Role of EPAC in axon determination

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Henry David Thoreau

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LIST OF ABBREVIATIONS

AC:	Adenylate cyclase
AKAP:	A-kinase anchoring protein
AMP:	Adenosine monophosphate
AMPK:	5' AMP-activated protein kinase
AnkG	Ankyrin G
ATP:	Adenosín trifosfato
BDNF:	Brain-derived neurotrophic factor
BSA:	Bovine serum albumi
C terminal:	Carboxy-terminal
cAMP:	Cyclic AMP
CA	Constituvely active
Ca2+ :	Calcium ion
CDC25:	Cell division cycle 25
Cdc42:	Cell division control protein 42 homolog
cDNA:	Complementary deoxyribonucleic acid
cGMP:	Guanosine 3'5' cyclic monophosphate
CNS:	Central nervous system
cNBD:	Cyclic-nucleotide-binding domain
CNG:	Cyclic nucleotide-gated
DAG:	Diacylglycerol
DEP:	Dishevelled, Egl-10, Pleckstrin domain
DMEM:	Dulbecco's modified Eagle Medium
DMSO:	Dimethylsulfoxide
DN	Dominant negative
E17/18:	Embryonic day 17/18
E. coli:	Escherichia coli
ECL:	Enhanced chemiluminiscence
EDTA:	Ethylenediamine tetraacetic acid
EGTA:	Ethylene glycol tetraacetic acid
EPAC:	Exchange protein directly activated by

	cAMP
ERK1/2:	Extracellular-signal-regulated kinases 1 / 2
ER:	Endoplasmatic reticulum
FRET:	Förster resonance energy transfer
GAP:	GTPase-Activating Proteins
GDP:	Guanosine diphosphate
GDI:	GDP-dissociation inhibitor
GEF:	Guanine nucleotide exchange factors
GFP:	Green fluorescent protein
GPCR:	G-protein coupled receptor
GST:	Glutathione S-transferase
GTP:	Guanine-triphosphate
GTPase:	Guanosine triphosphatases
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS:	Hanks balanced salt solution
HRP:	Horseradish peroxidase
IPTG:	Isopropyl-β-D-thiogalactopyranoside
kD:	Kilodalton
LKB1:	Liver kinase B1
MAP:	Microtubule associated protein
MEM:	Minimal essential medium
Min:	Minute(s)
mRNA:	Messenger ribonucleic acid
mTOR:	Mammalian Target of Rapamycin
N terminal:	Amino terminal
NGF:	Nerve growth factor
p:	Probability
PAGE:	Polyacrylamide gel Electrophoresis
PBS:	Phosphate buffered saline
PFA:	Paraformaldehyde

PAR3:	Partitioning defective 3 homolog
PAR6:	Partitioning defective 6 homolog
PDE:	Phosphodiesterase
PI3K:	Phosphatidylinositol 3-kinase
PKA:	PKA Protein kinase A
PKB:	Protein kinase B
PKD:	PKD Protein kinase D
PLC:	PLC phospholipase C
PM:	Plasma membrane
PVDF:	Polyvinylidene difluoride
RA:	Ras-association domain
Rac1:	Ras-related C3 botulinum toxin substrate 1
Rap1B:	Ras-related protein Rap-1B
RalGDS:	Ral guanine nucleotide dissociation stimulator
REM:	Ras-exchange motif
RhoA:	Ras homolog gene family, member A
RIPA:	Radio-Immunoprecipitation Assay
SEM:	Standard error of the mean
shRNA :	Short hairpin RNA
siRNA:	Small interfering RNA
SV:	Synaptic vesicle
SDS:	Sodium dodecyl sulfate
TEMED:	N,N,N',N'-Tetramethylethylenediamine
TIAM1:	T-cell lymphoma invasion and metastasis-inducing protein 1
TBS:	Tris buffered saline
Trk:	Tropomyosin-related kinase
VGLUT1:	Vesicular glutamate transporter 1
Wt:	Wild type
YFP:	Yellow Fluorescent Protein

RESUMEN

El establecimiento de una morfología polarizada axo-dendrítica a partir de una celula simétrica junto con la especialización funcional de los diferentes compartimentos celulares son esenciales para la función neuronal. Intensos estudios en los últimos 40 años sobre el proceso de polarización neuronal, han estado enfocados en entender los eventos secuenciales que ocurren en una neurona para promover el desarrollo de un axón y dendritas maduras. Los modelos in vitro han permitido estudiar neuronas aisladas y comprender los mecanismos celulares y moleculares de las etapas conducentes a la formación del axón. El desarrollo de polaridad neuronal requiere dos fenómenos activos y complementarios: una retroalimentación positiva que permite la elongación selectiva de una sola neurita para generar un axón; y una negativa que evite el crecimiento excesivo de las demás neuritas, requisitos que se cumplen con los segundos mensajeros AMPc y GMPc. Estas señales intrínsecas de polarización son acompañadas de la degradación selectiva de proteínas involucradas en polaridad por medio del sistema ubiquitina-proteosoma.

Entre los diversos mecanismos intrínsecos de polaridad neuronal, se ha demostrado que la GTPasa pequeña Rap1B tiene un rol pivotal para la generación del axón, ya que su forma activa se enriquece y activa en la neurita que se convertirá en el axón. Esta activación local de Rap1B podría estar bajo el control del segundo mensajero AMPc a través de la GEF dependiente de

AMPc para Rap1B, EPAC que cuenta con dos isoformas, EPAC1 y EPAC2, las cuales han sido estudiadas en diversos procesos neuronales. En esta tesis mostramos que la expresión de EPAC1 es mayor que la de EPAC2 en estadios tempranos de desarrollo de neuronas hipocampales. Adicionalmente, EPAC1 muestra una distribución somática y en los extremos de neuritas indiferenciadas, mientras que EPAC2 presenta una distribución subcellular homogénea.

La activación farmacológica de EPAC por su activador selectivo, 8-pCPT induce neuronas con múltiples axones positivos para marcadores axonales como Tau-1 o SMI-31. Estos axones muestran una distribución subcelular de Rap1B activo producto de la activación de EPAC. Adicionalmente neuronas en cultivos de largo plazo tratadas con 8-pCPT presentan marcadores de axones funcionales y maduros como ankG, sinaptofisina y VGLUT1. Este fenotipo de axones múltiples también fue corroborado por una ganancia de función genética de EPAC1.

Determinamos que la inhibición farmacológica de EPAC afecta la correcta polarización y que la inhibición de PKA no afecta la generación de axones múltiples producido por la activación de EPAC. Además EPAC no tiene efecto en la vía rio abajo dependiente de PKA.

Este conjunto de evidencias nos permite concluir que EPAC regula la activación de Rap1B durante la polarización neuronal, y ofrece un mecanismo alternativo y complementario para entender como el AMPc regula la adquisicion de polaridad neuronal.

SUMMARY

The establishment of an axo-dendritic polarized morphology and the functional specialization of these two different cellular components is critical for neuronal function. Intense studies conducted in the past 40 years regarding neuronal polarization have been focused in understanding the sequential events that occur in a neuron to extend matures axon and dendrites. In vitro models have allowed the study of the cellular and molecular mechanisms involved in axon formation. Two active and complementary events are required for the generation of polarity in neurons: a positive feedback which potentiates selective elongation of the neurite becoming the axon and a negative feedback loop which prevents excessive outgrowth of sibling neurites. Intracellular second messengers such as cAMP and cGMP coordinate these events. Along with these intrinsic polarizing signals, the selective degradation of proteins involved in acquisition of neuronal polarity, through the ubiquitin-proteasome system allows fine-tuning of polarity acquisition.

Amongst the many intrinsic signals involved in neuronal polarity acquisition it has been demonstrated that the small GTPase Rap1B has a pivotal role in the generation of the axon, as its active form is enriched in the neurite that will become the axon and selectively degraded in the rest of neurites. Thus local Rap1B activation could be regulated by the second messenger cAMP, through a cAMP-dependent-guanine exchange factor for Rap1B, termed EPAC. EPAC

proteins present two isoforms, namely EPAC1 and EPAC2, which have been studied in several neuronal processes.

In this thesis, we showed that the expression of EPAC1 is higher than EPAC2 at early stages of neuronal polarity. Additionally, EPAC1 present somatic distribution and is concentrated in the tip of the undifferentiated neurites, whereas EPAC2 presents a homogenous subcellular distribution. The pharmacological activation of EPAC using its selective activator 8-pCPT, induced neurons bearing multiples axons positive for axonal markers such as Tau -1 and SMI-31. These axons exhibit a subcellular distribution of active Rap1B upon EPAC activation. Moreover, long term-cultured neurons treated with 8-pCPT present markers of functional and mature axons such as AnkG, synaptophysin and VGLUT1. This phenotype of multiple axons was also corroborated by a genetic gain of function model for EPAC1. The pharmacological inhibition of EPAC proteins affects the correct polarization of neurons. PKA inhibition has no impact on the generation of multiple axons induced by EPAC activation. Moreover, EPAC does not affect the pathway downstream PKA.

All together these evidences allow us to conclude that EPAC regulate Rap1B activation during neuronal polarization, and offer an alternate and complementary mechanism to understand how cAMP control the acquisition of neuronal polarity.

INTRODUCTION

Neurons are highly polarized cells involved in the generation of functional networks based on the molecular, structural, morphological and functional properties of axons and dendrites, which begin early during development with the establishment of neuronal polarity. In the past 40 years 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 was first described by Santiago Ramón y Cajal as well as the termed neuronal polarization, in his law of Dynamic polarization (Ramón y Cajal, 1914, 1954) establish 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 brains of rodents to study the formation of the central nervous system (CNS). In particular, studying the neuronal migration. In this context, radial glia fibers plays a key role, providing a temporary scaffold and enforce 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)

towards the margin of the cerebral wall to form the primordial layer or preplate (PP), which is further split it into the superficial marginal zone (MZ) and the deeper subplate (SP). Preplate splitting define the margins for cortical plate formation (CP) which develops in an 'inside-out' pattern, where newly arrived neurons bypasses the subplate, lead to newest neurons 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 arise upon 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 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 evolved into a more complex morphology 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.

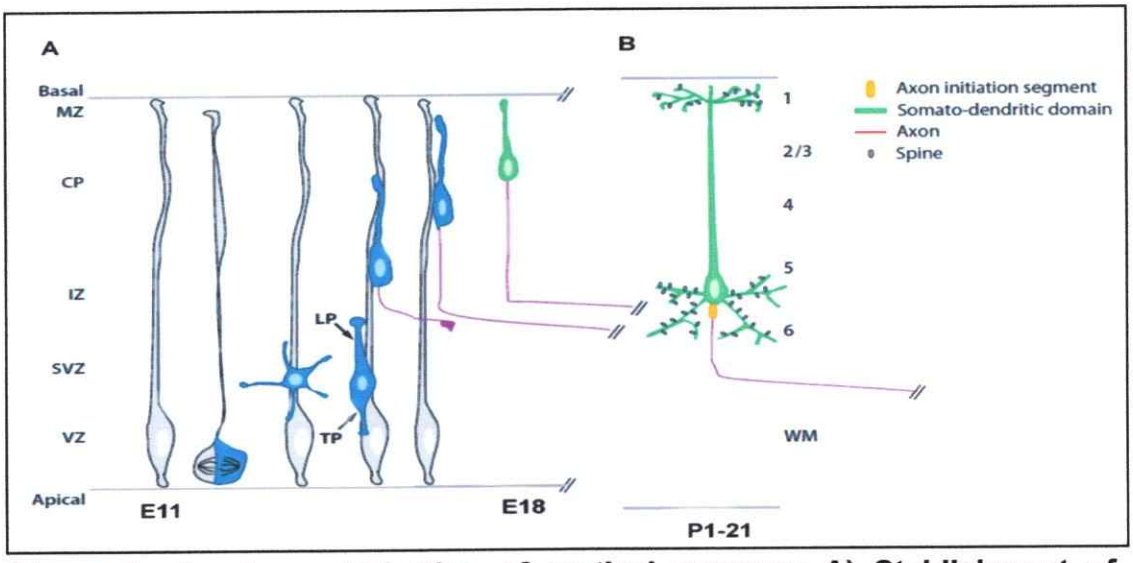

Figure 1: In vivo polarization of cortical neurons; A) Stablishment of mammalian neurons in vivo from E11-E18: axon-dendrite polarity of pyramidal neurons (blue cell) is derived from the polarized emergence of the trailing (TP) and leading processes (LP). B) At postnatal stages (P1-P21), pyramidal neurons 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))

In vitro neuronal polarization

The study of in vitro neuronal polarity started with the establishment of cultured primary neurons derived from rodent hippocampus at the end of the 70's by Banker and Cowan (Banker and Cowan, 1977b). 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., 1988b).

Hippocampal culture allow 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, providing a well suited model to physiological studies (Banker and Cowan, 1977a; 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 developed as in vivo neurons, showing defined axo-dendritic compartments and synapse formation (Banker and Cowan, 1977b; Kaech and Banker, 2006).

Ex vivo cultured hippocampal neurons undergoes dramatic morphological changes during their polarization, following a highly stereotyped sequence of developmental events which can be divided into five stages as described by Dotti and Banker (Dotti et al., 1988b): Stage I is defined soon after cells are plated upon substrate (Banker and Cowan, 1977a; Dotti et al., 1988a; Arimura and Kaibuchi, 2007), where neurons form a continuous actin-based structure all along 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) 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**).Later on, after 6-18 hours, this lamella coalesces at distinct spots around the cell periphery,

which leads to the protrusion of several "minor neurites" of 20-30 µm in length which are undergo intermittent growth and retraction over short distances which corresponds to the developmental Stage 2 (Dotti et al., 1988a; Barnes and Polleux, 2009; Polleux and Snider, 2010). At this stage, neurite tips are decorated with large growth cones important for motility. Cells remain in Stage 2 up to 36 h with subtle net growth of neurites, which produce a symmetric appearance, where all neurites possess the capacity to become axons or dendrites (Dotti et al., 1988b; 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 begin to extend rapidly and continuously than its siblings, becoming two to three times longer than other neurites. This fast growing neurite is the axon; while other neurites undergo brief bursts of slow growth and retraction and will further acquire dendritic identity (Dotti et al., 1988b; Kaech and Banker, 2006; Barnes and Polleux, 2009; Polleux and Snider, 2010). The transition between Stage 2 and Stage 3 is critical and a 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 do not occur synchronously across the cell population because half of the neurons in culture under optimal conditions reach Stage 3, 24 h after plating and 80% after 36-48h. (Kaech and Banker, 2006). Interestingly, such transition is the focus for most of 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, which are shorter and thicker than the axon (Polleux and Snider, 2010; Cáceres et al., 2012). At this stage, axonal and dendritic proteins are segregated indicating molecular polarization in axons and dendrites (Dotti et al., 1988a; 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 microtubules-associated proteins (MAPs) such as MAP2 (Kosik and Finch, 1987); while and 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., 1988b; Polleux and Snider, 2010; Cáceres et al., 2012). In addition at Stage 5, the assembly of the axonal initial segment, -a region where action potential is originated- (Kole and Stuart, 2012), indicate neuronal maturation required to allow the transmission of electrical activity (Segal, 1983; Bartlett and Banker, 1984) (Fig 2).

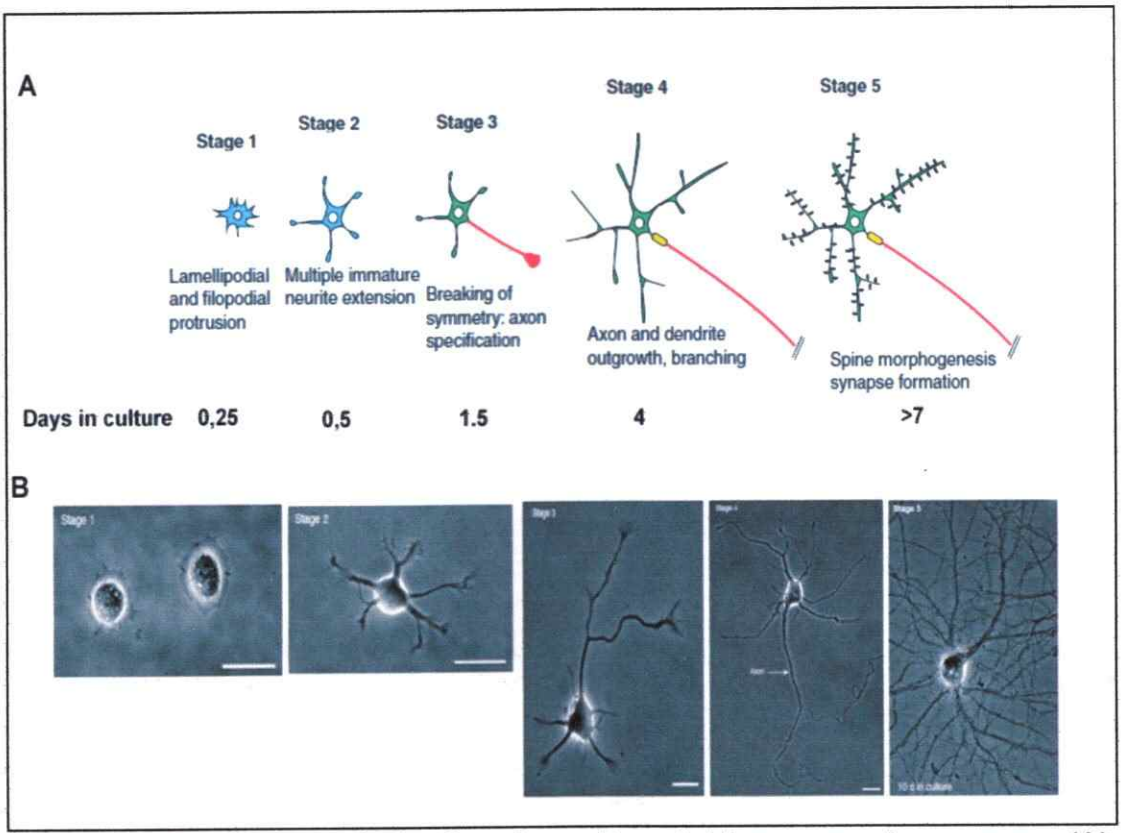

Figure 2: Neuronal polarization in cultured hippocampal neurons: (A) Hippocampal neurons change from round cells bearing lamellipodia (Stage 1) into multipolar cells (Stage 2). One neurite extends rapidly to become the axon (Stage 3). The remaining shorter neurites will become dendrites (Stage 4). This is followed by formation of dendritic spines, synapses and functional maturation and [polarization (Stages 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 (Kaech and Banker, 2006)

The development of neuronal polarity in cultured cells is very consistent from laboratory to laboratory. Moreover, primary culture of neurons has been used in 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

During 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 mechanotropic environmental signals and particular extracellular ligands as regulators of neuronal polarity

Some of the studies to address 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 giving 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 regulates asymmetric

growth of cortical neurons, acting as a repellent for axon and 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) has been reported to determine neuronal polarity and axon outgrowth independently 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 polarity in mammals is also linked to discrete sources of extracellular cues such as the family of neurotrophins which are involved in many different functions in the nervous system such as survival, neural development and neuronal functions in both 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 inmature neurites induce axon specification through tropomyosin receptor kinases (Trks) (Nakamuta et al., 2011). A similar approach were performed by Shelly and colleagues (Shelly et al., 2007) in cultured neurons growing over patterned substrate or in contact with coated-beads with BDNF causing that neurites of immature hippocampal neurons promoted axon specification. In these experiments, when two neurites contacted BDNF-source both become axons, leading to neurons bearing multiples axons. (Shelly et al., 2007). The mechanism by which BDNF signaling generated this changes in neurons may be related to a self-amplifying autocrine

response by triggering 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 that promotes anterograde transport of TrkB in the putative axon, further enhancing local BDNF/TrkB signaling. (Cheng et al., 2011a). 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., 2011; Cheng and Poo, 2012) inducing, for example, activation of the PI3K signaling in the neighbor neurons, suggesting that likely all this cues works in concert to determine neuronal polarity and genetic deletion or chemical inhibition in any one of them produce a weak or insignificant effect (Cheng and Poo, 2012) . These results suggest that the breaking symmetry likely involves a specific amplification signal that require the contribution of multiples factors but it is noteworthy that local contact of a neurite with any of this signal is enough to generate an axon and impair the remaining neurites to develop this structure (Nakamuta et al., 2011; 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 inmature 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 preferentially are incubated on substrate such as poly-L or poly-D- lysine, which promotes cell adhesion through ionic interactions (Kaech and Banker, 2006) with neurons allowing similar actions of in vivo environment such as the sensing, transduction and a cellular and molecular response.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 phenomenon: on one side, a positive feedback which permits the selective elongation of a single neurite that will later develop as the axon; and on the other hand, a negative signal which prevents the growing of the remaining neurites, which has been proposed as the principle of self-organization in neurons (Turing, 1990; Cheng and Poo, 2012). This principle predicts that a cell can lead to internal molecular changes that trigger the appearance of polarity using a combination of an enhanced local-activator that acts through positives feedback which increase its stochastic variations 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 polimerization, (Andersen and Bi, 2000; Arimura and Kaibuchi, 2007). And forth, there may be an opposite reaction induced by a global inhibitor which precludes the growing 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 exist 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 re-growing in plated cells (Menchon et al., 2011; Pollarolo et al., 2011; Cáceres et al., 2012).

Amongst the several intracellular signaling cascades involved in the generation of neuronal assimetry PI3K-Akt-GSK3β axis is a key player promoting neuronal polarity.

The 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 phosphorylate and activate Akt [also known as protein kinase B (PKB)] (Boudewijn 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 Phosphatase and tensin homologue deleted on chromosome 10 (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 high affinity (Hyvönen 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, which are important to promote 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 function such as cytoskeletal and membrane dynamics, gene transcription, cell polarity, and cell cycle progression. It has been identified more than 20 members, 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) whilst RhoA regulate 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, 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**).

Additionaly to the Rho GTPases there is another family of GTPases involve 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 as 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(Schwamborn and Püschel, 2004) Rap1B is initially present in all neurites of unpolarized neurons, but becomes accumulated to a single neurite during neuronal polarization due to its selective degradation by the ubiquitin ligase Smurf2 (Schwamborn et al., 2007). Puschel et al. (Schwamborn et al., 2007) suggested that Smurf2 mediates degradation of inactive (GDP-loaded Rab1B) and therefore protection from degradation at the tip of the future axon can be related with selective activation (Schwamborn et al., 2007). Interestingly, Rap1B function depends on PIP3 and seem 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 remained elusive (Fig 3B).

The tripartite complex named PAR6/PAR3/aPKC, which is conserved from worms to vertebrates, is essential for the determination of the axon. The complex is formed by two scaffold proteins PAR6 and PAR3, which interacts 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 et al., 2005); the tumor suppressor lethal giant larvae (lgl) (Plant et al., 2003); the ubiquitin ligases Smurf1 (Ozdamar et al., 2005; Cheng et al., 2011b) and Smurf2 (Schwamborn et al., 2007); the transforming growth factor receptor 1 (TGFβR1) (Ozdamar et al., 2005) and the active version of Cdc42 (Schwamborn and Püschel, 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 Püschel, 2004). Furthermore, Par complex is regulated downstream PI3K since inhibitors of PI3K prevent polarization and cause

mislocalization of Par3 and Par6 indicating that a correct localization and activity of Par complex are 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, which is produced by activation of membrane-bound or soluble adenylyl ciclases (ACs). Its production is generally initiated upon binding of a extracellular ligands to Gs protein-coupled receptors (William, 1999; Beavo and Brunton, 2002) (**Fig 4**). cAMP regulates fundamental physiologic 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**).

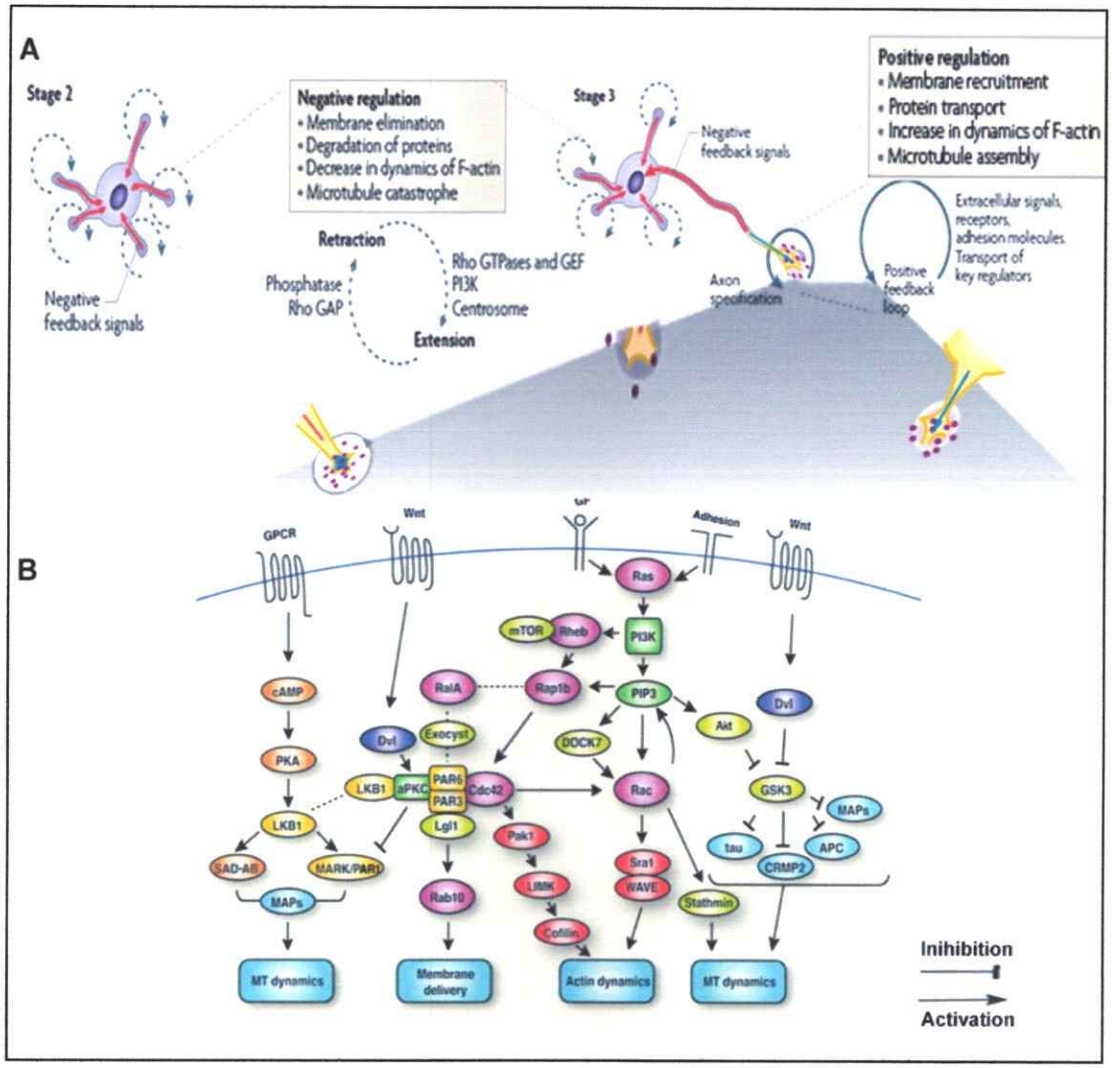

Figure 3: A tentative model of the principle of self-organization and the coordination of extracellular and intracellular signaling in axon formation. (A) Local amplification mechanisms for axon formation have random fluctuations within cytoplasmic axon determinants and growth-promoting activities. In 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 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)

Shelly et al (Shelly et al., 2010b) 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 stabilize 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., 2011b).

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 begun to change with the discovery of a family of novel cAMP effector proteins, named exchange protein directly activated by cAMP (EPAC) (De et al., 1998). 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 et al., 1998; Kawasaki et al., 1998a; Kawasaki et al., 1998b), with a cAMP-affinity similar to the PKA holoenzyme, suggesting that both effectors may respond to similar physiological concentration on this second messenger (Dao et al., 2006).

EPAC and PKA may not be the unique effector molecules acting downstream of cAMP signaling. A member of the A-kinase anchoring protein (AKAP) family expressed in the brain, namely AKAP150 would be important to fine-tune 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 coincident 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., 2008b) (Fig 4).

In addition, cAMP microdomains inside the cell are facilitated by local phosphodiesterases (PDEs) pools in particular cAMP-specifics PDEs (PDE4, PDE7, and PDE8) that regulate cAMP limiting its diffusion inside the cell by degradation (Xu et al., 2011). The PDEs together with the scaffold protein

AKAP, modifying the cAMP signalling in time and space localizing and facilitating crosstalks between its effectors (**Fig 4**).

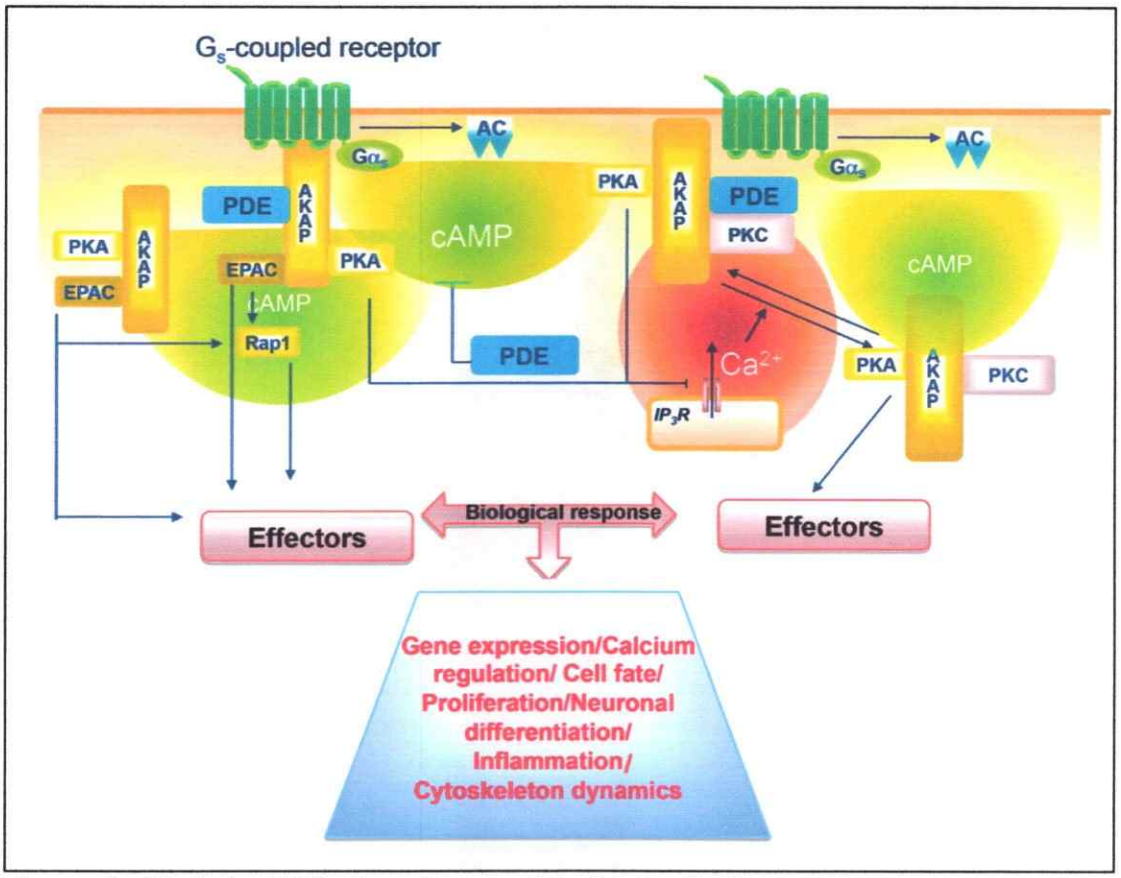

Figure 4: Global cAMP signaling and its biological response:

Generation of the second messenger cyclic AMP (cAMP) is initiated upon stimulation of Gs 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.

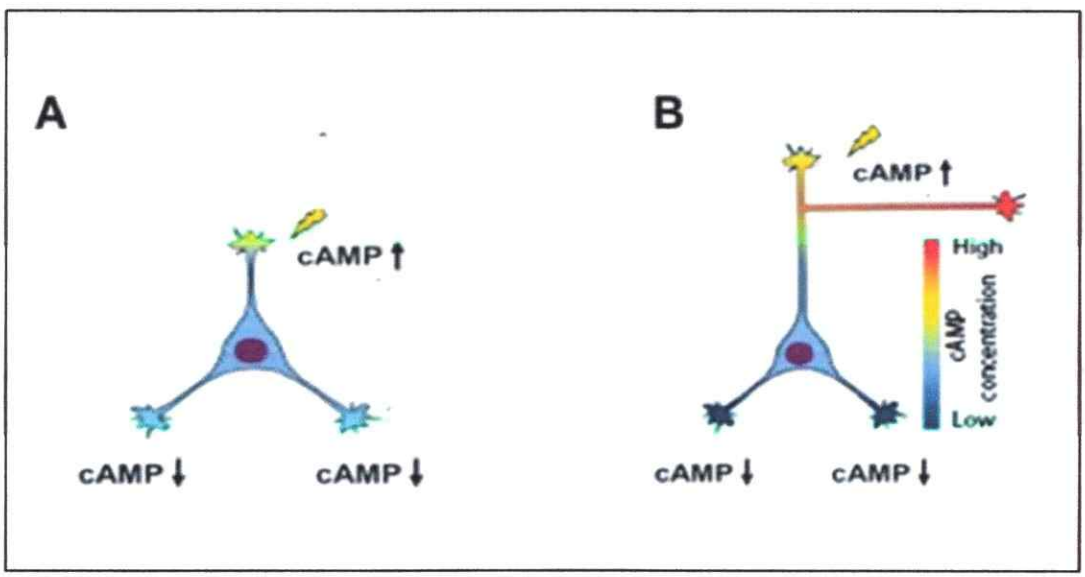

Figure 5: cAMP signals promote axonal growth.

(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)

EPAC: a new mediator of cAMP-signaling dependent.

The discovery of EPAC1 and EPAC2 has profoundly altered the prevailing idea on cAMP signaling, which historically had been associated only with PKA. The analysis of molecular mechanisms related to EPAC signaling, has shown that EPAC family regulate many physiological processes (Grandoch et al., 2010b; Schmidt et al., 2013) 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 present the c-AMP 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 isoform and contains the GEF activity that specifically activates Rap1 (**Fig 6A**). Nevertheless, EPAC2 contain 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 stabilize 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 that interact with active Ras (**Fig 6B**).

Although both EPAC1 and 2 present a RA domain, to date only EPAC2 show 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., 1998b; Bos, 2003, 2006) and in a greater extent in embryos (Murray and Shewan, 2008). Meanwhile; EPAC2 is expressed predominantly in adult nervous system, mainly in the brain cortex, hippocampus (specially CA3 and dentate gyrus), habenula and cerebellum, as well in adrenal gland (Kawasaki et al., 1998a; Kawasaki et al., 1998b; 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).

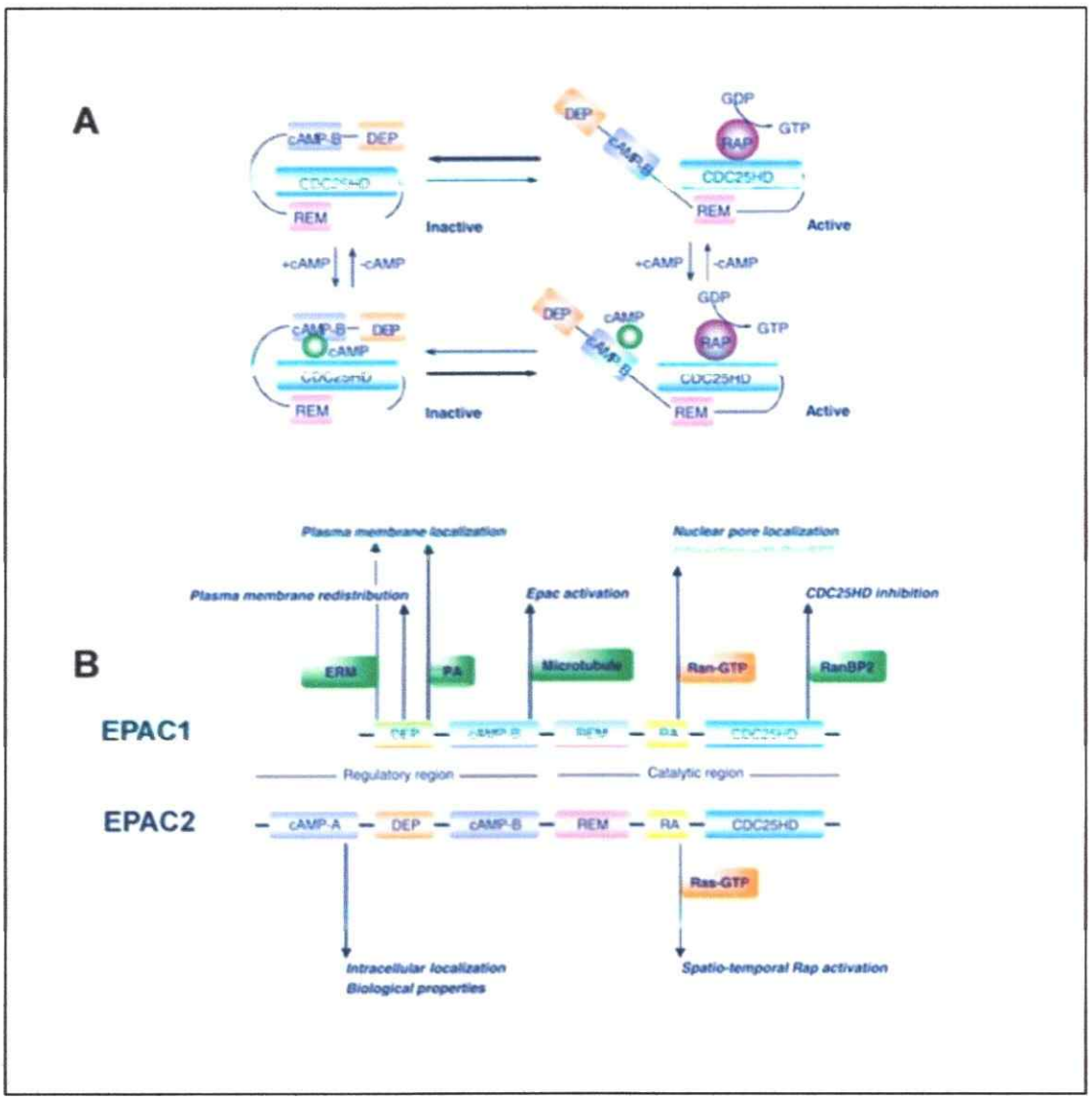

Figure 6: EPAC overview.

(A) 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))

Since changes in the concentration of cAMP will ultimately modify the functions of PKA and EPAC proteins, many efforts had been conducted in order to generate pharmacologic 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 antagonist for PKA. Rp-8-CPT-cAMP, inhibit 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**).

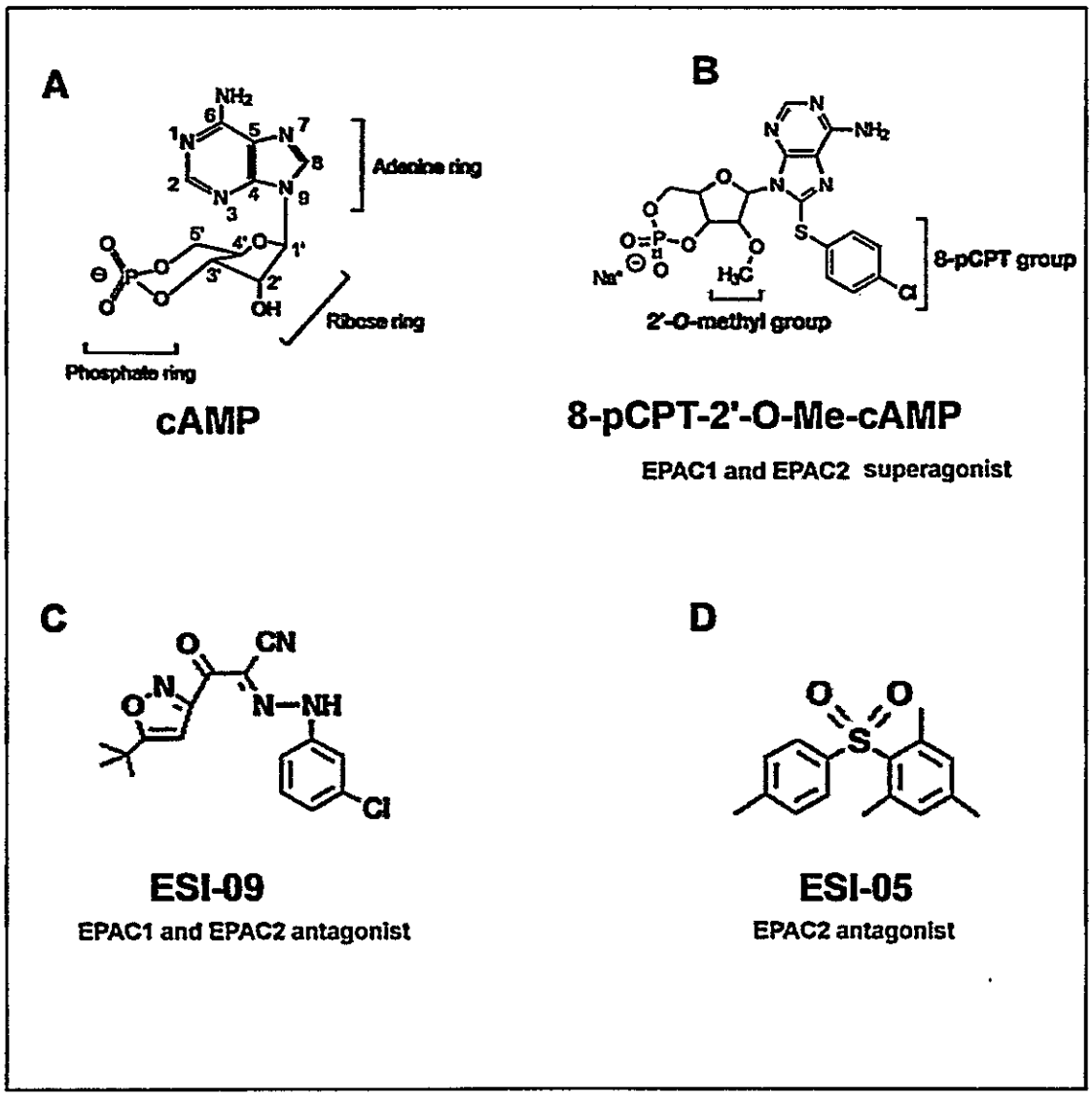

Figure 7: EPAC-selective agonist and antagonist.

(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 and I in in vitro assays. (Adapted from (Bos, 2003; Tsalkova et al., 2012b; Almahariq et al., 2013; Schmidt et al., 2013)

EPAC as mediator of neuronal polarity

Whether EPAC1 or EPAC2 have 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 showed that the ubiquitin ligase Smurf2 mediates Rap1B degradation in its inactive GDP-form but activation of Rap1B at the tip of nascent axon would protect it against proteasomal degradation (Schwamborn et al., 2007). This evidence, suggest that a necessary initial event would be the local activation of a Rap1B-GEF in the distal end of the neurite that will become the axon. EPAC proteins may serve this function.

Accordingly, Murray et al. (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 oscillates (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, with opposite effects on the levels of phosphorylation in this residue, through mechanisms which are still unclear (Nijholt et al., 2008a).

Molecular changes involved in neuronal polarization will ultimately modify cytoskeleton dynamics to provide a 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 interaction 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 of LC1 with PKA has not been demonstrated. Moreover, the interaction between EPAC and MAP1B is interesting since 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 activate Rac through the interaction between Rap1 and Rac GEFs, STEF, in CHO and Cos-1 cell 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 activate 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 cells differentiation triggered by neurotrophic actions of the pituitary adenylate cyclase-activating polypeptide (PACAP) signaling. PACAP-dependent mechanisms involved activation of ERK, p38 MAP kinase and Rit signaling pathway (Shi et al., 2006; Monaghan et al., 2008).

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.

Hypothesis:

EPAC family proteins (EPAC1 or/and EPAC2) are GEF proteins for Rap1B which are involved in axon specification and/or elongation in embryonic hippocampal neurons

General aim:

To determine whether EPAC1 or/and EPAC2 contributes to the determination and/or elongation of the axon in a model of hippocampal neuron cultures.

Specific aims:

1) To analyze the effect of loss and gain of function of EPAC1 and/or EPAC2 in axon formation

2) To determine the differential contribution and/or the possible redundancy of EPAC/PKA signaling in axon formation.

3) To assess how the loss and gain of function of EPAC1 and/or EPAC2 affect the Rap1B-dependent downstream signaling pathways.

MATERIALS AND METHODS

Animals

All animal experiments were performed with the approval of animal ethics commitee of Universidad de Chile.

Adult pregnant females *Sprague dawley* rats and adult females C57BJ6 wild-type mice were group-housed in cages with a light/dark cycle of 12 h and ad libitum feeding. The euthanasia was performed with Ether and Cervical dislocation.

Antibodies:

The following antibodies were used in this work: EPAC1 (Immunofluorescence 1:150, Rabbit Santa Cruz: H-70 sc-25632, Western blot 1:300, Mouse Cell Signaling: 5B1 #4155), EPAC2 (Immunofluorescence 1:150, Rabbit, Santa Cruz H-220 sc-25633, Western blot 1:300, Mouse, Cell Signaling: 5D3 #4156), Rap1B (1:300, Mouse, BD Biosciences, #610195), α-tubulin (1:10000, Mouse Sigma-Aldrich, #T6199), β-Actin (1:5000, sc-47778), β-III-tubulin (Tuj1) (1:1000, Mouse, Promega, # G1712A), MAP2 (1:500, Rabbit Millipore, AB5622), Tau1 (1: 500, Mouse, Millipore, MAB3420), SMI-31 (1:500, Mouse, Covance, #14835101), phospho-PKA Substrate (1:1000, Rabbit, Cell Signalling, 9624S),

RhoA (1:1000, Rabbit, Cytoskeleton #ARH03-A), AnkG (1:150, Mouse, Santa Cruz, sc-12719).

Anti-mouse (1:5000, Jackson Immuno Research, peroxidase-conjugated, Donkey, #15-035-150), Anti-rabbit (1:5000, Jackson Immuno Research, peroxidase-conjugated, Donkey #711-035-152).

For immunofluorescence experiments we used the following secondary antibodies: Anti-Mouse Alexa Fluor 488 (1:600, Donkey, Molecular Probes #A21202), Anti Rabbit Alexa Fluor 546 (1:600, Donkey, Molecular Probes, #A10040), Anti-Mouse Alexa Fluor 646 (1:600, Donkey, Molecular Probes, #31573).

Chemicals

DMSO (Sigma Aldrich, #472301), 8-pCPT-2'-O-Me-cAMP (8-pCPT, Biolog, #C041-05, Protein kinase inhibitor- (14-22) -amide, myristoylated (PKI, Tocris, # 2546), ESI-09 (biolog, #B133), Forskolin (Tocris, #1099), Lipofectamine 2000 (Invitrogen, #11668019), Protease Inhibitor Cocktail Tablets (Roche, #04693159001), Glutathione Sepharose 4B Media (Amersham, #17-0756-01), Pierce ECL Western Blotting Substrate (Thermo, #32106). PageRuler Plus Prestained Protein Ladder (Thermo scientific, #26619),

cAMP assay

Performed according to the protocol Cyclic AMP XPTM Assay Kit of Cell signalling Technology (4339).

DNA constructs

pmaxGFP (1µg/µl, Amaxa Nucleofector Kit), pCAG-RFP, Rap1GAP-RFP were kindly donated by John Cooper (Fred Hutchinson Cancer Research Center, Washington, USA) RalGDS-GFP was kindly provided by Johannes Bos (University of Utrecht, Utrecht, The Netherlands), Venus-vGLUT1 was a kind contribution of Nils Brose (Max Planck Institute, Goettingen, Germany). cDNAs encoding EPAC1 wild type (EPAC1 wt), EPAC1 dominant negative (Epac1 DN) and EPAC constituvely active (EPAC1 CA) in pcDNA vector and PGEX-RalGDS-RBD.

Cell cultures and transfection:

Primary culture of rat and mouse hippocampal and cortical neurons were prepared as described previously by Kaech & Banker (Kaech and Banker, 2006) (Fig 8). Briefly, hippocampal and cortical neurons were isolated from Sprague-Dawley rat embryos at embryonic day 18 (E18) or from the strain mice C57BJl6, the tissue were dissociated using 0.25% (w/v) trypsin (Gibco) for 30 min at 37°C, followed by trituration and mixing with a fire-polished pipette in plating medium containing MEM medium (Gibco), 0.6%D-glucose (Merk, # 108342), 10% horse serum and 1% penicillin-streptomycin (Invitrogen). Hippocampal cells were plated at 1×106 cells/mm2 in 6 mm tissue culture dishes coated with poly-L-lysine (1 mg/ml; Sigma-Aldrich) and in coverslips previously coated with poly-L-lysine (1 mg/ml) at 1,5×104 cells/cm2 in plating medium. After 1 h, the medium was replaced with Neurobasal maintenance medium (Gibco) supplemented with 2% B27 (Gibco), GlutaMAX (Gibco) and 1% of penicillin-streptomycin in the absence of serum. For biochemical experiments cortical were plated at 1×106

cells/mm2 in 100 mm or 60 mm tissue culture dishes coated with poly-D-lysine (1mg/ml) and hippocampal neurons were plated at 1×106 cells/mm2 in 60 mm tissue culture dishes, after 1 h of incubation the plating medium was replaced with Neurobasal maintenance medium supplemented with 2% B27 (Gibco), GlutaMAX (Gibco) and 1% of penicillin-streptomycin in the absence of serum. After 18 h 48 h and 72 h in culture, hippocampal neurons plated in coverslips were fixed for immunofluorescence and processed for protein extraction. Cortical neurons were maintained for 3 day and then used for Pulldown assay. N2A, N1E-115 and Cos7 cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Transfection of Hippocampal neurons were performed according instructions provided by Amaxa rat neurons Nucleofector Kit, (Amaxa biosystem, #VPG 1003) manufacturers program O-003, using 1,5 ×106 cells for transfection with Vglut and RFP plasmids (5 µg of DNA),. The RalGDS-GFP, wt Epac1, DN EPAC1, CA EPAC1, RFP, RapGAP-RFP plasmids (0,5 µg of DNA) were transfected using Neurobasal serum free medium and Lipofectamine 2000 (0,75 µL) following instructions provided by the manufacturer. The medium of neuron was exchanged after 2 h with Neurobasal maintenance medium and incubated for 48 h and 72 h.

Transfection of COS7 cells with RFP, wt Epac1, DN EPAC1, CA EPAC1 and Rap1GAP-RFP plasmids (8 µg of DNA) were using 4 µg of DNA on 100 mm dishes performed with OptiMEM (Gibco) médium and Lipofetamine 2000 according to the instructions. The medium was exchanged after 6 h with DMEM

fresh serum-containing medium and incubated for 48 h. The cultures were grown in a humidified culture incubator at 37°C, 5% CO2.

Treatments

PKA inhibition with PKI 14-22 amyde (20 µM), EPAC inhibition with ESI-09 (15 µM) and EPAC activation with 8-pCPT (10 µM) were performed on hippocampal cultures at 16 h after plating. Biochemical analyze of PKA inhibition was assessed 6 h after addition of inhibitors in hippocampal neurons. Cortical neurons were used for biochemical assay. Briefly, 8-pCPT, ESI-09 and PKI were dissolve in plain neurobasal medium and added for 30 minutes to the neurons then the cultures were prepared to perform Rap1B Pulldown.

N2A cells were treated for 10 h with 8-pCPT (10 µM), Forskolin (20 µM) and Forskolin+PKI14-22 amyde (20 µM) and then prepared for western blot analyze. The final DMSO concentration in all experiments was maintained lower than 0.1%.

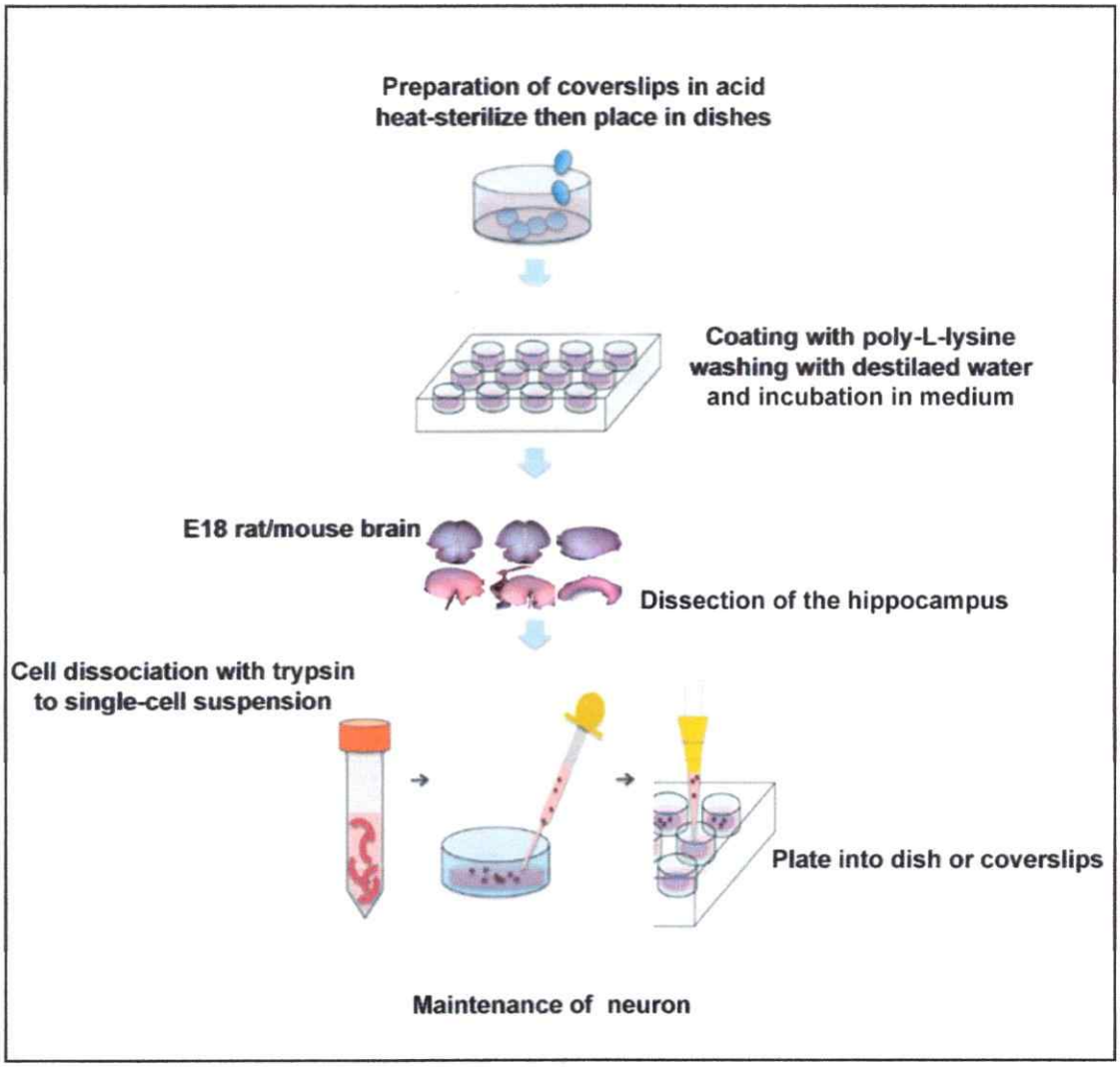

Figure 8: Overview of protocol for preparing hippocampal cultures.

Coverslips are treated, prepared and coated with poly-L-lysine. After washing, they are incubated in plating medium. Hippocampi are dissected; cells are trypsinated, dissociated and plated on dishes or coverslips. The neurons are maintained in special medium for embryonic tissue for up to 21 days. (Modified from (Beaudoin et al., 2012)

Immunofluorescence and images analyze

Hippocampal neurons were fixed at 18 h, 48 h and 72 h, with 4% (w/v) paraformaldehyde, 4% sucrose for 30 min at 37°C and washed with phosphate-buffered saline (PBS) three times for 5 min. The cells were incubated with PBS, 0.1% Triton X-100 for 5 min and then blocked with 5% (w/v) bovine serum albumin (BSA) in PBS for 1 h. After blocking, cells were incubated with primary antibodies diluted with 1% BSA in PBS overnight at 4°C, washed with PBS three times for 5 min and incubated with fluorescent secondary antibody for 1 h and washed with PBS three times for 5 min. For AnkG staining, neurons were permeabilized with 0.1% triton for 20 min at RT and blocked with 1% BSA for 1 h at RT. Neurons were incubated with antibodies against MAP-2 (1:500) and AnkG (dilution 1:50) overnight at 4°C. Later, secondary antibodies Alexa Fluor 546 anti-rabbit y Alexa Fluor 488 anti-mouse were incubated 1 h at RT. Neurons were washed for 1 h and Tau-1 antibody (dilution 1:500) was incubated overnight at 4°C. Secondary antibody Alexa Fluor 647 anti-mouse was incubated 1h at RT. Finally, samples were mounted on slides and examined using Zeiss LSM510 Meta confocal scanning microscope equipped with Plan-Apochromat 40x/1.3 N.A and Plan-Apochromat 63x/1.4 N.A objective lenses. Digital images were quantified using LSM 5 image browser and ImajeJ (NIH) for axonal length, fluorescence intensity and cell counting. Changes in phenotype of the neurons were measured by determining total axon length, percentage of polarization and neuron phenotype. For ·3DI reconstruction of vGlut1 axonal spots with Imaris Software package (Bitplane), Z-stacks images of 8 DIV Venus-

vGlut1 nucleofected neurons were acquired using a 63X objective in a resolution of 1024 x 1024 pixels and 3D images generated with the Surpass tool for filaments (Tau-1 positive axons) and spots (Venus-vGlut1 positive clusters). Automated number of spots quantification was performed with Matlab based "find spots close to filaments" plugin. For the purposes of this study we defined a polarized neuron, as a neuron displaying an axon >50µm or two-times longer than neuronal soma, with positive Tau1 or SMI-31 staining localized in a proximal-distal gradient along the shaft and negative for MAP2 staining.

GST fusion protein preparation and Rap1 activation pull down assay

Expression and purification of GST-conjugated proteins was performed as described (Henriquez et al., 2012) Briefly, BL21 (DE3) E. coli strains carrying GST-RalGDS-RBD plasmid were grown overnight in LB Ampicillin medium. The next day cultures were diluted 1:100 and grown in fresh medium until OD0.6 at 37°C. Then, 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration) was added. Two h after induction, cells were collected and lysed by sonication in lysis buffer A (50 mM Tris-HCl pH 8.0, 1% Triton X-100, 1 mM EDTA, 0.15 M NaCl, 25 mM NaF, 0.5 mM PMSF and 1× of protease inhibitor complex (Roche)). Cleared lysate was then purified by affinity with glutathione-Sepharose beads (Amersham). Loaded beads were washed ten times with lysis buffer B (lysis buffer A plus 300 mM NaCl) at 4°C. The GST fusion proteins were quantified and visualized in SDS-PAGE gels stained with Coomassie brilliant blue. For Rap1 activation assay, beads loaded with the RalGDS-RBD (Rap-binding domain of the Ral guanine nucleotide dissociation stimulator that binds

specifically to the RAP1-GTP but not to the inactive Rap1-GDP form) were incubated for 1 h at 4°C with 1 mg of fresh cortical neurons lysates from 3 days in vitro (3 DIV) or 1 mg of COS7 cells expressing EPAC constructs. Cell lysates were produced using fishing buffer (50 mM Tris-HCl pH 7.5, 10% glycerol, 1% Triton X-100, 200 mM NaCl, 10 mM MgCl2, 25 mM NaF, 1× protease inhibitor complex). The beads were washed three times with washing buffer (50 mM Tris-HCl pH 7.5, 30 mM MgCl2, 40 mM NaCl) and resuspended in SDS–PAGE sampling buffer. The levels of Rap1B -GTP was evaluated through western blot analysis and normalized against total Rap1B with the ImageJ program.

Western blot analysis

Neurons and cell lines grown on dishes were washed once with PBS and then incubated with RIPA (65 mM Tris, 155 mM NaCl, 1% Triton, 0.25% sodium deoxycholate, 1mM EDTA, pH 7.4, mixture of protease inhibitors, 5µg/ml Na3VO4, 20µM PMSF, 5mM NaF). Then cells were scraped from the plate and kept on ice for 15 min and finally centrifuged for 20 minutes at 14,000 RPM. Supernatant fractions were quantitated by the Bradford method, denatured in loading buffer by heating at 95 ° C for 5 minutes and subjected to SDS-PAGE, using 10% running gels for EPAC, 15 % for Rap1B and 12% for PKA substrates, Par6 and RhoA, then transferred to nitrocellulose membranes or PVDF membranes and continue with blocking with 5% of BSA for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C. The next day membranes were washed using TBST (50mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, pH 7,4), four times for 10 min and incubated

with HRP-conjugated secondary antibody for 1 h at room temperature. Finally, peroxidase activity in membrane blots was detected using photographic films quimioluminicente and SuperSignal West Pico western blotting substrate.

Statistical analysis

All data were represented as mean ± SEM of at least three independent experiments. Comparison between two groups was made using unpaired t test. Comparison among three or more groups were performed using one-way ANOVA analysis followed by Dunnetts or Tukey poshoc test, $p < 0.05$ was considered as the level of significance. Quantification was performed with GraphPad Prism (GraphPad Software, Inc.).

RESULTS

In order to assess the participation of EPAC proteins as potential Rap1B GEFs involved in neuronal polarization, we evaluate the expression and subcellular localization of EPAC isoforms. Since EPAC proteins are activated by cAMP we first evaluated the overall levels of this second messenger in polarizing neurons. We performed an affinity assay for cAMP concentration and results are shown in figure 9. The cAMP concentration measured showed no significant differences in the global concentration of cAMP between the stages, suggesting that local changes in cAMP concentration would trigger cellular responses involved in neuronal polarization.

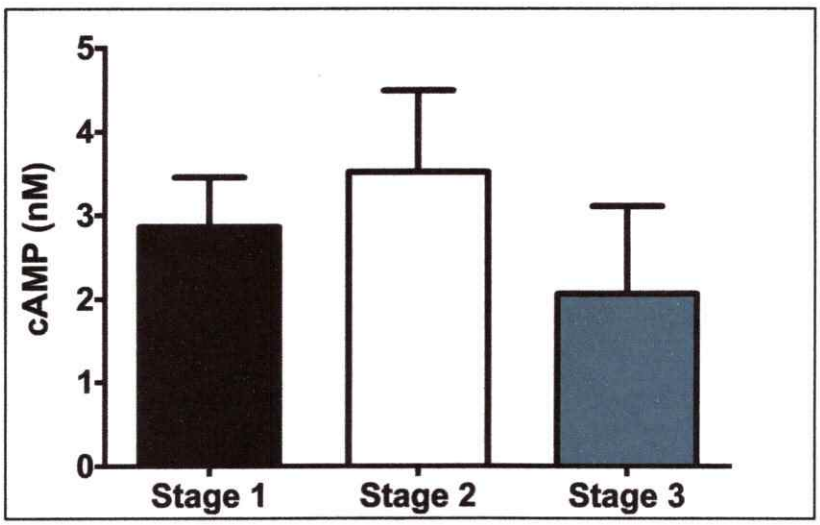

Figure 9: cAMP levels in hippocampal neurons during polarization. cAMP assay performed in hippocampal neurons at stage 1, stage 2 and stage. Stage 1 neurons were assayed after 14-16hours in culture, stage 2 neurons were assayed after 24 hours in culture and stage 3 neurons were assayed after 48 hours in culture. A slight different elevation of the concentration of cAMP between stage 1 and 2 (n.s.) and a reduction in stage 3 (n.s.) is shown. The error bars indicate the SEM of a triplicate experiment. (Stage 1, 2.86±0.6, stage 2: 3.52±1.0 and stage 3,: 2.06±1.1, n=3, One-Way ANOVA, Tukey multiple comparison test).

Expression of EPAC1 and EPAC2 during neuronal polarization

Previous studies on cultured hippocampal neurons showed that redistribution of active Rap1B to the tip of future axon and its selective degradation in the rest of the neurites, as a crucial step in the establishment of neuronal polarity (Schwamborn and Püschel, 2004; Schwamborn et al., 2007). Since Rap1 is the major downstream target of EPAC (Rehmann et al., 2006; Rehmann et al., 2008; Ponsioen et al., 2009), we first examined the expression and localization of both EPAC isoform in stage II (upolarized) and stage III (polarized) to assess whether EPAC isoforms distribution was similar to active Rab1B in neurons.

Western blotting of lysates from rat hippocampal neurons showed that both EPAC1 and EPAC2 are expressed in hippocampal neurons at stages II and III (Figure 10A and 11A), with no significant changes amongst stages (Figure 10B and 11B). However a significant protein abundance of EPAC1 over EPAC2 at stage II and stage III is observed ($p<0,05$, three experiments) (**Fig 12A, B**)

We then evaluated the subcellular distribution for EPAC1 and EPAC2 proteins in unpolarized and polarized cultured neurons (**Fig 10C and 11C**), using semi-quantitative immunofluorescence. Measurement of fluorescence intensity levels of EPAC1 (**Fig 10D**) and EPAC2 inmunostaining (**Fig 11D**) (normalized by Tuj1 staining) was analyzed at stage II (18 h in cultures) and we found that EPAC1 and EPAC2 were uniformly distributed in every neurite, but interesting fluorescence intensity of EPAC1 was significantly higher than EPAC2 (EPAC1; mean: 1,40 ±0,03; n=20 neurons, three experiments vs EPAC2; mean: 0,60±0,04, n=44 neurons, three experiments, p<0,0001) (**Fig 12C**). Next, we analyzed the fluorescence intensity at stage III (48 h in cultures). We found that EPAC1 was enriched at the tip of the longest neurite, which is the nascent axon (neurite 1: 3,43±0,36 vs neurite 2: 1,25±0,15; neurite 3: 1,39±0,20; neurite 4: 1,41±0,21; n=20 neurons, three independent experiments, p<0,0001) (**Fig 10D**). In contrast, EPAC2 distribution showed no significant differences amongst neurites (neurite 1: 0,53±0,05; neurite 2: 0,62±0,06; neurite 3: 0,058±0,05; neurite 4: 0,57±0,05; n=30 neurons, three independent experiments, ns, p>0,05), (**Fig 11D**). Furthermore, total neurites intensity of EPAC1 was higher than EPAC2 at stage III (EPAC1 1,87±0,52 vs EPAC2 0,58±0,02; n=20-30 neurons, three experiments) (**Fig 12C**).

These results suggest that EPAC1 is the predominant isoform of EPAC in polarizing neurons; its expression levels are higher than EPAC2 in hippocampal neurons and display a preferential accumulation in developing axon, providing a clue that EPAC1 could be involved in axon outgrowth.

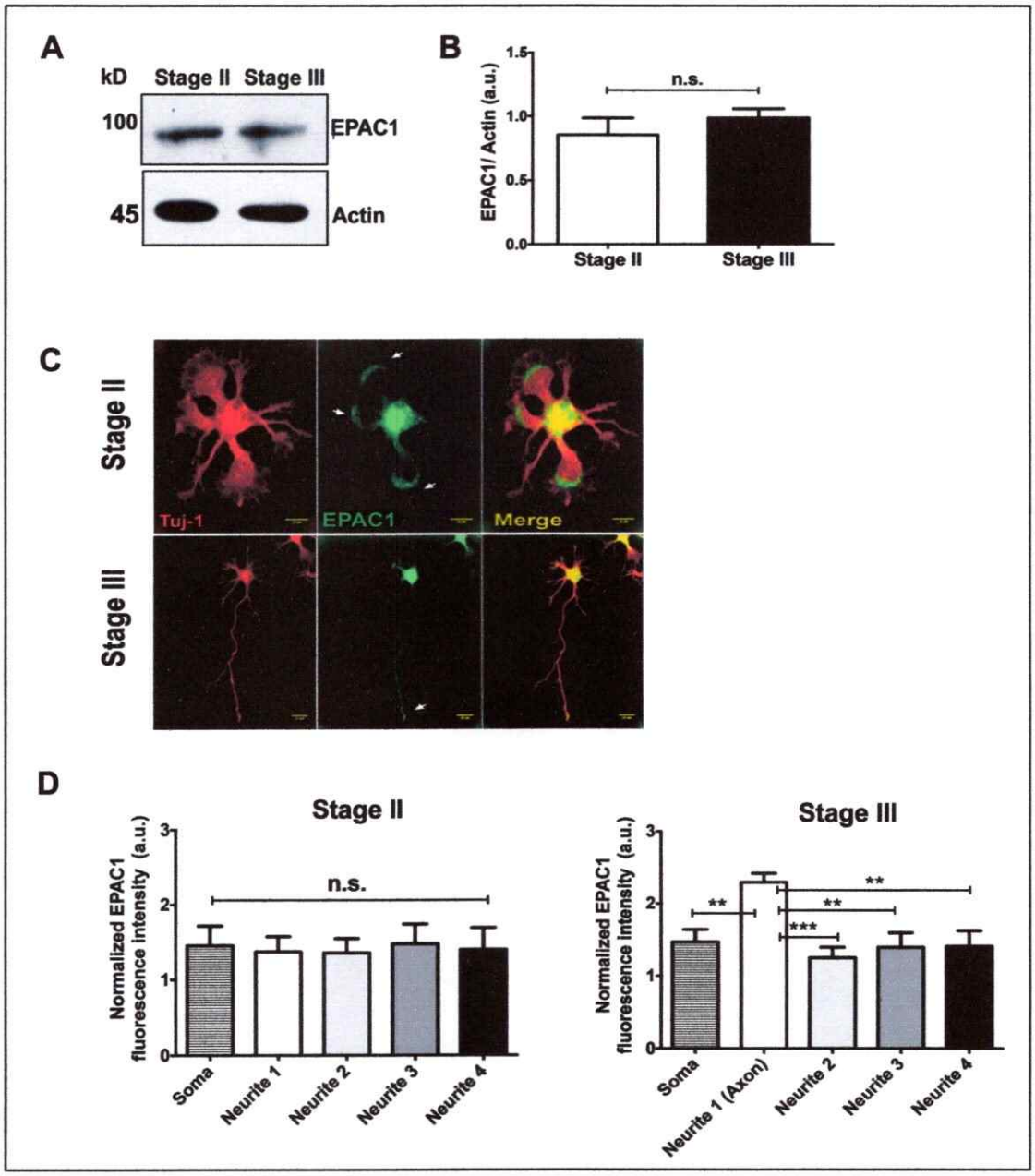

Figure 10: Expression and distribution of EPAC1 during development of cultured hippocampal neurons.

(A) EPAC1 immunoblot in cultured neurons at Stage II and III (B) Quantification of EPAC1 expression show no differences between stages (according to (Dotti et al., 1988a)) (Stage II: 0,86±0,13, Stage III: 0,98±0,1, Student's t tests, three

independent experiments). (C) EPAC1 distribution in cultured hippocampal at Stages II and III, show no preferential staining for EPAC1 in minor neurites in Stage II neurons (upper panel, white arrows). In contrast, EPAC1 is accumulated at the distal tip of growing axon in Stage III neurons (lower panel, white arrow) (D) Quantitative analysis of EPAC1 distribution in stage II showed no preference, while neurons in Stage III display increased EPAC1 immunostaining in the axon (n=20 neurons, Stage II: n.s., Stage III: Axon vs soma, ** p<0,01; Axon vs neurite 2, ***p<0,001; Axon vs neurite 3 **p<0,01; Axon vs neurite 4 **p<0,01, One-way ANOVA with Tukey's post hoc test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant, Scale bars: C upper panel 10 µm; lower panel 20 µm.

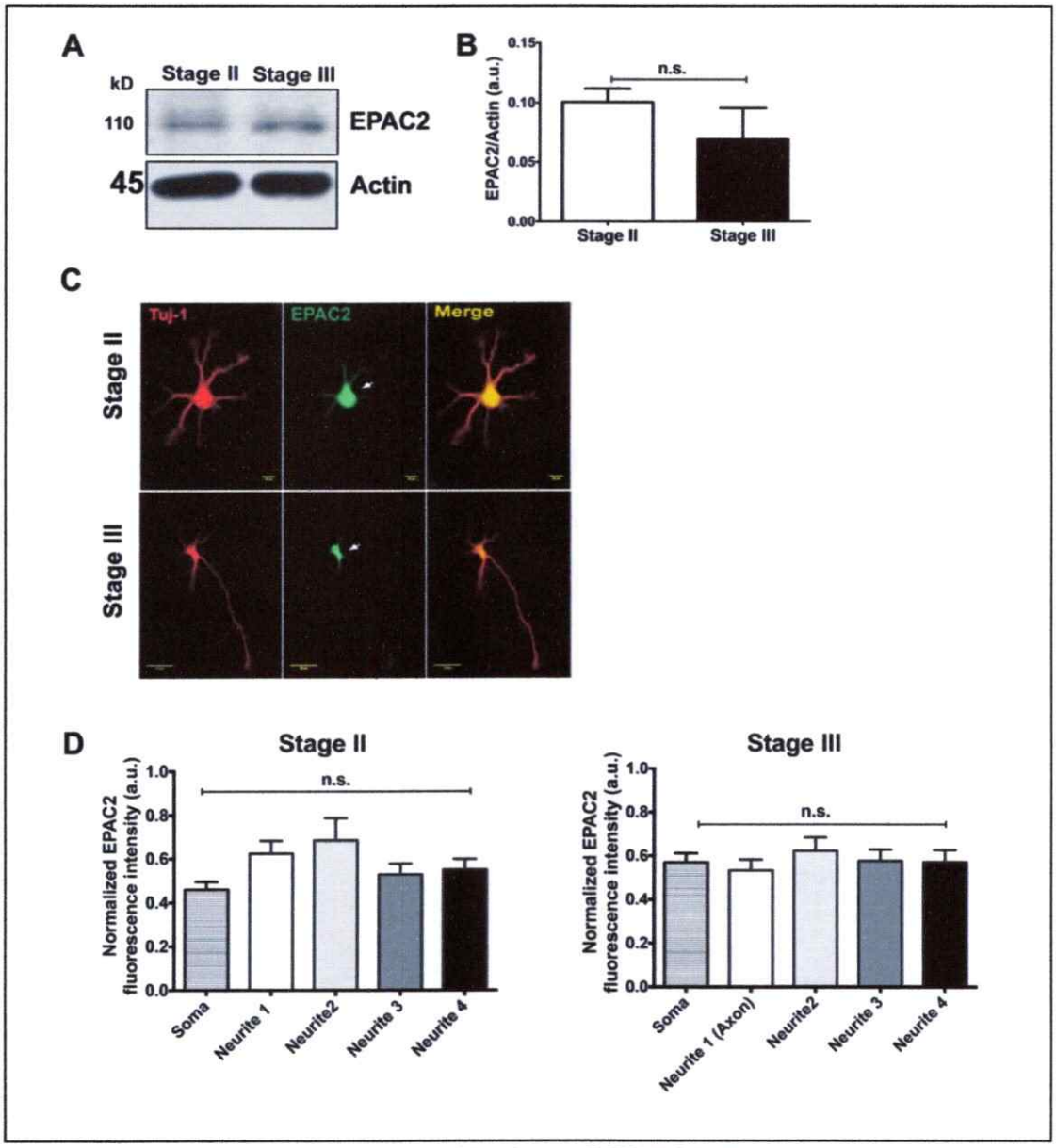

Figure 11: Expression and distribution of EPAC2 during development of cultured hippocampal neurons.

(A) EPAC2 immunoblot in cultured neurons at Stage II and III (B) Quantification of EPAC1 expression show no differences between stages (according to (Dotti et al., 1988a)) (Stage II: 0,86±0,13, Stage III: 0,98±0,1, Student's t tests, three independent experiments). (C) EPAC2 distribution in cultured hippocampal

neurons at stages II and III (white arrow), show faint immunoreactivity at both stages with no obvious subcellular enrichment in stage III neurons. (D) Quantitative analysis of EPAC2 distribution in both stage II and III revealed no enrichment on axons (n=30 neurons, Stage II: n.s., Stage III: n.s., One-way ANOVA with Tukey's post hoc test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant, Scale bars: C upper panel 10 µm; lower panel 20 µm.

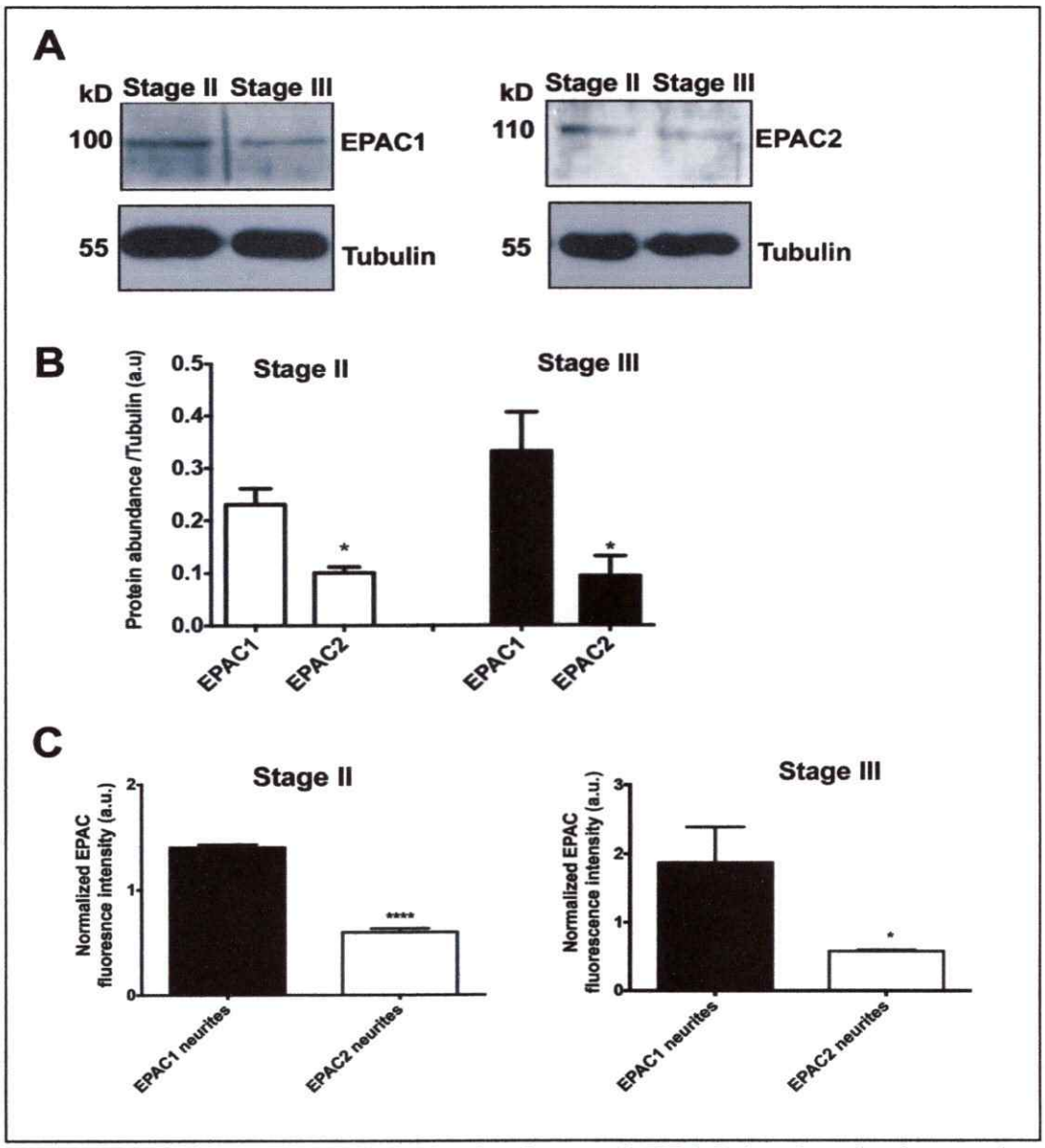

Figure 12: EPAC1 is the main isoform of EPAC in developing cultured neurons.

(A) Immunoblot assay revealed higher EPAC1 levels in both stage II and stage III as compared with EPAC2 (B) Quantification of EPAC1 and EPAC2 show hoigher protein abundance of EPAC1 over EPAC2 at stage II and III (EPAC1,

Stage II: 0,23±0,03 vs EPAC2 Stage II: 0,10±0,01, EPAC1 Stage III: 0,33±0,07 vs EPAC2 stage III: 0,09±0,04, * p<0,05, Student's t tests, three independent experiments). (C) EPAC1 total neurite inmunostaining is significant higher than EPAC2 at both stages (n=20-30 neurons, Student's t tests, *p<0,05, **** p<0,0001). Error bars indicate ±SEM..

In order to assess the role of EPAC activation in neuronal polarity and axonal elongation the next group of the experiment were conducted to analyze the effect of the pharmacological and genetic gain of function of EPAC and pharmacological loss of function EPAC in hippocampal neurons and to determine the relation of EPAC and its downstream effector Rap1B.

EPAC induced multiples axons through Rap1B activation

We initially set up pull down assays to evaluate pharmacological activation and inhibition of EPAC functions. We used a selective agonist, 8-pCPT, which activates EPAC but not PKA both in vitro and in vivo (Woolfrey et al., 2009; Lim et al., 2012), and the selective antagonist of both EPAC1 and EPAC2, ESI-09 and antagonist of EPAC2, ESI-05 (Tsalkova et al., 2012a; Tsalkova et al., 2012b). The inmunoblot analysis showed a significant increase in the fraction of GTP-bound Rap1B in cortical neurons treated with 10 µM 8-pCPT (1,53±0,13, p<0,05, three independent experiments) and a significant reduction of Rap1B activity in cultures treated with 15 µM of ESI-09 (0,58±0,12, p<0,05, three independent experiments) but not with 15 µM of ESI-05 (0,98±0,38, p>0,05, three independent experiments) (**Fig 13A, B**). These results suggest that 8-

pCPT stimulates Rap1 activity in neurons while ESI-09 inhibited Rap1 activation. Interestingly, ESI-05 the selective EPAC2 antagonist did not induced inactivation of Rap1B in cultured neurons, reinforcing the idea that EPAC1 would be the isoform involved in Rap1B activation in neurons.

We then examined the functional role of EPAC proteins during in vitro culture of hippocampal neurons prior to polarization. A single dose of 8-pCPT (10 µM) neurons were incubated in the presence of the drug to DIV 3. The results showed that most of vehicle-treated neurons formed one long Tau-1-positive axon and several shorter minor neurites MAP2 positive (**Fig. 14A**). In contrast, the 8pCPT-treated neurons displayed multiples axon-processes positives for two axonal markers hypophosphorylated Tau (epitope Tau-1) (**Fig 14A**) (DMSO: 5,83±1,83; n=76 neurons, 8-pCPT: 51,6±4,41 n=70 neurons, p<0,001, four independent experiments, **Fig 14C**) and mode I phosphorylated MAP1B (epitope SMI-31) (n=50 neurons, three independent experiments, **Fig 14B**). Moreover, neurons treated with 8-pCPT showed increased total axonal length compared to control group (**Fig 14C**. DMSO: 194,7±7,99, n=70 neurons vs 8-pCPT: 275,0±19,19, n=63 neurons, p<0,0001, four independent experiments).

In order to confirm that 8-pCPT was indeed activating Rap1B protein, we transfected hippocampal neurons with a GFP-fused Ral-GDS-RBD construct which is a reporter of active Rap1, previously used to assess subcellular localization of active Rap1 in several cell types (Bivona et al., 2004; Jordan et al., 2005; Kortholt et al., 2010). Neurons were transfected upon plating and further incubated with 8-pCPT (10 µM) at 16 h after plating and stained with

Tau1, to identify axonal compartment at 3 DIV (**Fig 15 A**). We quantified the fluorescence intensity of Ral-GDS-GFP in the last third of Tau1 positive axon and the results obtained show that transfected-control neurons display one Tau1 positive axon showing Ral-GDS-GFP fluorescence both along the axon shaft and cell body (**Fig 15B**). Whereas neurons treated with 8-pCPT exhibited multiples axons positive for Tau1 (**Fig 15B**) and the RalGDS-GFP signal was significantly concentrated to the distal end of each supernumerary axons (**Fig. 9G**, DMSO: 0,38±0,07, n=36 neurons vs 8-pCPT: 0,62±0,08, n=55 neurons, $p<0,05$, three independent experiments).

These data confirm that EPAC proteins mediate Rap1B activity, which is responsible of exacerbated neuronal polarity.

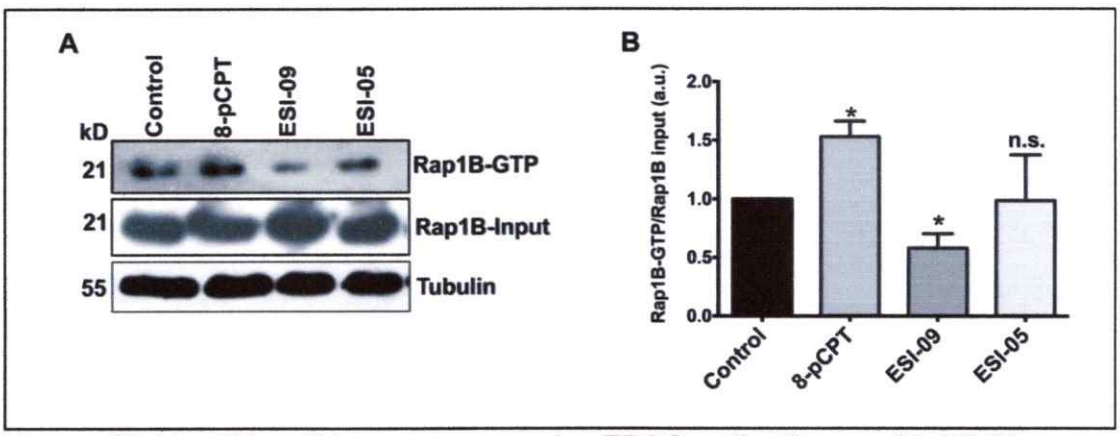

Figure 13: Rap1B pulldown assay under EPAC activation and inhibition.

Cortical neurons were incubated with EPAC specific agonist (8-pCPT) and antagonist (ESI-09 and ESI-05) and then activated Rap1b was evaluated by pull down. ESI-09 inhibits EPAC1, while ESI-05 inhibits EPAC1 and EPAC2. 8-pCPT increased Rap1B-GTP content as compared with control conditions. ESI-09 reduced Rap1B-GTP levels, while ESI-05 showed no dramatic decrease of

Rap1B-GTP. (B) Quantitative analyses revealed that EPAC agonist increased Rap1B activity almost 60%. In contrast ESI-09 decreased Rap1B activity to 50%, while ESI-05 showed no significant Rap1B-GTP reduction (n = 3; *p < 0,05, compared to control, Student's t tests, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant.

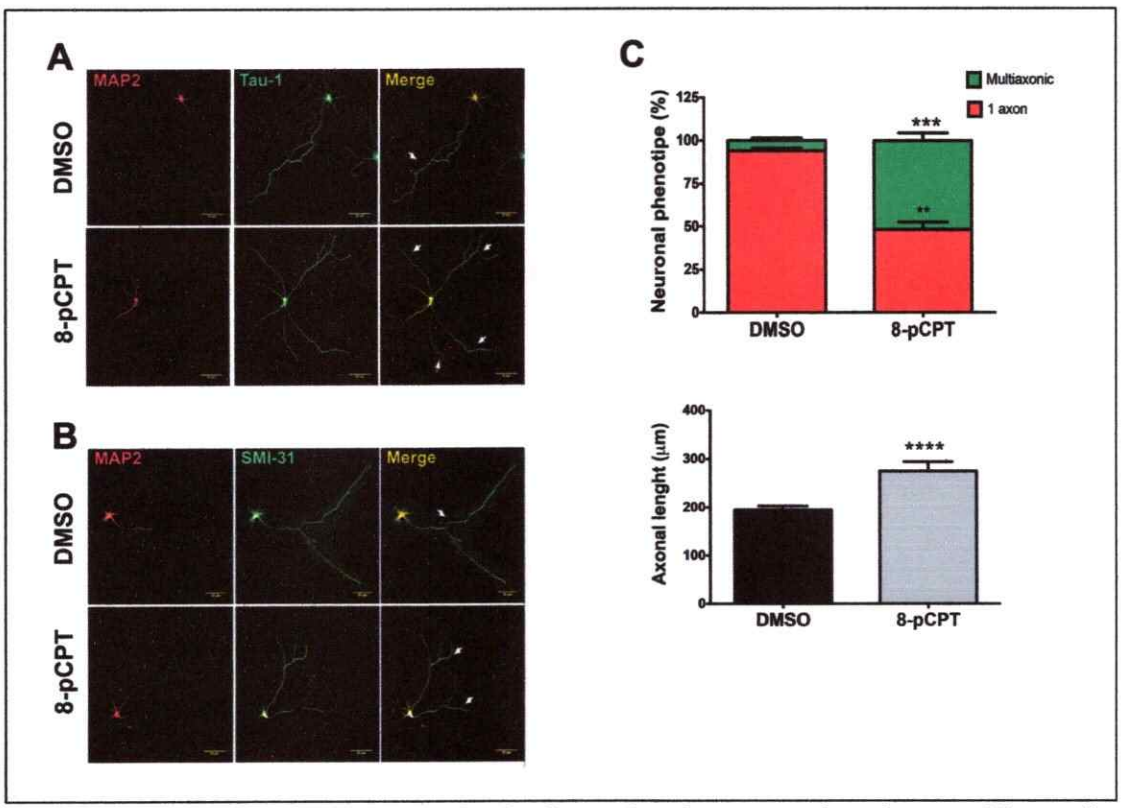

Figure 14: EPAC pharmacological activation results in multiaxonic neurons.

(A) DMSO control neurons cultured for 3 DIV display normal appearance with one long and thin neurite immunopositive for TAU staining (axon, in green, arrow) and several minor tapering neurites immunopositive for MAP2 staining (somatodendritic compartment, in red). 8-pCPT treated neurons display more

than one axon (arrows). (B) DMSO control neurons cultured for 3 DIV display normal appearance with one long and thin neurite immunopositive for phosphorylated MAP1B staining (axon, in green, arrow) and several minor tapering neurites immunopositive for MAP2 staining (somatodendritic compartment, in red). 8-pCPT treated neurons display more than one axon as indicated by phosphorylated MAP1B staining (arrows). (C) Quantitative experiments shows that 50% of neurons stimulated with EPAC agonist (8-pCPT) were multiaxonic. In addition, the mean total axonal length was also increased in neurons treated with EPAC agonist (n = 63-70 neurons; **p < 0,01, ***p < 0,001, ****p<0,0001, Student's t tests, four independent experiments) Error bars indicate ±SEM, Scale bars: A, B 50 µm

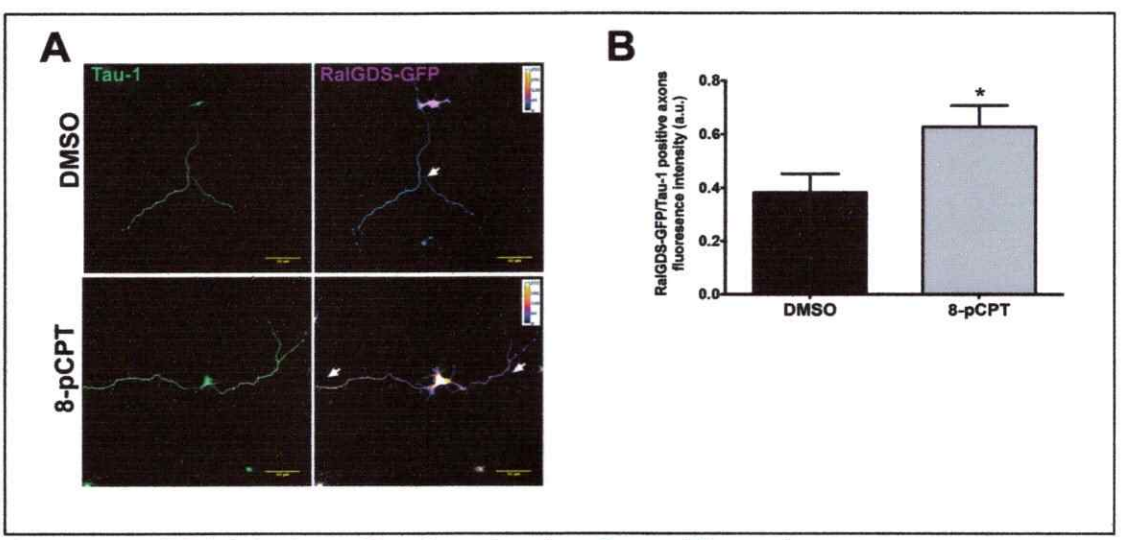

Figure 15: EPAC induce Rap1 activation in multiaxonic neurons

(A) DMSO control neurons show increased Rap1B activity in cell body and growing axon as revealed with Ral-GDS-GFP construct. 8-pCPT treated neurons showed increased Rap1B measured by Ral-GDS-GFP in neurites

immunopositive for TAU staining (arrows). (B) Quantitative analysis revealed higher ratio of Ral-GDS-GFP signaling to Tau-1 staining in neurons treated with EPAC agonist (n =36-55 neurons; *p < 0,05, Student's t tests, three independent experiments). Error bars indicate ±SEM, Scale bars: A 50 µm.

Activation of EPAC by 8-pCPT induce multiples axons positive for mature axonal markers.

In the next set of experiments we decided to test whether short-time effects induced by EPAC activation, namely induction of supernumerary axons, was maintained in long-term neuronal cultures. For this, we decided to evaluate the assembly of the axon initial segment (AIS) in long-term neurons treated with 8-pCPT. AIS is a physical and functional barrier between somatodendritic and axonal compartments, where Na+ and K+ channels are recruited to trigger action potential (Grubb and Burrone, 2010). The assembly of AIS represents the maturation and functional polarization of the axon (Rasband, 2010) For this aim, we used 10 DIV neurons to check whether neurons treated with 8-pCPT exhibit more than one Tau-1 positive process containing assembled AIS. We choose to evaluate the distribution of Ankyrin G (ankG) at the first 15-25 µm of the axon, since it has been shown to be essential for the organization of the AIS (Kordeli et al., 1995; Galiano et al., 2012). 8-pCPT was added to culture medium at 18 h in culture and neurons were fixed at day 10. Control and DMSO neurons revealed that in average 94.1±3.0% neurons display only one Tau-1 and AnkG positive axon, which was also negative for MAP2 staining (**Fig 16A, 16B**). In addition, AnkG was concentrated in a proximal region of each axon, a

characteristic pattern of AIS in cultured neurons (Kordeli et al., 1995; Galiano et al., 2012). In contrast, neurons treated with 8-pCPT exhibit in average 76.4±11.5% multiple axons, which were positive for Tau1 and AnkG and negative for MAP2 staining (Figure 16A, 16B). Next, to assess if these multiples axons were functional, we analyzed the distribution of the vesicular glutamate transporter, VGLUT1-Venus fusion construct, in neurons treated with 8-pCPT and cultured for 8 DIV before fixation. Previous studies have shown that VGLUT1 is a reliable indicator of synaptic vesicles loaded with glutamate (Bellocchio et al., 2000) even before synapses were formed (Sabo et al., 2006) and interestingly the overexpression of VGLUT1 constructs display the same subcellular distribution of the endogenous VGLUT1 protein characterized by a clear punctate pattern along the axon, in particular, presynaptic buttons (Wilson et al., 2005). Neurons were stained for Tau-1 and MAP2 and the distribution of VGLUT-Venus positive spots were quantified in a three-dimensional reconstruction of 100 µm along the axons (**Fig 17A, 17B**). The results showed, as expected, that transfected neurons under 8-pCPT treatments, display multiples axons Tau-1 positives (DMSO: n=12 neurons, three independent experiments, 8-pCPT: n=12 neurons, three independent experiments, **Fig. 17A**). Control neurons displayed 11.4±1.30 of VGLUT1-Venus positive spots per 100 µm of axonal length (**Fig 17C**). A very similar pattern was found in neurons bearing multiples axons that showed 11.5±1.12 VGLUT1-Venus positive spots per 100 µm of axonal length (**Fig. 17C**). In both cases, the distribution of VGLUT1-Venus was in punctate structures similar to those found in excitatory

neurons (Wilson et al., 2005), suggesting that multiple axons induced by EPAC activation might be functional. We further examined the expression and distribution of another axonal marker, synaptophysin in order to confirm the results obtained with AnkG and the overexpression of VGLUT in neurons with multiples axons induced by EPAC activation. Synaptophysin is a marker of synaptic density that predominantly show granular or punctate staining along axons that accumulate toward distal axon where it is involved in neurotransmitter release, being a good candidate of axon functionality (Fletcher et al., 1991b). We co-transfected neurons with red fluorescence protein (RFP) as a reporter gene to identified single neurons in confluent cultures. Next, cultures were treated with 8-pCPT and maintained for 8 DIV, fixed and performed immunocytochemistry for synaptophysin and MAP2. **Fig 18** show representative images of a control neuron with a single axon and a neuron treated with 8-pCPT showing multiples axon positive for synatophysin whose inmunostaining was very similar in both groups showing the cannonical punctate structures widely distributed along axons (Fletcher et al., 1991a).

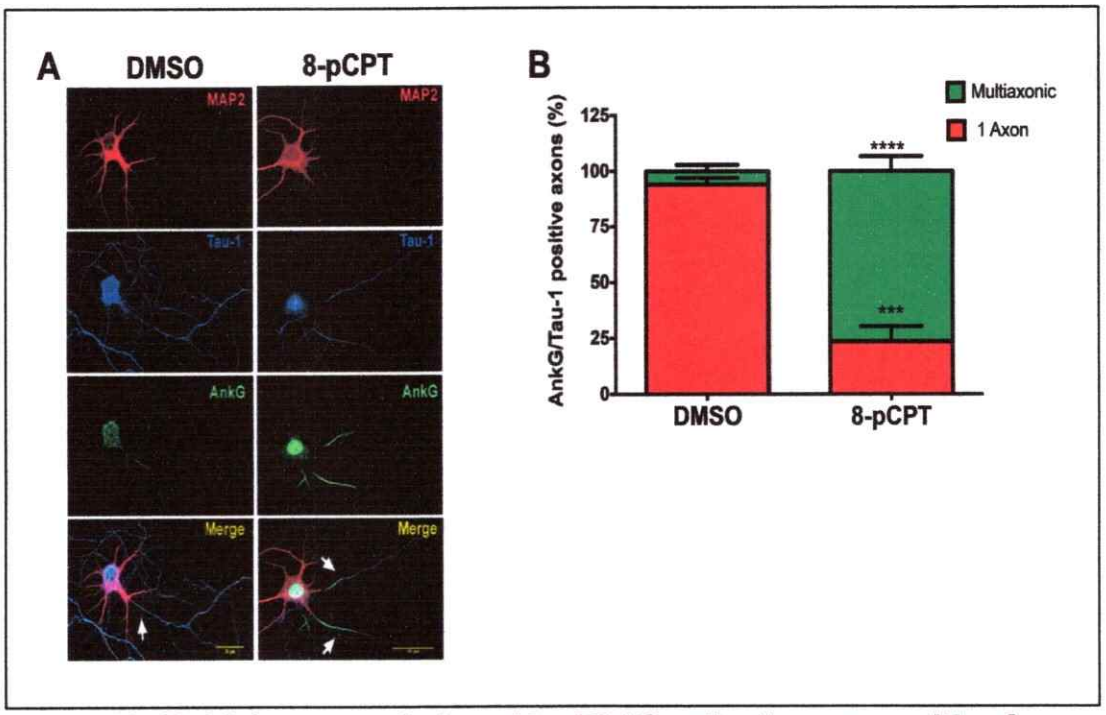

Figure 16: Multiples axons induced by EPAC activation are positive for AnkG.

(A) DMSO control neurons cultured for 10 DIV display an axonal initial segment (AIS), which is positive for ankG staining, and negative for MAP2 staining (arrow). Neurons treated with 8-pCPT displays more than axon at 10 DIV which display characteristic of mature axons, ankG and tau1 positive staining and MAP2 negative staining (arrows). (B) Quantitative analysis revealed a small fraction of neurons display more than one AIS in DMSO treated cells (less than 5 %), while 75% of neurons display more than one AIS in 8-pCPT treated samples (n = 60 neurons; ***p < 0,001, ****p<0,0001, Student's t tests, four independent experiments). Error bars indicate ±SEM, Scale bars: A 30 µm;

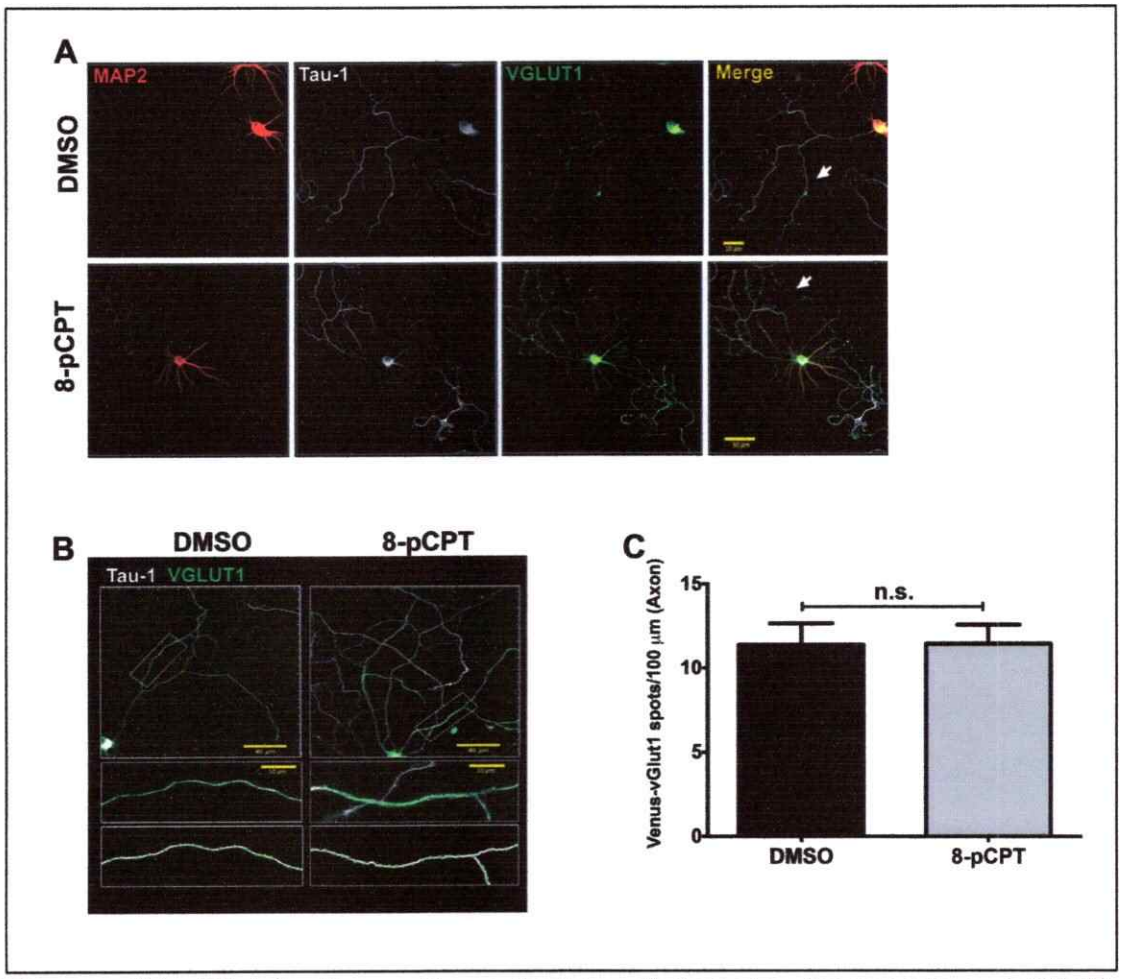

Figure 17: Distribution of vesicular glutamate transporter (vGluT) immunolabeling in multiples axons induced by EPAC activation.

(A) DMSO control neurons cultured for 10 DIV showed a punctate distribution of YFP-VGLUT1 (green) decorating the axon (tau1 positive in white and arrow) but not dendrites (MAP2, red) as expected for mature neurons. Similarly, neurons treated with 8-pCPT displayed more than one axon (TAU 1 positive and arrows), which contained punctate distribution of YFP-GLUT1 (green) in all axons and is absent from dendrites (MAP2, red). (B) Higher magnification of axons in DMSO and 8-pCPT neurons were used for z-stack reconstruction and semi-

automatized analyses of YFP-VGLUT puncta. Upper images show raw images of neurons cultured for 10 DIV. Middle panel show magnification indicated in white rectangle on upper panel. Lower panel showed filaments (TAU 1 staining in axons) and dots (YFP-VGLUT1 puncta staining). (C) Quantitation analyses revealed that DMSO and 8-pCPT display similar amounts of VGLUT1 puncta per 100 µm axon segment (n =12 neurons; n.s., Student's t tests, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant, Scale bars: A upper panel 20 µm, lower panel 50 µm; B upper panel 40 µm, lower panel 10 µm.

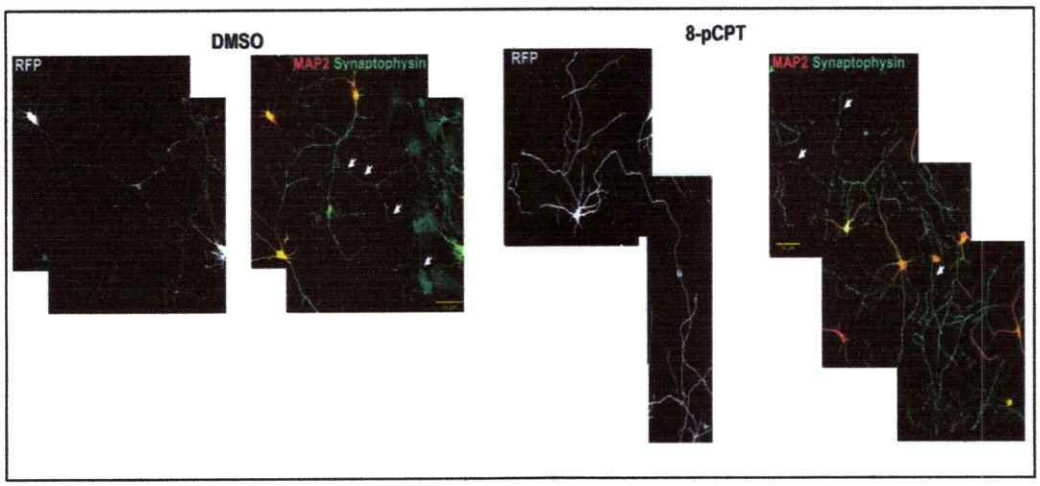

Figure 18: Distribution of synaptophysin in multiples axons induced by EPAC activation.

DMSO control neurons were transfected with RFP (white) as volume marker to allow individual identification of mature neurons. Control neurons show endogenous synaptophysin staining (green, white arrow) accumulated in a

highly branched axon, negative for MAP2 staining (red). 8-pCPT neurons display more than one highly branched axon (arrows), which was positive for synaptophysin staining (green) and negative for MAP2 staining (red). Scale bars: 50 µm.

Gain of function of EPAC1 induces multiples axons similar to 8-pCPT treated neurons

Previous work of Murray et al. showed that EPAC1 expression in brain tissue is higher at embryonic ages, whereas EPAC2 is barely detectable at this developmental stages (Murray and Shewan, 2008). Our results in Figures 10D and 11D showed that EPAC1 expression is also predominantly over EPAC2 in stages II and III of hippocampal neurons and biochemical analysis from Figure 13A showed that pharmacological inhibition of EPAC2 with ESI-05 did not impacted in the activity of Rap1B. We therefore wanted to test whether EPAC1 was sufficient to modify neuronal polarity. To verify this hypothesis, we first performed pull-down assays to examine Rap1 activation in response to different forms of EPAC1 (e.g wild type EPAC1, dominant negative EPAC1 and constituvely active EPAC1). COS 7 were transfected with EPAC1 constructs and additionally as a negative control, the Rap1 GTPase-activating-protein (GAP) (RFP-tagged-Rap1GAP) which acts as a GTPase activating protein, leading to Rap1 inactivation. Pull-down results revealed that the amount of GTP-bound Rap1 was increased in response to wild type EPAC1 and constituvely active EPAC1 and decreased by using either dominant negative form of EPAC1 and Rap1GAP (**Fig. 19A, 19B**). As expected, these results indicate that Rap1

activity is transiently activated or inhibited in response to different EPAC1 constructs. To further confirm the contribution of EPAC1 on the multiple axons phenotype in hippocampal neurons, we co-transfected neurons (2h after plating) with the reporter vector RFP and wild type, dominant negative, constituvely active vectors of EPAC1 or our negative control, RFP-RapGap, and neuronal polarity was evaluated at 3 DIV by measuring the length of axons and staining of the axon-specific marker, Tau1 and the somatodendritic marker MAP2 (**Fig. 20A**). Our data indicates that the overexpression of a wild type and constituvely active form of EPAC1 led neurons beating multiple axons (wild type EPAC1: 22±8.1%, n=53 neurons, constituvely active EPAC1: 36.7 ± 2,0%, n=80 neurons, three independent experiments, **Fig. 20B**). On the other hand, the overexpression of a dominant negative form of EPAC1 leads to neurons displaying mostly a single axon. A small percentage of neurons showed multiaxons but was not significantly different from the gain-of-function group, and was non significantly different from RFP control and Rap1GAP transfected neurons (RFP: 2.0±0.9%, n=50 neurons, Dominant negative EPAC1: 4.3 ±2.3%, n= 54 neurons, RapGAP: 3.0 ± 3.0%, n= 60 neurons, three independent experiments, p>0,05 **Fig. 20B**). Measurements of length axons average revealed that overexpression of constituvely active EPAC1 produced axons significantly longer than the RFP control whereas the dominant negative EPAC1 and Rap1GAP overexpression induced a significant reduction of axonal length compared to RFP control (RFP: 318.1±13.2 µm vs CA-EPAC1: 392.1±21.9 µm, p<0.05, DN-EPAC1: 207.7±19.8 µm, p<0.01, Rap1Gap: 195±13.5 µm p<0.001,

Fig 20C). These results are consistent with our previous pharmacological treatments of 8-pCPT from **Fig 14A** and **15A**, supporting that EPAC1 is sufficient to produce multiple axons during development of polarity in vitro through Rap1B.

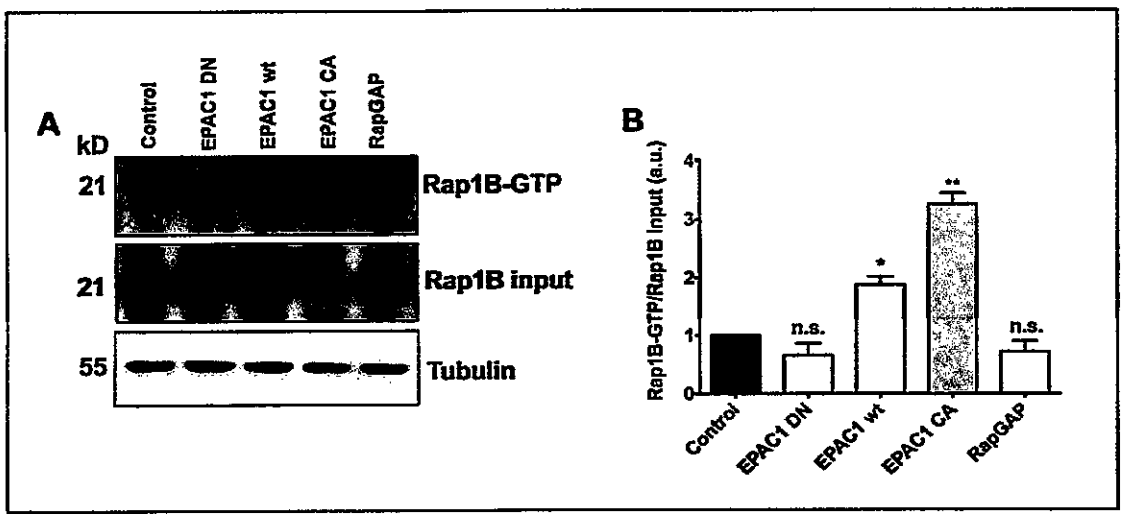

Figure 19: Effect of EPAC1 constructs on Rap1B activity.

(A) Rap1B activation assay was used to evaluate the effect of wild type (WT), constitutively active (CA) and dominant negative (DN) constructs of EPAC1 in COS7 cells. WT- and CA-EPAC1 induced robust Rap1B activation. In contrast, DN-EPAC1 constructs decrease Rap1B activity. In addition, cells were transfected with RapGAP constructs which inactivate Rap1B (B) Quantitative analysis reveals that WT- and CA-EPAC1 constructs significantly increased Rap1B activity (2- and 3-fold respectively) (DN Epac1: 0,7±0,20 wt Epac1: 1,9±0,14 Ca Epac1: 3,3±0,18 RapGAP1: 0,72±0,19 compared to normalized Control, *p<0,05, ****p<0,0001, One-way ANOVA with Dunnett's multiple

comparison post hoc test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant,

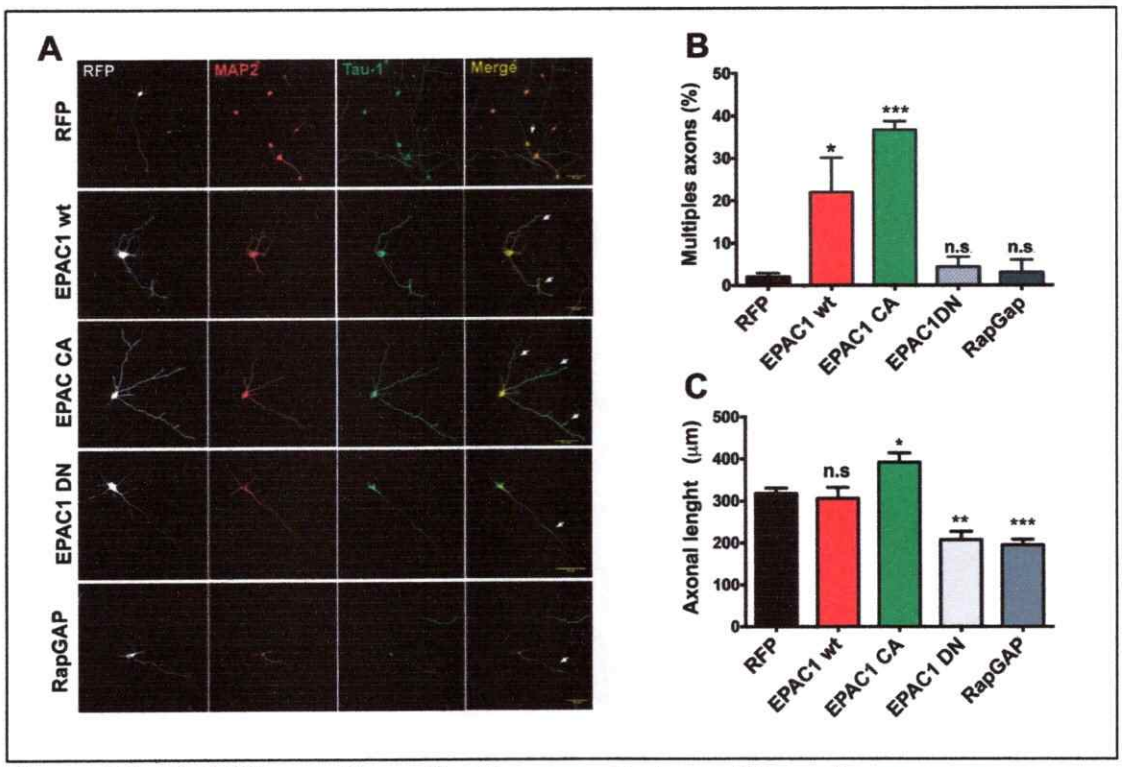

Figure 20: EPAC1 genetic activation results in multiaxonic neurons

Neurons were transfected with EPAC1 constructs along with RFP as volume marker. (A) Control neurons display a single axon positive for Tau-1 (green) and several minor neurites positive for MAP2 (red). WT- and CA-EPAC1 transfected neurons more than one axon (arrows) positive for Tau-1 (green) and negative for MAP2 (red). DN-EPAC displays a single axon (arrow) positive for Tau-1 (green) and negative for MAP2 (red). Rap-GAP transfected neuron show a single axon (arrow) positive for Tau-1 (green) and negative for MAP2 (red). (B) Quantitative analysis revealed that WT- and CA-EPAC1 significantly increased the amount of neurons displaying more than one axon (n=50-80 neurons,

*p<0,05, *p<0,001, n.s., compared to control, One-way ANOVA with Dunnett's multiple comparison post hoc test, three independent experiments) (C) Quantitative analysis of mean axonal length revealed that CA-EPAC significantly increased axonal length. In contrast, neurons expressing DN-EPAC1 and Rap-GAP showed shorter axons (n=50-80 neurons, *p<0,05, **p<0,01 ***p<0,001, n.s., compared to control, One-way ANOVA with Dunnett's multiple comparison post hoc test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant, Scale bars: A 50 μm.

Pharmacological inhibition of EPAC impairs axon development.

To clarify the role of EPAC in neuronal polarity, loss-of-function experiments were performed using a pharmacological approach with ESI-09, which inhibits cAMP-mediated activation of EPAC1 and EPAC2 but not PKA (Almahariq et al., 2013) We did not performed treatments with ESI-05 which selectively inhibit EPAC2 (Tsalkova et al., 2012b) because we did not see any significant changes in Rap1B activity in figure 13A and since EPAC2 is expressed at very low levels in rat hippocampal neurons we decide to continue our work only with ESI-09. Therefore, Hippocampal neuron were treated at 16 h after plating with 15 µM of ESI-09, and 8-pCPT (10 µM) incubated for 48 h, and further stained for axon and somatodendritic markers. Results shown in Figure 21A show that ESI-09 disrupted the formation of axons characterized by a weak Tau1 staining and very short axons. Quantitative analyses revealed a significantly reduction of the percentage of polarized neurons with the inhibitor, compared to those neuron

treated with vehicle and 8-pCPT (DMSO: 88.7±7.4%, n=50 neurons vs 8-pCPT: 100% n=50 neurons, n.s.; ESI-09: 31.8±2.5%, n=50 neurons, p<0,001, four independent experiments, **Fig 21B**). Regarding with the axonal elongation, the presence of ESI-09 induced neurons with short axons with an average length of 94,3 ±7,0 µm significantly reduced compared with the 151 ± 11 µm of controls neurons (**Fig 21C**). These results suggested that EPAC is required for axon elongation and specification during the establishment of neuronal polarity and reinforce the idea that Rap1B activation during neuronal polarity would be mainly through EPAC1.

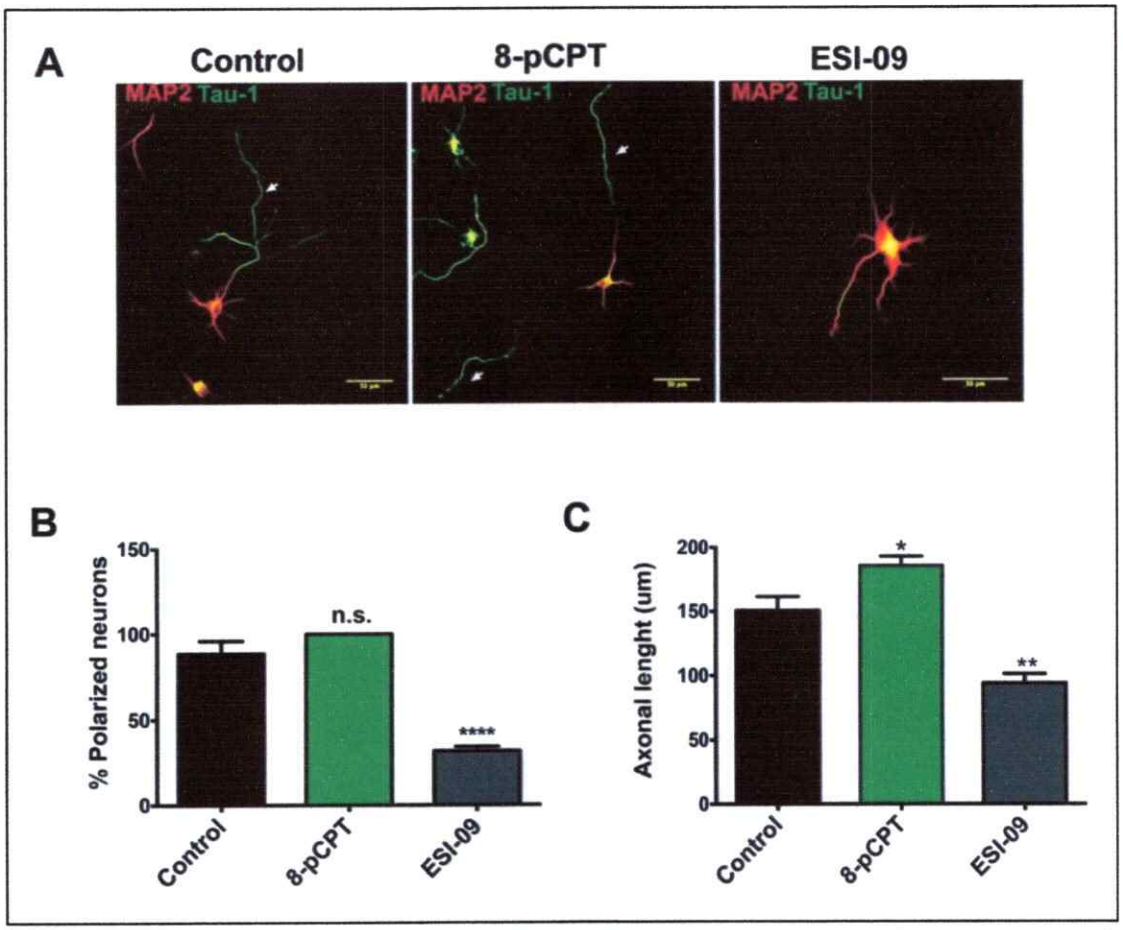

Figure 21: Loss of function of EPAC alters the polarity of hippocampal neurons.

(A) Neurons were treated with control vehicle, EPAC agonist (8-pCPT) or the antagonist of both EPAC1 and EPAC2 (ESI-09). DMSO treated neurons display a single axon immunostained with Tau-1 (green) and minor neurites stained with MAP2 (red). 8-pCPT treatment induced multiaxonic neurons positive for Tau-1 (green, arrows). ESI-09 treated neurons display shorter axons, which were equally stained with Tau-1 (green) and MAP2 (red). (B) Quantitative analysis of neurons correctly polarized revealed that ESI-09 impaired proper neuronal polarization (n=50 neurons, ***p<0,001, n.s., compared to control, One-way

ANOVA with Dunnett's multiple comparison post hoc test, three independent experiments). 8-pCPT neurons were 100% polarized. (C) Quantitative mean axonal length revealed increased axonal elongation in neurons treated with 8-pCPT. In contrast, ESI-09 induces axon shortening (n=50 neurons, *p<0,05, **p<0,01, compared to control, One-way ANOVA with Dunnett's multiple comparison post hoc test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant, Scale bars: A 50 µm.

The next set of experiments was designed to determinate whether EPAC role in neuronal polarization was independent of PKA signaling and to evaluate Rap1b downstream signaling.

Axon specification regulated by EPAC is independent on PKA signaling

Previous studies have been shown the role of the cAMP-PKA signaling in the establishment of neuronal polarity by at least two different complementary mechanisms. Elevation of cAMP induces increased LKB1 phosphorylation by PKA, promoting axon formation (Shelly et al., 2007; Shelly et al., 2010a). On the other hand, phosphorylation of the E3-ligase Smurf1 by PKA switches its selectivity for ubiquitination of Par6 or RhoA, promoting the accumulation of Par6 and degradation of RhoA in the axonal compartment (Cheng et al., 2011b). In this context, we tested the influence of PKA inhibition under gain of functions experiments of EPAC by 8-pCPT. For this approach we used the inhibitor PKI-(Myr-14-22)-amide (PKI) that binds to the free catalytic subunit of PKA and prevents phosphorylation of PKA targets (Dalton and Dewey, 2006) without nonspecific effects reported for other PKA inhibitors such as KT5720 and H89

(Murray, 2008). To test whether PKI can prevents phosphorylation of PKA targets. We added 20 µM of PKI alone or in combination with 10 µM of 8-pCPT to hippocampal, and next, western blotting of protein lysates were performed using an antibody that specifically recognizes nuclear and cytoplasmic PKA substrates (PKA-specific phospho-motifs ([RR]-X-[S*/T*])) (Bacallao and Monje, 2013) to analyze the levels of PKA inhibition (**Fig 22A**). Result showed that at this concentration, PKI reduce the phosphorylation of PKA substrates either alone or in combination 8-pCPT, compared to cultures treated with the vehicle (**Fig 22B**). We then confirmed the effect of PKA inhibition in the establishment of neuronal polarity. We treated hippocampal neurons at 16 h after plating with DMSO, 20 µM of PKI and a combination of 20 µM of PKI plus 10 µM of 8-pCPT, the cultures were fixed at 2 DIV and analyzed by immunofluorescence for neuronal phenotype (**Fig 23A**). Quantitative analyses revealed that PKI treatment did not significantly affected the neuronal polarization (DMSO: 91±4 %, n= 50 neurons; PKI: 89±4%, n=50 neurons, three independent experiments, n.s., **Fig 23B**) and the percentage of multiples axons were similar to those treated with the vehicle (DMSO: 8.4±3%, n=50 neurons; PKI: 4.2±2 n=50 neurons, three independent experiments, n.s., **Fig 23B**). Interestingly, PKA inhibition did not affected the generation of multiples axons produced by EPAC activation under 8-pCPT treatments. In fact the percentage of multiaxonic neurons were significantly higher in cultures treated with PKI plus 8-pCPT compared to PKI and DMSO groups and no significant different to those observed with neurons treated with 8-pCPT alone (PKI/8-pCPT: 36±5%, 52

±4.4%, n=50 neurons, three independent experiments, p<0,01, **Fig 23B**). In addition, a significant reduction in the length of the axons could be observed in neurons treated with PKI compared to the control and PKI/8-pCPT group (**Fig 23C**; DMSO: 196±9 µm, PKI: 102±7 µm, n=50 neurons, p<0,001, three independent experiments) Neurons treated with PKI/8-pCPT showed a length of 254±12 µm (n=50 neurons, p<0,001, three experiments, **Fig 23C**) significantly higher compared to PKI and control.

Altogether these results point out that inhibition of PKA did not interfere in the generation of multiple axons by EPAC and interestingly, inhibition of PKA only affects axon elongation but not determination.

Finally, we studied the effect of 8-pCPT on Par6 stabilization and RhoA degradation in neuro2A cells (N2A), a paradigm to assess the role of PKA-dependent Smurf1 phosphorylation (Cheng et al., 2011b) (**Fig 24**). We found incubation of N2A cells with 10 µM of 8-pCPT did not induce any obvious change in the overall levels of Par6 and RhoA (**Fig 24A**). In contrast, 20 µM forskolin selectively increased the level of Par6 and decreased the level of RhoA (**Fig 24A, 24B**). Forskolin effects upon Par6 and RhoA were abrogated when we used 20 µM PKI (**Fig 24A, 24B**). **Fig 25A** show the inmunoblot of a Cdc42 pulldown from cortical neurons treated with 8-pCPT (10 µM), 8pCPT plus PKI (20 µM) and 8-pCPT plus ESI-09 (15 µM). Quantitative analysis shows that 8-pCPT did not modify Cdc42 activity downstream of EPAC-Rap1B activation (**Fig 25B**). Moreover, we verified that neither EPAC nor PKA inhibitors modified Cdc42 activity in culture neurons (DMSO: 0.55 ±0.11, 8-pCPT: 0.60±0.11, 8-

pCPT+PKI: 0.52±0.09, 8-pCPT+ESi-09: 0,51±0.02, three independent experiments, **Fig 25B**). Altogether these experiments suggest that activation of Rap1B and Cdc42 would be independent events during neuronal polarization. Therefore, the cAMP-PKA-dependent pathway involved in selective stabilization and degradation of proteins, relevant to axon formation seems complementary to the cAMP-EPAC-Rap1B signaling in neuronal polarity, strengthening the idea that both signaling pathways cooperate in mediating axon formation and elongation.

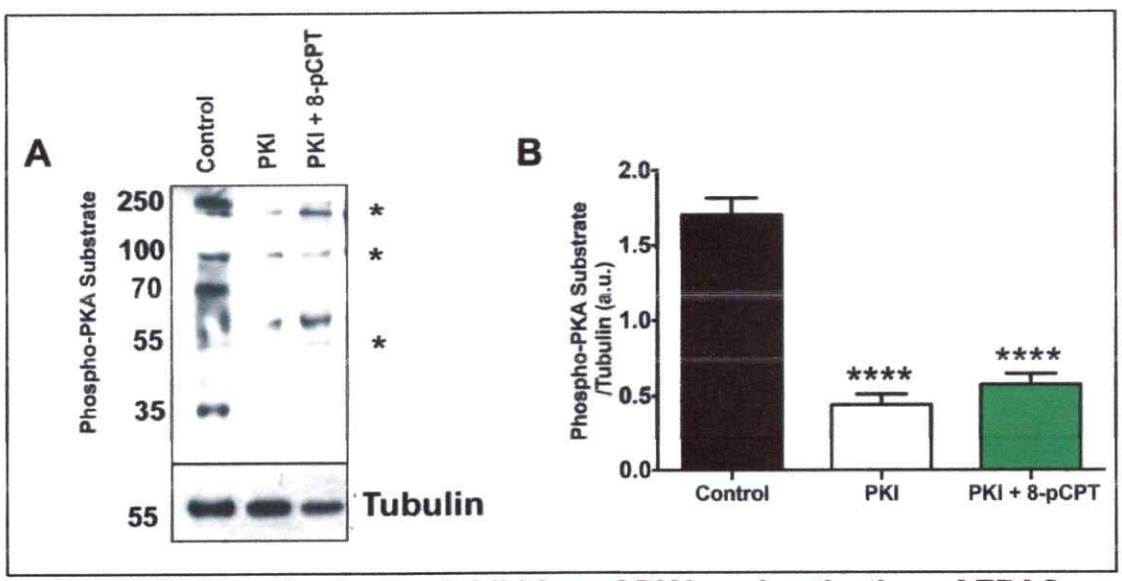

Figure 22: Effect of selective inhibition of PKA and activation of EPAC on phosphorylated PKA-specific substrates.

(A) Immunoblot of whole protein extracts from cultured neurons in the presence of vehicle (first lane), PKI (20 uM) a specific PKA inhibitor- (middle lane) and PKI in the presence of 8-pCPT (10 uM) (right lane) were incubated with an antibody directed that recognize phospho-PKA dependent epitopes. Vehicle lane displays a marked immunoreactivity against PKA-dependent epitopes. PKI treatment

induces a clear decrease in PKA-dependent epitopes (asterisks). Similarly, neurons treated with PKI+8-pCPT showed decrease immunoreactivity on PKA-dependent phosphoepitopes that was not affected by the presence of 8-pCPT. (B) Quantitative analysis revealed significant decreased on immunoreactivity of PKA-dependent epitopes in neurons treated with PKI or PKI+8-pCPT (**** p<0,0001, Student's t tests, three independent experiments). . Error bars indicate ±SEM.

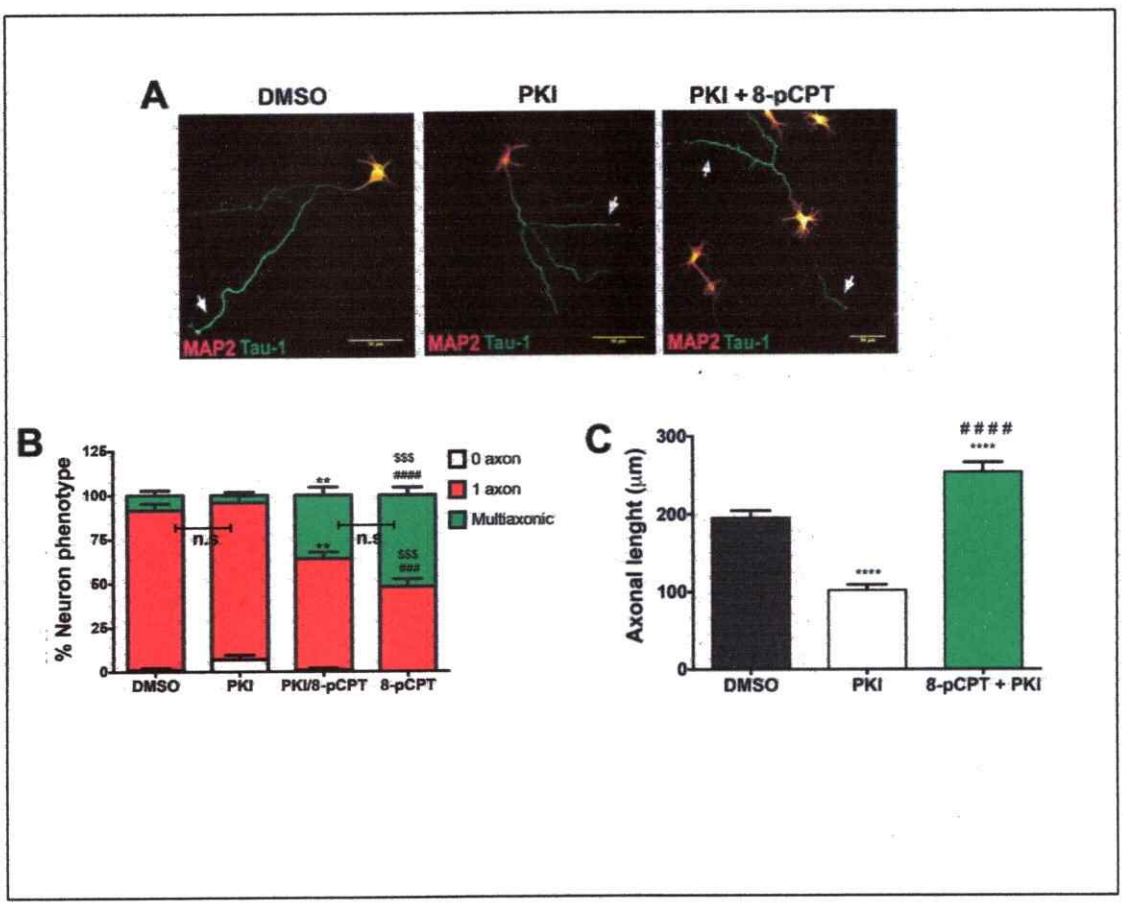

Figure 23: PKA inhibition did not impair the generation of multiples axon by EPAC activation.

(A) DMSO control and treated with PKI or PKI+8-pCPT neurons were assessed for neuronal polarization. Control neurons display a TAU positive staining for axon (green, arrow) and several minor neurites positive for MAP2 staining (red). Similarly, neurons treated with PKI showed a single axon positive for Tau-1 staining (green, arrow) and several minor neurites positive for MAP2 staining (red). In contrast, neurons incubated with PKI+8-pCPT display more than one axon positive for Tau-1 staining (green, arrows) and minor neurites positive for MAP2 (red). (B) Quantitative analysis revealed DMSO and PKI neurons display similar amounts of neurons showing axon and multiaxonic (less than 5%, green bars). In contrast, neurons treated with PKI+8-pCPT showed 35% of neurons showing more than one axon (green bars), no significant difference between PKI + 8-pCPT and 8-pCPT group was observed (n=50 neurons, **p<0,01, n.s., compared to control, 1 axon neurons, DMSO vs 8-pCPT ### p<0,001, PKI + 8-pCPT vs 8-pCPT $$$ p<0,001, Multiaxonic neurons, DMSO vs 8-pCPT #### p<0,0001 PKI + 8-pCPT vs 8-pCPT $$$ p<0,001, One-way ANOVA with Tukeys multiple comparison post hoc test, three independent experiments). (C) Quantitative mean axonal length revealed that neurons treated with PKI display shorter axons (white bars), while neurons treated with PKI+8-pCPT show axons longer than control neurons (green bars) (n=50 neurons, ****p<0,01 Control vs 8-pCPT, ####p<0,0001 8-pCPT vs PKI, One-way ANOVA with Tukey's post hoc

test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant, Scale bars: A 50 µm

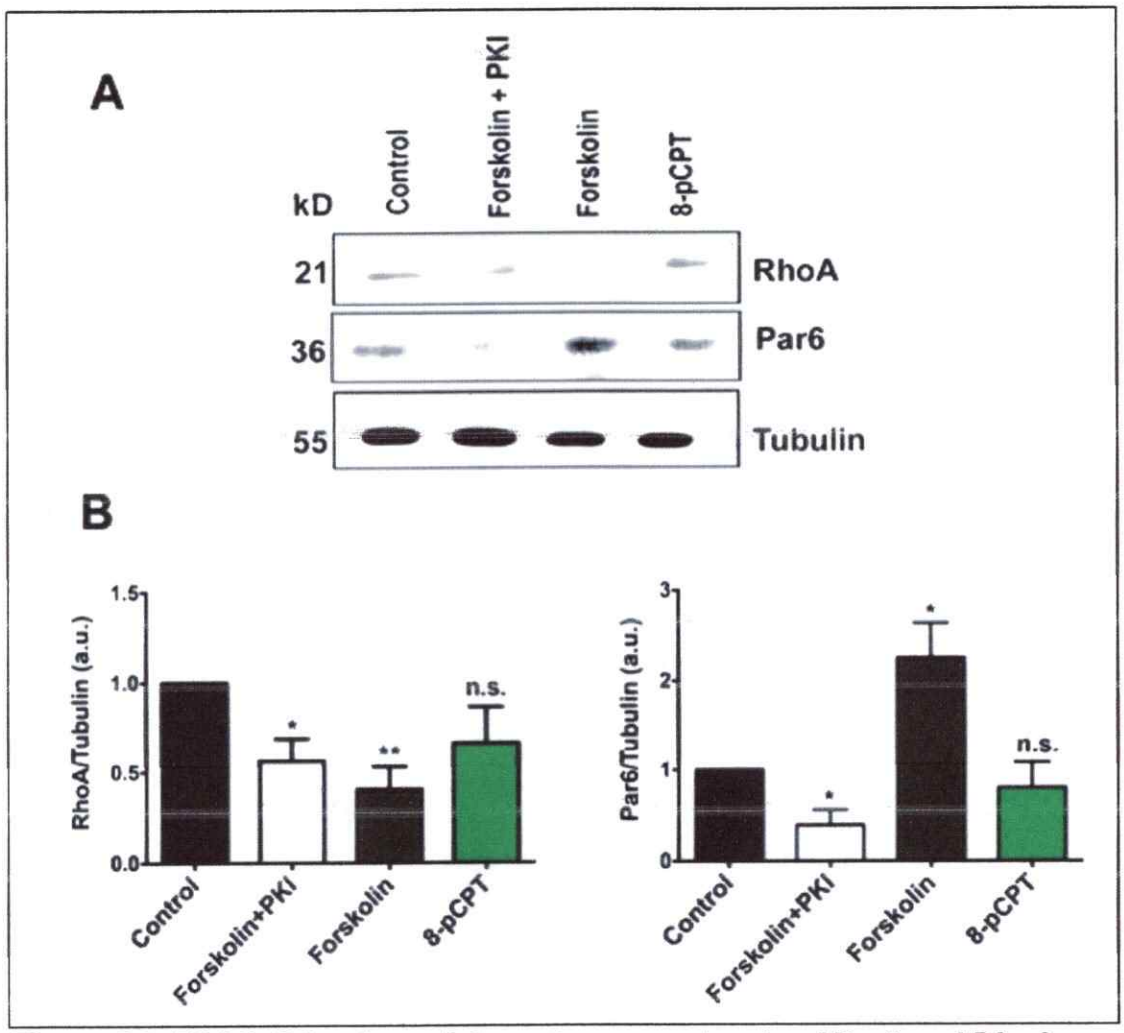

Figure 24: EPAC did not regulate endogenous levels of Par6 and RhoA.

PKA signaling involved in neuronal polarization was evaluated in control, PKI+forskolin, forskolin and 8-pCPT treated N2A neuroblastoma cells (according to Cheng et al 2011). (A) Forskolin treatment decreases RhoA levels and increase Par6 levels in N2A cells (third lane). These changes were abrogated in

the presence of PKI+forskolin (second lane). 8-pCPT treatments did not modify RhoA or Par6 levels in N2A cells (fourth lane). (B) Quantitation analysis revealed that overall changes in RhoA and Par6 were significant in forskolin treated cells (gray bars), and were reversed in the presence of PKI (white bars). 8-pCPT did not change either RhoA or Par6 in N2A cells (green bars) (normalized control vs RhoA; PKI/Forskolin: 0,56±0,12, Forskolin: 0,40±0,12, 8-pCPT: n.s., Par6; PKI/Forskolin: 0,39±0,2, Forskolin: 2,3±0,4, 8-pCPT: 0,80±0,3, *p<0,05, **p<0,01, Student's t tests, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant

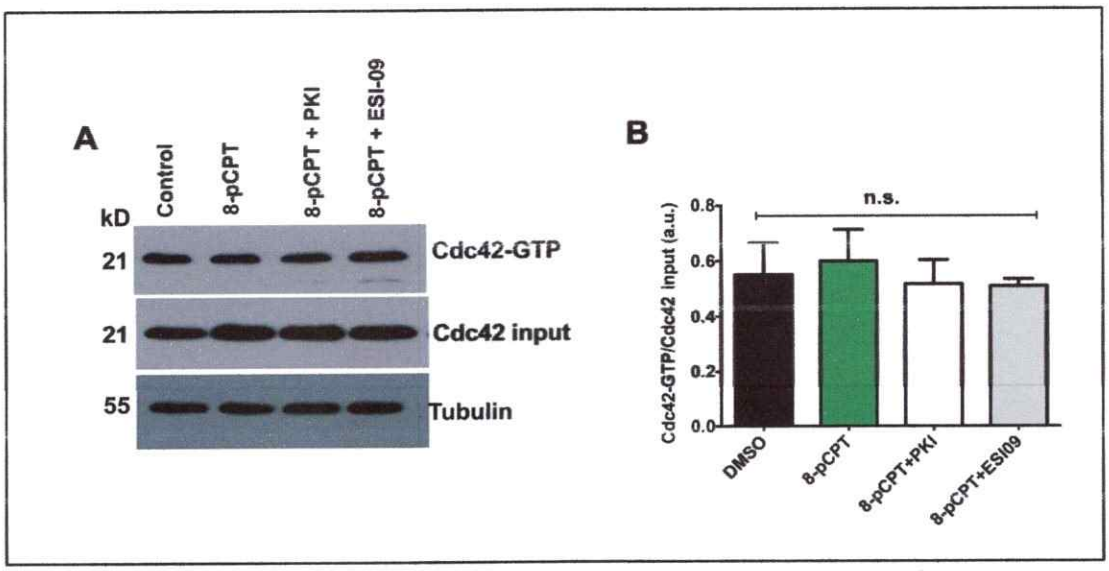

Figure 25: EPAC-Rap1B pathway did not induce Cdc42 activation.

(A) Cdc42-GTP levels were evaluated in neurons treated with 8-pCPT, 8-pCPT+PKI and 8-pCPT+ESI-09. 8-pCPT, 8-pCPT+PKI and 8-pCPT+ESI-09 did not induce Cdc42 increased activity. (B) Quantitation analyze show no significant difference in Cdc42 activity between all treatments compare to the control (Control: 0.55±0.11; 8-pCPT: 0.60±0.11; 8-pCPT+PKI: 0.52±0.10; 8-

pCPT+ESI-09:0.51±0,02, n.s, p>0,05, One-way ANOVA with Dunnett's post hoc test, three independent experiments). Error bars indicate ±SEM, n.s.= non-significant.

DISCUSSION

In this study, we propose EPAC is the GEF involved in Rap1B-dependent axon formation during neuronal polarization. Previously it was demostrated 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 attacted 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. Our results showed no significant variations amongst neurons on different stages of neuronal polarization. This may be related with the fact that most of cAMP local changes may not be evidenced using a biochemical approach considereing whole crude protein extracts. Local quantification of cAMP based on FRET probes present advantages related with spatio-temporal measurement in discrete domains in neurons, and could be usefull to overcome the apparent lack of sensitivity of our biochemical approach. In any case, the slight increase of cAMP observed at Stage II could activate downstream signaling 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 Püschel, 2004). It functions is related with a specific accumulation into a single neurite, which is achieved through Smurf2, an ubiquitin ligase that target GDP-bound Rap1B for proteasome degradation in neuronal compartments displaying low levels of GTP-bound Rap1B (Schwamborn et al., 2007). However, this degradative mechanisms need to act concertedly with molecular mechanisms leading to Rap1B activation. Since Rap1B is a small GTPase, such 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 (Schwamborn et al., 2007).

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) Similarly 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 III. 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). 3) Constituvely active forms of EPAC1 also induce the formation of supernumerary axons in cultured neurons; and 4) Selective inhibition of EPAC inhibits axon formation in cultured neurons. Noteworthy, in this work we used an improved form of EPAC activator (8-pCPT) that efficiently drive Rap1B activation in vitro and in vivo,

without any detectable effect upon PKA (Enserink et al., 2002; Rehmann et al., 2003). The presence of multiple neurites longer than their siblings showing positive Tau-1 and MAP1B staining and MAP2 negative staining highly suggests these neurites are axons. 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) involved in the generation of action potentials. Secondly, neurons displaying multiple axons show other mature axonal markers involved in synapse formation such as VGLUT1 and synaptophysin. 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 here presented (Wilson et al., 2005). Synaptophysin, is a synaptic vesicle protein that distribute along the axon, forming larges puncta structures that correspond to dense accumulation of vesicles within presynaptic specializations (Fletcher et al., 1991a). Synaptophysin clusters were present in supernumerary axons suggesting that neurons with exacerbated polarity could be functional. These results are consistent with previous evidences which assess the molecular identity of multiples axons by staining for synapsin I and performing recycling experiments using FM4-64 uptake (Schwamborn et al., 2007). We also examined the effects of EPAC loss of function during neuronal polarization. Previous studies showed the effect of Rap1A or Rap1B loss of functions. For instance, knockdown of

Rap1B by siRNA abrogates axon formation (Schwamborn and Püschel, 2004). Pharmacological and genetic EPAC1 inactivation blocked the presence of neurons displaying multiples axons and reduced average axonal length. Interestingly, axon formation was not impaired when we used dominant negative EPAC1 or Rap1GAP overexpression. These finding 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 involve in neuronal migration, stimulates tyrosine phosphorylation of C3G and activates Rap1(Ballif et al., 2004). Therefore, mutant mouse embryos lacking C3G present neurons with a multipolar morphology affecting a proper migration during cortex development, likely as 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, dual binding domains for calcium and diacylglycerol (DAG) and is required for striatal output neurons to respond to cholinergic cell activaton in adult brain (Crittenden et al., 2010) . This RapGEF is expressed in adult human brain hippocampus and enriched expression in rat brain basal ganglia pathways and their axon-terminal regions

(Kawasaki et al., 1998b) although there are not data of 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 phenomenons share with neuronal polarity(Carbo et al., 2010).

Additionaly is interesting stand out the role of EPAC2 regulating dendritic spine morphogenesis, spine dynamics, glutamate receptor trafficking and motility in adult cortical neurons. These mechanisms involve a complex between the postsynaptic adhesion protein neuroligin-3 (NL3), PSD-95 that increases EPAC2 activation and Rap1B activity. 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 operate downstream of PI3K increasing translation of Rap1B in the axon (Li et al., 2008).

Interestingly, *in vivo* experiments demonstrated that using the dominant negative form of Rap1A, Rap1A17N or Rap1A and 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 Püschel, 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 difference could be related with differences in the experimenal approaches on those studies.

Noteworthy, the activity of Cdc42 was not increased under 8-pCPT or ESI-09 treatments that previously were shown to modified Rap1B activity, suggesting that the sequential activitation of Rap1B and Cdc42 could be independent events (Schwamborn and Püschel, 2004). If, Rap1B and Cdc42 functions are molecularly not linked, what other mechanisms could be targeted by Rap1B activation and be involved in neuronal polarization? A possible answer to this question, position Rap1B activity upstream of a signaling pathway related to the Ras-like GTPase RalA signaling, which regulates neuronal polarity through the exocyst complex., which 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 is interestingly highlighting the role of the exocyst during neuronal polarity. Hence, it has been shown that the 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 bind IGF-1, a trophic factor required for axon formation in

hippocampal neurons, (Sosa et al., 2006). IGF-1 signalling activate 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.

Beside of 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. 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 signaling 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 mechanish that seems involved Rap1 (Jossin and Cooper, 2011).

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 and biochemical evidence shows a progressive interaction between the exocyst and the polarity complex during polarization, in

particular with Par3 and aPKC (Lalli, 2009), such interaction depending on RalA activation (Das et al., 2014).

Noteworthy, Sec15 exocyst subunit, colocalized selectively with the recycling endosome marker Rab11 and exhibited a GTP-dependent interaction with the Rab11 GTPase (Zhang et al., 2004). Rab11 regulate 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, and 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., 2011a) thus representing another positive-feedback loop in promoting TrkB anterograde transport. A possible role of EPAC-Rap1B in 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 signaling.

In this work we used recently developed EPAC pharmacological inhibitors,

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 μ M) is far below to those used in previous questioned work (Rehmann, 2013). A closely analysis of working conditions used by Rhemann, revealed important differences with Almaharig et al. work, 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 over time on EPAC but not as a selective inhibitor of EPAC competing with cAMP but affecting on the catalytic CDC25-homology domain of EPAC directly, which likely explain their apparent inhibitory effects and the impact on Rap1B activity. Nevertheless, recent works show no physiological side effects with ESI-09. Since ESI-09 antagonize 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 recapitulate the rickettsial infection effects found in *EPAC1* knockout mice (Gong et al., 2013). All these evidence allow us to conclude that ESI-09 indeed reduced EPAC1 activity, although more details in the physiological effects of this compound are

still necessary.

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 hippocampus and cortex in rat and mouse neurons. 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. The accumulation of a molecule or a protein complex into 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 Püschel, 2004; Schwamborn et al., 2007), Cdc42 (Schwamborn and Püschel, 2004), Par complex (Shi et al., 2003), pAkt (Yan et al., 2006) and LkB1/pLKB1 (Shelly et al., 2007). It is accepted that 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).

All along this study we focused our attention into 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 complementaty mechanisms. On the one hand, PKA phosphorylate Smurf1 in response to the neurotrophyn brain-derived neurotrophic factor (BDNF), reducing the degradation of a member of the polarity complex, Par6, an axonal

growth-promoter protein and increasing the degradation of the smallGTPase RhoA which is an axonal growth-inhibitor protein. On the other hand, PKA activate the liver kinase B1 (LKB1) that phosphorylates and activates the synapses of the amphid defective kynesin (SADK) and promote axonogenesis. Our results showed that EPAC activation with 8-pCPT did not affected PKA dependent signaling, since, treatments on neuroblastoma cell line, following the same criteria of cheng's work (2013), display levels of Par6 and Rhoa not significant different from the control cells which suggest that the changes seen in gain and loss of function of EPAC pathway correlated more with a pathway exclusively trough Rap1B. Another evidence for alternative cAMP pathway dependent on EPAC derive from the observation that inhibition of PKI by synthetic peptide does not impair the axon formation in rat neurons and the specific antagonist Rp-8-CPT-cAMPs does not impaired axon formation in mouse neurons either (data no shown). We determine that 20 μM of PKI 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 multiples axons when we combined EPAC activation with PKA inhibition. Although concentration of inhibitor and agonist for PKA were not sufficient to induced complete PKA inhibition of PKA, we avoid higher concentration for PKA inhibitor as those may induce artifacts as observed when using higher doses of other PKA inhibitors such as H89 and KT5220, which may exerts unwanted side effect affecting PKC and ERK functions(Murray, 2008).

Nevertheless PKI induced morphological changes in neurons, impairing axonal elongation in rat hippocampal neurons. Considering the evidences accumulated in this work, it seems reasonable to propose that cAMP sigaling related to axonal determination and elongation is contributed by both EPAC and PKA, through two apparent ihdependent mechanisms.

EPAC1 and EPAC2 bind cAMP with similar affinity as PKA holoenzyme, suggesting that both factors may respond to similar physiological concentration on this second messenger (Dao et al., 2006). The concerted functions of EPAC and PKA signaling had been shown to be dependent on cellular context and processes (Grandoch et al., 2010a). Activation of EPAC-dependent pathway 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; López De Jesús et al., 2006; Yoshimura et al., 2006b) and Rho/Cdc42/Rac1 (Schwamborn and Püschel, 2004; Moon et al., 2013). An additional regulation point for such complementary mechanism would be related with extracellular cues that trigger neuronal polarization such as BNDF elevates cytoplasmic cAMP leading to increased axonal elongation (Cheng et al., 2011a; Nakamuta et al., 2011). In contrast, NGF is not able to trigger axon elongation in cultured neurons, in spite of induce the activation of C3G another Rap1B-GEF (Nakamuta et al., 2011) reinforcing 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 Püschel, 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 for EPAC in axon determination, suggesting that their activity might be required during polarization in neuronal polarity thorugh Rap1B presenting EPAC1 as the GEF of Rap1B that 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 observed any changes in the activity of Cdc42. We think that an alternative pathway downstream EPAC-Rap1B might be the exocyst complex involving the smallGTPase RalA, thereby providing another link to membrane addition and cAMP signaling during axon elongation. Furthermore better tools are needed to elucidate the role of PKA inhibition in neuronal polarity. **Figure 26** shows the tentative model of EPAC in neuronal polarity

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

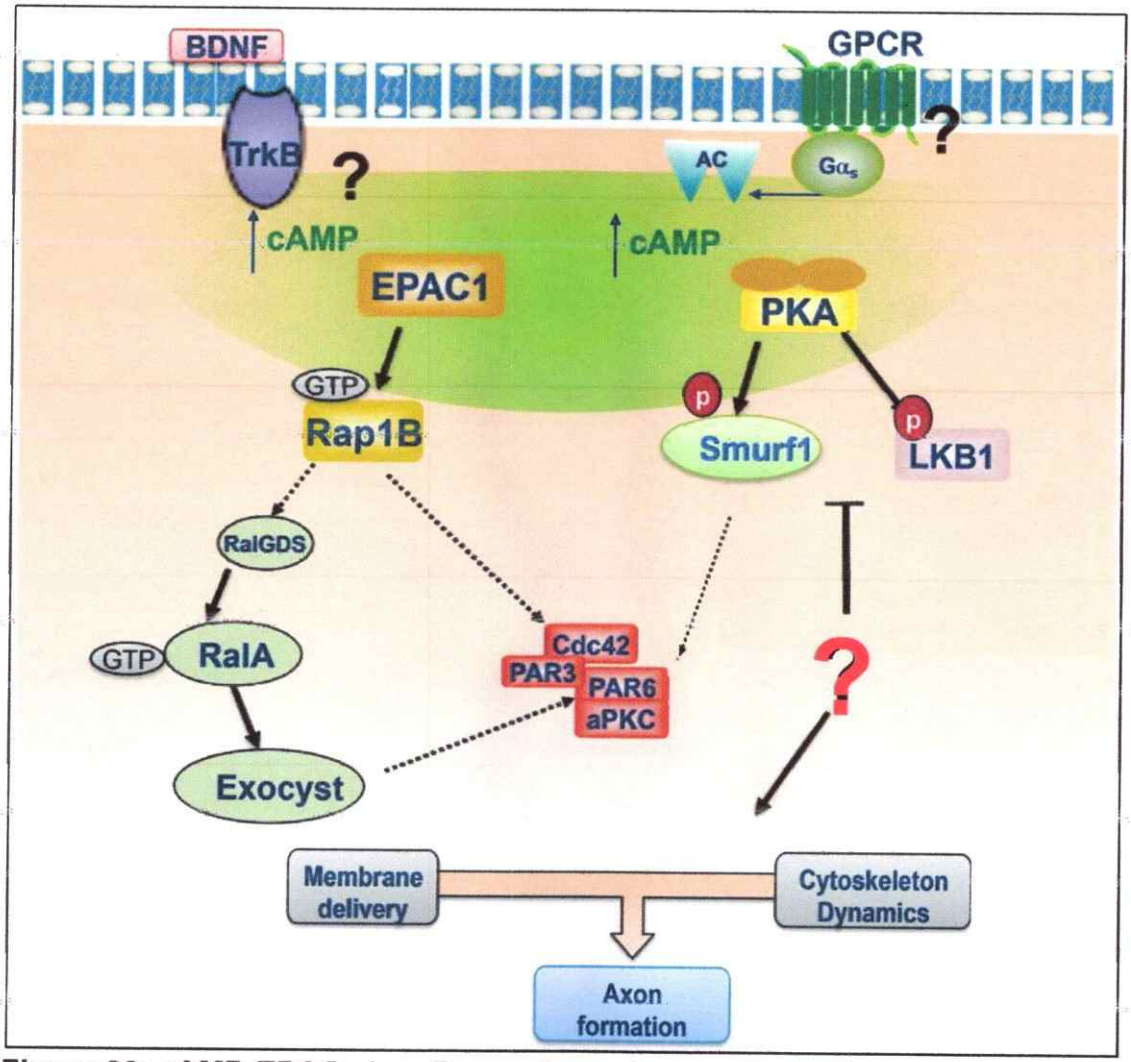

Figure 26: cAMP-EPAC signaling pathway in axon formation:

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 locally Rap1B, which regulate axon formation via the exocyst complex by RalA or the polarity complex trough Cdc42. Complementary PKA regulate axon formation trough Smurf1 and LKB1. Both pathway works together in cytoskeleton dynamics and membrane delivery to form the axon. Black question marks indicate that the mechanism by which

these receptors increased cAMP levels is unclear. Red question mark indicates that the mechanism that induced polarization after PKA inhibition is unclear.

CONCLUSION

The present evidence in this work highlight the role of EPAC in neuronal polarity thorugh its effector Rap1B. These results reopen a discussion that was stuck for many years, namely how Rap1B is protected in the tip of the axon that can lead to recruitment of the polarity complex and induce axon formation. We show that EPAC1 is the main isoform of EPAC involve in this process since the low levels of expression and the subcellular distribution of EPAC2.

The changes in the morphology induced by the pharmacological and genetic activation and inhibition of EPAC are interesting since activation produce multiples axon and the inhibition reduce the length and the number of neurons polarized a phenomenon share by several others regulators of polarity. Noteworthy the multiples axons induced by EPAC present mature neuronals markers such VGLUT1 and synapthophisin distribution and AnkG in the axonal initial segment that confirm the nature of these axon.

Additionaly, we showed that EPAC morphologycal changes are not affected by PKA inhibition and that this inhibition does not induce lost of polarization in the neurons, which lead to stablish that EPAC is a pathway complementary to PKA in cAMP signalling in embryonic neurons and that new work should address to

determinate the impact of PKA inhibition in the neuron.

Finally remains unclear how the changes in EPAC-Rap1B affected the global sequential activity of Cdc42 since we did not observed any changes in the activity of this small GTPases activating EPAC. To this end, we propose the use of FRET probes that might help to elucidate locally whether Cdc42 is activated in the axon. However the potential role of the exocyst complex are very interesting to elucidate the effect downstream EPAC-Rap1B signaling in neurons that would be interesting topics of future research.

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