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The E3 ubiquitin ligase Praja1 inhibits the development of a neuronal phenotype in PC12 cells

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„Alles ist einfacher, als man denken kann, zugleich verschränkter, als zu begreifen ist.“

Everything is more simple than one would imagine,

yet at the same time more complex than could be conceived.

Johann Wolfgang von Goethe: *Maximen und Reflexionen*

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Abstract

The process of ubiquitination and the subsequent degradation of proteins via the proteasome complex are of pivotal importance for intracellular homeostasis. Additionally, it has been established that different types of ubiquitination may influence activity states as well as intracellular distribution of proteins. With regard to the central nervous system, there is a growing body of evidence proposing an essential role of ubiquitination in development and plasticity. Praja1 is a ubiquitin ligase of the E3 type that is highly expressed in brain tissue, is up-regulated during embryonal development, and is increased in the basolateral amygdala during the memory consolidation phase following fear conditioning. To elucidate the role of Praja1 in the central nervous system, its influence on NGF-induced differentiation in rat phaeochromocytoma (PC12) cells, an established model system for investigating neuronal differentiation, has been studied. Following NGF stimulation of PC12 cells, an up-regulation of Praja1 could be observed. Furthermore, Praja1 co-localized with the neurotrophin receptor interacting MAGE homologue (NRAGE) and Smad3, both of which being known mediators of various signalling pathways relevant during neuronal development and differentiation. Importantly, the tetracycline-induced over-expression of Praja1 in stably transfected PC12 cell lines has led to a drastic reduction in NGF-induced neurite outgrowth, which has been associated with a proteasome-dependent decrease in NRAGE levels. These data suggest that Praja1 inhibits neuronal-like differentiation in PC12 cells through poly-ubiquitination and subsequent degradation of NRAGE.

Key words

Praja1, ubiquitin ligase, neuronal differentiation, PC12, NRAGE

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List of abbreviations

AEBSF	4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride
ANOVA	analysis of variance
APC	adenomatous polyposis coli
APS	ammonium persulfate
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
BSA	bovine serum albumin
BMP	bone morphogenic protein
cDNA	complimentary deoxyribonucleic acid
CKB	creatine kinase, brain
CMV	cytomegalovirus
CNS	central nervous system
COS7	CV-1 origin with SV40 genetic material; fibroblast-like cell line derived from African green monkey kidney
CRH	corticotropin-releasing hormone
CSP	cysteine string protein (= DnaJC5)
CT	cycle threshold
CV	coefficient of variation
Da	Dalton
DAPI	4',6-Diamidin-2-phenylindol
DIV	days *in vitro*
Dlx	distal-less homeobox
Dlxin	Dlx-interacting protein
DMDC	dimethyl-dicarbonate
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DnaJC5	DnaJ (Hsp40) homologue, subfamily C, member 5 (= CSP)
dNTP	deoxyribose-containing nucleosid triphosphates
DOC	deoxycholic acid
DUB	de-ubiquitinating enzyme
DZNep	3-deazaneplanocin A
E2F	eukaryotic family of transcription factors
EDTA	ethylene-diamine-tetraacetate
EED	embryonic ectoderm development
EFNB1	ephrin B1
EGFP	enhanced green fluorescent protein
ELF	embryonic liver fodrin
Erk	extracellular-regulated kinase
ESE	exonic splicer enhancer
EZH	enhancer of zeste homologue

FAM	6-carboxyfluorescein
FBS	foetal bovine serum
FOXP3	forkhead box P3
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GST	glutathione S-transferase
HA	hemagglutinin
HECT	homologue to E6-AP C-terminus
HEK293	human embryonic kidney cell line
HPA axis	hypothalamus–pituitary–adrenal axis
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis
JNK	cJun N-terminal kinase
LAS AF	Leica Application Suite Advanced Fluorescence software
LDCV	large dense core vesicle
MAD	mothers against decapentaplegic
MAGE	melanoma antigen
MAP	mitogen-associated protein
MAPK	mitogen-activated protein kinase
Mecp	methyl-CpG binding protein
MHD	MAGE homology domain
mRNA	messenger ribonucleic acid
msh	muscle segment homeodomain
Msx	msh homeobox
NCAM	neural cell adhesion molecule
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor “κ-light-chain-enhancer” of activated B cells
NGF	nerve growth factor
NILE	NGF-inducible large external glycoprotein
NP-40 Alternative	Nonylphenyl polyethylene glycol
NPY	neuropeptide Y
NRAGE	neurotrophin receptor-interacting MAGE homologue (rat)
	Synonyms: Dlxin-1 (mouse), MAGE-D1 (human)
NT-3/4/5	neurotrophins 3 to 5
$p75^{NTR}$	p75 pan-neurotrophin receptor
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC12	pheochromocytoma-derived cell line from the rat adrenal medulla
PCR	polymerase chain reaction
PDL	poly-D-lysine
PFA	para-formaldeyhde
PGK	phosphoglycerate kinase

PHD	plant homeodomain
PHGDH	phosphoglycerate dehydrogenase
PI3K	phosphoinositid-3 kinase
PKB	protein kinase B (= Akt)
PRC	polycomb repressive complex
PTSD	post-traumatic stress disorder
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real-time polymerase chain reaction
Ras	rat sarcoma
Rcf	relative centrifugal force
RESCUE	relative enhancer & silencer classification by unanimous enrichment
RING	really interesting new gene
RNA	ribonucleic acid
Ror	receptor-tyrosine-kinase-like orphan receptor
RQ	relative quantification
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate poly-acrylamide gel electrophoresis
S.E.M.	standard error of the mean
Slc18a	solute carrier family 18 vesicular mono-amine transporter (= VMAT)
SMA	small body size
Smad	Combination of SMA and MAD (see there)
SMC	structural maintenance of chromosomes
SSRI	selective serotonin re-uptake inhibitor
SUZ	suppressor of zeste
TBS	tris-buffered saline
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TNF	tumour necrosis factor
TRE	tetracycline-responsive element
Trk	tropomyosin-related kinase
UBE2	ubiquitin-protein ligase / ubiquitin-conjugating enzyme, type E2
UBE3	ubiquitin-protein ligase, type E3
UNC5H1	uncoordinated-5 homologue H1
UP	Ultra Pure
UPS	ubiquitin-proteasome system
VMAT	vesicular monoamine transporter (= Slc18a)
Wnt	wingless type

1. Zusammenfassende Darstellung

Für lange Zeit ist der Prozeß der Ubiquitinierung, d. h. die Markierung von Proteinen mit sogenannten „Ubiquitin"-Peptiden, nahezu ausschließlich im Zusammenhang mit der Halbwertzeit von Proteinen betrachtet worden. In der Tat führt das Anfügen längerer Ubiquitinketten (Polyubiquitinierung) zum Abbau des auf diese Weise markierten Proteins durch den Proteasomkomplex. Wie sich in den letzten Jahren jedoch zunehmend gezeigt hat, spielt insbesondere die post-translationale Modifikation durch kurze Ubiquitinketten oder einzelne Ubiquitinpeptide (Mono- und Multimonoubiquitinierung) im Rahmen intrazellulärer Transportvorgänge eine wichtige Rolle und hat sich als bedeutsam für die Aktivität einer Vielzahl von Signalwegen erwiesen. Die Erkenntnis, daß eine Störung der Ubiquitinierungsfunktion an der Pathogenese zahlreicher Erkrankungen und Syndrome beteiligt ist, hat zusätzlich dazu beigetragen, daß Ubiquitinierung mittlerweile als ein zentraler Vorgang für die Aufrechterhaltung der zellulären Homöostase sowie die Anpassung der Zelle an neue Gegebenheiten angesehen wird. Obschon die wesentlichen Abläufe im Rahmen der Ubiquitinierung von Proteinen bekannt sind, ist das Wissen über einen Großteil der an der Ubiquitinierung beteiligten Enzyme und über deren genaue Wirkung innerhalb der Zelle in den meisten Fällen nach wie vor gering.

Zu diesen Enzymen zählt auch die E3 Ubiquitinligase Praja1, die erstmals im Zusammenhang mit der Leberentwicklung von Mäusen beschrieben (Mishra et al. 1997) und in der Folge mit der Entstehung gastrointestinaler Tumoren in Verbindung gebracht worden ist (Mishra et al. 2005; Saha et al. 2006). Bereits frühzeitig ist zudem die mögliche Bedeutung von Praja1 für die neuronale Entwicklung diskutiert worden, da die Sequenz dieser Ubiquitinligase der von Neurodap1 (Nakayama et al. 1995), einem bekannten Faktor in der Entwicklung des zentralen Nervensystems, ähnelt. Die starke Expression von Praja1 im Gehirn (Yu et al. 2002) sowie ein Anstieg der Praja1-Expression in der Amygdala nach Furchtkonditionierung (Stork et al. 2001) haben dazu beigetragen, diesen Verdacht zu erhärten.

Um die postulierte Praja1-Funktion in neuronalen Zellen herauszuarbeiten, wurde in der vorliegenden Studie der Einfluß dieser Ubiquitinligase auf die durch den Wachstumsfaktor NGF induzierte Differenzierung von PC12-Zellen untersucht. Aufgrund ihrer Fähigkeit, unter Stimulation durch NGF einen Phänotyp ähnlich dem sympathischer Neurone zu entwickeln und in diesem Zusammenhang verstärkt neuritenartige Fortsätze auszubilden und neuronale Proteine zu exprimieren, ist diese Phäochromozytoma-Zellinie als Modellsystem für eine Differenzierung ähnlich der in Neuronen etabliert (Greene & Tischler 1976; vgl. McGuire & Greene 1980).

Neben dem immunzytochemischen Nachweis der verstärkten Expression charakteristischer Neurofilamente wie β3-tubulin und MAP2 nach NGF-Gabe als Beleg für ein suffizientes

NGF-Stimulationsprotokolls konnte gezeigt werden, daß die endogene *praja1*-Expression durch die Stimulierung der Zellen mit NGF innerhalb von drei Tagen auf das Dreifache gesteigert wird. Um die sich daraus ergebende Rolle von Praja1 während der Differenzierung besser zu verstehen, ist daraufhin die Morphologie von stabil transfizierten PC12-Zellen nach tetracylin-induzierter Praja1-Überexpression untersucht worden. Dabei hat sich eine massive Reduktion des durch NGF ausgelösten Neuritenwachstums in den PC12-Zellen gezeigt. Diese Wirkung des Praja1 scheint im wesentlichen unabhängig von extrazellulären Substraten zu sein, wenngleich Signalwege der integrin-vermittelten zellulären Adhäsion einen gewissen modulatorischen Einfluß auszuüben scheinen.

Frühere Studien haben nachweisen können, daß Praja1 das in mehreren Signalwegen eingebundene Protein NRAGE polyubiquitiniert und dessen proteasomalen Abbau induziert (Sasaki et al. 2002). NRAGE wiederum ist als Interaktionspartner der NGF-Rezeptoren $p75^{NTR}$ und TrkA in mehreren Untersuchungen mit Proliferationshemmung und Apoptose (z. B. Salehi et al. 2000) bzw. mit der neuronalen Differenzierung (Feng et al. 2010; Reddy et al. 2010) von PC12-Zellen in Zusammenhang gebracht worden. In Übereinstimmung mit diesen Befunden hat die Überexpression von Praja1 in dem hier verwendeten PC12-Modell zu einer Verringerung der NRAGE-Proteinlevel geführt, die durch Blockade des Proteasoms mittels Lactacystin verhindert werden konnte. Dies spricht dafür, daß der Praja1-vermittelte Abbau von NRAGE zu einer Störung der Signalwege führt, die die durch NGF hervorgerufene Differenzierung koordinieren. Hierzu zählen insbesondere von $p75^{NTR}$ und TrkA abhängige Kaskaden (vgl. Kaplan & Miller 2000; vgl. Arévalo & Wu 2006).

Da NRAGE in verschiedenen Studien eine proapoptotische Wirkung entfaltet hat (z. B. Salehi et al. 2000) und weil die Überexpression von Praja1 in Fibroblasten-Zellinien für Apoptose charakteristische morphologische Veränderungen hervorgerufen hat (Teuber et al. 2013), ist mittels Lumineszenz-Assay kontrolliert worden, inwieweit die beschriebene Hemmung der neuronalen Differenzierung von PC12-Zellen durch Praja1 auf eine beginnende Apoptose zurückzuführen sein könnte. Dabei hat sich jedoch keine Erhöhung der Aktivität der Caspasen 3 oder 7 in Praja1 überexprimierenden Kulturen gezeigt.

Aufgrund der mittels quantitativer real-time PCR ermittelten Expressionsmuster entsprechender Marker (Slc18a1, DnaJC5 und NPY) scheint es vielmehr, als würde Praja1 PC12-Zellen in Richtung eines sekretorischen Phänotyps drängen und gleichzeitig die neuronenartige Differenzierung inhibieren.

Die unterschiedlichen funktionalen Effekte der Praja1-Überexpression in PC12-Zellen und Fibroblasten-Linien sprechen dafür, daß die Wirkung von Praja1 wenigstens teilweise von der Zusammensetzung des jeweiligen, zelltypspezifischen Proteoms sowie den auf einer Zelle vorhandenen Rezeptoren abhängt. Dementsprechend könnten ähnliche oder gleiche

molekulare Interaktionen, wie beispielsweise der Abbau von NRAGE nach Ubiquitinierung durch Praja1, unterschiedliche Effekte in verschiedenen Gewebs- und Zelltypen hervorrufen.

Im Vorfeld dieser Studie sind zwei unterschiedliche Praja1-Isoformen als Folge alternativer Spleißvorgänge beschrieben worden: Praja1.1 und Praja1.2 (Mishra et al. 1997; Stork et al. 2001; Teuber et al. 2013). Die Transfektion mit einem *praja1.1*-Konstrukt führt aufgrund des alternativen Spleißens typischerweise zur Expression sowohl von Praja1.1 als auch von Praja1.2 in stöchiometrischen Verhältnissen, die denen in PC12-Zellen sowie im adulten Gehirn entsprechen. Dagegen führt die Transfektion mit *praja1.2* lediglich zur Expression von Praja1.2. Aufgrund der Expressionsdynamik *in vivo* ist vermutet worden, daß Praja1.2 insbesondere während der embryonalen Entwicklung von Bedeutung sein könnte. Die hier dargestellten Ergebnisse sprechen jedoch für eine insgesamt vergleichbare Funktion beider Isoformen, wenngleich einige subtile Unterschiede zu verzeichnen waren. So deutet sich an, daß Praja1.2 in geringerem Maße im Nucleus zu finden ist als Praja1.1, daß Praja1.2 weniger stark zu einer Vergrößerung der Zellfläche führt, was auf eine geringere Beeinflussung adhäsiver Signalwege hinweisen könnte, und daß Praja1.2 das Überleben der Zellen unter NGF-deprivierten Bedingungen fördern kann.

Zusammenfassend ist festzuhalten, daß die hier vorgelegte Untersuchung anhand eines Modellsystems erste konkrete Hinweise dafür liefert, daß die E3 Ubiquitinligase Praja1 als Mediator der neuronalen Differenzierung zu agieren vermag. Durch Regulation der Proteinlevel des Signalmoleküls NRAGE ist Praja1 imstande, u. a. BMP- und NGF-abhängige Signalwege zu beeinflussen. Diese wiederum sind mit anderen Signalkaskaden verknüpft, zum Beispiel denen der Wnt-Familie, so daß Grund zu der Annahme besteht, daß Praja1 eine wichtige Rolle während der Formierung und Entwicklung des zentralen Nervensystems spielen und darüber hinaus Einfluß auf Lernprozesse und die Gedächtniskonsolidierung nehmen könnte.

2. Introduction

While admiring nature makes most people value simplicity and regularly leads to feelings of awe, calmness, and tranquillity, insights into the processes lying beneath paradoxically reveal a most intricate interplay of seemingly uncountable factors, regardless of whether the dynamic change of mountain ranges, the patterns of sea wave potentiation and distribution, or the development and physiological function of biological species and individuals are investigated. To date, even the minutest observation in any scientific field uncovers new realms of complexity. This is no less true for the development not only of entire species or organisms, but also of their organs or even of single cells. It is, thus, hardly surprising that the development and differentiation of cells and tissues of neuronal, but also of non-neuronal background rely on closely intertwined cellular programmes in interplay with extracellular signals, such as diffusible factors, components of the extracellular matrix, and interactions with surrounding cells.

2.1 Ubiquitination

Among the intracellular processes, one that has gained growing attention within the past decades is that of ubiquitination. Its name refers to the observation that the targeted labelling of proteins with an 8.5 kDa peptide called “ubiquitin” has been found in all eukaryotic cells and is hence truly “ubiquitous”. Classically, attachment of ubiquitin peptide chains to proteins has only been considered as an important regulator of protein half-life, because ubiquitinated proteins have appeared to be solely prone for degradation by the proteolytic, multi-enzyme 26 S proteasome complex. However, it has become increasingly clear that ubiquitination is also pivotal for regulating processes such as receptor internalization and endocytosis, intracellular trafficking as well as transcriptional regulation. While targeting of proteins for proteasomal degradation has been commonly linked to poly-ubiquitination, i. e. the addition of four or more ubiquitin peptides in a chain, other functions of ubiquitination, like induction of receptor endocytosis and marking proteins or endosomes for lysosomal degradation, seem to rely on mono- or multi-mono-ubiquitination (cf. Glickman & Ciechanover 2002; cf. Murphey & Godenschwege 2002; cf. Mukhopadhyay & Riezman 2007; cf. Shenoy 2007).

Post-translational modification of a protein through ubiquitination requires several steps to occur. First of all, the ubiquitin peptide needs to be activated by an E1 ligase in an endo-thermic manner relying on the presence of adenosine triphosphate (ATP). Subsequently, the activated ubiquitin binds to an E2 ligase, which serves as a recognition partner for the numerous substrate-specific E3 ligases. The latter may then transfer the ubiquitin to lysine residues or, less frequently, to the amino terminus or to cysteine residues of a specific

protein, and by doing so regulate intracellular proteome homeostasis as well as signalling pathway activity (cf. Glickman & Ciechanover 2002).

Two major types of E3 ligases have been found to date: Those containing a homologue to E6-AP C-terminus (HECT) domain and those with a really interesting new gene (RING) motif, both of which are essential for their respective function as an E3 ligase. While the former covalently bind the activated ubiquitin before transferring it to their substrate, E3 ligases of the latter type appear to serve as scaffolding proteins with the cysteine- and histidine-rich, zinc-dependent RING motif creating spatial proximity between the substrate and the E2 ligase carrying an activated ubiquitin, which is then attached to the substrate by the E2 ligase itself (Lorick et al. 1999; cf. Glickman & Ciechanover 2002). The scaffolding function of RING E3 ligases may also depend on the formation of larger protein complexes, as has been shown on several occasions (cf. Glickman & Ciechanover 2002). Aside from these most prominent types of E3 ligases, other proteins containing several kinds of zinc finger domains, e. g. the plant homeodomain (PHD) motif, have been implicated to function as E3 ligases (cf. Coscoy & Ganem 2003). Furthermore, elongation of ubiquitin chains may be facilitated by another group of E3 ligases, often also referred to as E4 ligases, which contain a U-box, commonly recognized to be a modified RING domain (Koegl et al. 1999; Aravind & Koonin 2000; cf. Glickman & Ciechanover 2002). Degradation-inducing ubiquitin chains are usually linked via the lysine at position 48 in the ubiquitin sequence. In contrast, linkage via the lysine at position 63 has been found to trigger non-proteasomal pathways (cf. Glickman & Ciechanover 2002; Geetha et al. 2005; cf. Shenoy 2007).

Over the years, evidence implying important functions of ubiquitination during cell cycle control, development, and differentiation has accumulated, as also indicated by the manifold involvements in pathogenesis known to date; most prominently, mutations or aberrations of components of the ubiquitin-proteasome system (UPS) that may either induce stabilization of proto-oncogenes, e. g. several immediate early genes, or increase degradation of tumour suppressors, such as p53, may promote cancer development. With regard to the nervous system, several genetically determined conditions have been associated with altered UPS components. Angelman Syndrome has been among the first genetic disorders to be linked to an E3 ligase. A deletion in the maternal chromosome region 15q11-13, which comprises the *ubiquitin-protein ligase E3A* (*UBE3A*) gene, or direct mutation of the *UBE3A* gene have been found to cause Angelman Syndrome due to the parallel silencing of the corresponding paternal chromosome region through imprinting (Kishino et al. 1997; Matsuura et al. 1997). Autosomal recessive forms of juvenile Parkinsonism have been associated with another mutated E3 ligase called “Parkin” (Kitada et al. 1998; Moore 2006). Moreover, a less specified imbalance of proper ubiquitination has been implicated in a wide range of neuro-degenerative diseases like Alzheimer’s Disease, different kinds of ataxia, including Fragile X Associated Tremor / Ataxia Syndrome (Willemsen et al. 2003), Huntington’s Disease, or

other forms of Parkinson's Disease, although most of the exact pathogenic mechanisms are still unknown (cf. Glickman & Ciechanover 2002; cf. Johnston & Madura 2004; Meray & Lansbury 2007). Yet, there is good reason to attribute an important role to ubiquitination and E3 ligases in differentiating and mature neurons (cf. Murphey & Godenschwege 2002; cf. Glickman & Ciechanover 2002; cf. Johnston & Madura 2004; cf. Hernández et al. 2004). Accordingly, cell culture experiments have revealed elevated levels of ubiquitinated proteins during neuronal-like differentiation of phaeochromocytoma (PC12) cells as induced by the application of nerve growth factor (NGF) (see section 2.4 for an introduction to the PC12 model), while treatment of PC12 cells with the proteasome inhibitor lactacystin and subsequent accumulation of proteins could mimic NGF-induced neurite outgrowth in an apparently E3-ligase-dependent manner (Obin et al. 1999). Furthermore, NGF binding to its receptors and subsequent lysine-63 poly-ubiquitination of its high-affinity receptor have been shown to be pivotal for NGF-induced differentiation of PC12 cells (Geetha et al. 2005). Studies in primary neuronal cultures, on the other hand, have found adverse effects of proteasome inhibitor treatment on neuronal differentiation, resulting either in inhibition of neurite outgrowth or in neurite degeneration resembling a dying-back pattern in cultures that had already been allowed to differentiate (Laser et al. 2003). Nonetheless, ubiquitination and subsequent proteasomal degradation of synaptic proteins in primary neurons have been observed to play an important role during the activity-dependent reorganization of the postsynaptic density (Ehlers 2003) and with regard to general synaptogenesis as well as synaptic maintenance (cf. Johnston & Madura 2004). Furthermore, a profound role of the UPS during memory formation has been demonstrated in many different studies: Its induction in the rat hippocampus is needed for long-term memory formation, and inhibitory avoidance training leads to an increase in ubiquitination levels (Lopez-Salon et al. 2001). In accordance with the afore-mentioned findings by Ehlers, poly-ubiquitination-dependent degradation of postsynaptic proteins has been described in the hippocampus after retrieval of contextual fear memory, while proteasome blockade has been capable of preventing extinction of fear memory (Lee et al. 2008). Similarly, Jarome and collaborators have found evidence that protein degradation via the UPS is essential during synaptic reorganization and memory formation in the amygdala after fear conditioning (Jarome et al. 2011).

The key role of ubiquitination processes in mediating cellular activity may also be indicated by their tight control. Many E3 ligases have been found to be capable of auto-ubiquitination, hence self-regulating their activity. Furthermore, abundance of de-ubiquitinating enzymes (DUB), which may rescue poly-ubiquitinated proteins from proteasomal degradation, or mechanisms like SUMOylation, which can block lysine residues and prevent ubiquitination, indicate that a fine balance between ubiquitination and other modifications is needed to maintain cellular stability or to allow for particular changes to occur (cf. Johnston & Madura 2004; cf. Glickman & Ciechanover 2002).

2.2 The E3 ubiquitin ligase Praja1

Praja1, which means “birth” or “development” in Sanskrit, is an E3 ubiquitin ligase containing a RING motif (Lorick et al. 1999). Initially, *pja1* has been identified as a gene related to liver development (Mishra et al. 1997), but sequence similarity to Neurodap1 (Nakayama et al. 1995), which is also referred to as Praja2, and prominent expression in the brain (Yu et al. 2002) have also indicated an involvement in nervous system function, making Praja1 a candidate for the control of neuronal development and plasticity in the nervous system (Mishra et al. 1997; Stork et al. 2001; Loch et al. 2011). This notion is supported by the clinical observation that a deletion of the X-chromosomal region harbouring the *pja1* gene is found in patients with craniofrontonasal syndrome and appears to be associated with mild learning disabilities (Yu et al. 2002; Wieland et al. 2007). Furthermore, Praja1 has been found to be up-regulated in the basolateral amygdala (BLA) following fear conditioning (Stork et al. 2001) and has been reported to target various anti-apoptotic and brain developmental factors (Loch et al. 2011).

Alternative splicing of the murine *pja1* gene generates two transcript variants termed *praja1.1* (National Center for Biotechnology Reference Sequence: NM_001083110.1) and *praja1.2* (NCBI Reference Sequence: NM_008853.3) that code for two isoforms, hereafter referred to as Praja1.1 and Praja1.2, with predicted molecular weights of 64 kDa and 44 kDa, respectively (Teuber et al. 2013). Accordingly, ESEfinder (Cartegni 2003) and RESCUE-ESE (Fairbrother et al. 2002) online services have identified relatively strong SC-35 exonic splicing enhancer (ESE) consensus sequences, which are known to be important regulators of exon inclusion (Liu et al. 2000), in proximity to splice sites within the *pja1* gene. Analysis of *pja1* expression during development in mice by Anne Albrecht has revealed a 3.5-fold increase in Praja1 messenger ribonucleic acid (mRNA) levels between embryonic stages at day 7 (E7) and 11 (E11) post conception. Until E15, both *pja1* transcript variants show equal expression levels, but then levels of Praja1.2 mRNA decline, leaving Praja1.1 to be the dominating variant in adult mice with a ratio of about 3:1 compared to Praja1.2 in most tissues (Teuber et al. 2013). In general, *praja1* expression is found in a wide range of tissues in adult mice, most prominently in testes, but also in brain, spleen, lung, or liver; in contrast, kidney, heart, and skeletal muscle show only low levels of expression (Teuber et al. 2013).

A Praja1 association with microtubules, as indicated by a comparable cytosolic distribution, overlapping with filamentous structures, has been observed by Ryoji Fukabori in immuno-cytochemical experiments in neuronal-like differentiated PC12 cells after NGF stimulation. Disruption of this association by treatment with colchicine has pointed to a dependence on microtubule integrity (Teuber et al. 2013). Less frequently, association of Praja1 with micro-filaments has been found, in particular at sites of neurite outgrowth and in filopodia (Teuber et al. 2013).

Functionally, evidence from fibroblast-like COS7 cells has indicated a pro-apoptotic role of Praja1 by inducing micro-spike formation, cell rounding, and the development of pyknotic nuclei (Teuber et al. 2013).

2.2.1 Substrates of Praja1

Among the most promising substrates of Praja1 that could be identified to date is the rat neurotrophin receptor-interacting MAGE homologue (NRAGE), named melanoma antigen D1 (MAGE-D1) in human and distal-less homeobox (Dlx) interacting protein 1 (Dlxin-1) in mouse. For the sake of clarity, it will only be referred to as “NRAGE” in the following. NRAGE is a member of the class II MAGE family and serves as a multi-functional signalling molecule. It has generally been recognized for its involvement in NGF and bone morphogenic protein (BMP) signalling, but has recently also been implicated in receptor-tyrosine-kinase-like orphan receptor (Ror) 2 signalling as well as in cell adhesion mediated by axonal guidance receptors such as uncoordinated-5 homologue H1 (UNC5H1) or by cadherins; all of which are involved and appear to interact in regulating neuronal development (Iwasaki et al. 1999; Salehi et al. 2000; Bui et al. 2002; Ito et al. 2002; Althini et al. 2003; Matsuda et al. 2003; Williams et al. 2003; Lönn et al. 2005; cf. Nykjaer et al. 2005; Xue et al. 2005; Lai et al. 2012; cf. Mouri et al. 2013). Underpinning the versatile role of NRAGE in neuronal survival and differentiation, NRAGE-deficient mice present a peculiar phenotype combining elements of depression, autism, and Prader-Willi-Syndrome: Hyperphagia paired with reduced motor activity lead to progressive obesity and with regard to behaviour, these mice show a reduction in social interactions and memory performance, increased levels of anxiety and self-grooming as well as deficient sexual behaviour, which could be alleviated by administration of oxytocin, selective serotonin re-uptake inhibitors (SSRI), or tricyclic anti-depressants (Dombret et al. 2012; Mouri et al. 2012).

Following over-expression in human embryonic kidney (HEK) 293 cells, Sasaki *et alii* have found an increased ubiquitination of NRAGE by Praja1, paired with a reduction in NRAGE protein levels and NRAGE-dependent transcriptional activity (Sasaki et al. 2002; Masuda et al. 2001). *In vitro* experiments have additionally demonstrated direct binding of Praja1 to the MAGE homology domain (MHD) within the NRAGE sequence via a region close to the Praja1 RING domain (Sasaki et al. 2002). Interestingly, the expression pattern of murine NRAGE is very similar to that of Praja1. Just like the latter, NRAGE is up-regulated between E7 and E11 and is highly expressed in brain, liver, and testes, whereas skeletal muscle, kidney, spleen, and lung show low or undetectable expression levels (Masuda et al. 2001). In comparison, adult animals as well as differentiated PC12 cells present drastically reduced levels of NRAGE (Salehi et al. 2000; Masuda et al. 2001; Williams et al. 2003). So far, NRAGE has mainly been recognized for its pro-apoptotic role in various cell types (Salehi

et al. 2000; Jordan et al. 2001; Salehi et al. 2002; Kendall et al. 2005), but also for its alteration of neuronal differentiation of PC12 cells (Feng et al. 2010; Reddy et al. 2010).

Aside from NRAGE, Sasaki and collaborators have observed a ubiquitination-dependent degradation of muscle segment homeodomain (msh) homeobox (Msx) 2 – a pivotal factor in the differentiation of osteoblasts – caused by Praja1. However, because they had not found any evidence for direct binding of Praja1 to Msx2, they have speculated that a complex formed by Praja1, NRAGE, and Msx2 may serve to make Msx2 accessible for ubiquitination by Praja1 (Sasaki et al. 2002). From a clinical perspective, substitution of one single amino acid within the Msx2 protein sequence is known to cause autosomal dominant Boston-type craniosynostosis, which is characterized by skull defects and insufficient brain development. The mutated $Msx2_{P138H}$ also appears to enhance degradation of Msx2 by Praja1, potentially explaining the dominant-negative loss-of-function of this mutation (Yoon et al. 2008; Jabs et al. 1993).

Transforming growth factor β (TGF-β) signalling, which is known to be of importance for cell polarity, cell differentiation, and tumour suppression, is mediated by the family of so-called "Smad" proteins within the cell; an allusion to their *Caenorhabditis elegans* and *Drosophila melanogaster* homologues small body size (SMA) and mothers against decapentaplegic (MAD). The downstream transcriptional activity of Smad proteins is regulated by adaptor proteins such as embryonic liver fodrin (ELF) (Tang et al. 2003). Mishra's group has also revealed an inverse relationship between Praja1 and ELF protein levels in hepatocytes and two gastrointestinal cancer cell lines, which could be linked to ubiquitination of ELF by Praja1 (Mishra et al. 2005; Saha et al. 2006). This interaction and subsequent degradation of ELF is accelerated by TGF-β stimulation, upon which Praja1 also moves from a diffuse distribution in the cytoplasm to specific cell-to-cell contact points at the plasma membrane (Saha et al. 2006). In addition, a weak ubiquitination of Smad3 has been observed by Saha and collaborators; yet, the physiological relevance of this has not been addressed in their study (Saha et al. 2006). However, Praja1 over-expression has been demonstrated to be capable of disrupting TGF-β signalling and of increasing cell proliferation. This has led to the assumption that basal Praja1 expression might be of importance in a self-regulating, negative feedback loop in TGF-β signalling, but that increased Praja1 expression could be tumourigenic (Saha et al. 2006). Accordingly, Praja1 levels are known to be elevated in several gastrointestinal cancers, while up to 37.5 % of gastric cancers present decreased Smad3 levels (Han et al. 2004; Mishra et al. 2005; Saha et al. 2006).

In a comprehensive study of human proteomic profiles, Loch and colleagues have screened for other potential interaction partners of Praja1. They have identified several human E2 ubiquitin ligases that interact with Praja1, among those ubiquitin-conjugating enzyme E2D2 (UBE2D2), UBE2D3, UBE2E3, and UBE2K, with the first two showing the highest activity levels (Loch et al. 2011). Focussing on UBE2D3 as the partnering E2 ligase, they have

tested those human proteomic profiles for substrates, clustered their results for substrate function, and found that Praja1 appears to target factors playing a role in brain development, anti-apoptotic factors, proteins linked to kinase activity as well as proteins involved in RNA processing and transcription (Loch et al. 2011). The factors involved in brain development comprise phosphoglycerate dehydrogenase (PHGDH), needed during amino acid synthesis, and brain creatine kinase (CKB), which is important for energy homeostasis (Loch et al. 2011).

While NRAGE has not been on their arrays, Loch *et alii* have identified another member of the MAGE family, MAGE-B4, to be poly-ubiquitinated by Praja1 (Loch et al. 2011). Whereas MAGE-D1 is widely expressed throughout the body, MAGE-B4 expression has been strictly localized to foetal gonads and adult testes and appears be relevant during gametogenesis (Osterlund et al. 2000; Mäkelä et al. 2014).

Interestingly, Praja1 may also decrease levels of the polycomb repressive complex (PRC) 2 that methylates histone H3, hence modulating gene expression and influencing epigenetic modification (Zoabi et al. 2011). PRC2 consists of three components: the histone H3 methyl transferase enhancer of zeste homologue (EZH) 2, suppressor of zeste (SUZ) 12, and embryonic ectoderm development (EED). Praja1 may individually target any of the PRC2 components for poly-ubiquitination and subsequent degradation. However, whether the entire complex may also be a target of Praja1 has not been resolved, yet (Zoabi et al. 2011).

2.2.2 Regulation of Praja1 expression and its activity

So far, little is known about the regulators of Praja1 expression and activity. As could be demonstrated for a number of RING E3 ligases, Praja1 is capable of auto-ubiquitination in absence of substrates, indicating a tightly controlled self-regulation of its activity (Saha et al. 2006; Teuber et al. 2013).

Within recent years, however, two inductors of *pja1* gene expression have been identified. On the pharmacological side, the unselective histone methylation inhibitor 3-deazaneplanocin A (DZNep), known for decreasing PRC2 levels via induction of proteasomal degradation, has been found to do so by elevating levels of Praja1 (Miranda et al. 2009; Zoabi et al. 2011). Among the common transcription factors, forkhead box P3 (FOXP3), an important tumour suppressor, e. g. in mammarian cancer, has been observed to induce *pja1* expression in order to subsequently reduce EZH2 levels as well, thus regulating proliferation and cell survival (Shen et al. 2013).

Interestingly, Praja1 activity might also be modulated by interaction with MAGE proteins. Several MAGE family members have been found to bind to RING E3 ligases via their shared MHD and serve as modulators by increasing E3 ligase activity rather than being targeted for degradation by the UPS (Doyle et al. 2010). In that regard, MAGE-G1, best known for its

involvement in the chromatin re-organizing structural maintenance of chromosomes proteins (SMC) 5-6 complex (Taylor et al. 2008), has been proposed to be another interaction partner of Praja1 (Doyle et al. 2010). Notably, the murine homologue of MAGE-G1, expressed in brain, testes, ovaries, and kidney, might be involved in neurodevelopmental disorders such as Prader-Willi-Syndrome, has been demonstrated to inhibit proliferation in an osteosarcoma cell line, appears to suppress activity of the eukaryotic transcription factor E2F1, and interacts with the death domain of the p75 pan-neurotrophin receptor ($p75^{NTR}$) (Kuwako et al. 2004). Weaker interactions between Praja1 and the potentially cancerogenic factors MAGE-A2, MAGE-B18, and MAGE-C2 (the latter is only expressed in testes) have also been implicated (Doyle et al. 2010), although their physiological function and relevance are unknown to date.

2.3 The potential involvement of Praja1 in different signalling pathways

Based on the fact that Praja1 has been found to reduce levels of NRAGE, Msx2, and Smad3, it may be assumed that this E3 ubiquitin ligase might mediate the signalling cascades in which these proteins are involved, the most prominent of which shall be briefly introduced hereafter.

2.3.1 The basics of BMP signalling

Originally, NRAGE has been identified as an activator of Dlx5 transcriptional activity and hence as a mediating factor in BMP signalling (Masuda et al. 2001). BMP are members of the TGF-β family, originally recognized for their ability to induce bone or cartilage formation via Smad-dependent as well as Smad-independent signalling (Urist 1965; Reddi & Huggins 1972; Chen et al. 2012; Nishimura et al. 2012). More recently, BMP signalling has been linked to dendritic growth and synapse stabilization in neuronal cells (cf. Liu & Niswander 2005; Lee-Hoeflich et al. 2004; Eaton & Davis 2005; Meng et al. 2002; Endo et al. 2003). In addition, BMP has been shown to control key steps during the development of the central nervous system (CNS) by regulating cell fate, proliferation, and differentiation at distinct developmental stages (cf. Liu & Niswander 2005). Certain members of the BMP family have been demonstrated to induce neuronal-like differentiation in PC12 cells (cf. section 2.4) as well as differentiation of neurons producing γ-aminobutyric acid (GABA). At least in the latter, it seems as if development of a GABAergic phenotype relies on p38 and is independent of other signalling pathways that have been shown to play a role in neuronal differentiation, especially activation of extracellular-regulated kinase (Erk) 1/2, also named mitogen-activated protein kinase (MAPK) p42/p44 (Iwasaki et al. 1996; Iwasaki et al. 1999; Hattori et al. 1999). In contrast, other BMP family members only enhance NGF-induced neuritogenesis instead of inducing neuritogenesis themselves (Althini et al. 2003; Lönn et al. 2005).

BMP-binding to one of its receptors typically leads to the subsequent activation of receptor-specific Smad proteins (Smad2 and Smad3), which may then form a complex with Smad4,

translocate to the nucleus, and act as transcription factors (Piek et al. 2001; Lutz et al. 2004). This process is tightly regulated by intracellular inhibitory Smad proteins (Smad6 or Smad7), which lead to proteasomal degradation of the activated Smad complex, by phosphorylation through the rat sarcoma (Ras) and Erk1/2 pathway, or by negative feedback loops following BMP-induced gene expression (Kretzschmar et al. 1997; cf. Liu & Niswander 2005).

Among the genes induced by the BMP cascade are several homeobox genes of the highly conserved Msx family, which are known for contributing to neurogenesis and dorsoventral patterning during CNS development (Isshiki et al. 1997; Liu et al. 2004) and in turn regulate BMP signalling upon their expression (Alvarez Martinez et al. 2002; Binato et al. 2006; cf. Liu & Niswander 2005). One of them is *msx2* (Hussein et al. 2003; Nishimura et al. 2012), which has been identified as a target of Praja1, as previously stated. Remarkably, there is some indication that wingless-type (Wnt) signalling – another important signalling cascade in development and differentiation of the CNS – might also be able to induce expression of the *msx2* gene, indicating that pivotal signalling cascades in development such as BMP and Wnt signalling may be intertwined to some degree (Willert et al. 2002; Hussein et al. 2003; Riccomagno et al. 2005; Zhai et al. 2011; Chen et al. 2012).

Depending on an increase in Msx activity (Liu et al. 2004), BMP also induces expression of *dlx5*, a member of the Dlx family, which has first been identified in relation to development and fracture healing of the skeletal system, but has meanwhile been recognized for its promoting effects in neurogenesis, especially with regard to GABAergic interneurons, in which Dlx5 activity induces expression of the key enzyme glutamic acid decarboxylase (GAD) (cf. Masuda et al. 2001; Stühmer et al. 2002; Perera et al. 2004; Yu et al. 2011). Moreover, up-regulation of Dlx5 and Dlx6 in knock-out mice lacking the methyl-CpG binding protein (Mecp) 2 has linked Dlx5 to the X-chromosomal neurodevelopmental disorder called Rett Syndrome, which is caused by mutations in the *Mecp2* gene (cf. Wenk 1997; Horike et al. 2005).

Importantly, NRAGE binds to and stimulates transcriptional activity of Dlx5 via its N-terminal transcriptional activation domain (Masuda et al. 2001) – hence its naming as Dlx-interacting protein (Dlxin-1) in mice – and has therefore been proposed to influence the development of GABAergic neurons (Kuwajima et al. 2004). Furthermore, NRAGE could potentially contribute to the phenotype in *Mecp2*-null mice by activating the up-regulated Dlx5, although this has not been further studied, yet. However, while elevating expression levels of Msx2 and Dlx5, BMP stimulation has been insufficient to increase NRAGE expression (Masuda et al. 2001).

2.3.2 A brief introduction to neurotrophin signalling

Although BMP signalling has been identified to play a role in neuronal differentiation as well, first implications for a role of NRAGE during neuronal development have resulted from its involvement in NGF neurotrophin signalling.

The term “neurotrophin” refers to a family of five proteins, namely NGF, brain-derived neurotrophic factor (BDNF), and the neurotrophins 3 to 5 (NT-3, NT-4, NT-5), all of which seem to have derived from one common neurotrophin ancestor (cf. Murer et al. 2001). As the name implies, neurotrophins are important for the survival and differentiation of neurons, although it is important to note that effects of neurotrophins are not restricted to the nervous system (cf. Aloe et al. 1994; cf. Cirulli & Alleva 2009; cf. Gioiosa et al. 2009). Neurotrophins bind to two types of receptors: the high-affinity tropomyosin-related kinase (Trk) receptors with an intracellular tyrosine kinase activity and the low-affinity pan-neurotrophin receptor $p75^{NTR}$, a member of the tumour necrosis factor (TNF) super-family with a cytoplasmatic death domain. There are different subclasses of Trk receptors with varying specificity for the different neurotrophins and with NGF preferentially binding to TrkA (Meakin et al. 1992; cf. Meakin & Shooter 1992; cf. Kaplan & Miller 2000; cf. Murer et al. 2001; Tyler et al. 2002). Signalling solely via $p75^{NTR}$ requires binding of a neurotrophin homo-dimer, predominantly NGF, to the receptor. Typically, however, NGF binds to a homo-dimer of the TrkA receptor or, in presence of $p75^{NTR}$, to a hetero-dimer of TrkA and $p75^{NTR}$, in which the latter seems to enhance the affinity of NGF to TrkA (cf. Murer et al. 2001). Despite the well-established functional interaction of $p75^{NTR}$ and TrkA, early studies have not provided any evidence for direct binding of $p75^{NTR}$ to TrkA (cf. Meakin & Shooter 1992; Wehrman et al. 2007), mainly explaining the joint functional effects by ligand passing from $p75^{NTR}$ to TrkA, during which a short-lived hetero-complex linked by NGF would be formed (Mehta et al. 2012). In contrast, more recent findings have suggested an interaction of both receptors via their intracellular domains (Iacaruso et al. 2011; Matusica et al. 2013).

Pro-apoptotic effects of $p75^{NTR}$ upon neurotrophin stimulation and in absence of Trk receptors, for instance following seizure or withdrawal of trophic factors, have been reported to be primarily mediated by activation of cJun N-terminal kinase (JNK) and p53, whereas interaction of NGF with a hetero-dimer of $p75^{NTR}$ and TrkA promotes cell survival by activating nuclear factor “κ-light-chain-enhancer” of activated B cells (NF-κB) in addition to signalling via phosphatidylinositol-3 kinase (PI3K) and protein kinase B (PKB or Akt), a serine/threonine kinase (Yao & Cooper 1995; cf. Toker & Cantley 1997; Friedman 2000; cf. Kaplan & Miller 2000; Culmsee et al. 2002; Yeiser et al. 2004; cf. Arévalo & Wu 2006; cf. Cirulli & Alleva 2009; cf. Diarra et al. 2009; Geetha et al. 2012). While NF-κB has been implicated to be of importance during differentiation and synaptogenesis (Boersma et al. 2011), NGF-dependent neuronal differentiation appears to rely predominantly on activation of the Erk1/2 cascade upon TrkA stimulation (cf. Kaplan & Miller 2000; Piiper et al. 2002; cf. Arévalo & Wu 2006; cf. Cirulli & Alleva 2009), which may be enhanced by co-activation of $p75^{NTR}$ (Diolaiti et al. 2007; Matusica et al. 2013). With regard to synaptic plasticity, neurotrophin effects have been demonstrated to depend on protein synthesis following signalling pathway activation (Tyler et al. 2002; Yamada & Nabeshima 2003; Kang & Schuman 1996; Takei et al. 2001).

In accordance with these findings, NGF is capable of inducing neuronal-like differentiation and an elevation in neurofilament protein levels as well as of neural adhesion molecules in PC12 cells (also refer to section 2.4), which express both, $p75^{NTR}$ and TrkA (Greene & Tischler 1976; Doherty et al. 1987; Doherty et al. 1988; Mann et al. 1989; Grant et al. 1996). Importantly, blocking TrkA is known to completely abolish NGF-dependent neurite outgrowth in PC12 cells (Itoh et al. 1995), whereas continuous NGF exposure leads to a decrease in TrkA activity, probably due to receptor internalization (cf. Kaplan & Stephens 1994; Geetha et al. 2005).

Hippocampal neurons have been demonstrated to express NGF with expression levels depending on the general activity pattern and the developmental stage, peaking during early postnatal development (Korsching et al. 1985; Large et al. 1986; Zafra et al. 1990; Zafra et al. 1991; Lindholm et al. 1994; Berninger et al. 1995). On the receptor side, expression of $p75^{NTR}$ in the hippocampus is typically observed during late embryonic and in some studies also early postnatal development (Buck et al. 1988; Lu et al. 1989), but not in adults, in which $p75^{NTR}$ appears to be primarily expressed in cholinergic neurons (Kiss et al. 1988). Fittingly, survival of cholinergic neurons in the basal forebrain and the hippocampus as well as their capacity to increase synthesis and release of acetylcholine depends on NGF (Rylett et al. 1993; Moises et al. 1995; Dixon et al. 1997; Hellweg et al. 2002; Shimode et al. 2003; Berry et al. 2010; Mercerón-Martínez et al. 2013). Regarding TrkA levels, published data are contradictory: Although there have been reports of TrkA expression in primary hippocampal cultures for at least seven days *in vitro* (DIV7), with a maximum around DIV4, other studies could not replicate this expression dynamic (Culmsee et al. 2002).

Another brain region involved in learning and memory as well as behavioural control and known to be rich in NGF is the amygdala (Yan & Johnson 1988; Altar et al. 1991; Nishio et al. 1992; Yee et al. 2007). However, the exact roles of neurotrophin and in particular of NGF signalling in the amygdala have not been studied in comparable detail to date.

Nonetheless, the expression of NGF in hippocampus and amygdala supports the assumption that – aside from mediating neuronal development and differentiation – there might be a distinct role for neurotrophin signalling in anxiety behaviour and fear memory consolidation. Furthermore, a neuroprotective role of NGF is indicated by an increased expression of NGF in several brain regions following seizures and an up-regulation of NGF during inflammatory processes, for instance during acute attacks of multiple sclerosis patients (Gall & Isackson 1989; cf. Aloe et al. 1994).

Although NRAGE has been found to interact with the intracellular death domain of $p75^{NTR}$ (Salehi et al. 2000; cf. Nykjaer et al. 2005), there are controversial findings regarding its exact effects. Feng and co-workers, for instance, have found a decrease in NRAGE levels during NGF-induced neuronal differentiation of PC12 cells, paralleled by an up-regulation of

p75^{NTR} (Feng et al. 2010). In this context, they have seen a negative influence of NRAGE on Erk1/2 activation and TrkA expression, both of which are important for NGF-dependent neuronal-like differentiation in PC12 cells (Feng et al. 2010). In contrast, Reddy *et alii* have observed increased survival rates and accelerated neuronal-like differentiation of PC12 cells when over-expressing NRAGE and culturing cells in presence of NGF (Reddy et al. 2010). While enhanced neuritogenesis in their studies has relied on NRAGE interaction with TrkA (but not with p75^{NTR} alone) and subsequent Erk1/2 activation, early activation of the PKB pathway through TrkA signalling has mediated cell survival effects (Reddy et al. 2010).

These findings are thus implying a role of neurotrophins, their receptors, and the subsequent intracellular cascades as mediators of plasticity and neuroprotection. Clearly, the complex interaction of Trk receptors with p75^{NTR} as well as of subsequent signalling cascades are far from being understood, and it appears as if signalling via both receptor types can be somewhat synergistic as well as antagonistic, at least partially depending on extracellular neurotrophin levels, ratio of mature and precursor neurotrophins as well as on activity states of intracellular signalling cascades and on the respective cell type (cf. Kaplan & Miller 2000; cf. Arévalo & Wu 2006; cf. Cirulli & Alleva 2009). Moreover, the potential relevance of these NGF signalling pathways for and their varying balance in different cell types, brain regions, developmental stages, or with regard to behavioural phenomena have yet to be elucidated. Due to its involvement in NGF-induced intracellular signalling pathways and its influence on neuritogenesis in PC12 cells, NRAGE might serve as a key factor in mediating some of these effects.

2.3.3 Praja1 as a mediating factor in different signalling cascades

This brief summary of two different signalling pathways, which have their share in cell survival, differentiation, and development – partially on their own behalf, partially by close interaction – and that both make use of NRAGE at some point, unveils NRAGE to be at the focus of processes deciding a cell's fate.

NRAGE is highly expressed in the developing and adult nervous system, especially in neuronal progenitors and in early post-mitotic neurons, and is often – but not exclusively – co-expressed with p75^{NTR} (Barrett et al. 2005; Salehi et al. 2000; Kuwajima et al. 2004). Despite having been shown to be pro-apoptotic by mediating JNK and subsequent p53 activation as well as inducing cleavage of inhibitor of apoptosis (IAP) proteins (Salehi et al. 2000; Jordan et al. 2001; Kendall et al. 2005; Salehi et al. 2002), NRAGE has also been found to mediate neuronal differentiation of PC12 cells (Feng et al. 2010; Reddy et al. 2010), which endogenously express the NRAGE activators p75^{NTR} (Salehi et al. 2000) as well as TrkA (cf. Kaplan & Stephens 1994), which are both known to mediate NGF-induced effects such as cell survival, differentiation, or cell death (Nykjaer et al. 2005; Salehi et al. 2000). NRAGE binds to p75^{NTR} as well as to TrkA, and has been proposed to inhibit p75^{NTR}–TrkA

dimerization (Barrett et al. 2005). BMP signalling, on the other hand, has been described as an important pro-apoptotic pathway in neuronal progenitor cells. In such cells, NRAGE has been observed to facilitate pro-apoptotic p38 activation independent of Smad activity (Kendall et al. 2005), but has also been linked to the development of GABAergic neurons (Kuwajima et al. 2004).

As stated before, Praja1 is capable of decreasing the levels of NRAGE in a UPS-dependent manner. Control of NRAGE activity through Praja1 may thus contribute to achieving the delicate balance between different signalling pathways and provide an important mechanism for regulating neuronal differentiation in varying neuronal populations and at different stages of development. This effect might be further enhanced by Praja1-dependent degradation of other important signalling molecules, in particular Smad3.

2.4 PC12 – A model system for neuronal differentiation

In 1976, Greene and Tischler published their findings on a newly obtained single cell clonal line from a rat phaeochromocytoma, a mostly benign tumour of the adrenal medulla (Greene & Tischler 1976). As a reference to its origin, they named this cell line “PC12” and showed that these PC12 cells resemble the morphology of sympathetic neurons and are capable of producing varicose processes of up to 1,000 µm in length upon application of NGF. This was accompanied by a drastic reduction in proliferation (Greene & Tischler 1976). However, while sympathetic neurons depend on the presence of NGF for their survival, PC12 cells only do so in serum-free media, whereas serum-enriched medium sustains PC12 viability even in absence of NGF (Greene & Tischler 1976; Greene 1978).

Naïve PC12 cells have been found to produce the catecholamines norepinephrine and dopamine, whereas epinephrine has not been detected, indicating that they resemble a noradrenergic adrenal chromaffin phenotype (Greene & Tischler 1976). Following NGF treatment, relative catecholamine protein levels decline, although total protein levels of these catecholamines per cell appear to remain more or less stable, indicating a NGF-induced up-regulation of protein expression rather than a down-regulation of catecholamines (Greene & Tischler 1976). In general, NGF treatment of PC12 cells appears to primarily affect the quantitative proteome composition, although qualitative alterations have been observed as well, especially with regard to glycoproteins at the plasma membrane (McGuire et al. 1978; McGuire & Greene 1980; Obin et al. 1999). Notably, a qualitative overlap of neuron-specific glycoproteins in sympathetic neurons and naïve PC12 cells has become apparent, and up-regulation of protein expression as induced by NGF has been proven to diminish the quantitative gap in membrane glycoproteins between both cell populations (Lee et al. 1977; Lee et al. 1981). Aside from changing protein levels, protein activity is also modified by the application of NGF, which alters the overall phosphorylation status of PC12 cells and induces

expression as well as increased phosphorylation of certain high molecular weight proteins like mitogen-associated proteins (MAP), especially MAP1.2 (Greene et al. 1983; Aletta et al. 1988). This NGF-induced increase in levels of neurofilament proteins as well as of neural adhesion molecules has been confirmed in several subsequent studies (Doherty et al. 1987; Doherty et al. 1988; Mann et al. 1989; Grant et al. 1996).

As previously stated, the responsiveness of PC12 cells to NGF is based on their expression of the neurotrophin receptors $p75^{NTR}$ and TrkA (cf. Meakin & Shooter 1992) and blockade of signalling via TrkA has been shown to abolish NGF-dependent neurite outgrowth in PC12 cells, but not all of the NGF-induced proteome alterations (Itoh et al. 1995).

Like adrenal chromaffin cells, naïve PC12 cells present a secretory phenotype. As part of this, they produce large dense core vesicles (LDCV) that may contain mono-amines, especially catecholamines, but also neuropeptides, most prominently neuropeptide Y (NPY) (Greene & Tischler 1976; Walch-Solimena et al. 1993). While NPY expression is low or undetectable in naïve PC12 cells, NPY levels are drastically increased following NGF stimulation (Allen et al. 1984; Allen et al. 1987; Sabol & Higuchi 1990; Higuchi et al. 1992; Balbi & Allen 1994; Rajakumar et al. 1998). Depending on the expressed set of NPY receptors, this may then decrease or enhance catecholamine synthesis (DiMaggio et al. 1994; McCullough et al. 1998). Typical markers of LDCV are vesicular mono-amine transporters (VMAT), nowadays more commonly referred to as solute carrier family 18 vesicular mono-amine transporters (Slc18a) (Liu et al. 1994; Liu & Edwards 1997). Slc18a proteins are known to mediate quantal size of vesicles as well as mono-amine transmitter and neuropeptide storage (Pothos et al. 2000; Hoard et al. 2008). Slc18a member 1 (Slc18a1, formerly VMAT1) is confined to endocrine and paracrine cells, e. g. of the sympathetic nervous system, whereas Slc18a2 (formerly VMAT2) is predominantly found in sympathetic and aminergic neurons, but also in a subset of adrenal chromaffin cells (Weihe et al. 1994; Peter et al. 1995; Tillinger et al. 2010). Accordingly, naïve PC12 cells express Slc18a1 (Cordeiro et al. 2000b). So far, however, there is no evidence of Slc18a2 expression in naïve or NGF-treated PC12 cells, despite the development of a neuron-like phenotype in the latter (Weihe et al. 1996). DnaJ (Hsp40) homologue subfamily C member 5 (DnaJC5), which corresponds to cysteine string protein (CSP) in *Drosophila melanogaster*, is another mediator of vesicular transmission in PC12 cells (Burgoyne 1996; Bai et al. 2007; Evans et al. 2001; Cordeiro et al. 2000a) and has been shown to be capable of elevating mono-amine release (Chamberlain & Burgoyne 1998). Slc18a1 and DnaJC5 may hence serve as markers for a secretory PC12 phenotype, whereas an induction of NPY expression may indicate successful NGF stimulation.

An essential feature of neurons is their excitability by various mechanisms, which provides the basis for the formation of functional neuronal networks. Supporting the proposition of PC12 cells being a model system for studying primary neurons, NGF treatment has been found to increase the number of functional, voltage-dependent sodium channels at the

plasma membrane without altering its passive permeability, hence allowing for the generation of action potentials (Rudy et al. 1987; Bouron et al. 1999). Unstimulated PC12 cells, on the other hand, have been demonstrated to be electrically unexcitable (Rudy et al. 1987). In addition, NGF stimulation has been observed to elevate calcium influx as well as calcium release from intracellular stores in response to bradykinin, a peptide of the kininogen family that plays a role in diverse processes, e. g. neural pain transmission (Bush et al. 1991; Bouron et al. 1999). Moreover, membrane depolarization caused by elevated potassium levels in the medium is capable of preserving NGF-induced neurites as well as protein level and phosphorylation changes in a calcium-dependent manner even after NGF withdrawal (Teng & Greene 1993). However, elevated potassium levels do not promote further neurite elongation; nor do they induce differentiation in the first place or increase vitality of PC12 cells, as they do in sympathetic neurons (Teng & Greene 1993).

Based on these observations and the incapability of adrenal chromaffin cells to respond to NGF in a comparable manner, it has been assumed that PC12 cells have gained some degree of pluripotency, enabling them to develop either a secretory or a neuronal phenotype depending on the stimulation they receive (Greene & Tischler 1976). Although PC12 cells do not develop a phenotype identical to sympathetic or other neuronal populations, the findings gathered since establishing this cell line have repeatedly demonstrated the occurrence of neuronal key features in morphology and functionality upon NGF application. Hence, PC12 cells are recognized as a model system for NGF-induced neuronal-like development and differentiation. Considering the fact that primary neuronal cultures have been pre-exposed to NGF *in vivo* and are more difficult to obtain and maintain, the PC12 model is furthermore perceived as being especially valuable with regard to initial effects of NGF exposure (cf. McGuire & Greene 1980).

2.4.1 The influence of extracellular matrix components on PC12 cells

As is widely appreciated, extracellular matrix structure and composition do strongly influence activation of cellular pathways. The use of specific extracellular substrates may thus allow for assessing, in how far the observed effects depend on or are influenced by cell-to-cell or cell-to-matrix interactions.

PC12 cells have been demonstrated to interact with the collagens type I and IV as well as with laminin in a manner similar to that of sympathetic neurons (Tomaselli et al. 1987). All of these substrates have been extracted from neural tissues and are known to promote neurite outgrowth, cell survival, and cell attachment in various neuronal populations and in an integrin-dependent manner (Lander et al. 1985b; Tomaselli et al. 1986; cf. Tomaselli et al. 1987; cf. Powell & Kleinman 1997; Beaujean et al. 2003). Accordingly, the influence of laminin, collagen I, and collagen IV on PC12 cells is mediated by a functional hetero-dimer of β_1-integrin and α-integrin subunits (Tomaselli et al. 1987; Tomaselli et al. 1988; Tomaselli

et al. 1990). However, while laminin interacts with $\alpha_1\beta_1$- as well as with $\alpha_3\beta_1$-integrin, collagens type I and IV appear to only activate $\alpha_1\beta_1$-integrins (Tomaselli et al. 1990). In addition, several integrin-independent laminin receptors and signalling cascades have been described (cf. Powell & Kleinman 1997). In that regard it should be mentioned that PC12 cells have been found to secret low amounts of laminin (Lander et al. 1985a; Tomaselli et al. 1986), thus autonomously shaping their extracellular environment to an extent that has not been further specified to date.

Another important component of neural extracellular matrices is fibronectin. However, PC12 cells adhere poorly to fibronectin-coated surfaces, which appears to be due to low expression levels of the fibronectin receptor subunit α in this cell line (Tomaselli et al. 1987; Tomaselli et al. 1988).

Among the membrane glycoproteins that are up-regulated following NGF stimulation of PC12 cells is the so-called "NGF-inducible large external glycoprotein" (NILE), which has been shown to be identical to L1 (Bock et al. 1985), a member of the neural cell adhesion molecule (NCAM) family. This group of cell adhesion mediators has been proven to promote neurite outgrowth and to modulate cell-to-cell adhesion independent of calcium (Rathjen & Schachner 1984; Rathjen & Rutishauser 1984; Mann et al. 1989; cf. Baldwin et al. 1996; Webb et al. 2001). In consistence with these findings, induction of L1 in NGF-treated PC12 cells has been observed to depend on cell-to-cell interaction rather than on extracellular substrates or TrkA activation (Itoh et al. 1995). Seeding cells on L1-coated surfaces may thus serve as a control for the influence of cell-to-cell interactions on observed alterations by mimicking high cell densities.

To control for the specificity of potential substrate effects, poly-D-lysine (PDL) has been found to increase cellular adherence in an unspecific manner and independent of integrins (Brighton & Albelda 1992). Nonetheless, some experiments have indicated that neurite outgrowth – in contrast to general adherence – on PDL-coated surfaces might depend on some degree of integrin activation after all (Tomaselli et al. 1987).

2.5 Aim of this study

Against this backdrop, the study at hand has aimed at testing the hypothesis that the E3 ubiquitin ligase Praja1 is generally capable of influencing neuronal differentiation. To this end, the influence of Praja1 on NGF-induced differentiation of PC12 cells has been studied in the following manner.

First, endogenous expression and intracellular localization of Praja1 have been examined in NGF-treated PC12 cells. Upon this, the effect of Praja1 on neurite outgrowth and cellular morphology during NGF stimulation has been determined on various extracellular substrates using the two validated transcript variants of mouse *praja1*, which are coding for the two

isoforms referred to as Praja1.1 and Praja1.2, in tetracycline-inducible stably transfected PC12 cell lines.

To control for changes in the expression of phenotype markers in Praja1 over-expressing cells during NGF stimulation, immunocytochemistry as well as quantitative real-time PCR (qRT-PCR) have been applied.

Finally, a Praja1-dependent increase in poly-ubiquitination levels and specific degradation of NRAGE have been confirmed in the PC12 model system via Western blotting, linking the observed morphological effects of Praja1 on NGF-induced differentiation of PC12 cells to a reduction of NRAGE following over-expression of Praja1.

PC12 cells are insensitive to signalling via TGF-β, due to a lack of the required receptor. Nonetheless, the BMP signalling factor Smad3 may be activated and translocate to the nucleus independent of TGF-β signalling, instead induced by NGF-binding to TrkA (Lutz et al. 2004). To evaluate the possibility that the observed alterations in morphology might be at least partially caused by a Praja1-dependent reduction of Smad3, levels of this protein have also been assessed.

3. Materials and Methods

Complementing the following description of all methods applied in the course of experiments for this dissertational thesis, the suppliers of all devices and materials mentioned in this chapter are listed in detail in the appendix (see A.II).

3.1 Cell culture

PC12 cells have been cultured in 85 % Dulbecco's modified Eagle medium (DMEM), 10 % horse serum, and 5 % foetal bovine serum (FBS) (high serum condition). African green monkey kidney fibroblast-like (COS7) cells have been grown in 90 % DMEM and 10 % FBS.

3.1.1 Praja1 over-expression constructs

Isolation of *praja1* transcripts has been performed by Oliver Stork as previously described (Stork et al. 2001): Primer 5'-CTCGAGCCATGAGCCACCAGG-3' has been used to introduce an XhoI restriction site to the 5'-end of the open reading frame, allowing for in-frame cloning into the expression vector pEGFP-C1, which produces enhanced green fluorescence protein (EGFP), into the pCMV::HA vector, expressing hemagglutinin (HA) under a cytomegalovirus (CMV) promoter, or into pTRE2-hyg, a vector allowing for selection of transfected cells using hygromycin B as well as for conditional expression following tetracycline application due to its tetracycline-responsive element (TRE).

Transfections with pCMV::HA-Praja1.1, pCMV::HA-Praja1.2, or pCMV::HA as well as with pEGFP-Praja1.1, pEGFP-Praja1.2, or pEGFP-C1 for acute transfection experiments, and with pTRE::EGFP-Praja1.1, pTRE::EGFP-Praja1.2, or pTRE::EGFP for stable transfections have been performed using the GeneJammer reagent according to the manufacturer's instructions. Functionality of these constructs with regard to their capability of increasing ubiquitination and reducing NRAGE levels has been confirmed in COS7 cells by Sandra Vorwerk and Ryoji Fukabori (Teuber et al. 2013).

Ryoji Fukabori has selected stably transfected PC12 cells by applying 500 µg/ml of G418 for two months and 200 µg/ml of hygromycin B for another two months according to the manufacturer's protocol. They have then been maintained using 200 µg/ml of G418 and 100 µg/ml of hygromycin B.

Due to alternative splicing, transfection with *praja1.1* fusion constructs has generated both isoforms, Praja1.1 and Praja1.2 (henceforth referred to as Praja1.1/2), whereas *praja1.2* constructs produced only Praja1.2 in all cell types tested (PC12, COS7, HEK293) (Figure 1, Figure 2).

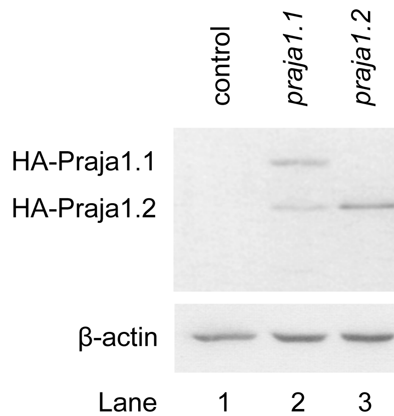


Figure 1: Alternative splicing following over-expression of Praja1. Western blotting has demonstrated the expression of Praja1 isoforms in COS7 cells. Immunostaining against the HA-tag has been used for visualization. While expression of *praja1.1* vectors produces HA-Praja1.1 and HA-Praja1.2 (lane 2), transfection with *praja1.2* constructs leads to the exclusive expression of HA-Praja1.2 (lane 3). Control transfection with pCMV::HA does not provide a signal due to the low molecular weight of HA (ca. 1 kDa) (lane 1). B-actin has served as loading control.

3.1.2 Differentiation of PC12 cells

For assessing proliferation, neuronal differentiation, apoptosis, and intracellular localization of Praja1 isoforms, acutely and stably transfected PC12 cells have been allowed to adhere to cover-slips (ca. 1.13 cm^2) coated with collagen I (13.3 $\mu g/cm^2$), collagen IV (13.3 $\mu g/cm^2$), or PDL (13.3 $\mu g/cm^2$), either further cultured under high serum conditions as stated above or in 99.6 % DMEM, 0.2 % horse serum, 0.2 % FBS (low serum condition). To evaluate substrate-dependence of Praja1 effects on neuronal differentiation, cover-slips coated with either laminin (5.3 $\mu g/cm^2$) or L1-Fc have also been tested. For L1-Fc coating, PDL-coated cover-slips have been treated with anti-human Fc-antibody solution (0.9 $\mu g/cm^2$), upon which human neurite growth protein L1 (0.9 $\mu g/cm^2$) has been added.

Neuronal-like differentiation of acutely and stably transfected PC12 cells has been induced by administering NGF (25 ng/ml) for up to four days.

Expression of the tetracycline-responsive tet-on system in stably transfected cells has been achieved by parallel application of doxycycline (1 µg/ml).

3.1.3 Determining the intracellular localization of Praja1

To evaluate the intracellular distribution of Praja1 constructs, localization indices have been calculated for each splice variant based on the distribution of constructs tagged with EGFP in each condition. If the nucleus has presented the predominant EGFP signal, the cell has been counted as +1, whereas –1 indicates a mostly cytosolic staining, and 0 represents an equal staining of nucleus and cytosol.

3.1.4 Determining apoptosis

The rate of apoptosis in stably transfected PC12 lines has been evaluated by applying a Caspase-Glo® 3/7 luminescence assay to cell lines after two days of treatment with NGF and doxycycline in the afore-mentioned manner and subsequent triplicate measurement in a micro-plate reader with the coefficient of variation (CV) commonly ranging from 0.1 to 7.4 for each triplicate.

3.2 Immunocytochemistry

3.2.1 Staining protocol

Cells have been fixated in 4 % para-formaldeyhde (PFA), before being permeabilized in 0.3 % Triton™ X-100 and washed in phosphate-buffered saline (PBS) (Table 1). Following immunocytochemical staining, cover-slips have been mounted on slides using Shandon Immu-Mount™.

PBS (pH 7.4)	
Sodium chloride (NaCl)	137 mM
Potassium chloride (KCl)	2.7 mM
Disodium hydrogen phosphate (Na_2HPO_4)	12 mM
Potassium dihydrogen phosphate (KH_2PO_4)	1.8 mM
Aqua destillata	solvent

Table 1: Formulation of phosphate-buffered saline. PBS has been prepared according to the formulation provided in this table. The molarities refer to final concentrations. Aqua destillata has been used as solvent and the pH has been adjusted to 7.4.

Fluorescence staining has been performed by blocking unspecific binding through application of 5 % donkey serum for 45 minutes at room temperature, followed by overnight incubation at 4°C with the primary antibody diluted in 2.5 % donkey serum. After washing, appropriate secondary antibodies in a 2 % bovine serum albumin (BSA) solution have been added for one hour at room temperature. Staining of the actin cytoskeleton has been achieved by adding rhodamine phalloidin (5 U/ml) 30 minutes before the end of incubation. Afterwards, incubation with 4',6-Diamidin-2-phenylindol (DAPI) (300 nM) for up to five minutes has been performed to stain all nuclei, thus allowing for an estimation of cell numbers, intracellular localization, and to visualize pyknotic nuclei as indicators of apoptotic cell death. PBS has been used as solvent for all dilutions mentioned above.

Immunocytochemical staining has been using the following primary antibodies: polyclonal anti-NRAGE (1:100), polyclonal anti-Smad3 (1:200), and monoclonal anti-α-tubulin (1:200). In some experiments, EGFP signals have been enhanced by additional antibody staining (1:500). Neuronal differentiation of PC12 cells has been confirmed by staining with anti-MAP2 (1:200) and anti-β3-tubulin (also named TuJ1; 1:200), each in combination with anti-α-tubulin.

Samples have been examined with epi-fluorescence microscopy and digital image capturing equipment. Subsequent analysis of digital images has relied on the Leica Application Suite Advanced Fluorescence (LAS AF) software and on ImageJ in the Fiji installation package (Schindelin et al. 2012).

3.2.2 Analysis of neurite outgrowth in PC12 cells

For analysis of neurite outgrowth, the proportion of stably transfected cells producing neurites of 25 μm or more in length has been determined after two and four days of NGF stimulation and the number of those neurites has been compared between groups. In absence of NGF, spontaneous neurite formation has been observed only rarely and has not exceeded 1 % in any of the stably transfected PC12 lines (percentage of cells with neurites for Praja1.1/2: 0.61 % ± 0.41 %, for Praja1.2: 0.66 % ± 0.88 %, for EGFP control: 0.35 % ± 0.06 %; data provided by Ryoji Fukabori and Oliver Stork). Cells have been counted as being differentiating if they had developed filopodia-like protrusions of at least 5 μm or neurites of at least 25 μm in length. To estimate matrix adhesion, the proportion of transfected cells showing a flattened appearance and spreading on the substrate (Stork et al. 2004) has been determined and put in relation to the number of rounded cells in each line.

3.3 Gene expression analysis

Gene expression has been evaluated in stably transfected PC12 cells after four days of incubation with NGF and doxycycline as described above.

3.3.1 Sample preparation and reverse transcription PCR

Sample RNA has been collected by detaching adherent cells through applying 0.25 % Trypsin/ethylenediaminetetraacetate (EDTA) for five minutes and while incubating at 37°C, subsequent gentle centrifugation to collect cells, and final re-suspension of cell pellets in ice-cold lysis buffer from the Cells-to-cDNA™ II kit. Following the manufacturer's instructions, the lysate has then been incubated at 75°C for ten minutes to inactivate RNases before being treated with 0.04 U/μl DNase I at 37°C for 15 minutes to degrade genomic deoxyribonucleic acid (DNA). Inactivation of DNase I has been achieved by incubating samples at 75°C for another five minutes. If further processing has not been performed directly after cell lysis, samples have been stored at –80°C.

Generation of complimentary DNA (cDNA) from template RNA has been performed following the protocol of the Sensiscript® kit. First, a reaction master mix has been prepared as listed in Table 2. The Sensiscript reverse transcriptase is a recombinant hetero-dimeric reverse transcriptase obtained from *Escherichia coli*.

Sensiscript® reaction master mix for cDNA synthesis	
10x Buffer RT	2.0 µl
Deoxyribose-containing nucleosid triphosphate (dNTP) mix (5 mM per dNTP)	2.0 µl
Oligo-$(dT)_{18}$ first strand primer (10 µM)	2.0 µl
RNase Inhibitor (20 U/µl)	0.5 µl
Sensiscript® reverse transcriptase	1.0 µl
RNase-free water	7.5 µl
Final volume of Sensiscript® reaction master mix:	15.0 µl

Table 2: Formulation of Sensiscript® reaction master mix. The reaction master mix for cDNA synthesis with the Sensicript® kit has been prepared according to this formulation and is sufficient for one 20 µl reaction (total volume after adding 5 µl of template RNA).

To denature any secondary structures, template RNA has been heated to 70°C for three minutes, followed by short incubation on ice and brief centrifugation. Then, 5 µl of template RNA have been added to 15 µl of master mix, upon which the entire reaction mix has been incubated at 37°C for 60 minutes to allow for reverse transcriptase polymerase chain reaction (PCR) to take place, resulting in double-strand cDNA. Obtained cDNA samples have then been diluted at a ratio of 1:5 in dimethyldicarbonate (DMDC) water and have been stored at –20 °C.

3.3.2 Quantitative real-time PCR protocol

In a second step, multiplex qRT-PCR using TaqMan® reagents and primer probes labelled with the fluorescence dyes 6-carboxyfluoresceine (FAM™) or VIC® has been performed to assess gene expression.

5 µl of TaqMan® Gene Expression Master Mix, which contains all necessary reagents for subsequent qRT-PCR (Table 3), have been mixed with 0.5 µl of TaqMan® gene expression assay for the respective target, 0.5 µl of the TaqMan® gene expression assay serving as internal control (see below), and 1 µl of DMDC water. Finally, 3 µl of cDNA have been added. Sample distribution on 96-well plates for qRT-PCR has been performed using a pipet robot.

In order to reduce carry-over contamination, samples have been uracil-N-glycosylated for two minutes at 50°C at the beginning of qRT-PCR to prevent re-amplification of previous PCR products. After this, double-strand cDNA has been denatured for ten minutes at 95°C, upon which amplification and real-time quantification have been performed over 50 cycles, each of which consisting of 15 seconds at 95°C for denaturation and one minute at 60°C for subsequent annealing and elongation.

Typically, fluorescence thresholds have been reached within 20 to 35 cycles. However, detection has occasionally required up to 40 cycles.

TaqMan® Gene Expression Master Mix
AmpliTaq Gold® DNA Polymerase
Ultra Pure (UP)
dNTP mix (including dTTP/dUTP)
Uracil-DNA glycosylase
Passive internal reference (Rox™ dye)

Table 3: Components of the TaqMan® Gene Expression Master Mix. According to the information provided by the manufacturer, the TaqMan® Gene Expression Master Mix contains all components necessary for qRT-PCR, except for template RNA and target primers. Ultra Pure (UP) allows for hot start activation and improves detection of bacterial targets, if applicable.

3.3.3 Data analysis

From each sample subjected to qRT-PCR, cycle thresholds (CT) have been extracted using the StepOne™ software and analysing the resulting fluorescence signal curves. The CT corresponds to the amplification cycle at which the fluorescence threshold is reached and the signal becomes distinguishable from baseline levels. If samples remain undetected, a CT of 50, corresponding to the maximum number of cycles, has been assumed to allow for subsequent comparison of groups using the ΔΔCT method (see below).

All samples have been measured as triplicates and mean values have been determined for each triplicate assay. In case of intra-assay standard deviations of 0.165 or more and two measurements with readings in closer proximity, single outliers have been excluded from triplicates to reduce intra-assay variation.

Sample comparison by relative quantification has relied on the ΔΔCT method (Schmittgen & Livak 2008). At first, target gene expression is normalized to the total cDNA content of each sample by using the expression levels of housekeeping genes, which have been checked to be unaffected by the experimental protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phospho-glycerate kinase (PGK) have been the two genes used as internal controls in this study.

Because VIC®-labelled GAPDH has been used, FAM™-labelled gene expression assays could be measured in parallel. The ΔCT_{GAPDH} per well has then been computed as follows:

$$\Delta \mathrm{CT}_{\mathrm{GAPDH}}^{\mathrm{target/PGK}} = \mathrm{CT}_{\mathrm{target/PGK}} - \mathrm{CT}_{\mathrm{GAPDH}}$$

Mean values for ΔCT_{GAPDH} have been calculated for each triplicate assay. In addition, PGK expression has been considered to further minimize methodical variation:

$$\Delta \mathrm{CT}_{\mathrm{target}} = \left(\mathrm{mean}\ \Delta \mathrm{CT}_{\mathrm{GAPDH}}^{\mathrm{target}}\right) - \left(\mathrm{mean}\ \Delta \mathrm{CT}_{\mathrm{GAPDH}}^{\mathrm{PGK}}\right)$$

The ΔCT_{target} represents the normalization of target gene expression levels in relation to the expression of GAPDH and PGK.

Statistical analysis has been conducted comparing these ΔCT_{target} values.

For better illustration, the relative quantification (RQ) value has been calculated based on mean ΔΔCT values, which result from normalizing ΔCT_{target} values in the over-expressing conditions to the according ΔCT_{target} in EGFP controls:

$$\Delta\Delta CT_{target} = \Delta CT_{target}^{Praja1\ over-expression} - \Delta CT_{target}^{EGFP\ control}$$

Relative quantities are then obtained by taking into account the exponential amplification during qRT-PCR:

$$RQ_{target} = 2^{-\Delta\Delta CT_{target}}$$

Thus, RQ represents relative target mRNA amounts in Praja1 over-expressing conditions as compared to the control condition, for which RQ values equal 1.

3.3.4 Gene expression assay for Praja1

Expression of *praja1* has been determined using a custom-made TaqMan® gene expression assay labelled "MPraja1_-RT6". Praja1 mRNA primers have had the following sequences: 5'-GTTGCTGTCAGGGTTACCTATCTG-3' for the forward primer and for the reverse primer 5'-GAACATTAACCCATGACATGCAACA-3'. For fluorescence detection, primers have been tagged with the FAM™-reporter (sequence: 5'-TCGGAAAGACAAAATTACT-3').

Specificity of MPraja1_-RT6 to praja1.1 and praja1.2 mRNA has been evaluated by testing against plasmid DNA of both *praja1* variants. As expected, both *praja1* plasmids have been recognized by MPraja1_-RT6 early during amplification and at comparable levels (CT = 8.69 for *praja1.1* and CT = 6.96 for *praja1.2*), whereas pEGFP-C1 control plasmids have remained undetected during 50 cycles of qRT-PCR.

3.4 Immunoblotting

3.4.1 Experimental designs

For the analysis of endogenous Praja1 expression, $1 * 10^6$ PC12 cells have been collected after NGF stimulation either for 30 minutes, two hours, or three days.

Poly-ubiquitination activity has been tested collecting $2 * 10^6$ cells from stably transfected PC12 cultures treated with NGF and doxycycline for two days.

In an additional set of experiments, poly-ubiquitinated proteins have accumulated in PC12 cells during eight hours of pre-treatment with lactacystin (1 µM). Beforehand, it had been ascertained through staining with propidium iodide that lactacystin treatment for 8 as well as for 24 hours would not lead to a significant increase of cell death (less than 1.5 % of cells with or without lactacystin).

3.4.2 Immunoblotting protocol

Cells have been suspended in lysis buffer (Table 4), incubated for 30 minutes at 4°C, and then centrifuged at 16,000 rcf and 4°C for another 30 minutes, after which the supernatant has been retained. Determination of protein concentrations has relied on the colorimetric DC™ Protein Assay, which is similar to the Lowry assay (Lowry et al. 1951), but provides improved compatibility with a wide range of detergents.

Protein lysis buffer	
Tris-(hydroxymethyl)-aminomethanhydrochlorid (Tris-HCl) (pH 7.4)	500.0 mM
EDTA	1.0 mM
NaCl	150.0 mM
Nonylphenyl Polyethylene Glycol (NP-40 Alternative)	1.0 %
Deoxycholic acid (DOC)	0.5 %
Sodium orthovanadate (Na_3VO_4)	2.0 mM
Sodium fluoride (NaF)	1.0 mM
4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)	1.0 mM
Aqua destillata	solvent
Protease inhibitor cocktail	1 tablet / 10 ml

Table 4: Formulation of the protein lysis buffer. The protein lysis buffer has been prepared according to the formulation provided in this table and comprised components improving the yield of phosphorylated proteins as well as of membrane-associated proteins. Final concentrations have been provided for each chemical.

For carrying out sodium dodecyl sulphate (SDS) poly-acrylamide gel electrophoresis (PAGE), lysed sample solutions have been diluted in loading buffer (Table 5) at a ratio of 3:1 and have been incubated for five minutes at 98°C for protein denaturation.

4x SDS-PAGE loading buffer	
Tris-HCl (pH 6.8)	250.00 mM
SDS	8.00 %
Glycerin	40.00 %
β-mercaptoethanol	20.00 %
Bromphenol blue	0.04 %
Aqua destillata	solvent

Table 5: Formulation of 4x SDS-PAGE loading buffer. Samples have been diluted in loading buffer before SDS-PAGE. Final concentrations have been provided for each chemical.

Proteins have been separated using SDS-PAGE (Table 6, Table 7), before they have been transferred to polyvinylidene difluoride (PVDF) membranes by Western blotting in a wet blotting chamber (Table 7) with 100 V applied for one hour.

Running gel			Stacking gel	
Acrylamide solution (37.5:1)	3.00 ml		Acrylamide solution (37.5:1)	0.42 ml
1.5 M Tris (pH 8.8)	2.25 ml		1.0 M Tris-HCl (pH 6.8)	0.32 ml
10 % SDS	90.00 µl		10 % SDS	25.00 µl
10 % ammonium persulfate (APS)	90.00 µl		10 % APS	25.00 µl
Aqua destillata	3.56 ml		Aqua destillata	1.70 ml
Tetramethylethylenediamine (TEMED)	9.00 µl		TEMED	2.50 µl

Table 6: Preparation of SDS gels. 1.5 mm thick 10 % SDS gels have been casted using the formulation provided in this table. After polymerization of the running gel, the solution for the stacking gel has been poured on top and the gel comb has been inserted. Pre-solutions (Tris, SDS, APS) have been using aqua destillata as solvent. Provided volumes are sufficient for one gel.

SDS-PAGE running buffer			Western blotting transfer buffer	
Tris (pH 8.3)	25 mM		Tris (pH 8-10)	25 mM
Glycine	250 mM		Glycine	192 mM
SDS	0.1 %		SDS	0.1 %
			Methanol	20 %
Aqua destillata	solvent		Aqua destillata	solvent

Table 7: Formulation of SDS-PAGE and Western blotting buffers. SDS-PAGE running buffer and Western blotting transfer buffer have been prepared according to the formulation provided in this table. Methanol is added to the Western blotting transfer buffer to decrease gel swelling and improve transfer of smaller and medium-sized proteins during wet blotting. SDS addition to the transfer buffer improves transfer of proteins with a molecular weight of 75 kDa or more in Western blotting.

Subsequently, membranes have been allowed to dry for 30 minutes and have then been re-activated by brief incubation in 100 % methanol (ca. ten seconds), followed by washing steps in distilled water and tris-buffered saline (TBS) (Table 8) for equilibration.

TBS	
Tris-HCl (pH 7.4)	50 mM
NaCl	150 mM
Aqua destillata	solvent

Table 8: Formulation of tris-buffered saline. TBS has been prepared according to the formulation provided in this table. Aqua destillata has been used as solvent.

After blocking of unspecific binding using 5 % milk-powder dissolved in TBS, blots have been incubated with different combinations of the following primary antibodies, diluted in a TBS solution containing 2.5 % milk powder and 0.1 % Tween® 20: polyclonal anti-ubiquitin (1:100), anti-NRAGE (1:2,000), anti-Smad3 (1:1,000), anti-β-actin (1:5,000), anti-α-tubulin (1:1,000), anti-GFP (1:5,000), or anti-HA (1:1,000). Anti-Praja1 serum has been generated against the peptide CRSPFASTRRRWDDSE and used at a dilution of 1:75 in the same manner as just described (Figure 2).

Signals have been detected with secondary antibodies coupled to horseradish peroxidase (HRP) (1:2,000–1:5,000) and ECL-plus chemiluminescence substrate, or with fluorescence antibodies IRDye® 680LT and IRDye® 800CW (1:15,000) for use in an Odyssey® scanner. Secondary antibodies have been diluted in a TBS solution containing 2.5 % milk powder, 0.1 % Tween® 20, and 0.02 % SDS.

Biochemical experiments have been performed at least in triplicate. Subsequent quantification has relied on the Odyssey® Application Software.

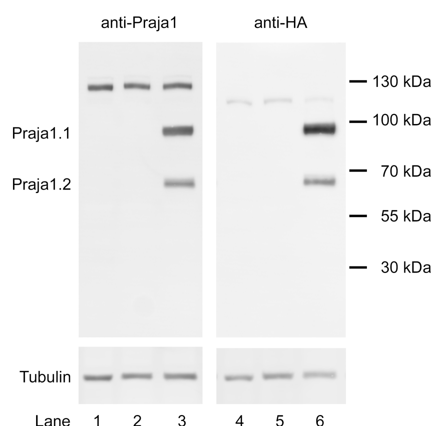


Figure 2: Specificity of anti-Praja1 serum. The expression of HA-Praja1.1 and HA-Praja1.2 from HA-tagged *praja1.1* (lane 3) has been detected in HEK293 cells using Praja1-specific anti-serum. The apparent molecular weight of ca. 95 kDa and 65 kDa, respectively, differs clearly from the predicted molecular weight of the two isoforms, but is in agreement with the previously reported reduced migration of Praja1 in SDS-PAGE (Mishra et al. 1997). The expression level ratio of isoforms is 3:1, resembling the ratio in most tissues (Teuber et al. 2013). Detection with anti-HA has confirmed the specificity of the Praja1 antiserum (lane 6). An unspecific signal has been detected at around 130 kDa in all lanes, including the mock control (lanes 1&4) and pCMV::HA transfected control cells (lanes 2&5). Due to its small molecular weight of ca. 1 kDa, the HA-tag itself could not be detected in lane 5. Detection of tubulin has served as loading control.

3.5 Statistical analysis

Quantitative data are presented as mean ± standard error of the mean (S.E.M.). An α-error at or below 0.05 has been considered to constitute statistically significant results. Statistical analyses have been carried out using SPSS (named PASW from 2009 to 2010).

For comparison of two groups, a two-tailed Student’s t-test has been applied, taking into account the homogeneity of variance according to Levene testing.

Three or more groups have been compared by one-way ANOVA. If significant differences between groups have been rendered by the ANOVA, post-hoc testing has been performed using the Tukey-HSD test for homogeneous and the Dunnett-T3 test for inhomogeneous variances. Homogeneity of variance has been assessed using the Brown-Forsythe test for skewed data sets and the Levene test for symmetric, normal distributions.

4. Results

As discussed above, a range of findings has implied a role of Praja1 during development and differentiation of neurons: the abundant expression of Praja1 in brain tissue (Yu et al. 2002), an up-regulation of Praja1 mRNA levels in neuronal cells of the BLA following fear conditioning (Stork et al. 2001), and the interaction of Praja1 with NRAGE (Sasaki et al. 2002), which is known to mediate various pathways involved in neuronal differentiation, as well as with other factors involved in the development of the nervous system. This hypothesis has been tested with the experimental approach described in the following.

4.1 The role of Praja1 in a model of neuronal development

4.1.1 Assessment of Praja1 expression following NGF stimulation in PC12 cells

The potential relevance of Praja1 in neuronal differentiation has been addressed by using naïve PC12 cells following NGF stimulation. Analysis of endogenous Praja1 protein levels throughout NGF-induced differentiation in PC12 cells has been conducted in collaboration with Daniel Lang and Bettina Müller. While only low levels of Praja1.1 and Praja1.2 have been observed during the initial phase of NGF stimulation, there was a three-fold increase in Praja1 protein levels after three days of NGF treatment ($F = 22.048$, $p = 0.002$ for Praja1.1; $F = 110.561$, $p = 0.000$ for Praja1.2; Table 11 in A.I.1, Figure 3).

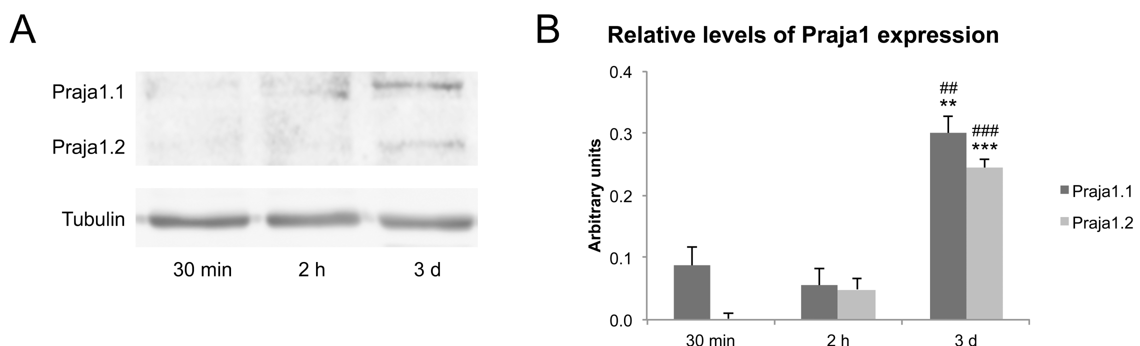


Figure 3: Up-regulation of Praja1 during neuronal differentiation of PC12 cells. (A) Immunoblot analysis has revealed an increased expression of endogenous Praja1.1 and Praja1.2 after three days of NGF treatment. (B) Expression levels (normalized to tubulin levels) after three days are increased more than three-fold compared to the initial phase of NGF stimulation. Values are presented as mean ± S.E.M. (N = 3). Statistical analysis has relied on one-way ANOVA with significant differences assumed for $p \leq 0.05$. Significance levels of post-hoc tests are shown for measurements after three days of NGF exposure compared to either 30 minutes (asterisks) or two hours (hashes) of NGF treatment. ** $p \leq 0.01$, *** $p \leq 0.001$; ## $p \leq 0.01$, ### $p \leq 0.001$.

4.1.2 The intracellular localization of Praja1

In order to identify the intracellular compartments in which Praja1 might exert its activity, the intracellular localization of this E3 ligase has been studied more closely. A prediction based on the amino acid sequence of both Praja1 isoforms using WoLF PSORT (Nakai & Horton 1999; Horton et al. 2007) has suggested a primarily nuclear localization based on structural

similarity to other proteins and identification of the nuclear localization sequence PRRRRTM at position 292 of Praja1.1 and position 108 of Praja1.2.

Indeed, Praja1.1/2 fusion proteins have displayed a nuclear staining, but relative expression levels have been higher in the cytosol than in the nucleus, whereas Praja1.2 and control constructs provided an almost exclusive cytosolic staining (Table 9). A dependence of the intracellular localization of Praja1 on PDL or collagen (type I or IV) coating as well as on differentiation states or NGF treatment of PC12 cells could not be observed.

	Praja1.1/2	Praja1.2	Control
Nuclear index	−0.49	−0.95	−1.00
CV	10.4	5.1	0.0
Number of evaluated cells	361	305	611

Table 9: Intracellular localization of Praja1 in PC12 cells. The intracellular localization of Praja1 has been assessed by calculating the nuclear index for cells maintained under one of the following conditions: grown on either PDL or collagen (type I or IV), and for each coating either with or without NGF treatment. Nuclear indices represent the pooled mean values of all conditions, because no differences could be observed based on coating or NGF treatment as indicated by the low CV between conditions.

4.1.3 Confirming the PC12 cellular differentiation model

As has been summarized in section 2.4, PC12 cells have been described as a model for neuronal-like differentiation on many occasions. To confirm applicability of this model with the experimental conditions planned for this study, neuronal-like differentiation of acutely and stably transfected PC12 cells has been determined after supplementation of NGF for up to four days. Development of a neuronal-like phenotype has been verified by controlling the levels of neuron-specific cellular markers such as β3-tubulin (also named “TuJ1”) and MAP2, which have both been drastically increased in PC12 cells following NGF treatment (Figure 4).

Interestingly, this experiment has conveyed the qualitative impression that over-expression of Praja1 isoforms might lead to a reduction in neuritogenesis, which has then been followed up on with subsequent trials allowing for proper quantitative assessment.

4.1.4 Inhibition of neuronal-like differentiation in PC12 cells by Praja1

Based on the up-regulation of endogenous Praja1 during NGF-induced differentiation and following confirmation of neuronal-like differentiation, cell soma morphology and neurite outgrowth have been determined after four days of NGF stimulation in stably transfected PC12 cell lines grown on PDL and expressing Praja1 isoforms in a doxycycline-inducible manner.

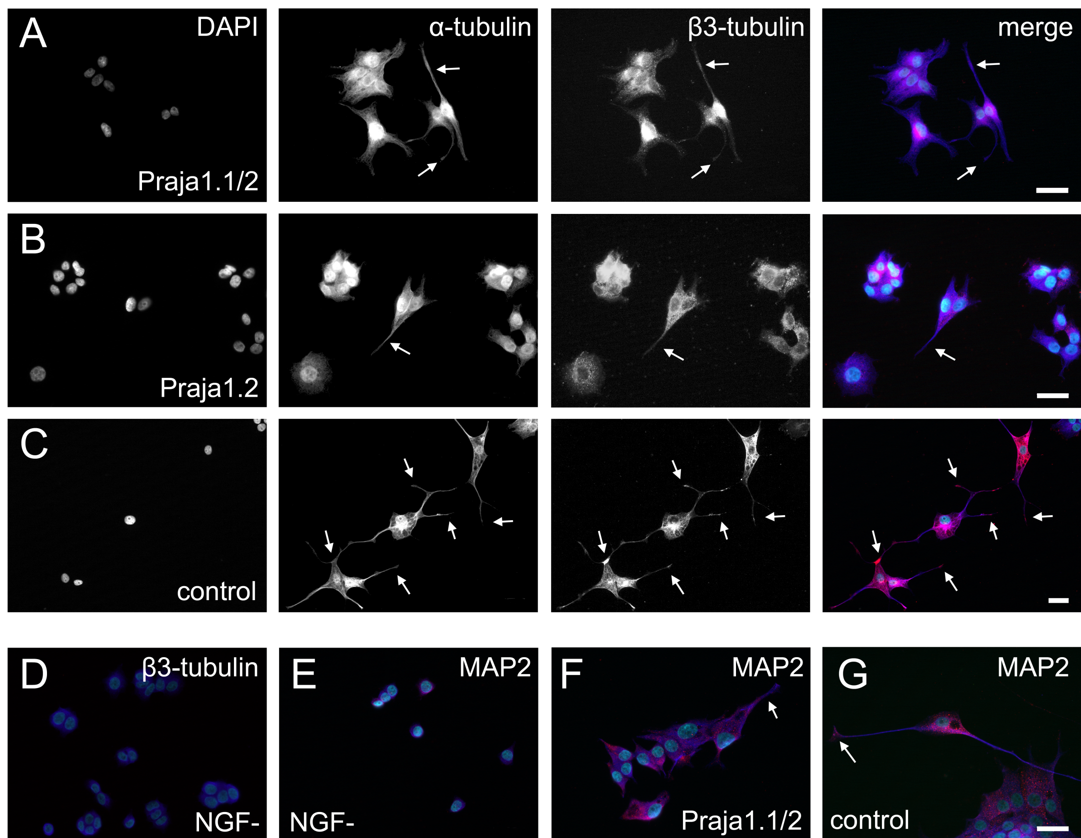


Figure 4: Confirming a neuronal-like differentiation of PC12 cells. PC12 cells of each stably transfected line have been stained with antibodies against the neuron-specific markers β3-tubulin (red) or MAP2 (red), each in combination with DAPI (cyan) and α-tubulin (blue) staining. (A) to (C) show separate and merged staining for cells over-expressing Praja1.1/2 (A) or Praja1.2 (B) as well as for control cells (C), each after NGF treatment. Arrows indicate the expression of β3-tubulin in neurites, which is particularly found at growth tips. (D) and (E) exemplify the lack of spontaneous differentiation in absence of NGF: No neurites are present and levels of β3-tubulin (D) or MAP2 (E) are almost undetectable. Cells over-expressing Praja1 isoforms have shown equal results. (F) and (G) present the MAP2 labelling of Praja1.1/2-expressing and of control cells after NGF treatment, which, in essence, are equivalent to staining of β3-tubulin. Bars: 25 µm.

This has revealed a significant impairment of NGF-induced neuronal-like differentiation in comparison to control cells when over-expressing either Praja1 transcript variant. There has been an almost three-fold reduction in the number of neurites per differentiating cell upon Praja1.1/2 over-expression and a more than eight-fold decrease following over-expression of Praja1.2 ($F = 27.735$, $p = 0.000$; Table 12 in A.I.2, Figure 5). This has been paralleled by a significant increase in the proportion of flattened cells when over-expressing Praja1.1/2, while over-expression of Praja1.2 has led to a similar, yet non-significant trend ($F = 4.546$, $p = 0.029$; Table 12 in A.I.2, Figure 5). After two days of NGF stimulation, the effect of Praja1 on neuronal-like differentiation in PC12 cells has been comparable, but not quite as distinct (data not shown).

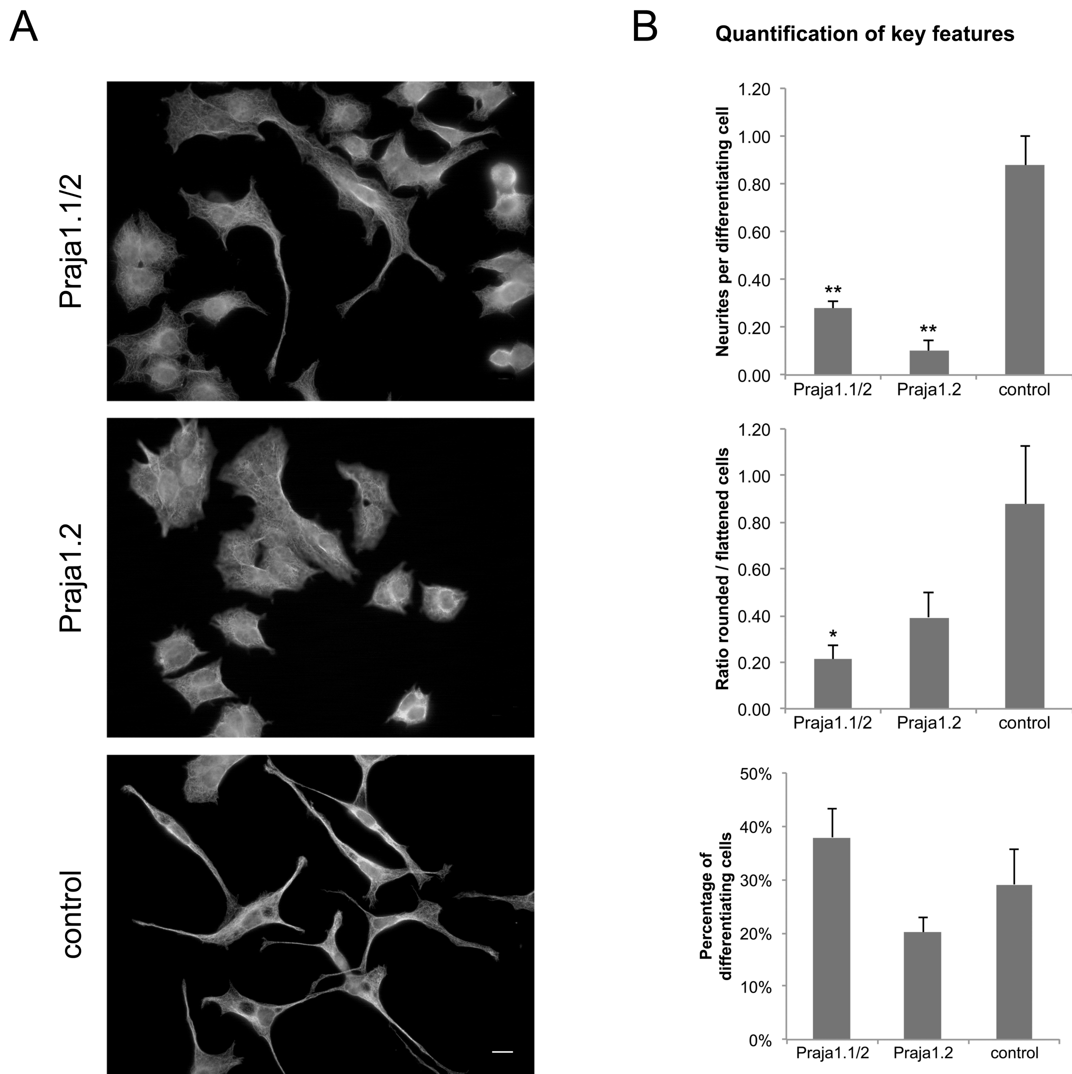


Figure 5: Inhibition of neuronal-like differentiation of PC12 cells by Praja1. (A) shows representative examples of PC12 cells stably expressing either Praja1.1/2, Praja1.2 alone, or control vectors upon doxycycline induction. For complete visualization, cells have been stained with an anti-α-tubulin antibody. (B) A reduced proportion of Praja1 over-expressing PC12 cells compared to control cells have developed neurites of 25 µm or more. At the same time, the attachment of cells to the substrate has been altered as indicated by a reduced ratio of rounded to flattened cells following over-expression of Praja1.1/2. Cells over-expressing Praja1.2 have shown a similar trend, but failed to reach significance. A generally comparable proportion of cells in all cell lines has been judged as being differentiating, i. e. showing a neuron-like morphology. All values are presented as mean ± S.E.M. * $p \leq 0.05$, ** $p \leq 0.01$ in post-hoc tests of an ANOVA (N = 6), when compared to controls. Bar: 10 µm.

To evaluate substrate specificity of this inhibitory effect of Praja1.1/2 on the development of a neuronal PC12 phenotype, other extracellular substrates like collagen IV, laminin, or the cell adhesion Fc-fusion fragment L1-Fc have been tested in a separate set of experiments, using the same paradigm as before. However, none of these substrates could abolish the inhibition of neurite outgrowth by Praja1.1/2 ($p = 0.001$ for collagen IV, $p = 0.007$ for laminin, $p = 0.002$ for laminin/PDL and L1-Fc, and $p = 0.000$ for PDL; Table 13 in A.I.2, Figure 6), indicating that it is largely independent of extracellular matrix components. Moreover, cell rounding has also been decreased on most substrates with the exception of collagen IV ($p = 0.017$ for laminin, $p = 0.032$ for laminin/PDL, $p = 0.011$ for L1-Fc, $p = 0.003$ for PDL,

and p = 0.837 for collagen IV; Table 13 in A.I.2, Figure 6). However, while the rounding ratio for Praja1.1/2-expressing cells has been fairly comparable to that of the other groups, control cells on collagen IV (and less pronounced on laminin/PDL) have shown a profound reduction in the number of rounded cells compared to other substrates ($F = 6.341$, $p = 0.000$; Table 14 in A.I.2, Figure 6). Notably, Praja1 over-expression in PC12 cells has not caused a reduction in the number of filopodia-like protrusions (5–25 µm) per differentiating cells on any substrate tested (data not shown).

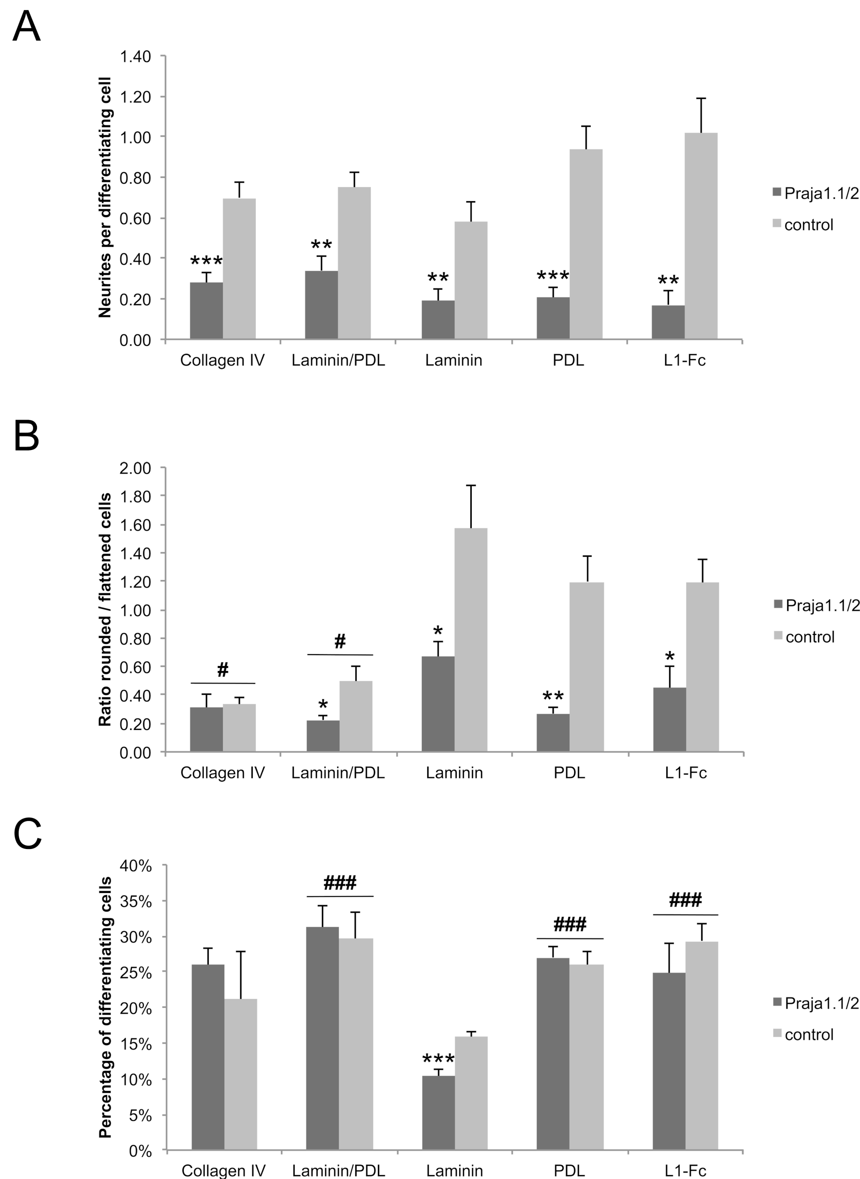


Figure 6: Substrate independence of Praja1 effects. (A) Reduced growth of neurites ≥ 25 µm has been observed in cells over-expressing Praja1.1/2, regardless of the substrate used. (B) Cell rounding has been reduced on all tested substrates except for collagen IV. Laminin/PDL and collagen IV have shown decreased ratios in comparison to laminin, which is mainly caused by a reduced ratio in control cells. (C) The overall proportion of differentiating cells has generally not been affected by Praja1 over-expression, except on laminin (but not on laminin/PDL). Presented values are mean ± S.E.M. For comparison of Praja1.1/2 and control cells, levels of significance (asterisks) have been assessed using a two-tailed Student's t-test, taking into account homogeneity of variance by applying Levene testing and performing t-testing accordingly; *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$. Differences between coatings have been assessed by one-way ANOVA and appropriate post-hoc testing with each significance level (hashes) presented in comparison to laminin; ### $p \leq 0.001$, # $p \leq 0.05$.

On the other hand, an equal percentage of PC12 cells expressing either Praja1.1/2 or control vectors have been judged as differentiating when grown on collagen IV, PDL, or L1-Fc. Yet, when grown on laminin, cells have generally shown lower levels of differentiation (F = 9.048, p = 0.000; Table 14 in A.I.2), with Praja1.1/2 even amplifying this decline in differentiation induction (p = 0.001 compared to control; Table 13 in A.I.2). It should be pointed out that this suppressive laminin effect could be abolished by co-coating of laminin with PDL (Figure 6, cf. Table 14 in A.I.2).

4.1.5 Praja1 effects on apoptosis in differentiating PC12 cells

Being part of several pro-apoptotic signalling pathways, NRAGE has commonly been implied to play a role in mediating apoptotic effects and influencing cell survival during development and differentiation of neuronal cells (cf. e. g. Mouri et al. 2013). In addition, cell rounding and loss of cell area are morphological features of apoptosis, and Praja1 has been observed to induce apoptosis in fibroblast-like COS7 cells (Teuber et al. 2013). Hence, to ascertain that the observed effects following Praja1 expression in PC12 cells have not been associated with an increased apoptosis rate, activation of caspases 3 and 7 – key components of the intracellular apoptosis cascade – has been measured in stably transfected PC12 cells after doxycycline induction of Praja1.1/2, Praja1.2, or EGFP expression, either maintained with or without NGF.

Generally, PC12 cells without NGF stimulation have provided higher caspase 3/7 activation levels. As might be expected from other data presented in the literature (Bui et al. 2002; Culmsee et al. 2002), application of NGF has reduced pro-apoptotic caspase 3/7 activation to some extent (for Praja1.1/2 by 38 %, for Praja1.2 by 25 %, and for EGFP control by 47 %), although this difference has failed to reach significance (Table 10).

	Praja1.1/2	**Praja1.2**	**EGFP control**	**Brown-Forsythe**	**One-way ANOVA**	
	Mean ± S.E.M.	***Mean ± S.E.M.***	***Mean ± S.E.M.***	***p-value***	$F_{0.95}(2,9) = 4.26$	***p-value***
+ NGF	69,869.71 ± 16,816.56	43,128.50 ± 7,480.46	72,677.00 ± 18,722.84	0.368	≥ 1.158	0.357
– NGF	112,511.08 ± 13,819.17	57,841.13 ± 7,579.94	136,817.21 ± 25,739.04	0.054	≤ 5.388	0.029
Post-hoc test	***p-value***	***p-value***	***Procedure***			
– NGF	0.603	0.026	Tukey-HSD			

Table 10: Praja1 effects on caspase 3/7 activation in PC12 cells. Activation of caspases 3 and 7 has been assessed using a luminescence assay after two days with or without NGF stimulation. Measured values from four independent experiments per group (N = 4) are noted as mean ± S.E.M. of arbitrary relative luminescence units. Significant differences have been assumed for p ≤ 0.05 in a one-way ANOVA. Presented post-hoc p-values are each in comparison to EGFP control cells. Post-hoc comparison procedures have been chosen depending on the result of the Brown-Forsythe test.

Importantly, no significant differences in caspase 3/7 activation have been found between NGF-stimulated PC12 cells over-expressing either Praja1 or the control construct (F = 1.158, p = 0.357; Table 10). It should be pointed out, however, that Praja1.2, but not Praja1.1/2, could mimic the NGF-induced decrease in caspase 3/7 activation in a significant manner in the absence of NGF (F = 5.388, p = 0.029; Table 10).

4.1.6 Changes of gene expression in PC12 cells over-expressing Praja1

Having observed the Praja1-dependent blockade of neurite outgrowth in NGF-stimulated PC12 cells, NPY, Slc18a1, and DnaJC5 mRNA levels (cf. section 2.4) have been measured in stably transfected PC12 cell lines via qRT-PCR to assess, whether over-expression of Praja1 would induce a general de-differentiation or whether a secretory phenotype would be maintained instead of promoting a neuronal one. Gene expression has been evaluated after four days of NGF stimulation as described above.

Praja1 over-expression in either stably transfected PC12 cell line has been accompanied by an almost 10-fold increase of Slc18a1 expression compared to control cells (F = 42.494, p = 0.000; Table 15 in A.I.3, Figure 7). DnaJC5 mRNA levels, on the other hand, have shown a weak increase following over-expression of Praja1, but have failed to provide significant differences compared to controls cells (F = 3.513, p = 0.098; Table 15 in A.I.3, Figure 7). Strikingly, over-expression of either Praja1 construct has also led to a strong induction of NPY expression (F = 20.664, p = 0.002; Table 15 in A.I.3, Figure 7).

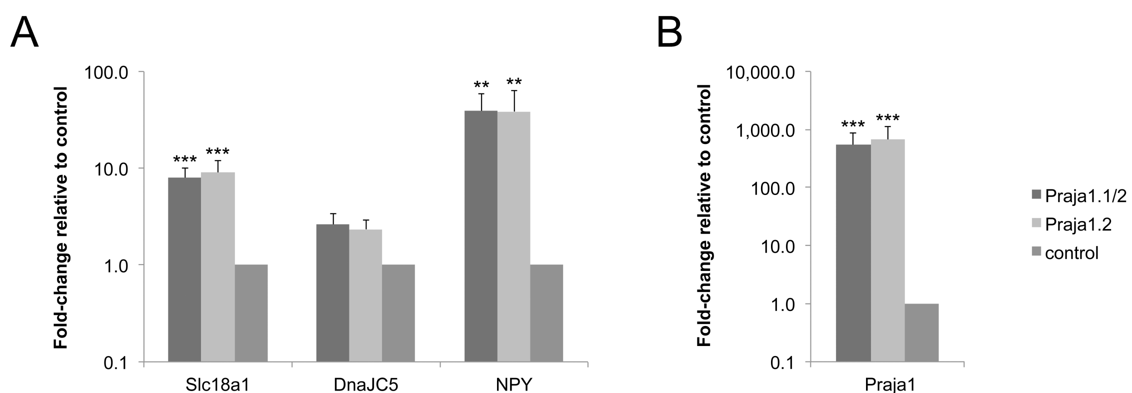


Figure 7: Influence of Praja1 on mRNA levels of secretory phenotype markers. (A) While over-expression of Praja1 isoforms does induce expression of DnaJC5 only weakly, levels of Slc18a1 mRNA are significantly elevated following Praja1 over-expression. The same has been observed for the expression of NPY, which has not been detectable in control cells upon NGF treatment. In this case, a CT of 50 has been assumed to allow for further quantitative comparison based on the ΔΔCT method (see section 3.3.3). (B) To confirm successful Praja1 over-expression in stably transfected tetracycline-inducible PC12 cells, Praja1 mRNA levels have also been measured and have been increased significantly in the appropriate cell lines following doxycycline treatment. Values are presented as mean multiples of control mRNA levels (RQ) ± S.E.M. for either stably transfected Praja1 cell line. Due to the RQ method, control mRNA levels equal 1 (no S.E.M. provided). Significance levels have been calculated for ΔCT values in a one-way ANOVA followed by appropriate post-hoc testing. ** p ≤ 0.01 and *** p ≤ 0.001 compared to controls.

Sufficient functionality of the tetracycline-induced Praja1 over-expression system in stably transfected PC12 cell lines has been confirmed by measuring Praja1 mRNA levels, which have been 600-fold higher in Praja1 over-expressing than in control cell lines ($F = 50.412$, $p = 0.000$; Table 15 in A.I.3, Figure 7).

4.2 Praja1 effects on NRAGE

Because NRAGE has been found to be an important mediator of neurotrophin signalling via $p75^{NTR}$ and TrkA, which are known for regulating cell survival and neuronal differentiation (see section 2.3.2), and has been shown to be an interaction partner and substrate of Praja1 (Sasaki et al. 2002), it is the most promising candidate for explaining the inhibitory effect of Praja1 on NGF-dependent differentiation in PC12 cells. Due to the fact that Smad3 has also been implicated to be ubiquitinated by Praja1 (Saha et al. 2006) and is known to be involved in some of the same signalling pathways as NRAGE (cf. section 2.3.1), Smad3 has been evaluated in addition.

4.2.1 Co-localization of Praja1 and NRAGE in PC12 cells

In accordance with the proposed interaction of Praja1 with the signalling molecules NRAGE and Smad3, a co-localization of Praja1.1/2 with NRAGE as well as with Smad3 has been observed in the nucleus, along filamentous structures in the cytosol, and at distinct points at the plasma membrane (Figure 8).

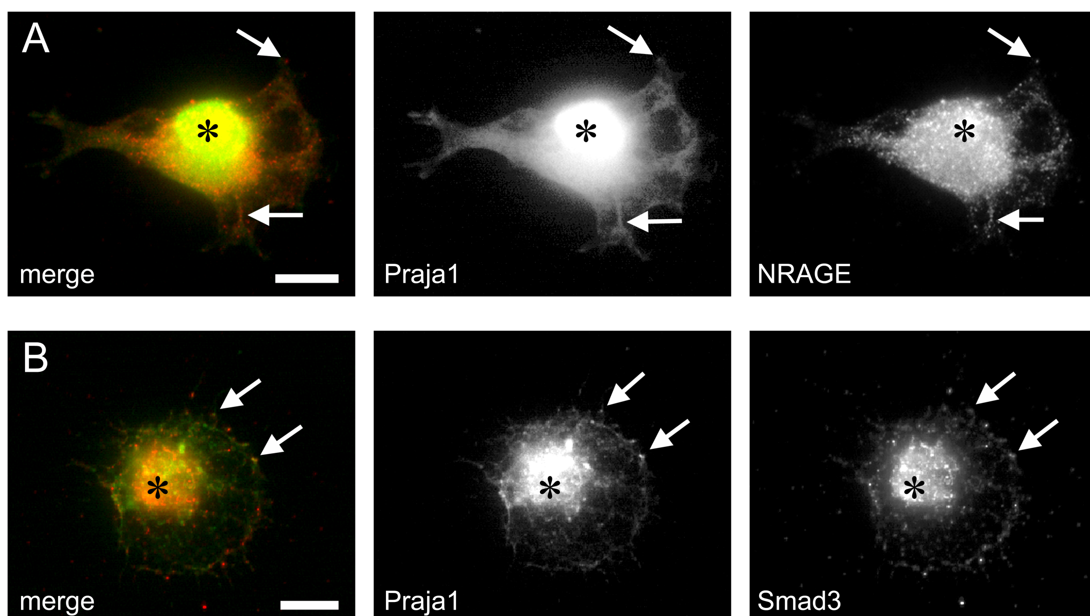


Figure 8: Co-localization of Praja1 with its putative substrates NRAGE and Smad3. (A) EGFP-Praja1.1/2 (green) has co-localized with NRAGE (red) in the nucleus (asterisk), at distinct positions at the plasma membrane, and along filamentous structures in the cytosol (arrows). (B) Co-localization of Smad3 (red) and EGFP-Praja1.1/2 (green) has also been observed in the nucleus (asterisk) and at few points at the plasma membrane (arrow). In contrast to the subtle differences in intracellular distribution between both *praja1* constructs (see section 4.1.2), no distinction in co-localization has been found between the transcript variants *praja1.1* and *praja1.2*. Bars: 10 µm.

4.2.2 Praja1-dependent ubiquitination and degradation of NRAGE in PC12 cells

As demonstrated in HEK293 cells by Sasaki and colleagues (Sasaki et al. 2002), Praja1 is capable of directly binding to NRAGE, leading to its ubiquitination in a RING-H2-dependent manner and reducing NRAGE-mediated transcriptional effects.

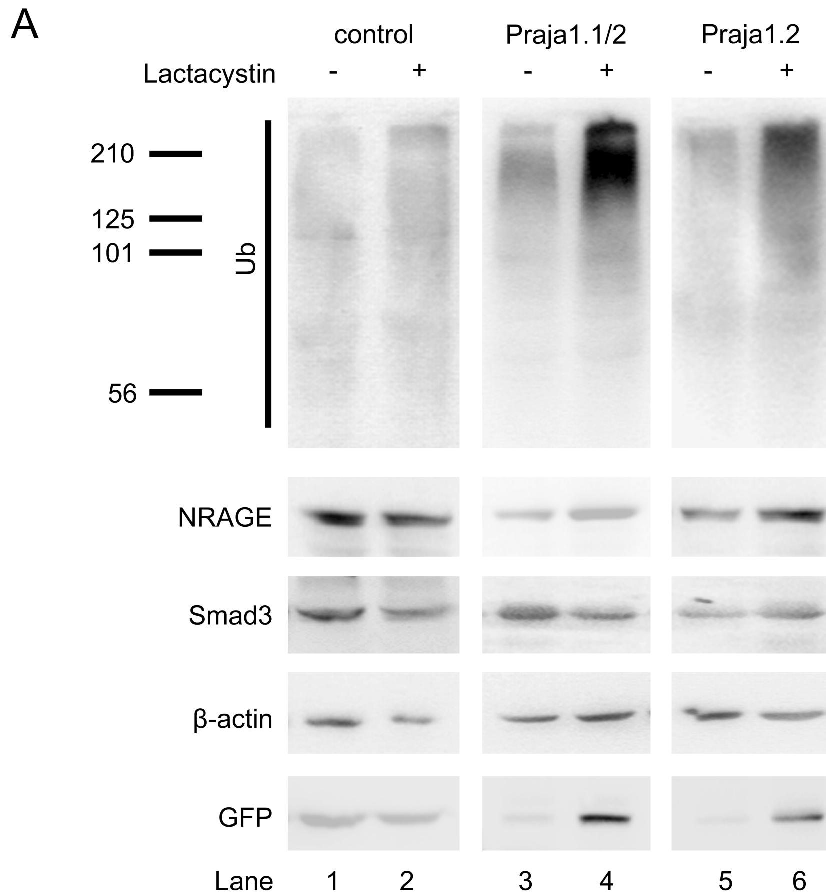
To evaluate, whether Praja1 exerts comparable effects in the NGF-induced neuronal-like PC12 model system, general ubiquitination activity and endogenous NRAGE protein levels have been studied in collaboration with Bettina Müller. For unveiling potential high turnover effects, experiments have been performed in presence and absence of the proteasome inhibitor lactacystin in the stably transfected PC12 cell model.

Increased poly-ubiquitination compared to controls has been detected in PC12 cells stably expressing either EGFP-Praja1.1/2 or EGFP-Praja1.2 in a tetracycline-dependent manner (Figure 9). However, these effects have primarily been observed following treatment with lactacystin, which has led to a more pronounced accumulation of poly-ubiquitinated proteins in these cells.

Substantiating previous findings, protein levels of NRAGE have been significantly reduced in both Praja1 over-expressing cell lines ($F = 7.250$, $p = 0.013$; Table 16 in A.I.4, Figure 9), which has been abolished by treatment with lactacystin ($F = 0.420$, $p = 0.669$; Table 16 in A.I.4, Figure 9).

Confirming earlier hypotheses on an auto-regulation of Praja1 through the UPS (Saha et al. 2006), EGFP-Praja1.1/2 as well as EGFP-Praja1.2, but not EGFP controls, have also been accumulating during lactacystin treatment (Figure 9).

Importantly, Smad3 levels, which have been tested to control for the specificity of the observed effects, have not been affected by Praja1 over-expression in an analogous manner (Figure 9).



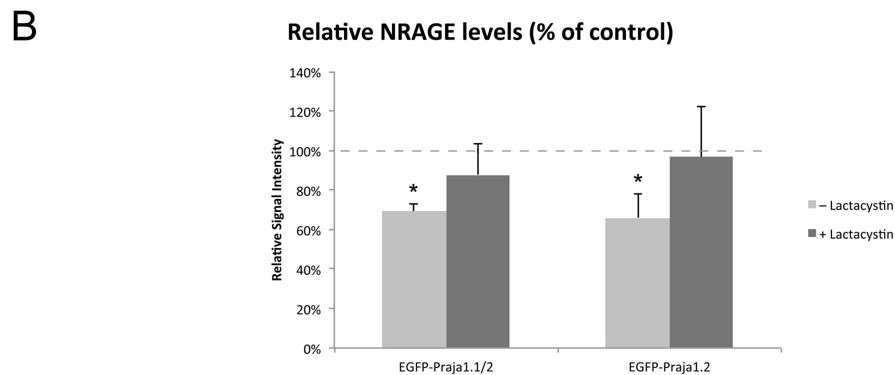


Figure 9: Poly-ubiquitination and NRAGE expression levels in PC12 cells. (A) Immunoblot analysis has revealed enhanced total protein ubiquitination in cells expressing EGFP-Praja1.1/2 (lanes 3&4) or EGFP-Praja1.2 (lanes 5&6) compared to EGFP-transfected controls (lanes 1&2). However, this effect has only become clearly apparent after blockade of proteasomal degradation with lactacystin and subsequent accumulation of ubiquitinated proteins (lanes 4&6). Endogenous NRAGE has been reduced by Praja1 over-expression (lanes 3&5) in a manner sensitive to application of lactacystin, whereas no comparable change in Smad3 protein levels has been observed. In agreement with its self-regulating ability of auto-ubiquitination, levels of EGFP-Praja1.1/2 and EGFP-Praja1.2 have been strongly increased following lactacystin treatment. (B) Quantitative analysis of Western blots (N = 6) has revealed a significant reduction of NRAGE upon Praja1 over-expression that could be prevented by application of the proteasome inhibitor lactacystin. B-actin has served as loading control. Mean values ± S.E.M. presented in (B) have been calculated after normalization to control-transfected cells of the respective lactacystin treatment. Significance levels are in comparison to normalized controls (100 %) and have been obtained by one-way ANOVA and subsequent post-hoc testing, if applicable. * $p \leq 0.05$.

5. Discussion

Initially, the E3 ubiquitin ligase Praja1 has mainly been implicated in liver development (Mishra et al. 1997). Later findings have also implied a role in the aetiology of gastric cancer (Mishra et al. 2005; Saha et al. 2006). However, its structural similarity to Neurodap1, which is strikingly also named Praja2, has suggested a role in the nervous system from early on (Mishra et al. 1997; Sasaki et al. 2002). This impression has been further encouraged by the observation of an up-regulation of Praja1 in the BLA following fear conditioning (Stork et al. 2001) and by the dynamic expression of *praja1* mRNA in brain and other mouse tissues at different developmental stages (Teuber et al. 2013). Yet, the precise cellular functions as well as the physiological role of Praja1 have still remained to be elucidated.

In previous experiments, two splice variants of murine *praja1* had been identified, which appear to be generated through a differential intron retention mechanism using canonical U2 splice donor and splice acceptor sites located within the second exon (Stork et al. 2001; Teuber et al. 2013). It has been found that the *in vivo* ratio of *praja1.1* to *praja1.2* changes from roughly 1:1 in early development to 3:1 in most adult tissues, including the brain. This may suggest an enhanced developmental expression and function of *praja1.2* (Teuber et al. 2013). To study the role of Praja1 in NGF-dependent differentiation, stably transfected PC12 lines have been generated that express either transcript variant in a tetracycline-inducible manner with *praja1.1* constructs expressing Praja1.1 and Praja1.2 due to alternative splicing and *praja1.2* constructs expressing Praja1.2 only.

5.1 Praja1 co-localizes with cytoskeleton elements and with NRAGE

For both transcript variants, Praja1 has been localized predominantly in the cytosol, where it partially associated with microtubules and filopodial actin filaments (Teuber et al. 2013). Notably, Praja1.1/2 has also been located in the nucleus, whereas Praja1.2 alone as well as control cells have presented an almost exclusive cytosolic distribution. One might thus speculate, whether Praja1.1 might be more likely to translocate into the nucleus, whereas Praja1.2 would do that on rare occasions only, therefore largely contributing to the overall more cytosolic staining of *praja1.1* constructs, which are generating Praja1.1/2.

Unfortunately, none of the currently available constructs and antibodies allow for verifying this hypothesis. So far, antibodies have only been generated to assess total Praja1 levels. This has been sufficient for evaluating differential influences of the two splice variants in Western blotting, during which their respective molecular weights lead to two distinct bands. In addition, isoform-specific gene expression assays have been designed to measure mRNA levels of either isoform. However, to address the question of differences in the intracellular distribution of each isoform, antibodies should be created that specifically target sequences

that are part of Praja1.1, but not of Praja1.2 after alternative splicing. In addition, targeted mutations within the SC-35 ESE consensus sequence or at other splice sites in the *praja1* sequence could be considered to modulate alternative splicing, thus potentially generating a construct that will exclusively express Praja1.1 and will hence enable to address the exact influence of either isoform more precisely in future studies.

From a functional perspective, the intracellular distribution of Praja1 in conjunction with its partial co-localization with NRAGE and Smad3 at cytoskeletal structures, at few, but distinct positions at the plasma membrane as well as in the nucleus may suggest a role of Praja1 in cytoskeletal re-arrangement and in signalling cascades of cytoskeleton-associated factors to the nucleus, most prominently neurotrophin and BMP signalling, which rely on NRAGE and Smad3 for intracellular signal transduction, as has been discussed (see section 2.3). Notably, the occasional association of Praja1 with microfilaments at sites of neurite outgrowth (Teuber et al. 2013) should be examined more closely in light of this study.

5.2 Praja1 inhibits NGF-induced neurite outgrowth in PC12 cells by targeting NRAGE for proteasomal degradation

Up-regulation of endogenous Praja1 expression after three days of NGF stimulation has suggested a role of Praja1 during the progression of NGF-induced PC12 cell differentiation, rather than during its initiation. Accordingly, over-expression of either Praja1.1/2 or Praja1.2 alone has resulted in a profound reduction of neurite outgrowth independent of extracellular substrates, while the number of filopodia per differentiating cell has not changed following over-expression of Praja1, further portending that initial differentiation might be unaltered. Moreover, Praja1 over-expression has affected cell attachment, as indicated by enhanced cell spreading and thus a greater proportion of flattened cells on most extracellular substrates tested. This is in line with a potential role of Praja1 in cytoskeletal re-arrangement as well as in modulation of transcriptional activity, as mentioned above.

In support of previous studies on the special importance of extracellular matrix components, in particular laminin, for the NGF-dependent initiation of neurite outgrowth in PC12 cells (Fujii et al. 1982; Attiah et al. 2003), cells cultured on laminin in this study have presented a reduced number of differentiating cells compared to all other substrates investigated. The observation that over-expression of Praja1.1/2 diminishes the proportion of differentiating cells on laminin even further might point to a specific influence of Praja1.1/2 on signalling cascades following laminin-induced co-activation of $\alpha_1\beta_1$- and $\alpha_3\beta_1$-integrin; with $\alpha_3\beta_1$-integrin potentially being the crucial factor, because collagen IV is known to activate $\alpha_1\beta_1$-integrin only (Tomaselli et al. 1990) and has not shown similar results following over-expression of Praja1.1/2. Alternatively, integrin-independent laminin receptors could also play a certain role in this context (cf. Powell & Kleinman 1997).

Previously, Praja1 has been shown to precipitate NRAGE in a glutathione S-transferase (GST) pull-down and to occur in a complex with NRAGE and Msx2 in HEK293 cells (Sasaki et al. 2002; Kuwajima et al. 2004). In addition, NRAGE has been reported to stimulate neuronal differentiation and neurite outgrowth in PC12 cells (Reddy et al. 2010). It has thus been hypothesized that Praja1-mediated ubiquitination and proteasomal degradation of NRAGE may explain the Praja1 effect on neuritogenesis.

Indeed, the over-expression of either one of the Praja1 variants has induced a reduction of endogenous NRAGE in PC12 cells in a lactacystin-sensitive manner, hence confirming results from previous studies in non-neuronal cell lines in a neuronal-like cell culture model. Furthermore, these findings support those by Reddy and colleagues, who have observed an increase in NGF-induced neuronal differentiation upon stable over-expression of NRAGE in PC12 cells (Reddy et al. 2010). In accordance, over-expression of other $p75^{NTR}$-associated MAGE family members such as necdin (Salehi et al. 2000; Bronfman et al. 2003) has been shown to increase NGF-induced and TrkA-dependent neurite growth in PC12 cells induced by or in co-operation with endogenous NRAGE (Tcherpakov et al. 2002; Bronfman et al. 2003; Reddy et al. 2010). On the other hand, Feng and co-workers have reported opposite effects, namely a reduction of neuronal differentiation following NRAGE over-expression (Feng et al. 2010). Interestingly, however, they have found a gradually increasing reduction in endogenous NRAGE protein levels, which has been starting about two days after on-set of NGF treatment and has eventually led to a complete depletion of NRAGE after NGF stimulation for two weeks (Feng et al. 2010). Notably, NRAGE mRNA levels have been unaltered within the first two days (Feng et al. 2010), implying a post-transcriptional cause for this reduction rather than a down-regulation of gene expression. In light of the repeatedly demonstrated interaction of NRAGE and Praja1 as well as the up-regulation of endogenous Praja1 in a comparable time line following treatment with NGF – as has been shown in this study –, Praja1 might well be the factor causing the post-transcriptional decrease in NRAGE protein levels observed by Feng and collaborators.

Importantly, previous studies have demonstrated that $p75^{NTR}$-induced and JNK-mediated apoptosis – rather than differentiation – would be facilitated by NRAGE in various cell types, including PC12 cells (Salehi et al. 2000; Salehi et al. 2002). In contrast, Praja1 has been reported to also target several anti-apoptotic factors according to a micro-array screening (Loch et al. 2011). Therefore, it is important to note that over-expression of Praja1 in the presented PC12 cell model has not been associated with an induction of apoptosis as indicated by an unaltered activity of the caspases 3 and 7. To the contrary, Praja1.2 appears to even promote cell survival in PC12 cells grown in absence of NGF. However, an earlier study in fibroblastic COS7 cells after acute transfection with Praja1 constructs had revealed the emergence of distinct morphological changes comprising common features of apoptosis: cell rounding and decrease of cell area in combination with increased micro-spike formation

and the development of pyknotic nuclei (Teuber et al. 2013). This difference between both cell lines could be due to different receptor repertoires. While Praja1 might contribute to regulating $p75^{NTR}$ and TrkA receptor function upon binding of NGF in differentiating PC12 cells (Masoudi et al. 2009), lack of these receptors in most non-neuronal cell lines, such as COS7, would shift pathway activity patterns in these cells, thus promoting differing biological effects like apoptosis.

It should be noted that based on the present data, modulation of other known or putative Praja1 substrates cannot be entirely excluded from contributing to the observed changes in PC12 morphology upon Praja1 over-expression. However, with regard to the two most prominent alternative substrates, Msx2 has previously been demonstrated to depend on presence of NRAGE for ubiquitination by Praja1 (Sasaki et al. 2002), whereas no evidence for an influence of Praja1 on Smad3 levels – as proposed in a previous study (Saha et al. 2006) – could be identified in the examined cellular model. Thus, although other putative Praja1 targets should certainly be considered in future studies, it is reasonable to assume that modulation of NRAGE levels and activity by Praja1 is pivotal in explaining the current findings.

Because the focus of this study has been on earlier stages of neuronal-like differentiation of NGF-treated PC12 cells, Praja1 levels and function have yet to be checked in mature cultures, e. g. after two weeks *in vitro*. At later stages of differentiation, Praja1 might well exert differing functions or endogenous Praja1 may be down-regulated again. However, knowing about this general influence of Praja1 on neuronal-like differentiation in the PC12 model system, it appears preferable to conduct such continuing studies in primary neuronal cultures to investigate influences of Praja1 on early growth (DIV7) as well as synaptogenesis (DIV14) and synapse modification (DIV21).

5.3 Praja1 promotes a secretory phenotype in PC12 cells

Application of NGF is known to promote a shift of PC12 morphology from a secretory to a neuronal-like phenotype. The development of a neuronal-like morphology and the increased expression of typical neuronal markers, namely β3-tubulin and MAP2, upon NGF stimulation have been confirmed in the presented cellular PC12 model.

Gene expression levels of three markers for a secretory phenotype have been measured to assess, whether the Praja1-induced inhibition of neurite outgrowth might be associated with a general de-differentiation. While levels of DnaJC5, a mediator of vesicular transmission (Burgoyne 1996; Chamberlain & Burgoyne 1998; Cordeiro et al. 2000a; Evans et al. 2001; Bai et al. 2007), have only shown a weak, yet non-significant increase following Praja1 over-expression, mRNA levels of the LDCV-associated Slc18a1 (Liu et al. 1994; Liu & Edwards 1997), important for mono-amine transmitter and neuropeptide storage (Pothos

et al. 2000; Hoard et al. 2008), have clearly been elevated upon Praja1 induction. Moreover, expression of NPY, which has been reported to be up-regulated upon NGF treatment of PC12 cells (Allen et al. 1984; Allen et al. 1987; Sabol & Higuchi 1990; Higuchi et al. 1992; Balbi & Allen 1994; Rajakumar et al. 1998), but which may nonetheless promote a secretory phenotype depending on the expressed NPY receptor repertoire (McCullough et al. 1998; DiMaggio et al. 1994), has been strongly induced following over-expression of Praja1. At variance with findings presented in the literature, however, NPY mRNA has not been detected in control cells after NGF stimulation. Although the exact reason for this could not be addressed in the context of this work, it should be investigated further in the future.

Taken together, the observed alterations in gene expression and the morphologic changes upon over-expression of Praja1 suggest that rather than causing a general de-differentiation, Praja1 is shifting PC12 cells to a secretory phenotype while largely blocking development of a neuronal-like morphology.

This is further complemented by the qualitative impression that levels of β3-tubulin, but not of MAP2 have been reduced in Praja1 over-expressing PC12 cells undergoing stimulation with NGF (cf. Figure 4 in section 4.1.3). Prospective experiments should therefore aim at quantifying a range of typical neuronal markers during neuronal differentiation and following modulation of Praja1 expression to allow for precise evaluation of this suspicion.

5.4 Proposing a role for Praja1 in neuronal signalling pathways

5.4.1 Praja1 and NRAGE in the nervous system

NRAGE has been shown to modulate the function of cell adhesion molecules, their interaction with the cytoskeleton, as well as signalling to the nucleus (Williams et al. 2003; Kuwajima et al. 2004; Kuwajima et al. 2006; Xue et al. 2005). NRAGE is furthermore known to be critically involved in the differentiation of GABAergic neurons in the forebrain (Kuwajima et al. 2006) and appears to be generally involved in neuronal differentiation processes (Reddy et al. 2010; Feng et al. 2010).

Strikingly, Praja1 and NRAGE show similar patterns of expression during early development, but also in a large number of adult tissues, including the brain (Stork et al. 2001; Masuda et al. 2001; Teuber et al. 2013). NRAGE and Praja1 also exist at high levels in the adult hippocampus and amygdala (Stork et al. 2001; Barrett et al. 2005).

In conjunction with the capability of Praja1 to regulate NRAGE protein levels and hence modulate NRAGE-mediated activation of the Dlx5 transcription factor complex (Sasaki et al. 2002; Saha et al. 2006), these findings imply that changes in the expression or interaction of these signalling molecules due to activity of Praja1 may contribute to neuronal plasticity

and information storage in various brain regions during development, but also during learning and memory formation at later stages; a notion that is further supported by the study at hand.

5.4.2 Praja1 in BMP and NGF signalling

As previously stated, NRAGE is one of the key factors in BMP and NGF signalling. With regard to BMP signalling, the putative Praja1 target Smad3 (Saha et al. 2006) also plays an important role. Albeit it should be kept in mind that Smad3 activation in PC12 cells is induced by TrkA due to a lack of proper BMP receptors (Lutz et al. 2004), the fact that Praja1 failed to alter Smad3 levels in the experiments presented here indicates that at least in PC12 cells, Praja1 modulation of downstream BMP signalling pathways could be independent of Smad3, rather relying on non-canonical BMP cascades, such as that via NRAGE and Dlx5 (cf. Kendall et al. 2005). To evaluate, whether Praja1 might generally be less likely to directly affect canonical, Smad-dependent BMP signalling during development and differentiation of the CNS, other time points as well as potential changes in activity states of Smad3 need to be checked in different model systems in the future. Interestingly, the capability of Praja1 to reduce levels of Msx2 and modulate Dlx5 activation – both of which are increasingly expressed upon BMP signalling – might point to a role of Praja1 in controlling or counteracting activation of BMP cascades during the precisely timed processes of development, differentiation, and synaptic re-organization.

Induction of neuronal-like differentiation in PC12 cells relies on the activation of a $p75^{NTR}$ and TrkA hetero-dimer or a TrkA homo-dimer upon NGF binding. Subsequently, a range of intracellular cascades is activated with differentiation mainly depending on TrkA-induced Erk1/2 activation, further enhanced by NF-κB signalling following $p75^{NTR}$ stimulation (e. g. cf. Kaplan & Miller 2000; cf. Arévalo & Chao 2005). In contrast, activation of $p75^{NTR}$ alone, for example through neurotrophin homo-dimers or precursors, has been found to cause growth arrest and to promote apoptosis via activation of JNK and p53 in several neuronal cell types (e. g. cf. Kaplan & Miller 2000; cf. Arévalo & Chao 2005).

Although initially thought to only bind to $p75^{NTR}$, NRAGE has meanwhile been shown to also interact with TrkA (Barrett et al. 2005; Reddy et al. 2010; Feng et al. 2010). Yet, while Reddy and colleagues have observed cell cycle arrest and a TrkA-dependent induction of neurite outgrowth with subsequent Erk1/2 activation, which has appeared to partially rely on NRAGE levels (Reddy et al. 2010), Barrett and collaborators have proposed a blockade of $p75^{NTR}$/TrkA hetero-dimer formation due to NRAGE binding to both receptors, thus blocking promoting effects on differentiation through hetero-dimer formation (Barrett et al. 2005) and subjecting TrkA to increased degradation (Feng et al. 2010). Accordingly, the reduction of neuronal differentiation following NRAGE over-expression, as reported by Feng *et alii*, has been associated with decreased Erk1/2 signalling and TrkA protein levels, whereas NRAGE knock-down has led to an enhancement in NGF-induced Erk1/2 activation

(Feng et al. 2010). These contradictory findings have been discussed as being due to different experimental approaches, as Feng and co-workers have used transient transfection or transduction under low serum conditions during their experiments (Feng et al. 2010), while Reddy *et alii* have established cell lines stably over-expressing NRAGE, which have been maintained under high serum conditions and supplemented with Ham's nutrient mixture F-12 (Reddy et al. 2010). From early on, serum and nutrient content in the growth medium has been known to influence activity of various intracellular signalling cascades in PC12 cells (e. g. Greene & Tischler 1976; Greene 1978). Furthermore, it is commonly accepted that constitutive stable over-expression may cause subsequent compensatory adaptations in transfected cells. Hence, initial conditions and pathway activation patterns in PC12 cells could have been different in both referenced studies, potentially explaining their differing effects. However, although the experiments summarized in this study have been conducted using a conditionally inducible instead of a constitutive over-expression system under low serum conditions, they do seem to emphasize a positive contribution of NRAGE to neurite outgrowth. Instead, actual $p75^{NTR}$ expression levels in relation to TrkA levels could help to understand the opposing findings as Feng and collaborators have observed a substantial up-regulation of $p75^{NTR}$ within 48 hours of NGF treatment (Feng et al. 2010). Although $p75^{NTR}$ protein dynamics have not been measured in a comparable manner neither by Reddy and colleagues (Reddy et al. 2010) nor in this study, Brann *et alii* have previously demonstrated that increasing $p75^{NTR}$ levels in primary hippocampal neurons may lead to a shift from neurite-promoting to JNK-mediated pro-apoptotic effects of NGF after two days of stimulation (Brann et al. 2002). The observed down-regulation of endogenous NRAGE in parallel to the up-regulation of $p75^{NTR}$ in the PC12 model used by Feng and co-workers (Feng et al. 2010) might then primarily prevent apoptosis, therefore allowing for sufficient stimulation of proper neurite outgrowth through TrkA-dependent Erk1/2 and possibly $p75^{NTR}$-induced NF-κB signalling. Considering varying reports on the dynamics of $p75^{NTR}$ and TrkA expression in PC12 cells, differences in the relative amounts of either NGF receptor as well as in accompanying NRAGE levels might account for the contradictory findings on the NRAGE influence on neurite outgrowth. In conjunction with the study at hand, it may hence be hypothesized that NRAGE indeed promotes cell cycle arrest or even apoptosis upon $p75^{NTR}$ activation, while also modifying TrkA-induced differentiation via Erk1/2. The exact nature of this modulation appears to depend on relative NGF receptor levels and on activity states of various intracellular signalling cascades. By controlling the levels of NRAGE and other intracellular signalling factors (cf. Loch et al. 2011), Praja1 may thus contribute to regulating the fine balance between $p75^{NTR}$ and TrkA receptor function upon binding of NGF in differentiating PC12 cells (Masoudi et al. 2009).

Specifying the precise mechanisms and the balance of intracellular signalling pathway activity that enable Praja1 to cause such effects will be a matter of future studies. This

should also include addressing the physiological regulation of Praja1 activity. In that regard, confirming the previously indicated (Saha et al. 2006) auto-ubiquitination capability of Praja1 in the course of this study might point to an important negative feedback loop for regulating activity of this ubiquitin ligase. Aside from auto-ubiquitination as a measure of self-limitation, inductors of *praja1* expression like FoxP3 as well as potential enhancers of Praja1 activity, such as MAGE-G1, should be evaluated more closely and with regard to their contribution in neuronal cells.

5.4.3 B-catenin – Thinking one step further

Trying to understand the alluded multitude of complex signalling pathway interactions, especially the proposed influence of NRAGE on one of the key factors in so-called canonical Wnt signalling, β-catenin (cf. Niehrs 2012), appears to be of special importance, since the latter has been implicated in neuronal development and differentiation as well as in fear behaviour and memory consolidation on several occasions (Maguschak & Ressler 2008; Maguschak & Ressler 2011).

Normally, β-catenin is found predominantly at the plasma membrane, where it is forming a complex with E-cadherin and α-catenin; the former being an important factor in cell-to-cell adhesion (Nagafuchi & Takeichi 1988; cf. Adams & Nelson 1998; Cailliez & Lavery 2006), thus influencing differentiation, proliferation, and migration of cells. Moreover, studies in *Drosophila melanogaster* have implied a role of the E-cadherin/β-catenin complex (named Shotgun and Armadillo, respectively, in *Drosophila*) in controlling axonal tract branching and trajectory in the larval brain (Fung et al. 2009). It has been widely proposed that α- and β-catenin link E-cadherin to the actin cytoskeleton (cf. Adams & Nelson 1998; Pokutta & Weis 2000) and that the strength of intercellular adhesion via E-cadherin as well as of E-cadherin binding to the cytoskeleton appear to be modulated by its cytoplasmic domain (Nagafuchi & Takeichi 1988) and by β-catenin binding to E-cadherin (Rimm et al. 1995; Yamada et al. 2005; Drees et al. 2005; Catimel et al. 2006), although the exact interaction between these three factors and their joint effects on the stability of cell-to-cell adhesions still need to be clarified.

NRAGE has been demonstrated to disrupt this complex formed by E-cadherin and β-catenin, hence influencing cell-to-cell adhesion and leading to alterations of the cytoskeleton (Xue et al. 2005). In addition, β-catenin translocation to the nucleus and subsequent induction of β-catenin-dependent transcription have been found to be promoted by NRAGE through protecting cytoplasmic β-catenin from immediate degradation (Xue et al. 2005). In turn, this could mean that by controlling the levels of NRAGE, Praja1 might also be capable of modulating cell-to-cell adhesion and β-catenin-dependent transcriptional activity, which would also affect expression of key factors in BMP signalling, such as *msx2* (Willert et al. 2002; Hussein et al. 2003; Zhai et al. 2011).

As a matter of fact, there already is a preliminary body of evidence substantiating this hypothesis. First, the punctual co-localization of Praja1 and NRAGE at the plasma membrane and at peripheral cytoskeleton structures might also be, at least partially, a co-localization with E-cadherin/β-catenin complexes and would fit well with previous observations of a co-localization of Praja1 with specific cell-to-cell contact points at the plasma membrane (Saha et al. 2006); something that is the subject of current studies in continuation of the work presented here. Second, the promotion of a flattened appearance of PC12 cells might indicate an influence of Praja1 on cellular adhesion; although based on the results of this study, it would not necessarily be specific to either cell-to-matrix or cell-to-cell adhesion. Third, in a first set of experiments investigating β-catenin-dependent transcriptional activity upon over-expression of Praja1, a sound inhibition of β-catenin-induced transcription has been noticed.

5.4.4 Ror signalling – A new receptor to consider

A direct influence of Wnt signalling on NRAGE as well as β-catenin activity, on the other hand, appears to be mediated through Ror, mammalian receptors with tyrosine kinase activity and thus similar to the family of Trk receptors. A variety of developmental processes, especially of the genital, skeletal, and cardiovascular systems, has been demonstrated to be impaired in *Ror2*-deficient mice (Liu, Ross, et al. 2007; Liu, Bhat, et al. 2007; Lai et al. 2012). Accordingly, Ror2 is widely expressed in the early embryonic phase, whereas its expression becomes more restricted during late embryonic and postnatal development (Al-Shawi et al. 2001; Matsuda et al. 2001). Interestingly, this down-regulation has occurred around E12, and would therefore take place right after the up-regulation of Praja1 (Teuber et al. 2013) and NRAGE (Masuda et al. 2001) between E7 and E11.

Ror2 is known to modulate JNK activation in several different pathways (Kraus et al. 2003; Crossthwaite et al. 2004) and has been implicated to play a role in tumour progression and metastasis (Lai et al. 2012; O'Connell et al. 2010; Ren et al. 2011). Ror2 has been shown to be a mediating factor in Wnt5a-induced signalling and cell migration (Yamamoto et al. 2007; O'Connell et al. 2010; Liu et al. 2008) through modulating actin re-organization and thereby mediating filopodia formation (Nishita et al. 2006). Although Ror2 activation by Wnt5a has been found to induce so-called non-canonical, while suppressing β-catenin-dependent canonical Wnt signalling (Yamamoto et al. 2007), Ror2 has also been observed to enhance canonical Wnt1 signalling (Lai et al. 2012). These seemingly contradicting findings might, however, be also due to different proteome and receptor repertoires in different cell types. It seems noteworthy that although Wnt3a is capable of binding to Ror2, it fails to induce tyrosine kinase phosphorylation (Liu et al. 2008), making it a potential inhibitor of Ror2-mediated signalling.

NRAGE has been shown to associate with Ror2 at its cytoplasmic C-terminal region, which enables Ror2 to affect Msx2 and Dlx5 transcriptional activity, most likely by regulating the intracellular NRAGE distribution (Lai et al. 2012; Matsuda et al. 2003). Moreover, in view of the NRAGE capability to disrupt E-cadherin/β-catenin complexes and promote nuclear translocation of β-catenin (Xue et al. 2005), Ror2 induction of β-catenin-independent non-canonical and parallel suppression of β-catenin-dependent canonical Wnt signalling in several cell types might be explained by the reduction of cytoplasmic NRAGE. In addition, a Ror2-induced, pro-oncogenic increase in cell migration could be abolished by binding of NRAGE to Ror2 (Lai et al. 2012), which appears to be due to a stabilization of intercellular adhesion. Furthermore, dominant brachydactylia type B – a known heritable skeletal disorder in humans that has been linked to Ror2 – is characterized by a shortened Ror2 missing the cytoplasmic C-terminal region important for NRAGE binding (Lai et al. 2012; Afzal & Jeffery 2003; Matsuda et al. 2003), providing further evidence for the important role of NRAGE in Ror2 signalling cascades.

5.5 Potential clinical implications

In summary, current findings on the influence of Praja1 on intracellular homeostasis and signalling cascades make Praja1 a potential candidate in a variety of pathological instances, among those in particular developmental disorders, cancerogenesis, as well as cognitive impairments and psychopathologies. Hence, the following chapter is dedicated to outlining the body of evidence supporting this postulate in more detail.

5.5.1 A potential role for Praja1 in genetic syndromes and developmental disorders

So far, Praja1 has only been directly linked to one genetic syndrome: craniofrontonasal syndrome. This condition is usually caused by deletion or mutation of the X-chromosomal *ephrin B1* (*EFNB1*) gene and is characterized by craniofrontonasal dysplasia, asymmetric body development, and abnormalities of fingers and toes. In a subset of patients, larger deletions also affect neighbouring regions of the *EFNB1* gene, which include the *PJA1* gene. It is noteworthy that patients suffering craniofrontonasal syndrome usually show an unaltered mental performance. Additional deletion of *PJA1*, however, has been implicated to account for some cases of mild learning disabilities (Wieland et al. 2007). Adding to the understanding of Praja1 as being a potential factor in proper mental development, Yu and colleagues have found the *PJA1* genetic location to be in a region that has been linked to several disorders of the X-linked mental retardation spectrum, although the exact causative genes within that region have not been identified to date (Yu et al. 2002).

NRAGE itself has not been shown to cause any developmental disorders or syndromes. Yet, it is known to bind to necdin, which has been linked to the development of Prader-Willi-Syndrome (Kuwajima et al. 2006). Accordingly, NRAGE-deficient mice present a phenotype

that comprises hallmarks of Prader-Willi-Syndrome: hyperphagia, reduced motor activity, and progressive obesity (Dombret et al. 2012).

In this regard, it is worth mentioning that MAGE-G1, a MAGE family member that has been implicated to enhance activity of Praja1 (Doyle et al. 2010), has also been proposed to be involved in the aetiology of Prader-Willi-Syndrome (Kuwako et al. 2004).

Substitution of one amino acid in another potential target of Praja1, Msx2, is known to cause autosomal dominant Boston-type craniosynostosis. Aside from skull defects, this condition is accompanied by insufficient brain development. Initially, this mutated Msx2, $Msx2_{P138H}$, has been assumed to be a gain-of-function mutation due to its dominant inheritance pattern and an increased DNA binding capability. More recent evidence, however, has suggested a dominant negative role for this mutation, essentially resulting in a loss of function, because it drastically increases degradation of $Msx2_{P138H}$, but also of wild-type Msx2, seemingly mediated by Praja1 (Yoon et al. 2008).

Moreover, Rett Syndrome, another neurodevelopmental pathology which is characterized by mutations in the *Mecp2* gene, has been found to be associated with an increase in Dlx5 levels (Horike et al. 2005). Although current data provide little reason to assume that Praja1 would cause this up-regulation of Dlx5 in Rett Syndrome models, induction of an increased Praja1 activity and subsequent NRAGE degradation might, in consequence, decrease Dlx5 activation and subsequent Dlx5-induced transcriptional activity, hence potentially alleviating some of the impairments in Rett Syndrome, especially with regard to the development of the GABAergic system.

In conclusion, the potential importance of Praja1 in developmental disorders and genetic syndromes relies primarily on circumstantial evidence at this point. Yet, there appears to be a sound foundation for further investigating its involvement in the aetiology of mental impairments associated with the X-chromosome, of Prader-Willi-Syndrome as well as of Boston-type craniosynostosis, but also its potential capacity to alleviate at least some of the symptoms of Rett Syndrome.

5.5.2 Praja1 in tumourigenesis

From early on, an involvement of Praja1 in the pathogenesis of cancerous tumours has been suggested and there is a diverse variety of evidence in support of this hypothesis. As stated above, Praja1 levels are known to be elevated in a range of gastrointestinal cancers. At the same time, more than a third of gastric cancers present decreased Smad3 levels (Han et al. 2004; Mishra et al. 2005; Saha et al. 2006). Although over-expression of Praja1 in PC12 cells has not changed Smad3 levels in contrast to previous findings by Saha *et alii* (Saha et al. 2006), varying intracellular programmes might modulate Praja1-dependent ubiquitination of Smad3 depending on the respective cell type. Moreover, Praja1 has been demonstrated to

disrupt ELF-mediated TGF-β signalling independent of its influence on Smad3 (Mishra et al. 2005; Saha et al. 2006).

Importantly, Praja1 has also been found to induce cell proliferation (Saha et al. 2006), an essential feature of tumourigenesis. Increased proliferation in the course of oncogenic transformation is often accompanied by improper desmosome formation and hence impaired cell-to-cell adhesions. In agreement with this, sporadic forms of colon cancer as well as poorly differentiated forms of gastric cancer, which are typically associated with a higher risk of carcinoma infiltration and metastases, characteristically show, among others, a loss of E-cadherin and catenin in up to 50 % of cases (cf. Ming 1998). This might be partially due to TGF-β signalling via Smad3, which may induce E-cadherin suppressors and promote translocation of β-catenin into the nucleus (Cano et al. 2000; Carver et al. 2001; Peinado et al. 2003). Furthermore, characteristic mutations leading to hyper-activity of β-catenin, for instance of the *adenomatous polyposis coli* (*APC*) gene, are found in a wide range of gastrointestinal tumour entities (White et al. 2012; Krausova & Korinek 2014). The disruption of E-cadherin/β-catenin complexes at the plasma membrane by NRAGE followed by an increase in β-catenin-induced transcriptional activity (Xue et al. 2005) might then further contribute to a loss of proper intercellular connections and an increase in β-catenin activity. In this context, however, Praja1 would appear to counteract cancerogenic TGF-β as well as NRAGE effects rather than exerting a pro-oncogenic influence. Interestingly, activation of Smad4 – also by TGF-β – has been observed to increase E-cadherin levels and induce subsequent α- and β-catenin recruitment to the cell membrane, thus strengthening cell-to-cell adhesions (Müller et al. 2002).

When taking into account the general capability of Praja1 to disrupt ELF-mediated TGF-β signalling and promote cell proliferation (Mishra et al. 2005; Saha et al. 2006), there is good reason to postulate a tumourigenic role of Praja1 through impairing TGF-β signalling. At the same time, disruption of TGF-β signalling together with degradation of NRAGE by Praja1 might exert tumour-suppressing effects by indirectly stabilizing cell-to-cell adhesions and decreasing β-catenin-induced transcriptional activity. Praja1 might hence play a Janus-faced role in the course of tumour development, possibly depending on the conjuncture of critical pro- and proto-oncogenic mutations as well as their ability to shift intracellular signalling cascade interplay for acting as either a tumour promoter or tumour suppressor.

Although current findings on the involvement of Praja1 in tumour development are almost exclusively linked to gastrointestinal tumours, it appears valid to assume that at least some of the interactions and modulations of signalling cascades might also be involved in the pathogenesis of neural and neuronal tumours.

5.5.3 Potential influences of Praja1 on behaviour and cognition

To date, not much is known about the influence of Praja1 on behaviour and cognition. Yet, fair assumptions may be drawn from what has been established on Praja1 and its putative targets.

First, the afore-mentioned up-regulation of Praja1 in the BLA following fear conditioning (Stork et al. 2001) in conjunction with the presented inhibition of neurite outgrowth by Praja1 and its potential effects in mediating cytoskeletal re-arrangement, which is necessary during synaptic re-organization, strongly imply a role of Praja1 during memory consolidation. This hypothesis is currently investigated by according behavioural experiments following virally induced knock-down of Praja1 *in vivo* as well as by adequate Praja1 manipulation in primary neuronal cultures.

Furthermore, there is strong evidence for assuming a distinct role of neurotrophin signalling in anxiety behaviour and consolidation of fear memory. Stress or fear, for instance, have been found to increase NGF levels in the amygdala, thus modulating behavioural responses in various ways (Rattiner, Davis & Ressler 2004; Rattiner, Davis, French, et al. 2004; Yee et al. 2007; cf. Cirulli & Alleva 2009; cf. Gioiosa et al. 2009; cf. Alleva & Francia 2009; Lakshminarasimhan & Chattarji 2012). Based on the observation that NGF blood serum levels are increased during the experience of psycho-social stress as well as in the course of positive social bonding, it has been proposed that NGF might serve to compensate for at least some of the negative effects in stressful situations (cf. Cirulli & Alleva 2009; cf. Alleva & Francia 2009; cf. Gioiosa et al. 2009), which might also account for the fact that increasing NGF levels may indicate a positive response during cognitive behavioural therapy in patients with generalized anxiety disorder (Jockers-Scherübl et al. 2007). This is further supported by findings of decreased NGF levels in different brain regions during long-term depression, associated with neuronal loss and decreased neurogenesis in animal models of depression (cf. Gioiosa et al. 2009) and normalized through pharmacotherapy with antidepressants (Hellweg et al. 2002; cf. Gioiosa et al. 2009). In addition, NGF has been found to directly exert anti-depressant effects through shifting the general network activity by down-regulating the expression of certain receptors pivotal for neurotransmission, for instance receptors for GABA, dopamine, serotonin, somatostatin, or glycine (McGeary et al. 2011).

An influence of Praja1 on these neurotrophin-dependent behavioural effects would most likely be mediated through its regulation of NRAGE. Indeed, NRAGE-deficient mice have not only been reported to show symptoms resembling key elements of Prader-Willi-Syndrome (see above), but they also exhibit behavioural features typical of depression and autism: reduced social interaction and memory performance, increased levels of self-grooming and anxiety as well as deficient sexual behaviour. Concurrently, alleviation of these alterations

in behaviour could be achieved by administration of tricyclic antidepressants, oxytocin, or SSRI (Dombret et al. 2012; Mouri et al. 2012).

Moreover, β-catenin has been implicated in fear behaviour and memory consolidation (Maguschak & Ressler 2008; Maguschak & Ressler 2011), as mentioned before. The influence of NRAGE on the stability of intercellular adhesions and its capability of inducing downstream canonical Wnt signalling by stabilizing β-catenin may thus provide an additional mechanism of NRAGE-mediated alterations in cellular re-organization processes, such as during learning processes and memory formation.

Based on this brief over-view on potential influences of Praja1 on some of the pathways contributing to fear and anxiety behaviour as well as to memory formation, one could even speculate, whether Praja1 might contribute to the aetiology of post-traumatic stress disorder (PTSD). This severe anxiety disorder may develop after exposure to extreme traumatic stressors and is often associated with a feeling of helplessness or lack of control. Apart from anxiety and exaggerated startle, PTSD is characterized by dissociative symptoms, flashbacks, sleep disturbances, deficits in concentration as well as vegetative symptoms like increased heart rates (cf. Foa et al. 2006; cf. Javidi & Yadollahie 2012; cf. Pitman et al. 2012). Although more than 50 % of the population experience at least one traumatic event, life-time prevalence of PTSD is ranging between 0.3 % and 7.8 % only (Kessler et al. 1995; cf. Javidi & Yadollahie 2012). Certain risk factors have been discussed, among those female gender and younger age (cf. Javidi & Yadollahie 2012; cf. Pitman et al. 2012), experience of childhood abuse, leading to difficulties in emotional regulation (Stevens et al. 2013), or maternal inexperience, as seen in experiments with certain murine strains (Dahlhoff et al. 2010). Furthermore, epigenetic influences and polygenic inheritance patterns have been investigated, but current findings are rather indecisive and often unspecific for PTSD (cf. Pitman et al. 2012). Hence, the mechanisms, which contribute to the consolidation of acute post-traumatic symptoms and the development of PTSD in some people, but not in others remain largely unknown.

Yet, the conception that PTSD appears to be a consequence of inefficient extinction of traumatic mnemic contents has led to identifying anatomical and functional changes in PTSD patients and animal models that affect several brain regions commonly associated with memory consolidation and fear behaviour: hippocampus, amygdala, and ventromedial prefrontal cortex (cf. Pitman et al. 2012).

Moreover, the pathognomonic hyper-reactivity of the sympathetic nervous system appears to rely on an imbalance of the hypothalamus–pituitary–adrenal (HPA) axis via increased levels of the anxiogenic corticotropin-releasing hormone (CRH) as well as a stress-induced reduction of NPY, which may inhibit the release of noradrenalin from the adrenal medulla (Maes et al. 1999; Rasmusson et al. 2000; Cohen et al. 2012; cf. Pitman et al. 2012). In

recent years, evidence suggesting that systemic changes of NGF levels are related to an interaction with the HPA axis has been accumulating. NGF expression has been described to depend on glucocorticoid hormone levels (cf. Cirulli & Alleva 2009). In return, NGF has been found to increase glucocorticoid secretion (cf. Cirulli & Alleva 2009; cf. Gioiosa et al. 2009). In agreement with this, traumatic events early in life have been shown to be capable of causing a lasting dysbalance in the HPA axis (cf. Alleva & Francia 2009), while alterations of brain development as caused by juvenile stress appear to raise susceptibility to mood or anxiety disorders (Agid et al. 1999; cf. Cirulli & Alleva 2009).

Furthermore, increased β-catenin degradation in the hippocampus as well as stabilization of β-catenin in the BLA have been linked to vulnerability to PTSD in certain murine strains, which have also shown altered activity levels of other Wnt signalling pathway components (Dahlhoff et al. 2010).

Although a coherent picture of a potential Praja1 involvement in PTSD pathogenesis is still to be drawn, Praja1 does appear to contribute to all signalling cascades, which have been implicated to contribute to the development of PTSD. Praja1 has been shown to mediate NRAGE-dependent neurotrophin signalling, which seems to be key in the aetiology of PTSD. Praja1 has also been found to induce NPY expression in adrenal medulla PC12 cells, while a reduction of NPY levels in the adrenal medulla has been observed in PTSD patients. Finally, by altering NRAGE levels Praja1 might even be capable of counteracting some of the aberrant β-catenin activity that has been associated with vulnerability to PTSD. Most likely, there will not be a general up- or down-regulation of Praja1 in PTSD, but there might be alterations of Praja1 activity specific to certain cell types, tissues, or brain regions that could contribute to PTSD-associated changes one way or another.

6. Conclusion

Over the past decade, ubiquitination has been increasingly appreciated as a process that does not only regulate protein half-life, but also intracellular trafficking and signalling pathway activity by controlling intracellular homeostasis on many levels. This growing awareness of ubiquitination was paralleled and further promoted by discoveries, which have put enzymes mediating ubiquitination processes in key positions with regard to the aetiology of a wide range of pathologies.

Despite an increased interest, however, most enzymes playing a role during ubiquitination are not well investigated to date. Among those is the E3 ubiquitin ligase Praja1, which has first been described in the context of liver development and has later been implicated to be involved in the development of gastrointestinal tumours. Due to its sequence similarity to Neurodap1, which had already been known to play a role during the development of the CNS, its abundant expression in brain tissue, and its up-regulation in the BLA after fear conditioning, Praja1 has also been proposed to be of relevance to neuronal development.

For addressing this potential function of Praja1 in neuronal tissue, the work at hand has investigated the effects of Praja1 on NGF-induced neuronal-like differentiation in PC12 cells. As could be demonstrated, the expression of endogenous *praja1* is indeed induced during stimulation of PC12 cells with NGF. Moreover, Praja1 over-expression has been observed to drastically suppress NGF-induced neurite outgrowth in PC12 cells, most likely through poly-ubiquitination and subsequent degradation of the signalling molecule NRAGE, which is involved in BMP signalling as well as in NGF signalling via $p75^{NTR}$ and TrkA. Although integrin-dependent cell adhesion may have some modulatory influence, these Praja1 effects have been found to be largely independent of extracellular substrates. Furthermore, in contrast to previous findings in non-neuronal cell lines, Praja1 has not induced apoptosis in PC12 cells. It rather appears as if Praja1 would shift PC12 cells to a secretory phenotype, as indicated by an elevated expression of according markers. Taken together, the findings of this study strongly suggest that at least some of the functional effects of Praja1 depend on a cell's proteome composition as well as receptor repertoire. In this case, similar or identical molecular interactions such as the degradation of NRAGE following ubiquitination by Praja1 could induce diverging physiologic effects in different tissues and cell populations.

Previous work has identified two different Praja1 isoforms, Praja1.1 and Praja1.2, resulting from alternative splicing events. Cells transfected with *praja1.1* constructs over-express both, Praja1.1 and Praja1.2, in a stoichiometry similar to the endogenous expression in the adult brain as well as in naïve PC12 cells, whereas cells transfected with *praja1.2* generated only Praja1.2. The latter might play a distinct role during development due to the dynamics of its expression. However, the evidence presented here suggests a generally

comparable function of both Praja1 isoforms. Nonetheless, few subtle distinctions could be observed. It seems as if Praja1.2 would be less capable of translocating into the nucleus than Praja1.1, induced slightly less cell spreading, but could promote cell survival under NGF-deprived conditions.

In conclusion, this work provides first evidence for the concept of Praja1 being a mediator in neuronal differentiation. Taking into account the pathways that Praja1 seems to modulate through its activity, there is good reason to assume that this ubiquitin ligase might be of importance for proper formation and later re-organization of the CNS, especially with regard to the GABAergic system, and that it could influence learning processes as well as memory consolidation. In consequence, Praja1 might be a relevant factor for the aetiology of various pathologies, in particular genetic syndromes (e. g. X-linked mental retardation syndromes, Prader-Willi-Syndrome, or Rett Syndrome) as well as anxiety disorders and PTSD, but also neoplastic transformation.

7. References

1. Adams, C.L. & Nelson, W.J., 1998. Cytomechanics of cadherin-mediated cell-cell adhesion. *Current Opinion in Cell Biology*, 10(5), pp.572–7.
2. Afzal, A.R. & Jeffery, S., 2003. One gene, two phenotypes: ROR2 mutations in autosomal recessive Robinow syndrome and autosomal dominant brachydactyly type B. *Human Mutation*, 22(1), pp.1–11.
3. Agid, O. et al., 1999. Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. *Behavioural Pharmacology*, 4(2), pp.163–72.
4. Al-Shawi, R. et al., 2001. Expression of the Ror1 and Ror2 receptor tyrosine kinase genes during mouse development. *Development Genes and Evolution*, 211(4), pp.161–171.
5. Aletta, J.M. et al., 1988. Nerve growth factor regulates both the phosphorylation and steady-state levels of microtubule-associated protein 1.2 (MAP1.2). *The Journal of Cell Biology*, 106(5), pp.1573–81.
6. Allen, J.M. et al., 1984. Neuropeptide Y (NPY) in PC12 phaeochromocytoma cultures: responses to dexamethasone and nerve growth factor. *Neuroscience Letters*, 46(3), pp.291–6.
7. Allen, J.M., Martin, J.B. & Heinrich, G., 1987. Neuropeptide Y gene expression in PC12 cells and its regulation by nerve growth factor: a model for developmental regulation. *Brain Research*, 427(1), pp.39–43.
8. Alleva, E. & Francia, N., 2009. Psychiatric vulnerability: suggestions from animal models and role of neurotrophins. *Neuroscience and Biobehavioral Reviews*, 33(4), pp.525–36.
9. Aloe, L. et al., 1994. Nerve growth factor and autoimmune diseases. *Autoimmunity*, 19(2), pp.141–50.
10. Altar, C.A. et al., 1991. Recombinant human nerve growth factor is biologically active and labels novel high-affinity binding sites in rat brain. *Proceedings of the National Academy of Sciences*, 88(1), pp.281–5.
11. Althini, S. et al., 2003. Bone morphogenetic protein signalling in NGF-stimulated PC12 cells. *Biochemical and Biophysical Research Communications*, 307(3), pp.632–639.
12. Alvarez Martinez, C.E. et al., 2002. Characterization of a Smad motif similar to Drosophila mad in the mouse Msx 1 promoter. *Biochemical and Biophysical Research Communications*, 291(3), pp.655–62.
13. Aravind, L. & Koonin, E.V., 2000. The U box is a modified RING finger - a common domain in ubiquitination. *Current Biology*, 10(4), pp.R132–4.
14. Arévalo, J.C. & Chao, M. V, 2005. Axonal growth: where neurotrophins meet Wnts. *Current Opinion in Cell Biology*, 17(2), pp.112–5.
15. Arévalo, J.C. & Wu, S.H., 2006. Neurotrophin signaling: many exciting surprises! *Cellular and Molecular Life Sciences*, 63(13), pp.1523–37.
16. Attiah, D.G., Kopher, R.A. & Desai, T.A., 2003. Characterization of PC12 cell proliferation and differentiation-stimulated by ECM adhesion proteins and neurotrophic factors. *Journal of Materials Science: Materials in Medicine*, 14(11), pp.1005–9.
17. Bai, L., Swayne, L.A. & Braun, J.E.A., 2007. The CSPalpha/G protein complex in PC12 cells. *Biochemical and Biophysical Research Communications*, 352(1), pp.123–9.
18. Balbi, D. & Allen, J.M., 1994. Role of protein kinase C in mediating NGF effect on neuropeptide Y expression in PC12 cells. *Molecular Brain Research*, 23(4), pp.310–6.

19. Baldwin, T.J. et al., 1996. Elucidation of the molecular actions of NCAM and structurally related cell adhesion molecules. *Journal of Cellular Biochemistry*, 61(4), pp.502–13.
20. Barrett, G.L. et al., 2005. Co-expression of the P75 neurotrophin receptor and neurotrophin receptor-interacting melanoma antigen homolog in the mature rat brain. *Neuroscience*, 133(2), pp.381–92.
21. Beaujean, D. et al., 2003. Combinatorial code of growth factors and neuropeptides define neuroendocrine differentiation in PC12 cells. *Experimental Neurology*, 184(1), pp.348–58.
22. Berninger, B. et al., 1995. GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro. *Development*, 121(8), pp.2327–35.
23. Berry, A. et al., 2010. Conjunctivally administered NGF antibody reduces pain sensitivity and anxiety-like behavioral responses in aged female mice. *Behavioural Brain Research*, 210(2), pp.284–7.
24. Binato, R. et al., 2006. SMAD 8 binding to mice Msx1 basal promoter is required for transcriptional activation. *The Biochemical Journal*, 393(Pt 1), pp.141–50.
25. Bock, E. et al., 1985. Demonstration of immunochemical identity between the nerve growth factor-inducible large external (NILE) glycoprotein and the cell adhesion molecule L1. *The EMBO Journal*, 4(11), pp.2765–8.
26. Boersma, M.C.H. et al., 2011. A requirement for nuclear factor-kappaB in developmental and plasticity-associated synaptogenesis. *The Journal of Neuroscience*, 31(14), pp.5414–25.
27. Bouron, A., Becker, C. & Porzig, H., 1999. Functional expression of voltage-gated Na+ and Ca2+ channels during neuronal differentiation of PC12 cells with nerve growth factor or forskolin. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 359(5), pp.370–7.
28. Brann, A.B. et al., 2002. Nerve growth factor-induced p75-mediated death of cultured hippocampal neurons is age-dependent and transduced through ceramide generated by neutral sphingomyelinase. *The Journal of Biological Chemistry*, 277(12), pp.9812–8.
29. Brighton, C.T. & Albelda, S.M., 1992. Identification of integrin cell-substratum adhesion receptors on cultured rat bone cells. *Journal of Orthopaedic Research*, 10(6), pp.766–73.
30. Bronfman, F.C. et al., 2003. Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. *The Journal of Neuroscience*, 23(8), pp.3209–20.
31. Buck, C.R. et al., 1988. Differential expression of the nerve growth factor receptor gene in multiple brain areas. *Brain Research. Developmental Brain Research*, 44(2), pp.259–68.
32. Bui, N.T. et al., 2002. p75 neurotrophin receptor is required for constitutive and NGF-induced survival signalling in PC12 cells and rat hippocampal neurones. *Journal of Neurochemistry*, 81(3), pp.594–605.
33. Burgoyne, R.D., 1996. Identification of a Novel Cysteine String Protein Variant and Expression of Cysteine String Proteins in Non-neuronal Cells. *Journal of Biological Chemistry*, 271(13), pp.7320–7323.
34. Bush, A.B. et al., 1991. Nerve growth factor potentiates bradykinin-induced calcium influx and release in PC12 cells. *Journal of Neurochemistry*, 57(2), pp.562–74.
35. Cailliez, F. & Lavery, R., 2006. Dynamics and stability of E-cadherin dimers. *Biophysical Journal*, 91(11), pp.3964–71.
36. Cano, A. et al., 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature Cell Biology*, 2(2), pp.76–83.
37. Cartegni, L., 2003. ESEfinder: a web resource to identify exonic splicing enhancers. *Nucleic Acids Research*, 31(13), pp.3568–3571.

38. Carver, E.A. et al., 2001. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Molecular and Cellular Biology*, 21(23), pp.8184–8.

39. Catimel, B. et al., 2006. In situ phosphorylation of immobilized receptors on biosensor surfaces: application to E-cadherin/beta-catenin interactions. *Analytical Biochemistry*, 357(2), pp.277–88.

40. Chamberlain, L.H. & Burgoyne, R.D., 1998. Cysteine string protein functions directly in regulated exocytosis. *Molecular Biology of the Cell*, 9(8), pp.2259–67.

41. Chen, G., Deng, C. & Li, Y.-P., 2012. TGF-β and BMP signaling in osteoblast differentiation and bone formation. *International Journal of Biological Sciences*, 8(2), pp.272–88.

42. Cirulli, F. & Alleva, E., 2009. The NGF saga: from animal models of psychosocial stress to stress-related psychopathology. *Frontiers in Neuroendocrinology*, 30(3), pp.379–95.

43. Cohen, H. et al., 2012. The neuropeptide Y (NPY)-ergic system is associated with behavioral resilience to stress exposure in an animal model of post-traumatic stress disorder. *Neuropsychopharmacology*, 37(2), pp.350–63.

44. Cordeiro, M.L., Umbach, J.A. & Gundersen, C.B., 2000a. Lithium ions enhance cysteine string protein gene expression in vivo and in vitro. *Journal of Neurochemistry*, 74(6), pp.2365–72.

45. Cordeiro, M.L., Umbach, J.A. & Gundersen, C.B., 2000b. Lithium ions up-regulate mRNAs encoding dense-core vesicle proteins in nerve growth factor-differentiated PC12 cells. *Journal of Neurochemistry*, 75(6), pp.2622–5.

46. Coscoy, L. & Ganem, D., 2003. PHD domains and E3 ubiquitin ligases: viruses make the connection. *Trends in Cell Biology*, 13(1), pp.7–12.

47. Crossthwaite, A.J., Valli, H. & Williams, R.J., 2004. Inhibiting Src family tyrosine kinase activity blocks glutamate signalling to ERK1/2 and Akt/PKB but not JNK in cultured striatal neurones. *Journal of Neurochemistry*, 88(5), pp.1127–1139.

48. Culmsee, C. et al., 2002. Nerve growth factor survival signaling in cultured hippocampal neurons is mediated through TrkA and requires the common neurotrophin receptor P75. *Neuroscience*, 115(4), pp.1089–108.

49. Dahlhoff, M. et al., 2010. AKT/GSK-3beta/beta-catenin signalling within hippocampus and amygdala reflects genetically determined differences in posttraumatic stress disorder like symptoms. *Neuroscience*, 169(3), pp.1216–26.

50. Diarra, A. et al., 2009. Signaling of the neurotrophin receptor p75 in relation to Alzheimer's disease. *Biochemical and Biophysical Research Communications*, 390(3), pp.352–6.

51. DiMaggio, D.A., Farah, J.M. & Westfall, T.C., 1994. Effects of differentiation on neuropeptide-Y receptors and responses in rat pheochromocytoma cells. *Endocrinology*, 134(2), pp.719–27.

52. Diolaiti, D. et al., 2007. Functional cooperation between TrkA and p75(NTR) accelerates neuronal differentiation by increased transcription of GAP-43 and p21(CIP/WAF) genes via ERK1/2 and AP-1 activities. *Experimental Cell Research*, 313(14), pp.2980–92.

53. Dixon, C.E. et al., 1997. Nerve growth factor attenuates cholinergic deficits following traumatic brain injury in rats. *Experimental Neurology*, 146(2), pp.479–90.

54. Doherty, P., Mann, D. a & Walsh, F.S., 1988. Comparison of the effects of NGF, activators of protein kinase C, and a calcium ionophore on the expression of Thy-1 and N-CAM in PC12 cell cultures. *The Journal of Cell Biology*, 107(1), pp.333–40.

55. Doherty, P., Mann, D.A. & Walsh, F.S., 1987. Cholera toxin and dibutyryl cyclic AMP inhibit the expression of neurofilament protein induced by nerve growth factor in cultures of naive and primed PC12 cells. *Journal of Neurochemistry*, 49(6), pp.1676–87.

56. Dombret, C. et al., 2012. Loss of Maged1 results in obesity, deficits of social interactions, impaired sexual behavior and severe alteration of mature oxytocin production in the hypothalamus. *Human Molecular Genetics*, 21(21), pp.4703–17.

57. Doyle, J.M. et al., 2010. MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. *Molecular Cell*, 39(6), pp.963–74.

58. Drees, F. et al., 2005. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell*, 123(5), pp.903–15.

59. Eaton, B.A. & Davis, G.W., 2005. LIM Kinase1 controls synaptic stability downstream of the type II BMP receptor. *Neuron*, 47(5), pp.695–708.

60. Ehlers, M.D., 2003. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nature Neuroscience*, 6(3), pp.231–42.

61. Endo, M. et al., 2003. Control of growth cone motility and morphology by LIM kinase and Slingshot via phosphorylation and dephosphorylation of cofilin. *The Journal of Neuroscience*, 23(7), pp.2527–37.

62. Evans, G.J. et al., 2001. Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis. *The Journal of Biological Chemistry*, 276(51), pp.47877–85.

63. Fairbrother, W.G. et al., 2002. Predictive identification of exonic splicing enhancers in human genes. *Science*, 297(5583), pp.1007–13.

64. Feng, Z. et al., 2010. NRAGE is a negative regulator of nerve growth factor-stimulated neurite outgrowth in PC12 cells mediated through TrkA-ERK signaling. *Journal of Neuroscience Research*, 88(8), pp.1822–8.

65. Foa, E.B., Stein, D.J. & McFarlane, A.C., 2006. Symptomatology and psychopathology of mental health problems after disaster. *The Journal of Clinical Psychiatry*, 67 Suppl 2, pp.15–25.

66. Friedman, W.J., 2000. Neurotrophins induce death of hippocampal neurons via the p75 receptor. *The Journal of Neuroscience*, 20(17), pp.6340–6.

67. Fujii, D.K. et al., 1982. Neurite outgrowth and protein synthesis by PC12 cells as a function of substratum and nerve growth factor. *The Journal of Neuroscience*, 2(8), pp.1157–75.

68. Fung, S. et al., 2009. Drosophila E-cadherin and its binding partner Armadillo/ beta-catenin are required for axonal pathway choices in the developing larval brain. *Developmental Biology*, 332(2), pp.371–82.

69. Gall, C.M. & Isackson, P.J., 1989. Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. *Science*, 245(4919), pp.758–61.

70. Geetha, T. et al., 2012. Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival. *Biochemical and Biophysical Research Communications*, 421(2), pp.286–90.

71. Geetha, T., Jiang, J. & Wooten, M.W., 2005. Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Molecular Cell*, 20(2), pp.301–12.

72. Gioiosa, L., Iannitelli, A. & Aloe, L., 2009. Stress, anxiety schizophrenia and neurotrophic factors: the pioneer studies with nerve growth factor. *Rivista di Psichiatria*, 44(2), pp.88–94.

73. Glickman, M.H. & Ciechanover, A., 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiological Reviews*, 82(2), pp.373–428.

74. Goethe, J.W. von, 2006. *Maximen und Reflexionen.* H. Koopmann, ed., München: C.H.Beck / Deutscher Taschenbuch Verlag. §525.

75. Grant, N.J. et al., 1996. Glucocorticoids and nerve growth factor differentially modulate cell adhesion molecule L1 expression in PC12 cells. *Journal of Neurochemistry*, 66(4), pp.1400–8.

76. Greene, L.A., 1978. Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *The Journal of Cell Biology*, 78(3), pp.747–55.

77. Greene, L.A., Liem, R.K. & Shelanski, M.L., 1983. Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. *The Journal of Cell Biology*, 96(1), pp.76–83.

78. Greene, L.A. & Tischler, A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of America*, 73(7), pp.2424–8.

79. Han, S.-U. et al., 2004. Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene*, 23(7), pp.1333–41.

80. Hattori, A. et al., 1999. Bone morphogenetic protein-2 promotes survival and differentiation of striatal GABAergic neurons in the absence of glial cell proliferation. *The Journal of Biological Chemistry*, 72(6), pp.2264–71.

81. Hellweg, R. et al., 2002. Subchronic treatment with lithium increases nerve growth factor content in distinct brain regions of adult rats. *Molecular Psychiatry*, 7(6), pp.604–8.

82. Hernández, F. et al., 2004. Testing the ubiquitin-proteasome hypothesis of neurodegeneration in vivo. *Trends in Neurosciences*, 27(2), pp.66–9.

83. Higuchi, H., Nakano, K. & Miki, N., 1992. Identification of NGF-response element in the rat neuropeptide Y gene and induction of the binding proteins. *Biochemical and Biophysical Research Communications*, 189(3), pp.1553–60.

84. Hoard, J.L. et al., 2008. Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norepinephrine transporters, and the neurotrophin receptors tropomyosin-related kinase A and p75. *Neuroscience*, 156(1), pp.129–42.

85. Horike, S. et al., 2005. Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nature Genetics*, 37(1), pp.31–40.

86. Horton, P. et al., 2007. WoLF PSORT: protein localization predictor. *Nucleic Acids Research*, 35(Web Server issue), pp.W585–7.

87. Hussein, S.M., Duff, E.K. & Sirard, C., 2003. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. *The Journal of Biological Chemistry*, 278(49), pp.48805–14.

88. Iacaruso, M.F. et al., 2011. Structural model for p75(NTR)-TrkA intracellular domain interaction: a combined FRET and bioinformatics study. *Journal of Molecular Biology*, 414(5), pp.681–98.

89. Isshiki, T., Takeichi, M. & Nose, A., 1997. The role of the msh homeobox gene during Drosophila neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development*, 124(16), pp.3099–109.

90. Ito, H., Nomoto, H. & Furukawa, S., 2002. Role of low-affinity p75 receptor in nerve growth factor-inducible growth arrest of PC12 cells. *Journal of Neuroscience Research*, 69(5), pp.653–61.

91. Itoh, K., Brackenbury, R. & Akeson, R.A., 1995. Induction of L1 mRNA in PC12 cells by NGF is modulated by cell-cell contact and does not require the high-affinity NGF receptor. *The Journal of Neuroscience*, 15(3 Pt 2), pp.2504–12.

92. Iwasaki, S. et al., 1996. Characterization of the bone morphogenetic protein-2 as a neurotrophic factor. Induction of neuronal differentiation of PC12 cells in the absence of mitogen-activated protein kinase activation. *The Journal of Biological Chemistry*, 271(29), pp.17360–5.

93. Iwasaki, S. et al., 1999. Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. *The Journal of Biological Chemistry*, 274(37), pp.26503–10.

94. Jabs, E.W. et al., 1993. A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. *Cell*, 75(3), pp.443–50.

95. Jarome, T.J. et al., 2011. Activity dependent protein degradation is critical for the formation and stability of fear memory in the amygdala. E. M. C. Skoulakis, ed. *PLoS ONE*, 6(9), p.e24349.

96. Javidi, H. & Yadollahie, M., 2012. Post-traumatic Stress Disorder. *The International Journal of Occupational and Environmental Medicine*, 3(1), pp.2–9.

97. Jockers-Scherübl, M.C. et al., 2007. Nerve growth factor serum concentrations rise after successful cognitive-behavioural therapy of generalized anxiety disorder. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 31(1), pp.200–4.

98. Johnston, J.A. & Madura, K., 2004. Rings, chains and ladders: ubiquitin goes to work in the neuron. *Progress in Neurobiology*, 73(4), pp.227–57.

99. Jordan, B.W. et al., 2001. Neurotrophin receptor-interacting mage homologue is an inducible inhibitor of apoptosis protein-interacting protein that augments cell death. *The Journal of Biological Chemistry*, 276(43), pp.39985–9.

100. Kang, H. & Schuman, E.M., 1996. A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science*, 273(5280), pp.1402–6.

101. Kaplan, D.R. & Miller, F.D., 2000. Neurotrophin signal transduction in the nervous system. *Current Opinion in Neurobiology*, 10(3), pp.381–91.

102. Kaplan, D.R. & Stephens, R.M., 1994. Neurotrophin signal transduction by the Trk receptor. *Journal of Neurobiology*, 25(11), pp.1404–17.

103. Kendall, S.E. et al., 2005. NRAGE mediates p38 activation and neural progenitor apoptosis via the bone morphogenetic protein signaling cascade. *Molecular and Cellular Biology*, 25(17), pp.7711–24.

104. Kessler, R.C. et al., 1995. Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of General Psychiatry*, 52(12), pp.1048–60.

105. Kishino, T., Lalande, M. & Wagstaff, J., 1997. UBE3A/E6-AP mutations cause Angelman syndrome. *Nature Genetics*, 15(1), pp.70–3.

106. Kiss, J., McGovern, J. & Patel, A.J., 1988. Immunohistochemical localization of cells containing nerve growth factor receptors in the different regions of the adult rat forebrain. *Neuroscience*, 27(3), pp.731–48.

107. Kitada, T. et al., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, 392(6676), pp.605–8.

108. Koegl, M. et al., 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell*, 96(5), pp.635–44.

109. Korsching, S. et al., 1985. Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *The EMBO Journal*, 4(6), pp.1389–93.

110. Kraus, S. et al., 2003. c-Src is activated by the epidermal growth factor receptor in a pathway that mediates JNK and ERK activation by gonadotropin-releasing hormone in COS7 cells. *The Journal of Biological Chemistry*, 278(35), pp.32618–30.

111. Krausova, M. & Korinek, V., 2014. Wnt signaling in adult intestinal stem cells and cancer. *Cellular Signalling*, 26(3), pp.570–579.

112. Kretzschmar, M., Doody, J. & Massagué, J., 1997. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature*, 389(6651), pp.618–22.

113. Kuwajima, T. et al., 2004. Necdin interacts with the Msx2 homeodomain protein via MAGE-D1 to promote myogenic differentiation of C2C12 cells. *The Journal of Biological Chemistry*, 279(39), pp.40484–93.

114. Kuwajima, T., Nishimura, I. & Yoshikawa, K., 2006. Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins. *The Journal of Neuroscience*, 26(20), pp.5383–92.

115. Kuwako, K., Taniura, H. & Yoshikawa, K., 2004. Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor. *The Journal of Biological Chemistry*, 279(3), pp.1703–12.

116. Lai, S.-S. et al., 2012. Ror2-Src signaling in metastasis of mouse melanoma cells is inhibited by NRAGE. *Cancer Genetics*, 205(11), pp.552–62.

117. Lakshminarasimhan, H. & Chattarji, S., 2012. Stress leads to contrasting effects on the levels of brain derived neurotrophic factor in the hippocampus and amygdala. *PLoS ONE*, 7(1), p.e30481.

118. Lander, A.D., Fujii, D.K. & Reichardt, L.F., 1985a. Laminin is associated with the “neurite outgrowth-promoting factors” found in conditioned media. *Proceedings of the National Academy of Sciences of the United States of America*, 82(7), pp.2183–7.

119. Lander, A.D., Fujii, D.K. & Reichardt, L.F., 1985b. Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. *The Journal of Cell Biology*, 101(3), pp.898–913.

120. Large, T.H. et al., 1986. Nerve growth factor gene expression in the developing rat brain. *Science*, 234(4774), pp.352–5.

121. Laser, H. et al., 2003. Proteasome inhibition arrests neurite outgrowth and causes “dying-back” degeneration in primary culture. *Journal of Neuroscience Research*, 74(6), pp.906–16.

122. Lee, S.-H. et al., 2008. Synaptic protein degradation underlies destabilization of retrieved fear memory. *Science*, 319(5867), pp.1253–6.

123. Lee, V., Shelanski, M.L. & Greene, L.A., 1977. Specific neural and adrenal medullary antigens detected by antisera to clonal PC12 pheochromocytoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, 74(11), pp.5021–5.

124. Lee, V.M., Greene, L. & Shelanski, M.L., 1981. Identification of neural and adrenal medullary surface membrane glycoproteins recognized by antisera to cultured rat sympathetic neurons and PC12 pheochromocytoma cells. *Neuroscience*, 6(12), pp.2773–86.

125. Lee-Hoeflich, S.T. et al., 2004. Activation of LIMK1 by binding to the BMP receptor, BMPRII, regulates BMP-dependent dendritogenesis. *The EMBO Journal*, 23(24), pp.4792–801.

126. Lindholm, D. et al., 1994. Brain-derived neurotrophic factor and neurotrophin-4 increase neurotrophin-3 expression in the rat hippocampus. *International Journal of Developmental Neuroscience*, 12(8), pp.745–51.

127. Liu, A. & Niswander, L.A., 2005. Bone morphogenetic protein signalling and vertebrate nervous system development. *Nature Reviews. Neuroscience*, 6(12), pp.945–54.

128. Liu, H.X. et al., 2000. Exonic splicing enhancer motif recognized by human SC35 under splicing conditions. *Molecular and Cellular Biology*, 20(3), pp.1063–71.

129. Liu, Y., Ross, J.F., et al., 2007. Homodimerization of Ror2 tyrosine kinase receptor induces 14-3-3(beta) phosphorylation and promotes osteoblast differentiation and bone formation. *Molecular Endocrinology*, 21(12), pp.3050–61.

130. Liu, Y. et al., 1994. Preferential localization of a vesicular monoamine transporter to dense core vesicles in PC12 cells. *The Journal of Cell Biology*, 127(5), pp.1419–33.

131. Liu, Y., Bhat, R.A., et al., 2007. The orphan receptor tyrosine kinase Ror2 promotes osteoblast differentiation and enhances ex vivo bone formation. *Molecular Endocrinology*, 21(2), pp.376–87.

132. Liu, Y. et al., 2008. Wnt5a induces homodimerization and activation of Ror2 receptor tyrosine kinase. *Journal of Cellular Biochemistry*, 105(2), pp.497–502.

133. Liu, Y. & Edwards, R.H., 1997. Differential localization of vesicular acetylcholine and monoamine transporters in PC12 cells but not CHO cells. *The Journal of Cell Biology*, 139(4), pp.907–16.

134. Liu, Y., Helms, A.W. & Johnson, J.E., 2004. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. *Development*, 131(5), pp.1017–28.

135. Loch, C.M., Eddins, M.J. & Strickler, J.E., 2011. Protein microarrays for the identification of praja1 e3 ubiquitin ligase substrates. *Cell Biochemistry and Biophysics*, 60(1-2), pp.127–35.

136. Lönn, P. et al., 2005. BMP enhances transcriptional responses to NGF during PC12 cell differentiation. *Neurochemical Research*, 30(6-7), pp.753–65.

137. Lopez-Salon, M. et al., 2001. The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *The European Journal of Neuroscience*, 14(11), pp.1820–6.

138. Lorick, K.L. et al., 1999. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proceedings of the National Academy of Sciences*, 96(20), pp.11364–9.

139. Lowry, O.H. et al., 1951. Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, 193(1), pp.265–75.

140. Lu, B. et al., 1989. Expression of NGF and NGF receptor mRNAs in the developing brain: evidence for local delivery and action of NGF. *Experimental Neurology*, 104(3), pp.191–9.

141. Lutz, M. et al., 2004. Nerve growth factor mediates activation of the Smad pathway in PC12 cells. *European Journal of Biochemistry*, 271(5), pp.920–31.

142. Maes, M. et al., 1999. Serotonergic and noradrenergic markers of post-traumatic stress disorder with and without major depression. *Neuropsychopharmacology*, 20(2), pp.188–97.

143. Maguschak, K.A. & Ressler, K.J., 2008. Beta-catenin is required for memory consolidation. *Nature Neuroscience*, 11(11), pp.1319–26.

144. Maguschak, K.A. & Ressler, K.J., 2011. Wnt signaling in amygdala-dependent learning and memory. *The Journal of Neuroscience*, 31(37), pp.13057–67.

145. Mäkelä, J.A. et al., 2014. Reconstruction of mouse testicular cellular microenvironments in long-term seminiferous tubule culture. *PLoS ONE*, 9(3), p.e90088.

146. Mann, D.A., Doherty, P. & Walsh, F.S., 1989. Increased intracellular cyclic AMP differentially modulates nerve growth factor induction of three neuronal recognition molecules involved in neurite outgrowth. *Journal of Neurochemistry*, 53(5), pp.1581–8.

147. Masoudi, R. et al., 2009. Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. *The Journal of Biological Chemistry*, 284(27), pp.18424–33.

148. Masuda, Y. et al., 2001. Dlxin-1, a novel protein that binds Dlx5 and regulates its transcriptional function. *The Journal of Biological Chemistry*, 276(7), pp.5331–8.

149. Matsuda, T. et al., 2001. Expression of the receptor tyrosine kinase genes, Ror1 and Ror2, during mouse development. *Mechanisms of Development*, 105(1-2), pp.153–6.

150. Matsuda, T. et al., 2003. The receptor tyrosine kinase Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution. *The Journal of Biological Chemistry*, 278(31), pp.29057–64.

151. Matsuura, T. et al., 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nature Genetics*, 15(1), pp.74–7.

152. Matusica, D. et al., 2013. An intracellular domain fragment of the p75 neurotrophin receptor (p75(NTR)) enhances tropomyosin receptor kinase A (TrkA) receptor function. *The Journal of Biological Chemistry*, 288(16), pp.11144–54.

153. McCullough, L.A., Egan, T.M. & Westfall, T.C., 1998. Neuropeptide Y inhibition of calcium channels in PC-12 pheochromocytoma cells. *The American Journal of Physiology*, 274(5 Pt 1), pp.C1290–7.

154. McGeary, J.E. et al., 2011. Effects of nerve growth factor (NGF), fluoxetine, and amitriptyline on gene expression profiles in rat brain. *Neuropeptides*, 45(5), pp.317–22.

155. McGuire, J.C. & Greene, L.A., 1980. Stimulation by nerve growth factor of specific protein synthesis in rat PC12 pheochromocytoma cells. *Neuroscience*, 5(1), pp.179–89.

156. McGuire, J.C., Greene, L.A. & Furano, A. V, 1978. NGF stimulates incorporation of fucose or glucosamine into an external glycoprotein in cultured rat PC12 pheochromocytoma cells. *Cell*, 15(2), pp.357–65.

157. Meakin, S.O. et al., 1992. The rat trk protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 89(6), pp.2374–8.

158. Meakin, S.O. & Shooter, E.M., 1992. The nerve growth factor family of receptors. *Trends in Neurosciences*, 15(9), pp.323–31.

159. Mehta, H.M., Woo, S.B. & Neet, K.E., 2012. Comparison of nerve growth factor receptor binding models using heterodimeric muteins. *Journal of Neuroscience Research*, 90(12), pp.2259–71.

160. Meng, Y. et al., 2002. Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron*, 35(1), pp.121–33.

161. Meray, R.K. & Lansbury, P.T., 2007. Reversible monoubiquitination regulates the Parkinson disease-associated ubiquitin hydrolase UCH-L1. *The Journal of Biological Chemistry*, 282(14), pp.10567–75.

162. Mercerón-Martínez, D. et al., 2013. Hippocampal neurotrophins after stimulation of the basolateral amygdala, and memory improvement in lesioned rats. *Restorative Neurology and Neuroscience*, 31(2), pp.189–97.

163. Ming, S.-C., 1998. Cellular and molecular pathology of gastric carcinoma and precursor lesions: A critical review. *Gastric Cancer*, 1(1), pp.31–50.

164. Miranda, T.B. et al., 2009. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. *Molecular Cancer Therapeutics*, 8(6), pp.1579–88.

165. Mishra, L. et al., 1997. Praja1, a novel gene encoding a RING-H2 motif in mouse development. *Oncogene*, 15(19), pp.2361–8.

166. Mishra, L., Katuri, V. & Evans, S., 2005. The role of PRAJA and ELF in TGF-beta signaling and gastric cancer. *Cancer Biology & Therapy*, 4(7), pp.694–9.

167. Moises, H.C. et al., 1995. Nerve growth factor facilitates cholinergic neurotransmission between nucleus basalis and the amygdala in rat: an electrophysiological analysis. *The Journal of Neuroscience*, 15(12), pp.8131–42.

168. Moore, D.J., 2006. Parkin: a multifaceted ubiquitin ligase. *Biochemical Society Transactions*, 34(Pt 5), pp.749–53.

169. Mouri, A. et al., 2012. MAGE-D1 regulates expression of depression-like behavior through serotonin transporter ubiquitylation. *The Journal of Neuroscience*, 32(13), pp.4562–80.

170. Mouri, A. et al., 2013. The roles of MAGE-D1 in the neuronal functions and pathology of the central nervous system. *Reviews in the Neurosciences*, 24(1), pp.61–70.

171. Mukhopadhyay, D. & Riezman, H., 2007. Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science*, 315(5809), pp.201–5.

172. Müller, N. et al., 2002. Smad4 induces the tumor suppressor E-cadherin and P-cadherin in colon carcinoma cells. *Oncogene*, 21(39), pp.6049–58.

173. Murer, M.G., Yan, Q. & Raisman-Vozari, R., 2001. Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Progress in Neurobiology*, 63(1), pp.71–124.

174. Murphey, R.K. & Godenschwege, T.A., 2002. New roles for ubiquitin in the assembly and function of neuronal circuits. *Neuron*, 36(1), pp.5–8.

175. Nagafuchi, A. & Takeichi, M., 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *The EMBO Journal*, 7(12), pp.3679–84.

176. Nakai, K. & Horton, P., 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends in Biochemical Sciences*, 24(1), pp.34–6.

177. Nakayama, M. et al., 1995. A novel RING-H2 motif protein downregulated by axotomy: its characteristic localization at the postsynaptic density of axosomatic synapse. *The Journal of Neuroscience*, 15(7 Pt 2), pp.5238–48.

178. Niehrs, C., 2012. The complex world of WNT receptor signalling. *Nature Reviews. Molecular Cell Biology*, 13(12), pp.767–79.

179. Nishimura, R. et al., 2012. Regulation of bone and cartilage development by network between BMP signalling and transcription factors. *Journal of Biochemistry*, 151(3), pp.247–54.

180. Nishio, T., Akiguchi, I. & Furukawa, S., 1992. Detailed distribution of nerve growth factor in rat brain determined by a highly sensitive enzyme immunoassay. *Experimental Neurology*, 116(1), pp.76–84.

181. Nishita, M. et al., 2006. Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration. *The Journal of Cell Biology*, 175(4), pp.555–62.

182. Nykjaer, A., Willnow, T.E. & Petersen, C.M., 2005. p75NTR--live or let die. *Current Opinion in Neurobiology*, 15(1), pp.49–57.

183. O'Connell, M.P. et al., 2010. The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma. *Oncogene*, 29(1), pp.34–44.

184. Obin, M. et al., 1999. Neurite outgrowth in PC12 cells. Distinguishing the roles of ubiquitylation and ubiquitin-dependent proteolysis. *The Journal of Biological Chemistry*, 274(17), pp.11789–95.

185. Osterlund, C. et al., 2000. Mage-b4, a novel melanoma antigen (MAGE) gene specifically expressed during germ cell differentiation. *Cancer Research*, 60(4), pp.1054–61.

186. Peinado, H., Quintanilla, M. & Cano, A., 2003. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *The Journal of Biological Chemistry*, 278(23), pp.21113–23.

187. Perera, M. et al., 2004. Defective neuronogenesis in the absence of Dlx5. *Molecular and Cellular Neurosciences*, 25(1), pp.153–61.

188. Peter, D. et al., 1995. Differential expression of two vesicular monoamine transporters. *The Journal of Neuroscience*, 15(9), pp.6179–88.

189. Piek, E. et al., 2001. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *The Journal of Biological Chemistry*, 276(23), pp.19945–53.

190. Piiper, A. et al., 2002. Cyclic AMP induces transactivation of the receptors for epidermal growth factor and nerve growth factor, thereby modulating activation of MAP kinase, Akt, and neurite outgrowth in PC12 cells. *The Journal of Biological Chemistry*, 277(46), pp.43623–30.

191. Pitman, R.K. et al., 2012. Biological studies of post-traumatic stress disorder. *Nature Reviews Neuroscience*, 13(11), pp.769–87.

192. Pokutta, S. & Weis, W.I., 2000. Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Molecular Cell*, 5(3), pp.533–43.

193. Pothos, E.N. et al., 2000. Synaptic vesicle transporter expression regulates vesicle phenotype and quantal size. *The Journal of Neuroscience*, 20(19), pp.7297–306.

194. Powell, S.K. & Kleinman, H.K., 1997. Neuronal laminins and their cellular receptors. *The International Journal of Biochemistry & Cell Biology*, 29(3), pp.401–14.

195. Rajakumar, P.A., Westfall, T.C. & Devaskar, S.U., 1998. Neuropeptide Y gene expression in immortalized rat hippocampal and pheochromocytoma-12 cell lines. *Regulatory Peptides*, 73(2), pp.123–31.

196. Rasmusson, A.M. et al., 2000. Low baseline and yohimbine-stimulated plasma neuropeptide Y (NPY) levels in combat-related PTSD. *Biological Psychiatry*, 47(6), pp.526–39.

197. Rathjen, F.G. & Rutishauser, U., 1984. Comparison of two cell surface molecules involved in neural cell adhesion. *The EMBO Journal*, 3(2), pp.461–5.

198. Rathjen, F.G. & Schachner, M., 1984. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *The EMBO Journal*, 3(1), pp.1–10.

199. Rattiner, L.M., Davis, M., French, C.T., et al., 2004. Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. *The Journal of Neuroscience*, 24(20), pp.4796–806.

200. Rattiner, L.M., Davis, M. & Ressler, K.J., 2004. Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. *Learning & Memory*, 11(6), pp.727–31.

201. Reddi, A.H. & Huggins, C., 1972. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. *Proceedings of the National Academy of Sciences of the United States of America*, 69(6), pp.1601–5.

202. Reddy, E.M. et al., 2010. Dlxin-1, a MAGE family protein, induces accelerated neurite outgrowth and cell survival by enhanced and early activation of MEK and Akt signalling pathways in PC12 cells. *Experimental Cell Research*, 316(14), pp.2220–36.

203. Ren, D., Minami, Y. & Nishita, M., 2011. Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition. *Genes to Cells*, 16(3), pp.304–15.

204. Riccomagno, M.M., Takada, S. & Epstein, D.J., 2005. Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh. *Genes & Development*, 19(13), pp.1612–23.

205. Rimm, D.L. et al., 1995. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proceedings of the National Academy of Sciences of the United States of America*, 92(19), pp.8813–7.

206. Rudy, B. et al., 1987. Nerve growth factor increases the number of functional Na channels and induces TTX-resistant Na channels in PC12 pheochromocytoma cells. *The Journal of Neuroscience*, 7(6), pp.1613–25.

207. Rylett, R.J. et al., 1993. Acetylcholine synthesis and release following continuous intracerebral administration of NGF in adult and aged Fischer-344 rats. *The Journal of Neuroscience*, 13(9), pp.3956–63.

208. Sabol, S.L. & Higuchi, H., 1990. Transcriptional regulation of the neuropeptide Y gene by nerve growth factor: antagonism by glucocorticoids and potentiation by adenosine 3',5'-monophosphate and phorbol ester. *Molecular Endocrinology*, 4(3), pp.384–92.

209. Saha, T. et al., 2006. RING finger-dependent ubiquitination by PRAJA is dependent on TGF-beta and potentially defines the functional status of the tumor suppressor ELF. *Oncogene*, 25(5), pp.693–705.

210. Salehi, A.H. et al., 2000. NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. *Neuron*, 27(2), pp.279–88.

211. Salehi, A.H., Xanthoudakis, S. & Barker, P.A., 2002. NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway. *The Journal of Biological Chemistry*, 277(50), pp.48043–50.

212. Sasaki, A. et al., 2002. A RING finger protein Praja1 regulates Dlx5-dependent transcription through its ubiquitin ligase activity for the Dlx/Msx-interacting MAGE/Necdin family protein, Dlxin-1. *The Journal of Biological Chemistry*, 277(25), pp.22541–6.

213. Schindelin, J. et al., 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7), pp.676–82.

214. Schmittgen, T.D. & Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, 3(6), pp.1101–1108.

215. Shen, Z. et al., 2013. Downregulation of Ezh2 methyltransferase by FOXP3: new insight of FOXP3 into chromatin remodeling? *Biochimica et Biophysica Acta*, 1833(10), pp.2190–200.

216. Shenoy, S.K., 2007. Seven-transmembrane receptors and ubiquitination. *Circulation Research*, 100(8), pp.1142–54.

217. Shimode, H., Ueki, A. & Morita, Y., 2003. Nerve growth factor attenuates hippocampal cholinergic deficits and operant learning impairment in rats with entorhinal cortex lesions. *Behavioural Pharmacology*, 14(3), pp.179–90.

218. Stevens, N.R. et al., 2013. Emotion regulation difficulties, low social support, and interpersonal violence mediate the link between childhood abuse and posttraumatic stress symptoms. *Behavior Therapy*, 44(1), pp.152–61.

219. Stork, O. et al., 2001. Identification of genes expressed in the amygdala during the formation of fear memory. *Learning & Memory*, 8(4), pp.209–19.

220. Stork, O. et al., 2004. Neuronal functions of the novel serine/threonine kinase Ndr2. *The Journal of Biological Chemistry*, 279(44), pp.45773–81.

221. Stühmer, T. et al., 2002. Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. *Development*, 129(1), pp.245–52.

222. Takei, N. et al., 2001. Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes: comparison with the effects of insulin. *The Journal of Biological Chemistry*, 276(46), pp.42818–25.

223. Tang, Y. et al., 2003. Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. *Science*, 299(5606), pp.574–7.

224. Taylor, E.M. et al., 2008. Identification of the proteins, including MAGEG1, that make up the human SMC5-6 protein complex. *Molecular and Cellular Biology*, 28(4), pp.1197–206.

225. Tcherpakov, M. et al., 2002. The p75 neurotrophin receptor interacts with multiple MAGE proteins. *The Journal of Biological Chemistry*, 277(51), pp.49101–4.

226. Teng, K.K. & Greene, L.A., 1993. Depolarization maintains neurites and priming of PC12 cells after nerve growth factor withdrawal. *The Journal of Neuroscience*, 13(7), pp.3124–35.

227. Teuber, J. et al., 2013. The Ubiquitin Ligase Praja1 Reduces NRAGE Expression and Inhibits Neuronal Differentiation of PC12 Cells. B. Bardoni, ed. *PLoS ONE*, 8(5), p.e63067.

228. Tillinger, A. et al., 2010. Vesicular monoamine transporters (VMATs) in adrenal chromaffin cells: stress-triggered induction of VMAT2 and expression in epinephrine synthesizing cells. *Cellular and Molecular Neurobiology*, 30(8), pp.1459–65.

229. Toker, A. & Cantley, L.C., 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature*, 387(6634), pp.673–6.

230. Tomaselli, K.J. et al., 1990. A neuronal cell line (PC12) expresses two beta 1-class integrins-alpha 1 beta 1 and alpha 3 beta 1-that recognize different neurite outgrowth-promoting domains in laminin. *Neuron*, 5(5), pp.651–62.

231. Tomaselli, K.J., Damsky, C.H. & Reichardt, L.F., 1987. Interactions of a neuronal cell line (PC12) with laminin, collagen IV, and fibronectin: identification of integrin-related glycoproteins involved in attachment and process outgrowth. *The Journal of Cell Biology*, 105(5), pp.2347–58.

232. Tomaselli, K.J., Damsky, C.H. & Reichardt, L.F., 1988. Purification and characterization of mammalian integrins expressed by a rat neuronal cell line (PC12): evidence that they function as alpha/beta heterodimeric receptors for laminin and type IV collagen. *The Journal of Cell Biology*, 107(3), pp.1241–52.

233. Tomaselli, K.J., Reichardt, L.F. & Bixby, J.L., 1986. Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. *The Journal of Cell Biology*, 103(6 Pt 2), pp.2659–72.

234. Tyler, W.J. et al., 2002. From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learning & Memory*, 9(5), pp.224–37.

235. Urist, M.R., 1965. Bone: Formation by Autoinduction. *Science*, 150(3698), pp.893–9.

236. Walch-Solimena, C. et al., 1993. Synaptotagmin: a membrane constituent of neuropeptide-containing large dense-core vesicles. *The Journal of Neuroscience*, 13(9), pp.3895–903.

237. Webb, K. et al., 2001. Substrate-bound human recombinant L1 selectively promotes neuronal attachment and outgrowth in the presence of astrocytes and fibroblasts. *Biomaterials*, 22(10), pp.1017–28.

238. Wehrman, T. et al., 2007. Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron*, 53(1), pp.25–38.

239. Weihe, E. et al., 1994. Localization of vesicular monoamine transporter isoforms (VMAT1 and VMAT2) to endocrine cells and neurons in rat. *Journal of Molecular Neuroscience*, 5(3), pp.149–64.

240. Weihe, E. et al., 1996. Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles. *Proceedings of the National Academy of Sciences of the United States of America*, 93(8), pp.3547–52.

241. Wenk, G.L., 1997. Rett syndrome: neurobiological changes underlying specific symptoms. *Progress in Neurobiology*, 51(4), pp.383–91.

242. White, B.D., Chien, A.J. & Dawson, D.W., 2012. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. *Gastroenterology*, 142(2), pp.219–232.

243. Wieland, I. et al., 2007. Contiguous gene deletions involving EFNB1, OPHN1, PJA1 and EDA in patients with craniofrontonasal syndrome. *Clinical Genetics*, 72(6), pp.506–16.

244. Willemsen, R. et al., 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. *Human Molecular Genetics*, 12(9), pp.949–59.

245. Willert, J. et al., 2002. A transcriptional response to Wnt protein in human embryonic carcinoma cells. *BMC Developmental Biology*, 2, p.8.

246. Williams, M.E. et al., 2003. UNC5H1 induces apoptosis via its juxtamembrane region through an interaction with NRAGE. *The Journal of Biological Chemistry*, 278(19), pp.17483–90.

247. Xue, B. et al., 2005. Human NRAGE disrupts E-cadherin/beta-catenin regulated homotypic cell-cell adhesion. *Biochemical and Biophysical Research Communications*, 336(1), pp.247–51.

248. Yamada, K. & Nabeshima, T., 2003. Brain-derived neurotrophic factor/TrkB signaling in memory processes. *Journal of Pharmacological Sciences*, 91(4), pp.267–70.

249. Yamada, S. et al., 2005. Deconstructing the cadherin-catenin-actin complex. *Cell*, 123(5), pp.889–901.

250. Yamamoto, H. et al., 2007. Wnt5a modulates glycogen synthase kinase 3 to induce phosphorylation of receptor tyrosine kinase Ror2. *Genes to Cells*, 12(11), pp.1215–23.

251. Yan, Q. & Johnson, E.M., 1988. An immunohistochemical study of the nerve growth factor receptor in developing rats. *The Journal of Neuroscience*, 8(9), pp.3481–98.

252. Yao, R. & Cooper, G.M., 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science*, 267(5206), pp.2003–6.

253. Yee, B.K. et al., 2007. Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice. *Journal of Neural Transmission*, 114(4), pp.431–44.

254. Yeiser, E.C. et al., 2004. Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice. *The Journal of Neuroscience*, 24(46), pp.10521–9.

255. Yoon, W.-J. et al., 2008. The Boston-type craniosynostosis mutation MSX2 (P148H) results in enhanced susceptibility of MSX2 to ubiquitin-dependent degradation. *The Journal of Biological Chemistry*, 283(47), pp.32751–61.

256. Yu, M. et al., 2011. Activity of dlx5a/dlx6a regulatory elements during zebrafish GABAergic neuron development. *International Journal of Developmental Neuroscience*, 29(7), pp.681–91.

257. Yu, P. et al., 2002. PJA1, encoding a RING-H2 finger ubiquitin ligase, is a novel human X chromosome gene abundantly expressed in brain. *Genomics*, 79(6), pp.869–74.

258. Zafra, F. et al., 1990. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *The EMBO Journal*, 9(11), pp.3545–50.

259. Zafra, F. et al., 1991. Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America*, 88(22), pp.10037–41.

260. Zhai, Y. et al., 2011. MSX2 is an oncogenic downstream target of activated WNT signaling in ovarian endometrioid adenocarcinoma. *Oncogene*, 30, pp.4152–4162.

261. Zoabi, M. et al., 2011. PRAJA1 is a ubiquitin ligase for the polycomb repressive complex 2 proteins. *Biochemical and Biophysical Research Communications*, 408(3), pp.393–8.

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Statement of interest (Ehrenerklärung)

Ich erkläre, dass ich die an der Medizinischen Fakultät der Otto-von-Guericke-Universität Magdeburg zur Promotion eingereichte Dissertation mit dem Titel

The E3 ubiquitin ligase Praja1 inhibits the development
of a neuronal phenotype in PC12 cells

in der Abteilung Genetik & Molekulare Neurobiologie am Institut für Biologie der Fakultät für Naturwissenschaften ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 9. Februar 2015

Jan Teuber

Appendix

A.I Supplemental Data

A.I.1 Up-regulation of endogenous Praja1 in PC12 cells following application of NGF

	30 min	2 h	3 d	Levene	One-way ANOVA	
	Mean ± S.E.M.	*Mean ± S.E.M.*	*Mean ± S.E.M.*	*p-value*	$F_{0.95}(2,6) = 5.14$	*p-value*
Praja1.1	0.09 ± 0.03	0.05 ± 0.03	0.30 ± 0.03	0.990	≤ 22.048	0.002
Praja1.2	−0.05 ± 0.01	0.05 ± 0.02	0.25 ± 0.01	0.493	≤ 110.561	0.000
Post-hoc test	*p-value*	*p-value*	*Procedure*			
Praja1.1	0.004	0.002	Tukey-HSD			
Praja1.2	0.000	0.000	Tukey-HSD			

Table 11: Up-regulation of endogenous Praja1 following NGF stimulation of PC12 cells. Immunoblots of PC12 cell lysates obtained after 30 minutes, two hours, or three days of NGF stimulation (N = 3) have been quantified with the LI-COR Odyssey® system. Intensities of respective Praja1 signals have then been normalized to according tubulin intensities to control for total protein content. Stated means ± S.E.M. have been computed from the resulting values. Statistical analysis has relied on one-way ANOVA with significant differences assumed for $p \leq 0.05$. Presented post-hoc p-values are each in comparison to measurements after three days of NGF exposure. Post-hoc comparison procedures have been chosen depending on the results of the Levene test.

A.I.2 Influence of Praja1 on neurite outgrowth in PC12 cells

	Praja1.1/2	Praja1.2	Control	Brown-Forsythe	One-way ANOVA	
	Mean ± S.E.M.	*Mean ± S.E.M.*	*Mean ± S.E.M.*	*p-value*	$F_{0.95}(2,15) = 3.68$	*p-value*
Neurites per differentiating cell	0.28 ± 0.03	0.10 ± 0.04	0.88 ± 0.12	0.000	≤ 27.735	0.000
Rounding ratio	0.21 ± 0.06	0.39 ± 0.11	0.88 ± 0.25	0.051	≤ 4.546	0.029
Percentage of differentiating cells	0.38 ± 0.06	0.20 ± 0.03	0.29 ± 0.07	0.096	≥ 2.909	0.086
Post-hoc test	*p-value*	*p-value*	*Procedure*			
Neurites per differentiating cell	0.010	0.002	Dunnett-T3			
Rounding ratio	0.027	0.119	Tukey-HSD			

Table 12: Praja1 effects on neuronal-like differentiation of PC12 cells. Different parameters of cell morphology have been assessed after four days of NGF stimulation. Measured values from six independent experiments per group (N = 6) are noted as mean ± S.E.M. Significant differences have been assumed for $p \leq 0.05$ in a one-way ANOVA. Presented post-hoc p-values are each in comparison to control cells. Post-hoc comparison procedures have been chosen depending on the result of the Brown-Forsythe test.

	Praja1.1/2	Control	Levene	Student's t-test		
	Mean ± S.E.M.	*Mean ± S.E.M.*	*p-value*	*T*	*df*	*p-value*
Collagen IV						
Neurites per differentiating cell	0.28 ± 0.05	0.70 ± 0.08	0.195	−4.409	10	0.001
Rounding ratio	0.31 ± 0.09	0.34 ± 0.05	0.469	−0.212	10	0.837
Percentage of differentiating cells	0.26 ± 0.02	0.21 ± 0.07	0.029	0.696	6.158	0.512
Laminin/PDL						
Neurites per differentiating cell	0.34 ± 0.07	0.75 ± 0.08	0.974	−3.799	14	0.002
Rounding ratio	0.22 ± 0.03	0.50 ± 0.11	0.051	−2.380	14	0.032
Percentage of differentiating cells	0.31 ± 0.03	0.30 ± 0.04	0.790	0.374	14	0.733
Laminin						
Neurites per differentiating cell	0.19 ± 0.06	0.58 ± 0.10	0.117	−3.349	10	0.007
Rounding ratio	0.67 ± 0.10	1.57 ± 0.30	0.261	−2.865	10	0.017
Percentage of differentiating cells	0.10 ± 0.01	0.16 ± 0.01	0.330	−4.533	10	0.001
PDL						
Neurites per differentiating cell	0.21 + 0.05	0.94 + 0.12	0.257	−5.675	10	0.000
Rounding ratio	0.27 ± 0.04	1.20 ± 0.18	0.023	−4.939	5.557	0.003
Percentage of differentiating cells	0.27 ± 0.02	0.26 ± 0.02	0.591	0.411	10	0.690
L1-Fc						
Neurites per differentiating cell	0.17 ± 0.07	1.02 ± 0.17	0.107	−4.318	9	0.002
Rounding ratio	0.45 ± 0.15	1.19 ± 0.17	0.679	−3.285	8	0.011
Percentage of differentiating cells	0.25 ± 0.04	0.29 ± 0.02	0.383	−0.991	9	0.348

Table 13: Substrate-specificity of Praja1-dependent inhibition of neurite outgrowth. The listed morphological parameters have been evaluated following NGF stimulation. Since there are no overall differences between two and four days of NGF treatment, both groups have been pooled. Measured values from at least five independent experiments per substrate (N = 8 for Laminin/PDL, N = 5 for Praja1.1/2-expressing cells on L1-Fc, N = 6 for all others) are noted as mean ± S.E.M. Significant differences have been assumed for $p \leq 0.05$. Comparison of groups has relied on a two-tailed Student's t-test after checking the homogeneity of variances by applying the Levene test and calculating t-test p-values accordingly.

	Collagen IV	L1-Fc	Laminin / PDL	PDL	Laminin	Brown-Forsythe	One-way ANOVA	
	Mean ± S.E.M.	***Mean ± S.E.M.***	***Mean ± S.E.M.***	***Mean ± S.E.M.***	***Mean ± S.E.M.***	***p-value***	$F_{0.95}(4,58) \cong 2.53$	***p-value***
Neurites per differentiating cell	0.49 ± 0.08	0.63 ± 0.16	0.54 ± 0.07	0.57 ± 0.13	0.39 ± 0.08	0.594	≥ 0.745	0.565
Rounding ratio	0.32 ± 0.05	0.82 ± 0.16	0.36 ± 0.07	0.73 ± 0.17	1.12 ± 0.20	0.001	≤ 6.341	0.000
Percentage of differentiating cells	0.24 ± 0.03	0.27 ± 0.02	0.30 ± 0.02	0.27 ± 0.01	0.13 ± 0.01	0.000	≤ 9.048	0.000
Post-hoc test	***p-value***	***p-value***	***p-value***	***p-value***	***Procedure***			
Rounding ratio	0.020	0.925	0.028	0.762	Dunnett-T3			
Percentage of differentiating cells	0.091	0.001	0.000	0.000	Dunnett-T3			

Table 14: Influence of different substrates on PC12 morphology. The listed morphological parameters have been evaluated following NGF stimulation. Since there are no overall differences between two and four days of NGF treatment, both groups have been pooled. Additionally, Praja1.1/2 over-expressing and control cells have been pooled for assessing substrate-dependent differences only. Measured values from at least five independent experiments per substrate (N = 8 for Laminin/PDL, N = 5 for Praja1.1/2-expressing cells on L1-Fc, N = 6 for all others) are noted as mean ± S.E.M. Significant differences have been assumed for $p \leq 0.05$ in a one-way ANOVA followed by appropriate post-hoc testing with post-hoc comparison procedures chosen depending on the result of the Brown-Forsythe test.

A.I.3 Influence of Praja1 on gene expression of selected phenotype markers

	Praja1.1/2	Praja1.2	Control	Levene	One-way ANOVA	
	Mean ± S.E.M.	*Mean ± S.E.M.*	*Mean ± S.E.M.*	*p-value*	$F_{0.95}(2,6) = 5.14$	*p-value*
Praja1	−6.59 ± 0.78	−6.79 ± 0.74	1.80 ± 0.51	0.591	≤ 50.412	0.000
Slc18a1	1.76 ± 0.23	1.67 ± 0.24	4.66 ± 0.31	0.684	≤ 42.494	0.000
DnaJC5	7.08 ± 0.13	7.23 ± 0.34	8.33 ± 0.52	0.186	≥ 3.513	0.098
NPY	8.25 ± 0.28	8.73 ± 0.36	13.13 ± 0.92	0.152	≤ 20.664	0.002
Post-hoc test	*p-value*	*p-value*	*Procedure*			
Praja1	0.000	0.000	Tukey-HSD			
Slc18a1	0.001	0.000	Tukey-HSD			
NPY	0.003	0.005	Tukey-HSD			

Table 15: Influence of Praja1 over-expression on expression of selected genes. Gene expression in stably transfected PC12 cells has been measured after four days of NGF stimulation using quantitative real-time PCR and relative quantification methods. Measured CT values of target genes have been normalized using GAPDH and PGK as internal controls before performing statistical analysis. Presented values are mean ΔCT values (after normalization) from three independent experiments per group (N = 3) ± S.E.M. Due to the normalization method, higher mean values indicate lower mRNA amounts. Significant differences have been assumed for $p \leq 0.05$ in a one-way ANOVA. Presented post-hoc p-values are each in comparison to control cells. Post-hoc comparison procedures have been chosen depending on the results of the Levene test.

A.I.4 Reduction of NRAGE expression following over-expression of Praja1

	Praja1.1/2	Praja1.2	Control	Levene	One-way ANOVA	
	Mean ± S.E.M.	*Mean ± S.E.M.*	*Mean ± S.E.M.*	*p-value*	$F_{0.95}(2,9) = 4.26$	*p-value*
– Lactacystin	0.94 ± 0.07	0.87 ± 0.12	1.36 ± 0.10	0.137	≤ 7.250	0.013
+ Lactacystin	0.90 ± 0.11	0.96 ± 0.18	1.08 ± 0.11	0.167	≥ 0.420	0.669
Post-hoc test	*p-value*	*p-value*	*Procedure*			
– Lactacystin	0.034	0.017	Tukey-HSD			

Table 16: Reduction of NRAGE expression following over-expression of Praja1. Immunoblots of PC12 cell lysates obtained after two days of NGF stimulation (N = 4) have been quantified with the Odyssey® system by LI-COR. Intensities of respective NRAGE signals have then been normalized using the according β-actin signal to control for total protein content. From the resulting values, mean ± S.E.M. have been computed as stated. Statistical analysis has relied on one-way ANOVA with significant differences assumed for $p \leq 0.05$. Presented post-hoc p-values are each in comparison to control cells. Post-hoc comparison procedures have been chosen depending on the results of the Levene test.

A.II Materials and suppliers

A.II.1 Water

Aqua destillata (double-distilled) has been produced using the on-site purification system Astacus by membraPure (Bodenheim / Germany).

To obtain RNase-free water, double-distilled water has been treated with 0.1 % DMDC, stirred for three hours, and subsequently autoclaved.

A.II.2 Molecular weight markers

Molecular weight markers used in SDS-PAGE and Western blotting:

PageRuler Prestained Protein Ladder	Thermo Fisher Scientific, Waltham MA / USA
PageRuler Precision Plus Prestained Protein Ladder	Thermo Fisher Scientific, Waltham MA / USA

A.II.3 Kits and assays

Amersham ECL Plus Detection Reagents	GE Healthcare, Little Chalfont / UK
Caspase-Glo® 3/7 Assay	Promega, Madison WI / USA
Cells-to-cDNA™ II Kit	Ambion®, Huntington / UK *Only the following kit component has been used:* *Cell Lysis II Buffer*
DC™ Protein Assay	Bio-Rad, Hercules CA / USA
PureLink™ HiPure Plasmid Midiprep Kit	Invitrogen™, Carlsbad CA / USA
Sensiscript® Reverse Transcription Kit	QIAGEN, Hilden / Germany *Contained reagents:* *Sensiscript reverse transcriptase* *Buffer RT (10x)* *dNTP mix (5 mM each)* *RNase-free water*
RETROscript® Reverse Transcription Kit	Ambion®, Huntington / UK *Only the following kit component has been used:* *Oligo(dT)$_{18}$ primers*
SUPERase-In™ RNase Inhibitor	Ambion®, Huntington / UK
TaqMan® Gene Expression Reagents	Applied Biosystems®, Foster City CA / USA *TaqMan® Gene Expression Master Mix* *Inventoried Assays:* *Slc18a1 (VMAT1): Rn00461866_m1* *DnaJC5 (CSP): Rn00577363_m1* *NPY: Rn00561681_m1* *PGK: Rn00821429_g1* *GADPH: 4352338E* *Assay-by-Design:* *MPraja1_-RT6*

A.II.4 Chemicals and reagents (except kits, assays, and cell culture)

Acrylamide solution (37.5:1)	Carl Roth®, Karlsruhe / Germany
APS	Serva, Heidelberg / Germany
Β-mercaptoethanol	Serva, Heidelberg / Germany
Bromophenol blue	Serva, Heidelberg / Germany
BSA	Carl Roth®, Karlsruhe / Germany
DAPI	Merck, Darmstadt / Germany
DMDC	Sigma-Aldrich, Saint Louis MO / USA
DNase I	Roche, Mannheim / Germany
DOC	Fluka® (Sigma-Aldrich), Buchs / Switzerland
Donkey serum	Linaris, Dossenheim / Germany
EDTA	Carl Roth®, Karlsruhe / Germany
Ethanol (96 %)	Carl Roth®, Karlsruhe / Germany
Glycerol	Carl Roth®, Karlsruhe / Germany
Glycin	Serva, Heidelberg / Germany
Immersol™ 518 F	Zeiss, Oberkochen / Germany
Isopropanol	Carl Roth®, Karlsruhe / Germany
KCl	Fluka®, Buchs / Switzerland
KH_2PO_4	Sigma-Aldrich, Seelze / Germany
Methanol	Carl Roth®, Karlsruhe / Germany
Milk powder	Carl Roth®, Karlsruhe / Germany
NaCl	Carl Roth®, Karlsruhe / Germany
NaF	Carl Roth®, Karlsruhe / Germany
Na_3VO_4	Sigma-Aldrich, Saint Louis MO / USA
Na_2HPO_4	Carl Roth®, Karlsruhe / Germany
NP-40 Alternative	Calbiochem®, San Diego CA / USA
PFA	Carl Roth®, Karlsruhe / Germany
Potassium chloride	Carl Roth®, Karlsruhe / Germany
Propidium iodide	Invitrogen™, Carlsbad CA / USA
Proteinase Inhibitor Tablets	Pierce Biotechnology, Rockford IL / USA
Rhodamine phalloidin	Life Technologies, Eugene OR / USA
SDS	Serva, Heidelberg / Germany
Shandon Immu-Mount™	Richard-Allan Scientific™, Kalamazoo MI / USA
TEMED	Carl Roth®, Karlsruhe / Germany
Tris-HCl PUFFERAN®	Carl Roth®, Karlsruhe / Germany

Tris PUFFERAN®	Carl Roth®, Karlsruhe / Germany
Triton™ X-100	Sigma-Aldrich, Saint Louis MO / USA
Tween® 20	Carl Roth®, Karlsruhe / Germany

A.II.5 Cell culture

Bacillol® plus	Bode Chemie, Hamburg / Germany
COS7 cells (DSMZ No. ACC 60)	DSMZ, Braunschweig / Germany
Collagen I	Sigma-Aldrich, Saint Louis MO / USA
Collagen IV	Sigma-Aldrich, Saint Louis MO / USA
DMEM	Gibco®, Carlsbad CA / USA
Doxycycline	Sigma-Aldrich, Saint Louis MO / USA
Foetal bovine serum	Gibco®, Carlsbad CA / USA
G418 (Geneticin)	Invitrogen™, Carlsbad CA / USA
GeneJammer	Stratagene, La Jolla CA / USA
HEK293 cells (DSMZ No. ACC 305)	DSMZ, Braunschweig / Germany
Horse serum	Gibco®, Carlsbad CA / USA
Hygromycin B	Gibco®, Carlsbad CA / USA
L1, human recombinant protein (GF220)	Chemicon® (Millipore), Billerica MA / USA
Lactacystin	Sigma-Aldrich, Saint Louis MO / USA
Laminin	BD Bioscience, Paolo Alto CA / USA
NGF (2.5 S)	Invitrogen™, Carlsbad CA / USA
PC12 cells (DSMZ No. ACC 159)	DSMZ, Braunschweig / Germany
PBS	Gibco®, Carlsbad CA / USA
PDL	Sigma-Aldrich, Saint Louis MO / USA
Penicillin–Streptomycin–Glutamine	Gibco®, Carlsbad CA / USA
Trypsin/EDTA	Gibco®, Carlsbad CA / USA

A.II.6 Vectors

pEGFP-C1	BD Bioscience, Paolo Alto CA / USA
pCMV::HA	BD Bioscience, Paolo Alto CA / USA
pTRE2-hyg	BD Bioscience, Paolo Alto CA / USA

A.II.7 Antibodies

Alexa Fluor 488 (A11055)	Invitrogen™, Carlsbad CA / USA
Alexa Fluor 555 (A31570 & A31572)	Invitrogen™, Carlsbad CA / USA
Alexa Fluor 647 (A31571)	Invitrogen™, Carlsbad CA / USA
A-tubulin (T6199)	Sigma-Aldrich, Saint Louis MO / USA

B-actin (ab6276)	Abcam®, Cambridge / UK
B-tubulin (926-42211)	LI-COR®, Lincoln NE / USA
B3-tubulin (TuJ1) (5568S)	Cell Signaling Technology®, Danvers MA / USA
Fc IgG (MAB1302)	Chemicon® (Millipore), Billerica MA / USA
GFP (Ab6556)	Abcam®, Cambridge / UK
HA-tag, clone 114-C2-7 (05-902R)	Millipore, Billerica MA / USA
HRP-coupled immunoglobulins (P0160 & P0447)	DAKO, Copenhagen / Denmark
IRDye® 680LT (926-68020)	LI-COR®, Lincoln NE / USA
IRDye® 800CW (926-32211)	LI-COR®, Lincoln NE / USA
MAP2 (Ab32454)	Abcam®, Cambridge / UK
NRAGE (07-394)	Upstate® (Millipore), Lake Placid NY / USA
Praja1 anti-serum (custom-made)	PINEDA Antibody Service, Berlin / Germany
Smad3 (ab28379)	Abcam®, Cambridge / UK
Ubiquitin (U0508)	Sigma-Aldrich, Saint Louis MO / USA

A.II.8 Instruments and consumables

Autoclaves & oven:

DB-23	Systec, Wettenberg / Germany
FP 53	Binder, Tuttlingen / Germany
VX-120	Systec, Wettenberg / Germany

Cell culture incubators:

C60	Labotect, Göttingen / Germany
C200	Labotect, Göttingen / Germany

Centrifuges:

5415D	Eppendorf, Hamburg / Germany
5415R	Eppendorf, Hamburg / Germany
5424	Eppendorf, Hamburg / Germany
5430	Eppendorf, Hamburg / Germany
5810R	Eppendorf, Hamburg / Germany
Galaxy Mini	VWR™ International, Radnor PA / USA

Freezers & fridges:

Apollo® & BIOSAFE® nitrogen storage	Cryotherm, Kirchen (Sieg) / Germany
KU 2407	Liebherr, Ochsenhausen / Germany
GN 2756	Liebherr, Ochsenhausen / Germany
GU 4506	Liebherr, Ochsenhausen / Germany

TS 606-G/4-i	WTW, Weilheim / Germany
Ultra-Low (V.I.P.® Series)	Sanyo, Moriguchi / Japan

Glassware:

Beakers	DURAN Group, Wertheim (Main) / Germany
Cover-slips	Carl Roth®, Karlsruhe / Germany
Erlenmeyer flasks	DURAN Group, Wertheim (Main) / Germany
Glass bottles	DURAN Group, Wertheim (Main) / Germany
Graduated cylinders	DURAN Group, Wertheim (Main) / Germany
Microscope slides	Carl Roth®, Karlsruhe / Germany

Instruments:

Eclipse TS100 & TS100-F	Nikon, Chiyoda (Tokyo) / Japan
EpMotion® 5070 pipetting robot	Eppendorf, Hamburg / Germany
Infinite® M200 microplate reader	Tecan, Männedorf / Switzerland
Leica DMI6000CS microscope	Leica, Wetzlar / Germany
Mini-PROTEAN® Electrophoresis System	Bio-Rad, Hercules CA / USA
Odyssey® Infrared Imaging System	LI-COR®, Lincoln NE / USA
StepOnePlus™	Applied Biosystems®, Foster City CA / USA
Veriti® Thermal Cycler	Applied Biosystems®, Foster City CA / USA

Pipettes:

Accu-jet® pro	Brand, Wertheim (Main) / Germany
Costar® Stripette® (10 ml, 25 ml, 50 ml)	Corning, Corning NY / USA
Pasteur capillary pipettes	WU, Mainz / Germany
Pasteur pipettes (disposable)	Carl Roth®, Karlsruhe / Germany
Pipettes	Brand, Wertheim (Main) / Germany
Pipette tips	Brand, Wertheim (Main) / Germany
Pipette tips with filter	Brand, Wertheim (Main) / Germany

Plastic ware:

Adhesive qPCR folie	Sarstedt, Nümbrecht / Germany
Cell culture flasks (ventilated caps)	Corning, Corning NY / USA
Cell culture dishes	Corning, Corning NY / USA
CentriStar™ tubes (15 ml, 50 ml)	Corning, Corning NY / USA
Costar® cell culture cluster (well plates)	Corning, Corning NY / USA
MicroAmp® Fast Reaction Tubes	Applied Biosystems®, Foster City CA / USA
MicroAmp® 8-cap strip	Applied Biosystems®, Foster City CA / USA

MicroAmp® Fast Optical 96-well plate	Applied Biosystems®, Foster City CA / USA
Object slide box	Carl Roth®, Karlsruhe / Germany
Safe lock tubes (0.5 ml, 1.5 ml, 2 ml)	Sarstedt, Nümbrecht / Germany

Safety hoods:

Biowizard Silver Class II Safety Cabinet	Kojair Tech Oy, Vilpulla / Finland
Captair® bio	erlab, Düsseldorf / Germany

Scales:

TE 153S	Sartorius, Göttingen / Germany
TE 2101	Sartorius, Göttingen / Germany
TE 212	Sartorius, Göttingen / Germany

Shakers, stirrers & rotators:

Dual-Action Shaker KL2	Edmund Bühler, Hechingen / Germany
HS 260 basic shaker	IKA®, Staufen / Germany
Innova™ 4200 incubator shaker	New Brunswick Scientific, Edison NJ / USA
Lab dancer S40	VWR™ International, Radnor PA / USA
RCT basic magnetic stirrer	IKA®, Staufen / Germany
Tube rotator SB3	stuart equipment (Bibby Scientific), Stone / UK

Thermo mixers & water baths:

A100 & A103 water bath	Lauda-Brinkmann, Delran NJ / USA
Mixing Block MB-102	BIOER, Hangzhou / China
Thermomixer comfort	Eppendorf, Hamburg / Germany
WB 10 water bath	P-D Industriegesellschaft, Dresden / Germany

Others:

AF200 ice machine	Scotsman®, Vernon Hills IL / USA
Aluminium foil	neoLab®, Heidelberg / Germany
Immobilon®-FL PVDF membrane	Millipore, Billerica MA / USA
inoLab® pH 720 ph meter	WTW, Weilheim / Germany
Lab timers	Carl Roth®, Karlsruhe / Germany
Parafilm “M”	BEMIS, Neenah WI / USA
Power-Pac 300	Bio-Rad, Hercules CA / USA
Power Source 300V	VWR™ International, Radnor PA / USA
VaccuSafe comfort vacuum pump	IBS Integra Biosciences, Fernwald / Germany

A.II.9 Software (excluding instrument-specific software)

Data analysis:

Excel® 2008 & 2011 (MacOffice)	Microsoft®, Redmond WA / USA
Fiji (ImageJ distribution package)	http://fiji.sc/Fiji
SPSS (versions 16 through 21)	IBM, Armonk NY / USA

Data management & presentation:

FileMaker® Pro 10 Advanced	FileMaker, Santa Clara CA / USA
Mendeley PDF and reference manager	Elsevier, Amsterdam / Netherlands
Photoshop CS6 Extended	Adobe Systems, San Jose CA / USA
PowerPoint® 2008 & 2011 (MacOffice)	Microsoft®, Redmond WA / USA
Word® 2008 & 2011 (MacOffice)	Microsoft®, Redmond WA / USA

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