UNIVERSITA' DEGLI STUDI DI MILANO

Dottorato di Ricerca in Scienze Farmacologiche Sperimentali e Cliniche

XXX CICLO

Arhgap22 disruption affects Rac1 signaling pathway and results in altered formation and function of glutamatergic synapses in mouse hippocampus

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RIASSUNTO

La regolazione del citoscheletro actinico ad opera delle RhoGTPasi è fondamentale per la morfogenesi neuronale, in particolare per l'estensione e la ramificazione dei neuriti, la sinaptogenesi e la plasticità sinaptica. La disregolazione delle RhoGTPasi provoca disfunzioni a livello neuronale tra cui disabilità intellettiva, schizofrenia e malattia di Alzheimer.

Rac1 è un membro della famiglia delle RhoGTPasi ed è stato dimostrato regolare positivamente la dendritogenesi e la formazione e la maturazione delle spine dendritiche. Come altre RhoGTPasi, l'attività di Rac1 è regolata principalmente da due tipi di molecole: GEFs e GAPs. La proteina Arhgap22 è una specifica Rac1-GAP che promuove l'inattivazione di Rac1. Anche se è stato precedentamente dimostrato che Arhgap22 è trascritta in diversi organu murini tra cui il cervello, le sue funzioni a livello neuronale nel topo non sono ancora state ancora approfondite.

In questo lavoro abbiamo dimostrato che Arhgap22 è espressa nel cervello del topo in una precisa finestra spazio-temporale. Inoltre, approfittando di un modello animale di topo knock out (KO) per Arhgap22, abbiamo descritto gli effetti del suo silenziamento in neuroni ippocampali. *In vivo*, l'assenza di Arhgap22 ha causato un aumento del livello di Rac1 attivato e delle vie a valle da esso regolate, con un conseguente aumento della densità delle spine dendritiche nella regione CA1 dell'ippocampo. Inoltre, topi KO hanno esibito une riduzione di recettori AMPA nelle spine dendritichee e questa alterazione è stata riflessa dalla compromissione dell'induzione e del mantenimento del potenziamento a lungo termine (LTP). I topi Arhgap22 KO hanno mostrato anche difetti a livello comportamentale, in particolare esibendo defict in abilità cognitive e ridotti comportamenti ansiosi.

Riassumendo, i risultati di questo lavoro suggeriscono che Arhgap22 è un regolatore chiave della segnalazione di Rac1 e che influisce sulla maturazione delle sinapsi eccitatorie, della plasticità sinaptica e delle funzioni cognitive.

ABSTRACT

The regulation of actin cytoskeleton operated by RhoGTPases is crucial for neuronal morphogenesis, especially for neurite elongation and branching, synaptogenesis and synaptic plasticity. Dysregulation of RhoGTPases leads to neuronal dysfunctions including intellectual disability, schizofrenia and Alzheimer disease.

Rac1 is a member of the RhoGTPase family and it has been demonstrated to positively regulate dendritogenesis and dendritic spines formation and maturation. As well as other RhoGTPases, Rac1 activity is regulated principally by two kinds of molecules: GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins). Arhgap22 protein is a specific Rac1-Gap that promotes the inactivation of Rac1. Although it was previously reported that Arhgap22 transcripts is present in murine brain, its functions in neurons have not been studied yet.

Here, we reported that Arhgap22 is expressed in mouse brain in a precise spatio-temporal window. Moreover, taking advantage of an animal mouse model knock out (KO) for Arhgap22, we described the effects of its silencing in hippocampal neurons. *In vivo*, Arhgap22 disruption led to an increase level of activated Rac1 and its downstream pathways, with a subsequent increase in dendritic spine density in CA1 region of hippocampus. Additionally, Arhgap22 lacking mice presented reduced AMPA receptors in the post-synaptic density of excitatory synapses and this alteration was reflected by the impairment in the induction and mainteinance of long-term potentiation (LTP). Arhgap22 KO mice presented also defects in cognitive tasks and decreased anxiety-like behaviours.

In a nutshell, the results of this work suggested that Arhgap22 is a key regulator of Rac1 signaling and that affects the maturation of excitatory synapses, synaptic plasticity and cognitive functions.

1. INTRODUCTION

1.1 Small G proteins

Small G proteins represent a superfamily of small (20-24 kDa) ubiquitary proteins that have been found in all eukaryotes, from yeast to human. The most important member of this family is Ras (Rat Sarcoma). First identified as a viral oncogene that causes osteosarcoma in rats (Shih et al., 1979), Ras was then demonstrated to be present also in human cells.

Up to now, more than 100 different small G proteins have been identified and they are divided into 5 family: Ras, Rho, Rab, Sar1/Arf and Ran.

In general, each family has peculiar characteristics and functions: for example, Ras proteins orchestrate the regulation of gene expression, Rho members are involved in the organization and remodeling of actin cytoskeleton, Rab1 and Sar1/Arf family regulate vesicular transport while Ran proteins mediate nucleus-cytoplasm translocation and the organization of microtubules during different steps of cell cycle (Takai et al., 2001).

The name G proteins derives from their capacity to bind guanosine diphosphate (GDP) and guanosine triphosphate (GTP) molecules. Small G polypeptides work as molecular switches that continuously cycle between an active state, when bound to GTP, and an inactive state, when bound to GDP (Hall, 1990). The GTP/GDP exchange is mediated by an external stimulus that activates intracellular transduction pathways whereas the inactivation is due to GTP hydrolysis. This tightly regulated mechanism of action is fundamental for their correct functioning (Takai et al., 2001).

1.1.1 Introduction to Rho GTPases

The Rho GTPases family belongs to the Ras-related small GTPases superfamily. It is composed by 20 proteins classified into 8 subfamilies depending on their aminoacidic sequence homology (Fig.1) (Azzarelli et al., 2015). The first member was identified in 1985 during studies on Ras-correlated genes. (Madaule & Axel, 1985).

As for others small G proteins, Rho GTPases can cycle between active and inactive states. When they are inactive, Rho GTPases are localized in the cytosol but, after activation, they translocate to the plasmatic membrane or organelles which they bind to by means of lipidic modifications. Lipidic groups are post-translationally added to a cysteine residue by enzymes that recognize the CAAX motif present at the C-terminus of Rho GTPase proteins (Casey & Seabra, 1996; Winter-Vann & Casey, 2005). Different lipidic groups determine different subcellular localization of the proteins: for example, RhoB is normally bound to endomembrane system if geranyl-geranylated but it localizes to plasmatic membrane if farnesylated (Azzarelli et al., 2015); (Lebowitz et al., 1995).

	Rho subfamilies	Rho members	Identity	Structure
Classical Rho GTPases	Rho	RhoA		
		RhoB	84%	
		RhoC	92%	
	Rac	Rac1		
		Rac2	92%	
		Rac3	93%	
		RhoG	72%	
	Cdc42	Cdc42		
		TC10 (RhoQ)	62%	
		TCL (RhoJ)	55%	
	Rif	Rif (RhoF)		
		RhoD	50%	
Atypical Rho GTPases	Rnd	Rnd1		
		Rnd2	51%	
		Rnd3 (RhoE)	58%	
	RhoUV	RhoU (Wrch1, Chp2)		
		RhoV (Chp, Wrch2)	54%	
	RhoH	RhoH (TTF)		
	RhoBTB	RhoBTB1		
		RhoBTB2	66%	

Figure 1. Classification of Rho GTPases. Rho GTPases family members are reported in the table and classified in 8 subgroups. The column identity shows the percentage of homology with the first member of the family. In the column structure, the schematic representations of general protein structures are shown. (from Azzarelli et al., 2015)

1.1.2 Rho GTPases regulation

RhoGTPases act as molecular switch cycling between an "active" state, when bounded to GTP, and an "inactive" state, when bounded to GDP (Fig.2) (Hodge & Ridley, 2016); (Jaffe & Hall, 2005). Their activity is regulated predominantly by three groups of proteins:

(i) Guanine nucleotide exchange factors (GEFs) that act promoting exchange of GDP with GTP and are considered positive modulators.

One of the most important GEF is the oncogene Dbl. This protein contains a region of 200 amino acids called Dbl homology (DH) that is present near the Pleckstrin Homology (PH) domain. The concomitant presence of these regions is a common characteristic fundamental for the catalytic activity of all GEFs (Rossman et al., 2005).

(ii) GTPase activating proteins (GAPs) enhance the intrinsic activity of RhoGTPases thereby inactivating them (Garrett et al., 1989).

(iii) Guanine nucleotide dissociation inhibitors (GDI) whose activity is to block the activation of RhoGTPases. In fact, in the inactive state Rho GTPases form a complex with GDI proteins. In this way, GDI sequestrate them, preventing their translocation to the membranes or their binding to GEFs. Notably, to activate Rho GTPases is necessary a stimulus that induces GDIs dissociation (Bustelo et al., 2007).

Up to now, more than 70 GAPs and 80 GEFs proteins have been identified. These findings suggest that the regulation of RhoGTPases is a very complex mechanism: in fact, different activities at different subcellular localizations could modulate several signal pathways at different spatiotemporal levels.

Although GTP/GDP switching is the principal mechanism of regulation, some of the components of RhoGTPases family follow an alternative mechanism of activation/inactivation and they are called atypical GTPases. These proteins mainly stay in a constitutive GTP-bound state: either they constitutively prevent nucleotide exchange activity (ex. RhoU) (Shutes., 2004) or they possess amino-acid substitution in the catalytic domain that prevent the GTPase activity (ex. Rnd protein) (Nobes et al., 1998).

Other mechanisms of RhoGTPases regulation (of both typical and atypical RhoGTPases as well as their regulators) comprehend post-translational modifications such as phosphorylation and ubiquitynation. Generally speaking, phosphorylation enhances catalytic activity of RhoGTPases while ubiquitynation is fundamental for the modulation of RhoGTPases proteins expression levels (Hodge & Ridley, 2016).

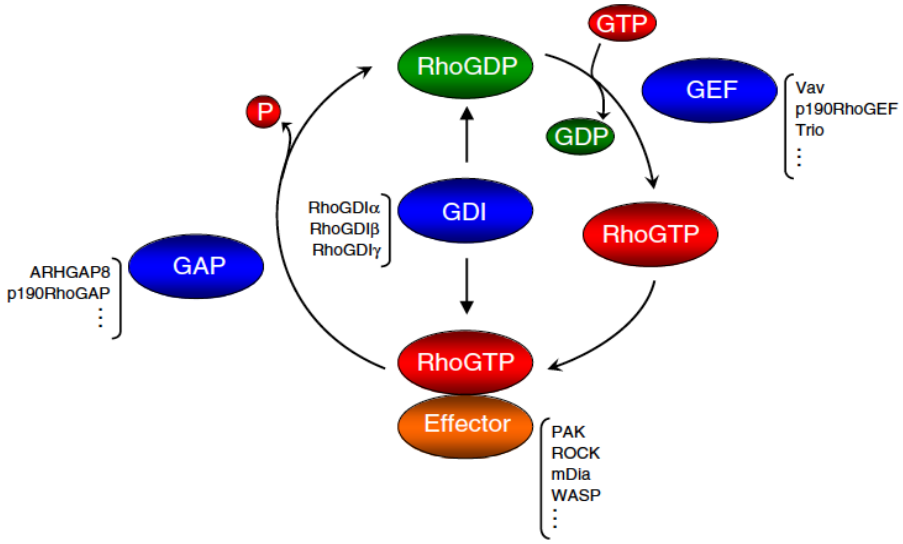


Fig.2 Basic Rho GTPase activation cycle. The exchange between GDP and GTP and RhoGTPases activation is mediated by GEFs. In their active form, RhoGTPases bind to different effectors and perform their functions. GAP proteins enhanced the hydrolysis of GTP to inactivate the protein. RhoGDI proteins sequester the protein in the cytoplasm, preventing their function. Some examples of GEFs, GAPs and effectors are reported (Vega and Ridley, 2008).

1.1.3 RhoGTPases main functions

Ras homologous member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42) proteins are the most studied and characterized RhoGTPases. They have more than 50 effectors and they control several complex signal transduction pathways (Hall, 2005).

The following sections describe some of the main functions of Rho, Rac1 and Cdc42 (Fig.3).

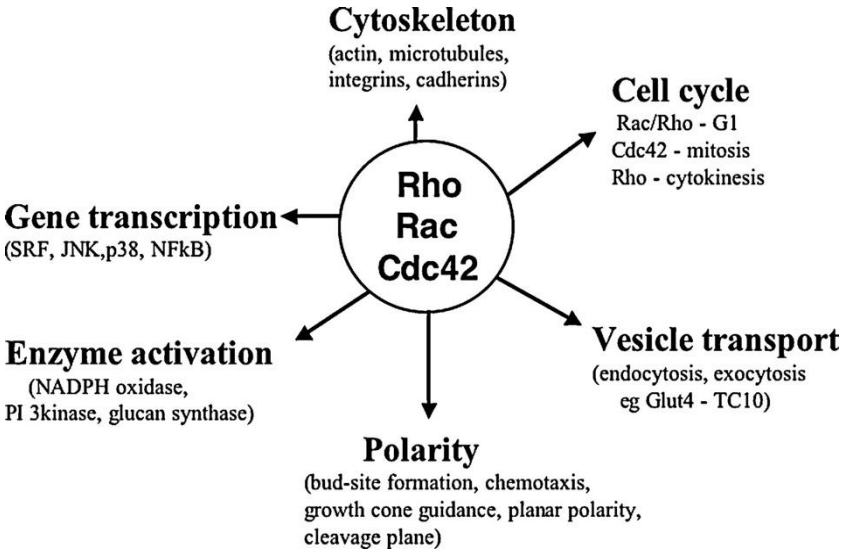


Fig.3 RhoGTPases cellular functions. The scheme summarizes the principal cellular functions of RhoGTPases. The principal function is the regulation of the cytoskeleton. In addition, it has been demonstrated that RhoGTPases participate in several cellular processes such as gene transcription, cell cycle, vesicle transport and enzymatic activities such as phosphoinositide 3-kinase and NADPH oxidase (from Hall,2005).

1.1.3.1 Actin cytoskeleton remodeling

The polymerization and the nucleation of F-actin after external stimuli is principally mediated by Rho GTPases in eukaryotic species.

The role of Rac1 and RhoA was first described by Ridley and colleagues (Ridley & Hall, 1992; Ridley et al., 1992). Several studies on fibroblasts demonstrate that RhoA induces the formation of actin-myosin contractile fibers. On the other hand, stimulation by extrinsic (PDGF and insulin) or intrinsic inputs (activated Ras) leads

to the activation of Rac1 and subsequent formation actin-rich lamellipodia and membrane ruffles. It was also demonstrated that active Cdc42 induces the formation of filopodia.

Different effectors of RhoGTPases have been identified so far. The RhoA binding protein mDia, for example, is a formin which orchestrate the polymerization of F-actin filament from the barbed ends. On the other hand, ROCK (Rho-associated kinase) inactivates through phosphorylation myosin light chain promoting myosin II activity. The activation of both mDia and ROCK is a key point for the formation of the contractile actin-myosin fibers.

For what concerns Rac1 downstream activity, its binding with WAVE (WASP velprolin homologous) leads to the release of WAVE from an inactive complex and to the interaction with Arp2/3 protein. This latter mediates the elongation of actin from the ends of pre-existing filaments to produce new branched filaments. Arp2/3 activity is regulated not only by WAVE interaction but also by the binding with WASP (Wiskott–Aldrich syndrome protein) which is a direct partner of Cdc42.

ADF/Cofilin induces severing and filaments disassembly through the dissociation of G-actin from minus ends. Cofilin activity is mediated by phosphorylation and dephosphorylation. In particular, dephosphorylation induces cofilin activation whereas phosphorylation promotes its inactivation. Different enzymes are responsibles of cofillin phosphorylation but the most important are LIM kinases (LIMK) that could be activated by PAK (Cdc42/Rac1-dependent) or ROCK (Rho-dependent) (Andrianantoandro & Pollard, 2006).

In a nutshell, the three pathways regulate (potitively or negatively) each other suggesting that a cross-talk is necessary to correctly orchestrate actin cytoskeleton dynamics (Hall, 2005).

1.1.3.2 Regulation of gene expression

Rho GTPases affect different cellular pathways that are known to modulate gene expression: SRF, JNK, NF-κB and MAPK/ERK at different extent depending on the cell type.

Different RhoGTPases could modulate the same pathway with different strenght. For example, the activity of the transcription factor SRF (serum response factor) is mainly regulated by RhoA-GTP while Rac1 and Cdc42 have very low effect on it. In fact, after Rho activation, changes in actin cytoskeleton dynamics induce the translocation of SRF to the nucleus. Although the activation of all RhoGTPases positively regulates actin polymerization, Cdc42 and Rac1 induce a smaller effect on SRF localization in the nucleus (Miralles et al,, 2003).

Moreover, some gene expression pathways are regulated by actin-independent mechanisms. JNK and MAP/ERK are activated by direct or indirect (through scaffold proteins) binding with MAP kinases. It is well known that Rac1 and Cdc42 are key regulators of MAP/ERK signaling. In fact, after stimulation, the active forms of Rac1 and Cdc42 bind to PAK kinases that can phosphorylate and activate RAF and MEK followed by ERK1/2 activation (L. Van Aelst & D'Souza-Schorey, 1997).

1.1.3.3 Cell cycle

RhoGTPases control different aspect of cell cycle progression. In particular, they are important for the regulation of cyclin-dependent kinases activity during G1 phase and for the organization of cytoskeleton during mitosis. For what concerns G1 phase, Rac1 and Cdc42 works together to increase the expression of Cyclin D1, a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6 which induces the transition from G1 phase to S phase (Etienne-Manneville & Hall, 2002).

1.2 Rho GTPases in central nervous system

Brain is an organ that regulate crytical processes including learning and memory. The development of central nervous system (CNS) is a complex mechanism that starts with the migration of newborn neurons and continues with the formation of specialized processes to the final differentiation and formation of functional connections. All these events need regulated activation and re-modeling of actin cytoskeleton and signal transduction and it is widely accepted that Rho GTPases are deeply involved in these events (Govek et al., 2005).

1.2.1 Excitatory synapses

The word synapse indicates a structure that allows the transmission of a chemical or electrical stimulus from a neuron to another. Each synapse is basically composed by (Fig.4):

(i) a pre-synaptic terminal
(ii) a cleft
(iii) a post-synaptic terminal

The pre-synaptic side is the terminal part of the axon. It is called “bouton” : it could be identified by the presence of neurotransmitter-containing synaptic vesicles and by a thickening of the membrane called “active zone” (Li & Sheng, 2003; Südhof, 2012). The active zone is the portion of the bouton that interacts with the post-synapse: it is particularly enriched in synaptic vesicles (some of them are docked to the membrane and ready for fusion) and in voltage-gated channels that are necessary for the fusion of vesicles and neurotransmitter release (Li & Sheng, 2003).

On the post-synaptic side, there is the post-synaptic density (PSD), an electron-dense structure composed by transmembrane and scaffolding proteins. It could be considered a sort of specialized organelle: it promotes the interaction between pre- and post-synaptic sides and it allows the clustering of receptors at the membrane. In this way, PSD has an active role in promoting tha activation of signal transducing pathways following an excitatory stimulus (Sheng & Kim, 2011).

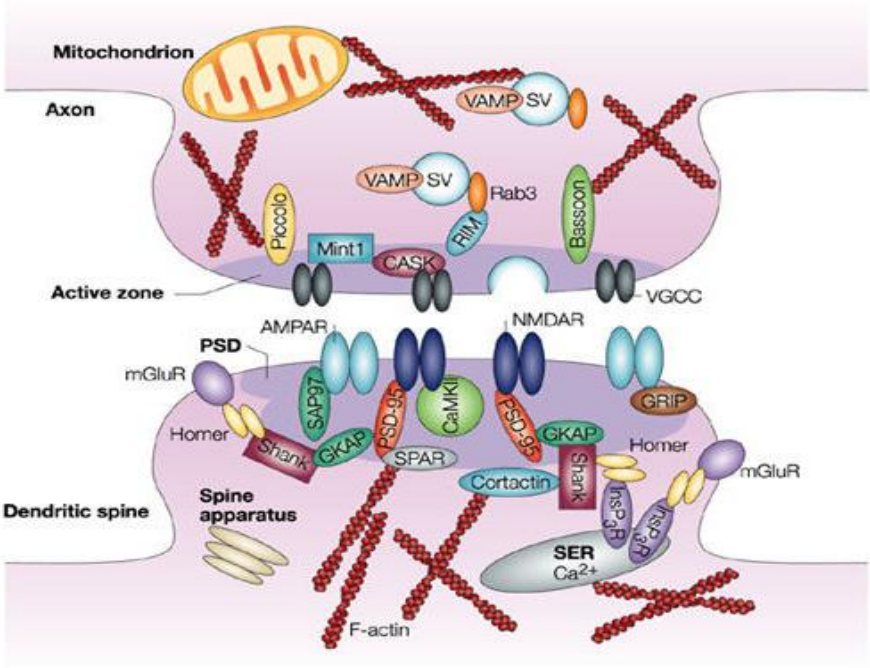


Fig.4 Schematic representation of a mature excitatory synapse. The panel shows some of the most important components of both pre-synaptic bouton (up) and the post-synaptic compartment of a dendritic spine (down) (from Li and Sheng, 2003).

The PSD contains mainly glutamate receptors (AMPAR, NMDAR) and scaffold proteins that hold together the PSD, other postsynaptic receptors and adhesion molecules, enzymes and cytoskeletal components. The molecular composition of PSD changes between different brain areas and during development. For example, in rodent brain the expression levels of many PSD proteins (PSD-95, CaMKIIα, and AMPA receptor subunits) increase during development, with a peak at ~2–4 weeks after birth which corresponds to the maturation of synapses in the brain (Sans et al., 2000). However, the remodeling of PSD composition is a mechanism conserved also in mature naurons at basal conditions and it shows larger changes in response to activity (Sheng & Kim, 2011).

The pre-synaptic active zone and the PSD are separated by a gap of 20/25 nm, the synaptic cleft. A huge number of cell adhesion molecules maintain pre- and post-synaptic compartments bound together (Scheiffele, 2003).

1.2.2 Dendritic spines

Dendritic spines are small (0,5-2 μm in length but up to 6 μm in the CA3 region of the hippocampus) membrane protrusions that contain the essential post-synaptic density components, actin cytoskeleton and supporting organelles. Spines are found at a density of 1-10 spines per μm of dendrite length and they receive most of the excitatory signals in the mature mammalian brain (Sheng & Kim, 2011);(Rochefort & Konnerth, 2012).

During the early phases of synaptogenesis, a great number of filopodia normally extend from dendrites and soon after (10 minutes) retract. Probably this dynamic process depends on the need to favour the encounter between the pre- and post-terminals. Once a contact is created, a synapse can be formed and it follows different maturation steps. (Calabrese et al., 2006).

Each spine is basically composed by a dilatated tip called “head” connected to the dendrite by a “neck”. Dendritic spines are classified into different classes on the base of their head shape and dimension (Fig.4) (Peters & Kaiserman-Abramof, 1970):

(i) Mushroom spines. They present a large head and a narrow neck and represent the mature state of dendritic spines

(ii) Thin spines that have a smaller head (compared to mushroom) and narrow neck and are considered immature spines

(iii) Stubby spines that do not present a constriction between the head and the dendritic shaft

(iv) Cup-shaped spines

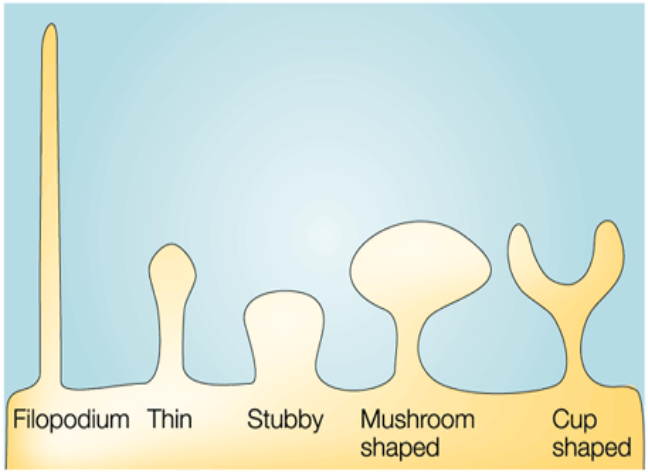


Fig.5 Dendritic spines classification. The cartoon represents the current classification of dendritic spines based on their morphology (from Hering and Sheng, 2001).

Dendritic spines are highly diverse. Changes in size as well as density reflect changes in the strength of synaptic transmission: mushroom spines are generally more stable, present higher number of glutamate receptors and create stronger connections. On the contrary, thin spines are less stable and present weaker synaptic connections (Matsuzaki et al., 2004).

In vivo timelapse experiments strongly contributed to study dendritic spines turn over in mouse brain. Basically, a large fraction of mushroom spines is persistent with a lifetime up to several months. Despite this, a subset of spines can change their shapes or number in response to stimuli, especially during post-natal brain development. This mechanism semms to be necessary to translate short-term changes in synaptic activity into long lasting alterations in the structure and function of excitatory synapses. (Hering & Sheng, 2001).

The variations in the number and in the shape of dendritic spines could be present not only in physiological but also in pathological conditions. It has been demonstrated that alterations in spine density and shape are associated with several disorders with environmental or genetic causes (Fig.6) (Hering & Sheng, 2001). Considering the pathological alterations of spines, the most common condition is intellectual disability that affects about 2–3% of children and young adults, as suggested for the first time in the 1970s (Purpura, 1974).

Factor/stimulus	Associated change in spine density
Visual deprivation	Reduction in spine numbers and spines with abnormal morphology along apical shafts of pyramidal cells in the rabbit visual cortex
Visual stimulation	Increased spine density in the rat visual cortex
Rearing in complex/ 'enriched' environment	Increased spine density in the CA1 region of the hippocampus or dorsolateral striatum of the rat
Hibernation	40% loss of spines in the squirrel hippocampus during hibernation; spine numbers are recovered within hours of arousal
Sex steroid hormones	Female rats have more spines than males in a subset of hypothalamic nuclei and in the CA1 region of the hippocampus; spine density changes with the oestrus cycle in females
Stress	Spine density is enhanced in the hippocampus of male rats, but reduced in the female hippocampus in response to an acute stressful event
Fragile-X syndrome	Spines are abnormally long and thin, and of increased density in the cerebral cortex
Down's syndrome	Spine density is markedly decreased in the hippocampus and cortex
Epilepsy	Spine density is decreased on hippocampal and neocortical pyramidal cells

Figure 6. Factors that alter spine density. This scheme resume different environmental and genetic factors that are known to cause alteration in dendritic spine density in different brain area. (from Hearing and Sheng, 2001)

1.2.3 Synaptic plasticity

Synaptic plasticity represents the activity-dependent modifications of strength and efficacy of pre-existing synapses and it represents the molecular mechanism that allows to transform transient experience in persistent memories (Citri & Malenka, 2008).

The first evidence of synaptic plasticity was provided in 1970 by Bliss and colleagues who demonstrated that repetitive stimulation of excitatory synapses in hippocampus lead to a potentiation of excitatory synapses strength (Bliss & Gardner-Medwin, 1973). This permanent potentiation is called long-term potentiation (LTP) and it is considered the principal mechanism at the basis of learning and memory. A different form of long-term plasticity is represented by long-term depression (LTD), a long-lasting decrease in synaptic strenght due to a brief patterned stimulation (Citri & Malenka, 2008).

1.2.3.1 Long-term potentiation (LTP)

Since its first demonstration in 1973, LTP has been considered as the cellular paradigm for storage information in the brain.

Most LTP studies focused their attention on hippocampus and in particular on the synapses between the Schaffer collateral and CA1 pyramidal cells, although it has been demonstrated that the general characteristics of this process are maintained in different area of mammal brain. One peculiarity is that LTP is input-specific and associative: repetitive activation induces tha strengthening of a specific synapse but not other synapses of the same neuron, although the increase in synaptic strength of one set of synapses can facilitate LTP at an independent adjacent active synapse in a restricted temporal window (Malenka & Nicoll, 1999)

It has been demonstrated that two classes of glutamate receptors are essential for LTP: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic) receptors and NMDA (N-methyl-D-aspartate) receptors. AMPA receptors are channels permeable to Na^+ and K^+ and they are responsible for most of basal excitatory synaptic transmission. In basl conditions, NMDA receptors channels are blocked by extracellular Mg^{2+}. When the post-synaptic membrane is depolarized during the early phase of LTP, the $Mg2^+$ is released from the channel, NMDARs are activated and they induce a strong influx of Na^+ and $Ca2^+$ which is necessary for LTP.

One of the effector of NMDA receptor activation is CaMKII, a kinase that regulate AMPA receptors in two different ways: on one hand, it phosphorylates AMPA receptors (particularly GLUA1 subunit) localized at plasma membrane, increasing their single-channel conductance. On the other hand, CaMKII induces the insertion of new AMPA receptors at synapse (Barria et al.,1997).

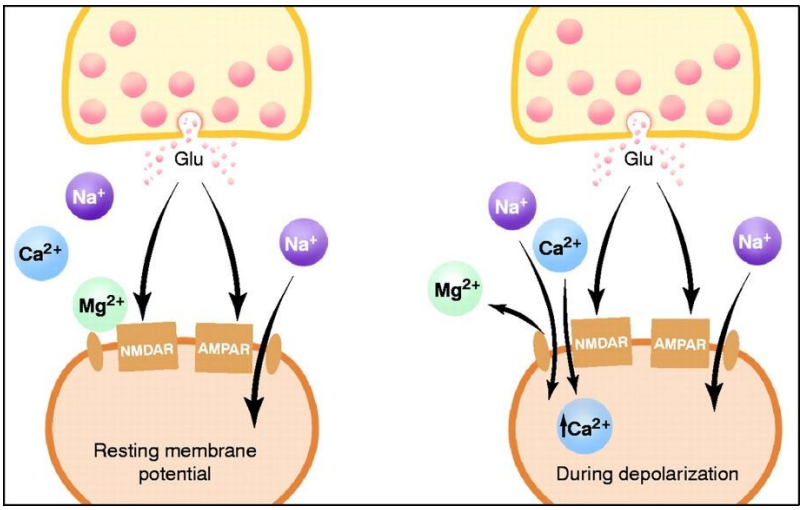


Fig.7 Long-term potentiation model In baseline conditions, glutamate binds to AMPA and NMDA receptors but it activates only AMPARs because NMDA pore is blocked by the presence of Mg^{2+}. After AMPA-dependent depolarization, Mg^{2+} is released from the channels and NMDA is activated. These events promote the onset of LTP (From Malenka and Nicoll,1999).

An important feature of dendritic spines is that their volume and density can be dynamically regulated. In fact, the induction of long-term potentiation (LTP) promote changes in spine head dimensions through the clustering of AMPA receptors at membrane level. Moreover, LTP induces the rapid formation of new spines/filopodia. These observations suggest a model in which NMDARs recruit AMPARs to developing synapses regulating spine growth and maturation. This model predicts that loss of NMDARs should lead to smaller AMPA-mediated currents and smaller spines (Citri & Malenka, 2008).

1.2.4 Rho GTPases and dendritic protrusion

Dendrites are protrusion that generates from the neural cell body and divides into different branches to form connections with other neurons. The architecture of dendritic tree affects the propagation of signals and determines the connectivity of neurons.

Formation of the dendritic arbor is a very dynamic mechanism: in fact, filopodia continuously originate from dendritic branching site and they could be elongated and stabilized as well as retracted. During dendritogenesis, the major component of

dendrites is actin while, in later phases, microtubules invade dendrites to promote stabilization and leading to actin re-organization close to plasma membrane (Linda Van Aelst & Cline, 2004).

Since F-actin is the major structural component of growing dendrites, the involvement of the Rho GTPases in dendrite growth and remodeling has been intensively studied both in vertebrates and invertebrates.

RhoA is considered a negative modulator of dendrite extension. In fact, *in vitro* studies demonstrated that the over-expression of constitutively active (CA) RhoA strongly induces the retraction of dendritic branches while RhoA silencing and its dominant negative (DN) mutant lead to the increase of dendritic length in mouse and rat cultured neurons and in *Xenopus* (Ahnert-Hilger et al., 2004); (Ruchhoeft et al., 1999).

In contrast, Rac1 and Cdc42 (to a lesser extent) have a positive effect on dendritic branching and remodeling. In *Xenopus* retinal ganglion cells (RGCs) as well as in mouse and rat cortical neurons the dendritic arbors present a reduced complexity after over-expression of dominant negative mutant forms of Rac1 and Cdc42 while the CA form of Rac1 promotes the opposite effect (Ruchhoeft et al., 1999); (Hayashi et al., 2002).

Moreover, studies in *Xenopus* tectal neurons demonstrated that crosstalk between different components of Rho GTPases is necessary for the correct growth and complexity of dendritic arbor: RhoA positive effect on dendritic growth is promoted by Rac1 activation and Cdc42 inhibition, whereas Rac1 is inhibited by activation of RhoA (Fig.7).

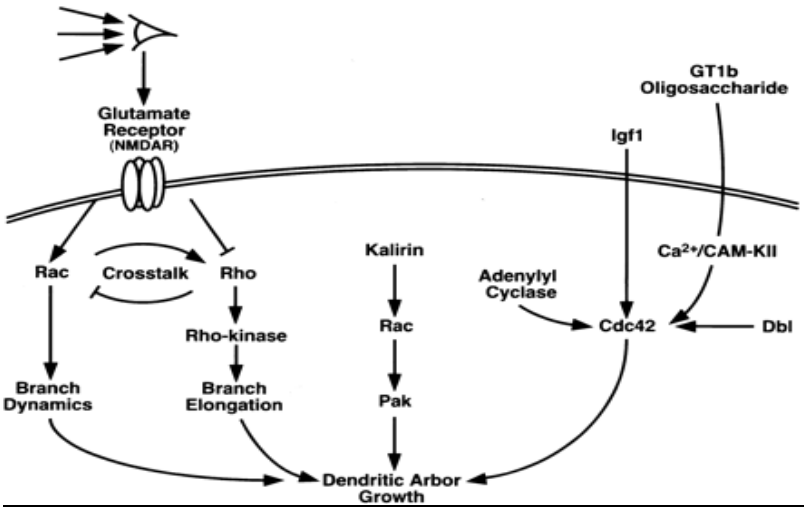


Fig. 7 Regulation of dendritic arborization through RhoGTPases signaling. The image represented a simplified scheme of the activation of RhoGTPases by different stimuli and their effect on dendritic growth and branching (from Govek et al, 2005).

1.2.5 Rho GTPases and dendritic spines

The size, motility and stability of dendritic spines are strictly dependent on actin cytoskeleton and Rho GTPases family has a predominant role in its regulation (Govek et al., 2005).

As in dendritogenesis, RhoA seems to be a negative regulator of spines formation. In fact, CA RhoA expression in mouse and rat cultured neurons leads to a reduction in spine density and length, whereas its inhibition induces an increase in spine density (Nakayama et al., 2000; Tashiro et al., 2000).

For what concerning Rac1, different studies demonstrated its role in the formation, maturation and maintenance of dendritic spines. *In vivo* studies show that CA Rac1 in Purkinje cells of the cerebellum induces an increased number of spines with the formation of peculiar structures that are composed by numerous little spines (Luo et al., 1996). The results in spine density was reconfirmed *in vitro* by experiments on hippocampal neurons. Transfection of rat/mouse cultured neurons with Rac1 DN, as expected, causes a decrease in spine density although spine length is increased. Moreover, different studies demonstrated that the expression of Rac1 DN could transform part of already existing spines into filopodia protrusion. The down-regulation of Rac1 induces a reduction in spine density but also in spine head dimension and in spine stability (Ayumu Tashiro & Yuste, 2004).

Cdc42 role in dendritic spine is less characterized than that of Rac1 and RhoA. Analysis on rodent cultured neurons revealed that Cdc42 CA and DN have no effect on spine density/length (Tashiro et al., 2000) while *in vivo* studies show that reduced Cdc42 protein expression is associated with reduced cortical pyramidal neuron spine density (Cheng et al., 2003). Moreover, silencing of Cdc42 in Drosophila visual system causes a reduction in the density of spines (Scott et al., 2003). Altogether these results indicate that Cdc42 could be important in dendritic spines formation and maintenance in certain cell types.

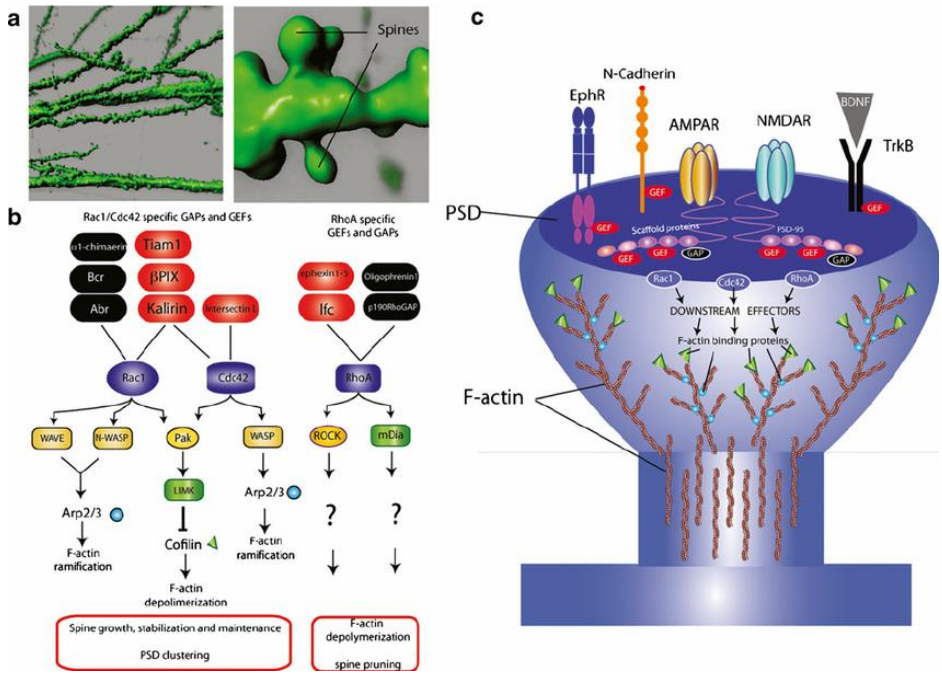


Fig. 8 Contribution of RhoGTPases in dendritic spines generation. (a) 3D reconstruction of dendritic spines. (b) Scheme of RhoGTPases (violet) and their activators (red) and inhibitor (black) as well as effectors (yellow and green) that mediate the reorganization of actin in dendritic spines (c) Representation of a simplified dendritic spine. GAPs and GEFs proteins bind to different classes of membrane receptors and scaffold proteins to regulate RhoGTPases activity in response to external stimuli. RhoGTPases through their effectors regulate actin spine dynamics (from Bolognin et al., 2014).

1.3 Rac1 GEFs and GAPs in nervous system

1.3.1 Rac1 GEFs

Since the necessity to deeply regulate Rac1 activity in neurons, it appears clear that dysregulation in GEF proteins activity could be associated to alteration in neuronal development, spine morphology and density. Here we report some example of Rac1-GEFS and their effect *in vitro* and *in vivo* models.

Kalirin-7

KALRN gene encodes for several protein isoforms via alternative splicing. Kalirin-7 is the prevalent isoform in mature neurons and its expression is developmentally regulated through all synaptogenesis process. Kalirin-7 is mainly located at the post-synaptic compartments of excitatory synapses through the interaction with different scaffold proteins (PSD95, SAP97, SAP102). These interactions allow Kalirin-7 to be part of receptor and signaling molecules complexes that transduces different inputs, leading to Rac1 activation (Penzes et al., 2001); (Penzes & Jones, 2008).

Being mainly expressed at late developmental stages, Kalirin-7 seems to be important more for dendritic spines maturation and remodeling than for their genesis.

Its role as dendritic spine regulator has been demonstrated both *in vitro* and *in vivo*. Cultured cortical and hippocampal neurons are affected by overexpression or silencing of Kalirin-7: while overexpression induces an increased number of dendritic spines, downregulation leads to reduced dendritic spines density and loss of excitatory synapses (Penzes et al., 2003). On the other hand, characterization of Kalirin7 isoform KO mice found morphological and behavioral defects. KO mice exhibit decreased spine density at hippocampal level, impairment in LTP and reduced anxiety-like behavior. (Ma et al., 2008)

Kalirin-7 interacts also with NMDA and AMPA receptors regulating activity-dependent modifications of dendritic structures. In particular, NMDA activation induces CaMKII-dependent phosphorylation of Kalirin-7 on tyrosine and threonine residues, leading to the clustering at membrane level and increased GEF activity. Its

activation lead to cytoskeletal remodeling of dendritic spines and promotes the insertion of AMPA GLUA1 subunit in the membrane at least *in vitro* (Xie et al., 2007).

TIAM1

Tiam1 (T-lymphoma invasion and metastasis 1) is a multi-domain protein that positively regulates Rac1 activity. The protein is present in the whole brain during development at high levels while in adult mouse brain it is expressed only in specific areas such as hippocampus and cerebellum (Ehler et al.,1997). Tolias and colleagues demonstrated that Tiam1 is expressed preferentially in the post-synaptic density. Even if at lower levels, Tiam1 is present also at the pre-synaptic terminal (Tolias et al., 2005).

Like Kalirin-7, Tiam1 interacts with several synaptic receptors as NMDARs and it is activated by phosphorylation by CaMKII. *In vitro*, glutamate application induces an increase in Tiam 1 GEF activity, Rac1 activation and in spine density whereas SiRNA-mediated silencing in cortical and hippocampal cultured neurons leads to a reduced number of spines (Tolias et al., 2005)

Moreover, Tiam1 interacts with BCR (breakpoint cluster region), a Rac GAP present at synapses, and the two proteins compete for Rac1 interaction. This regulator complex works to maintain a balance between facilitation and inhibition of Rac1 activity, fundamental for spine structures dynamics (Um et al., 2014).

αPIX and βPIX

αPIX and βPIX are members of PIX (p21-activated kinase(PAK) interacting exchange factor) family.

βPIX (also called Arhgef7) is expressed in the whole brain during development and in defined area (hippocampus, cerebellum) in adult brain. The protein is present both in excitatory and inhibitory synapses. At excitatory synapses, βPIX interacts with different scaffold proteins including Shank and GIT1, an Arf-GAP molecule that acts recluting βPIX at synapses and therefore modulating Rac1 activation. Rac1-GTP promotes PAK activation and Pak-mediated MLC phosphorylation that is necessary

for spine formation (Park et al., 2003);(Zhang, 2005). Like the others GEFs as Kalirin-7 and Tiam1, βPIX activity is promoted by neural activity (in particular NMDARs stimulation) and downregulation of NMDA receptors in cultured neurons results in loss of dendritic spines and electrophysiological alterations (reduced mEPSCs) (Saneyoshi et al., 2008).

Notably, βPIX and GIT1 are present also in inhibitory synapses where the complex regulates the stability of $GABA_A$ receptor. The presence in both excitatory and inhibitory synapse provide an evidence of the role of βPIX in modulating synaptic transmission (Smith et al., 2014).

1.3.2 Rac1 GAPs

The first Rho-GAP protein has been identified by Garret and colleagues in 1989 and up to know more than 70 Rho GAP proteins have been found (Garrett et al., 1989).

The promotion of intrinsic GTPase activity of RhoGTPases is due to the presence of a conserved domain composed approximately by 170 amino-acidic residues which is called GAP domain. In addition to GAP domain, all Rho GAPs proteins contain different domains important to specify their subcellular localization and protein-protein interactions.

The high number of Rho GAP proteins reflects their importance in the regulation of RhoGTPases activity and suggests that each Rho GTPase needs to be tightly regulated both spatially and temporally. A great number of Rho GAP proteins is ubiquitary expressed while others have a tissue specific distribution. Moreover, different GAPs could affect only a specific Rho GTPase or more (Tcherkezian & Lamarche-Vane, 2007).

Since the interest in Rac1 specific GAPs, here we report some example of their function in neuronal development focusing on dendritic spines.

BCR

BCR (breakpoint cluster region protein) and ABR (active BCR-related) proteins are abundantly expressed in brain and show selective GAP activity for Rac1 both *in vitro* and *in vivo*.

BCR /ABR souvle KO mice have been generated. Increased levels of activated Rac1 and its downstream effectors Pak1/3 and pERK1/2 have been detected in double KO mice. Consequently, dendritic spine density in hippocampal neurons was increased and, although no changes in baseline synaptic transmission were detected, KO mice presented an impairment in LTP maintenance that was behaviorally represented by reduced performance in spatial and object recognition tasks (Um et al., 2014).

srGAP2/3

Among the srGAP (Slit-Robo GTPase-activating proteins) family, srGAP2 and 3 are inhibitor of Rac1 activation (Endris et al., 2011;) (Fossati et al., 2016).

SrGAP2A mediates Rac1-dependent neurite extension and branching and promotes the maturation of both inhibitory and excitatory synapses while limiting the density of both types of synapses via its Rac1-GAP activity (Fossati et al., 2016; Guerrier et al., 2009).

SrGAP3 is considered a key element in the maintenance of correct dendritic spine density. Different srGAP3 KO mouse models have been characterized: while total KO mice present alterations in spine morphology (longer spines) but not in spine number, conditional KO mice show reduced dendritic spine density (Waltereit et al., 2012). These results suggest that a developmentally regulation of protein expression regulates different aspect of its activity.

1.4 Arhgap family

The ARHGAP family genes encode GAPs for ARH (Aplysia Ras-related homologue, also called Rho) proteins; they are located on different chromosomes

and, thanks to differential splicing, express different protein isoforms (Nakamura, 2013).

The first Arhgaps proteins (Arhgap24 and Arhgap25) have been identified through in silico analysis on the human genome although a precise description of their activity has been defined more recently (Katoh & Katoh, 2004).

Different ARHGAPs proteins are encoded by genes located on different chromosomes (for example ARHGAP24 on chromosome 4; ARHGAP22 on chromosome 10; ARHGAP25 on chromosome 2 in human).

The different members of the family share a common structure:

- PH (pleckstrin homology)
- GAP activity region
- a spacer
- C-terminal CC (coiled-coil)

PH domain is predicted to be present in all Arhgap proteins based on bio-informatics analysis, although no evidences of its function have been reported. The presence of this domain could have important implication in the subcellular localization of Arhgap proteins being a substrate for lipidic post-translational modifications. GAP domain possesses catalytic activity and stimulates RhoGTPases intrinsic hydrolysis of GTP while CC terminal coiled-coil domain mediates protein-protein interaction.

Thanks to alternative splicing, different variants are generated for each protein: usually there are longer isoforms that contain all the domain described above whereas the shorter isoforms usually lack the N-terminus PH domain (Nakamura, 2013).

1.4.1 Arhgap22

Arhgap22 (alias RhoGAP2) protein is the product of a gene localized on chromosome 10 in human/14 in mouse. It is ubiquitary expressed with prevalence in highly vascularized tissues (Aitsebaomo et al., 2004).

The long form of the protein contains three conserved domains:

- an N-terminal pleckstrin homology (PH) domain (residues 38 to 147) that is computationally predicted to bind phospholipids
- a RhoGAP domain (residues 140 to 376) with catalytic activity
- C-terminal coiled-coil domain (residues 607 to 687) thought to be important for protein-protein interactions
-

Shorter forms (p68RacGAP) deriving from alternative splicing lack functional PH domain (Aitsebaomo et al., 2004);(Hu et al., 2012); (Nakamura, 2013).

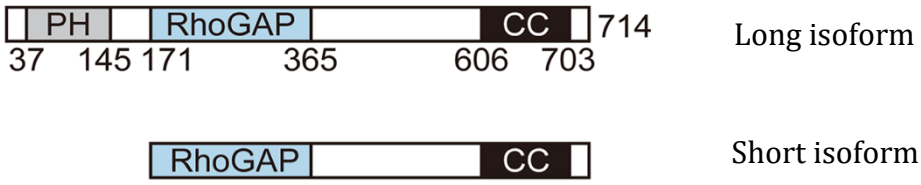


Fig.9 Schematic representation of Arhgap22 protein. The figure represents the structure of both long and short isoforms of Arhgap22. The two variants share the catalytic domain (RhoGAP) and the coiled-coil C-terminus (CC) while only the long isoform posseses the pleckistin homology domain (PH). (Modified from Mori et al., 2014).

Arhgap22 GAP activity has been demonstrated to specifically stimulate the hydrolysis of Rac1-bound GTP *in vitro* without any effect on Cdc42 or Rho. Accordingly, silencing of Arhgap22 leads to an increase in cellular levels of Rac1 GTP (Aitsebaomo et al., 2004); (Sanz-Moreno et al., 2008).

Arhgap222 was identified as a critical cytoskeleton regulator, having an important role in regulating cell motility and mesenchymal transition trough downregulation of Rac1 activity downstream of RhoA activation. In fact, suppression of its expression is thought to be associated with amoeboid movement of tumor cells (Sanz-Moreno et al., 2008).

A previous study has also reported that Arhgap22 is an Akt substrate (S16 and S395) that binds 14-3-3 proteins in response to insulin. 14-3-3 proteins play an important role in insulin and growth factor-dependent changes in cell motility, regulating cell migration but also in neuronal transmission. A hypothesis is that the interaction of 14-3-3 with Arhgap22 inhibits its activity, modulating the GTP loading of Rac1, leading to localized changes in actin turnover and cell movement (Hu et al., 2012).

Arhgap22 seems to have functions besides its activity on Rac1 regulation and actin cytoskeleton. In fact, the splice variant lacking a PH domain translocate from cytoplasm into nucleus in endothelial cells when co-expressed with Vezf1 (vascular endothelial zinc finger-1), inhibiting its positive transcriptional activity for the endothelin-1 promoter. These evidences suggested that Arhgap22 (at least one isoform) have a role in the regulation of cellular transcription (Aitsebaomo et al., 2004).

Moreover, through a yeast two-hybrid screening, Arhgap22 was identified as a new intracellular partner of IL1RAPL1, a gene associated with cognitive impairment ranging from non-syndromic X-linked intellectual disability to autism. IL1RAPL1 and Arhgap22 interaction is required for the ability of IL1RAPL1 to induce dendritic spine formation in cultured hippocampal neurons (Valnegri et al., 2011).

Recently, Mori and colleagues demonstrated that Arhgap22 localizes at endosomes through its C-terminal tail in A7 cells. The authors speculate about a possible role in protein endosomal recycling although Arhgap22 ShRNA-mediated silencing do not affect the recycling of transferrin receptor (Mori et al., 2014).

These studies had revealed the role of RhoGAP2 in regulating actin organization, regulation of transcription as well as protein trafficking.

2. Aim of the work

ArhGAP22 is a member of Arhgap family. It is ubiquitary expressed, with a prevalence in highly vascularized tissues (Aitsebaomo et al., 2004). Its GAP activity has been demonstrated to specifically inhibit the RhoGTPase Rac1 *in vitro* (Sanz-Moreno et al., 2008, Aitsebaomo et al., 2004).

Although ArhGAP22 activity on actin cytoskeleton, cell movement and morphology has been observed, specific mechanism and functional consequences of its activity haven't been demonstrated yet. In particular, since Arhgap22 inhibits Rac1 activity, a protein involved in cytoskeleton remodeling and in dendritic spines morphology (Etienne-Manneville & Hall, 2002); (Negishi & Katoh, 2005), we wondered if Arhgap22 has a role in the formation and maintenance of excitatory synapses.

Thus, the main aim of this project is to define molecular and functional mechanisms underlying biological properties of Arhgap22 in nervous system.

The first part of the project was aimed to define Arhgap22 expression in murine tissues. In particular, we investigated expression level of Arhgap22 transcripts in different murine organs with specific interest in the spatio-temporal distribution in mouse brain.

Presence of Arhgap22 in brain and in post-compartment of post-synapses (Valnegri et al., 2011) allowed us to speculate about its direct role in synaptic functioning. Therefore, the second aim of the work was to investigate the role of Arhgap22 in synapse organization and functioning by means of an animal model for Arhgap222 knock down which represent an ideal model to investigate how potential neuronal abnormalities due to loss of function can affect synapse formation and synaptic network organization *in vivo.* To address these aims, we characterize the phenotype of our models through morphological, biochemical and functional analyses.

3. Materials and methods

3.1. Arhgap22 KO mouse generation

Mutant *Arhgap22* mice were generated using a gene-trapping technique. Mice (strain C57BL/6) were generated from an embryonic stem (ES) cell line (Texas Institute for Genomic Medicine, TIGM). The retroviral (Omni- Bank Vector 76) cassette contained a splice acceptor sequence (SA) followed by a 5′ selectable marker –geo (a functional fusion between the beta galactosidase and neomycin resistance genes) for identification of successful gene trap events, followed by a poly- adenylation signal (pA). Insertion of the retroviral vector into the *Arhgap22* gene (intron 3) led to the splicing of the endogenous first 3 exons and the cassette to produce a truncated transcript of Arhgap22 gene. 3′ RACE was used to verify the insertion of the cassette into the correct genomic location. The ES cell clone, containing the retroviral cassette in the *Arhgap22* gene, was microinjected into C57BL/6 host blastocysts to generate chimeras using standard procedures. Chimeric males were bred to C57BL/6 wild types females for germ line transmission of the mutant Arhgap22 allele.

3.2. Mouse genotyping

Genomic DNA was extracted and then amplified by PCR using the commercial REDExtract-N-Amp™ Tissue PCR Kit (Sigma Aldrich) following the protocol suggested by provider. Briefly, DNA was released from mouse tail (approximately $1mm^3$) by incubating the sample with a solution composed of "extraction solution" and "tissue preparation solution" at room temperature for 10 minutes, followed by 3 min at 95°C and addition of "neutralization solution". Extracted DNA was then mixed with REDExtract Reaction Mix (2X ready mix containing buffer, salts, dNTPs, and *Taq* polymerase) and specific primers (0,4 μM) for following amplification.

Primers sequences:

Arhgap22 Fw primer	5’ TATTAAGCTTACTGGGCCTGTGTCCGT 3’
Arhgap22 Rev primer	5’ GTGCCTCATCACCCTCGGCCCTTCATT 3’
V76 Rev primer	5’ CCAATAAACCCTCTTGCAGTTGC 3’

Each PCR sample was prepared as reported:

Reagent	**Volume**
Water	4 µL
Extract-N-Amp PCR reaction mix	10 µL
Arhgap22 FW primer (0.4 µM)	2 µL
Arhgap22 Rev primer (0.4 µM)	1 µL
V76 Rev primer	1 µL
DNA	4 µL
Total volume	22 µL

PCR parameters:

Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	94 °C	3 minutes	1
Denaturation	94 °C	30 sec	30-35
Annealing	55 °C	30 sec	
Extension	72 °C	40 sec	

Final Extension	72 °C	10 minutes	1

After this step, samples were loaded on 1,5% agarose 1×TAE (40 mM Tris, 20 mM acetic acid and 1 mM EDTA pH 8) gels. PCR products were visualized using SYBR Safe DNA Gel Stain (Life Technologies).

3.3. mRNA extraction from mouse tissues

Mouse mRNA was extracted from tissues using Nucleozol Reagent (Macherey Nagel). Briefly, each tissue (max 30 mg) was mechanically homogenized in 500 µl of Nucleozol and centrifuged at 12000 g for 5 min. Supernatant was then transferred to a tube containing 200 µl of RNAse-free water and incubated at RT for 15 min. After being centrifuged for 15 min at 12000g, solubilized RNA was precipitated by incubation with 500 µl of isopropanol for 10 min and subsequent centrifugation for 10 min at 12000g. After 2 washing steps with 75% ethanol, RNA was resuspended in 30 µl of RNase-free water and quantified by spectrophotometer.

3.4 cDNA synthesis and Real-Time PCR

Extracted mRNA was used to synthetize cDNA using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher).

Each PCR sample was prepared as reported:

mRNA	1,5 µg
VILO reaction mix 5X	4 µl
SuperScriptVILO retrotrascrittase	2 µl
RNase-free water	Up to 20 µl

Retrotrascription parameters:

25° C	10 min
42° C	60 min
85° C	5 min

Prior to use in RT-PCR experiments, cDNa was diluted up to 1:2.

To perform RT-PCR assay, we used the Applied Biosystems 7000 Real-Time thermocycler. For each sample, we prepared in triplicate a mix containing:

RNA/Water	2,5 µl
SYBR Green master mix	10 µl
F primer (400nM final)	1,25
Rev 97 primer (400nM final)	1,25
Water	5 µl

Parallel PCR reactions was performed with (i) α-actin specific primers as an housekeeping control gene (ii) water as negative control.

The sequences of primers (Sigma Aldrich) were the following:

RT Arhgap22 Fw97	5’ TTCGGCCACAGATAGAGGAT 3’
RT Arhgap22 Rev97	5’ GTCATCAGATGCTGAACCAGAG 3’
RT α-actin Fw	5’ AGATGACCCAGATCATGTTTGAGA 3’
RT α-actin Rev	5’ CCTCGTAGATGGGCACAGTGT 3’

RT-PCR parameters:

Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95 °C	10 min	1
Denaturation	95 °C	15 sec	40
Annealing/Extension	60 °C	1 min	
Dissociation stage	95° C 60° C 95° C	15 sec 20 sec 15 sec	1

Data were analyzed using ABI PRISM 7000 software to calculate the C_T value of each sample normalized on α-actin controls.

3.5 mRNA *in situ* hybridization

Wild type animals at age E14.5 or P0 were used for mRNA *in situ* hybridization experiments. After fixation in 4% paraformaldehyde (PFA) overnight, brains were washed in washing buffer (5 mM sodium phosphate-buffered 0.9% saline) and

equilibrated in 30% sucrose. Brains were then embedded in optimal cutting temperature compound (OCT) and cut at cryostat. Sections 11 µm-thick were mounted on poly-lisinated glass (Thermo scientific).

Hybridization was performed with Digoxigenin (DIG)-labeled riboprobes based on the antisense sequence of Arhgap22 gene. Sections were permeabilized with 3 µg/ml proteinase K, washed in PBS, and acetylated with 1.3% triethanolamine and 0.25% acetic anhydrate at RT. They were then pre-hybridized in 50% formamide at 60 °C, hybridized with the DIG-labeled probes for 16 hrs and incubated with an anti-DIG-alkaline phosphatese (AP) conjugated antibody (Roche). After developing with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT-BCIP) (Sigma), images were recorded.

3.6 Rac1-GTP pull-down

Rac1 activity in mouse brain was assessed by pull down experiments using GST-Pak, a fusion protein that bound only the active form of Rac1. Briefly, WT and KO brains (4 per genotype) were collected and homogenized in Rac Buffer (10% glycerol, 100mM NaCl, 10mM $MgCl_2$, 50mM Tris HCl ph7.4, 10% triton, 1:1000 protease inhibitor). Lysates were then centrifuged at 12000g for 10 min at 4°C. Supernatants were incubated with 40 µl of S-transferase PAK glutathione coupled Sepharose 4B beads (GE Healthcare) for 30 min at 4°C. After 3 washes with Rac buffer, resins were resuspended in sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl). Bounded protein as well as inputs were loaded and analyzed by western blotting using monoclonal Rac1 antibody (Abcam). Signals were acquired with Li-COR Odissey instrument and quantified using Image Studio software.

3.7 Golgi staining

Golgi staining was performed using FD Rapid GolgiStainTM Kit (FD Neurotechnologies inc.). Adult WT and KO male mice (3 per genotype) were deeply anesthetized and trans-cardiacally perfused with saline solution (0,9%). Brains

were then washed in MilliQ water and immersed in impregnation solution (solution A 50% and solution B 50%) for 14 days in the dark at room temperature. After several washes in MilliQ water, brains were put in solution C for 3 days at room temperature and consequently cut in 100 μm coronal sections with a vibratome (Leica VT1000S). Slices were mounted on gelatin-coated glasses with solution C and maintained at room temperature in the dark, allowing them to dry naturally. Sections were then washed in MilliQ water and placed in working solution (Solution D 25%, Solution E 25%) for 10 minutes. Subsequently, slices were washed and dehydrate with increasing concentrations of ethanol (50, 70,95 and 100%) for 4 minutes each and, as last step, with xylene (3 times,4 minutes each). At the end of the procedures, slides were mounted with a coverslip using Permount mounting medium (Fisher Scientific). Acquisition of the stained neurons from the CA1 and DG region of hippocampus was performed using a Spinning Disk Confocal Microscope. Stacks were collected every 0.5 μm using 63× objective. Analysis of the dendritic spine density was performed using RECONSTRUCT software.

3.8 Hippocampal crude synaptosomes preparation

Brains from WT and KO adult male mice were removed from cranium and dissected. Hippocampi were homogenized with glass-teflon homogenizer in cold HEPES/sucrose buffer (4mM HEPES pH 7.4, 0.32 M sucrose, 1:1000 protease inhibitor) and centrifuged at 1000 g at 4°C for 10 minutes to remove cellular debris and nuclear fraction (P1). Supernatant (S1) were re-centrifuged at 10000 g at 4°C for 15 minutes. Resulting supernatant contained mainly cytosolic compartment while the pellet was composed by crude synaptosomes (P2). After resuspension in 10 volumes of HEPES/sucrose buffer, pellet was centrifuged at 10000g for 15 minutes at 4°C to purify the preparation. P2 fractions were then quantified with bicinchoninic acid assay (BCA) (Euroclone) and stored at –80°C until use.

3.9 SDS-PAGE and western blot analysis

Samples were loaded on poly-acrilammide gels at variable concentrations (range 7,5%-15%). After separation, proteins were transferred on nitrocellulose membranes 0.2 μm (GE Healthcare) in blotting buffer (250 mM Tris HCl, 1.92 M glycine, 20% methanol) at 1000 mA for 2 hours. Membranes were incubated with blocking buffer (5% dry non-fat milk, TBS-Tween 0.1%) for 1h at room temperature. After that, primary antibody staining was performed for 14 hours at 4°C.

Diluition of primary antibodies in blocking buffer:

Antibody	Diluition	Producer
Rac1	1:500	Abcam
Tubulin	1:40000	Sigma Aldrich
GAPDH	1:4000	Santa Cruz Biotechnologies
GLUA1	1:1000	Millipore
GLUA2/3	1:1000	Gift from dr Cecilia Gotti
GLUN1	1:500	Gift from dr Cecilia Gotti
GLUN2A	1:1500	Sigma Aldrich
GLUN2B	1:1000	Millipore
GLUK2	1:1000	Prestige
CASK1	1:2500	NeuromAb
Arp2/3	1:500	Santa Cruz Biotechnology

WAVE	1:100	Cell signaling
PAK	1:1000	Cell signaling
pPAK	1:1000	Cell signaling
PSD95	1:2500	Neuromab
PICK1	1:1000	Neuromab
GRIP1	1:2000	BD transduction laboratories
VGAT	1:1000	Synaptic System
Synaptophysin	1:5000	Synaptic System

After 3 washes (10 minutes each) with blocking buffer, membranes were incubated with secondary antibodies (700 IRdye-αmouse/800 IRdye-αrabbit, 1:7500, Li-COR) for 1 hour at room temperature and then washed 6 times in TBS-Tween 0.1% and 3 times in TBS (10 minutes each). After complete drying of nitrocellulose, immunorective bands were detected by Li-COR Odissey instrument (Li-COR) and quantified with ImageStudio software.

3.10 Electrophysiological recording

Adult Arhgap22 WT and KO male mice (at least 3 *per* genotype) were used for electrophysiological recordings. Coronal hippocampal slices (thickness, 400 μm) were prepared with a vibratome VT1000 S (Leica) and then incubated for 40 min at room temperature in aCSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl2, 25 mM glucose, and 26 mM NaHCO3; pH 7.3, equilibrated with 95% O2 and 5% CO2) for fEPSPs recordings. Slices were immersed in aCSF at a rate of ~2 ml/min at 32°C of temperature in a recording chamber.

fEPSPs were evoked by stimulation (0.05 Hz of frequency) of Schaffer collateral and recorded from the dendritic field of the CA1 pyramidal neurons of the hippocampus.

fEPSPs were acquired at 20 kHz and filtered at 5 kHz.

Input–output (I–O) curves were obtained by measuring the slope of fEPSPs evoked in response to stimulation with increasing intensity (0–1.0 mA). Stimulus strength was adjusted to give 50% maximal response and long-term potentiation was stimulated by high frequency stimulation (HFS) (100 stimuli at 250 Hz).

For Paired Pulse ratio (PPR) experiments pairs of stimuli were delivered at 50 ms intervals every 20 s (0.05 Hz) and PPRs were calculated by dividing the amplitude of the second response by the first one.

3.11 Behavioral tests

All the behavioral tests were performed on adult Arhgap22 WT and KO male mice (at least 5 *per* genotype). Animals were kept in 12h light/dark cycle with food and water *ad libitium*.

3.11.1 General health assessment

Animals were evaluated once a week during behavioral experiment to evaluate their general health status by measuring body weight and food intake.

3.11.2 Spontaneous motor activity

Mice locomotion integrity was evaluated by recording animals in automated activity cage (43 cm × 43 cm × 32 cm) (Ugo Basile, Varese, Italy). Cumulative horizontal and vertical beams breaks were counted for 180 minutes.

3.11.3 Balance beam

Balance beam test was used to assess mice balance and coordination. Mice were trained and tested to cross circular beams of different diameters (6mm and 12 mm). Latency to traverse each beam was recorded.

3.11.4 Wire hanging

Muscle strength was evaluated by positioning the mouse on the top of a wire cage lid. After shaking the lid to give the mouse the possibility to grip to the wire, the operator turned upside down the lid. Latency to fall off the wire was recorded.

3.11.5 Marble burying

Marble burying test was used to evaluated anxiety-like behavior in mice. Briefly, animals were placed in a cage filled with 5 cm depth of bedding and 12 marbles regularly positioned. The number of buried and the latency to bury were measured.

3.11.6 Elevated plus maze

Elevated plus maze was the second paradigm used to evaluate anxiety-like behavior. The apparatus presented a cross shape (two opposite open arms and two opposite enclosed arms separated by a central platform) and was positioned 50 cm far from the floor in a quiet room. After 20 min adaptation, mice were placed individually in the center of the apparatus. The number of total and open arm entries and the time spent in open arms were recorded for 10 min.

3.11.7 Novel object recognition

Novel object recognition test was divided into two phases: the familiarization phase and the object recognition phase. The familiarization phase consisted in 20 min of habituation in a cage with two different objects. After that, animals were returned to the home cage and the experiment was repeated at different time points (5 min, 120 min, and 24 h later) but every time one of the familiar object was replaced by a novel one. Object recognition was achieved when mice stayed within 0.5 cm from the novel object with the nose toward the object.

3.11.7 Spatial object recognition

Spatial object recognition test was divided into two distinct phases: the familiarization phase and the object recognition phase. In the first phase, ,

two different objects were placed at different corners of the arena. Mice were able to explore the objects for 20 minutes. After the familiarization phase, animals were returned to the home cage. After 5 and 120 minutes, animals were put again in the arena but every time one of the two object was relocated. The discrimination index was calculated as described by Pitsikas et al. 2001.

3.11.8 T-maze

T-maze test was performed to evaluate hippocampal dependent spatial working memory in mice. Before the experimemt, mice were food deprived (24h). Then, animals were placed in the start arm of a maze with a T shape where they could choose between entering either the left or the right arm. In the acquisition phase (10 days), the left arm was used as the reinforcing area as it contained food. The percentage of animals and number of days to reach the criterion (80% of correct choices for 3 days) was calculated. Each mouse that reached the criterion for acquisition was then tested using a reversal phase in which the reinforcer arm was changed.

3.11.9 Water maze

Water maze paradigm was used as described by Morris et al.1982. The maze consisted in a tank (diameter 1.5 m) containing water and a hidden platform placed about 1cm below the water surface. The pool was divided into four quadrants: NW, NE, SW and SE as reference. Several posters and furniture that provided visual cues were mounted near the maze. During the acquisition phase, mice began the experiment from different points of the pool and while the platform position was maintained (4 trials/day for 4 days). 24 h after the last trial the platform was removed from the pool and a probe test was performed. Escape latency during acquisition phase and the time spent in the target zone of the maze during probe phase were recorded.

3.12 Statistical analysis

For all analysis, we used at least 3 mice *per* genotype and each experiment was repeated at least three times. Results were described as mean ± standard error of the mean (SEM). Statistical analyses were performed using Prism 6 software (GraphPad, San Diego, CA), except for electrophysiological analysis performed using Statview 5.0 (SAS Institute, Cary, NC, USA, www.statview.com). Statistical significance was determined by Student's t test or by ANOVA followed by Bonferroni's post hoc tests for multiple comparison. The level of significance was described as: *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

4. Results

4.1 Arhgap22 is expressed in cortex and hippocampus in mouse brain

Since little is known about Arhgap22 protein distribution in mouse, we evaluated its expression in murine tissues. First, we tried different commercial and home-made antibodies directed against Arhgap22 protein but, unfortunately, we could not obtain any specific signal in western blot experiments (data not shown). Thus, we analyzed Arhgap22 mRNA expression by Real Time PCR (RT-PCR). Quantification of Arhgap22 mRNA levels indicated that the transcript was expressed in several mouse tissues including brain (Fig.10a). We also analyzed different brain area and we found that Arhgap22 mRNA was expressed at low levels in cerebellum while it was particularly abundant in cortex and hippocampus (Fig.10b). These results are coherent with *in vitro* data that showed the expression of Arhgap22 protein in the post synaptic compartments of excitatory synapses of both cortical and hippocampal cultured neurons (Valnegri et al., 2011).

From a temporal point of view, we analyzed the expression of Arhgap22 mRNA by RT-PCR during different stages of mouse brain development. In total brain, the transcript was present at lower levels at embryonic stage, it increased during post-natal life with a peak at P14 and it was maintained at constant levels during adult life (Fig.10c). The lower expression of Arhgap22 transcript during fetal life compared to post-natal life was also confirmed by mRNA *in situ* hybridization experiments performed in E14,5 and P0 mice (Fig.10d).

In summary, we observed that Arhgap22 is a protein expressed in cortex and hippocampus during the peak of synaptogenesis in mice (Sans et al., 2000). Moreover, it is known from literature that Arhgap22 is a negative regulator of Rac1 activity, a key element in the proper formation of dendritic spines. Given these premises, we could speculate that the protein plays an active role in the formation and maturation of synapses. To corroborate this hypothesis, we decided to perform

the following experiments in hippocampus because hippocampal pyramidal neurons are one of the best model for the study of dendritic spines.

4.2 Arhgap22 KO mice validation

To examine Arhgap22 function in murine brain, we generated Arhgap22 Knock Out (KO) mouse by gene-trapping technology (Fig.11a). Briefly, the ES cell clone (Texas A&M Institute for Genomic Medicine (TIGM)) containing the retroviral insertion cassette V76 in the Arhgap22 gene was microinjected into C57BL/6 host blastocysts to generate germ line chimeras. Chimeric males were bred to C57BL/6 WT females for germ line transmission of the mutant Arhgap22 allele according to mendelian genetics.

To confirm the disruption of the functional Arhgap22 gene in the KO mice, mutant and normal alleles were amplified by PCR. The specific Arhgap22 normal allele (400 bp band) was detected in wild type and heterozygous animals, while mutated product (200 bp band) was amplified in heterozygous and knock out mice (Fig.2b). Genotyping results were confirmed by RT-PCR on total brain lysates. KO mice showed the almost complete absence of Arhgap22 transcript (Fig.11c). We explained the presence of low residual levels of Arhgap22 transcript with the synthesis of processed non-coding transcripts that lacks the first 3 exons (and consequentially the retroviral insertion cassette) as suggested by bio-informatics analyses (ENSEMBL database).

4.3 Arhgap22 mutant mice presented a good general state of health

A first characterization of Arhgap22 mutant mice indicated that they presented a small decrease in body weight (WT 591.600 ± 7.419 n=4, KO 552.700± 10.730 n=5, Student t-test * $p<0.05$) reflected by a slightly reduced food intake (WT 15.980 ± 0.284 n=4, KO 14.660 ± 0.402 n=5, Student t-test * $p<0.05$) (Fig.12a, b). To better understand if animals had a suffering phenotype and if they could be used for subsequent experiments, we evaluated other parameters normally used to describe the state of mice health such as locomotor activity (Fig.12c), motor coordination and

balance (Fig.12d), muscular strength (Fig.12e). Arhgap22 KO mice did not present gross abnormalities compared to wild type littermates.

Moreover, since we were particularly interested in brain function, we also analyzed KO and WT brains: we observed no altered phenotype since gross anatomy and dimension of brain were comparable between the two groups (Fig.12f).

In conclusion, this first phenotypical characterization of Arhgap22 deficient mice indicated that the animals presented a good general health state, comparable with that of WT.

4.4 Arhgap22 KO mice showed hyper-activated Rac1 and altered downstream pathways

Since it was demonstrated that Arhgap22 is a selective inhibitor of Rac1 activity *in vitro* (Sanz-Moreno et al., 2008), we wondered if the absence of the protein had the same activity *in vivo* or if the effect could be eliminated by compensatory mechanisms. To answer this question, we performed GST pull down experiments. Briefly, we used as bait the GST tagged CRIB domain of Pak1 protein, known to selectively bind the active form of Rac1 (Rac1-GTP). As shown by the western blot image (Fig.13a) and relative quantification (Fig.13b), KO mice presented a significative increase in the levels of Rac1-GTP compared to WT (WT 1.000 ± 0.007, KO 1.620 ± 0.060, Student t-test ** $p<0.01$).

Since the evident increase in Rac1-GTP levels, we wondered if mutated animals presented alterations also in the pathways regulated by Rac1. We performed western blot experiments on hippocampal lysates and we evaluated the expression of downstream effector proteins WAVE, Arp2/3 and Pak1. Western blot image and the relative quantification (Fig.13c) showed that there was an increased level of WAVE (WT 1.000 ± 0.061 n=5, KO 1.24 ± 0.065 n=5, Student t-test * $p<0.05$) and Arp2/3 (WT 1.000± 0.054 n=4, KO 1.271 ± 0.089 n=4, Student t-test * $p<0.05$) proteins and in the active form of PAK (phosphorylated form) (WT 1.000 ± 0.072 n=5, KO 1.321 ± 0.085 n=5, Student t-test * $p<0.05$), suggesting an overall over-

activation of the pathways regulated by Rac1 in knock out compared to wild type animals .

4.5 Hyper-activation of Rac1 induced an increase number of dendritic spines in Arhgap22 KO mouse hippocampus

The role of Rac1 and its downstream pathway in the regulation of actin cytoskeleton, fundamental for dendritic spines formation and maturation, is well known. Moreover, different mouse models that presented the disruption of different Rac-GAPs encoding genes had strong alterations in spinogenesis as well as in maturation of dendritic spines in cortex and hippocampus (Sarowar et al., 2016; Valdez et al., 2016).

Therefore, we evaluated spine density in excitatory neurons from hippocampal areas CA1 and DG in Golgi staining experiments. Our results indicated that KO mice had an increase in dendritic spine density respect to the control animals (Fig.14a, 14b) (WT 1.000 ±0.065, KO 1.402 ± 0.14, Student t-test * $p<0.05$), accordingly to previous results obtained in mutant mice with increased levels of Rac1-GTP.

Altogether these data demonstrated that Arhgap22 KO mice presented a consistent dysregulation of Rac1 pathway, with strong effect on dendritic spines number. Since the correlation between alteration of both dendritic spine density and structure with synaptic activity, we are going to perform detailed analyses of dendritic spine morphology by electron microscopy experiments. Our goal is to characterize the ultrastructure of dendritic spines in order to determine their morphology as well as other parameters such as the integrity of the post synaptic density.

4.6 Arhgap22 disruption causes alteration in molecular composition of dendritic spines

Since we demonstrated that Arhgap22 KO mice presented an alteration in dendritic spine number, we decided to analyze also the molecular composition of synapses. For this reason, we performed western blot experiments on crude synaptosomes

prepared from hippocampi of WT and KO adult mice. We evaluated a panel of synaptic markers including: glutamate receptors, post synaptic density (PSD) scaffold proteins and pre-synaptic markers (Fig.15). We found that KO mice presented reduced levels of GLUA1(WT 1.00±0.121 n=4, KO 0.640± 0.041, n=4, Student t-test * $p<0.05$) and GLUA2/3 (WT 1.000±0.061 n=4, KO 0.800± 0.043 n=4, Student t-test * $p<0.05$) AMPA receptors subunits compared to WT littermates while there were no statistical differences for all the other proteins analyzed. Although no statistical results were achieved for other markers, we noticed a negative trend for different glutamate receptors subunits, probably indicating a general defect in the composition of synapses.

These results were quite unexpected considering that we found that mutated animals had an increased spine density compared to WT mice. The possible explanation is that KO mice possess an alteration in the morphology of spines: the presence of an increased number of immature spines as well of dysmorphic spines could lead to an increased number of silenced or non-functional spines with a reduced number of glutamate receptor at synapses.

4.7 Arhgap22 loss of function causes Long Term Potentiation (LTP) impairment in mutant mice

Since we found evident defects in the number of spines and in their molecular composition, we wondered if these alterations could have also a functional effect. We therefore decided to analyze physiological properties of hippocampal synapses in Arhgap22 WT and KO mice.

First of all, we evaluated if the functionality of CA1-CA3 baseline transmission was somehow compromised. To do that, we stimulated Schaffer collateral (SC) fibers at increasing stimulus intensity and registered field excitatory post-synaptic Potentials (fEPSPs). That allowed us to evaluate the input-output relationship, a measure of the strength of synapses. As shown in figure 16a, WT and KO animals had similar responses, suggesting that CA3-CA1 synapses were normal in KO compared to WTs.

To characterize the functionality of pre-synaptic terminals, we evaluated the Pair Pulse Ratio (PPR). PPR was used as read-out of the release probability of neurotransmitter (glutamate). KO mice presented values comparable to those of WT animals (Fig.16b). Therefore, we could conclude that KO animals did not present defects at pre-synaptic levels.

Morphological and biochemical analysis suggested that Arhgap22 deficient mice had a strong alteration at post-synaptic levels. In particular, since GLUA1 and GLU2/3 containing AMPA receptors trafficking at the synapses is fundamental for the generation of LTP and KO mice show decreased levels of these subunits, we decided to study if KO mice had defects in long term plasticity. Indeed, mutant mice presented strong impairment in LTP induction after high frequency stimulation of Schaffer collateral fibers of hippocampus (Fig.16c) (WT 209.2 ± 24.8, KO 121.3 ± 7.52, N=6, Student t-test **p<0,01).

4.6 Arhgap22 KO mice presented learning /memory deficits and reduced anxiety-like behavior

Analysis of morphological, biochemical and electrophysiological properties suggested that Arhgap22 mice could present also behavioral alterations. We previously assessed that mice general health status and locomotor activity were comparable to those of WT thus we decided to study deeper the behavioral phenotype of Arhgap22 mutant mice. In particular, considering the impairment in LTP, one of the fundamental mechanism at the basis of learning and memory, we tested WT and KO mice for cognitive tests.

Firstly, we monitored WT and KO littermates to examine their explorative and emotional behaviors using a dark/light box, emergence tests and activity cages. The results showed no differences between the genotypes and indicated that both had intact explorative and emotional behaviors (data not shown). Next, episodic and spatial memory were tested by novel object recognition, spatial object recognition, water maze and T-maze tests.

For what concern novel object recognition, KO mice presented reduced discrimination index compared to WT at 5, 30, 120 min after familiarization compared to WT (Fig.17a, left), (5 min: WT: 0.2167 ± 0.0606, KO −0.2025 ± 0.0741; 30 min: WT 0.2320 ± 0.0330, KO −0.0900 ± 0.0987; 120 min: WT: 0.2790 ± 0.0638, KO: −0.0540 ± 0.0840; n = 10 per group , differences between genotypes **P < 0.01, ***P < 0.001, two-way ANOVA). This result suggests that the alteration in hippocampal functions are reflected by defects in recognition memory.

In the spatial object recognition task (Fig.17a, right), WT mice explored more the spatially displaced object compared with the object that remained stationary during the recognition trial, whereas KO mice spent the same amount of time exploring both objects [5 min: WT 0.2975 ± 0.0536, KO −0.2100 ± 0.1460; 120 min: WT 0.1938 ± 0.0454, KO −0.0900 ± 0.06071, n= 9 per group, differences between genotypes, two-way ANOVA **P < 0.01]. This suggested that Arhgap22 KO mice were not able to discriminate between the two objects.

During T-maze acquisition phase, KO mice showed an increased latency to reach the criterion compared to WT (Fig.17b) (WT 4 ± 0.02582, KO: 6.6 ± 1.03, n= 6 per group, two-way ANOVA *P < 0.05). On the contrary, we did not find significant difference between genotypes during the reversal phase although a trend was detectable.

When we tested animals in Water maze test, a hippocampal function-depending assay, mutant mice showed an increased latency (although not significant) to find the platform during the acquisition phase. During the probe test, Arhgap22 KO mice spent less time in the target quadrant compared with WT mice (WT 51.875 ± 0.048331, KO: 14.82 ± 6.2982, n= 6 per group, Student-t test **P < 0.01) (Fig.17c).

The results obtained by cognitive tests demonstrated that Arhgap22 KO mice presented a functional impairment in learning and memory formation.

Therefore, we decided to evaluate animals also in elevated plus maze and marble burying tests, that are generally used to study anxiety-related behavior. In elevated plus maze test, KO mice entered more frequently (WT 39.330 ± 3.199, KO: 66.180 ± 5.117, n= 6 per group, Student-t test **P < 0,01) and spent more time in the open arm compared to WT (18.600, ± 2.185, KO: 62.270 ± 9.474, n= 6 per group, Student-t test ***P < 0,001) even if the number of total arm entry was comparable between

genotypes (Fig.17d), suggesting that KO mice are less anxious. As last experiment, we performed marble burying assay (Fig.17e). WT animals buried a major number of marble (WT 10.000 ± 1.633, KO 3,714 ± 0.8081 n= 7 per group, Student-t test **P < 0.01) and had a lower latency to bury compared to KO animals (WT 186.500 ± 77.690, KO 479.700 ± 98.520 n= 6 per group, Student-t test * P < 0.05).

These last data suggested that Arhgap22 deficient mice had reduced anxiety-related behavior, probably caused by a general incapability to understand which kind of external factor could be dangerous and thus to avoid.

5. Figure appendix

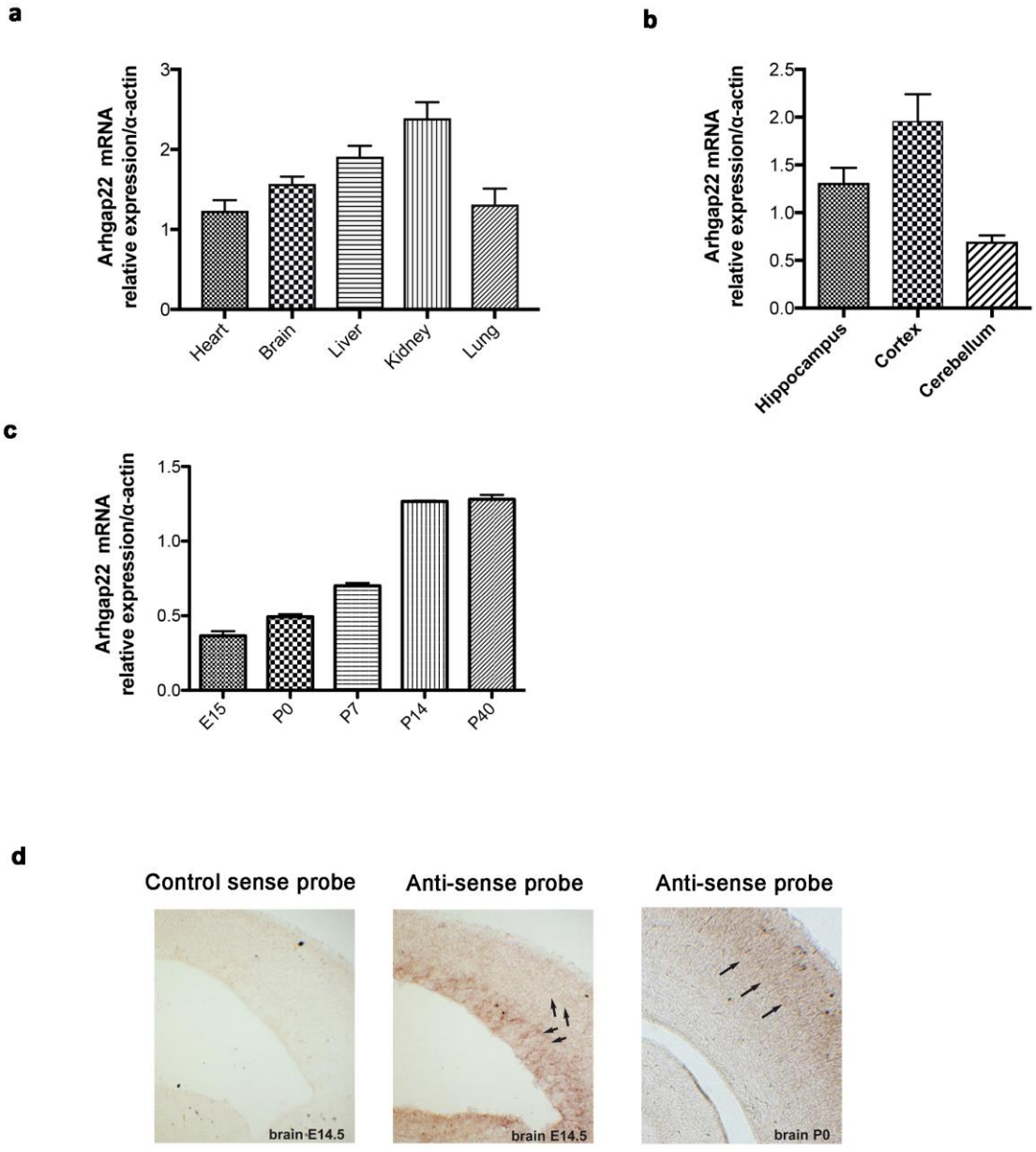
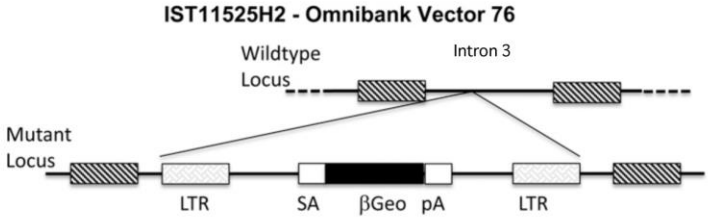
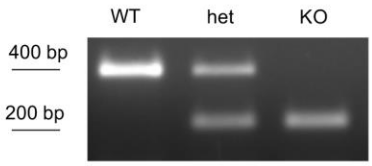


Fig.10 Arhgap22 mRNA expression in mouse. (a) Arhgap22 mRNA relative expression in different murine organ. Arhgap22 is particularly expressed in kidney, liver and brain. Values are normalized on α-actin. Error bars indicate ± s.e.m. (b) Arhgap22 mRNA expression in different brain area of adult mouse. Arhgap22 transcript is express in cortex and hippocampus while is expressed at lower extent in cerebellum. Values are normalized on α-actin. Error bars indicate ± s.e.m (c) Arhgap22 mRNA expression at different time-point: embrional day 15(E15, post-natal day 0 (P0), 7 (P7), 14 (P14), 40 (P40). The higher degree of expression is present a P14 and P40. Values are normalized on α-actin. Error bars indicate ± s.e.m. (d) *In situ* hybridization with a probe specific for *ArhGAP22* mRNA (anti-sense probe) and negative control (control sense probe). The image show magnification of cortex from coronal section of E14.5 and P0 mouse brain. Black arrows indicate labeled neurons.

a


IST11525H2 - Omnibank Vector 76
Wildtype
Locus
Intron 3
Mutant
Locus
LTR
SA
βGeo
pA
LTR

b


WT
het
KO
400 bp
200 bp

c

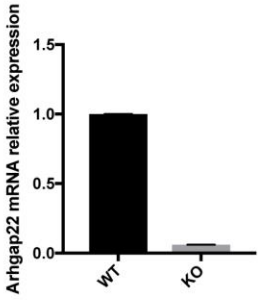

Arhgap22 mRNA relative expression
1.5
1.0
0.5
0.0
WT
KO

Fig.11 Generation of Arhgap22 KO mouse. (a) Schematic representation of the vector used to randomly insert into Arhgap22 wild typelocus (intron 4). The gene-trap cassette includes the following elements: 5′ and 3′ flankinglong terminal repeats (LTR), splicing acceptor (SA), βGeo marker (βGal and Neo fusion)and a polyadenylation site.(b) Characteristic genotyping PCR bands of the resulting phenotypes.Arhgap22Fw/Arhgap22Rev genotyping primer pairs hybridize on intron 4 at either side ofthe insertion point resulting in amplification only from the wild-type allele, whereas theArhgap22/V76R pairs result in amplification from the targeted allel. (c) Arhgap22 mRNA expression in WT and KO mice brain. KO mice show the almost complete absence of Arhgap22 transcript. Values are normalized on α –actin. Error bars indicate ± s.e.m.

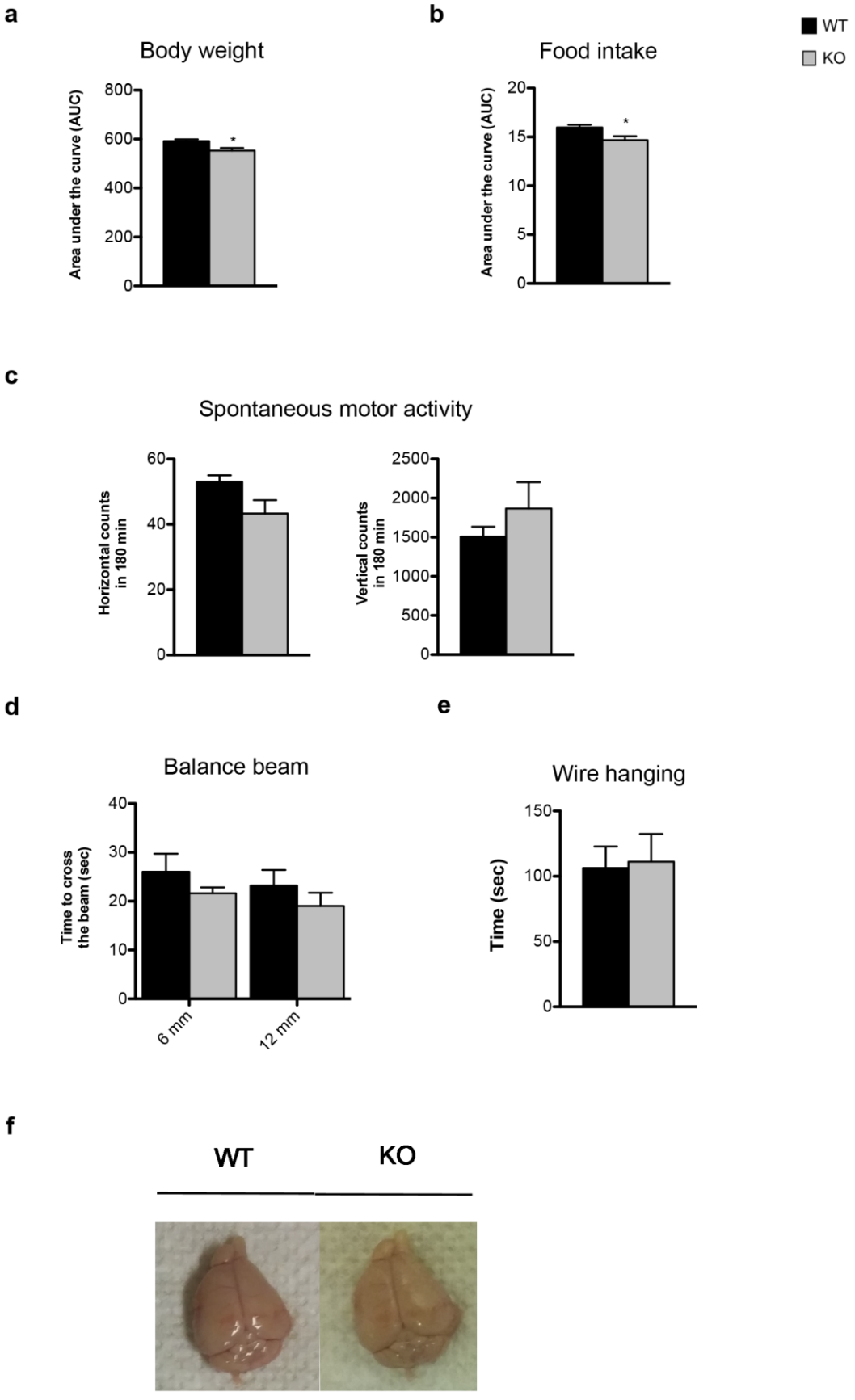

a
Body weight
Area under the curve (AUC)
800
600
400
200
0
*
b
Food intake
Area under the curve (AUC)
20
15
10
5
0
*
WT
KO
c
Spontaneous motor activity
Horizontal counts in 180 min
60
40
20
0
Vertical counts in 180 min
2500
2000
1500
1000
500
0
d
Balance beam
Time to cross the beam (sec)
40
30
20
10
0
6 mm
12 mm
e
Wire hanging
Time (sec)
150
100
50
0
f
WT
KO

Fig.12 Arhgap22 KO mice has a good general health state. Arhgap22 WT and Ko have been tested to asses their general health state. (a), (b) KO mice present decrease in body weight and food intake (Student t-test, *P <0.05). (a) Arhgap22 WT e KO mice were tested to evaluate also general motor functions. No differences were detected in spontaneous motor activity (c), balance beam (d) and wire hanging (e). (f) Photographs of WT (left) and KO (right) mouse brains. Gross anatomy and size were similar between the two genotypes. Error bars indicate ± s.e.m.

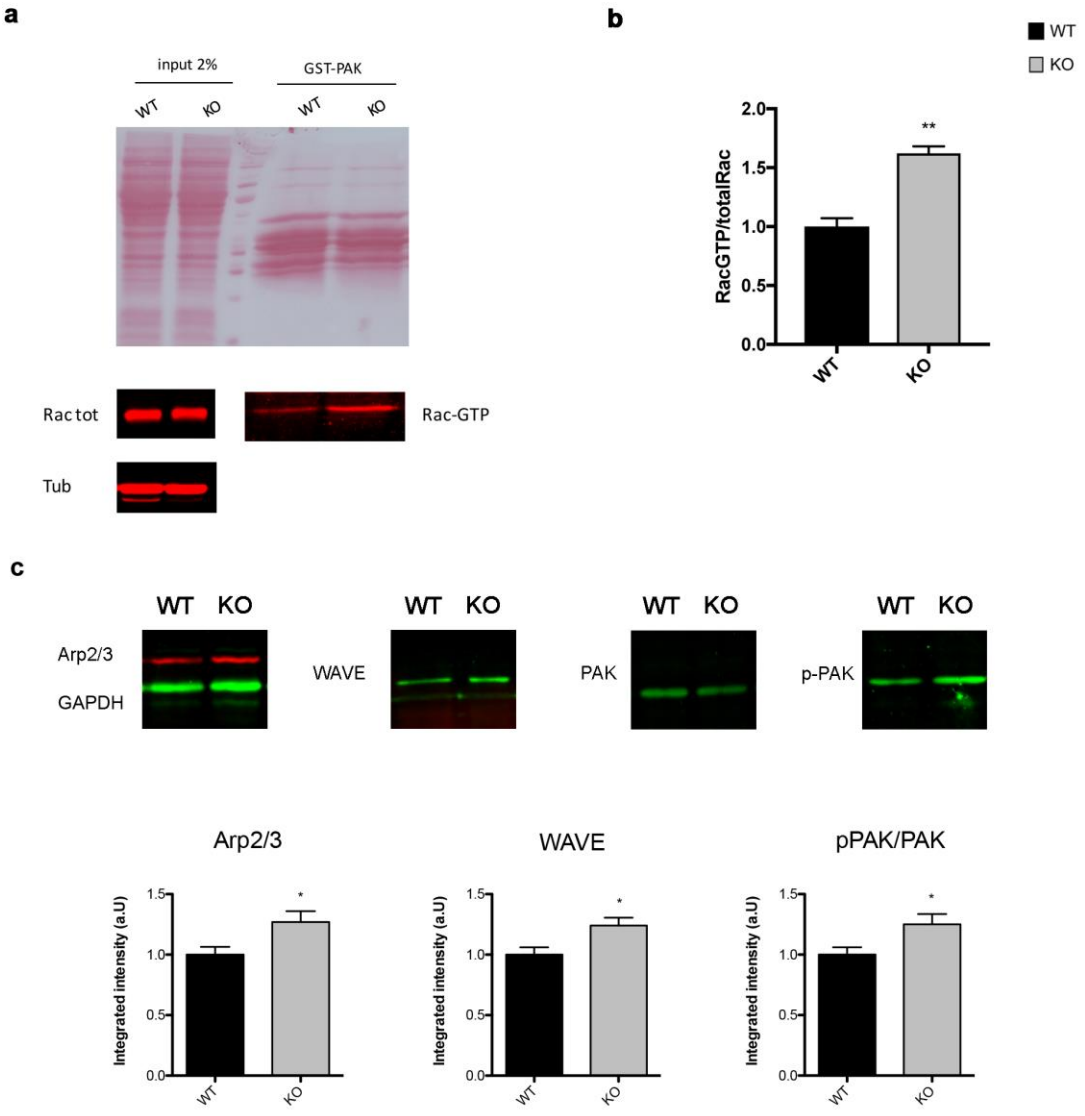
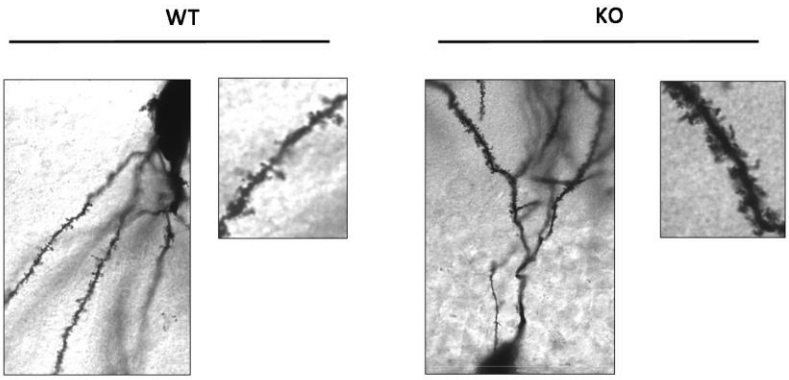

a
input 2%
GST-PAK
WT
KO
WT
KO
Rac tot
Rac-GTP
Tub
b
WT
KO
RacGTP/totalRac
2.0
1.5
1.0
0.5
0.0
**
WT
KO
c
WT KO
Arp2/3
GAPDH
WT KO
WAVE
WT KO
PAK
WT KO
p-PAK
Arp2/3
WAVE
pPAK/PAK
Integrated intensity (a.U)
1.5
1.0
0.5
0.0
*
WT
KO

Fig.13 Arhgap22 mutant mice present hyper-activated Rac1 pathway in brain. (a) Activated Rac1 GST-pulldown on hippocampal lysates. Red ponceau (up) show the amount of lysates and GSt-CRIB beads used for the experiment. Western blot of total Rac1 (Rac tot) and active Rac1 (Rac-GTP) are shown. Tubulin was used as internal control. (b) Quantification of the level of active Rac1 normalized on total Rac1 protein. Ko animals present an elvated level of Rac1 GTP (Student t-test, **P<0,01). (c) Western blot analyses on hippocampal lysates of WT and KO mice. Levels od Rac1 downstream effectors Arp2/3, WAVE, PAK1 and pPAK1 have been quantified and normalized on GAPDH (up). Arp2/3 and WAVE protein levels and the ratio between the active(phosporilated) and inactive form of PAK1 are increased in KO mice compared to WT(down) (Student t-test, *P<0,05). Error bars indicate ± s.e.m.

a



b

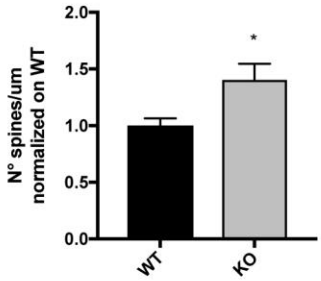
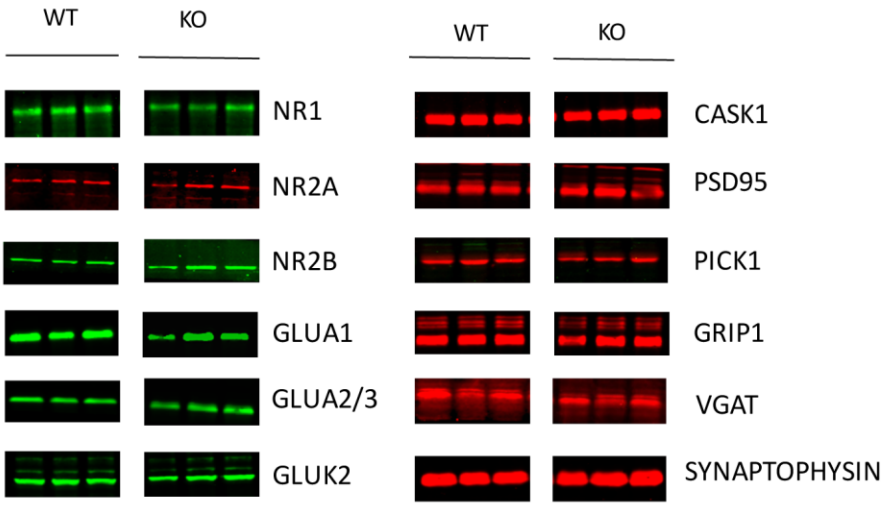


Fig.14 Arhgap22 mutant mice have an increased spine density in hippocampus. (a) Representative Golgi staining images of hippocampal CA1 neurons and high magnification of dendrites segments from WT (left) and KO (right) mice. (b) Dendritic spine density measurement indicates increased number of dendritic spines in KO mice compared to WT mice (student t-test,*P<0,05). Error bars indicate ± s.e.m.


WT
KO
WT
KO
NR1
NR2A
NR2B
GLUA1
GLUA2/3
GLUK2
CASK1
PSD95
PICK1
GRIP1
VGAT
SYNAPTOPHYSIN

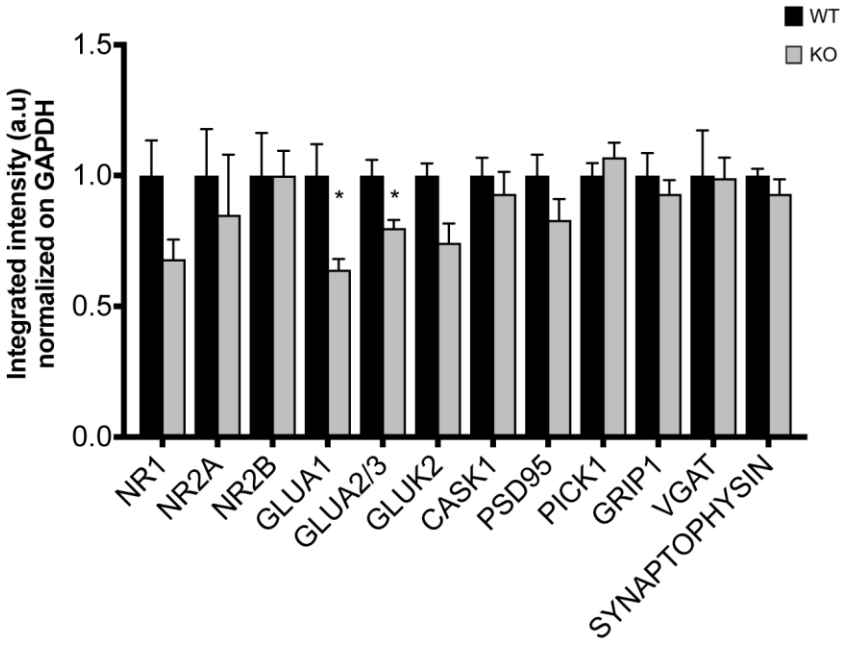

WT
KO
Integrated intensity (a.u) normalized on GAPDH
1.5
1.0
0.5
0.0
*
*
NR1
NR2A
NR2B
GLUA1
GLUA2/3
GLUK2
CASK1
PSD95
PICK1
GRIP1
VGAT
SYNAPTOPHYSIN

Fig.15 Arhgap22 silencing induces alteration in the molecular composition of excitatory synapses. Representative western blots (up) and histograms (bottom) showing the quantification of synaptic markers on crude hippocampal synaptosomes from adult Arhgap22 WT and KO mice. Densimeter analysis performed with Li-Cor technology show that GLUA1 and GLUA2/3 proteins are significantly reduced in Arhgap22 KO mice compared to WT (Student t-test, * $P < 0.05$). No statistically significant differences were found for the other synaptic markers analysed. Error bars indicate ± s.e.m.

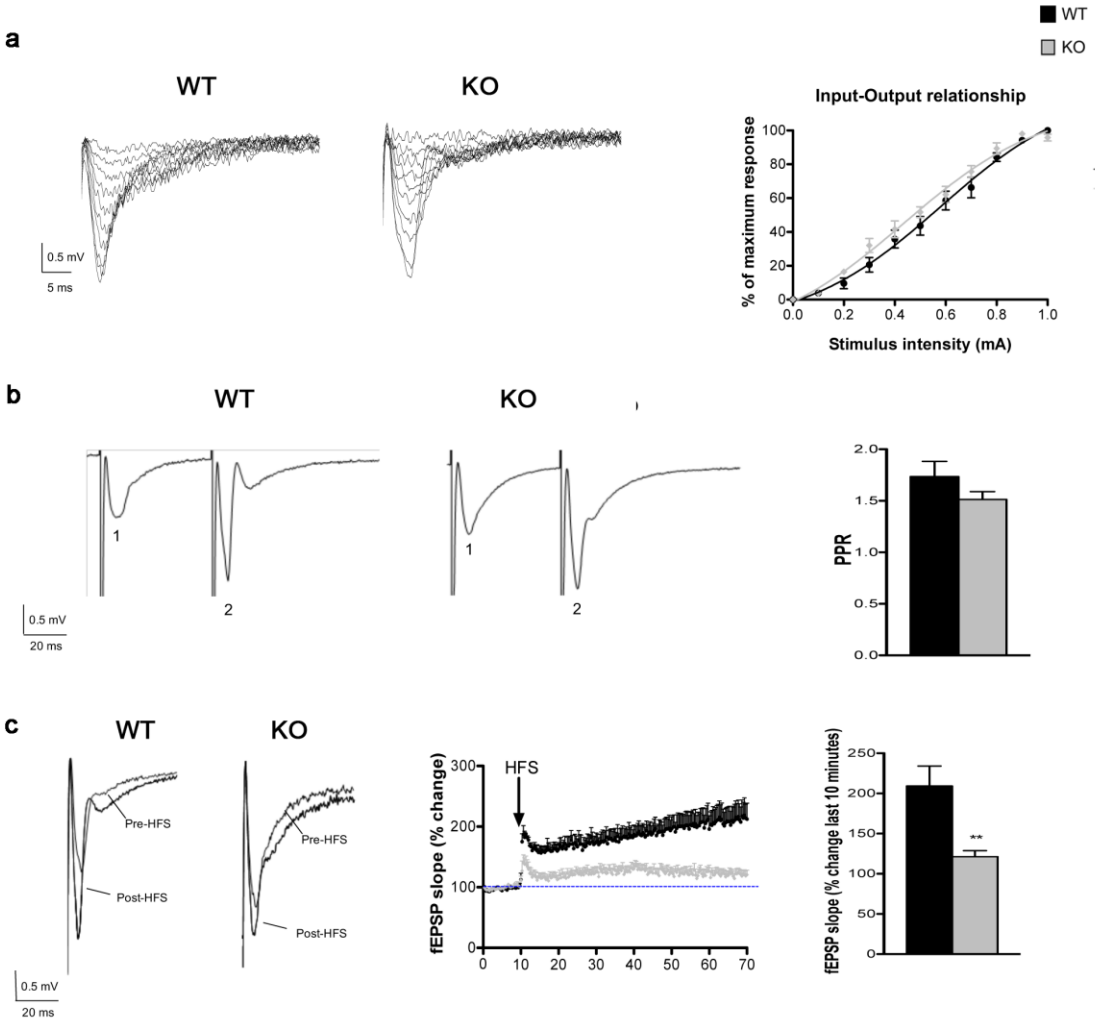

a
WT
KO
WT
KO
0.5 mV
5 ms
Input-Output relationship
% of maximum response
Stimulus intensity (mA)
b
WT
KO
1
2
1
2
0.5 mV
20 ms
PPR
c
WT
KO
Pre-HFS
Post-HFS
Pre-HFS
Post-HFS
0.5 mV
20 ms
HFS
fEPSP slope (% change)
fEPSP slope (% change last 10 minutes)
**

Fig.16 Arhgap22 loss of function causes LTP impairment (a) Representative traces of fEPSPs recorded from Arhgap22 WT and KO mice hippocampal CA1 and quantification of the input/output relationship. (b) Representative traces and quantification of paired pulse ratio experiments showing no differences in the glutammate release probability between genotypes. Error bars indicate ± s.e.m. (c) fEPSPs recordings and slope quantification show impairment in LTP induction at Schaffer's collaterals-CA1 synapses in Arhgap22 KO mice compared to WT. (unpaired t-test, **P < 0.01). Error bars indicate ± s.e.m.

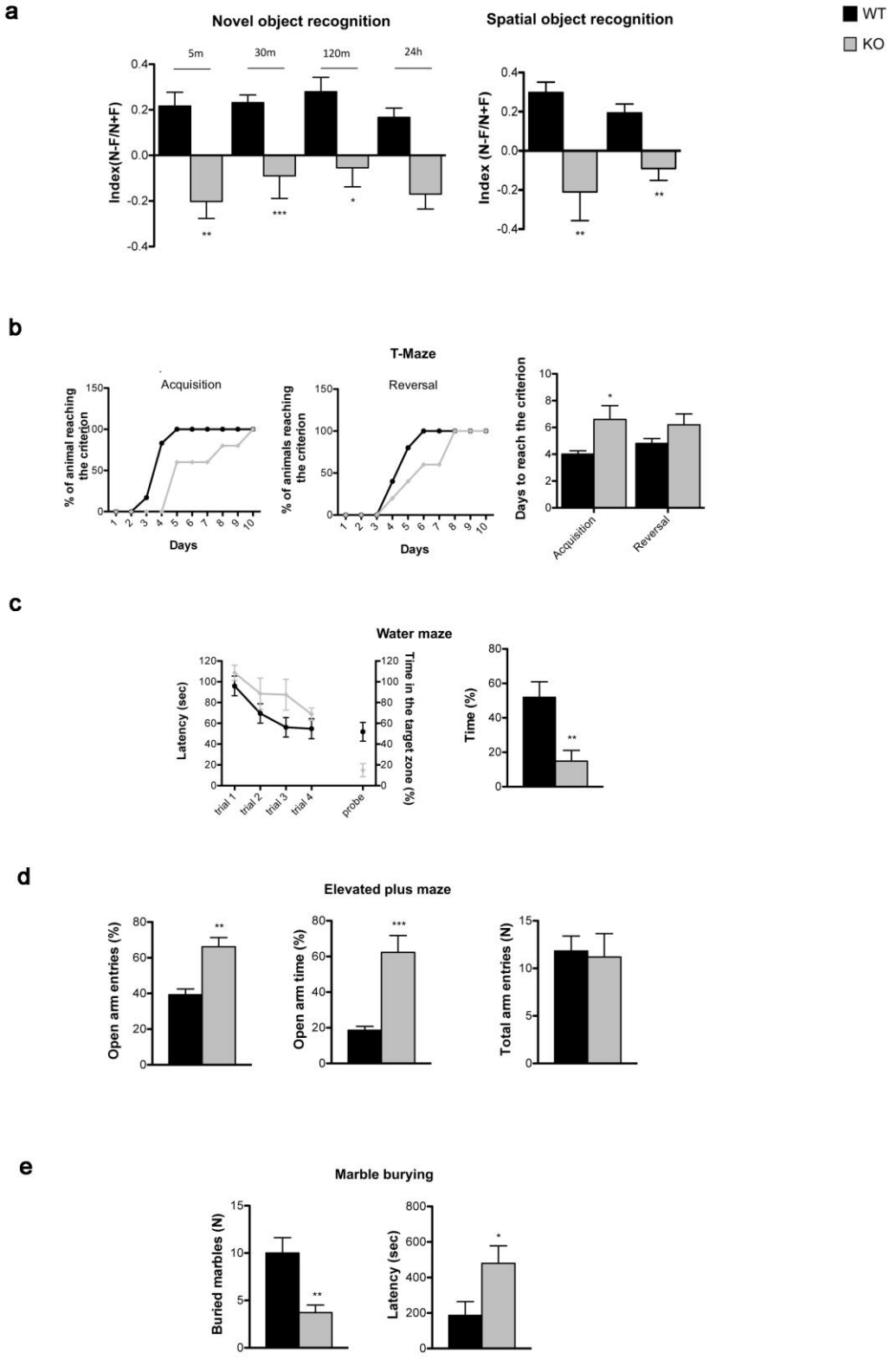

a
Novel object recognition
Spatial object recognition
WT
KO
5m
30m
120m
24h
Index(N-F/N+F)
Index (N-F/N+F)
b
T-Maze
Acquisition
Reversal
% of animal reaching the criterion
% of animals reaching the criterion
Days
Days to reach the criterion
Acquisition
Reversal
c
Water maze
Latency (sec)
Time in the target zone (%)
trial 1
trial 2
trial 3
trial 4
probe
Time (%)
d
Elevated plus maze
Open arm entries (%)
Open arm time (%)
Total arm entries (N)
e
Marble burying
Buried marbles (N)
Latency (sec)

Fig.17 RhoGAP2 mice present learning/memory deficiency and reduced anxiety-like behaviours. (a) Arhgap22 KO mice presente altered capability for both episodic (novel object) and spatial memory (spatial object recognition). (Two way ANOVA,Bonferroni test, * P<0.05, **P < 0.01; *** P < 0.001). Error bars indicate ± s.e.m. (b) Arhgap22 KO mice present a significant impairment during T-Maze acquisition phase (Student t-test, *P <0.05) but non in reversal phase. Error bars indicate ±s.e.m. (c) Arhgap22 KO mice spent more time trying to reach the target zone during water maze test (Student t-test, ** P < 0.01). Error bars indicate ± s.e.m. Anxiety-like behaviour is evaluated in Arhgap22 WT mice compared to KO (d,e). (e) Time spent in the open arm and number of entries in the open arm indicate that KO mice are less anxious than WT mice (Student t-test, **P < 0.01; *** P < 0.001). Error bars indicate ± s.e.m. (e) Marble burying test presents reduced number of marbles buried and an increase latency to the first burial (unpaired t-test, *P < 0.05; ** P < 0.01). Error bars indicate ± s.e.m.

6. Discussion

In this work, we demonstrated for the first time the effects of Arhgap22 loss in mouse brain. We proposed that Arhgap22 absence induced, at least in hippocampus, the hyper-activation of Rac1 and its downstream effectors. The result of this hyper-activation was an altered regulation of actin cytoskeleton and consequently the increase in dendritic spines density. In parallel to morphological modifications, Arhgap22 deficient synapses presented reduced levels of GLUA1 and GLUA2/3 subunits of AMPA receptors that are known to be mediators of excitatory signaling and synaptic plasticity in hippocampus. Moreover, synapses defects induced functional alterations: Arhgap22 KO mice presented an impairment in long term potentiation, the biological base of learning and memory, processes that are partially destroyed in Arhgap22 mutant mice.

6.1 Arhgap22 disruption induced altered dendritic spines properties

Since little is known about Arhgap22, we firstly investigated Arhgap22 expression in mouse. We confirmed the distribution of its transcript in different mouse tissues including brain as firstly described in 2004 (Aitsebaomo et al., 2004). Focusing on brain, evidences of Arhgap22 expression in cortex, hippocampus and cerebellum recapitulated the findings obtained *in vitro* by Valnegri and colleagues (Valnegri et al., 2010). We observed a specific spatial and temporal distribution of Arhgap22 in mouse brain: its transcript was expressed at higher level in cortex and hippocampus compared to cerebellum, particularly during the peak of synaptogenesis and throughout adult life. This particular expression pattern is common to some Rac1 GEF and GAP proteins (Ma et al., 2003; Tcherkezian and Lamarche-Vane, 2007) while others are more expressed during embryonic life. This suggest that they could solve different functions during different stages of neuronal development.

The fact that Arhgap22 was hughly expressed in the last steps of neuronal development could indicate that it may have a more predominant role in the maturation and maintenance of dendritic spines rather than in their formation. Therefore, we hypothesized that its silencing could cause defects in the number of dendritic spines as well as in their morphology. Results from literature demonstrated that a tightly regulatory of spines is necessary for the correct formation of functional excitatory synapses and alterations in both morphology and spine density are associated with different pathological phenotypes including intellectual disability (Purpura et al., 1974).

Since previous works demonstrated that Arhgap22 silencing leads to the activation of Rac1, we evaluated the levels of Rac1-GTP and its effectors that are known to mediate actin polymerization in dendritic spines. As expected, Arhgap22 absence led to the over-activation of Rac1 and downstream pathway also *in vivo*. Thus, we analyzed dendritic spines density which is one of the parameters that is mostly affected in neurons expressing constitutively active forms of Rac1 both *in vitro* and *in vivo.* As expected, Arhgap22 KO mice presented an increase in dendritic spines density in hippocampus which is coherent with the phenotype of neurons which present elevated Rac1-GTP (Luo et al., 1996). Moreover, the same effects on dendritic spine density were observed also in studies focused on animal models knocked out for Rac1 GAPs (Um et al., 2014).

What emerged from both *in vitro* and *in vivo* studies was that the hyper-activation of Rac1 had profound effects also on the morphology of spines even thhough with different results. In fact, while mouse models with unbalanced Rac1 activation mostly presented an increase in mature spines and dysmorphic spines, *in vitro* studies suggested that enhanced levels of Rac1-GTP promotes filopodia-like protrusion. These different results could be explained by transgene expression level and by relative timing of transfection compared to neuronal differentiation. Additionally, in complete KO animals, neurons probably counterbalanced the constant high level of Rac1 with compensatory mechanisms, thus leading to a reduced filopodial responsiveness to stimulation. With regard to Rac1-GAPs, the effect of their depletion in dendritic spine density and morphology is likely to be

dependent on the temporal window of expression and on the set of functions specific to each protein.

It is accepted that the maturation of dendritic spines is not exclusively dependent on the regulation of the actinic cytoskeleton but also on synaptic accumulation of specific receptors. N-methyl-D-aspartate receptor subunit 2B (NR2B)/ N-methyl-D-aspartate receptor subunit 2A (NR2A) developmental switch is the process that promotes the maturation of excitatory synapses through the insertion of AMPA receptors in the plasmatic membrane (Hall et al., 2007). It has been demonstrated that the Rac1 promotes the clustering of AMPA receptors at synapses inducing the maturation of dendritic spines (Wiens et al.,2005); (Um et al., 2014). Unexpectedly, SRGAP2A, a Rac1-GAP protein, favored the maturation of dendritic spines and synaptic accumulation of AMPA receptors through the interaction iwith different members of PSD (Fossati et al., 2016). The authors suggested a new model in which, on the one hand, the protein regulated the density of dendritic spines through its GAP activity, while on the other hand it promoted the maturation of spines through interaction with synaptic proteins.

The analysis of Arhgap22 mutant mice demonstrated that KO mice have an increased density of dendritic spines with a concomitant reduction in the expression levels of AMPA receptor subunits 1 and 2/3 (GLUA1, GLUA2/3) at synapses. These results are in line with those reported by Fossati and colleagues and suggest that in our model dendritic spines, even if increased in number, are more immature. All things considered, we could speculate that Arhgap22 normally mediates the insertion of AMPA receptors in the post-synaptic membrane although we still do not know which is the precise mechanism. We hypothesized three possible options: (i) Arhgap22 binds to PSD scaffolding protein and induces the clustering of AMPA similarly to SRGAP2A (ii) Arhgap22 is translocated to the nucleus in response to stimuli (as demonstrated by Aitsebaomo et al., 2004) and mediates the transcription of AMPA receptors or other protein involved in receptor trafficking, (iii) Arhgap22 regulates the trafficking of AMPA receptors through its interaction with the endosomal compartments (Mori et al., 2014). To identify which is the mechanism in charge of this effects, we will perform different new experiments as AMPARs bis(sulfosuccinimidyl)suberate (BS3) crosslinking on mice hippocampi to evaluate

if Arhgap22 depletion induces defects in the total level of protein or in their targeting to the membrane. Moreover, we are planning an antibody feeding assay (Rizzolio & Tamagnone, 2017) on mouse cultured neurons (WT and KO) to evaluate endocytosis and intracellular trafficking of individual membrane receptors in living neurons.

For what concerns the morphology of spines, we could not define the degree of maturation of dendritic spines in KO animals through the Golgi staining experiment we performed. Indeed, this technique have limitations in terms of image resolution. Our aim is to analyze in detail the morphology of dendritic spines in Arhgap22 KO mice through staining of brains with fluorescent neurotracers and subsequent confocal imaging acquisition. Moreover, electron microscopy will allow us to evaluate the ultrastructure of excitatory synapses in our animal model.

To summarize this first part, we demonstrated that Arhgap22 disruption had serious consequences on dendritic spines density and levels of AMPA receptors at synaptic level caused by hyper-activated Rac1 signaling. More experiments are requested to confirm our theory concerning the promotion of maturation of synaptic spines by Arhgap22.

6.2 Arhgap22 deficient mice presented functional defects associated with learning and memory

To address if Arhgap22 deletion functionally affected excitatory synapses, we decided to study electrophysiological and behavioral properties of Arhgap22 KO animals. As previously described, biochemical and morphological analyses on mutant mice revealed a consistent increase in dendritic spine density with a reduced level of AMPA receptors at synaptic level.

We initially evaluated the synaptic activity through Input/Output ratio experiments. Arhgap22 KO mice presented no alterations in basal transmission compared to their littermates. Additionally, the glutamate release probability (PPR) was evaluated and no differences were found between genotypes, suggesting no alteration in the pre-synaptic compartments of Arhgap22 KO mice.

When we evaluated long-term potentiation, a biological mechanism that strictly requires the presence and activation of AMPA receptors in response to stimuli (Malenka and Nicoll,1999), we found that Arhga22 KO mice presented a strong impairment in generating and maintaining LTP. This result is coherent with the reduction of GLUA1 and GLUA2/3 subunits observed in Arhgap22 deficient animals.

Reduction in AMPARs should normally affects the functionality of synapses since excitatory transmission in hippocampal neurons is mainly regulated by these receptors (Hjelmstad et al., 1999). It was quite surprising that in our animal model decrease in AMPA receptors at synapses had an effect on LTP but not on basal transmission. However, similar situations have been already described in literature (Wang et al., 2011). Our hypothesis is that in KO mice basal excitatory transmission is maintained because, despite the presence of weaker synapses, the increase in the total number of spines could compensate the reduction of GLUA1 and GLUA2/3 subunits at synapse. On the contrary, when a strong stimulus is provided, the amount of AMPA receptors at single synapse is not sufficient to induce the activation of NMDA receptors. Therefore, we hypothesized that, in Arhgap22 KO mice, AMPARs trafficking impairment is present making neurons not able to sustain the LTP response.

LTP is the cellular mechanism that underlies the processes of learning and memory. Our data showed that Arhgap22 KO mice present deficits in novel and spatial object recognition, T-Maze test and in the probe phase of Water maze test, common paradigms used to evaluate hippocampus-dependent learning and memory. These results were totally coherent with the electrophysiological data on LTP. Moreover, they have been already described in other animal models with deletion of Rac1 regulator proteins (Oh et al., 2010);(Bai et al., 2015) and were comparable to those found when Rac1 activation is inhibited. Examples are mutation in genes such as αPix/ARHGEF6 and PAK3 whose disruption is associated with intellectual disability (Nadif Kasri & Van Aelst, 2008; Newey, Velamoor, Govek, & Van Aelst, 2005). All these data confirmed that the equilibrium in the activity of Rac1 is fundamental for

the correct establishment of synaptic plasticity and cognitive functions. This perspective may also shed light on how defective regulation of Rac1 leads to cognitive dysfunction, an important question in the field of intellectual disability.

6.3 Future perspectives

Concerning the future, our aim is to complete the characterization of mice phenotype. In particular, the first goal is to clarify the morphology of dendritic spines and to demonstrate how Arhgap22 disruption could impair the levels of AMPA receptors at the synapses. Moreover, we are going to study in detail the electrophysiological properties of Arhgap22 deficient neurons to create a bridge between the biochemical/morphological and functional evidences.

After the conclusion of this part, we are planning to perform pharmacological rescue experiments. At the moment, several molecules have been demonstrated to specifically target Rac1, inhibiting its activity. One of the most studied compound is NSC23766 [*N*6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydro-chloride], a small, highly soluble and membrane permeable molecule. Both *in vitro* and *in vivo* studies demonstrated the efficacy of this compound in regulating the levels of Rac1 activation in different cellular population including neurons (Bongmba et al., 2011). Our idea is that, bringing active Rac1 levels back to normal, we will be able to rescue at least partially the phenotype of Arhgap22 KO mice. Moreover, these experiments will help us to better understand which mouse defects are closely dependent on Rac1 signaling and which are dependent on specific Arhgap22 functions in mouse brain.

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