKAP. We showed that the differentiation of N1E115 is higher in the presence of Dibutyryl-cAMP (DB-cAMP). Analysis of the
expression of microtubule regulating proteins in the differentiated cells revealed that most of the microtubules promoting factors were up regulated during neuronal differentiation. Additionally, we found the activation of PKA and EPAC synergistically induced neuronal differentiation. Moreover, inhibition of EPAC and disruption of the interaction PKA-AKAP reduced the effect induced by DB-cAMP. Altogether, these results show that changes during neuronal differentiation involve both cAMP players, in particular EPAC and Rap1B. Additionally, these two cAMP players likely have a similar role during this process, as was shown in neurons. Finally, the neuronal differentiation was accompanied by microtubule stabilizing factors, which suggests a connection between cAMP signaling and these proteins.

In chapter 7, we discuss about the role of EPAC in neuronal polarization and the future perspective of this work.
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General Introduction


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