LPA SIGNALLING DURING AXON GROWTH: RULE FOR PRG-1/ RAS GRF-2 INTERACTION

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Contents

Abbreviations

The most recurrent abbreviations are alphabetically listed below. However, they are fully written with the abbreviation in brackets whenever thought to be needed.

-/-	knock out
aa	amino acid
ATX	Autotaxin
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CA 1-3	*Cornu Ammonis 1-3*
Da	Dalton
DAG	Diacylglycerol
dg	*dentate gyrus*
DIV	Day *in vitro*
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphatasa
E	Embryonal
ECL	Enhanced Chemiluminescence
ERK	Extracellular Signal Regulated Kinase
FEM	*Forschungseinrichtung für Experimentelle Medizin* (Animal facilities)
Fig.	Figure
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GPCR	G Protein Coupled Receptor
GTP	Guanosine Triphosphate
h.	hours
h	human
HA	Hemagglutinin
HPRT	Hypoxanthine Phosphoribosyl Transferase
IB	Immuno Blot
IP	Immunoprecipitation
LB	Lysis Buffer
LPA	Lysophosphatidic Acid
LPC	Lysophophatidylcholine
LPL	Lysophospholipid
LPP	Lipid Phosphate Phosphatase
LTD	Long Term Depression

LTP	Long Term Potentiation
min.	minutes
MACS®	Magnetic Activated Cell Sorting
MAG	Monoacylglycerol
MAPK	Mitogen Activated Protein Kinase
mRNA	messenger Ribonucleic Acid
NGS	Normal Goat Serum
ns	no significant
nt	nucleotide
o.n.	over night
p-	phospho-
P	Postnatal
PA	Phosphatidic Acid
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide-3 Kinase
PIns(4,5)P2	Phosphatidylinositol 4,5 bisphosphate
PIns(3,4,5)P3	Phosphatidylinositol 3,4,5 trisphosphate
PLC	Phospholipase C
p.n.	primary neurons
PRG	Plasticity Related Gene
q RT PCR	quantitative Real Time PCR
RNA	Ribonucleic Acid
rpm	revolutions per minute
r.t.	room temperature
sec.	seconds
S1P	Sphingosine 1 Phosphate
SN	Supernatant
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Suppl.	Supplementary
Tab.	Table
TRP	Thrombin Receptor Peptide
w.t.	wild type
WB	Western Blot

1 Abstract

1.1 English Abstract

Plasticity Related Gene-1 (PRG-1) is a brain-specific membrane protein and the first identified member of the Plasticity Related Gene family (PRG-1-5). PRGs belong to the Lipid Phosphate Phosphatase (LPP) superfamily whose members have an extracellular ectoenzymatic activity known to dephosphorylate Lysophosphatidic Acid (LPA) into its inactive monomers (Monoacylglycerol (MAG) and phosphate). PRG-1 is also known to act specifically at the excitatory synapse on hippocampal neurons and has been recently proposed as an important player in the modulatory control of hippocampal excitability by means of non-enzymatic control of extracellular LPA concentration. The non-enzymatic control occurs at the synaptic level but its exact process is not clarified yet.

The interaction between PRG-1 and the Ras-specific exchange factor: Ras Guanosine Release Factor-2 (Ras GRF-2) has been identified. PRG-1/Ras GRF-2 interaction takes place not only after overexpression of both proteins in mammalian cells, but also in cortical primary neuronal cultures when analysing endogenous PRG-1/Ras GRF-2 interaction. Furthermore, the endogenous PRG-1/Ras GRF-2 interaction is disrupted after extracellular LPA application and not after the application of the Thrombin activator: Thrombin Receptors Peptide (TRP) known to have a similar effect on cell fate but through distinct receptors. It has also been found that PRG-1 protein-protein interaction controls the intracellular levels of the active protooncogene N-Ras and indeed the N-Ras activation is regulated depending on the extracellular concentration of LPA. To assess the intracellular signalling cascade, phosphorylation of Mitogen Activated Protein Kinase (MAPK) has been analysed; enhanced MEK/ERK activation but no p38 phosphorylation could be detected in cell culture after LPA application. And finally, significant axon elongation could be demonstrated after increasing the protein level of active N-Ras in primary neurons.

Presented results will conclude showing PRG-1 as a Ras-cascade controller, during brain development mainly through Ras GRF-2 and depending on extracellular LPA presence. In the final schematic representation will be showed how extracellular LPA controls, in turn, Ras GRF-2 resulting in a decreased amount of active N-Ras protein known to induce axon growth during neuronal differentiation as well as to be deregulated in tumors.

Newly presented data are the first results on PRG-1 function after its discovery on 2003 and the obtention of the PRG-$1^{-/-}$ animals on 2009. They represent a significant step further towards the understanding of the protein, its function during embryonal brain development but also its role in pathologies such as brain injury or cancer.

1.2 German Abstract

Plasticity Related Gene-1 weist eine weitgehend gehirnspezifische Expression auf. Das PRG-1 Protein wurde als erstes Mitglied der Plasticity Related Genes Familie (PRG-1-5) identifiziert. Die PRG-Familie gehört zu den Lipid Phosphatase/Phosphotransferasen (LPP), die an der Regulation des LPA-Spiegels beteiligt sind. LPP Familienmitglieder haben eine ektoenzymatische Aktivität, die LPA zu zwei inaktiven Monomere, Monoacylglicerol (MAG) und Phosphat (P), degradiert. PRG-1 ist ein Protein, das die hippocampale Erregbarkeit über die Regulation des LPA-Spiegels moduliert. Es wird vermutet, dass PRG-1 einen nicht-enzymatischen Einfluss auf Synapsen hat, welcher abhängig von der extrazellulären LPA-Konzentration ist.

Im Rahmen dieser Arbeit wurde die Interaktion zwischen PRG-1 und Ras-specific Exchange Factor: Ras Guanosine Release Factor-2 (Ras GRF-2) untersucht. Diese Wechselbeziehung wurde sowohl mittels Überexpression beider Proteine in Säugerzellen, als auch durch eine endogene Expression in kortikalen Neuronen analysiert. Darüber hinaus konnte hierbei LPA als spezifischer extrinsischer Faktor, der die Interaktion unterbricht, nachgewiesen werden. Anders als Thrombin, welches einen vergleichbaren Effekt auf Zellen hat wie LPA, jedoch nur LPA die Interaktion unterbindet und Thrombin keinen Einfluss auf diese Interaktion nimmt. Des Weiteren wurde bewiesen, dass PRG-1 Protein-Protein Interaktionen durch extrazellulläres LPA reguliert werden und gleichzeitig die Expression des intrazellulär onkogen aktiven N-Ras beeinflussen. Um zu kontrollieren ob PRG-1 als Membranprotein auf extrinsische Faktoren, wie zum Beispiel LPA, reagiert und somit eine intrazelluläre Signalkaskade auslöst, wurde die Phosphorylierung der Mitogen Aktivated Protein Kinase (MAPK) untersucht. Dabei konnte gezeigt werden, dass eine MEK/ERK Aktivierung, in Gegenzätz zur p38 Phosphorylierung, nach LPA Behandlung vorhanden ist. Detaillierte morphologische Analysen an N-Ras überexprimierenden primären Neuronen zeigten eine Verlängerung der Axone.

Die im Verlauf dieser Arbeit generierten Daten zeigen PRG-1 als Regulator der Ras-Kaskade in Abhängigkeit von extrazellulärem LPA und Ras GRF-2. Aufgrund der Zusammenfassung ergibt sich die Hypothese, dass extrazelluläres LPA indirekt Ras GRF-2 beeinflusst. Dieses führt zu einer Abnahme von aktivem N-Ras, welches das Auswachsen der Axone während der neuronalen Differenzierung begünstigt und die Entwicklung von Tumoren negativ beeinflussen kann.

Diese zum ersten Mal präsentierten Daten sind die ersten Resultate zur Funktion von PRG-1 seit seiner Entdeckung im Jahre 2003 und der Herstellung von PRG-$1^{-/-}$ im Jahre 2009. Die Daten liefern einen Fortschritt im Verständnis der Funktion von PRG-1 während der embryonalen Gehirnentwicklung und dessen Rolle bei pathologischen Veränderungen, wie zum Beispel Gehirnverletzungen oder Krebs.

2 Introduction

Neuroscience is currently one of the most rapidly growing areas of science. Towards the end of the 20th century, the study of the brain moved from a peripheral position with both the biological and psychological sciences to become an interdisciplinary field that is now central within each discipline. Today, neuroscience spans a wide range of research and endeavours from molecular biology of nerve cells to biological basis of normal and disordered behaviour, emotion, and cognition [1].

One mainstone of neuroscience is the study of the neuronal development. This field draws on neuroscience and developmental biology to provide insight into the cellular and molecular mechanisms by which complex nervous systems develop [2]. It comprises the processes which generates and continuously shapes and reshapes the nervous system from early stages of embryogenesis to the final years of life.

2.1 Neural development

The brain emerges during embryonic development from a sheet of cells called the neural plate that invaginates to form the neural tube, an early embryonic structure [1]. The most anterior part of the neural tube is called the telencephalon, which expands rapidly due to cell proliferation, and gives rise to the brain. Gradually some of the cells stop dividing and differentiate into the main cellular components of the brain: neurons and glial cells [3]. The newly generated neurons migrate to different parts of the developing brain to self-organize into different brain structures. In Fig. 1, there is a schema in which selected types of neurons in three CNS structures are shown as an example of variety of neuron type and morphology depending on their location and the final brain structure. Once the neurons have reached their regional positions, they extend axons and dendrites allowing them to communicate with other neurons via synapses. Synaptic communication between neurons leads to the establishment of functional neural circuits that mediate sensory and motor processing, and underlie behavior [1].

Some landmarks of neural development include the birth and differentiation of neurons from stem cell precursors, the migration of immature neurons from their birthplaces in the embryo to their final positions, outgrowth of axons and dendrites from neurons (Fig. 2), guidance of the motile growth cone through the embryo towards postsynaptic partners, the generation of synapses between these axons and their postsynaptic partners, and finally the lifelong changes in synapses, which are thought to underlie learning and memory.

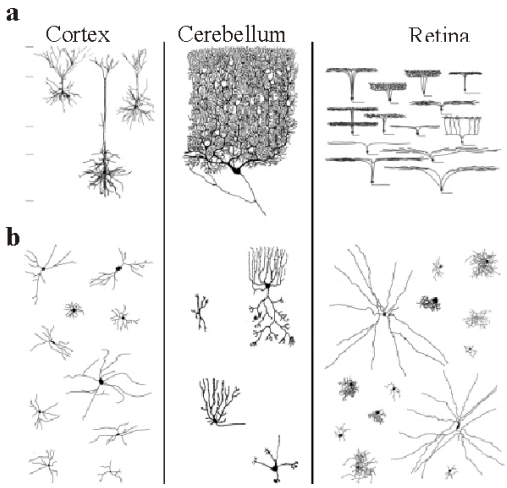


Figure 1: Schematic representation of selected types of neuron in three different CNS structures. **a)** Projection neurons **b)** intrinsic neurons. The projection neurons of the cortex are pyramidal cells, and the intrinsic cells are simply called interneurons. The projection neuron of the cerebellum is the Purkinje cell, and the intrinsic cells are the granule, basket, stellate and Golgi cells.The projection neurons of the retina are the retinal ganglion cells, and the intrinsic cells are a diverse collection of bipolar and amacrine cells. The drawings are simplified, with the goal of conveying the most important features of each cell type. Modified from Masland et al. 2004.

Among those landmarks, one of the most important points during development is the specific guidance of axons to their targets [4]. The question about how axons succeed in navigating through the embryonic environment to targets, which in some cases can be many centimeters away, has been largely studied [3]. Axons appear to be guided along their appropriate trajectories by their responses to selectively distributed molecular signals within the developing embryo. The mature conformation of the central nervous system is accomplished through the precise and also coordinated behaviour of neuronal cells [3].

Studies over the past three decades have established primary neuronal culture as *in vitro* system for brain development (Fig. 2).

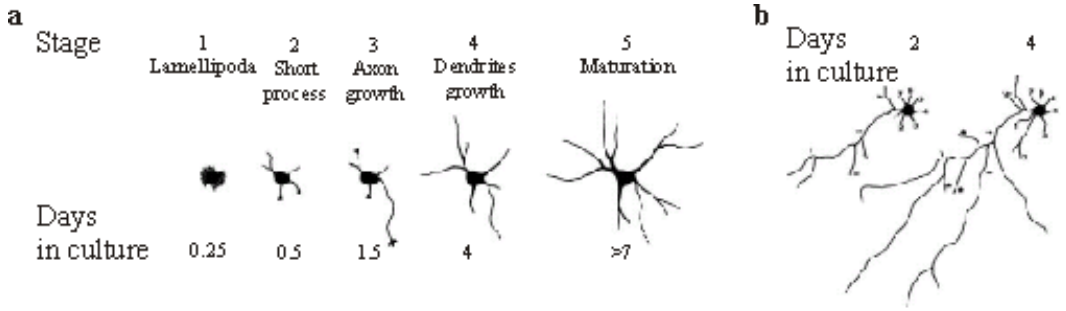


Figure 2: Schematic representation of a primary neuron development in culture. **a)** Neuron development in neuron stages and days in culture. **b)** Branching development in a neuron in culture. Modified from Dotti et al. 1988.

Those methods have led to the view that axon guidance involves the coordinate action of four types of cues: short range, long range, positive or negative cues. These guidance cues are signalling molecules that influence the cell biological mechanisms by which growth cones extend, turn and retract [2]. In recent years, the roles of an interesting new class of biologically active molecules, lysophospholipids [5, 6, 7], has begun to be unveiled.

2.2 Lysophospholipids

Lysophospholipids (LPLs) are membrane-derived signalling molecules produced by phospholipases that exhibit diverse biological activities and, as a class, represent a major constituent of the brain [8]. LPLs and their receptors have been found in a wide range of tissues and cell types indicating their importance in many physiological processes including reproduction [9], vascular development [10] and nervous system [11].

They have recently become a focus of attention since it was discovered that in addition to their role in phospholipid metabolism they function as second messengers, exhibiting a broad range of biological activities in their own right [12]. Phospholipids interfere with intracellular signal transduction either by their intra- or extracellular action coupling to nuclear receptors or membrane proteins like G-Protein-Coupled Receptors (GPCRs) [6]. For example, it is now accepted that bioactive lipids increased wound healing, tumor growth, metastasis and angiogenesis [13]. And deregulation of phospholipid metabolism is involved in the progression of glioblastoma and various other neurological, neurodegenerative and psychiatric disorders such as Alzheimer's disease, multiple sclerosis, ischaemia, epilepsy, prion diseases or traumatic brain injury [14, 15].

2.2.1 Lysophospholipids in the brain development

In the last few years, brain-specific lipid functions gained relevance in neuroscience [16]. Lipidomics and, in particular, neural lipidomics are opening a new area of interdisciplinary research connecting neuroscience, medicine and biochemistry with the unexpected lipid/protein interaction. Brain lipidomics became an important field mainly because high lipid concentrations are detectable in the mammalian brain, and lipid signalling in neurons is now thought to play an important role for the development and function of the Central Nervous System (CNS) [15, 17]. Moreover, the nervous system contains the largest diversity of lipid classes and lipid molecular species.

The diversified neuronal lipids play multiple specific roles in the nervous system other than their general functions. In fact, lipid signalling in the nervous system is apparently much complicated than a second messenger does in a cell as recently reviewed by Piomelli [16].

In brain lipidomics, the bioactive phosphates Lysophosphatidic Acid (LPA) and Sphingosine 1-Phosphate (S1P) receive special attention because of their neuronal functions [14]; they are not only essential structural compounds preserving the integrity and flexibility of cellular membranes, but they also act as signalling mediators in the vital cellular processes, such as proliferation, differentiation survival [16]. And they have also been linked to pathological situations such as cancer [18, 19].

- **Lysophosphatidic Acid**

Lysophosphatidic Acid (LPA) is a bioactive lipid present in biological fluids. In fact, LPA is the smallest and the structurally simplest bioactive phospholipid. There is LPA in serum in

a micromolar range and can be detected also in other body fluids like cerebrospinal fluid [20, 21]. LPA itself is not only known to stimulate tissue cell growth, survival, differentiation and motility [22] but also, to be produced by at least two distinct enzymatic mechanisms (Fig. 3).

One mechanism is the hydrolysis of Phosphatidic Acid (PA) by soluble Phospholipase A2 (sPLA2), which cleaves the fatty acyl chain at the sn-2 position, or hydrolysis by Phospholipase A1 (PLA1), which cleaves the fatty acyl chain at the sn-1 position of the glycerol backbone (see left part of Fig. 3). The other mechanism involves the hydrolysis of Lysophosphatidylcholine (LPC) by ATX, which liberates the hydrophilic headgroup (Choline) [23] (see right part of Fig. 3).

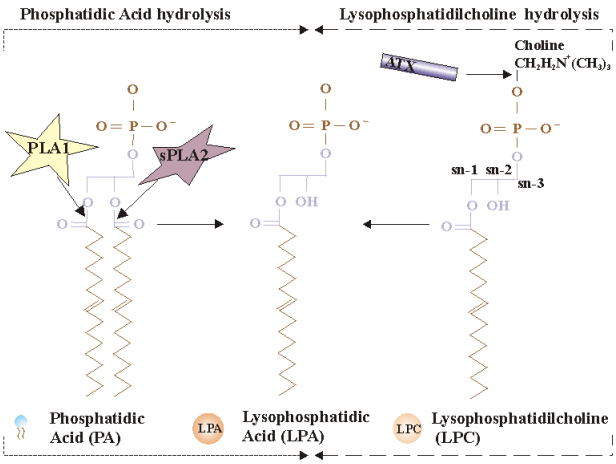


Figure 3: Lysophosphatidic acid production through enzymatic mechanisms. LPA can be produced by at least two distinct enzymatic mechanisms. On the left side, hydrolysis of phosphatidic acid (PA) by either soluble phospholipase A2 (sPLA2) cleaving the fatty acyl chain at the sn-2 position, or hydrolysis by phospholipase A1 (PLA1) cleaving the fatty acyl chain at the sn-1 position of the glycerol backbone. On the right side, hydrolysis of lysophosphatidylcholine (LPC) by ATX, which liberates the hydrophilic headgroup (choline).

LPA production by Autotaxin (ATX) [5, 24] is one of the most important mechanism. In this mechanism, see Fig. 4, LPA concentration is tight controlled and kept stable in a physiological range. To do so, the system has: on the one hand, the tight control of LPA biosynthesis by ATX which belongs to the ecto-nucleotide pyrophosphatase / phosphodiesterase family and catalyzes the conversion of LPC to LPA [5, 25, 26]. LPA is then produced upon release of Lysophophatidylcholine (LPC) from activated cells like platelets and neurons [27], injured corneal tissue, adipocytes, neurones and trough several biochemical mechanisms [25] On the other hand, there is the LPA catabolism modulated by Lipid Phosphate Phosphatases (LPPs) known to hydrolyse LPA to Monoacylglycerol (MAG) (Fig. 4) [28].

Studies have showed that LPA levels are increased during development as well as on brain pathological conditions, for example: cerebral ischaemia in response to injury, or following disruption of the Blood Brain Barrier (BBB) [7]. Other investigators have detected elevated LPA levels in serum and ascites under ovarian cancer and atherosclerotic lesion [22]. Extracellular levels of LPA are essential and tightly regulated during development. This is showed by the fact that ATX knock out ($ATX^{-/-}$) mutants exhibit failure of neuronal tube closure while extracellular LPA application rescued the severe compromised neurite outgrowth of the $ATX^{-/-}$ implants [29].

LPA was first found to activate the so called Ras / Mitogen Activated Protein Kinase (MAPK) pathway, which controls cell proliferation, differentiation and survival in response to numerous extracellular stimuli [30, 31]. The early mechanisms that mediates activation of this pathway in

cells stimulated with LPA are not completely defined yet [32]. However, LPA signalling has been proved to be involved during brain development in neuronal precursor cell proliferation and cortical layer formation [33]. Nowadays it is clear that the majority of documented responses for extracellular LPA are attributable to the activation of seven specific transmembrane domain G protein-coupled receptors (GPCRs) [18]. Those receptors were first called Endothelial cell Differentiation Gene (EDG 1-8) but afterwards renamed as LPA receptors 1-5 ($LPA_{1\text{-}5}$) (Fig. 4) [34].

In addition to LPA-induced fibroblast proliferation, platelet aggregation and tumor cell invasiveness [35], LPA signalling is thought to play a pivotal role in brain development and aging, as well as in regeneration of neural lesions, considering comparably high LPA concentrations in the CNS [36].

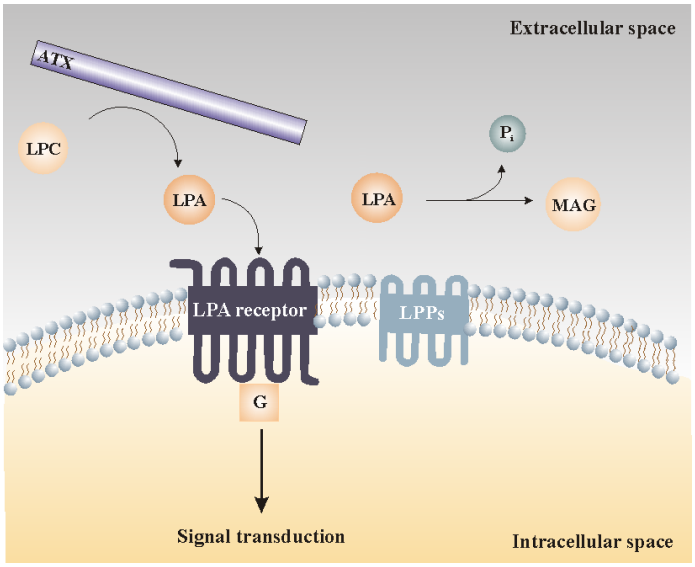


Figure 4: Lysophosphatidic acid metabolism. LPA biosynthesis is controlled by Autotaxin (ATX) converting extracellular Lysophophatidylcholine (LPC) into LPA, whereas the Lipid Phosphate Phosphatases (LPPs) dephosphorylate LPA to Monoacylglycerol (MAG) and thereby modulate the affinity to specific membrane receptors (LPA receptors) that couple intracellular G proteins (G) initiating several signal transduction pathways.

Similar to many of the inhibitory protein axon guidance cues, LPA and S1P have been showed to be inhibitory *in vitro* to neural cell lines, as well as some really young primary neurons in culture, often causing growth cone collapse and neurite retraction [37]. More information about the biological effects of LPA with their references, remarks and the cell type in which LPA effects were proved can be found in the appendix (Suppl. Tab. A).

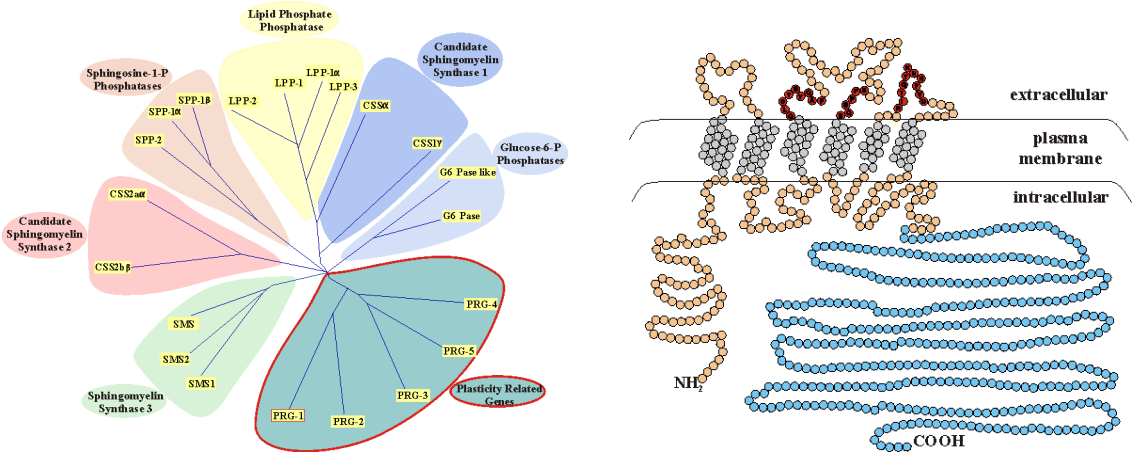
Taken together, lysophsopholipids are active cues considered as potential candidates for guiding axons during brain development [4] which activate LPA receptors [8] and are mainly degraded to inactive monomers by Lipid Phosphate Phosphatases [6, 28, 38].

2.2.2 Lipid Phosphate Phosphatases

Lipid Phosphate Phosphatases (LPPs) [28, 38] are a family of integral membrane glycoproteins, which are known to degrade lipid phosphates either on the cell surface or inside the cell, to regulate cell signalling under physiological as well as pathological conditions [6] and which include five groups of homologous proteins. These homologous families are: Sphingosine-1-P phosphates (S1PPs), Lipid phosphate phosphatases (LPPs), Candidate Sphingomyelin Synthase (CSS1s and CSS2s), Glucose-6-P Phosphatases (G6Pases), Plasticity Related Genes (PRGs) and Sphingomyelin Synthase 3 (SMSs) (Fig. 5a).

LPPs have increasingly attracted interest because of their involvement in modulating the spe-

cific signalling of bioactive lipid phosphates such as LPA in the context of CNS development [21, 39], specifically in cell migration, mitogenesis, neurite retraction [35], and also studies on lysophospholipids have frequently linked lysophospholipids to cancer [40]. This modulation is due to the fact that bioactive lipid phosphates are key factors in initiating receptor directed signalling cascades [18, 39] and, therefore, participate in diverse cellular processes such as cell proliferation, neurite retraction, cortical development and regulation of exocytosis [17, 33].



(a) Phylogenetic analysis of the human LPP superfamily.

(b) Predicted structure after hydrofobicity studies of human PRG-1, one member of the LPP superfamily.

Figure 5: Lipid Phosphate Phosphatases (LPP) superfamily.

Generally, LPP superfamily members have an ubiquitous expression pattern [41] but, interestingly, only one LPP subfamily has brain restricted expression [42]. This subfamily is the Plasticity Related Gene family (PRGs). PRGs were first published on 2003 and the family is compound by five high homologous members named PRG-1/5 (For an overview on the homology between all five human PRG protein sequences see Suppl. Fig. A).

- **Plasticity Related Gene-1**

Plasticity Related Gene-1 (PRG-1) was first discovered after differential screening of a lesioned hippocampus cDNA library [42]. So far, it is known that PRG-1 is vertebrate, brain and neuron-specific. Its expression is located in plasma membranes of outgrowing axons in which is supposed to act as biolipid activity modulator because PRG-1 facilitates axonal outgrowth during regenerative sprouting and, interestingly, attenuates axon collapse induced by LPA and facilitates outgrowth in the hippocampus [42]. PRG-1 gene is highly conserved in vertebrate species, including humans, and protein expression begins perinatally, is strongly expressed in hippocampal neurons and rapidly upregulated after brain lesion [42, 43].

PRG-1 hydrophobicity analysis predicts similar protein structure to those other members of the LPP family. In the concrete, *in silico* analysis of PRG-1 predicts six membrane-spanning regions and, according to the structural models of LPP orientation in the plasma membrane, N-

and C-terminal extensions positioned in the cytoplasmic side (Fig. 5b). PRG-1 has high homology to other members of the LPP family although the amino acid residues, which have been showed to be essential for ecto-enzyme activity in other LPP family proteins, are only partially conserved (Fig. 5b red-coloured balls). These domains enable the LPPs to dephosphorylate a variety of phosphorylated lipid substrates, such as Phosphatidic Acid (PA), Lysophospatidic Acid (LPA) or Sphingosine 1-Phosphate (S1P) [38]. Unlike the other members of the PRGs family, the second half of the PRG-1 protein consists of a long hydrophilic domain of around 400 amino acids (aa) (See blue tail in Fig. 5b).

Recently, the PRG-$1^{-/-}$ have been obtained [43]. First studies of the knock out animals show that PRG-1 gene deletion leads to epileptic seizures. PRG-1 deficiency results in pathological network synchronisation, probably due to a pathological increase of excitatory synaptic transmission. In fact, the excitatory transmission was significantly increased in CA1 pyramidal neurons obtained from PRG-$1^{-/-}$ whereas inhibitory events were not altered in those cell types [43].

A table with extended information about PRG-1 is added in the appendix (Suppl. Tab. B) including published and refereed data as well as data obtained during the development of the thesis.

2.3 Guanosine Exchange Factors

Guanine Exchange Factors: GEFs [44] are components of intracellular signalling networks; they compound a big family of proteins known to be Ras protein activators. The main function of this family of proteins is to regulate the exchange between inactive Ras (Ras-GDP) and the active Ras form: Ras-GTP (Fig. 7a).

There are hundreds of GEFs described (some examples are refereed in the Tab. 1), thus far exhibit varying degrees of specificity, some are able to activate multiple G proteins of different families and others only can activate one specific isoform [45] .

Table 1: Examples of Guanosine Exchange Factors

Name	Species	Reference
Dedicator Of Cytokinesis (DOCK)	from Drosophila to Mammalian	reviewed by Miymoto and Yamaucho 2010
GNOM	Arabidopsis	reviewed by Richer et al. 2010
MR-GEFs	Mammalian	Reghun et al. 2000
Ras GRFs	Mammalian	Guerrero et al. 1996
Son of Sevenless (SOS)	Mammalian	reviewed by Nimnual and Bar-Sagi 2010
Vav	Mammalian	reviewed by Bonnefoy-Bérard et al. 1996

Among all the GEFs, the Ras Guanosine Release Factors (Ras GRFs) are a mammalian GEFs subfamily composed of two members: Ras GRF-1 and Ras GRF-2.

2.3.1 Ras Guanosine Release Factors

Ras Guanosine Release Factors (Ras GRFs) are seen as signalling adapters and integrators because of their coupling to various signalling processes at the cell membrane to Ras and because their changes inside the cell [46].

Ras GRF-1 is expressed at high levels in the brain, and particularly in the hippocampus [47, 48], although traces can also be detected in other tissues [49]. Although Ras GRF-2 exhibits a more widespread expression pattern including spleen or lung [50], it is also expressed at high levels in the CNS in neuronal cells but not in glia.

Both proteins, Ras GRF-1 and Ras GRF-2, share many similarities in expression profile, physiology and structure, but some differences have been reported [51]. First, they have their high structural homology broken only towards the C-terminus of both proteins (Fig. 6 red box or see the homology between both protein mouse sequences in Suppl. Fig. B). Second, in hippocampal neurons Ras GRF-1 is known to contribute to Long Term Depression (LTD) meanwhile Ras GRF-2 contributes to Long Term Potentiation (LTP) [52]. Third, clear differences have been reported downstream within the signalling cascade in MAPK cascade were Ras GRF-1 has been clearly linked to p38 phosphorylation [53] meanwhile Ras GRF-2 downstream signal in the MAPK cascade is known to be produced through ERK and MEK phosphorylation [54]. These differences may imply heterogeneity of both isoforms in functions as for example in regulating synaptic plasticity [49, 51].

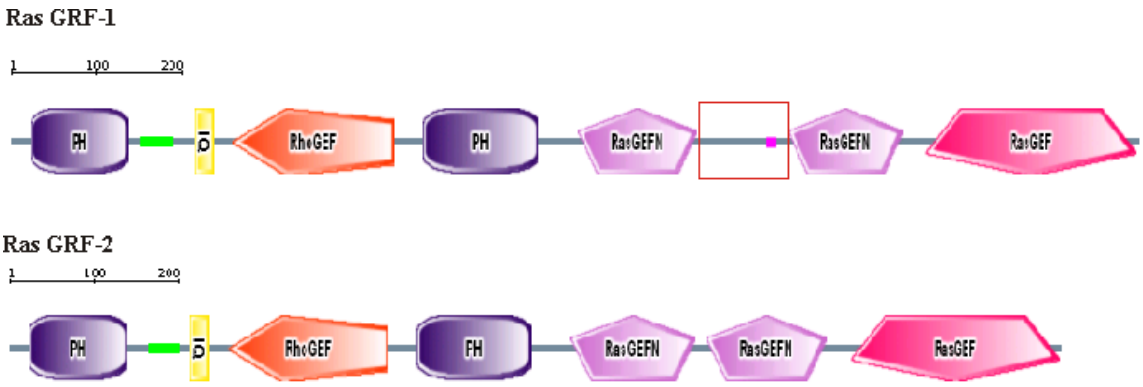


Figure 6: Ras GRFs domains *in silico.* *In silico* domain analysis of both Ras Guanosine Release Factors. The analysis has been done using the on line web page of SMART [55] and the main difference between both proteins is marked with a red box.

More information of Ras GRF-1 can be found in the appendix (see Suppl. Tab. C) and for Ras GRF-2 also supplementary information in a table format is added in the appendix (Suppl. Tab. D). Both tables are done following the same format and the same points to allow easy comprehension and comparison between both proteins and attempt to complete the overview of PRG-1 and GRFs. However, only Ras GRF-2 will be further studied during the project.

- **Ras Guanosine Release Factor-2**

Ras GRF-2 is a Guanosine Exchange Factor discovered when comparing *Saccharomices Cerevisiae* ras activators and mouse cDNA [47] and known to be expressed in juvenile rat brain [48] and in different human tissues as well as in some cell lines [49]. Its final cloning and characterisation was done in 1997 [50] and afterwards has been linked to MAPK [54] as well as to LTP [52]. Recently the Ras GRF-2 knock out (Ras GRF-$2^{-/-}$) have been obtained and showed viability, reached sexual maturity and showed similar fertility levels as wild type litter mates [56].

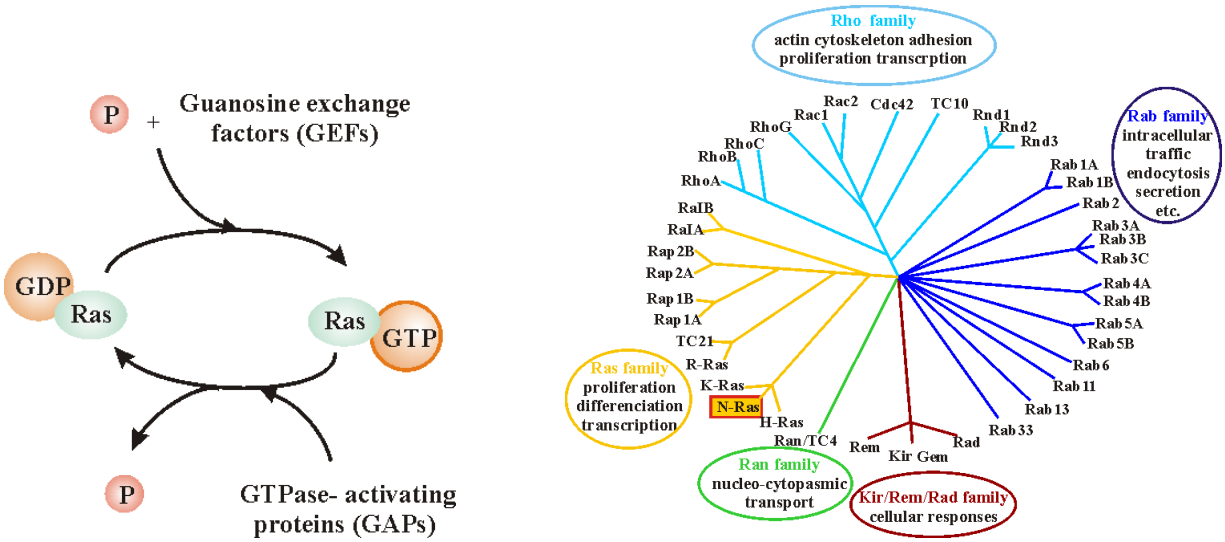
2.4 Small GTPase superfamily

Small GTPase proteins belong to a large family of monomeric guanine nucleotide-binding proteins [57]. They are often referred to as protooncogenes product because they were first discovered as transforming products of a group of related retroviruses [58]. They are all single-chain polypeptides of 189 amino acids (aa) in length that bound to the plasma membranes of the cell by posttranslational lipid attachments at their C-terminus [58].

Small GTPase proteins function as molecular switches whose activity depends on whether they are bound to Guanine Diphosphate (GDP) (“off” position) or Guanine Triphosphate (GTP) (“on” position) (Fig. 7a) [57]. Inactive GDP-bound proteins are activated by interaction with members of Guanine nucleotide Exchange Factors (GEFs), which catalyze the release of GDP. Lost GDP is then rapidly replaced by GTP. This exchange of GDP for GTP results in an allosteric change in two key regions of the GTPase that enables the binding of a variety of different effector proteins when Ras is in its GTP-bound configuration. Various GTPase Activating Proteins (GAPs) bind to small GTPases in their GTP-bound state and act as negative regulators, enhancing the low intrinsic GTPase activity of the proteins. Intrinsic GTPase results in hydrolysis of GTP to GDP and causes an allosteric change of the small GTPases to its inactive state (Fig. 7a) [59].

The small GTPase proteins are archetypes of a large superfamily. All members share sequence homology, the superfamily includes over 150 small GTPases and it falls into five distinct subfamilies called Ras, Rho, Rab, Ran and Kir/Rem/Rad (Fig. 7b) [59]. Within each subfamily, the homologies are rather strong. Indeed, beyond the more immediate subgroups of superfamily, all these proteins share some limited sequence homologies with short “fingerprint” sequences present in the bacterial elongation factors and also in the alpha-subunits of the heterotrimeric G proteins. The presence of these short motifs, appropriately distributed along the chain of the protein, can be taken as a fairly sure indication that it will be a GTPase [58].

Therefore, small GTPases are positioned as a central molecular switches in the coordinated regulation of multiple biological outcomes, they interact with a variety of downstream targets to exert its effects through cellular signalling pathways that ultimately influence the cell fate [60, 61].



(a) Small GTPase proteins activation general schema.

(b) Small GTPase superfamily phylogenetic tree including the main known functions of each subfamily.

Figure 7: Small GTPase proteins.

2.4.1 Ras family

The Ras subfamily (an abbreviation of RAt Sarcoma) is a protein subfamily of small GTPases that are involved in cellular signal transduction [61] (see yellow branches on Fig. 7b). Activation of Ras signalling causes cell growth, differentiation and survival [61, 62, 63]. The sequence of the all Ras family proteins are closely related (see the homology between mouse Ras protein sequences in Suppl. Fig. C). For example, the first 164 aa of human H-Ras and chicken Ras differ in only 2 positions, and the sequence of the first 80 aa of human N-Ras and *Drosophila* D-Ras are identical. All those close similarities are due to many conservative substitutions [58].

Because of their essential function in a variety of differentiation processes and signal transduction, including the regulation of cell growth, vesicle movement, cell survival, apoptosis, cytoskeleton, they have to be tight regulated and any change on their control leads to cancer [64]. In fact, Ras proteins are continuously associated with the membrane via C-terminal lipidation and additional interactions in both their inactive and active forms [65]. This association, as well as the targeting of specific Ras isoforms to plasma membrane microdomains and to intracellular organelles, have recently been implicated in Ras signalling and have shown oncogenic potential [66]. Moreover, this compartmentalisation is believed to underlie their biological differences [65].

Ras proteins are at the center of a network of interacting pathways, are activated and modulated directly and indirectly by several receptors and, in turn, have its influence on a large number of downstream processes [57]. Indeed, it is amazing that a protein as small as Ras can interact with so many other proteins and thus, contribute to intracellular changes [46]. Among functions directly linked to Ras there are the activation of the protein kinase Raf and the activation of phosphatidylinositol 3-kinase. They are the first members of the chain sequence of phosphorylating enzymes that lead to the activation of Extracellular Receptor Kinase (ERK) and they enhance to

the transcription of genes controlled through the Serum Response Element (SRE). Ras roles in vertebrates may vary between cell types or may depend on the stimulus and its timing [61]. Their roles may also depend on the number of receptors, the concentration of the ligand and the cytosolic concentration of the different adapters [57].

Inside the cell Ras proteins have to be tightly controlled as any small deregulation leads to tumor cells. In fact, the incidence of mutated Ras protein varies among different tumor types but, just as an example, 90 % of human pancreatic adenocarcinomas and 50 % of colon adenocarcinomas are associated with Ras mutations [67] and, in particular for N-Ras, N-Ras mutations are found in melanoma, hepatocellular carcinoma, hematological malignancies [46] and neuroblastoma [68].

Among the Ras family several members are comprised: Ha- [69], K- [70], and N-Ras [63, 71] (see yellow branches in Fig. 7b). N-Ras was discovered as a transforming gene product having sequence homology to the other Ras proteins present in a neuroblastoma cell line [72].

- **N-Ras**

N-Ras is one of the members of the Ras subfamily (see yellow branches and red square in Fig. 7b), is a protooncogene discovered in neuroblastoma cells [72] and known to play a key role in cellular regulation [63]. It is also known to induce neuronal differentiation in PC12 cells [71], to be up-regulated in several tumors [68] and to be rapidly activated in fibroblast after alpha-Thrombin [73].

Interestingly, N-Ras is known not to be essential for fertility, haematopoiesis neither for development. However, N-Ras$^{-/-}$ mice present defects in the development of cell subsets and their function [59] such as the upregulation of some immunity-related genes and the upregulation of several *loci* involved in apoptosis [74].

In its GTP bound form, N-Ras activates a variety of downstream target proteins and signalling pathways [71]. One activated signalling pathway is the Mitogen Activated Protein Kinase Cascade (MAPK cascade) of which the ERK pathway has been to date thoroughly characterised [75], although p38 phosphorylation pathway has also been reported [63].

2.5 Mitogen activated kinase

Cell signalling is part of a complex system of communication that governs basic cellular activities and coordinates cell actions [58]. In fact, the ability of cells to perceive and correctly respond to their microenvironment is the basis of development, tissue repair and immunity as well as normal tissue homeostasis [6] and any mistakes in the cellular information processing can lead to diseases such as autoimmunity, diabetes or cancer [67].

The events following the activation of mammalian Ras lead to the activation of a series of kinases culminating in kinase pathway. This was originally discovered as a serine/threonine phosphorylating activity present in the cytosol of EGF-treated cells and finally given the name Mitogen Activated Protein Kinase (MAPK). The Mitogen Activated Protein Kinase (MAPK) pathway is

a signal transduction pathway that couples intracellular responses to the binding of extracellular molecules such as growth factors to cell surface receptors. At the same time, it is one of the most studied high complex signal transduction pathways [31, 54, 61]. The MAPK protein is a protein kinase that can attach phosphate to target proteins, and, thereby, altering the gene transcription and, ultimately, the cell cycle progression [76, 77]. Phosphorylation at a given site on a given receptor may facilitate or inhibit transcription and given the number of potential sites and the variety of kinases and receptors, the picture is complicated because its complexity and variety of protein components [58, 78]. However, a basic diagram of the simplified pathway is found in Figure 8. Within the figure, only two major components of the pathway are detailed; one downstream signalling from Ras is the one in which Raf, ERK and MEK phosphorylation are involved. This pathway it is also called ERK pathway and this signalling has been proved to be downstream Ras GRF-2 [53, 54] and has been showed to be involved in activity-dependent dendrite differentiation [76, 79]. The other downstream signalling detailed is the one linked to Ras GRF-1 and known to involve different MEK proteins and p38 phosphorylation, which have been linked to induce transcription factors directly linked to apoptosis [63]. This pathway is usually named p38 pathway.

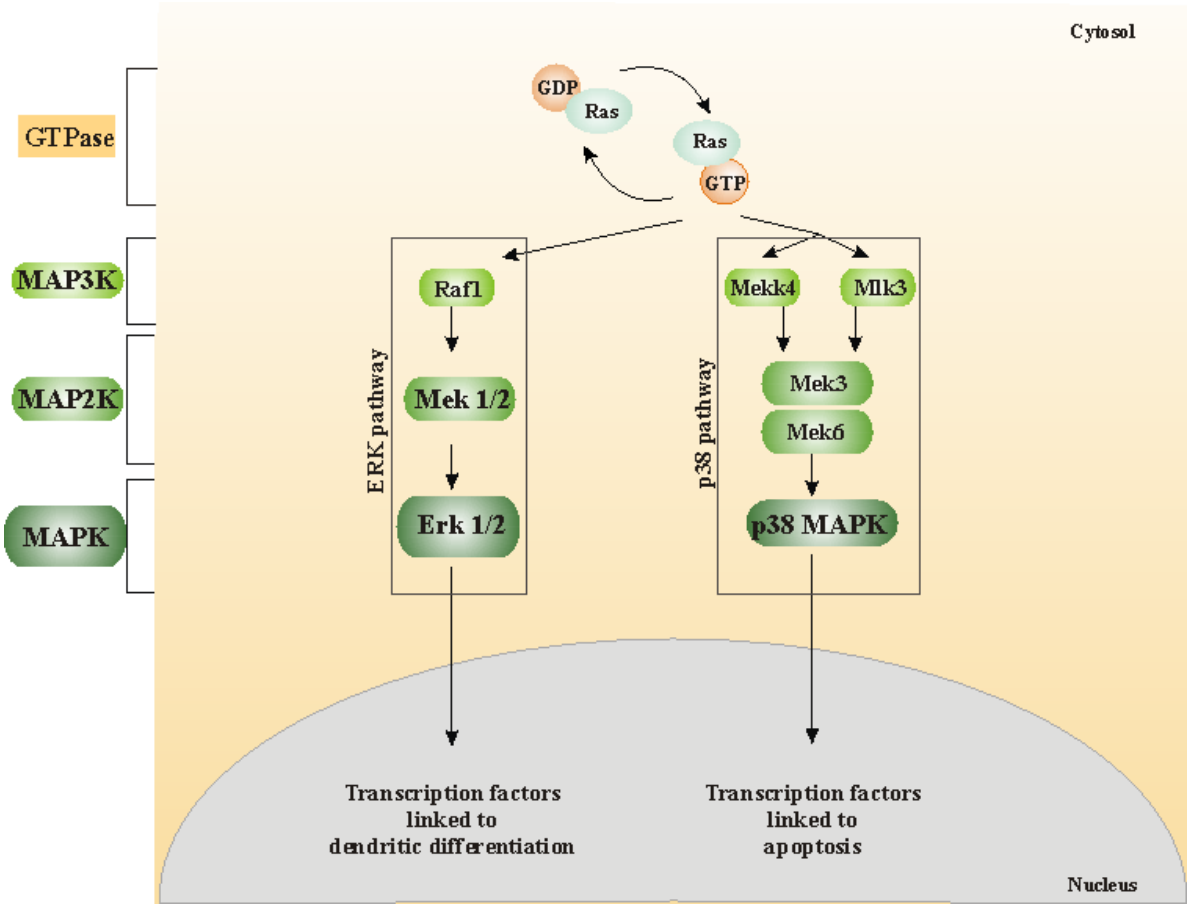


Figure 8: MAPK signalling. Simplified schematic representation for kinase phosphorylation downstream Ras proteins. ERK pathway bibliographically linked to Ras GRF-2 [53, 54] and to transcription factors linked to dendritic differentiation as well as p38 pathway whose link to Apoptotic transcription factors and Ras GRF-1 is also published [53].

Ras proteins, GEFs, MAPK and LPPs families have been separately discovered and studied [11, 30, 50, 61]. The present project links several families, connecting some of their protein members (e. g. PRG-1 [42], Ras GRF-2 [50] and N-Ras [71]) within brain development and in particular in axon growth. Moreover, the analysis of the link between those proteins is done in situations which the increase of LPA concentration present in biological fluids [37] is essential. In such situations, for example during brain development, after a brain lesion and in pathogenic conditions, the role and the interaction between PRG-1, Ras GRF-2 and N-Ras becomes essential.

3 Aims of the project

The major aim of this thesis was to investigate the relation between PRG-1 and a putative interaction partner Ras GRF-2, as well as the possible mechanisms controlling its interaction. Moreover, a part of the work was focused on the characterisation of and effects of the downstream signal of PRG-1, analysing N-Ras activation as well as MAPK phosphorylation and finally the possible phenotype of the whole system.

In the pursuit of this goal the following questions were addressed and experimental approaches adopted:

1. How are PRG-1 and Ras GRF-2 expressed?
   (a) mRNA expression during development, done using *in situ* hybridization in brain slices and q RT PCR of different mouse brain areas in different developmental time points
   (b) colocalisation studies at tissue and cellular level using immunofluorescence techniques
2. Is there an interaction between PRG-1 and Ras GRF-2?
   (a) interaction analysis using coimmunoprecipitation assays in HEK cells after overexpressing both proteins of interest
   (b) coimmunoprecipitation assays using cortical primary neurons culture in order to analyse the endogenous relation between PRG-1 and Ras GRF-2
3. How does the interaction work?
   (a) extracellular application of active cues (e.g. LPA) and analysis of the effect over the interaction using coimmunoprecipitation assays and comparing pretreated cells with the non treated ones
   (b) mapping the interaction within PRG-1 sequence first producing PRG-1 deletion constructs and then analysing their ability to perform the interaction
   (c) study of the affinity of PRG-1, Ras GRF-2 and PRG-1 deletion construct towards membrane lipids using lipid affinity membranes and purified recombinant proteins
4. Is PRG-1 changing Ras activity within the cell?
   (a) Ras activity analysis using precipitation assays after overexpressing PRG-1
   (b) endogenous Ras activity assay in primary neuron culture and comparing between pretreated and non treated primary neurons

5. Is there any change in the intracellular signalling downstream Ras proteins after pretreatment?

   (a) MEK / ERK phosphorylation studies using Western Blot

   (b) p38 phosphorylation studies using Western Blot

6. Is there any functional consequence?

   (a) morphological analysis of N-Ras transfected neurons

4 Materials and methods

4.1 Materials

4.1.1 Products purchased

Generally, plastic material and consumable supplies are purchased from Applied Biosystems, BD Biosciences, Corning, Eppendorf, Nunc and Sarstedt. However, a more detailed list of products, the company which supplied them and the method in which they are used is listed hereunder in a table format.

Table 2: Products purchased used for:

experiments with animals

Product	**Source**	**Methods in which it is used**
[alpha-^{35}S]-dATP	DuPont NEN	radioactive label
BioSpin6 Chromatography Columns	Bio-Rad	*in situ* hybridization
Bromphenolblau	Roth	tissue staining
desoxynucleotide transferase	Boehringer Mannheim	*in situ* hybridization
Dithiothreitol (DTT)	Roth	*in situ* hybridization
Entellan	Merck-Germany	finishing brain slices
Ethanol	Berkel Ahk	dehydration/hydration tissue
Formamide	Roth	*in situ* hybridization
Glutaraldehyde	Serva	perfusion
Ketamine	Actavis	anaesthetize
NaCl (0.9 %)	Gambro	perfusion
Paraformaldeyde (PFA)	Roth	tissue fixation
Oligonucleotide proves	Metabion	sonde for hybridization
Rompun 2 %	Bayer	anaesthetize
Sucrose (D+)	Roth	tissue fixation
Vetranquis 1 %	Sanofi	anaesthetize
Xylol	Roth	brain slices

molecular biology methods

Product	Source	Methods in which it is used
Agarose	Roth Lonza	for cloning and further processing for control digestions
Ampicilin	Roth	used for bacterial growing
dNTPs Mix	Bioline	PCR
Hyperladder I	Bioline	molecular weight marker for agarose's gel
Kanamycin	Roth	used for bacterial growing
Lambda DNA marker	Housemade	molecular weight marker for agarose's gel
PCR primers	Metabion	PCR
pcDNA 3.1 (+)	Invitrogen	cloning
pEGFP, pECFP, pEYFP	Invitrogen	cloning
RNase Away^{TM}	Roth	mRNA extraction
Tris	Roth	include on solutions
TRIzol®	Invitrogen	mRNA extraction
Tryptone	Roth	bacterial growing
XL10-Gold *E.coli*	Stratagene	transformation for plasmid amplification
XL-1 Blue *E.coli*	Stratagene	transformation for plasmid amplification
Yeast extract	Roth	bacterial growing

cellular biology methods

Product	Source	Methods in which it is used
Acetone	JT Baker	protein precipitation
Albumin Bovine Fraction V	Serva	immunohistochemistry
Amerscham ECL	GE Healthcare	detection reagent for IB

Product	**Source**	**Methods in which it is used**
Amerscham Hyperfilm^{TM} ECL	GE Healthcare	light sensitive films for IB
B-27 Supplement	GIBCO	supplement for p.n. culture
beta-mercaptoethanol	Roth	for SDS-PAGE
Borate acid	Roth	plastic precoating
BSA	Serva	lipid blots blocking solution
CaCl	Roth	cell pretreatment
ColorPlus Prestained Protein Marker	New England Biolabs	weight marker for WB
Complete protease inhibitor cocktail tablets	Roche	protease inhibitor to complement any cell lysis
Cytosine arabinoside (CytA)	Sigma	cortical primary neurons culture
EDTA	Roth	cellular lysis
Fetal Bovine Serum	PAN	cell culture supplement for media
Glycerol ($C_3H_8O_3$)	Roth	immunoprecipitation buffer
Glutamine (L) , 200 mM	Invitrogen	HEK cells culture supplement
HEPES ($C_8H_{18}N_2O_4$ S)	Sigma	HEK cells transient transfection
KCl	Merck	cell pretreatment
Ki16425	Sigma	cell pretreatment
Lipid dot blots 6002	Echelon Biosciences	membrane protein studies
LPA (Oleoyl-l-alpha-Lysophosphatidic acid sodium salt)	Sigma	cell culture pretreatments
$MgCl_2$	Merck	include on solutions
Milk powder (non fatty)	Roth	membrane blocking for WB
Methanol	Roth	membrane blotting for WB
Mouse Serum (Normal)	Dako	immunoprecipitation (IP) assays
NaCl	Merck	include on solutions
NaF	Sigma	include on solutions
Nonidet P40 (NP40)	AppliChem	cell lysis buffer
Penicillin-Streptomycin (Pen/Strep)	PAN	media supplement for cell culture

Product	Source	Methods in which it is used
PhosphoStop (phosphatase inhibitor cocktail tablets)	Roche	phosphatase inhibitor to complement any cell lysis
Poly-L-lysine	Sigma	p.n. culture
Ponceau S Red	Roth	WB
Protan BA 85 Nitrocellulose	Whatman	Western Blot (WB) membrane
Protein G Sepharose^{TM}4 Fast Flow	GE Healthcare	IP assays
Rotiphoreses® Gel A	Roth	acrylamide/bisacrylamide electrophoresis gel
Rotiphoreses® Gel B 2 % bisacrylamide solution	Roth	acrylamide/bisacrylamide electrophoresis gel
TEMED	Roth	acrylamide/bisacrylamide electrophoresis gel
Triton®X 100	Sigma	cellular lysis
TRP (Thrombin Receptor activating Peptide)	custom-made *peptide sequence is: SFLLRN*	cell culture pretreatments
Tween®20	Roth	cellular lysis

microscopy

Product	Source	Methods in which it is used
Hoechst dye	Sigma	DNA labeling for microscopy
immunomount	Thermo Fischer	closing imunocitochemistry slices
Na_2HPO_4	Roth	immunos
Saponin	Sigma	immunos

Enzymes and buffers

Table 3: Purchased enzymes and buffers

Enzyme	Source
10X Buffer	FastDigest
10X goTag buffer	Promega
10X Herculase buffer	Stratagene
Bam H I	FastDigest
Bgl I	FastDigest
Bgl II	FastDigest
Cla I	FastDigest
goTag	Promega

Enzyme	Source
Herculase	Stratagene
Hind III	FastDigest
Kpn I	FastDigest
Not I	FastDigest
T4 Ligase	BioLabs
SAP	Roche
Xho I	FastDigest

Antibodies

Primary antibodies used during experiments are listed in the following table (Tab. 4). The working concentration, the company that supplied them and the technique for which they are used are detailed in the same table. The first antibodies are diluted in PBS unless otherwise noted.

Table 4: Primary antibodies

Name	**Species** (clonality)	**Supplier**	**Application** (dilution)
Anti-ß actin	Mouse, monoclonal (clone AC-15)	Sigma	IB (1:5,000)
Anti-ATPase	Mouse, Monoclonal	Abcam	IB (1:2,500)
Anti-cMyc	Mouse, monoclonal (Clone 9E10)	Bioscience	IP, IB (1:1000)
Anti-Flag	Mouse, monoclonal (clone M2)	Sigma	IP, IB (1:500)
Anti-GFP	Rabbit, polyclonal	Abcam	IF (1:1,500)
Anti-HA	Rat, monoclonal (clone 3F10)	Roche	IP, IB (1:500), IF (1:500)
Anti-MAP2	Mouse, monoclonal	Sigma	IF (1:1,000)
Anti-MEK1/2*	Rabbit, polyclonal	Cell signalling	IB (1:1,000)

Anti-p-MEK1/2* (Ser 217/221)	Rabbit, polyclonal	Cell signalling	IB (1:1,000)
Anti-N-Ras	Mouse, monoclonal (F155-227)	Sigma	IB (1:500)
Anti-p44/42 MAP Kinase* *(Anti-Erk1/2)*	Rabbit, polyclonal	Cell signalling	IB (1:1,000)
Anti-MAP Kinase activated* *(Anti-p-Erk1/2)*	Activated mouse, monoclonal (clone MAPK-YT)	Sigma	IB (1:10,000)
Anti-p38 MAP Kinase (Thr 180/Tyr 182)*	Rabbit, polyclonal	Cell signalling	IB (1:1,000)
Anti-p-p38 MAP Kinase*	Rabbit, polyclonal	Cell signalling	IB (1:1,000)
Anti-PRG-1	Rabbit, polyclonal recognises aa 624-639 from NP_808332	Housemade	IP, IB (1:1,000), IF (1:500)
Anti-Ras GRF-1	Rabbit, polyclonal	New England Biolabs	IP, IB (1:1,000)
Anti-Ras GRF-1	Rabbit, polyclonal	PTG	IP, IB (1:1,000)
Anti-Ras GRF-2	Goat, polyclonal	Santa Cruz	IP, IB (1:500)
Anti-Tau	Mouse, monoclonal	Chemicon	IF (1:1,000)
Anti-ßIII Tubulin	Mouse, monoclonal (clone TUJ1)	Covance	IF (1:1,500)

IF: Immunofluorescence; IP: Immunoprecipitation; IB: Immuno Blot.

** Diluted in PBS Tween instead of PBS.*

The secondary antibodies used are also listed in table format (Tab. 5). In the same table, the working concentration of the antibodies, the company who purchased them as well as the technique in which they are used is detailed.

Table 5: Secondary antibodies

Name	**Species**	**Supplier**	**Application** (dilution)
Alexa Fluor® 488 Anti-Rabbit	Goat	Molecular Probes.	IF (1:1,000)
Alexa Fluor® 568 Anti-Rabbit	Goat	Molecular Probes	IF (1:1,000)
Alexa Fluor® 568 Anti-Mouse	Goat	Molecular Probes	IF (1:1,000)
Alexa Fluor® 488 Anti-Goat	Donkey	Molecular Probes.	IF (1:1,000)
Alexa Fluor® 568 Anti-Goat	Donkey	Molecular Probes	IF (1:1,000)
ECLTM-HRP-conjugated Anti-Mouse	Sheep	GE Healthcare	IB (1:5,000)
ECLTM-HRP-conjugated Anti-Rabbit	Donkey	GE Healthcare	IB (1:5,000)
ECLTM-HRP-conjugated Anti-Rat	Goat	GE Healthcare	IB (1:5,000)
Peroxidase-conjugated Anti-Goat	Rabbit	Sigma	IB (1:5,000)

IF: Immunofluorescence; IB: Immuno Blot.

4.1.2 Solutions and buffers

Table 6: Solutions and buffers used for:

experiments with animals

Name	**Ingredients**	**Methods in which it is used**
Hybridization Buffer (HB)	50 % formamide, 10 mM Tris-HCl pH 8.0, 10 mM PB pH 7.2, 2x SSC, 5 mM EDTA pH 8.0, 10 % dextran sulphate, 10 mM dithiothreitol (DTT), 1 mM beta-mercaptoethanol and 200 ng/µl tRNA	*in situ* hybridization
Ketamine cocktail	*for mouse:* 50 mg/ml Ketamine, 20 mg/ml Rompun *for rats it is also added:* 10 mg/ml Vetranguis	anaesthetize
PFA (4 %)	4 % Paraformaldehyde diluted in water and 0.2 M PB (1:1)	cell fixation
Phosphate buffered saline (PB)	0.1 M K_2HPO_4/ KH_2PO_4	*in situ* hybridization
Saline solution	0.9 % NaCl	blood washing

Name	Ingredients	Methods in which it is used
SSC (20X)	3 M NaCl; 0.3 M $C_6H_5O_7.3Na\ .2H_2O$	*in situ* hybridization

molecular biology methods

Name	Ingredients	Methods in which it is used
Agar plates	10 g NaCl, 10 g tryptone, 5 g yeast extract, 20 g agar in petri dishes *autoclaved and supplemented with antibiotics.*	growing bacteria in petri dishes
DYT medium	16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl (pH 7.0) in 1 liter H_2O *this solution has to be autoclaved and antibiotics is added before use.*	growing bacteria in suspension
Mini -A solution	10 mM EDTA (pH 8), 25 mM Tris (pH 8), 5 g NaCl (pH 7.0) in 1 liter H_2O	DNA purification (miniprep)
Mini-B solution	1 % SDS, 0.2 M NaOH	DNA purification (miniprep)
Mini-C solution	5 M KCH_3COO , 11.5 ml $C_2H_4O_2$ diluted in 100 ml H_2O	DNA purification (miniprep)
TBE buffer (stock 10X)	108 g Tris, 55 g H_3BO_3, 40 ml EDTA (0.5 M, pH 8) in 1 liter H_2O	agarose gel separation
TBE buffer with Guanosine (stock 10X)	108 g Tris, 55 g H_3BO_3, 40 ml EDTA (0.5 M, pH 8) with 2.8 g guanosine in 1 liter H_2O	agarose gel separation while DNA cloning

cellular biology methods

Name	Ingredients	Methods in which it is used
Blocking solution for WB	5 % milk powder diluted in 1X PBS or PBS-T	WB
Blotting buffer for WB	14.42 g glycine, 3 g Tris dissolved in 700 ml H_2O with 200 ml methanol	WB
Borate Buffer	0.1 M Borate acid pH 8.5	for precoating of plates and

Name	Ingredients	Methods in which it is used
(BB)	*this solution is used after sterilization.*	coverslips before cell plating
HEPES buffer for LPA	50 µM HEPES, 138 µM NaCl, 2.7 µM KCl, 1 µM $CaCl_2$, 1 µM $MgCl_2$ and 1 % BSA	for LPA stocks for cellular pretreatments
HEPES for transfection	50 µM HEPES, 138 µM NaCl, 2.7 µM KCl and pH: 7.15	transient transfection of HEK cells
Calcium chloride solution (2 M $CaCl_2$)	29.4 g $CaCl_2$ x 2 H_2O in 100 ml Milipore II *this solution is used after sterile filtration under the bench*	transient transfection of HEK cells
Immunoprecipitation buffer* (IPB)	50 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 % NP40, 5 mM EDTA, 2.5 $MgCl_2$, 10 % glycerol	IP
Lysis buffer-A* (LB-A)	20 mM Tris, pH 7.5; 100 mM NaCl; 10 mM NaF; 10 mM $MgCl_2$; 1 mM Na_3VO_4; 10 % [wt/vol] glycerol; 1 % [wt/vol] Triton X-100;	cell lysis for Ras activity assay in HEK cells
Lysis buffer-B* (LB-B)	20 mM Tris-HCl with pH:7.5; 0.25 M Sucrose; 1 M EGTA; 1 M EDTA	membrane/cytosol fraction separation assays
PBS buffer	8 g NaCl, 0.2 g KCl, 0.24 g $K_2H_2PO_4$ 1.44 g Na_2HPO_4-$2H_2O$ in 1 liter H_2O	WB and immunos
PBS-Tween	PBS with 0.1 % Tween	WB and immunos
SDS buffer	62.5 mM Tris-HCl, 2 % SDS, 10 % Sucrose 50 % Bromphenolblau (BPB)	loading buffer for gel electrophoresis

microscopy

Name	Ingredients	Methods in which it is used
Blocking solution	0.1 % Saponin, 10 % FCS *diluted in PB*	imunofluorescence
PB	0.1 M Na_2HPO_4/ NaH_2PO_4	washing steps

* *These buffers are used to lysate the cells and are complemented before use with protease and, if needed, phosphatase inhibitors.*

All solutions are prepared using distilled water unless otherwise specified.

Cell culture media

Table 7: Cell culture media

Medium	Supplier	Use	Supplemented with
Distilled water	GIBCO	p.n.	-
DMEM *without piruvate*	PAN	HEK cells and COS 7	10 % FCS, 1 % Pen/strep, 1 % L-Glutamine
DMEM *with piruvate*	PAN	N1E-115 cells	10 % FCS, 1 % Pen/strep,
HBSS	GIBCO	p.n.	-
Neurobasal A	GIBCO	p.n.	2 % B27, 0.5 mM Glutamine
MEM	GIBCO	p.n.	10 % Horse Serum, 1 % Pen/Strep, Glucose
PBS	GIBCO	p.n.	-
RPMI	GIBCO	DU 145 cells	10 % FCS, 1 % Pen/strep

Kits

Table 8: Purchased kits used for:

molecular biology methods

Kit	Source	Methods in which it is used
High capacity cDNA Reverse Transcription Kit	Applied Biosystems	cDNA generation for quantitative Real Time PCR
NucleoSpin® Extract II	Clontech	DNA extraction from agarose
Plasmid Maxi	Quiagen	DNA purification (maxiprep)
TaqMan® Universal PCR Master Mix	Applied Biosystems	quantitative Real Time PCR
TopoTA cloning	Invitrogen	cloning

cellular biology methods

Kit	Source	Methods in which it is used
Active GTPase Pull-Down and Detection Kit	Thermo Fisher Scientific	Ras activity assay for p.n.
BCA Protein Assay Kit	Thermo Fisher Scientific	protein quantification
Effectene Tranfection Reagent	Qiagen	p.n. transient transfection
µMACS^{TM}epitope-tagged protein isolation	Miltenyi Biotec	protein purification

4.1.3 Equipments

Table 9: Equipment required for:

molecular biology methods

Name	Source	Methods in which it is used
ABI PRISM® thermal cycler	Applyed Biosystems	quantitative Real Time PCR
Biomate 3 spectrometer	Thermo	mRNA and DNA quantification

cellular biology methods

Name	Source	Methods in which it is used
Centrifuge 5415R	Eppendorf	cell lysis
Optima MAX-XP ultracentrifuge	Beckman Coulter	membrane/cytosol fraction separation assays
Perfect Blue™ Dual Gel System	PeqLab	acrylamide/bisacrylamide electrophoresis gel
Scanner	Kyocera	scanning pictures and WB
Semi dry-blotter	PeqLab	WB
Synergy 2 plate reader	BioTek	protein quantification

microscopy

Name	Source	Methods in which it is used
Microtom HM 650 V	Thermo Scientific	tissue cutting
Leica TCS SL confocal microscopy	Leica	cell and tissue microscopy
Olympus BX50 fluorescence microscopy	Olympus	cell and tissue microscopy

4.1.4 Softwares

Table 10: Softwares required for:

molecular biology methods

Name	Source	Methods in which it is used
DNASIS® MAX	Hitashi Software Engineering	checking DNA sequence
7500 Fast System SDS	Applyed Biosystems	quantitative RT-PCR
GraphPadPRISM^{TM}	GraphPad Software	statistical data analysis
Microsoft office	Windows	

cellular biology methods

Name	Source	Methods in which it is used
EXPASY	Swiss Institute of Bioinformatics http://expasy.org/	*in silico* study of proteins
GraphPadPRISM^{TM}	GraphPad Software	statistical data analysis
ImageJ	public domain but developed by National Institute of Health	IB quantification
Office	Microsoft	
SMART database	EMBL Heidelberg http://smart.embl-heidelberg.de/	*in silico* study of proteins

microscopy

Name	Source	Methods in which it is used
Leica confocal	Leica	to obtain cell pictures
MetaMorph	Molecular Devices	p.n. morphology studies

4.2 Methods

4.2.1 Animals

Mice lines

Animals are obtained from *Forschungseinrichtung für experimentelle Medizin (FEM)* (Berlin, Germany). All experiments are done in accordance with the local animal protection regulations. The animals are housed under standard laboratory conditions and all procedures are performed in agreement with the German law (in congruence with 86/609/EEC), on the use of laboratory animals as well as on the Federation of European Laboratory Animal Science Associations (FELASA). All efforts were made to minimize the number of animals used. The various animal lines that are used are hereunder detailed with their reference on Table 11.

Table 11: Animals used for the experiments

Animal	Reference
BalbC	reviewed by Potter 1985 [80]
C57BL/6	Harrison et al. 1978 [81]
LPA_2 $^{-/-}$	Contos et al. 2002 [82]
PRG-1 $^{-/-}$	Trimbuch et al. 2009 [43]

Mouse and rat perfusion

Set up perfusion pump - Perfusion set and perfusion needle are attached. A first run of about 100 ml of normal tap water through the tubing is done to remove any residue. The open end of the perfusion tube is placed in a tube with 0.9 % saline solution which is afterwards changed to cold 4 % paraformaldehyde (PFA) solution while perfusing. The volume of solution should be scaled to the size of the animal; usually 200 ml is sufficient for one animal. The pumping system has to be adjusted to a slow steady drip (20 ml/min.).

Set up surgery - The set up is set with scissors, forceps and clamps; the animal is given an appropriate amount of anesthetic and, only once the animal is under anesthesia and unresponsive for pinch-response, the animal is placed and fixed on the operating table with its back down. The process begins with an incision through the abdomen the length of the diaphragm. Then, the connective tissue at the bottom of the diaphragm is cut and the thoracic cavity is opened up with scissors. The animal is fixed open to expose the heart and to provide drainage for blood and fluids. While steadily holding the still beating heart with forceps, the needle is inserted directly into the protrusion of the left ventricle about 5 mm. The needle position is clamped in place near the point of entry. The valve is then switched on to allow slow, steady flow of around 20 ml/min. of 0.9 % saline solution and a cut in the atrium is made to ensure solution is flowing freely. When the blood has been cleared from the body, the pumped solution is changed to 4 % paraformaldehyde (PFA) solution taking special care not to introduce air bubbles while transferring from one solution to the other. Perfusion is almost complete when there is a spontaneous movement and lightness colour of the liver. Then the perfusion is stopped and tissue of interest excised, placed in vials containing the same fixation solution and fixed at 4 °C before proceeding.

Mouse brain *in situ* hybridization

Antisense oligonucleotides complementary to the mouse sequence of Ras GRF-2 are used for hybridization. Oligonucleotide probes are small, generally around 40-50 base-pairs, and are produced synthetically by automated chemical synthesis, in this case Metabion. The specificity is confirmed by a BLAST GenBank [83] search to rule out cross hybridization with other genes. Horizontal cryostat sections (20 µm) are fixed in 4 % PFA, washed in 0.1 M PB (pH 7.4) and

dehydrated through an ascending series of ethanol (50, 60, 75, 90 and 100 %).

The oligonucleotides are end-labeled using terminal desoxynucleotide transferase and [alpha-^{35}S]-dATP. Probe labeling is performed for 10 minutes (min.) at 37 °C. The radioactive probes are purified using BioSpin6 Chromatography Columns and 100,000-200,000 counts per minute (cpm) labeled oligonucleotides are diluted in HB and 50 µl solution are added per brain section. Hybridization is performed for 16 hours (h.) at 42 °C in a humidified chamber, after which the slides are washed as follows: 1 x 60 min. in 0.1 x SSC at 56 °C and 1 x 5 min. in 0.05X SSC at room temperature (r.t.). Finally, the sections are rinsed in water at r.t. and dehydrated in consecutive washes of 50, 75 and 96 % ethanol. For autoradiography, the slides are exposed to light sensitive films for 15 days.

No signals should be detected on the control sections, which are hybridized either with the specific antisense oligonucleotide when the unlabeled oligonucleotide is added in 100-fold surplus or with sense probes of the respective oligonucleotides. Following the exposure, the sections are rehydrated using a decreasing ethanol series, washed in PB, counterstained with Toluidinblau, dehydrated through an ascending series of ethanol, flat-embedded with Entellan and coverslipped. The sections are then digitally photographed.

4.2.2 Molecular biology

Yeast two-hybrid screening [1]

The DNA encoding the C-Terminus of PRG-1 was used as a bait for CytoTrap system from Stratagene. For this purpose, DNA encoding the protein of interest (C-Terminus of h PRG-1 as bait protein) is cloned into the pSos vector Multicloning Site (MCS), generating a fusion protein of hSos and the C-Terminus of human (h) PRG-1 protein. DNA encoding an expression library is cloned into the pMyr vector MCS and expressed as a fusion protein with a myristylation sequence that anchors the fusion protein to the plasma membrane. These fusion proteins are coexpressed in the cdc25H yeast strain, and the yeast cells are incubated at the restrictive temperature of 37 °C. If the bait and target proteins physically interact, the hSos protein is recruited to the membrane, thereby activating the Ras signalling pathway and allowing the cdc25H yeast strain to grow at 37 °C. Positive colonies are sequenced.

Constructs

Ras GRF-2, N-Ras and N-Ras ^{D12} constructs were kindly provided by Dr. Piero Crespo (*Instituto de Investigaciones Biomédicas Alberto Sols*, Madrid, Spain) and are cloned into suitable vectors for experiments such as pires2 EGFP or pEGFP vectors using PCR technique. PRG-1 was already established in the laboratory and HA-PRG-1 and HA-PRG-1 deletion constructs are cloned into the pcDNA3.1(+) expression vector using Kpn 1 and Not 1 restriction sites. For the mem-HA-PRG-1

[1] This experiment was done before I began with the project but it has been included because of its importance for the project.

deletion construct, membrane tag was cloned in front of the HA-PRG-1 delta -975 construct. The tag was amplified from the commercial vector mem-eGFP (see primers in Supp. Tab G) and introduced in frame upstream the fusion protein. Primers used for cloning are listed in supplementary table G in the appendix. The constructs are frequently checked through sequencing.

There is a more detailed list of all the constructs used; their name, the insert they contain, the used vector bone, their restriction sites and sizes are detailed in the appendix as a supplementary information. (Suppl. Tab. E)

Mutants clonation (PCR, TopoTA, plasmidic DNA extraction, ligation, transformation and clone analysis)

Mutants and constructs used in the project are cloned following this general protocol:

First, a Polymerase Chain Reaction **(PCR)** is conducted to amplify the fragment of interest. The constructs are amplified by a reverse transcription-polymerase chain reaction from cDNA or from a plasmid using specific primers. Primers used for HA-PRG-1 mutants are detailed in the appendix as supplementary information in the table G. The specific oligonucleotides needed for each construct are self designed for each construct and synthesized by Metabion.

Second, the amplification products are ran in an agarose gel and purified by NucleoSpin Extract II. Then, the purified amplification fragment is **cloned in a TopoTA vector**, cultivated in a petri dish to obtain single colonies and grown in DYT. The extraction of plasmidic DNA from a single colony bacteria culture is as follows: for **plasmidic DNA extraction**, bacteria are pelleted through centrifugation, supernatant is removed and pellet is resuspended in 200 µl Mini-A. Once the bacteria are resuspended and, if necessary, vortexed, 200 µl Mini-B are added. Tubes are inverted 6-8 times and incubated at r.t. for up to 5 min. The lysis reaction is stopped through the addition of 200 µl of Mini-C and tubes are gently turned overhead. The genomic DNA precipitate is pelleted after 20 min. at top speed centrifugation and the supernatant is transfered into new tube. The plasmid DNA is precipitated by adding 900 µl of isopropanol and collected through 20 min. top speed centrifugation. The pellet is cleaned with 1 ml Etanol 70 % followed by a short centrifuge at top speed, then dried on air. The obtained plasmidic DNA is resuspended with 30 µl of pure water. After the obtention of the plasmid, the sequence is first checked through restriction enzymes and only positive clones sequence is checked.

Table 12: Ligation reaction mix

Product	Volume:
10X buffer	2,5 µl
Vector	5 µl
Insert	15 µl
T4 - Ligase	1 µl
H_2O	1,5 µl

Third, the **fragment of interest is cloned into the chosen mammalian expression vector**; in this project it was usually pCDNA3.1 (+) zeo expression vector or a fluorescent expression vector such as pEGFP. The **ligation** consist of mixing a ligation reaction of 25 µl (for detail see Tab. 12) over night (o.n.) at 16 °C. On the next day a **transformation** of the total ligation into 750 µl of competent bacteria (XL10-

Gold) is done incubating bacteria and ligation product on ice for 30 min., followed by heat shock 30 sec. at 42 °C, then put on ice for 2 min. After the heat shock, 700µl DYT media are added and the bacteria are incubated at 37 °C for 1 h. with vigorous shaking. The bacteria are then plated into a petri dish in low density to allow a single colony culture and grown o.n. at 37 °C. The next day the single colonies are gown in 5 ml cultures and kept o.n. at 37 °C.

Forth, the **single colonies are cultivated** in 5 ml DYT (with its specific antibiotic) and the extraction of the plasmids is carried out using Mini-A, Mini-B and Mini-C miniprep solutions as already explained. The plasmid content is analysed through a restriction enzyme digestion and, finally, the bacterial clones seen to contain the fragment of interest are sequenced (by MWG) and preserved at - 80°C for further use in 1.5 ml cryotubes where sterile glycerol is added in a ratio of 1:2.

mRNA, cDNA and quantitative Real Time PCR (q RT PCR)

After killing the mice, brains are dissected and minimum of 3 different animals are used for each sample. Samples are frozen at -80°C at least o.n. and just after removing them from the -80°C TRIzol® reagent is added. For **mRNA**, around 18 x 10^6 primary neurons 7 or 14 DIV as well as brain tissue samples are homogenized in TRIzol® reagent and the total RNA is purified according to the TRIzol® protocol. RNA concentrations are determined using an spectrophotometer. A High Capacity cDNA Reverse Transcription Kit is used to generate total **cDNA** for the quantitative Real Time PCR (q RT PCR) For that purpose, 5 µg of the total mRNA from each sample are treated as indicated in the manufacturer's recommendations of the Reverse Transcriptase Kit.

For **q RT PCR**, the TaqMan® Universal PCR Master Mix Kit is used for the TaqMan Assay and the reactions are performed in a 96 well Optical Reaction Plate from Applied Biosystems. The gene expression assays employed are alphabetically listed in table 13 and the housekeeping genes used for that set of experiments were Glyceraldehyde - 3 - Phosphate Dehydrogenase (GAPDH) and Hypoxanthinephophoribosyl- Transferase (HPRT).

Table 13: Alphabetically listed gene expression assays employed for quantitative Real Time PCR

Gene	Assay ID	Supplier
GAPDH	Mm99999915_g1	Applied Biosystems
HPRT	Housemade (for primer sequence see Suppl. Tab. F)	
N-Ras	Mm01308659_m1	Applied Biosystems
PRG-1	Mm00724102_m1	Applied Biosystems
Ras GRF-1	Mm00441097_m1	Applied Biosystems
Ras GRF-2	Mm00485561_m1	Applied Biosystems

The reactions are detected using the ABI PRISM™ 700 Sequence Detection System. Primers efficiency was checked before performing any experiment. Results are analysed using 7500 Fast

System software settling the cycle threshold (ct) at 0.3 and a manual base line between cycle 3 to 15. In addition, the results are discarded if the standard deviation between triplicates is higher than 0.5.

4.2.3 Cellular biology

Protein quantification

BCA Protein Assay Kit is used for protein measurement. As per the manufacturer's instructions, 200 µl of BCA Working Reagent (1 part of BCA Reagent B diluted in 50 parts of BCA Reagent A) are pipetted in triplicates into a 96 well plate, then 25 µl of BSA standards or the unknown samples (diluted 1:25 and 1:50 in 0.9 % NaCl) are added and the plate is incubated for 30 min. at 37 °C. The well absorbance is measured at 562 nm on the plate reader. The average 562 nm absorbance measurement of the Blank standard replicates is subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates. A standard curve is generated by plotting the average Blank-corrected 562 nm measurement for each BSA standard versus its concentration in µg/ml. The protein concentration of the unknown samples is determined by extrapolation using the obtained standard curve.

Cell culture and transient transfection

The HEK 293, DUC 145, N1E-115 and COS 7 cells lines have been bought to *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DSMZ) (see Tab. 14). All cell lines are cultured under sterile conditions with their specific medium (see Tab. 7) and are kept at 37 °C with 5 % CO_2 in an incubator as standard incubation conditions

Table 14: Cells used for the experiments

Animal	Source	Reference	Origins
COS 7	DMSZ	ACC60	monkey kidney
DU 145	DMSZ	ACC261	human prostate carcinoma
HEK 293	DMSZ	ACC635	human epithelium kidney
N1E-115	already in the lab		mouse neuroblastoma cell line

The most used cell line for this project was the HEK 293 cell line. More detailed information about its proceedings is included hereunder. HEK cells are cultured under sterile conditions in Dulbecco's Minimal Essential Medium (DMEM), supplemented with 10 % Fetal Bovine Serum (FBS), 1 % L-glutamine and 1 % penicillin-streptomycin. The cells are kept at 37 °C with 5 % CO_2 in an incubator. For the transient transfection 1 x 10^6 HEK 293 cells are plated on precoated 9 cm culture dishes; 24 h. after plating, cells are transiently transfected with 10 µg of DNA encoding for the proteins specified in each case by calcium phosphate precipitation, as previously described [84].

Incubated for 30 h. in standard conditions prior to cell lysis, protein isolation or fixation and staining. .

Cortical primary neurons culture and transient transfection

Cortical primary neurons are prepared from mice at embryonic day 18 (+/- 0.5 days), as previously described [85]. The neurons are cultured in neurobasal A medium, supplemented with 2 % B27 and 0.5 mM L-glutamine. For Imunoprecipitation (IP) and signalling studies, neurons are plated on 14 cm culture poly-L-lysine-coated petri dishes at a density of 5 x 10^6 cells in 30 ml growth medium and cultured with medium renewal every 2-3 days for a total of 7 or 14 Days *in vitro* (DIV). For immunofluorescence purposes, 150,000 cells in growth medium are plated on 18 mm poly-L-lysine-coated glass coverslips in 12 well plates. The transient transfection of p.n. is performed using Effectene Transfection Reagent from Qiagen and following the protocol established by the company. For mRNA extractions CytA is added 2 days before mRNA extraction to ensure culture purity.

Cell pretreatments

LPA stimulation - 1-Oleoyl-Lysophosphatidic Acid (LPA) and fatty acid free BSA are employed for the stimulation medium. There are different species of LPA because of the various acyl chain lengths, their degrees of saturation and their positions on the glycerophosphate backbone, however we use the 1-oleoyl-Lysophosphatidic Acid as this is the commonly used in the laboratory [8] in combination with fatty acid free BSA as detailed on table 2 for the stimulation medium. LPA is dissolved in HEPES buffer for LPA (see Tab. 6) and 2.6 mM stocks are stored, until use, at -20 °C in glass tubes to avoid plastic contact reported to induce lipid loss [86]. Neurons are stimulated with a final concentration of 10 µM LPA in the growth medium and incubated at 37 °C and 5 % CO_2 for 10 min. The medium is then replaced from the culture dishes and the cells are immediately fixed for immunocitochemistry or lysed and used for coimmunoprecipitation assays or signalling.

TRP stimulation - Thrombin Receptor activating Peptide (TRP) used is a custom-made peptide whose sequence is: SFLLRN. The TRP is kept at -20 °C, diluted in water and at 12.5 mM stock solution. Neurons are pretreated with 25 µM TRP included in the growth medium and incubated at 37 °C and 5 % CO_2 for 10 min. prior to lysis.

Calcium stimulation - Cultured neurons are treated with $CaCl_2$ at a final concentration of 50 mM. The calcium is added and neurons are incubated at 37 °C and 5 % CO_2 for 10 min. prior to lysis.

Ki 16425 stimulation - Crystalline solid Ki 16425 is dissolved in DMSO for a stock solution of 33.6 mM and kept at -20 °C. Neurons are stimulated with 10 µM Ki 16425 at 37 °C and 5 % CO_2 for 10 min. to block LPA receptors before any further pretreatment and cellular lysis.

Immunoprecipitation (IP) and signalling

30 h. after the transfection for the HEK 293 cells or 7-14 DIV for the neurons, cells are either non-treated or treated either 10µM LPA is applied and cells are incubated for 10 min at 37°C and the control set of cells are treated either with 25 µM TRP, 10 µM Ki16425 or 50 mM $CaCl_2$. The pretreatment is applied and cells are incubated for 10 min. at 37 °C. Incubation is stopped by placing the culture dishes on ice and the neurons are suspended in PBS and lysed by adding Immunoprecipitation Buffer (IPB) (Tab. 6) with a protease inhibitor cocktail and a phosphoStop (Tab. 2). The suspension is first homogenized and subsequently sonicated, followed by centrifugation at 1,000 g for 5 min. to discard the nuclear debris. The supernatant is either used for signalling studies or for Immunoprecipitation (IP) assays.

For **immunoprecipitation (IP) studies**, the supernatant is incubated at 4 °C and end-over-end rotation with precleared protein G beads for 1 h., followed by a short and soft centrifugation (1 min. at < 0.8 rpm). The supernatant is incubated o.n. with the antibody (5 µl of antibody when using: cMyc, Flag, PRG-1 or 15 µl of Ras GRF-2 antibody) or with no antibody as a control. Next day 20 µl precleared beads are added to the sample and incubated 1 h. at 4 °C and over head rotation, washed 3 times with IPB and centrifugated shortly. Finally the samples are analysed by SDS-PAGE electrophoresis and immunoblotting.

For **the signalling assay**, the total amount of protein on the supernatant is quantified using BCA Protein Assay Kit (see protein quantification) and 20 µg per lane are analysed in a Western Blot (WB) for ERK pathway phosphorylation studies; meanwhile, up to 40 mg/lane are loaded in the SDS-PAGE for p38 pathway phosphorylation studies.

µMACS

30-48 h. after the transient transfection, the HEK 293 cells are washed twice with ice-cold PBS prior to scraping, lysing and purifying the protein purification. The procedures for lysis and protein purification are followed according to the manufacturer's instructions for HA tagged proteins µMACS™ Kit. Finally, 10-20 µl of the purified protein are used for the WB and 35 µl for lipid blots.

Ras activity assays

The **activation status of Ras on the transfected HEK 293 cells**[2] is assayed using the Glutathione S-Transferase (GST) fusion protein of the Ras-Binding Domain (RBD) of Raf-1 (posi-

[2]Ras experiments in the HEK cells overexpressed system were done in collaboration with Prof. Offermans and performed by Dr. Swiercz.

tions 1 to 149), which has a high affinity for GTP-loaded and therefore, affinity for active Ras [87]. The activation status of Ras in HEK 293 cells is assayed as previously described [88]. Briefly: the HEK 293 cells are starved, treated with LPA and lysed in the Lysis Buffer A (see in Tab. 6). Cleared lysates are then incubated with a Ras-binding domain of Raf-1 fused to GST (GST-RBD) for 1 h. at 4 °C. Precipitated, active Ras is analysed in WB using anti-N-Ras antibody.

The **activation status of Ras proteins on primary neurons** is assayed using the Active GTPase Pull-Down and Detection Kit. According to the manufacturer's instructions, 750 µg protein lysate are used for each sample (sample itself, positive control and negative control). The precipitated Ras is analysed by SDS-PAGE, followed by IB using 1 mg of a monoclonal N-Ras antibody per ml.

Western Blot (SDS-PAGE and Immunoblotting)

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) - SDS-PAGE is performed using a vertical PeqLab system with 10-12 % acrylamide/bisacrylamide gels for the analysis of small signalling kinases or 7.5 % acrylamide/bisacrylamide gels for the separation of bigger proteins such as the ones obtained with the IP assays. Protein samples are prepared with SDS buffer (Tab. 6) and 10 % beta-mercaptoethanol, then cooked for five minutes at 95 °C, then beta-mercaptoethanol is readded and samples are loaded onto SDS-PAGE gel.

Immunoblotting (IB) - The separated proteins are transferred electrophoretically onto nitrocellulose membranes using a semi-dry blotter. The blotting result is checked by Ponceau S Red staining before the membranes are incubated in blocking solution (3 % non-fatty milk) for one hour at r.t. Depending on the antibody, 1X PBS or PBS-T is used to prepare the blocking solution as well as for washing. The primary antibodies are diluted in PBS, PBS-T or a blocking solution to the appropriate concentration (Tab. 4) and incubated with the membrane o.n. at 4 °C under continuous rotation. After washing three times with PBS or PBS-T, the membranes are incubated with horseradish peroxidase-conjugated secondary antibody (Tab. 5) diluted in PBS, PBS-T or blocking solution depending on the primary antibody. The incubation of the secondary antibody is performed either o.n. at 4 °C or for several hours at r.t., both incubations under constant rotation. Finally, the blots are washed three times with PBS or PBS-T for at least 1 h. in total, then incubated for 2 min. with ECL reagent and exposed to light sensitive films.

Lipids binding assay

Lipid dot blot assays are performed according to the manufacturers instructions. Briefly, the 6002 membranes are blocked in PBS-T 3 % BSA for 1 h. at r.t. Then, 35 µl of purified HA-PRG-1 full length, HA-Ras GRF-2, HA-PRG-1 delta 1-975 or mem-HA-PRG-1 delta 1-975 in PBS-T solution are added and incubated o.n. at 4 °C in blocking solution. The dot blots are washed and incubated with anti-HA antibody, followed by goat anti-rat HRP all in PBS-Teen. Finally,

ECL reagent is added and their signal is developed using photosensitive film exposition. As a control, one extra lipid membrane is incubated with a protein purification solution from µMACS Kit without any protein and further treated as any other membrane, with first antibody incubation, second antibody incubation and ECL to prove no cross linking between the materials used and the lipid membranes.

Membrane / cytosol fraction separation

30 h. after the HEK 293 cells transfection, the culture dishes are washed with PBS and lysed by adding Lysis Buffer B (LB-B) (Tab. 6) with freshly added protease inhibitor cocktail (Tab. 2). The suspension is sonicated (3 times, 3 sec. each time), followed by 30 min. incubation on ice and ultracentrifugation. The pellet is resuspended in 100-150 µl of LB-B with 1 % Triton X-100 and incubated 30 min. on ice. After incubation, the samples are centrifuged for 5 min. at 5,000 rpm and its supernatant (SN2) corresponds to the membrane protein. The total amount of protein on both supernatants (SN1: cytosol fraction and SN2: membrane fraction) are quantified using BCA and 20 µg per lane are used for WB analysis.

4.2.4 Microscopy

Immunocytochemistry

5 DIV Primary neurons are washed with filtered PBS and fixed using 4 % PFA with 15 % Sucrose for 5 min. After 3 washing steps with filtered PBS during 10 min., the cells are permeabilized using a solution containing 0.1 % Triton X-100 and 0.1 % $C_6H_5Na_3O_7$ during 3 min. at 4 °C and blocked o.n. at 4 °C using 20 % FCS diluted in PBS. The next day, the first antibody diluted in 5 % FCS in PBS is incubated for 2 h. at r.t., followed by three washing steps with filtered PBS and 1 h. incubation in darkness with the secondary antibodies linked to fluorescent dyes. After staining, Hoechst dye is added and incubated 5 min. at r.t. Finally the cells are fixed with Immunomount into new coverslips. For antibody information, primary antibodies are detailed in table 4 and secondary antibodies are listed in table 5.

Immunohistochemistry

After the brain fixation (at 4 °C in 4 % PFA o.n.) the brain is placed in a petri dish and fixed with 4 % agarose's gel. Once agarose is gelified the brain is cut in the vibratom as required. For presented experiments, the brain is cut transversally with 30-40 µm thickness at the region of interest. The staining of the slices after cutting is performed as follows: first, the brain slices are blocked o.n. at 4 °C with blocking solution (see Tab. 6). Second, the first antibody incubation (first antibodies are listed in table 4) takes place o.n. at 4 °C. Third, the slices are washed with PB followed by secondary antibody incubation (secondary antibodies are listed in table 5) for 2 h. at r.t. in darkness. Fourth, slices are washed several times with PB. Fifth, short incubation is

conducted with Hoechst staining diluted 1/10,000 during 5 min. and at r.t. to stain cell nucleus. Finally, the brain slices are placed on a clear glass slide, air dried and closed with immunomount and a coverslip. The closed slides are kept at 4 °C until microscopy.

Scholl analysis

The length of the longest neurite is measured using Metamorph software and, depending on the staining, the number of neurites or dendrites are counted. For branching studies, pictures from the EGFP channel of transfected neurons are printed out and overlaid with a calibrated Scholl analysis circle patterns. Scholl analysis circle patterns have a radii increasing with 10 µm per circle, starting at a radius of 10 µm until a maximum radius of 70 µm. First 10 µm circle is overlaid with the cell nucleus and then all crossing points between branches and circles counted. Statistical analysis is done using GraphPrism 4.

Confocal microscopy

A Leica TCS SL confocal microscope was used. The 488 nm line of an argon lase is used for excitation of Alexa 488 whereas an helium-neon laser is used for excitation of Alexa 594. For Hoechst staining a cube 405 nm is used. Spatial z-steaks are taken with each image with a line average and frame average of 3 pictures and averaged along the z-axis. Excitations and recording of associated channels are done sequentially to prevent dye cross-talk.

4.2.5 Data and statistical analysis

Data analysis is performed using GraphPrism 4. Data are reported as mean +/- SEM, which are also presented as error bars in the figures. Statistical significance is assessed using the two tailed student's t-test for paired or unpaired data at the given significance level (p) where: *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$ and no significant (ns) ns $p > 0.05$.

5 Results

A Yeast two-hybrid screening had been previously conducted in the laboratory[3] using the unique PRG-1 C-Terminus as a bait for a Yeast two-hybrid screening (Cyto - Trap XR from Stratagene). The screening yielded several putative interaction candidates (data not showed) and among all the putative interaction partners Ras GRF-2 was found.

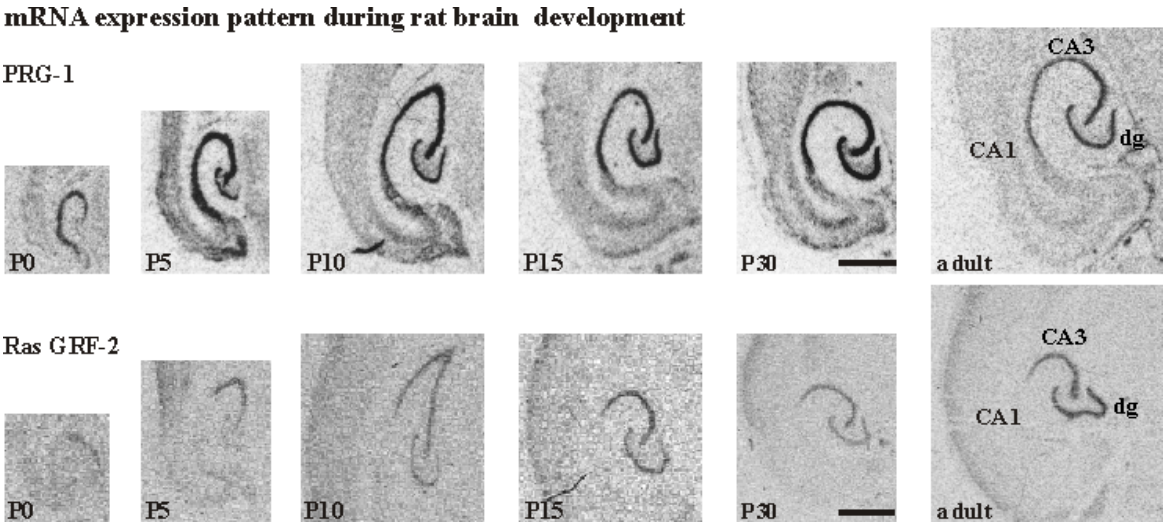
5.1 Expression studies

In order to have an interaction, proteins should be expressed at the same time and at the same location. Protein components are synthesised and assembled into the membranes of cell extensions through pathways of membrane biogenesis that have been elucidated primarily in non neuronal cells [1]. However, some adaptations of these general mechanisms have been necessary, due to the specific requirements of cells in the nervous system. The distribution to specific loci of organelles, receptors, ion channels and other proteins is critical to normal neuronal function and essential when postulating interaction between two proteins [89]. Therefore expression studies were done and they were performed not only at different levels (cells and tissue) and at different developmental stages but also using several technical approaches studying mRNA levels (5.1.1, 5.1.2) and protein levels (5.1.3) [90].

5.1.1 *In situ* hybridization of Ras GRF-2

The first approach used was the *in situ* hybridization to check mRNA levels of Ras GRF-2 in the rat brain during development in order to compare the mRNA expression of the putative interaction candidate with PRG-1 mRNA expression published in 2003 [42]. The obtained mRNA signals for Ras GRF-2 are clearly located in the hippocampus (mainly in the *dentate gyrus* - dg - as well as in the *cornu ammonis 3* - CA3 [91]) and show an increase after birth until adulthood (Fig. 9).

[3]This experiment was done before I began with the project but it has been included in the materials and methods and it is firstly mentioned in the results because of its importance for the project.



CA: *cornu ammonis*; dg: *dentate gyrus* [91]

Figure 9: Expression studies using *in situ* hybridization. *In situ* hybridization results for PRG-1 (Bräuer et al. 2003) and Ras GRF-2 in rat brain from birth (Postnatal Day 0 - P0) until adulthood. Scale bar is 1 cm.

5.1.2 quantitative RT PCR

The mRNA level of PRG-1 and Ras GRF-2, as well as N-Ras expression, has been studied during mouse brain development and in cortical primary neurons, as detailed in Figure 10 and the Supplementary Figure D in the appendix. All set of experiments were done using two housekeeping genes, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Hypoxanthine Phosphoribosyl Transferase (HPRT). However, hierunder only results obtained using GAPDH will be presented because a slight expression variation of HPRT was detected during brain development and therefore this gene could not longer be considered as housekeeping gene for the current approach.

On the one hand, mRNA levels were analysed during brain development using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as a housekeeping gene. PRG-1 and Ras GRF-2 expression typically features a progressive increase during embrional stages, followed by a maximum level around birth and a plateau thereafter. In contrast, N-Ras mRNA levels are higher during embryo development than after birth, when levels tend to decrease. The project work has been done mainly with cortical neurons, therefore, mRNA studies from cortex are showed in Figure 10a. However, mRNA levels in other brain structures, such as bulbus olfactory or hippocampus, were also studied and showed the same expression pattern but with a weaker signal than in the cortex. The mRNA levels of PRG-1, Ras GRF-2 and N-Ras in other brain structures are detailed as a supplementary figure in the appendix (Suppl. Fig. D).

On the other hand, mRNA levels in cortical primary neurons have been also measured. PRG-1 and N-Ras mRNA are significantly higher in 7 Days *in vitro* (DIV) neurons than in 14 DIV, while Ras GRF-2 expression level is similar in both time points (Fig. 10b).

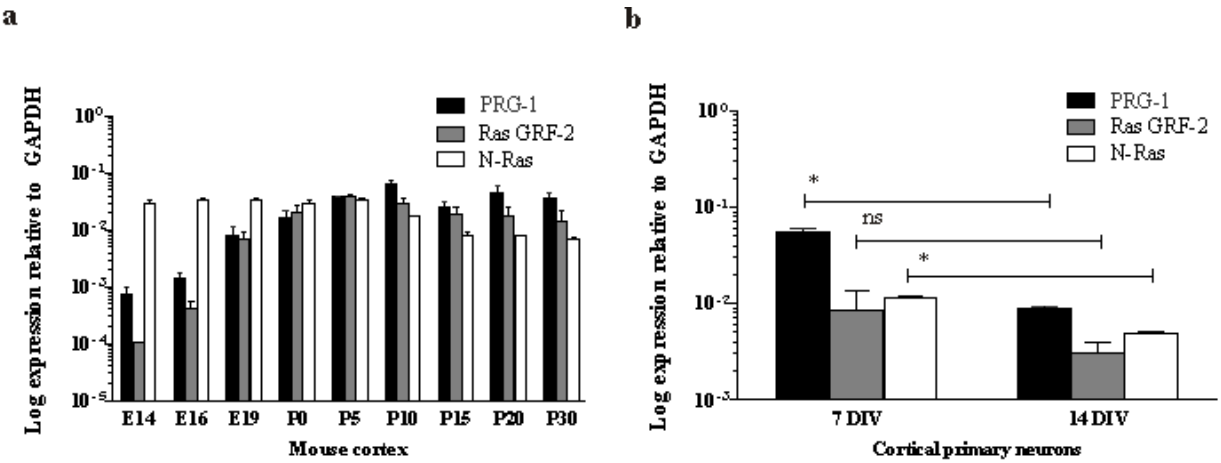


Figure 10: Expression studies using quantitative RT PCR. a) mRNA expression pattern during development in neocortex mouse brain of PRG-1, Ras GRF-2 and N-Ras, using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as a Housekeeping gene (n=9). **b)** mRNA expression in 7 DIV and 14 DIV cortical primary neurons of PRG-1, Ras GRF-2 and N-Ras relative to GAPDH (n=9). Values correspond to mean +/- SEM and statistical analysis was performed using two-tailed t-test and p values are considered: *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$ and no significant (ns) ns $p > 0.05$.

5.1.3 Immunofluorescence

Immunofluorescence can be used to determinate whether two proteins share the same location within a cell at a defined time point. Immunofluorescence is a non invasive method that preserves the physiological conditions under which proteins may interact in a true cell environment. Therefore, it is generally accepted that if two endogenous proteins are located in the same region there is a possibility that they may bind to each other. Endogenous colocalisation is used in conjunction with other techniques to characterise protein-protein interaction in a true mammalian environment [90].

Endogenous Ras GRF-2 and PRG-1 expression were analysed at protein level not only in mouse brain tissue (Fig. 11a) but also in cortical primary neurons (Fig. 11b). In the brain tissue (Fig. 11a), the colocalisation is detected in structures such as cortex, *dentate gyrus* and CA3 [91] and at the neuron level, the colocalisation between both proteins is showed at the plasma membrane (Fig. 11b).

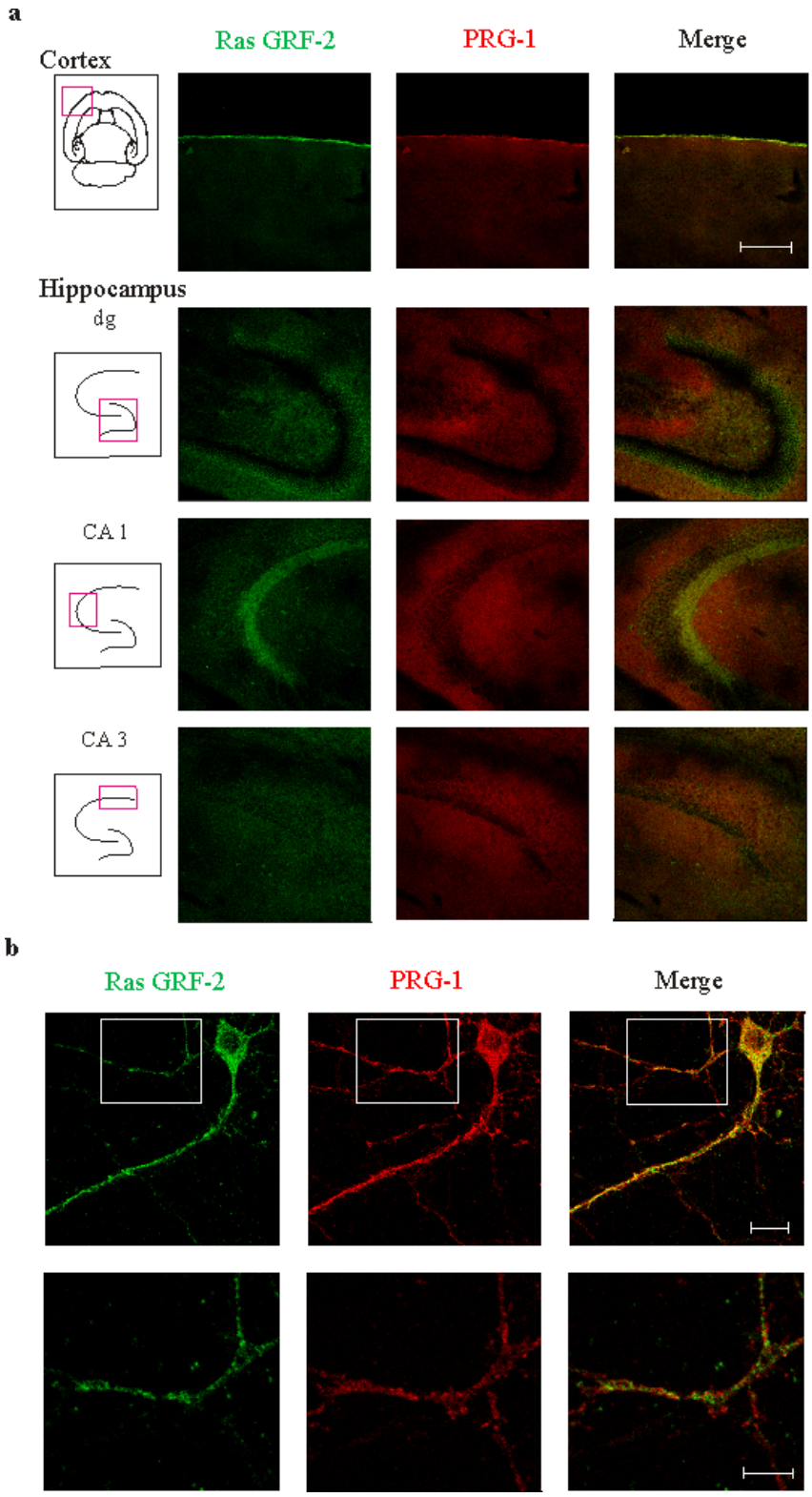


Figure 11: Expression studies using immunofluorescence techniques. a) PRG-1 and Ras GRF-2 coexpression and colocalisation in mouse adult brain; endogenous staining of Ras GRF-2 (in green) and PRG-1 (in red) in P15 mouse brain sagittal slices at cortex and hippocampus (dg: *dentate gyrus*; CA: *cornu ammonis*) [91]. Scale bare is 200 µm. **b)** PRG-1 and Ras GRF-2 coexpression and colocalisation in cortical primary neurons culture; endogenous staining of Ras GRF-2 (in green) and PRG-1 (in red) on 12 DIV cortical primary neurons. Scale bar for first raw is 10 µm and for the second raw is 5 µm.

5.2 Interaction studies

The study of a possible interaction between PRG-1 and its putative interaction partner Ras GRF-2 can began once it is proved that both elements of study are expressed at the same time and colocalise at protein level in the brain and in fact, they do so at the plasma membrane of neurons. Interaction studies can be done overexpressing the constructs (5.2.1) or checking within models in which the protein of interest are endogenously expressed (5.2.2, 5.2.3, 5.2.4).

5.2.1 Interaction in HEK cells

As already mentioned, a yeast two-hybrid screening had been previously conducted in the laboratory[4] using the unique PRG-1 C-Terminus as a bait. In order to validate the results from the yeast two-hybrid screening in mammalian cells, the binding properties between the fusion proteins cMyc-PRG-1 and Flag-Ras GRF-2 were analysed in an immunoprecipitation assay using transiently transfected HEK 293 cells (Fig. 12).

In this first approach, the interaction studies were done in both directions using anti-Flag as well as anti-cMyc for the immunoprecipitation (IP) and checking the immunoprecipitated protein and the coimmunoprecipitated protein at the Immuno Blots (IB). Both IP assays show an interaction between PRG-1 and Ras GRF-2 and none of them shows crosslinking between the protein lysate and the protein G sepharose used within the IP protocol.

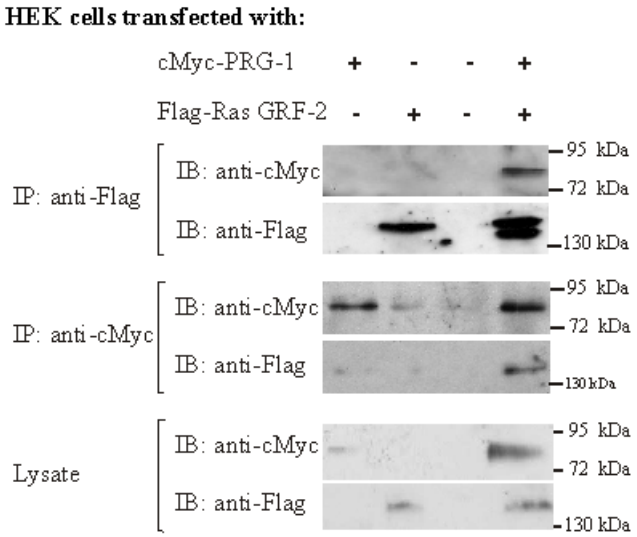


Figure 12: Interaction between PRG-1 and Ras GRF-2 in HEK cells. Total lysates of HEK 293 cells transient transfected with either one or both tagged constructs (cMyc-PRG-1 and Flag-Ras GRF-2) were immunoprecipitated using anti-Flag (IP: anti-Flag) or anti-cMyc (IP: anti-cMyc) antibodies. The samples were loaded in SDS-Page and Immuno Blots (IB) developed using anti-Flag (IB: anti-Flag) or anti-cMyc (IB: anti-cMyc) antibodies. IB show interaction between Flag-Ras GRF-2 and cMyc-PRG-1 in both experiments and no cross reactivity between beads and constructs on single transfected lanes. Total lysates (Lysate) are added at the bottom of the figure as transfection controls.

[4]This experiment was done before I began with the project but it has been included because of its importance for the project.

5.2.2 Endogenous interaction in cortical primary neurons

- **Using PRG-1 antibody**

To confirm the interaction detected in an overexpressed system, a second set of IPs was done using a system in which both proteins of interest are endogenously expressed. For that approach the IP assays were performed using cortical primary neurons lysate and only antibody against PRG-1.

14 DIV cortical primary neurons were used to analyse the endogenous interaction between PRG-1 and Ras GRF-2 (Fig. 13a, first two lanes) and as depicted in this figure, endogenous interaction was detected in wild type animals. However, for further understanding of the endogenous interaction and in order to delete possible cross-linking between PRG-1 and other LPA receptors, the same interaction hypothesis was analysed using cortical primary neurons which have no LPA receptor. LPA_2 receptor is the strongly expressed LPA receptor in neurons (Personal communication from Dr. Kieselmann) and therefore, cortical primary neurons from LPA_2 receptor knock out ($LPA_2^{-/-}$) animals were taken (Fig. 13b, first two lanes). Thus demonstrating that the same positive result for PRG-1 and Ras GRF 2 interaction as wild type cortical primary neurons was obtained.

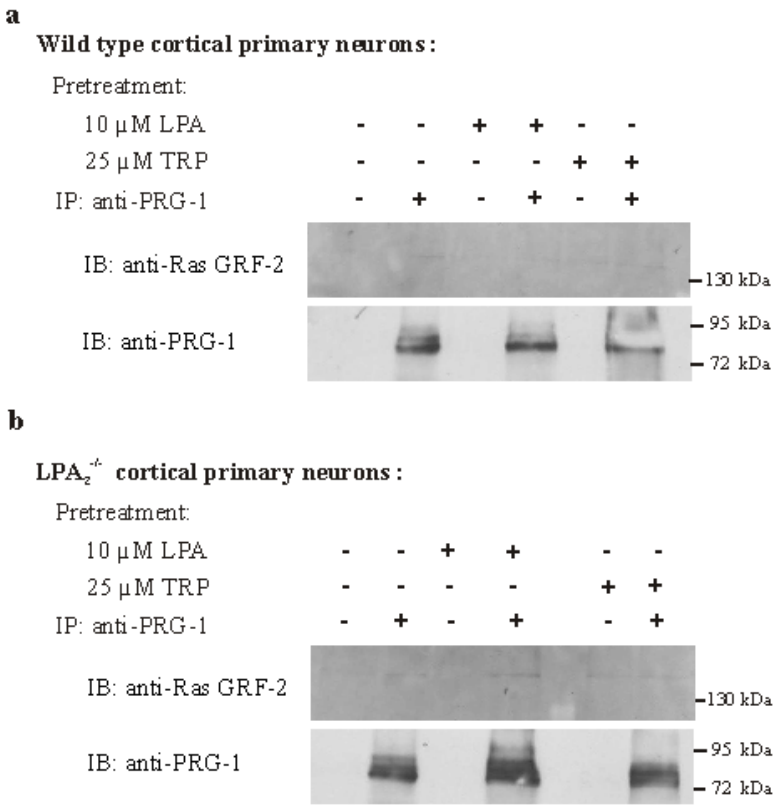


Figure 13: Endogenous interaction between PRG-1 and Ras GRF-2 using PRG-1 antibody. a) Interaction in wild type cortical primary neurons. Endogenous interaction in 14 DIV neurons with LPA as well as after TRP treatment and without any pretreatment. Immunoprecipitation using PRG-1 antibody and immunoblots using PRG-1 as well as Ras GRF-2 antibodies. **b)** Interaction in $LPA_2^{-/-}$ cortical primary neurons. Endogenous interaction in 14 DIV neurons obtained from $LPA_2^{-/-}$ animals; interaction studies without any pretreatment or with either LPA or TRP pretreatment. Immunoprecipitation using PRG-1 antibody and immunoblots using PRG-1 as well as Ras GRF-2 antibodies. Each blot of the figure is representative for at least three independent experiments.

Wild type and $LPA_2^{-/-}$ cortical primary neurons show endogenous interaction between PRG-1 and Ras GRF-2, when immunoprecipitating with PRG-1 antibody.

- **Using Ras GRF-2 antibody**

Interaction studies are typically done in both directions, using antibodies against both proteins of interest for two separate sets of immunoprecipitaion (IP) experiments. Such an analysis was conducted in the HEK cells where the interaction could be proved using antibodies against both tags (5.2.1). Endogenous IP assays showed so far were done only using PRG-1 homemade antibodies (Fig. 12, Fig. 13).

Interaction studies using Ras GRF-2 antibody were also carried out. However, when using Ras GRF-2 antibody, the interaction could not be consistently assessed (Fig. 14). In some situations it was possible to detect interaction, if the amount of Ras GRF-2 antibody used was twice the usual value (Fig. 14a lane 4), but in other assays the interaction was not reproducible (Fig. 14b lane 3 and 6).

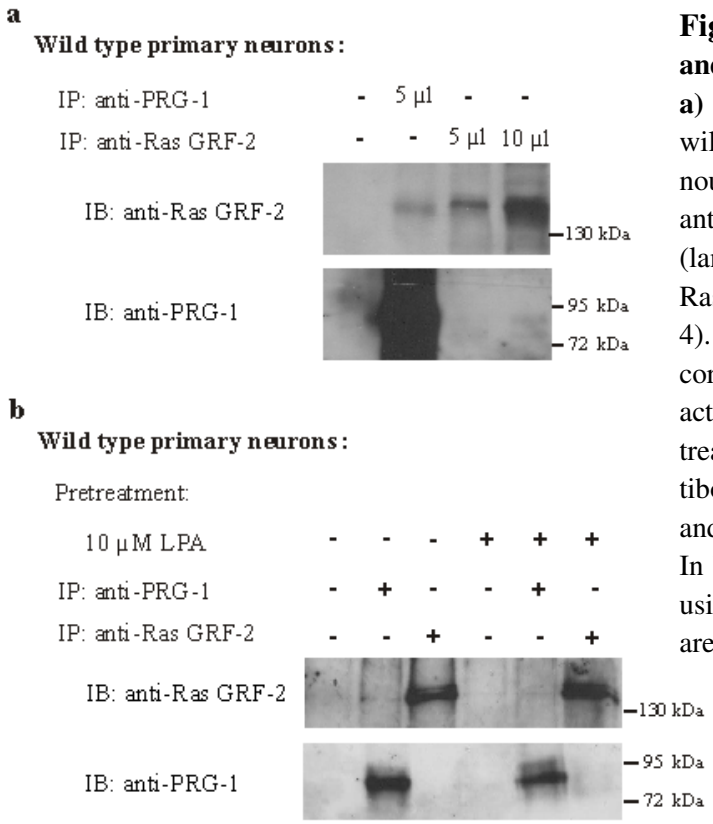


Figure 14: Interaction between PRG-1 and Ras GRF-2 using Ras GRF-2 antibody. **a)** PRG-1/Ras GRF-2 interaction in cortical wild type cortical primary neurons. Endogenous interaction in 14 DIV neurons without antibody (lane 1) with 5 µl PRG-1 antibody (lane 2) and with increasing concentrations of Ras GRF-2 antibody: 5 µl or 10 µl (lane 3 and 4). **b)** Interaction studies in cortical wild type cortical primary neurons. Endogenous interaction in 14 DIV neurons with or without LPA treatment. Immunoprecipitation using no antibody (lane 1 and 4), PRG-1 antibody (lane 2 and 5) or Ras GRF-2 antibody (lane 3 and 6). In both panels Immuno Blots (IB) developed using Ras GRF-2 as well as PRG-1 antibodies are showed.

It is interesting to note that when using $LPA_2^{-/-}$ brain areas the interaction could be proved in both directions (Fig. 16). However, the inconsistency on the immunoprecipitation of PRG-1 using Ras GRF-2 antibody will have to be further commented while developing the discussion.

5.2.3 Endogenous interaction disruption in cortical primary neurons after pretreatments

- **Pretreatment with extracellular LPA application**

In order to examine the potential role of the interaction, and knowing not only that LPA has been previously showed to prevent neurite retraction in PRG-1 transfected neuroblastoma cells [42] but also that PRG-1 was postulated as phospholipid controller in the brain [43], the effects of LPA on the interaction detected in cortical primary neurons were studied.

Extracellular LPA application in cortical primary neurons induces disruption of PRG-1/Ras GRF-2 interaction, as depicted in figure 13a (lanes 3 and 4). In fact, after extracellular LPA application PRG-1 is detected but the amount of coimmunoprecipitated Ras GRF-2 decreases.

In order to define whether the disruption of the interaction between PRG-1 and Ras GRF-2 is dependent only on the LPA application, a parallel set of experiments was conducted using the same method but with different neuron pretreatment, using Thrombin Receptor activating Peptide (TRP) instead of LPA. TRP was chosen for control purposes because Thrombin and LPA were the first GPCR agonists showed to stimulate Ras GTPs [92].

The interaction does not change when cortical primary neurons were pretreated with TRP instead of LPA, the results are showed in figure 13a (last two lanes). Therefore, extracellular application of TRP does not affect the PRG-1/Ras GRF-2 interaction, which is consistent with the hypothesis of a specific effect between the extracellular application of LPA and PRG-1/Ras GRF-2 interaction disruption.

The analysis of PRG-1/Ras GRF-2 interaction on cortical primary neurons obtained from $LPA_2^{-/-}$ animals is showed in figure 13b. The results after LPA treatment can be seen on lanes 3 and 4, and the result after control treatment using extracellular application of TRP (lanes 5 and 6) of $LPA_2^{-/-}$ animals also are depicted. The results indicate that there is no effect of $LPA_2^{-/-}$ in the studies interaction between PRG-1 and Ras GRF-2.

Generally, all endogenous IP studies presented here showed interaction between PRG-1 and Ras GRF-2 and, indeed, they show an interaction disruption only after extracellular LPA application and no change on the interaction can be detected after TRP application independently of the animals used (w.t or $LPA_2^{-/-}$).

- **Pretreatment with extracellular Ki 16425 application**

A part form the $LPA_2^{-/-}$ approach one can determinate the specificity of the interaction using chemical blockers. To determinate whether the disruption of the interaction between PRG-1 and Ras GRF-2 was specific for the LPA application, I performed a parallel set of experiments in which the same immunoprecipitation method was used except that before the pretreatment with LPA an LPA receptor antagonist - Ki 16425 - was applied. Ki 16425 is a LPA receptor antagonist known to exhibit Ki values of 0.34, 6.5 and 0.93 μM for the human LPA_1, LPA_2, and LPA_3 receptors [34]. Interestingly, LPA_2 receptor is the LPA receptor more expressed within brain cortical neurons and application of Ki 16425, at 10 μM, has been significantly proved to block the response of a variety of cancer cell lines to LPA-induced cell migration [93].

This chemical approach proves no direct dependency of the interacting partners (PRG-1 and Ras GRF-2) to the LPA receptors because when LPA_2 receptor is chemically blocked (Figure 15 lanes 5,6,7 and 8) the same interaction results between completely untreated cells (lanes 1,2,3 and 4) and Ki 16425 pretreated cells are obtained.

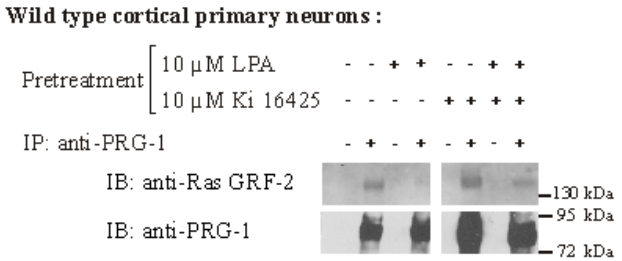


Figure 15: Endogenous interaction between PRG-1 and Ras GRF-2 using PRG-1 antibody and LPA receptor blockers. Interaction in wild type cortical primary neurons using a LPA_2 antagonist: Ki 16425. Endogenous interaction in 14 DIV neurons with and without Ki 16425 treatment followed with LPA treatment. Immunoprecipitation using PRG-1 antibody and immunoblots using PRG-1 as well as Ras GRF-2 antibodies. Each blot of the figure is representative for at least three independent experiments.

These interaction studies with LPA receptor antagonist correlate with the interaction studies done using LPA_2 $^{-/-}$; both approaches showed the same results, namely that the PRG-1/Ras GRF-2 interaction was disrupted specifically after extracellular application of LPA and not modified after blocking LPA receptors.

5.2.4 Interaction studies using brain tissue

Interaction assays at organ level were carried out using PRG-1 and Ras GRF-2 antibodies from wild type animals as well as from $LPA_2^{-/-}$ animals.

- **Brain tissue from wild type animals**

In wild type whole brains no interaction was detected (Fig. 16). No signal can bee seen while performing immunoprecipitation (IP) with Ras GRF-2 (Fig. 16 lane 2) and analysing PRG-1 and no signal can be detected neither when IP was done using PRG-1 (Fig. 16 lane 3) and Ras GRF-2 was analysed.

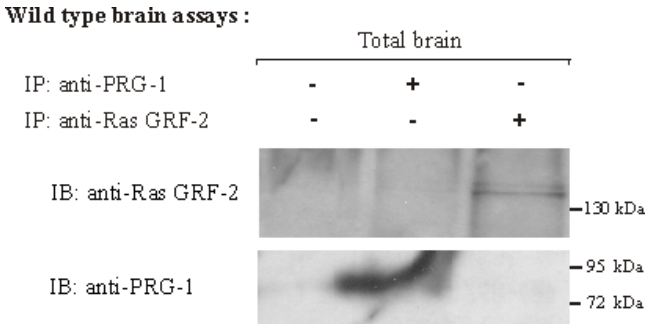


Figure 16: Interaction studies using wild type adult mouse brain. PRG-1/Ras GRF-2 interaction in brain obtained from adult wild type animals. Endogenous interaction in brain without antibody (lane 1), with PRG-1 antibody (lane 2) and with Ras GRF-2 antibody (lane 3). Immuno Blots (IB) using Ras GRF-2 as well as PRG-1 antibodies are showed.

- **Brain tissue from $LPA_2^{-/-}$ animals**

In $LPA_2^{-/-}$ brains separating different brain areas the interaction was detected in both directions, using PRG-1 antibody and Ras GRF-2 (Fig. 17). When analysing endogenous interaction within $LPA_2^{-/-}$ brains in specific brain areas, such as hippocampus and cortex, interaction can be detected in both directions. Immunoprecipitates of PRG-1 show Ras GRF-2 (Fig. 17 lanes 2 and 5) and the ones in which IP was done with Ras GRF-2 antibody were PRG-1 positive (Fig. 17 lanes 3 and 6).

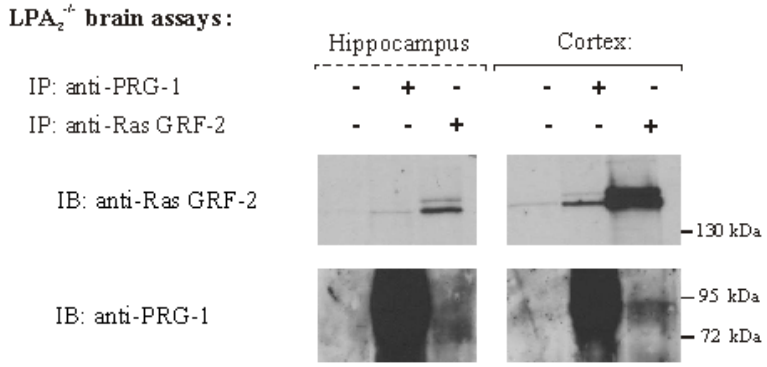


Figure 17: Interaction studies using adult $LPA_2^{-/-}$ mouse brain. Interaction studies in adult brain sections obtained from $LPA_2^{-/-}$ animals. Endogenous interaction in hippocampus or cortex obtained from $LPA_2^{-/-}$ animals. Immunoprecipitation using no antibody (lanes 1 and 4), PRG-1 antibody (lanes 2 and 5) or Ras GRF-2 antibody (lanes 3 and 6). Immuno Blots (IB) using Ras GRF-2 as well as PRG-1 antibodies are showed.

An important point to develop within the discussion will be the controversy due to the positive immunoprecipitation results obtained using either cortical primary neurons or some parts of the brain and against the negative results obtained while using total brain lysates.

5.2.5 Mapping the molecular interaction between PRG-1 and Ras GRF-2

Systematic screening through several databases was done during the project in search for any possible updated information linked to homologies which could provide some directions to the putative domains involved in protein interactions and therefore, to sheld light into the characterisation of the point where PRG-1/Ras GRF-2 interaction occurs. It is very important to note that, so far, only recently a single publication has postulated a non classical Calcium binding domain within the unique large PRG-1 C-terminus[94] and a N-ethylmaleimide Sensitive Fusion (NSF) protein interaction (personal communication from Mrs. Geist), thus leaving the intracellular structure of PRG-1 as a big challenge.

In order to map the interaction, several deletion mutants of PRG-1 were cloned using PCR techniques (see schematic view of all deletion constructs used for that purpose in Figure 18 and the primers used for cloning as supplementary information in the appendix in Table G). Because of the lack of classical domains within the C-terminus of PRG-1 three different deletions were done, namely, deleting 1/3, 2/3 or the whole of the C-terminus.

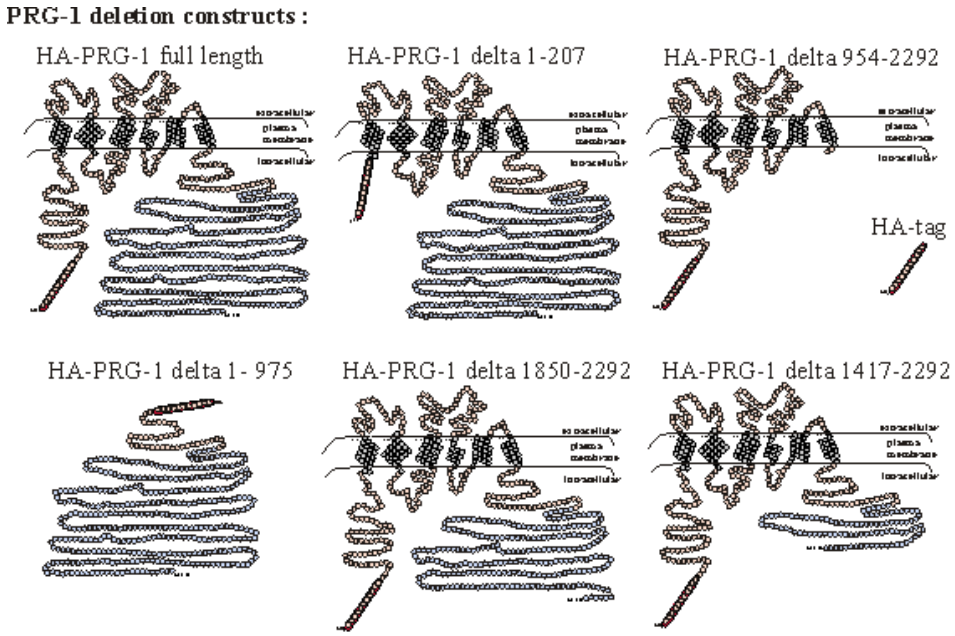


Figure 18: PRG-1 deletion constructs schema. PRG-1 deletion mutants were done using PCR and have an HA-tag on its N-terminus. On each mutant one part of PRG-1 has been deleted in order to allow the identification of the essential region for the interaction with Ras GRF-2. The primers used for cloning the deletion constructs can be found in the Suppl. Tab. G

HA tagged PRG-1 mutants lacking the N-terminal (HA-PRG-1 delta 1-207), the C-terminal (HA-PRG-1 delta 954-2292), 1/3 or 2/3 of the C-terminal (HA-PRG-1 delta 1850-2292 and HA-PRG-1 delta 1417-2292 respectively) or having only the C-terminal fragment of PRG-1 (HA-PRG-1 delta 1-975) were cotransfected in HEK 293 cells with Flag-Ras GRF-2. HA-PRG-1 mutants capacity of interaction with full length Flag-Ras GRF-2 was tested through immunoprecipitating HA-tag proteins with µMACS® (Fig. 19).

PRG-1 mutants lacking 2/3 and 1/3 part of the C-terminal or the whole C-terminal were found to be still capable of interacting with Flag-Ras GRF-2 as effectively as full length PRG-1 does (Fig. 19 lanes 1, 3, 5 and 6). In contrast, PRG-1 mutants composed with only the C-Terminus or only missing the N-terminus did not interact with Flag-Ras GRF-2 (Fig. 19 lanes 2 and 4). The transfection efficiency of all double transfections can be checked on the bottom Immuno Blots (IB) of the Figure 19 where total cell lysates are showed and Flag-Ras GRF-2 expression is detected in all lanes.

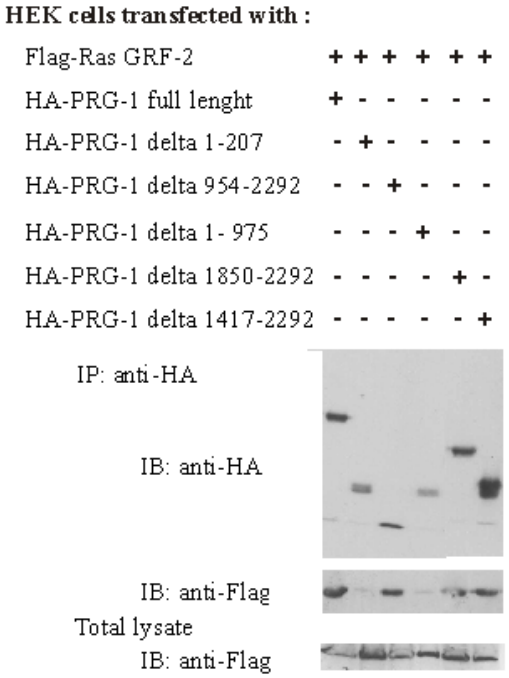


Figure 19: Mapping PRG-1/Ras GRF-2 interaction using several PRG-1 deletion constructs. Interaction studies between PRG-1 mutants and Ras GRF-2; HEK cells were cotransfected with one of the HA-tagged PRG-1 mutants and Flag-Ras GRF-2 and its pulldown using HA antibody was analysed using HA antibody as well as Flag antibody. At the bottom of the panel, total lysate developed using Flag antibody shows proper expression of Flag-Ras GRF-2 in all lanes.

5.2.6 Deletion constructs expression and location

To study the controversy of the interaction mapping results in which two constructs with all C-Terminus showed no interaction, the cellular localization of all constructs was studied using two different approaches, through cell fractioning and through immunohistochemistry. For this purpose all constructs were overexpressed and their location at the plasma membrane studied.

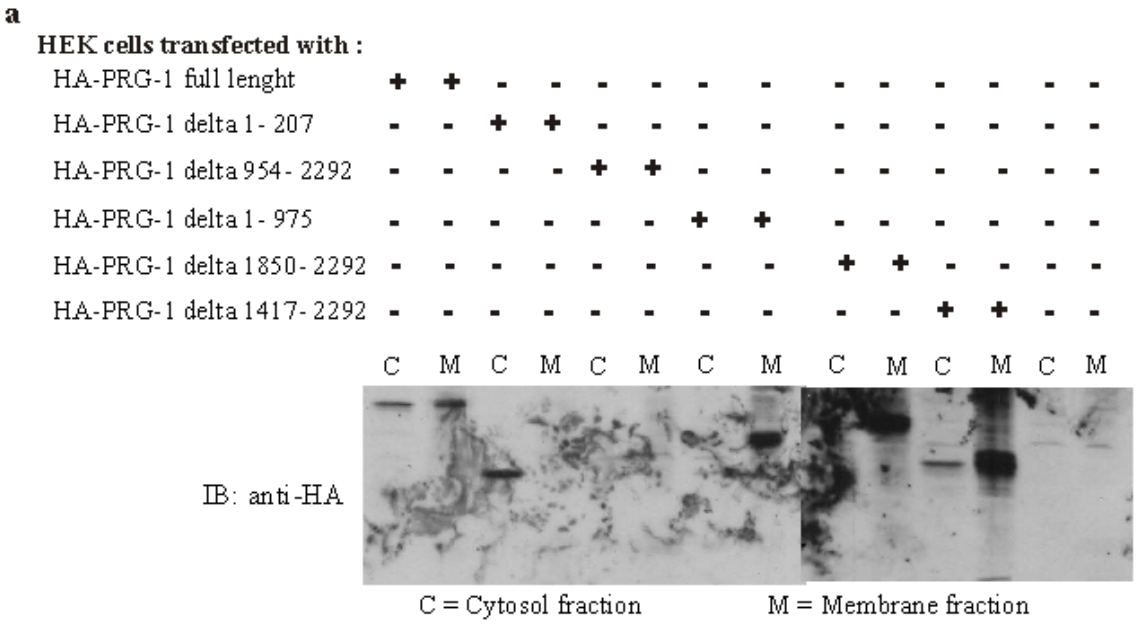
On the one hand, HEK 293 cells overexpressing one PRG-1 mutant construct were prepared, lysated and ultracentrifuged to separate the cytosol fraction (C) from the membrane fraction (M). The results are depicted on Figure 20a. Interestingly, HA-PRG-1 delta 1-207 construct does not reach the plasma membrane; this mutant is detected only in the cytosolic fraction (Fig. 20a lane 3) and no trace of the protein appears on the membrane fraction (Fig. 20a lane 4). Therefore, HA-PRG-1 delta 1-207 has no option to interact with Flag-Ras GRF-2. Meanwhile, the other construct which does not show interaction (HA-PRG-1 delta 1-975) is properly expressed and appears only in the membrane fraction (Fig. 20a lanes 7 and 8). However, the membrane fraction includes also other membranes a part form the plasma membrane and that can be studied with immunofluorescence.

On the other hand, to clarify the exact expression of all PRG-1 mutant constructs an immunofluorescent approach was used. HEK 293 were transiently transfected and stained with a plasma membrane marker ATPase[5] in order to localise the construct within the cell.

In Figure 20b, confocal images of HEK cells transiently transfected with one of both contro-

[5] ATPase are a class of enzymes that catalyse the composition of ATP to ADP and a free phosphate ion. They are integral membrane proteins anchored within biological membranes and therefore are suitable to be used as a membrane marker.

versial constructs, plus one extra construct in which PRG-1 delta 1-975 is specifically membrane tagged, are detailed. For HA-PRG-1 delta 1-207 there is clearly no colocalisation, between the membrane marker and the construct expression, corroborating the result of the cellular fractioning experiment where the construct is detected only in the cytosolic fraction. For HA-PRG-1 delta 1-975, instead, some colocalisation is detected although the major signal appears around the cellular nucleus, probably in the Endoplasmic Reticulum (ER) rather than at the plasma membrane.



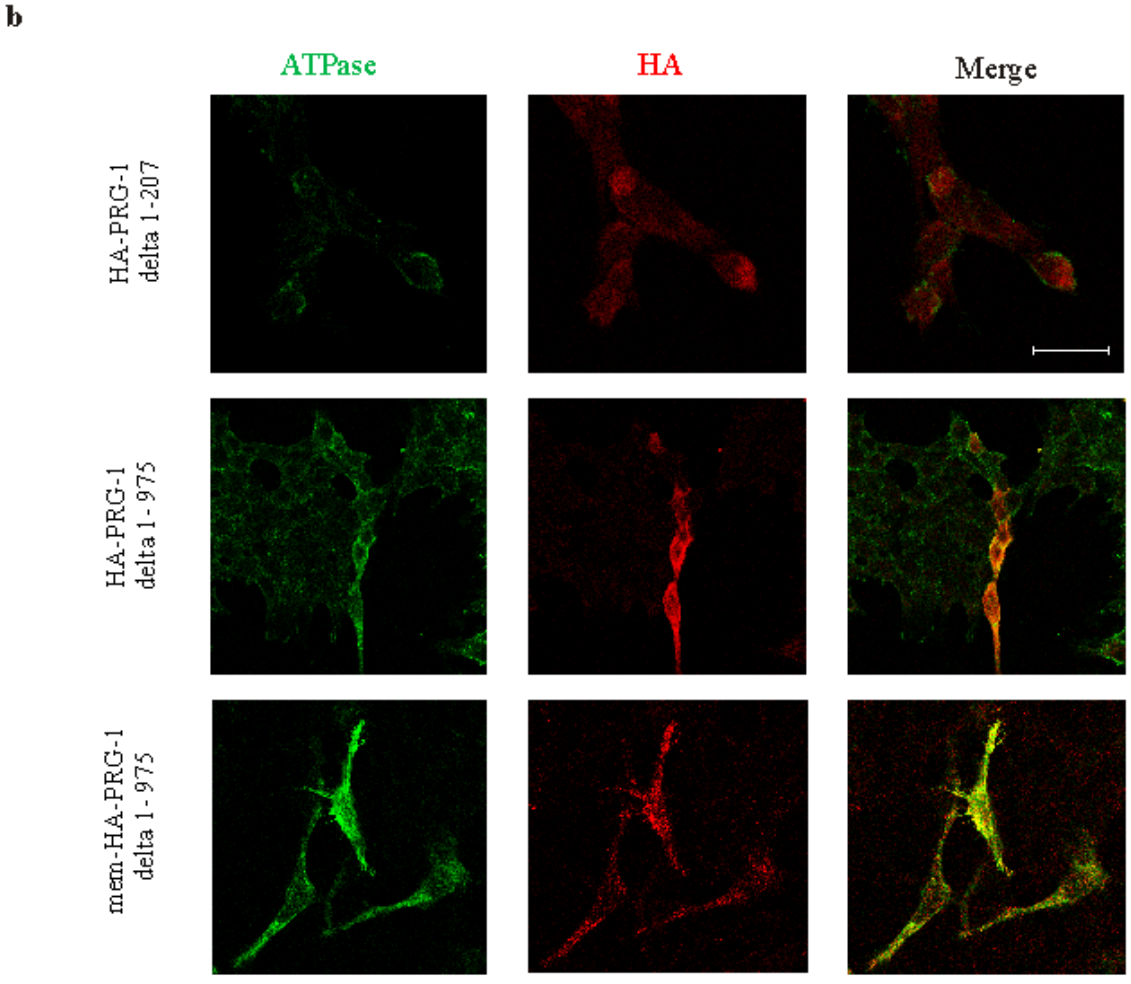


Figure 20: PRG-1 mutant constructs localisation within HEK cells after transient transfection. a) Expression studies. HA-PRG-1 mutants expression and localisation either on the cytosol (C) or at the membrane (M) fraction was assessed using ultracentrifugation. **b)** Immunofluorescence expression studies of HA-PRG-1 delta 1-207, HA-PRG-1 delta 1-975 and mem-HA-PRG-1 delta 1-975 stained with anti-HA (in red) and in colocalisation with the plasma membrane marker ATPase (in green). Scale bar represents 30 µm.

5.2.7 Phosphatidic Acid (PA) mediates PRG-1/Ras GRF-2 interaction

After the difficulties that appeared while mapping the interaction, another approach towards the mapping was taken. The new direction was taken to investigate the affinity of PRG-1, Ras GRF-2 and PRG-1 delta 1-975 towards specific membrane lipids, assuming a role of the membrane components on the studied protein-protein interaction. HA-PRG-1 full length, HA-PRG-1 delta 1-975, mem-HA-PRG-1 delta 1-975 as well as HA-Ras GRF-2 protein were purified from HEK 293 cells and later incubated with commercially available lipid dot blots which contain a known pattern of phosphoinositides and phospholipids (Fig. 21a). Results should bring light into protein binding pattern towards membrane lipids.

The full length PRG-1 binds to a variety of lipids, with its highest binding to Phosphatidic Acid (PA) and a lower but detectable binding to PtdIns(4)P and PtdIns(3,4,5)P3 (Fig. 21b see recombinant HA-PRG-1 full length). The Ras GRF-2 protein displayed specific binding only for PA even though the exposition times were increased (Fig. 21b see recombinant HA-Ras GRF-2 full length). Interestingly, testing HA-PRG-1 delta 1-975 did not show specificity to any lipid in particular even after using increased exposition times (Fig. 21b see recombinant HA-PRG-1 delta 1-975), showing no pattern like the control membrane in which protein solution without purified protein was used for first incubation. Interestingly, testing mem-HA-PRG-1 delta 1-975 (Fig. 21b see recombinant mem-HA-PRG-1 delta 1-975) construct show an affinity pattern completely different form full length PRG-1 and Ras GRF-2 namely affinity towards PtdIns(4,5)P2.

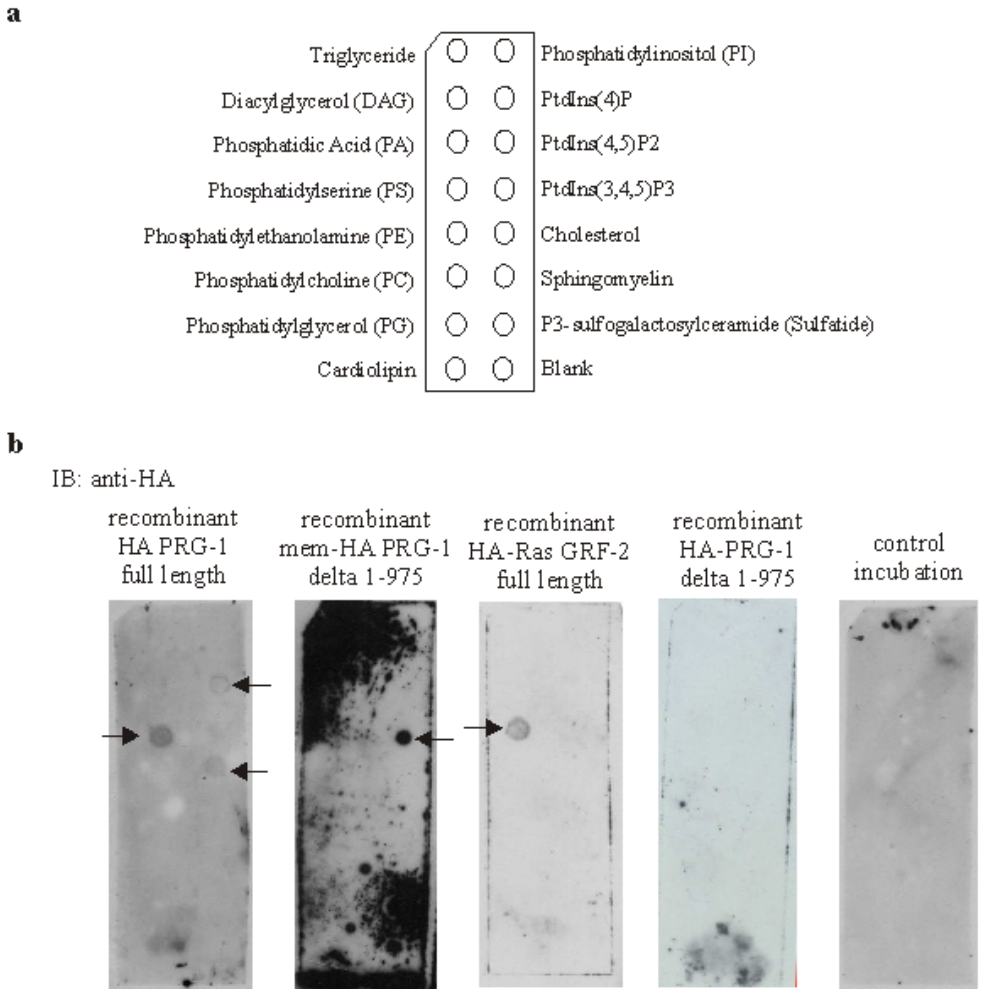


Figure 21: Protein-lipid affinity using lipid blots. **a)** Schema of the commercial lipid dot blots where the studied lipids and their location are detailed. **b)** Purified recombinant protein (HA-PRG-1 full length, HA-PRG-1 delta 1-975 and also HA-Ras GRF-2) or only the control solution (control incubation) was incubated with commercial lipid dot blots to study affinity between protein and different lipids which typically compound the cellular plasma membrane.

Therefore, both interacting proteins, PRG-1 and Ras GRF-2, have the same particular affinity to Phosphatidic Acid and not to other lipid patterns and the non interacting PRG-1 delta 1-975 construct shows no similar pattern indication, once more, of no specificity for plasma membrane.

5.3 Intracellular signalling cascades

Almost all aspects of neuronal function, from its maturation during development, to its growth and survival, cytoskeletal organisation, gene expression, neurotransmission, and use dependent modulation, are dependent on intracellular signalling initiated at the cell surface [58]. The response of neurons to neurotransmitters, growth factors and other signalling molecules is determined by their complement of expressed receptors and pathways that transduce and transmit these signals to intracellular compartments and the enzymes, ion channels, and cytoskeleton proteins that ultimately mediate the effects of the neurotransmitters [1]. Cellular responses are further determined

by the concentration and localisation of signal transduction components.

Signals are mediated through second messengers activating proteins that modify cellular processes and gene transcription. Phosphorylation of key intracellular proteins, ion channels, and enzymes activates diverse, highly regulated cellular processes [58]. Within cellular signalling two approaches have been taken. One approach has been the analysis of Ras activity (5.3.1) and the other one has been the MAPK signalling study (5.3.2).

5.3.1 Ras activity

Ras activity has been a point of interest mainly due to its link to GEFs [44, 50, 95] but also because Ras proteins are known to induce activation of signalling pathways involved in cell growth [65, 87].

- **PRG-1 overexpression inhibits N-Ras** [6]

To assess whether the interaction between PRG-1 and Ras GRF-2 has consequences on Ras protein activation, a Ras active set of pull downs was done. Several Ras proteins (PAN-Ras, R-Ras, H-Ras and N-Ras) were checked (data not showed). Among them, only N-Ras shows a significant difference between PRG-1 transfected cells (Fig. 22 lane 3) and control transfections done with EGFP and PRG-2 constructs (Fig. 22 lane 2 and 4).

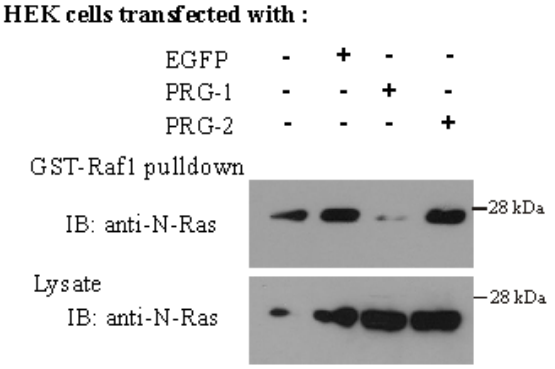


Figure 22: Ras activity in HEK cells. Using GST-Raf1 pulldown on total lysate of HEK 293 cells non transfected or transient transfected either with PRG-1, PRG-2 or EGFP. In the second Immuno Blot (IB) the total amount of N-Ras protein in the cell lysate as a control. IB were developed using N-Ras antibody.

Active-N-Ras is showed in Figure 22, where it can be seen that PRG-1 overexpression in HEK 293 cells leads to a decreased detection of active N-Ras protein (Fig. 22 lane 3) in comparison to both controls (empty EGFP vector and another PRG family member such as PRG-2) as well as comparing to non transfected cells (Fig. 22 lanes 1, 2 and 4).

[6] Ras experiments in the HEK cells overexpressed system were done in collaboration with Prof. Offermanns and performed by Dr. Swiercz.

- **PRG-1 overexpression inhibits N-Ras depending on extracellular LPA** [7]

HEK 293 cells were double transfected with PRG-1 and N-Ras and Ras activation analysis were performed with and without LPA treatment (Fig. 23). In this case, extracellular application of LPA leads to an increased detection of active N-Ras (Fig. 23). Interestingly, the LPA effect can be enhanced (Fig. 23 first two lanes) if cells are starved before the processing. The enhancement of the N-Ras activity is due to the hidden LPA included in the cell culture medium because of the serum supplement. Total lysate is showed on the bottom of the figure as loading control.

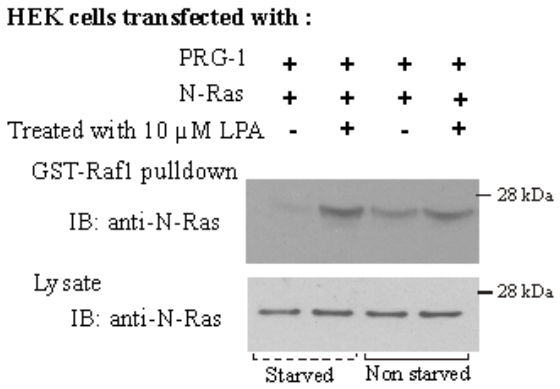


Figure 23: Ras activity in HEK cells after LPA application. HEK cells transiently co-transfected with PRG-1 and N-Ras with or without extracellular LPA and with or without starving before pretreatment. First is showed GST-Raf1 pulldown on total lysate developed using N-Ras antibody. Below, is showed the total amount of N-Ras protein in the cell lysate as a control.

- **Extracellular LPA application affects endogenous N-Ras protein activation level**

LPA applied extracellularly is also modifying the N-Ras activation in cortical primary neurons. When using w.t. cortical primary neurons, the analysis of the amount of active N-Ras in normal conditions or after extracellular application of LPA (Fig. 24a) shows an increase detection of active N-Ras after LPA application. Similar results are detected when analysing the amount of active N-Ras obtained from $LPA_2^{-/-}$ cortical primary neurons (Fig. 24b).

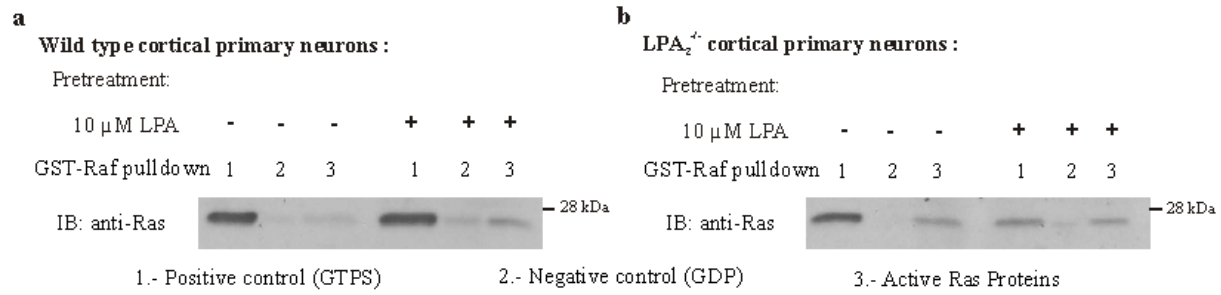


Figure 24: Endogenous Ras activity in cortical primary neurons. a) Study of the Ras activity in non treated or after LPA treated 14 DIV wild type cortical primary neurons. **b)** Study of the Ras activity in 14 DIV $LPA_2^{-/-}$ cortical primary neurons pretreated with extracellular LPA or non treated. Results are representative of at least 3 separate experiments.

[7] Ras experiments in the HEK cells overexpressed system were done in collaboration with Prof. Offermanns and performed by Dr. Swiercz.

5.3.2 MAPK signalling phosphorylation

Downstream signalling of N-Ras is known to happen through MAPK phosphorylation cascade [63, 75]. Protein phosphorylation and dephosphorylation are key processes that regulate cellular function [58]. They play a fundamental role in mediating signal transduction initiated by neurotransmitters, neuropeptides, growth factors, hormones and other signalling molecules [1]. The functional state of many proteins is modified by phosphorylation-dephosphorylation, the most ubiquitous posttranscriptional modification in eukaryotes. A fifth of all proteins may serve as targets for kinases and phoshatases. Phosphorylation or dephosphorylation can rapidly modify the function of enzymes, structural and regulatory proteins, receptors or ion channels taking part in diverse processes without a need to change the level of their expression [58].

At that point, phosphorylation changes in cortical primary neurons with and without extracellular LPA application (Fig. 25a and b) were compared. As explained in the introduction (Fig. 8, from the several kinase cascades, only MEK/ERK and p38 have been linked to GEFs [53, 54]. Therefore, some phosphorylation studies with MEK/ERK[8] as well as p38 phosphorylation were performed to analyse the effect of LPA downstream PRG-1 and Ras GRF-2 interaction.

In cortical primary neurons, the phosphorylation status of MEK, ERK and p38 were compared with and without extracellular LPA application (Fig. 25a). Extracellular application of LPA in cortical primary neurons induced an increase of MEK and ERK phosphorylation but did not induce any change in p38 phosphorylation which had to be confirmed with a control pretreatment of $CaCl_2$ (Fig. 25a). Western blot quantification (Fig. 25b) of p38 bands showed no significant difference between the intensities of non-phosphorylated and phosphorylated bands (p: 0.4035), whereas a significant difference was detected for MEK (p: 0.00396) and ERK (p: 0.0003) phosphorylation in neurons with and without extracellular application of LPA.

[8] ERK pathway studies were performed by the student Mrs. Bardehle during her Master thesis under my supervision.

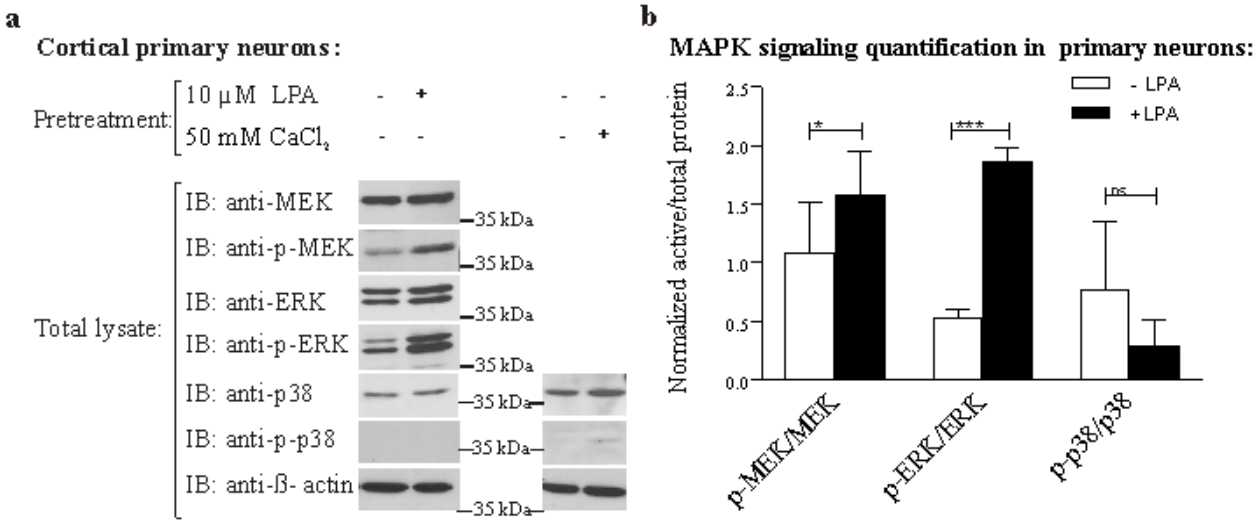


Figure 25: MAPK signalling. a) Effect of exogenous LPA on signalling kinases in 7 DIV cortical primary neurons. Western blot (WB) analysis of total and phosphorylated MEK, ERK and p38 depending on extracellular LPA $CaCl_2$-stimulated neurons on signalling kinases p38 as signalling cascade control to prove p38 phosphorylation within the model. At the bottom of the panel Western Blots are developed against Beta-actin to show an equal protein loading. Each blot presented in the whole figure is representative for at least three independent experiments. **b)** Pixel quantification. Quantification of MEK, ERK and p38 immunoreactivities using ImageJ software indicated activation in 7 DIV neurons in response to exogenous LPA application. Bar graphs represent the ratio of phosphor- MEK, ERK or p38 relative to total MEK, ERK or p38, respectively; *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$ and ns $p > 0.05$.

This confirms that LPA activates N-Ras, which, in turn, affects specific N-Ras downstream signals, such as MEK and ERK phosphorylation, but not p38 phosphorylation levels.

5.4 Functional consequences

During the development of the project, an interaction has been proved as well as its effect onto the control system of the N-Ras protooncogene. Therefore, a functional consequence was plausible and even expected. In order to assess any phenotype change of N-Ras at the cellular level, morphological studies of transfected young cortical primary neurons were performed. For this purpose, a detailed morphological study of neurons overexpressing either N-Ras, the constitutive active mutant (N-Ras ^{D12}) or the empty vector were carried out. Transfected cortical primary neurons were stained with a dendritic marker (MAP2)[9] (Fig. 26a) or with an axon marker (Tau)[10] (Fig. 26b). The first approach seemed to show changes in maturation of cells.

[9]MAP2 are neuron-specific cytoskeleton proteins enriched in dendrites where they are implicated in the stabilization of the dendritic tree during development and, therefore, can be used as a dendritic marker.

[10]Tau proteins are microtubules stabilizer proteins expressed only in axons and not in dendrites. Therefore, can be used as axon markers.

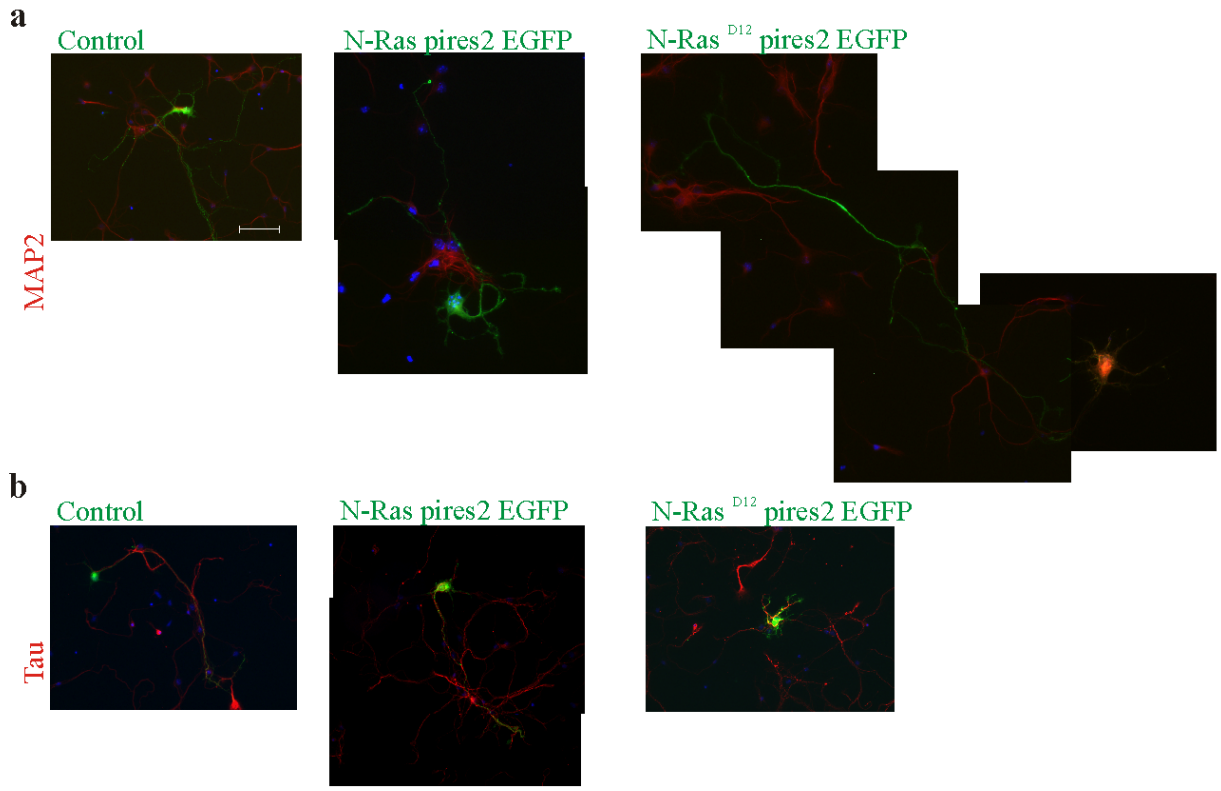


Figure 26: N-Ras overexpression in cortical primary neurons. Immunocitochemistry results of young cortical primary neurons after N-Ras overexpression. **a)** Cortical primary neurons transfected with N-Ras or N-Ras ^{D12} EGFP (green) and costained with dendrite marker (MAP2) in red. **b)** Cortical primary neurons transfected with N-Ras or its constitutive active mutant N-Ras ^{D12} (in green) and costained with an axon marker (TAU) in red. For both panels scale bare represents 100 µm.

However, when a detailed anatomic analysis of the neurons[11] was performed, an astonishing and significant difference in the axon length was obtained. On 7 DIV cortical primary neurons and for all three transfections constructs no significant change was detected in the number of Tau or MAP2 positive neurites.

Detailed Scholl analysis of both stainings (Tau and MAP2) revealed no apparent changes, due to N-Ras overexpression, in the axon number (Fig. 27a), in the dendrite number (Fig. 27b) neither in the branching patterns (Fig. 27d). Interestingly, in N-Ras ^{D12} transfected neurons, the longest neurite, representing the future axon, was significantly larger (Fig. 27c) compared to axons of the other transfected neurons. When analyzing neurons transfected only with N-Ras, axon length was not significantly greater, but instead appeared to be exactly halfway between control transfected neurons and neurons transfected with constitutive active construct N-Ras ^{D12}. This was an additional indication in our study that an increase in N-Ras induces larger axons.

N-Ras may not have an influence on neuronal complexity but it is an important factor promoting axonal outgrowth, the same as PRG-1 whose signalling has already been linked a regulatory function in synaptic plasticity.

[11]The morphological analysis was performed by the student Mr. Hoffmann under my supervision during his lab rotation within his Master thesis.

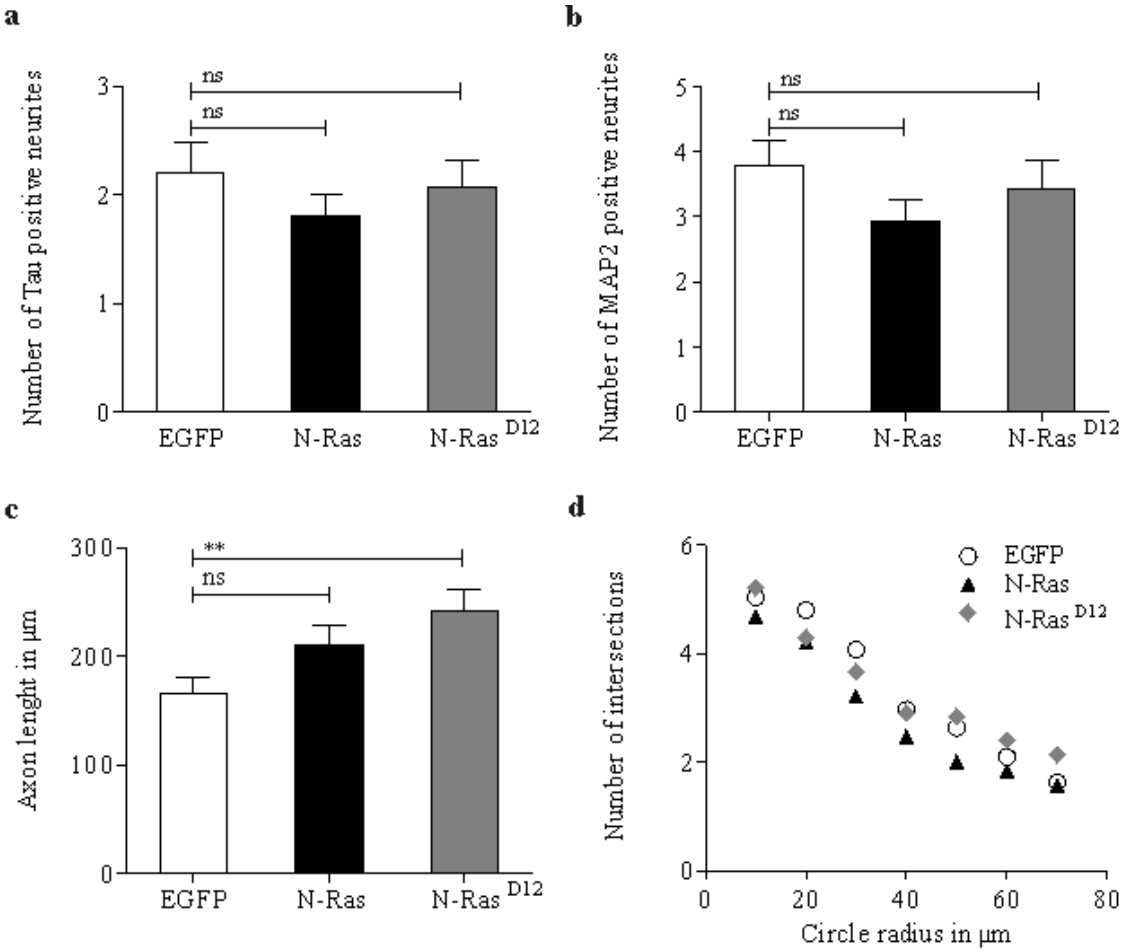


Figure 27: Morphological analysis of neurons. Analysis of 7 DIV, five days after N-Ras overexpression. **a)** Axon studies. Were TAU positive neurites analysis is represented (n=15). **b)** MAP2 (dendrite marker) positive elongations are showed in a graphic format (n=15). **c)** Axon length. The largest neurite was measured using Metamorph (n=30). **d)** Scholl analysis was done on all transfected cells (n=30). Values correspond to mean of three independent experiments, are represented SEM +/- and are analysed using two paired t-test. *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$ and ns $p > 0.05$.

6 Discussion

The present findings make an important contribution to our, as yet, limited understanding of function and signalling of PRG-1. Whereas previous results showed that PRG-1 controls axonal growth during regeneration [42], the present study elucidates the role of PRG-1 during neuronal differentiation. I show an interaction between PRG-1 and Ras GRF-2 which disruption us triggered after increase of LPA concentration at the extracellular matrix. The interaction, as well as its disruption, occurring at PA microdomains in the plasma membrane. LPA concentration change sets into motion an extensive internal signalling cascade, which I could prove is linked to N-Ras activation, MAPK protein phosphorylation and, finally, controls specifically the axonal outgrowth.

The expression analysis of PRG-1 and Ras GRF-2 have been able to prove colocalisation crucially found to be located within the plasma membrane and their coexpression in a regulated way during brain development, when there is active axonal growth [2]. The coexpression was detected at mRNA levels were PRG-1 and Ras GRF-2 detected patterns are in clear accordance with periods characterised by axonal growth in brain [2]. Generally, one would expect related proteins to have similar pattern, as it happens between PRG-1 and Ras GRF-2, but measurements for N-Ras, have proved this not to be the case. However, if PRG-1 and Ras GRF-2 are not inducing N-Ras activation but controlling it, the obtained mRNA expression pattern would be fitting. Then, PRG-1 and Ras GRF-2 could be the N-Ras negative regulators and their tight regulation is essential to control the N-Ras when brain maturation is finished [1].

PRG-1 / Ras GRF-2 interaction

The coimmunoprecipitation analysis identified an interaction between PRG-1 and Ras GRF-2. In fact, the coimmunoprecipitation studies showed a reproducible interaction in an overexpression system and in cortical primary neurons obtained from either wild type animals or from knock out animals missing one of the most expressed LPA receptors in neurons, as LPA_2 receptor (Dr. Kieselmann personal communication).

Unfortunately, the interaction was not consistently proved when using the Ras GRF-2 antibody presumably due to some problems with the antibody [90]. Mainly, the high similarity between Ras GRF-1 and -2 (see the homology within mouse sequences in the Suppl. Fig. B and the homology references on Suppl. Tab. C and D) induces problems on antibody specificity [53] which is the reason why people generally work either with Ras GRF-$1^{-/-}$ and Ras GRF-$2^{-/-}$ [56] or overexpressing the GEFs proteins [95, 96]. However, one can be sure that the detected interaction is happening between Ras GRF-2 and PRG-1. Firstly because when overexpressing both constructs the interaction could be proved in both directions. And, second, because the downstream signalling analysis included two main signals of the MAPK cascade. The one linked to Ras GRF-2 (ERK Pathway) [53, 54], which showed activation after LPA treatment, and the one linked to Ras GRF-1(the p38 pathway) [53], that showed no change on its phosphorylation status after LPA treatment. Ras GRF-2 plays an interesting role in this interaction, not only because it is expressed neuronaly

and acts as a signalling adapter via Ras proteins, but also because it can only be activated after relocalisation to the plasma membrane [50, 57]. Another difficulty faced with the immunoprecipitation assays appeared when using tissue lysate for coimmunoprecipitation. When the whole brain tissue was used, no interaction could be observed. This failure to immunoprecipitate could be attributed to the fact that proteins of study are not double expressed at the same levels in all brain structures as shown in this project within the mRNA studies during brain development neither in all cell structures [50]. Therefore, when all brain lysate is used, the concentration of proteins of interest is decreased because of the introduction of different cell types in which PRG-1/Ras GRF-2 interaction is not happening (as for example Glia cells where Ras GRF-2 is not expressed at all [50]). That effect can be corroborated when using small morphologically differentiated brain regions such as cortex or hippocampus because then, the interaction was observed without further troubles. Such result proves the need of high concentration of both proteins and, at the same time, validates the primary neurons culture as a good *in vivo* system for endogenous interaction studies.

Interestingly, no classical domain has yet been characterized within PRG-1's large and unique C-terminus [55] (see protein domains within Suppl. Tab. B) except in one recent publication which presented a non classical calmodulin-binding domain within the C-Terminus of PRG-1 [94] and the freshly found NSF interaction (personal communication from Mrs. Geist). After establishing the interaction, and because the few available information about PRG-1 domains, I tried to locate a sequence linked to PRG-1/Ras GRF-2 interaction within PRG-1 sequence. Unfortunately, studies using mutant constructs, in which different deletions had been made, were not conclusive. Constructs made did not show any possible sequence location for the interaction. Given the fact that the constructs, whose interaction was lost, showed other possible reasons to fail interacting, either due to not proper expression at the plasma membrane or to failure within the delivery from the Endoplasmatic Reticulum (ER) to the plasma membrane. Therefore, PRG-1/Ras GRF-2 interaction occurs due to the protein conformation and final 3D structure or through an unknown scaffolding protein rather than within a determinate protein sequence of PRG-1 C-Terminus.

The absence of classical domains [55] supports the hypothesis of the interaction happening according to the 3D structure rather to a domain within the protein sequence. Studies were done to clarify if there is an unknown scaffolding protein lipid. Lipid dot assays showed Phosphatidic Acid (PA) as a common element towards which PRG-1 and Ras GRF-2 showed specificity and high affinity. Thus, PA could be a key element in the PRG-1/Ras GRF-2 interaction and could even be the unknown scaffolding protein holding PRG-1 and Ras GRF-2 together. Actually, PA is the simplest membrane phospholipid and it is also a central intermediate point for the synthesis of membrane and storage lipids [97]. Recently, PA has emerged as part of a new class of lipid mediators and has been associated with cell proliferation, survival signalling, cell transformation, tumor progression and differentiation [98]. PA lipids have three main functions: firstly, PA alters membrane structure; secondly, it acts as a messenger by means of specific interactions with proteins; and thirdly, it tethers a protein to a membrane and/or modulates its catalytic activity [98]. The last postulated function of PA has been linked to the PH domains [98, 99] and Ras GRF-2 has

two of such domains within its sequence [50]. Although PH domains are usually characterised as lipid-binding regions, they also participate in protein-protein interactions [99] and recent studies have proved that the PH domain is required for inducing exchange in members of the Ras superfamily [96]. Therefore, I propose the PH domain, included in Ras GRF-2 (see protein domains within Sup. Tab. D) as essential for GEFs to reach microdomains, in which geranyl-geranylated GTPases reside, or as a mediator of the direct interaction between Ras GRFs and specifically modified GTPases.

Hypothetically, the PA would be fulfilling either some or all of their main known functions [98]. On the one hand, they could be gathering PRG-1, Ras GRF-2 and N-Ras proteins and acting as key elements in localising all the proteins therefore changing the plasma membrane concentration. On the other hand, the PA could be acting as a second messenger by means of their lipid-protein interaction with PRG-1 and Ras GRF-2. Finally, after tethering PRG-1, Ras GRF-2 and N-Ras, PA microdomains could be controlling N-Ras activity, because its activity is enabled only after translocation to PA microdomains [96]. N-Ras translocation to the PA microdomains is possible because the phospholipids in the membrane are spaced apart, which decreases the lateral pressure in the membrane underneath. Then, the lipid chain modifications of Ras can fill the volume below the protein backbone and the binding to the lipid membrane stabilises the structure of the Ras C-terminus [100].

In the final hypothesis of my thesis (see diagram on Fig. 28), plasma membrane microdomains mainly containing PA are agglutinating PRG-1, proteins with PH domains such as Ras GRF-2 [54] which are known to interact with several different phosphoinositides typically only with the headgroup and not involving significant membrane insertion of the domain, and posttranscriptionally modified N-Ras proteins [101] and enabling their interaction at specific areas of the plasma membrane.

Comparison of the PA-binding regions has, so far, not revealed a high sequence similarity among proteins specifically interacting with PA, except for the presence of basic amino acid residues that bind the acidic lipid. Therefore, a specific structural fold, rather than a simple electrostatic interaction is required for a PA-protein interaction [98]. The use of intracellular PA reporters or sensors is being explored currently, to detect PA changes in living cells. In this method, a PA-biding protein motif is fused to a fluorescent protein and the fluorescent proteins are used to image PA dynamics in living cells. Further steps to prove the presented hypothesis could be done using, for example, the phospholipid uptake experiment [43]. In the uptake experiments, the colocalisation of PA with PRG-1, Ras GRF-2 or N-Ras can be followed. Ideally, and considering that LPA can be produced from PA through hydrolisis (see Fig. 3), adding the life image approach to study the changes induced in lipid-protein interaction and location after extracellular LPA increase.

Extracellular element triggering PRG-1 / Ras GRF-2 interaction disruption

Further studies on the interaction between PRG-1 and Ras GRF-2 proved that the interaction is compromised after extracellular LPA application. The specificity of LPA to compromise the interaction was ensured by the fact that extracellular application of Thrombin Receptor activating Peptide (TRP), known to activate Thrombin receptors and to affect on cell shape in a way similar to LPA [27], showed no effect on the interaction.

Presented data could not clarify if LPA is directly binding PRG-1 or not. PRG-1 belongs to LPP superfamily but its ectoenzymatic activity is still controversial [102, 103]. The controversy lays mainly on two points of the ectoenzymatic activity. First point still under discussion is the fact that the consensus sequence that defines LPPs and which is critical for the catalysis and for the LPA hydrolysis is not conserved for PRG-1 (see red marks in Fig 5b and in Suppl. Fig. A). Thus, the group of Morris claimed that PRG-1 can not be included within the LPP superfamily [102]. The second critical point is the ectoenzymatic activity itself, because the group of Morris could not produce the ectoenzymatic activity [102], but Bäuer et al. did demonstrate the significant increase in monoacylglycerol (MAG) resulting from PRG-1 overexpression [103]. And they did so using the neuronal cell line N1E-115 which, when transfected with PRG-1-GFP fusion construct and exposed to LPA, generates increased extracellular LPA degradation products and protects against LPA neurite collapse in a dose-dependent fashion, which demonstrates that PRG-1 had interfered with lipid phosphate signalling. However, if LPA receptors are inactivated - either because of the application of chemical products such as Ki 16425, or because of using animals which do not express the LPA receptors (e.g. $LPA_2^{-/-}$) - the interaction and its dependency on LPA remains unchanged proving the link between them to be rather direct and extracellular than through LPA receptors or intracellular.

The lack of PRG-1 in neurons significantly limits uptake of bioactive lipids [43]. Therefore, there is reason to assume that apart from its potential residual enzymatic activity, PRG-1 is effective controlling the levels of LPA at the synapse by nonenzymatic mechanisms. PRG-1 function may have evolved from classical dephosphorylation performed by LPPs to mechanisms such as sensor like or transporter like activities. The here presented data proves that Ras GRF-2 is an interacting partner of PRG-1 and that extracellular LPA concentration is the sole disrupting element. Therefore, the data discussed so far is a clear support to the recently proposed alternative mechanism for PRG-1-mediated attenuation of LPA signalling linked to neuritogenesis in phospholipid-rich environment [6], because there is an interaction, and the relation between proteins is presented depending directly on extracellular concentration of LPA, independently of the controversy linked to the still unanswered point of weather LPA is directly degraded by PRG-1 or not [102, 103].

When proposing LPA as the specific disrupting element, it is important to remember: firstly, that LPA is tightly controlled during development where changes to extracellular LPA concentrations are essential for normal brain development [29] and vascular development, specifically, in the formation of vascular structures, such as the blood-brain barrier [104]. And secondly, that LPA

is also involved in both pathological and physiological states [19], including neuropathy [105] or cancer [22] where the bioactive lipid has even been suggested as a prognosis marker [106]. Thus, the results presented here are likely to have implications not only for development but also for pathology.

Intracellular effects of PRG-1/Ras GRF-2 interaction disruption

The extracellular increase of LPA concentration compromises PRG-1/Ras GRF-2 interaction. Obviously, triggering the interaction disruption has intracellular effects and those effects have been proved to be over N-Ras activation and through ERK pathway phosphorylation.

I have proved the link between extracellular LPA and N-Ras activation in both cortical primary neurons and HEK cells, whereby in the latter, direct overexpression of PRG-1 inhibited active N-Ras. This and the fact that extracellular LPA application switches N-Ras from inactive to active conformation, can only be explained if PRG-1 is understood as a negative regulator of the whole system.

Presented data suggests that while PRG-1 and Ras GRF-2 are interacting at the plasma membrane, the amount of active N-Ras is lower (see Fig. 28a) than when the interaction is disrupted by increased extracellular LPA concentration (see Fig. 28b). This role is new for Ras GRF-2 but not necessarily unexpected, because GEFs are known to be signalling adapters and integrators, coupling various processes at the cell membrane [46], and have been previously linked to several Ras proteins [62, 95, 96] where they have been involved in axon specification and neural polarization of hippocampal neurons [107], in neuronal differentiation in PC12 cells [108] or in promoting neuronal differentiation [62].

Downstream of the activation of Ras proteins is typically followed by Mitogen Activated Protein Kinases (MAPK) phosphorylation and the current study provides clear biochemical evidence that the LPA effect on MAPK signalling cascades is mainly concentrated on ERK phosphorylation rather than p38. ERK pathway phosphorylation has been already linked to two important elements of the project. First, Ras GRF-2 induction of Long Term Potentiation (LTP) [53] has been described to occur through ERK pathway and, second, axon growth has been proved to be regulated by ERK phosphorylation in chicken retinal neurons [77]. The absence of any signal of phosphorylation of the p38 pathway is also an interesting result because of the link between p38 phosphorylation and Ras GRF-1 [53] which proves once more the specificity of the interaction between PRG-1 and Ras GRF-2.

Functional consequences

Based on the obtained data, early stage primary neurons were transfected with N-Ras or constitutively active N-Ras (N-Ras^{D12}) constructs and were subjected to detailed morphological analysis trying to focus onto any possible morphological change. Statistically significant results were obtained only when N-Ras^{D12} constructs were overexpressed showing no further morphological

modifications within neurons rather than axon length. In fact, with N-Ras transfection the axon length was exactly between the control and the constitutive transfection samples; corresponding to the reality of the transfection nature were N-Ras transfected cells need the cellular mechanism to have active protein while N-Ras^{D12} produces constitutive active protein [62] and therefore, transfected neurons are able to show the effects quicker and more direct than the cells transfected with the no mutated construct.

The results presented here are in accordance with the first publication of PRG-1 [103], in which, Bräuer et al. presented PRG-1 and postulated this novel brain specific protein to be involved in axon growth because transcripts were detected during development [103]. Here I have been able to prove the intracellular link between PRG-1 and axon growth and, specifically, present a direct relation between PRG-1 and N-Ras protein activation finally effecting the axon growth.

The link between PRG-1 and regenerative sprouting of the first PRG-1 paper has not been further analysed. However there are several new publications which keep pointing towards the same idea; on the one hand, a strong upregulation of ATX after brain damage [109]. On the other hand, the strong need of LPA for a proper brain development [29]. The need of LPA and the upregulation of ATX suggest that the LPA level is possibly also increased not only during development but also after a lesion, during sprouting [2] . Then, LPA could act as a chemo-attracted factor for immune system cells and neuronal precursors and/or LPA could function as a repulsion factor on sprouting axons. If this last option is considered, one can take once more the recently proposed alternative mechanism for PRG-1-mediated attenuation of LPA signalling linked to neuritogenesis in phospholipid-rich environment [6] because PRG-1 attenuates axon colapse generally linked to extracellular LPA application [24].

Hypothetical scheme and upshot

Small pieces of a larger system in which PRG-1 is finally presented as a controller of phospholipid mediated signalling in the brain during development have been detailed, but a general overview as a final conclusion is needed.

Taken all the presented and discussed data together, a hypothetical system can be described from the extracellular inductor until the intracellular cascade is triggered; from the proteins location within the neuronal plasma membrane to the protein phosphorylation and from promoting the protein activation to changing the neuronal phenotype. To facilitate comprehension, the final hypothesis has been drown as a diagram (Fig. 28) where the two main situations (active/inacitve) are detailed.

The system is generally inactive and its activation depends on the extracellular concentration of LPA. When the system is inactive (Fig. 28a), there is an interaction between PRG-1 and Ras GRF-2 at the plasma membrane of neurons [57]. There, both proteins are located and agglutinated in PA microdomains fulfilling their main duties in terms of membrane structure, acting as a specific

messenger and as a scaffolding protein [98]. While PRG-1 is kidnapping Ras GRF-2 at the plasma membrane N-Ras activation is avoided.

When the extracellular LPA concentration increases due to the development [7] or due to pathologies (e.g. cancer [6]), the system is activated and the extracellular LPA increase triggers the disruption of the PRG-1/Ras GRF2 interaction. The interaction disruption between PRG-1 and Ras GRF-2 leads to a free cytoplasmic Ras GRF-2, located close to N-Ras (also agglutinated around PA domains [98]). Then, Ras GRF-2 is free to work as exchange factor [50] and it induces N-Ras activation. In the downstream signalling, active N-Ras induces MEK and ERK phosphorylation [54], and the final consequence of all the activation signal is on the axonal outgrowth. Therefore, PRG-1 has its final influence on neuronal length in one possible physiological role for PRG-1.

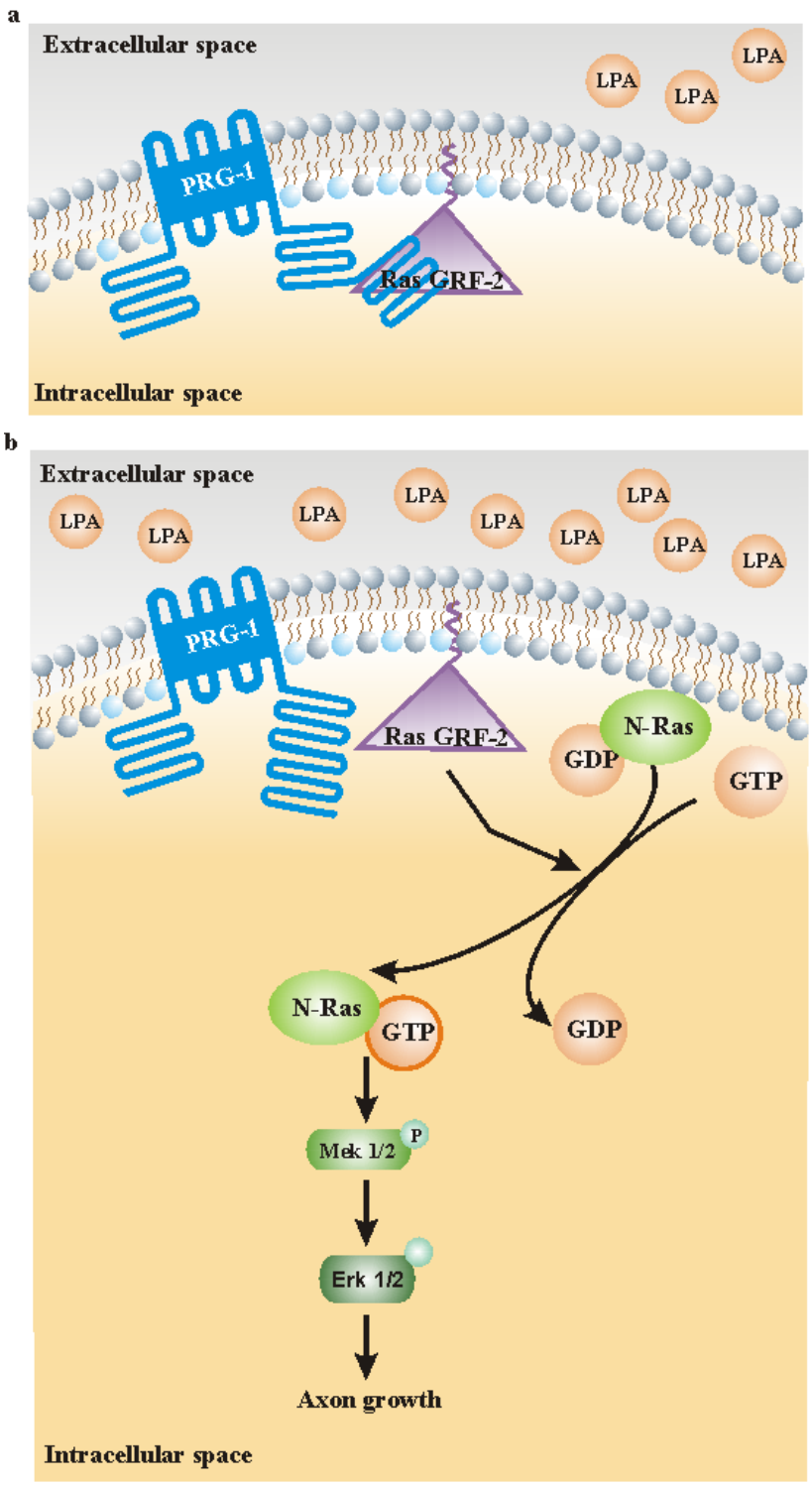


Figure 28: Final hypothesis. Sum up schema for PRG-1/Ras GRF-2 interaction and its intracellular signalling. **a)** inactive situation **b)** active situation due to an increase of extracellular LPA. In both panels all elements include their abbreviation except the sky blue lipids which represent Phosphatidic Acid.

The novel results provide the molecular clues that PRG-1 has an important regulatory function in the axonal growth during development. Specifically, PRG-1 activates N-Ras during development and does so through Ras GRF-2 and lipid metabolism. I show for the first time that PRG-1 is a Ras-cascade controller acting mainly during development, while LPA concentration increases [39]. The findings are in accordance with current data, in particular, the one showing that PRG-1 is regulated either during brain development or after a lesion [42], both periods being clearly characterized by active axonal growth [110]. Results are also in accordance with the recently proposed role of PRG-1 as an important player in the modulatory control of hippocampal excitability due to a non-enzymatic control of extracellular LPA at the synaptic level [43].

This research project leads to new insights into the molecular mechanisms of axon outgrowth, which might have implications during development as well as for the treatment of neurodegenerative diseases, but also into cancer, where LPA is already studied and PRG-1 expression could be used as a prognostic factor. Furthermore, similar studies are already underway to clarify this function and to shed light onto this new neural lipidomic approach, in which lipid-protein interactions represent the first step of a large-scale signalling cascade, occurring mainly during development but also under pathological conditions.

As a matter of fact, recently published data have provided insight into the role of LPA in pathological conditions such as cancer [13, 22, 111, 112]. Interestingly, the expression of other proteins during development, analysed as part of the project, have also been linked to cancer in the CNS, such as neuroblastoma in the case of N-Ras [68], or glioblastoma in the case of ERK phosphorylations [113].

Therefore, although PRG-1's function as a negative regulator for N-Ras is clear, key questions remain. Addressing these questions will contribute to our understanding of PRGs and should also provide insight not only into biological events such as development and the consequences of brain injury, but also into cancer, in which LPA is upregulated and detectable in biological fluids.

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[113] N. A. Lokker, C. M. Sullivan, S. J. Hollenbach, M. A. Israel, and N. A. Giese. Platelet-derived growth factor (pdgf) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel pdgf-c and pdgf-d ligands may play a role in the development of brain tumors. *Cancer Res*, 62:3729–35, 2002.

[114] European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB), and the Protein Information Resource (PIR). Extracted from the website: http://www.uniprot.org/.

[115] Swiss Institute of Bioinformatics (SIB). Extracted from the website:http://expasy.org/.

[116] Cambridge European Bioinformatics Institute. Wellcome Trust Genome Campus. Hinxton. Extracted from the website: http://www.ebi.ac.uk/interpro/.

List of Figures

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A Appendix

A.1 Supplementary information

Figures

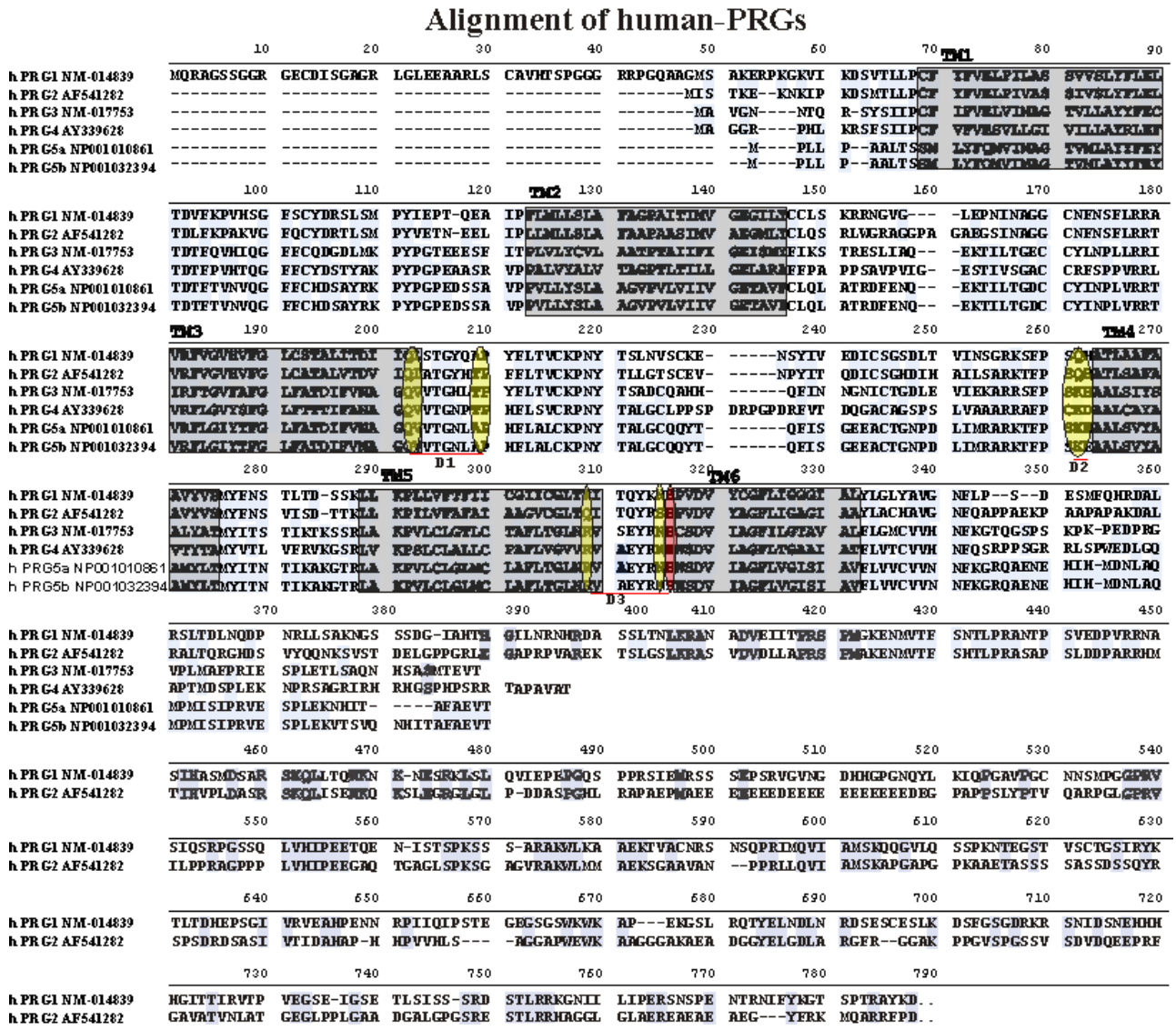

Figure A: Homology analysis between protein sequences of the human PRG family members. Protein sequences are compared and homologies are marked in blue, transmembranal domains are numbered (TM1-6) and have grey boxes, the ectoenzymatic domains are underlined in red (D1-3) and the essential amino acids for ectoenzymatic activity are extra showed in yellow when conserved and in red when differing from standard LPPs sequence.

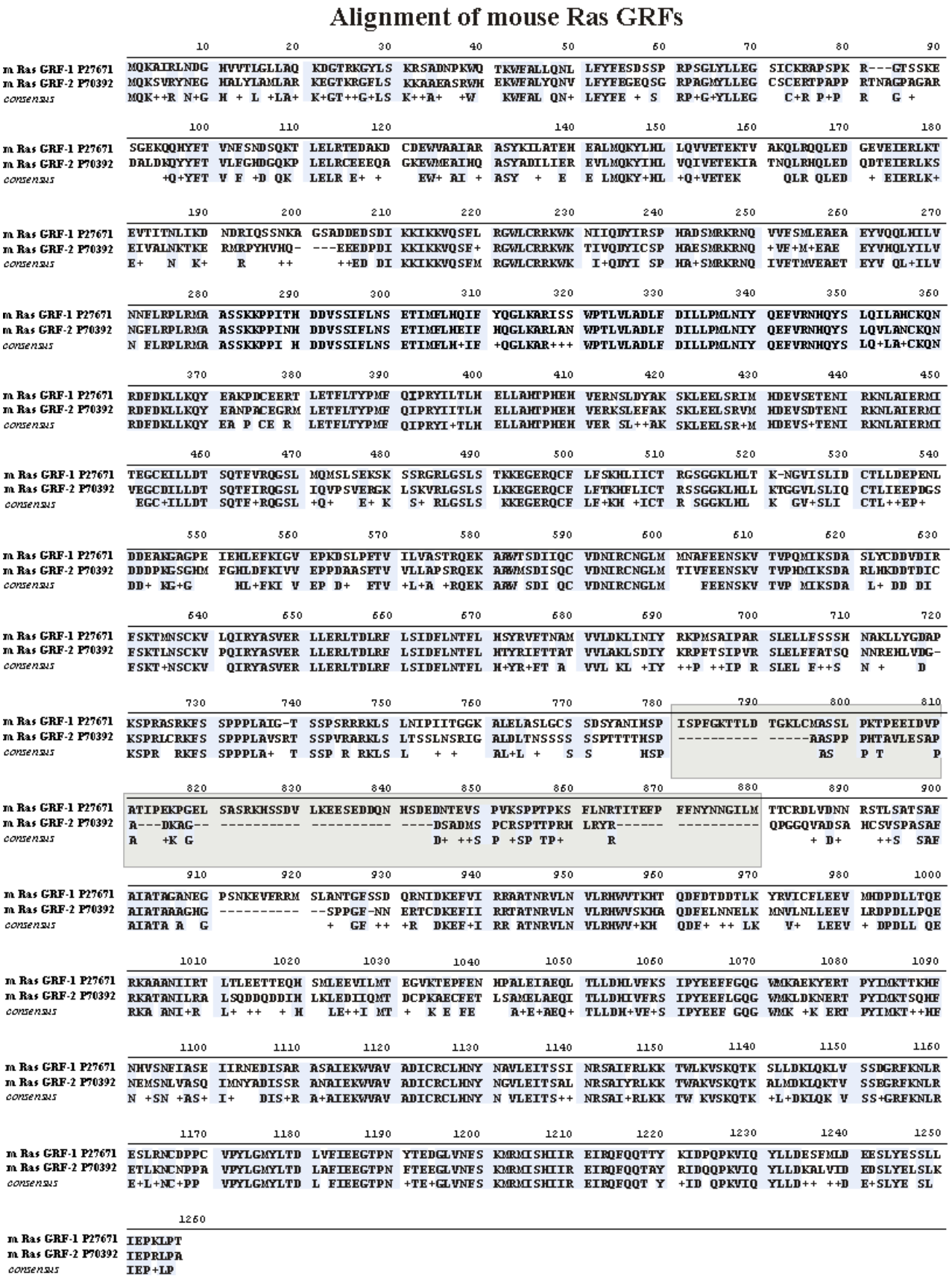

Figure B: Homology analysis between protein sequences of mouse Ras GEFs. The protein sequence are compared and homology is in blue while the main diverging sequence has a grey square. A third sequence, the *consensus*, is added including not only the conserved amino acids but also the conserved amino acid groups considering them positive matches (+)

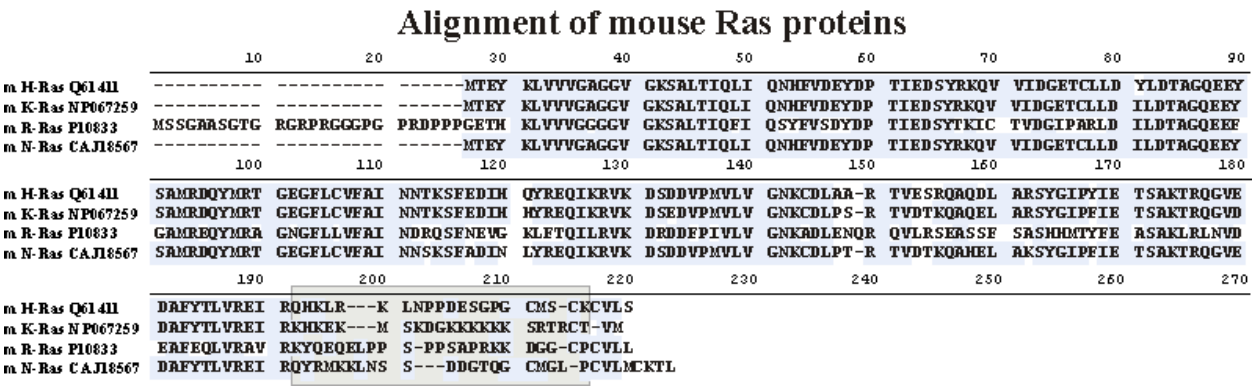

Figure C: Homology analysis between protein sequences of mouse Ras proteins. Protein sequence are compared, homology is signalized in blue and hypervariable region is signalised with a grey box.

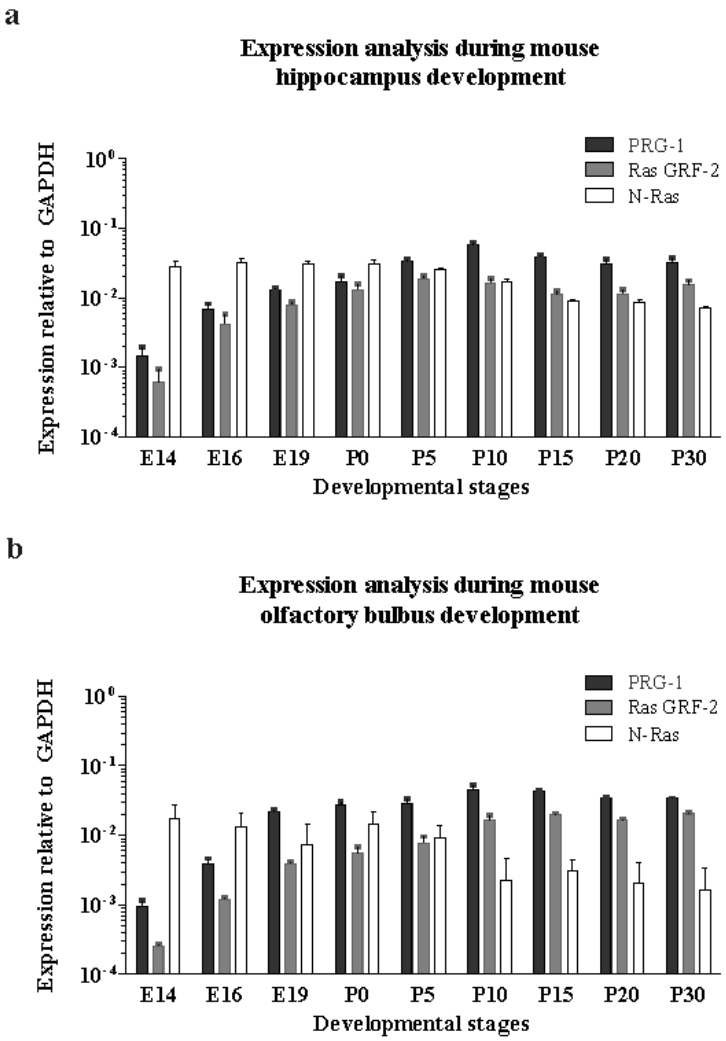

Figure D: q RT PCR supplementary expression studies. **a)** mRNA expression pattern of PRG-1, Ras GRF-2 and N-Ras during mouse hippocampus development. **b)** PRG-1, Ras GRF-2 and N-Ras mRNA pattern in bulbus olfactorius during development. Both graphics are done using GAPDH as Housekeeping. Values correspond to mean +/- SEM and statistical analysis was performed using two-tailed t-test and p values are considered: *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$ and no significant (ns) ns $p > 0.05$. (n=3).

Tables

Table A: Biological effects of Lysophospholipid Acid (LPA)

Effect	**Cell type**, ***remarks***	**References**
Cell clustering	Schwann cells	Weiner et al. 2001
Cell contraction	Smooth muscle cells, myofibroblasts N1E-115 and NG-108-16 neuronal cells	Jalink, Moolenaar et al. 1993 Kranenburg et al. 1997 Jalink, Eichholtz et al. 1993
Cell division	Diverse cell types (e.g. Fibroblasts)	Moolenaar et al. 1990 van Corven et al. 1992
Cell migration	Diverse cell types (normal and transformed)	Jalink et al. 1993
Cell proliferation	Many normal and transformed cells	van Corven et al. 1989
Cell survival (rescue from apoptosis)	Schwann cells, leukocytes, intestinal epithelial cells, osteoblasts, mesangial cells, hepatocytes	Taghavi et al. 2008
Cerebral cortex growth and folding *in vitro*	Cerebral cortex *due to reduced cell* *death and enhanced terminal mitosis* *of progenitor cells*	Kingsbury et al. 2003
Increased endothelial permeability	Brain capillar endothelial cells macrovascular endothelial cells	Schulze et al. 1997 Nieuw Amerongen et al. 2000
Inhibition of gap junctional communication	Hepatoma cells, fibroblasts Cultured brain endothelial cells	Postma et al. 1998 Schulze et al. 1997
Inhibition/reversal of differentiation	Neuroblastoma and glioma cells, vascular smooth muscle cells *morphology, loss of contractility*	Hayashi et al. 2001
Maturation of mammalian oocytes	Mammalian oocytes, *dependent on adjacent cumulus cells*	Hinokio et al. 2002
Membrane, chloride mediated, depolarization	Neuronal and non neuronal cells	Dubin et al. 1999 Postma et al. 2001
Neurite retraction, growth cone collapse, repulsive growth cone turning, cell rounding	Neuroblastoma and PC12 cells, cortical neuroblasts, chick DRG neurons, xenopus (retinal growth cones and spinal neurons), astrocytes	Fukushima et al. 2000 Campbell and Holt 2001 Yuan et al. 2003 Remakers and Moolenaar 1998
Platelet and platelet- monocyte aggregation	Blood cells apparent involvement of ADP receptors	Haseruck et al. 2004
Tumor cell invasion *in vitro*	Carcinoma, hepatoma and T- limphoma cells mammary tumorogenesis	Westermann et al. 1998 Jonkers et al. 2009
Wound healing *in vivo*	Skin, intestinal epithelium	Malazs et al. 2001, Sturm and Dignass 2002, Brindley 2004

Table B: PRG-1 information

Information		**References**
Species:	- Mouse	MGI ID: 106530
Genomic information	- Chromosome: 3	NCBI Entrez GeneID: 229791 Ensembl Gene ID: ENSMUSG00000044667
Protein information	- 766 aa (83 kDa)	NCBI accession NP_808332 UniProt Q7TME0
Protein domains	- 6 transmembranal domains - 1 putative ectoenzymatic site linked to LPA	on line databases such as: SMART [55] UniProt [114] EXPASY [115] EMBL-EBI [116]
	- non classical calmodulin domain within the C-Terminus	Tokumitsu et al. 2010
	- NSF interaction within the C-Terminus	Mrs. Geist (personal communication)
Process/Function:	- PRG-1 facilitates axonal outgrowth during development and regenerative sprouting, - attenuates axon collapse induced by LPA - facilitates outgrowth in the hippocampus	Bräuer et al. 2003
	- atenuates LPA signalling linked to neurogenesis in phosphlipid-rich environments	Brindley et al. 2004
Signalling pathway:	- it is still non-clarified mechanism. - controls axon lenght throught N-Ras activation, MEK/ERK pathway phosphorylation.	Brindley et al. 2004 Mrs. Soriguera (unpublished)
Expression		
in tissue	- brain expression, - in prostata human cancer	Bräuer et al. 2003 Mr. Rocha (unpublished)
in cells	- in neurons and not in microgliya or astrocytes for cell lines:	Dr. Keiselman (unpublished)
	expression in: DU-145	Dr. Rocha (unpublished)
	no expression in: NSC-34 N1E-115 or HEK cells	Dr. Keiselman (unpublished)
Intracellular localisation	- Membrane of outgrowing axons - spines of mature neurons	Bräuer et al. 2003 Dr. Keiselman (unpublished)

Information		References
Posttranscriptional modifications	-There are no proven posttranscriptional modifications, althought there are some putative posttranscriptional modifications sites and other PRG family members do have them (e.g glycosylation on PRG-3)	SMART [55] Dr. Velmans (unpublished)
Homology to	- mouse PRG-2 (AAP57767): 49 % identity (63 % matches) - human PRG-1 (AAP57770): 95 % identities (97 % matches)	using protein BLAST2 sequence using protein BLAST2 sequence
PRG-1$^{-/-}$ mouse phenotype	- homozygous mice exhibit: - reduced body weight - seizures - hiperexcitabilitiy of evoked EPSCs - premature lethality around 3 weeks	Thrimbuch et al. 2009

Table C: Ras GRF-1 information

Information		References
Species:	- Mouse	MGI ID: 99694
Genomic information	- Chromosome: 9 Location: E3.1	NCBI Entrez GeneID: 19417 Ensembl Gene ID: ENSMUSG00000032356
Protein information	- 1262 aa (144kDa)	NCBI accession NP_035375 UniProt P27671
Protein domains	- IQ calmodulin-binding region - Dbl homology (DH) domain - Guanine nucleotide exchange factor for Ras-like GTPases, N-terminal - Guanine-nucleotide dissociation stimulator, CDC24, conserved site - Pleckstrin-like Guanine-nucleotide dissociation stimulator CDC25 - Ras guanine nucleotide exchange factor - Pleckstrin homology-type (PH)	on line databases such as: SMART [55] UniProt [114] EXPASY [115] EMBL-EBI [116]
Process/Function:	- Activation by GPCR or dependent on calcium/calmodulin - Activation of Ras signalling cascade - Can activate plasma membrane and reticular, but not Golgi-associated, H-Ras - Regulation of neuronal synaptic plasticity	Mattingly et al. 1996, Zippel et al. 2000 and Farnsworth et al. 1995 Guerrero et al 1996 Arozena et al. 2004

Information		References
	- Synaptic transmission	
	- Cell proliferation	Finkbeiner et al. 1996
	- Hippocampal-dependent learning and memory	Giese et al. 2001
	- Interaction with Ras GRF-2	Anborgh et al. 1999
	- Development and maintenance of normal beta-cell number and function	Font de Mora et al. 2003
	- Functions in synaptic plasticity by contributing to the induction of long term depression	Li et al. 2006
Signalling pathway:	- Ras/MAPK cascade through p38	Li et al. 2006
	- These results suggest that Ras-GRF has the capacity to mediate Ras activation initiated by signals using heterotrimeric G proteins	Shou et al. 1995
	- Activates p21 Ras in response to GPCR	Zippel 1996
	- Calcium signals involved in postsynaptic signalling	Farnsworth et al. 1995
	- LPA signalling: Calcium-dependent Ras GRF-1 activation	Zippel et al. 2000 Farnsworth et al. 1995
	- Farnesylated TC21 can be activated by both Ras GRF-1 and Ras GRF-2	Calvo et al. 2009
Expression		
in tissue	- Brain specific; neurons of CNS	Zippel et al.1996
	- mRNA levels high in hippo CA1, CA3, DG and spinal cord	Martegani et al. 1992
	- Protein high levels in hippo, cortex, cerebellum, pancreatic islets and beta cells	Zippel et al. 1997 Arava et al. 1999
	- Expression increases sharply in first few day after birth in parallel with maturation of synaptic connections and neuronal differentiation	Font de Mora et al. 2003
	- Present in adult mouse brain	Forlani et al. 2006
	- Present in cerebral cortex of rat	Yang et al. 2003
in cells	- Localized at synaptic junctions and enriched in postsynaptic densities	Sturani et al. 1997

Information		References
	- Cell bodies of motor/sensory neurons (grey matter spinal cord)	
	- Present in granular cells (cerebellum), hippocampal neurons	Zippel et al. 1997
	- Absent in Gila, astrocytes, neuroblastoma cell lines, dorsal root ganglia	
Intracellular localisation	- Cell body and dendrites of pyramidal cells (cortex/hippo) as punctuated structures;	Sturani et al. 1997
	- Postsynaptic sites (PSD)	
	- Colocalisation with microtubules in neuroblastoma cells	Forlani et al. 2006
Posttranscriptional modifications	- Phosphorylation on several Ser/Thr/Tyr by CaMKII (*in vitro* phosphorylation of ectopically expressed Ras GRF-1 under basal conditions)	Sturani et al. 1997
	- Phospho-Ras GRF-1 is associated with calmodulin *Note: Phosphorylation*	Zippel et al. 1997
	enhances exchange activity	Mattingly et al. 1996
Homology to	- Lit.: Overall homology close to 80 %;	Forlani et al. 2006
	- mouse Ras GRF-2 (NP_033053): 63 % identity	using BLAST2 sequence
	- homo sapiens Ras GRF-1 (NP_002882): 83 % identities	using BLAST2 sequence
Ras GRF-1$^{-/-}$ mouse phenotype	- hippocampal-dependent learning and memory impaired in the process of memory consolidation	Giese 2001
	- abnormal amygdala function in long-term plasticity	Brambilla et al. 1997
	- intact learning and short-term memory	
	- increased lipid catabolism, reduction of beta-cells	Font de Mora 2003

Table D: Ras GRF-2 information

Information		**References**
Species:	- Mouse	MGI ID: 109137
Genomic information	- Chromosome: 13; Location: C3	NCBI Entrez GeneID: 19418 Ensembl Gene ID: ENSMUSG00000021708
Protein information	- 1189 aa (135 KDa)	NCBI accession NP_033053 UniProt P70392
Protein domains	- Guanine nucleotide exchange factor for Ras-like small GTPases - Dbl-homologous (DH) domain - Pleckstrin homology (PH) domain - Guanine nucleotide exchange factor for Ras-like GTPases - N-terminal to the Ras GEF domain (Cdc25-like)	on line databases such as: SMART [55] UniProt [114] EXPASY [115] EMBL-EBI [116]
Process and function	- Functions as a calcium-regulated nucleotide exchange factor activating: Ras and RAC1 through the exchange of bound GDP for GTP	Anborgh et al. 1999
	- Can activate plasma membrane and reticular, but not Golgi-associated, H-Ras	Arozena et al. 2004
	- Preferentially activates H-Ras *in vivo* compared to R-Ras based on their different types of prenylation	Gotoh et al. 2001
	- Functions in synaptic plasticity by contributing to the long term potentiation induction	Li et al. 2006
Signalling pathway	- Linked to ERK/MEK phosphorylation	Fan et al. 1998 and Li et al. 2006
	- Farnesylated TC21 can be activated by both Ras GRF-1 and Ras GRF-2, whereas geranyl-geranylated TC21 is unresponsive to Ras GRF-2	Calvo et al. 2009
	- Activates Ras-dependent and Rac-dependent MAPK pathways	Fan et al. 1998

Information		References
Expression		
in tissue	- Present in adult mouse brain and also in the nucleus of the solitary tract	Forlani et al. 2006, Guerrero et al. 1996 and Fam et al. 1997
	- Not observed in the hippocampus (at protein level)[12]	Fernández-Medarde et al. 2002.
in cells	- several tumor cell lines	Guerrero et al. 1996
Intracellular localisation	- Cytoplasm and cell membrane - Endoplasmic reticulum membrane - Found both at cell periphery and along the axon of neurons *Note: Translocates to membranes when activated.*	Kasavapany et al. 2004 Arozena et al. 2004 Downward 1996
Posttranscriptional modifications	- Phosphorylated by CDK5 leads to down regulation - Ubiquitinated upon interaction with Ras leading to degradation through the 26 S proteasome	Kesavapany et al. 2004 de Hoog et al. 2001
Homology to	- Lit.: Overall homology close to 80 % - mouse Ras GRF-1 (NP_035375): 63 % identity - homo sapiens Ras GRF-2 (NP_008840): 90 % identities	Forlani et al. 2006 using BLAST2 sequence using BLAST2 sequence
Ras GRF-2$^{-/-}$ mouse phenotype	- Mice do not display overt phenotype - Targeted disruption of Ras GRF-2 shows dispensability for mouse growth and development	Fernández-Medarde et al. 2002

[12] During the development of this project, endogenous Ras GRF-2 protein could be detected in hippocampus as well as in primary neurons (Fig. 14 and 16).

Table E: Constructs used in the project

Name	Insert	Vector	Restriction enzymes	Fragment sizes
HA PRG-1	HA h PRG-1	pCDNA3.1(+) zeo	Kpn1 and Not1	2.2 and 5.5 kbp
HA PRG-1 delta His	HA h PRG-1 delta His	pCDNA3.1(+) zeo	Kpn1 and Not1	2.2 and 5.5 kbp
HA PRG-1 delta 1-975	HA h PRG-1 delta 1-975	pCDNA3.1(+) zeo	Kpn1 and Not1	1.3 and 5.5 kbp
HA PRG-1 1-207	HA h PRG-1 1-207	pCDNA3.1(+) zeo	Kpn1 and Not1	1.1 and 5.5 kbp
HA PRG-1 954-2292	HA h PRG-1 954-2292	pCDNA3.1(+) zeo	Kpn1 and Not1	0.9 and 5.5 kbp
HA PRG-1 1417-2292	HA h PRG-1 1417-2292	pCDNA3.1(+) zeo	Kpn1 and Not1	1.4 and 5.5 kbp
HA PRG-1 1850-2292	HA h PRG-1 1850-2292	pCDNA3.1(+) zeo	Kpn1 and Not1	1.8 and 5.5 kbp
PRG1 in pECFP	h PRG-1	pECFP C1 and N1	Bgl 2 and BamH1	2.3 and 4.7 kbp
PRG1 in pEYFP	h PRG-1	pEYFP C1 and N1	Bgl 2 and BamH1	2.3 and 4.7 kbp
Ras GRF-2 in pECFP	mRas GRF-2	pECFP C1 and N1	Bgl 2 and BamH1	3.3 and 4.7 kbp
Ras GRF-2 in pEYFP	mRas GRF-2	pEYFP C1 and N1	Bgl 2 and BamH1	3.3 and 4.7 kbp
pcDNA3 Flag Ras GRF-2	mRas GRF-2	pCDNA3.1(+) zeo	Bam H1 and Xho 1	3.3 and 5.4 Kbp
pCEFL N-Ras	N-Ras	pCEFL	Bam H1 and Eco R1	0.5 and 6.0 Kbp
pCEFL N-Ras ^{D12}	N-Ras ^{D12}	pCEFL	Bam H 1 and Not 1	0.9 and 6.0 Kbp
pCGLH - PRG-1-siRNA 848	PRG-1-siRNA 848	pCGLH	Kpn I and Bgl II	0.37 and 8 kbp

Table F: HPRT sequence of the housemade primers used for q RT PCR

Primer mix	
for:	ATC ATT ATG CCG AGG ATT TGG AA
rev:	TTG AGC ACA CAG AGG GCC A

Prove
TGG ACA GGA CTG AAA GAC TTG CTC GAG ATG

Table G: PCR primers used in order to clone HA-PRG-1 full length as well as all HA-PRG-1 deletion constructs

HA-PRG-1 full length & HA-PRG-1 delta His
Forward:
AT **GGT ACC**[1] **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCG TGC**[2]ATG CAG CGC GCT GGC TCC A
Reverse:
A**GC GGC CG**[3] C **CTA**[4]CAA TCC TTA TAA GCC CGT GTG GGG

HA-PRG-1 delta 1-975
Forward:
AT **GGT ACC**[1] **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCG TGC**[2]G CTG TGG GGA ATT TCC TGC CCA
Reverse:
A**GC GGC CG**[3] C **CTA**[4]CAA TCC TTA TAA GCC CGT GTG GGG

HA-PRG-1 delta 1-207
Forward:
AT **GGT ACC**[1] **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCG TGC**[2]T TTT ATT TCG TCG AGT TGC CT
Reverse:
A**GC GGC CG**[3] C **CTA**[4]CAA TCC TTA TAA GCC CGT GTG GGG

HA-PRG-1 delta 954-2292
Forward:
AT **GGT ACC**[1] **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCG TGC**[2]ATG CAG CGC GCT GGC TCC A
Reverse:
A**GC GGC CG**[3] C **CTA**[4] CAA GCC CAA GTA CAG TGC AAT

HA-PRG-1 delta 1417-2292
Forward:
AT **GGT ACC**[1] **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCG TGC**[2]ATG CAG CGC GCT GGC TCC A
Reverse:
A**GC GGC CG**[3] C **CTA**[4] TGA CTG CCA GGC TCA GGC TCT AT

HA-PRG-1 delta 1850-2292
Forward:
AT **GGT ACC**[1] **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCG TGC**[2]ATG CAG CGC GCT GGC TCC A
Reverse:
A**GC GGC CG**[3] C **CTA**[4] CTC ATG GTC TGT CAA GGT TTT ATA

[1]Kpn 1 restriction site. [2]HA sequence. [3] Not 1 restriction site. [4]Stop codon.

A.2 Curriculum Vitae

CURRICULUM IS NOT ADDED IN THE PDF FORMAT DUE TO DATA PRIVACY PROTECTION

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A.3 Publications and presentations

ORIGINAL PAPERS IN INTERNATIONAL REFEREED JOURNALS

Analysis of PRG-1/Ras GRF-2 interaction and signalling effects. **Soriguera A**, Swiercz JM, Offermanns S, Nitsch R, Bräuer A.U. (in process, currently working on one extra experiment to add into the ready manuscript)

PRG-1 is expressed in Prostate Cancer and is a candidate prognostic marker of earlier PSA relapse. Bräuer A.U., Savaskan N.E., Rocha L., **Soriguera A**, Nitsch R., Stephan C, Sers C, Fritzsche F.R., Dietel M., Jung K., Kristiansen G. (in process, closing the experimental part)

Subcellular distribution of PRG-3 is associated with neurite consolidation Velmans T, Battefeld A, Geist B, Soriguera A, Strauss U, Bräuer A.U. (in process, manuscript under revision)

The actin-binding protein alpha-actinin-1 interacts with the metabotropic glutamate receptor type 5b and modulates the cell surface expression and function of the receptor. (2007) Cabello N, Femelli R, Canela L, **Soriguera A**, Mallol J, Canela EI, Robbins MJ, Lluis C, Franco R, Mcllhinney J, Ciruela F. J Biol Chem. 282(16):12143-53

Heptaspanning membrane receptors and cytoskeletal/scaffolding protein: Focus on Adenosine, Dopamine and metabotropic glutamate receptors function (2005). Ciruela F, Canela L, Burgueño J, **Soriguera A**, Cabello N, Canela E I, Casadó V, Cortés A, Mallol J, Woods A, Ferré S, Lluis C and Franco R. Journal of Molecular Neuroscience 65 277-292

COMMUNICATIONS

PRG-1 regulates N-Ras activity depending on extracellular LPA levels during axon growth. (2010) **Soriguera A**. 7th International PhD Symposium Berlin Brain Days 2010 (2010) Buch, Germany (Oral Presentation)

LPA Signalling during axon growth: Role for PRG-1/Ras GRF-2. (2010) **Soriguera A**. NETWORKS, a joint graduate schools symposium (2010) Wandlitz, Germany (Oral Presentation)

Lysophospholipid acid controls axonal outgrowth via PRG-1/Ras GRF-2 interaction. (2010) **Soriguera A**, Bardehle S, Hoffamnn S. A,Swiercz J.M., Offermanns S, Chun J, Nitsch R, Bräuer A.U, 7th Forum European Neuroscience Society FENS (2010) Amsterdam, Netherlands (Poster Presentation)

Lysophospholipid acid controls axonal outgrowth via PRG-1/Ras GRF-2 interaction. (2010) **Soriguera A**, Bardehle S, Hoffamnn S. A,Swiercz J.M., Offermanns S, Chun J, Nitsch R, Bräuer A.U, Berlin Neuroscience Forum; Liebenswalde, Germany (Poster Presentation)

LPA Signalling during axon growth: Role for PRG-1/Ras GRF-2. (2009) **Soriguera A**. Fall symposium GRK 1123; Berlin, Germany (Oral Presentation)

Analysis of PRG-1/Ras GRF-2 interaction and signalling effects. (2008) **Soriguera A**, Swiercz JM, Offermanns S, Nitsch R, Bräuer A.U, Berlin Neuroscience Forum; Berlin, Germany (Poster Presentation)

Analysis of the PRG-1 signalling cascade (2007) **Soriguera A**, Swiercz JM, Offermanns J,

Nitsch R, Bräuer AU; FASEB Summer School: Lysophospholipid Mediators in Health and Disease, Tucson, USA (Poster presentation)

Plasticity-related gene-1 and Ras-specific exchange factor 2 interaction controls axonal outgrowth (2006) **Soriguera A**, Swiercz JM, Offermanns J, Savaskan NE, Nitsch R, Bräuer AU. 17th European Students' Conference; Berlin, Germany (Poster presentation)

Axonal outgrowth is controlled by plasticity-related gene-1 Ras-specific exchange factor 2 interaction (2006) **Soriguera A**, Swiercz JM, Offermanns J, Singh B, Grantyn R, Nitsch R, Bräuer AU; Berlin Neuroscience Forum; Berlin, Germany (Poster presentation)

Alpha Actinin roll in the protein G coupled receptors heterodimerization (2004) Cabello N*, **Soriguera A***, Canela L*, Mallol J, Canela E.I, Lluis C, Franco F and Ciruela F: XXVII SEEBBM Congress; Lleida, Spain (Poster Presentation)

**These authors contributed equally to the work*

A.4 Thesis Declaration

I, Anna Soriguera Farrés, declare that I have personally authored the Doctoral submitted with the title:

LPA signalling during axon growth: Rule for PRG-1 / Ras GRF-2 interaction

and that I have not used any other sources or material apart from clearly mentioned in the bibliography. Furthermore, I have not benefited from the (illegal) assistance of a third party and I have not partially reproduced any other work.

Date Signature

A.5 Erklärung

„Ich, Anna Soriguera Farrés, erkläre, dass ich die vorgelegte Dissertation mit dem Thema:

LPA signalling during axon growth: Rule for PRG-1 / Ras GRF-2 interaction

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift

I wish to express, once more, my gratitude to all who contributed to this thesis.

Thank you, Danke, Efcharistíes, Dzieki, Grazie, Merci, Gràcies!