Chapter 7:
General discussion and summary

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In this thesis, we propose EPAC is the GEF involved in Rap1B-dependent axon formation during neuronal polarization. Previously it was demonstrated that embryonic rat neurons display higher levels of cAMP than postnatal neurons (Cai et al., 2001). Similarly, cAMP levels decline with age in developing frog retinal axons. These axons are initially attracted to netrin-1, but in later stages of development are repelled (Shewan et al., 2002). Therefore, our first goal was to evaluate potential changes in the overall intracellular cAMP levels in culture neurons (chapter 3). Our results showed no significant variations amongst neurons on different stages of neuronal polarization. Our finding is most likely related to the fact that local cAMP changes may not be monitored using whole crude protein extracts as biochemical approach. Local quantification of cAMP based on FRET probes present advantages related to spatio-temporal measurements in discrete domains in neurons, and could be useful in overcoming the apparent lack of sensitivity of our biochemical approach. In any case, the slight increase of cAMP observed at Stage 2 could activate downstream signalling involved in breaking of neuronal symmetry. Further experiments using a novel EPAC-based cAMP FRET sensor are needed to elucidate this point (Klarenbeek and Jalink, 2014).

Rap1B activation is an essential step in determining neuronal polarity (Schwamborn and Puschel, 2004). Its function is related to a specific accumulation into a single neurite. However, the mechanisms that need to act concertedly to lead to Rap1B activation at the tip of the axon are not yet clear. Since Rap1B is a small GTPase, this mechanism relies on a cyclic transition
between active-GTP and inactive-GDP forms. It follows that accumulation of Rap1B-GTP will be dependent on the activity of specific Rap1B-GEF and/or Rap1GAPs (Bos et al., 2001).

Our data suggest that EPAC1 is well positioned to be the GEF involved in Rap1B activation during neuronal polarization due to the following findings: (1) Similar to other proteins involved in neuronal polarization, EPAC1 is present at the growth cones of every neurite at stage II, but becomes restricted to the axon in Stage 3 (chapter 3, 4); (2) The use of a specific agonist for EPAC resulted in the formation of neurons displaying multiple axons, showing increased spatio-temporal activation of endogenous Rap1B-GTP (Bivona et al., 2004) (chapter 4); (3) Constitutively, active forms of EPAC1 also induce the formation of supernumerary axons in cultured neurons (chapter 4); and (4) Selective inhibition, knockdown and knockout of EPAC1 inhibits axon formation in cultured neurons (chapter 4). Noteworthy, the same phenomenon was observed in the N1E-115 neuroblastoma cell line (chapter 6), when we activated EPAC with pharmacological tools, the N1E-115 acquired a neuronal phenotype, when we inhibited EPAC, the process of neuronal differenciation induced by cAMP was reversed.

In this study, we used an improved form of an EPAC activator (8-pCPT) that efficiently drives Rap1B activation in vitro and in vivo, without any detectable effect on PKA (Enserink et al., 2002; Rehmann et al., 2003a). The presence of multiple neurites longer than their siblings showing positive Tau-1 and MAP1B staining and negative MAP2 staining highly suggests these neurites are axons (chapter 4). Under our experimental conditions, we observed neurons displaying
more than one axon initial segment, characterized by AnkG staining. AnkG clustering is required to maintain neuronal polarity and recruit other proteins to the AIS and form a diffusion barrier (Rasband, 2010; Galiano et al., 2012), which is involved in the generation of action potentials. Secondly, neurons displaying multiple axons show other mature axonal markers, such as VGLUT1 and synaptophysin, which are involved in synapse formation. VGLUT1 expression is observed in postnatal and hippocampal neurons at 7-14 DIV with a characteristic puncta distribution along the axon (Melo et al., 2013), which is similar to the VGLUT1-GFP construct presented here (Wilson et al., 2005). Synaptophysin is a synaptic vesicle protein that distributes along the axon, forming larges puncta structures that correspond to dense accumulation of vesicles within presynaptic specializations (Fletcher et al., 1991b). Synaptophysin clusters were present in supernumerary axons, suggesting that neurons with exacerbated polarity could be functional. Our results are consistent with previous evidence, which assesses the molecular identity of multiples axons, also by means of synaptophysin staining and raising the possibility that the supernumerary axons can form synaptic contact (Inagaki et al., 2001). We also examined the effects of loss of EPAC function during neuronal polarization (chapter 4). Previous studies showed the effect of loss of Rap1A or Rap1B functions. For instance, knockdown of Rap1B by siRNA abrogates axon formation (Schwamborn and Puschel, 2004). Pharmacological and genetic EPAC1 inactivation blocked the presence of neurons displaying multiple axons and reduced average axonal length. Interestingly, axon formation was not impaired when we used dominant negative EPAC1 or Rap1GAP overexpression. Furthermore, the neurons did not show an
increment of EPAC2 in response to a genetic knockout of EPAC1. However, our finding that a dominant negative form of EPAC did not show the same effect could be linked to residual Rap activity, supporting normal polarization due to other Rap1B GEFs that can show functional redundancy to activate Rap1. For instance, C3G (Hisata et al., 2007) has been shown have a role in neuronal migration, since Reelin, a secreted extracellular matrix glycoprotein involved in neuronal migration, stimulates tyrosine phosphorylation of C3G and activates Rap1 (Ballif et al., 2004). Therefore, mutant mouse embryos lacking C3G present with neurons that have a multipolar morphology, affecting a proper migration during cortex development. This is likely a result of a lack of integrin and cadherin signalling in the absence of C3G (Voss et al., 2008; Franco et al., 2011). 

PDZ-GEF induced sustained activation of Rap1 at late endosomes and was involved in the NGF-induced neurite outgrowth in PC12 cells and the BDNF-induced axon outgrowth in rat hippocampal neurons, prolonging the activation time of the MAPK cascade which results in the up-regulation of gene expression (Hisata et al., 2007). CalDAG-GEFI, which has substrate specificity for Rap1A and dual binding domains for calcium and diacylglycerol (DAG), is required for striatal output neurons to respond to cholinergic cell activation in adult brain (Crittenden et al., 2010). This RapGEF is expressed in adult human brain hippocampi and has an enriched expression in rat brain basal ganglia pathways and their axon-terminal regions (Kawasaki et al., 1998c); although, there is currently no data about its role in hippocampal neurons. However, CalDAG-GEFI plays a role in neutrophil chemotaxis by a mechanism that involves F-actin distribution and cell polarization, a phenomenon shared with neuronal polarity (Carbo et al., 2010).
Additionaly, the role of EPAC2 in regulating dendritic spine morphogenesis, spine dynamics, glutamate receptor trafficking and motility in adult cortical neurons is of particular interest. These mechanisms involve a complex between the postsynaptic adhesion protein neuroligin-3 (NL3), PSD-95 that increases EPAC2 activation and Rap1B activity. Furthermore, our results suggest that EPAC2 is part of the cAMP effect in neuronal differentiation, since its inhibition reverses the differentiation caused by Db-cAMP (chapter 6). Therefore, a deep observation of EPAC2 and its interactor in neuronal polarization should be addressed.

Finally, another Ras family member, the small GTPase Rheb, and its target mTOR (mammalian target of rapamycin) may compensate EPAC inactivation since Rheb operates downstream of PI3K increasing translation of Rap1B in the axon (Li et al., 2008). Our data suggest that EPAC-Rap1B may be upstream of PI3K and PKC signalling (chapter 3). Future experiments should address the mechanism of these pathways in more detail.

Interestingly, in vivo experiments demonstrated that using the dominant negative form of Rap1A, Rap1A17N, or Rap1A with Rap1B knockdown does not prevent axonogenesis in cortical neurons (Jossin and Cooper, 2011). In contrast to the in vitro work performed by Schwamborn and colleagues (Schwamborn and Puschel, 2004) on hippocampal neurons; although other studies demonstrated the questionable use of Rap1AS17N as a dominant negative mutant in vivo (van den Berghe et al., 1997). Nevertheless, these differences could be related with differences in the experimental approaches on those studies.

Noteworthy, the activity of Cdc42 was not increased or reduced under 8-
pCPT or ESI-09 treatments that previously were shown to modified Rap1B activity (chapter 4), suggesting that the sequential activation of Rap1B and Cdc42 could be independent events (Schwamborn and Puschel, 2004). If, Rap1B and Cdc42 functions are not molecularly linked, what other mechanisms could be targeted by Rap1B activation and be involved in neuronal polarization? A possible answer to this question positions Rap1B activity upstream of a signalling pathway related to the Ras-like GTPase RalA signalling, which regulates neuronal polarity through the exocyst complex. This is involved in several polarization events in many cell types, such as bud growing in yeast, basolateral membrane delivery in epithelial cells and directed cell migration (He and Guo, 2009).

The exocyst complex is an evolutionarily conserved octameric protein complex (comprised of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p) and is required for vesicle targeting and tethering exocytic carriers to the plasma membrane in eukaryotic cells (Munson and Novick, 2006; Wang and Hsu, 2006) a critical process in cell polarization (Grindstaff et al., 1998b; Yeaman et al., 2001). In this context, the role of the exocyst during neuronal polarity is interestingly highlighted. Hence, it has been shown that exocyst subunits (the sec6/8 complex) are enriched in the growth cone and Participate in membrane addition and synaptogenesis in growing axons (Hazuka et al., 1999). Moreover, the exocyst complex has been involved with the IGF-1 receptor (IGF-1R), which binds IGF-1, a trophic factor required for axon formation in hippocampal neurons (Sosa et al., 2006). IGF-1 signalling activates the GTP-binding protein TC10, which triggers translocation to the plasma membrane of
the exocyst component exo70 in the distal axon and growth cone (Dupraz et al., 2009), controlling membrane expansion at the axonal growth via a cascade involving PI3K.

Besides the role of the exocyst in membrane addition, it has been shown that Rap1B may regulate this complex through RalA, a well-known small GTPase involved in membrane trafficking. Active RalA interact with the exocyst via Sec5 and is enriched in brain associated to synaptic vesicle. The Rap1 effector, RalGDS, activates RalA, which docks secretory vesicles to the exocyst complex and recycles E-Cadherin to epithelial cell-cell junctions (Grindstaff et al., 1998a). Moreover, in Drosophila neuroblasts, Rap1–Rgl–Ral signalling has been shown to regulate cortical polarity (Carmena et al., 2011) and Rap1B activation in the tip of developing axons leads to local activation of RalA in the same region, thereby accelerating membrane insertion and enhancing axon specification (Nakamura et al., 2013). Additionaly, RalA and the exocyst are involved in neuronal migration in cortical development though a mechanism that seems involved Rap1 (Jossin and Cooper, 2011).

The RalA-exocyst pathway was reported to regulate exocyst function in neurite branching (Lalli and Hall, 2005), and the exocyst accumulates at the tip of the future axon in Stage 3. Biochemical evidence shows a progressive interaction between the exocyst and the polarity complex during polarization, in Particular, with Par3 and aPKC (Lalli, 2009), depending on RalA activation (Das et al., 2014a).

Noteworthy, the Sec15 exocyst subunit colocalized selectively with the recycling endosome marker Rab11 and exhibited a GTP-dependent interaction
with Rab11 GTPase (Zhang et al., 2004). Rab11 regulates endosomal/plasma membrane interactions by controlling membrane traffic through recycling (Chen et al., 1998). Interestingly, BDNF regulates the dynamics of recycling endosomes by increasing the activity of Rab11 and recruiting Rab11-positive vesicles to dendrites. This higher activity of Rab11 led to increased dendritic branching and accumulation of TrkB in dendrites, enhancing sensitization to endogenous BDNF (Lazo et al., 2013). Interestingly, Rap1 has been shown to co-localize with E-cadherin at the Rab11-positive recycling endosome compartment (Balzac et al., 2005). Thus, the interaction of the Rap1-exocyst and BDNF-Rab11 pathway might open an alternative view to the local amplification mechanisms for axon formation proposed by Cheng et al. (Cheng et al., 2011b), thus representing another positive-feedback loop in promoting TrkB anterograde transport. A possible role of EPAC-Rap1B in the context of this process remains unknown. However, in our work, we showed an accumulation of RalGDS-GFP along the axon upon EPAC activation by 8-pCPT, which may give insight to the hypothesis of a regulation of the exorcyst in neuronal polarity probably through EPAC-Rap1B signalling.

In our work we used recently developed EPAC pharmacological inhibitors (chapter 4), which support the idea that EPAC1 is the GEF responsible for Rap1B activation. Our results are consistent with studies showing that ESI-09 is a potent EPAC inhibitor 100-fold selective on EPAC over PKA (Almahariq et al., 2013). Although it has been shown some in vitro artifacts with ESI-09, raising concerns about the selectivities for EPAC 1 and EPAC2, the concentration used in this work (15 μM) is far below those used in previous studies (Rehmann,
2013). A close analysis of working conditions used by Rhemann, revealed important differences from the work by Almahariq et al., who characterized ESI-09 in vitro and physiologically using Rap1 activity as a readout (Almahariq et al., 2013). It was shown that Rap1 activity is reduced in the presence of ESI-09, during pancreatic cancer cell migration, an effect that reproduced genetic inactivation using siRNA against EPAC. However, in the work of Rehman 2013, ESI-09 induces loss of exchange activity on EPAC over time, but not as a selective inhibitor of EPAC; ESI-09 competes with cAMP, having an affect on the catalytic CDC25-homology domain of EPAC directly, which likely explains its apparent inhibitory effects and impact on Rap1B activity. Nevertheless, recent studies show no physiological side effects of ESI-09. ESI-09 antagonizes myelin sheath formation and Schwann cell differentiation, which is linked to EPAC functions (Bacallao and Monje, 2013) and in vivo inhibition of EPAC with ESI-09 recapitulates the rickettsial infection effects found in EPAC1 knockout mice (Gong et al., 2013). Moreover, we used the EPAC1 inhibitor, CE3F4 (Courilleau et al., 2013) in N1E-115 cell lines during neuronal differentiation (chapter 6). We observed that the cells showed defects in differentiation when EPAC1 is inhibited, a future characterization of these tools will help to the study of EPAC. Nevertheless, all these evidence leads us to the conclusion that ESI-09 indeed reduced EPAC1 activity in neurons, although more details on the physiological effects of this compound are still to be discovered.

We cannot rule out the possibility that EPAC2 may also be involved in axonogenesis, since both EPAC1 and EPAC2 proteins are expressed throughout the brain, including in the hippocampus and cortex. However, our subcellular
distribution experiments and the differential effect of ESI-09 suggest that EPAC1 is most likely responsible for Rap1B activation during neuronal polarity, since its distribution and high level of expression in the transition between stage II and III is similar to other regulators and determinant of polarity that accumulate in a single neurite (chapter 3, 4). The accumulation of a molecule or a protein complex in a single neurite during the transition from a highly symmetric cell to a polarized neuron is a common molecular mechanism which is fulfilled by Rap1B (Schwamborn and Puschel, 2004), Cdc42 (Schwamborn and Puschel, 2004), Par complex (Shi et al., 2003), pAkt (Yan et al., 2006) and LKB1/pLKB1 (Shelly et al., 2007). It is accepted that the accumulation of proteins depends on their local turnover, positioning proteasome activity as a fundamental player to specifically enrich molecular determinants in neurons (Arimura and Kaibuchi, 2007).

Throughout our study, we focused our attention on EPAC functions. However, we also studied the cross talk between cAMP/PKA and cAMP/EPAC dependent pathways. PKA has been shown to regulate neuronal polarity by two complementatay mechanisms (chapter 3, 4). On the one hand, PKA phosphorylates Smurf1 in response to the neurotrophyn brain-derived neurotrophic factor (BDNF), reducing the degradation of a member of the polarity complex, Par6. Par6 is an axonal growth-promoter protein that increases the degradation of the smallGTPase RhoA, which is an axonal growth-inhibitor protein. On the other hand, PKA activates the liver kinase B1 (LKB1) that phosphorylates and activates the synapses of the amphid defective kinesin (SADK) and promotes axonogenesis. Our results show that EPAC activation with 8-pCPT did not affect PKA dependent signalling in treatments on the
neuroblastoma cell line, following the same criteria of Cheng’s work (2013). We found that levels of Par6 and Rhoa were not significantly different from the control cells, suggesting that the changes seen in gains and losses of function of the EPAC pathway correlated more with a pathway exclusively through Rap1B. Another point supporting the existence of an alternative cAMP pathway, dependent on EPAC, derives from the observation that the inhibition of PKI by synthetic peptide does not impair axon formation in rat neurons, and that the specific antagonist Rp-8-CPT-cAMPs does not impair axon formation in mouse neurons either (chapter 3, 4). We determine that 20 μM of PKI and Rp-8-CPT-cAMPs are enough to produce significant reductions on phosphorylation of total PKA-specific substrates alone or in the presence of 8-pCPT in neurons. In fact, we found neurons with multiple axons when we combined EPAC activation with PKA inhibition. Although concentrations of inhibitor and agonist for PKA were not sufficient to induce complete inhibition of PKA phosphorylation, we avoided using higher concentrations of PKA inhibitor. Higher concentrations may induce artifacts similar to those observed when using higher doses of other PKA inhibitors, such as H89 and KT5220 and cause unwanted side effect affecting PKC and ERK functions (Murray, 2008). Nevertheless, PKI induced morphological changes in neurons, impairing axonal elongation in rat hippocampal neurons. Interestingly, we found that neuronal differentiation in N1E-115 it can be drive by EPAC or PKA independently. Considering the evidence accumulated in this work, it seems reasonable to propose that cAMP signalling related to axonal determination and elongation is influenced by both EPAC and PKA, through two apparently independent mechanisms.
EPAC1 and EPAC2 bind cAMP with similar affinity as PKA holoenzyme, suggesting that both factors may respond to similar physiological concentrations of this second messenger (Dao et al., 2006). The concerted functions of EPAC and PKA signalling has been shown to be dependent on cellular context and processes (Grandoch et al., 2010a). Activation of EPAC-dependent pathways may target several molecules widely accepted to regulate axon formation and elongation such as, c-Jun N-terminal kinase (JNK) (Hochbaum et al., 2003; Oliva et al., 2006), the small GTPase Rit (Shi and Andres, 2005; Shi et al., 2006), the small GTPase Ras (Li et al., 2006; Lopez De Jesus et al., 2006; Yoshimura et al., 2006b) and Rho/Cdc42/Rac1 (Schwamborn and Puschel, 2004; Moon et al., 2013). An additional regulation point for such complementary mechanisms would be related to extracellular cues that trigger neuronal polarization, such as BNDF elevating cytoplasmic cAMP, leading to increased axonal elongation (Cheng et al., 2011b; Nakamuta et al., 2011a). In contrast, NGF is not able to trigger axon elongation in cultured neurons, despite the induction of the activation of C3G, another Rap1B-GEF (Nakamuta et al., 2011a). This reinforces present findings that position EPAC as the GEF involved in Rap1B activation during neuronal polarization. Finally, extrinsic or intrinsic mechanisms triggering neuronal polarity ultimately modify cytoskeleton dynamics. Further evidence should be addressed to study the impact of EPAC functions over Rho (Schwamborn and Puschel, 2004; Moon et al., 2013), Rac (Lin et al., 2000; Nishimura et al., 2005; Zaldua et al., 2007) and the polarity complex Par3/Par6/aPKC in hippocampal neurons.

In summary, we found a possible role of EPAC in axon determination, suggesting that their activity might be required during polarization in neuronal polarity.
thorough Rap1B presenting EPAC1 as the GEF of Rap1B. This could protect from Smurf2 degradation during polarization, activating Rap1B in the tip of the axon. However, the sequential activation of Rap1B and Cdc42 in neuronal polarity is likely local and not global, since we could not observe any changes in the activity of Cdc42. We think that an alternative pathway downstream of EPAC-Rap1B might be the exocyst complex involving the smallGTPase RalA, thereby providing another link to membrane addition and cAMP signalling during axon elongation. Furthermore, better tools are needed to elucidate the role of PKA inhibition in neuronal polarity. Figure 1 in this section shows the tentative model of EPAC in neuronal polarity.

To our knowledge, this is the first evidence of a molecular mechanism explaining Rap1B activation during neuronal polarity, which, in addition, links two complementary signalling cascades activated by cAMP.
Figure legend

Figure 1: cAMP-EPAC signalling pathway in axon formation:

In response to the activation of cell surface receptors by neutrophins or extracellular ligands, the levels of cAMP becomes increased, activating EPAC1 and PKA. EPAC activated Rap1B locally, which regulates axon formation via the exocyst complex by RaIA or the polarity complex trough Cdc42. Complementary PKA regulates axon formation trough Smurf1 and LKB1. Both pathways work together in cytoskeleton dynamics and membrane delivery to form the axon. Black question marks indicate that the mechanism by which these receptors increase cAMP levels is unclear. The red question mark indicates that the mechanism that induced polarization after PKA inhibition is unclear.
Figure 1
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