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des Fachbereichs Medizin der Philipps-Universität Marburg
AG Molekulare Neurowissenschaften
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in Zusammenarbeit mit dem

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Pituitary adenylate cyclase-activating polypeptide mediates differential signaling through PAC1 receptor splice variants and activates non-canonical cAMP dependent gene induction in the nervous system

Implications for homeostatic stress-responding

Inaugural-Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

dem Fachbereich Medizin der Philipps-Universität Marburg
vorgelegt von
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Marburg, 2011

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg am: 01.08.2011

Gedruckt mit Genehmigung des Fachbereichs.

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Contents

Summary

Pituitary adenylate cyclase-activating polypeptide (PACAP)-mediated activation of its G protein-coupled receptor PAC1 results in activation of the two G proteins Gs and Gq to alter second messenger generation and gene transcription in the nervous system, important for homeostatic responses to stress and injury. PAC1 occurs in different splice variants of the third intracellular loop, designated PAC1null, hop or hip, affecting second messenger generation as shown in non-neural cells. At the splanchnico-adrenomedullary synapse, PACAP is required for prolonged catecholamine secretion from chromaffin cells to restore homeostasis during prolonged periods of stress. In the central nervous system, PACAP is neuroprotective in neurodegenerative conditions associated with e.g., stroke.

In the present study, PAC1 splice variant-specific second messenger production and activation of homeostatic responses were investigated in neuro-endocrine and neural cells. Heterologous expression of the major PAC1 splice variant of adrenomedullary chromaffin cells, PAC1hop, in pheochromocytoma PC12-G cells reconstituted a PACAP-mediated Ca^{2+} and prolonged secretory response similar to the one observed in primary chromaffin cells. The Ca^{2+} response mediated by PAC1null was somewhat smaller and PAC1hip failed to couple to Ca^{2+}. Neither variant conferred prolonged catecholamine release, suggesting that expression of the hop cassette in the third intracellular loop of the receptor is required for sustained catecholamine release from neuroendocrine cells.

In neuroblastoma x glioma NG108-15 cells, heterologous expression of the PAC1hop, null and hip receptor conferred PACAP-mediated intracellular cAMP generation, while elevation of $[Ca^{2+}]_i$ occurred efficiently in PAC1hop- and to a lesser extent in PAC1null-expressing cells. Expression of PAC1hip did not confer an intracellular Ca^{2+} response, indicating that PAC1hop is the receptor variant most efficiently coupled to combinatorial signaling through cAMP and Ca^{2+}. PAC1hop-mediated signaling activated the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinases 1 and 2 (ERK1/2). Signaling to ERK proceeded through cAMP independently of the cAMP dependent protein kinase (PKA). PACAP induced transcription of the gene encoding the putative neuroprotectant stanniocalcin 1 (STC1), which has previously been implicated in neuronal resistance to hypoxic/

ischemic insult; gene induction proceeded through ERK but not PKA. Cyclic AMP generation by forskolin did not activate ERK in NG108-15 cells, but rather induced STC1 mRNA elevation through the canonical PKA dependent pathway. This suggests that activation of non-canonical cAMP signaling, mediating ERK-dependent gene induction, requires additional signaling through Ca^{2+} via PAC1hop in these cells.

Primary rat cortical neurons expressed predominantly the PAC1hop and null variants. Exposure of cortical neurons to PACAP resulted in elevation of the two second messengers cAMP and Ca^{2+}, activation of ERK1/2, and induction of STC1 gene transcription. PACAP-mediated ERK activation proceeded through cAMP but not PKA, and STC1 was induced via ERK but not PKA. Pharmacological stimulation of adenylate cyclases by forskolin also resulted in increased ERK phosphorylation and STC1 mRNA elevation independently of PKA. These results indicate that cAMP production alone is sufficient to activate ERK in differentiated cortical neurons, unlike in the less differentiated NG108-15 cell line. Induction of another PACAP target gene, brain-derived neurotrophic factor (BDNF), occurred through the canonical cAMP/PKA pathway.

PACAP has been shown by our laboratory and others to be neuroprotective against ischemia in rodent stroke models. To begin to define the mechanism of this neuroprotection, we employed two cell culture stroke models. Rat cortical neurons subjected to either oxygen-glucose-deprivation or glutamate-induced excitotoxicity underwent cell death as expected. However, treatment with PACAP did not increase neuronal survival in either of the two models, and STC1 over-expression also failed to increase resistance to neuronal cell death during glutamate-induced excitotoxicity. These data suggest that the protective effects of the neurotrophic peptide PACAP and the putative neuroprotectant STC1 during neurodegenerative conditions in vivo are mediated through cells absent in cultures of cortical neurons, such as glial cells.

In conclusion, the present study has demonstrated that expression of different PAC1 splice variants determines the degree of activation of two different second messenger pathways that may mediate different functional outcomes during stress-responding. PACAP mediates ERK activation and STC1 induction via non-canonical cAMP signaling. The selective pharmacological activation of this potentially neuroprotective pathway, which is different from the cAMP/PKA pathway critical for learning and memory, could have therapeutic implications for neuroprotection in vivo.

Zusammenfassung

Aktivierung des G-Protein-gekoppelten Rezeptors PAC1 durch PACAP (pituitary adenylate cyclase-activating polypeptide) resultiert in Aktivierung der beiden G-Proteine Gs und Gq und führt über die Produktion von Second Messengern (sekundären Botenstoffen) zur Aktivierung von Gentranskription, die essentiell für die homöostatische Stressantwort zu sein scheint. Durch differenzielles Splicing der dritten intrazellulären Schleife von PAC1 entstehen sogenannte PAC1null-, hop- und hip-Rezeptoren. In non-neuralen Zellen konnte gezeigt werden, dass die Expression verschiedener Rezeptor-Varianten die Generation von Second Messengern beeinflusst. Als Kotransmitter an der splanchnico-adrenomedullären Synapse ist PACAP für anhaltende Katecholamin-Freisetzung von chromaffinen Zellen unentbehrlich, welche zur Wiederherstellung der Homöostase während anhaltendem Stress dient. Im zentralen Nervensystem ist PACAP neuroprotektiv in Assoziation mit neurodegenerativen Erkrankungen wie z.B. Schlaganfall.

In der vorliegenden Arbeit wurde die Produktion von Second Messengern durch verschiedene PAC1-Splice-Varianten und die Aktivierung homöostatischer Antworten in neuroendokrinen und neuralen Zellen untersucht. Heterologe Expression von PAC1hop, der Haupt-Variante adrenomedullärer chromaffiner Zellen, in Phäochromozytom PC12-G Zellen, resultierte in einer PACAP-evozierten Ca^{2+}- und anhaltenden sekretorischen Antwort, ähnlich der in primären chromaffinen Zellen. Auch die Aktivierung von PAC1null, jedoch nicht von PAC1hip, vermittelte eine intrazelluläre Ca^{2+}-Antwort; diese war etwas geringer als die durch PAC1hop vermittelte. Weder PAC1null- noch PAC1hip-Aktivierung resultierte in anhaltender Katecholamin-Freisetzung. Dies zeigt die Notwendigkeit der Expression der hop-Kassette in der dritten intrazellulären Schleife des PAC1-Rezeptors für anhaltende Freisetzung von Katecholaminen aus neuroendokrinen Zellen.

Heterologe Expression von PAC1hop-, null- und hip-Rezeptoren in NG108-15 Zellen (ein Neuroblastom-Gliom Hybrid) resultierte in PACAP-aktivierter Produktion von zyklischem AMP (cAMP), während eine intrazelluläre Ca^{2+}-Antwort nur durch PAC1hop und etwas geringer durch PAC1null vermittelt wurde. Diese Ergebnisse deuten darauf hin, dass eine Signalkopplung an Ca^{2+} durch Expression der hop-Kassette erhöht und durch Expression der hip-Kassette aufgehoben wird; eine

effiziente duale Signaltransduktion durch cAMP und Ca^{2+} wird also nur durch die hop-Variante des PAC1-Rezeptors vermittelt. Diese resultierte in einer Aktivierung der MAP Kinasen (mitogen-activated protein kinases) ERK1/2 (extracellular signal-regulated kinases 1 and 2). Zyklisches AMP, jedoch nicht die cAMP-abhängige Proteinkinase (PKA) waren in die Aktivierung von ERK involviert. PACAP induzierte das putativ neuroprotektive Gen Stanniocalcin 1 (STC1), welches laut Literaturstand neuronale Resistenz bei hypoxischem/ischämischem Insult vermittelt; die Genaktivierung erfolgte via ERK, unabhängig von PKA. Direkte Stimulation von Adenylatzyklasen durch Forskolin hingegen löste keine ERK-Aktivierung aus und STC1-Induktion wurde durch kanonische (PKA-abhängige) Signaltransduktion vermittelt. In NG108-15 Zellen scheinen also zur Aktivierung von nicht-kanonischer cAMP-abhängiger Signaltransduktion, welche ERK-abhängige Geninduktion vermittelt, zusätzlich PAC1hop-aktivierte Ca^{2+}-Signalwege nötig zu sein.

Primäre kortikale Neuronen exprimierten hauptsächlich die PAC1hop- und PAC1null-Varianten. PACAP-Behandlung resultierte in einer intrazellulären Erhöhung von cAMP und Ca^{2+}, ERK-Aktivierung und STC1-Induktion. Die Aktivierung von ERK war cAMP-abhängig und PKA-unabhängig. STC1-Induktion wurde durch ERK und nicht durch PKA vermittelt. Forskolin-Behandlung resultierte ebenso in PKA-unabhängiger ERK-Aktivierung sowie STC1-Induktion. Dies zeigt, dass in differenzierten kortikalen Neuronen, im Gegensatz zu weniger differenzierten NG108-15 Zellen, gesteigerte cAMP-Produktion zur ERK-Aktivierung ausreicht. PACAP-vermittelte Induktion von brain-derived neurotrophic factor (BDNF) mRNA hingegen war PKA-abhängig.

Im Tiermodell konnte gezeigt werden, dass PACAP im Zusammenhang mit Schlaganfall neuroprotektiv ist. Um erste Einsichten in den Mechanismus dieser Neuroprotektion zu bekommen, wurden zwei Zellkultur-Modelle für Schlaganfall angewandt. Sauerstoff-Glukose-Deprivation und Glutamat-induzierte Excitotoxizität lösten, wie erwartet, in kortikaler Neuronenkultur Zelltod aus, der sich jedoch als PACAP-resistent herausstellte. Auch durch STC1-Überexpression konnte der durch Excitotoxizität ausgelöste Zelltod nicht reduziert werden. Diese Ergebnisse deuten darauf hin, dass die neuroprotektiven Effekte von PACAP und STC1 während neurodegenerativen Erkrankungen durch Zellen vermittelt werden, die in kortikaler Neuronenkultur fehlten, wie z.B. Gliazellen.

Insgesamt weisen die Befunde dieser Arbeit darauf hin, dass die Expression verschiedener PAC1-Varianten den Aktivierungsgrad zweier Second Messengers bedingt und damit den funktionellen Ausgang nach Stress-Einwirkung bestimmt. PACAP-vermittelte ERK-Aktivierung und STC1-Induktion via nicht kanonischer cAMP-abhängiger Signaltransduktion unterscheidet sich von der kanonischen PKA-vermittelten Signaltransduktion, welche in physiologischen Prozessen wie Gedächtniskonsolidierung von Bedeutung ist. Die selektive pharmakologische Aktivierung dieses potenziell neuroprotektiven nicht-kanonischen Signalweges hat therapeutische Implikation für Neuroprotektion in vivo.

Abbreviations

AC	adenylate cyclase
ACh	acetylcholine
AM ester	acetoxymethyl ester
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAPTA	bis(2-aminophenoxy)ethane tetraacetic acid
BBB	blood-brain barrier
BCC	bovine chromaffin cell
BDNF	brain-derived neurotrophic factor
bp	base pair
BSA	bovine serum albumin
C	cytosine
CA	catecholamine
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine 3′5′-monophosphate
cDNA	complementary DNA
CMV	cytomegalovirus
CNS	central nervous system
CREB	cAMP response element binding protein
Da	dalton
DCV	dense-core vesicle
ddAd	2′5′-dideoxyadenosine
DEPC	diethylpyrocarbonate
div	days in vitro
DMEM	Dulbecco’s modified Eagle’s medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EC_{50}	half maximal effective concentration

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
Env	envelope
Epac	exchange protein directly activated by cAMP
Epi	epinephrine
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
et al.	et alteres
EtBr	ethidium bromide
F	forward
FBS	fetal bovine serum
FCS	fetal calf serum
G protein	guanine nucleotide-binding protein
G	guanidine
GABA	γ-aminobutyric acid
Gag	group-specific antigen
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GHRH	growth hormone releasing hormone
GPCR	G protein-coupled receptor
GRK	GPCR kinase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HAT	hypoxanthine, aminopterine, thymidine
HEK	human embryonic kidney
HEPES	hydroxyethylpiperazine ethane sulfonic acid
HRP	horseradish peroxidase
i.c.v.	intracerebroventricular
i.v.	intravenous
IBMX	3-isobutyl-1-methylxanthine

ic3	third intracellular loop
ICS	intracellular stores
IL	interleukin
InsP	inositol phosphate
IPTG	isopropyl-β-D-thiogalactoside
IRES	internal ribosomal entry site
KRB	Krebs-Ringer buffer
LB	lysogeny broth
LDS	lithium dodecyl sulfate
LTR	long terminal repeat
M	marker
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MEK	MAPK/ERK kinase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MTT	dimethylthiazolyldiphenyltetrazoliumbromide
MuLV	murine leukemia virus
NE	norepinephrine
NG	neuroblastoma/glioma
NGF	nerve growth factor
NMDA	N-methyl-D-aspartic acid
OGD	oxygen-glucose-deprivation
PAC1	PACAP type 1 receptor
PACAP	pituitary adenylate cyclase-activating polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	phase contrast
PC	pheochromocytoma
PCR	polymerase chain reaction
PDE	phosphodiesterase
PI	phosphoinositide
PI	propidium iodide

PIP_2 or PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PLL	poly-L-lysine
pMCAO	permanent MCAO
PNS	peripheral nervous system
Pol	polymerase
PRP	PACAP-related peptide
R	reverse
Rev	regulator of virion
RNA	ribonucleic acid
RPM	revolutions per minute
RRP	readily releasable pool
RT	reverse transcriptase
RT	room temperature
RTK	receptor tyrosine kinase
SAPK2/p38	stress-activated protein kinase 2
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIN	self-inactivating
SOCC	store-operated calcium channel
SOCE	store-operated calcium entry
SSV	small synaptic vesicle
STC	stanniocalcin
TAE	tris acetate EDTA
TBI	traumatic brain injury
TH	tyrosine hydroxylase
TM	transmembrane
tMCAO	transient MCAO
U	unit
UV	ultra violet

VAChT	vesicular acetylcholine transporter
VGCC	voltage-gated calcium channel
VIP	vasoactive intestinal polypeptide
VPAC	VIP/PACAP receptor
VSV-G	vesicular stomatitis virus glycoprotein
WB	Western blot
x g	times gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. Introduction

1.1 Classical ‘fast’ versus neuropeptide ‘slow’ transmitters

Neuropeptides are widely distributed in the nervous system, where they have important functions as signaling molecules. They generally co-exist with classical neurotransmitters in the same neuron. Classical ‘fast’ neurotransmitters such as glutamate or acetylcholine (ACh) are released from small synaptic vesicles (SSVs), generating electrical signals by regulating membrane ion flux through ionotropic receptors or ligand-gated ion channels. Neuropeptide ‘slow’ transmitters such as pituitary adenylate cyclase-activating polypeptide (PACAP), in contrast, are released from large dense-core vesicles (LDCVs), bind to metabotropic G protein-coupled receptors (GPCRs)[a] and alter the generation of intracellular second messengers such as cyclic adenosine 3′5′-monophosphate (cAMP) and calcium involved in modulatory processes over longer time periods. Low-frequency activity leads to the release of classical neurotransmitters, whereas high-frequency stimulation is needed to trigger the release of neuropeptides, which is of particular importance when the nervous system is challenged (Hokfelt et al., 2003; Hokfelt et al., 2000).

1.2 Slow transmitters activate G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are the largest family of membrane proteins in vertebrates, comprising 3-5% of all gene-encoded proteins. They mediate the majority of physiological responses to neurotransmitters and hormones and to sensory stimuli such as photons and odorants. About half of the therapeutic drugs prescribed worldwide modulate the activity of GPCRs. GPCRs share a common molecular architecture of a seven-transmembrane configuration (hence the alternate name of seven-transmembrane receptors) and a common signaling mechanism through coupling to heterotrimeric G proteins and regulation of second messenger-generating enzymes (Gudermann et al., 1997).

[a] Glutamate and ACh bind to both ionotropic and metabotropic receptors, therefore activating fast and slow transmission depending on receptor interaction. Neuropeptides uniquely activate metabotropic receptors, acting as slow transmitters.

The GPCR superfamily consists of approximately 1,000 members. Mammalian GPCRs are divided into three main families (A, B and C) based on sequence and structural similarities. All members have seven membrane-spanning domains separated by alternating intracellular (ic1, ic2, ic3) and extracellular loops (ec1, ec2, ec3), and an extracellular amino-terminal and intracellular carboxy-terminal domain. Family A is the largest and most diverse group and includes the light-sensing rhodopsin, adrenergic receptors, the olfactory subgroup, chemokine receptors and the majority of peptide GPCRs, including galanin, neuropeptide Y (NPY), oxytocin, vasopressin, melanocortin and opioid receptors (Lee et al., 2002). The first crystal structure of a seven-transmembrane (7TM) receptor solved was that for rhodopsin (Palczewski et al., 2000), followed by those for adrenergic receptors (Cherezov et al., 2007; Warne et al., 2008). Family B consists of a small group of receptors (15 in all) with large N-termini, which are activated by the peptides secretin, glucagon, vasoactive intestinal polypeptide (VIP), PACAP, growth hormone releasing hormone (GHRH), glucagon, the glucagon-like peptides (GLP-1 and GLP-2), gastric inhibitory peptide (GIP), corticotropin-releasing hormone (CRH), urocortin, parathyroid hormone (PTH), tuberoinfundibular peptide of 39 residues (TIP39), calcitonin, calcitonin gene related peptide (CGRP) and adrenomedullin (Martin et al., 2005). The first cloned receptor of this class was the secretin receptor (Ishihara et al., 1991), which had the predicted 7TM topology but only little sequence homology with family A GPCRs and therefore became the archetypical member of the second GPCR class. Family C is the smallest group and includes receptors for metabotropic glutamate and GABA-B receptors functioning as dimers (Pierce et al., 2002).

1.2.1 GPCR-mediated signaling

Classical GPCR signaling is initiated by binding of ligands (such as hormones, neurotransmitters or sensory stimuli) inducing conformational changes in the receptor and increasing the affinity for G protein binding. The activated receptor acts as a guanine-nucleotide exchange factor (GEF) for the α-subunit of the heterotrimeric G protein catalyzing the exchange of GDP for GTP. The GTP-bound α-subunit and the tightly linked βγ-complex dissociate from each other and the receptor and regulate the activity of several effector proteins, including enzymes that regulate the generation of intracellular second messengers or ion channels. The multi-component signaling

system of GPCRs creates the potential for tremendous signal amplification within the cell. The intrinsic GTPase activity of the α-subunit returns the G protein to the inactive GDP-bound heterotrimeric state and ends the signaling cycle.

Multiple G protein subtypes exist, which are composed of three different subunits (α, β and γ) and are generally referred to by their α-subunit. Gα subunits are divided into four families, regulating the activity of different effectors that in turn generate different second messengers. Proteins of the Gαs family activate and proteins of the Gαi family inhibit a group of effector enzymes called adenylate cyclases (ACs) that catalyze the conversion of adenosine triphosphate (ATP) to the second messenger cAMP. Cyclic AMP activates the cAMP-dependent protein kinase (PKA), the canonical cAMP effector (Kuo and Greengard, 1969), and other so called non-canonical downstream effectors such as cyclic nucleotide-gated ion channels (Biel and Michalakis, 2009) and exchange proteins directly activated by cAMP (Epac1 and 2), also known as cAMP-GEFs, which regulate guanine nucleotide exchange of the small G protein Rap (de Rooij et al., 1998; Kawasaki et al., 1998). Nine different ACs with a common 12 membrane-spanning domain structure are known. All transmembrane ACs (tmACs) are activated by Gαs. AC I, III and VIII are in addition activated by calcium, whereas AC V and VI are inhibited by calcium. Another source for cAMP generation is the soluble AC (sAC), which is activated by calcium and bicarbonate (Hanoune and Defer, 2001). The Gαq family regulates the activity of four isoforms of phospholipase Cβ (PLCβ). PLCβ isoenzymes hydrolyze the membrane lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2 or PIP_2) into diacyl glycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3 or IP_3). IP_3 regulates the release of calcium from intracellular stores such as the endoplasmic reticulum (ER) through activation of IP_3 receptors. Membrane-bound DAG controls together with calcium the activity of several isoforms of protein kinase C (PKC). PKC and PKA are serine/threonine kinases phosphorylating hydroxyl groups of the amino acids serine and threonine in respective target proteins. The Gα12 family regulates the activity of RhoGEFs. Rho is a small, monomeric GTPase of the Ras superfamily, which together with other members of the Ras superfamily (Ras, Rab, Arf and Ran) regulates cellular processes such as proliferation, differentiation, apoptosis and cytoskeleton organization (Etienne-Manneville and Hall, 2002). Among targets of the βγ-heterodimer are ion channels, PLCβ isoenzymes, phospholipase A2 (PLA2) and

GPCR kinases (GRKs) (Fig. 1). To date 16 α, five β and 12 γ proteins have been cloned, the combination of which can give rise to more than 800 heterotrimeric G proteins. In addition to signaling through the α-subunit and the βγ-dimer, recent research indicates that alternate signaling mechanisms are available to GPCRs including the activation of extracellular signal-regulated kinase (ERK) and other downstream kinases via formation and internalization of signaling complexes within the cell (Luttrell, 2006; Neer, 1995; Ritter and Hall, 2009).

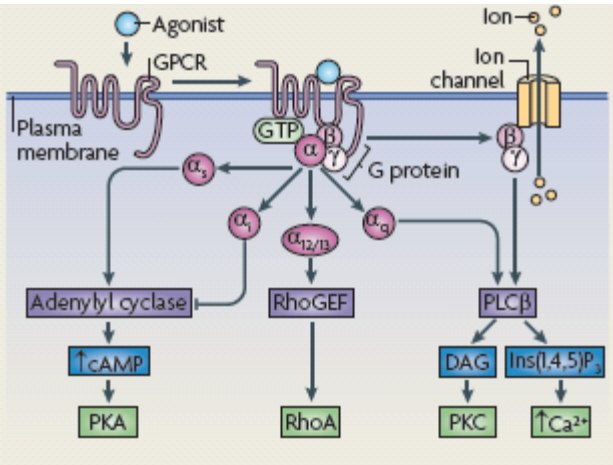


Figure 1: Classical GPCR-mediated signaling. An extracellular agonist binds to and activates the receptor. Activated G proteins regulate effector proteins such as enzymes (e.g., adenylate cyclases and PLCβ) and ion channels. Activated enzymes modulate downstream effectors directly or generate second messengers such as cAMP that further regulate certain downstream effectors (from Ritter and Hall, 2009).

1.2.2 Termination of G protein-mediated signaling

GPCR-mediated signaling is regulated at multiple levels, from second messengers to the receptor itself. The second messenger cAMP for example is degraded enzymatically by cAMP phosphodiesterases (PDEs). The intrinsic GTPase activity of the α-subunit of the G-protein is accelerated by GTPase-activating proteins (GAPs), that can be part of the effector itself or members of the family of RGS (regulators of G protein signaling) proteins, deactivating G proteins by allowing heterotrimers to reform.

Receptor function itself is negatively regulated through desensitization, a phenomenon where receptor signaling is rapidly dampened even in the continuous presence of the ligand. Second messenger-dependent protein kinases like PKA and

PKC can phosphorylate serine and threonine residues within cytoplasmic loops and the C-terminal tail of the receptor and directly impair G protein coupling. PKA- and PKC-mediated phosphorylation can also cause heterologous desensitization, in which kinase activation by one receptor leads to phosphorylation of another. GPCR kinases (GRKs) phosphorylate serine and threonine residues within the C-terminal tail and the third intracellular loop of the agonist-bound receptor (homologous desensitization) increasing the affinity for arrestin binding, which sterically inhibits further G protein binding. Moreover, β-arrestin 1 and 2 (which are the two ubiquitously expressed arrestins in contrast to the two visual arrestins in the retina) interact with clathrin and the clathrin adaptor AP2 to drive GPCR internalization into endosomes. Interestingly, β-arrestins not only regulate GPCR desensitization but also initiate distinct signaling pathways, including activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway. Following internalization, receptors can be trafficked to lysosomes, where they are degraded, or to recycling endosomes, where they are dephosphorylated and returned to the plasma membrane in their basal state. Finally, the expression of cell surface receptors is regulated at the transcriptional and translational level (Luttrell, 2006; Pierce et al., 2002).

1.3 Pituitary adenylate cyclase-activating polypeptide (PACAP)

1.3.1 PACAP, a neuropeptide slow transmitter

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a peptide that was isolated from ovine hypothalamus based on its ability to stimulate cAMP in rat anterior pituitary cells (Miyata et al., 1989). The mature peptide PACAP occurs in two C-terminally α-amidated forms, PACAP-27 and PACAP-38, with PACAP-27 being identical to the first 27 amino acids of PACAP-38. PACAP-27 has 68% sequence homology with vasoactive intestinal polypeptide (VIP) (Said and Mutt, 1970), identifying PACAP as a member of the VIP-secretin-GHRH-glucagon superfamily (Miyata et al., 1990) (Fig. 2). The first 27 amino acids of PACAP have almost been completely preserved through vertebrate evolution, from fish to mammals, and are responsible for its biological activity. Mature PACAP is generated through cleavage of the 176-amino acid prepro-protein by various prohormone convertases (PCs). Another 29-amino acid peptide, PACAP-related peptide (PRP), is

generated from prepro-PACAP, and exhibits only moderate homology with PACAP. The structure of the PACAP precursor is similar to that of the VIP precursor, which generates the 28-amino acid peptide VIP and the 27-amino acid VIP-related peptide histidine methionine (PHM) in humans or peptide histidine isoleucine (PHI) in rodents, sheep and chicken (Arimura, 1998; Mustafa and Eiden, 2006; Sherwood et al., 2000; Vaudry et al., 2009).

PACAP's central and C-terminal domain bind to the N-terminal domain of the receptor and the N-terminal domain of the peptide interacts with the juxtamembrane domains of the receptor, stimulating intracellular signaling. The N-terminally truncated form of PACAP, PACAP(6-38), is a potent receptor antagonist (Robberecht et al., 1992). Maxadilan, a naturally occurring vasodilator polypeptide of 61 amino acids, is a potent and selective PAC1 receptor agonist and was isolated from the salivary gland of the sand fly. Interestingly, maxadilan possesses no significant sequence homology with PACAP (Lerner et al., 1991; Moro and Lerner, 1997).

PACAP-38	**HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK-NH2**
PACAP-27	**.............................-NH2**
VIP	**...AV...N.T.L.........NSI.N-NH2**

Figure 2: Amino acid sequences of human PACAP and VIP (., amino acid identical with PACAP-38).

PACAP is widely expressed in the nervous system, e.g., the hypothalamus, cerebral cortex, amygdala, nucleus accumbens, hippocampus and cerebellum of the central nervous system, and in sensory neurons, sympathetic preganglionic neurons and parasympathetic pre- and postganglionic neurons of the peripheral nervous system. PACAP-38 is the predominant form expressed (Arimura et al., 1991; Ghatei et al., 1993; Hamelink et al., 2002b; Hannibal, 2002; Sundler et al., 1996). Moreover, PACAP is found in tumors, particularly in gliomas, neuroblastomas, pheochromocytomas, pancreatic carcinomas and pituitary tumors, where the stimulation of cAMP can lead to cell proliferation and tumorigenesis (Fahrenkrug et al., 1995; Takahashi et al., 1993a; Takahashi et al., 1993b). PACAP is present in the embryonic brain, where its concentration increases with prenatal maturation (Masuo et al., 1994).

1.3.2 PACAP receptors, members of the GPCR family B

As neuropeptides, PACAP and VIP bind to G protein-coupled receptors (GPCRs). The high sequence homology between these two peptides suggests that they can bind to common GPCRs. Three VIP/PACAP receptors have been identified named VPAC1, VPAC2 and PAC1, which are members of the family B of the GPCR superfamily (Harmar, 2001; Harmar et al., 1998; Ishihara et al., 1992; Lutz et al., 1993; Pisegna and Wank, 1993). VPAC1 and 2 bind VIP and PACAP with similar affinities (type II binding sites) whereas PAC1 binds PACAP with high affinity and VIP with much lower affinity (type I binding sites; Fig. 3).

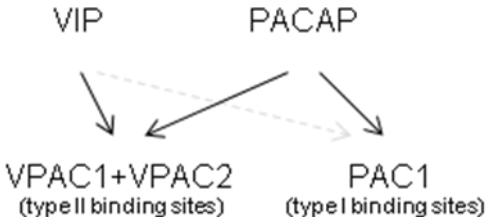
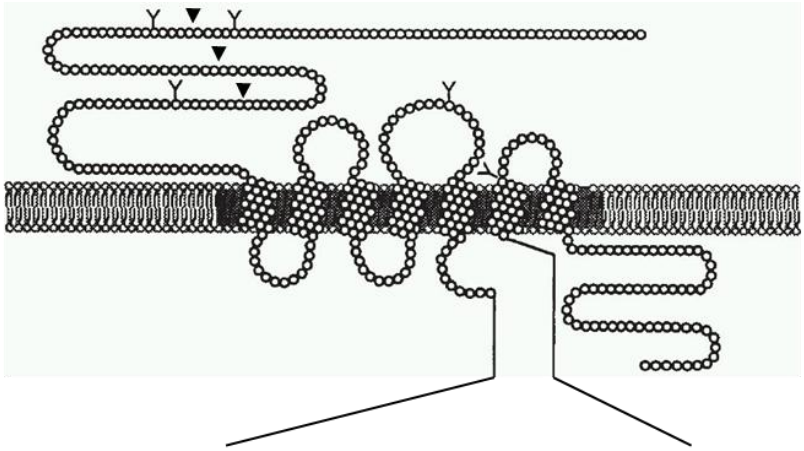


Figure 3: VIP and PACAP bind to three common GPCRs. VPAC1 and VPAC2 bind VIP and PACAP with similar affinities (type II binding sites), whereas PAC1 is a PACAP-preferring receptor (type I binding sites).

Several isoforms of the PAC1 receptor have been identified in vivo. These are mainly generated through alternative splicing within two regions of the PAC1 gene: the N-terminus and the third intracellular loop (ic3). N-terminal variants result from deletions (21 or 57 amino acids) at the N-terminal extracellular domain affecting ligand binding and the relative potencies of the ligands in second messenger stimulation (Dautzenberg et al., 1999; Pantaloni et al., 1996). In the rat testis a 24-amino acid N-terminal insertion (designated 3a) has been identified (Daniel et al., 2001). Ic3 variants result from the presence or absence of different insertions at the C-terminal end of the loop, a domain thought to be crucial for interaction with G proteins. Each insertion, designated hip and hop, consists of an 84-bp cassette. The alternative use of two contiguous consensus splice acceptor sites at the 5`-end of the hop cassette generates hop1 and hop2. The hip cassette can be included together with the hop cassette to give rise to hiphop. The null form does not contain any insert. The hop cassette encodes a consensus motif for phosphorylation by PKC (Spengler et al., 1993) (Fig. 4).



```
hop1:  TM5-QKLQSPDMGGNESSIYFSCVQKCYCKPQRAQQHSCKMSELSTITLRLARS-TM6
hop2:  TM5-QKLQSPDMGGNESSIYF-CVQKCYCKPQRAQQHSCKMSELSTITLRLARS-TM6
hip:   TM5-QKLQSPDMGGNESSIYLTNLRLRVPKKTREDPLPVPSDQHSPPFLRLARS-TM6
null:  TM5-QKLQSPDMGGNESSIYL----------------------------RLARS-TM6
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Figure 4: Amino acid sequences of the third intracellular loop of rat PAC1. Alternative splicing gives rise to hop1, hop2, hip, null, hiphop1 and hiphop2 (not shown; PKC phosphorylation site is underlined; arrowheads indicate the splice sites for the generation of the 21 and 57 amino acid N-terminal deletions (designated short and very short N-terminus, respectively); Y marks N-glycosylation sites; TM5 and 6: transmembrane domain 5 and 6; modified from Spengler et al., 1993).

Type I and II binding sites and their corresponding receptor transcripts are located throughout the central and peripheral nervous system. Both types of binding sites are found in early embryonic stages throughout the nervous system and their density increases during embryonic and early postnatal development. Type I sites are generally more abundant than type II sites. Brain structures with high concentrations of type I binding sites and PAC1 receptor expression include the cerebral and cerebellar cortex, hypothalamus, thalamus, hippocampus, olfactory bulb, amygdala, substantia nigra and brain stem. Type II binding sites show complementary distribution; VPAC1 is predominantly expressed in the cerebral cortex and hippocampus and strong VPAC2 expression is found in thalamus, hypothalamus and amygdala (Hashimoto et al., 1993; Joo et al., 2004; Sheward et al., 1995; Shioda et al., 1997). The predominant forms of the PAC1 receptor in the brain are the ic3-splice variants containing the hop cassette (PAC1hop) and without any cassette (PAC1null). The embryonic brain expresses high levels of receptors containing a short N-terminus

lacking 21 amino acids, whereas the receptor with a full-length N-terminus is the predominant form in the adult brain. Interestingly, the short N-terminus shows higher sequence homology with other members of the class B GPCR subfamily and increases the potency of VIP at the receptor (Cavallaro et al., 1996; Dautzenberg et al., 1999; Harmar, 2001; Lutz et al., 2006; Pantaloni et al., 1996; Pisegna and Wank, 1996; Zhou et al., 2000). Receptors are not only present on neurons but are also expressed in glial cells (Grimaldi and Cavallaro, 1999; Pilzer and Gozes, 2006; Tatsuno et al., 1991). Moreover, PACAP receptors are expressed in the anterior and posterior lobe of the pituitary gland, the adrenal medulla (Hashimoto et al., 1996; Mazzocchi et al., 2002; Moller and Sundler, 1996; Shioda et al., 2000) and many other tissues and organs including the retina, placenta and testis as well as cells of the digestive, respiratory, cardiovascular and immune system. The PAC1hop1 receptor with a full-length N-terminus is the predominant variant expressed in chromaffin cells of the adrenal medulla (Mustafa et al., 2007; Nogi et al., 1997; Ushiyama et al., 2007). PACAP receptors are also widely expressed in tumor cells from e.g., pheochromocytomas and neuroblastomas (Waschek et al., 1995; Watanabe et al., 1990). The very widespread distribution of PACAP and its receptors as well as their expression during ontogenesis is highly supportive of the fact that PACAP-mediated signaling is involved in the regulation of a plethora of biological functions (Mustafa and Eiden, 2006; Vaudry et al., 2009; Vaudry et al., 2000b).

1.3.3 PACAP-mediated signaling

All PACAP receptors regulate cAMP generation by coupling to adenylate cyclases (ACs) through Gαs (Pisegna and Wank, 1993; Spengler et al., 1993; Usdin et al., 1994). Moreover, stimulation of other second messengers, including phospholipase C (PLC), calcium and phospholipase D (PLD) have been reported (Delporte et al., 1995; Dickson et al., 2006; MacKenzie et al., 2001; MacKenzie et al., 1996; McCulloch et al., 2001; Ronaldson et al., 2002; Spengler et al., 1993; Ulrich et al., 1998). Specifically, coupling to PLCβ varies among the different PAC1 receptors alternatively spliced in the third intracellular loop. Inositol phosphate (IP) production by coupling to PLCβ through Gαq is more efficacious in PAC1hop and null compared to hip receptors (Lutz et al., 2006; Pisegna and Wank, 1996; Spengler et al., 1993).

The mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1 and 2 (ERK1/2) are important mediators in PACAP signaling to the nucleus. ERK activity is regulated through a three-component signaling cassette: the MAPKKK Raf phosphorylates the MAPKK MEK1/2, which in turn phosphorylates the MAPK ERK1/2. Sustained ERK activation by PACAP mediating neuronal differentiation requires activation of the Ras-related small G protein Rap1 and subsequent stimulation of B-Raf, the neuronal Raf isoform (Barrie et al., 1997; Lazarovici et al., 1998; Ravni et al., 2008).

Differentiation by nerve growth factor (NGF) in PC12 cells is also mediated through prolonged ERK activation, whereas proliferation by epidermal growth factor (EGF) is mediated through transient ERK activation. The neurotrophic factors NGF and EGF activate receptor tyrosine kinases (RTKs) leading to the stimulation of Ras and Rap1, inducing the activation of B-Raf (Kao et al., 2001; York et al., 1998). Signaling to ERK is determined by the transduction pathway used by the receptor but also by the level of receptor expression. The same growth factor receptor can stimulate proliferation or differentiation depending on cell context and expression level (Greene and Tischler, 1976; Marshall, 1995; Murphy and Blenis, 2006; Santos et al., 2007).

Protein kinase A (PKA) stimulates the activation of B-Raf through Rap1 leading to neuronal differentiation (Grewal et al., 2000; Vossler et al., 1997), however, cAMP-mediated activation of B-Raf can also occur independently of the canonical cAMP effector PKA (Dumaz and Marais, 2005). PACAP-mediated neuronal differentiation through cAMP, Rap1 and ERK1/2 can also proceed independently of PKA (Ravni et al., 2008).

1.3.4 Pleiotropic biological functions of PACAP

1.3.4.2 Hypothalamic and behavioral functions

The high expression of PACAP and its receptors in the hypothalamus indicates that PACAP regulates hypothalamic functions. Abundantly expressed in the paraventricular and supraoptic nucleus, it stimulates oxytocin and vasopressin release in the neural lobe of the pituitary. PACAP also modulates hypothalamic releasing hormones that regulate hormone secretion from the anterior pituitary; e.g., PACAP can activate corticotropin releasing hormone (CRH) neurons in the paraventricular

nucleus (PVN), therefore stimulating the release of adrenocorticotropic hormone (ACTH) in anterior pituitary cells to increase glucocorticoid levels and enhance stress responses (Stroth and Eiden, 2010). In addition to regulating stress responses at the level of the hypothalamo-pituitary-adrenal (HPA) axis, PACAP acts as a co-transmitter at the sympathoadrenal synapse, controlling adrenomedullary catecholamine synthesis and secretion during prolonged splanchnic nerve stimulation in response to metabolic stress (hypoglycemia). (Hamelink et al., 2002b; Stroth et al., 2011). PACAP and its receptors are expressed in neurons of the arcuate nucleus, which secrete neuropeptide Y (NPY) and α-melanocyte-stimulating hormone (α-MSH), playing a role in the regulation of food intake. PACAP is expressed in nerve fibers of the suprachiasmatic nucleus (SCN) of the hypothalamus corresponding to the projection of the retinohypothalamic tract and in nerve fibers in the pineal gland, thereby regulating circadian rhythmicity. PACAP is also expressed in cells of the preoptic nucleus, which play an important role in the regulation of body temperature (Hannibal, 2002).

PACAP's widespread central expression suggests a regulatory role in various physiological and behavioral processes. This is supported by the phenotypes of PACAP- and PACAP receptor-deficient mice. PACAP- and PAC1-deficient mice show altered psychomotor behaviors, such as increased locomotor activity in novel environments and reduced anxiety-like behavior, abnormal social behavior, and impairments in hippocampal long-term potentiation (LTP) and learning and memory (Hashimoto et al., 2006; Hashimoto et al., 2001; Matsuyama et al., 2003; Nicot et al., 2004; Otto et al., 2001a; Otto et al., 2001b).

1.3.4.3 Neurotrophic and cytoprotective functions

In the developing nervous system, PACAP functions as a neurotrophic factor promoting cell survival and neurite outgrowth in a variety of cell types, including cerebellar granule cells (Gonzalez et al., 1997; Vaudry et al., 2000a), cortical neuroblasts (Lu and DiCicco-Bloom, 1997) and dorsal root ganglion neurons (Lioudyno et al., 1998). PACAP also promotes survival and regeneration in the mature nervous system and inhibits apoptotic cell death under paraphysiological and pathophysiological conditions, such as stroke (Brenneman, 2007; Waschek, 2002). In vivo, endogenous and exogenous PACAP reduce infarct volume and ameliorate

neurological defects after permanent middle cerebral artery occlusion (pMCAO) (Chen et al., 2006; Ohtaki et al., 2006; Reglodi et al., 2002; Tamas et al., 2002). PACAP reduces the effect of focal and global transient ischemia even when given up to several hours post-insult (Reglodi et al., 2000; Stetler et al., 2010; Uchida et al., 1996). Moreover, PACAP crosses the blood-brain-barrier (BBB) via saturable peptide transport and has neuroprotective effects after ischemia not only when administered intracerebroventricularly (i.c.v.) but also when given intravenously (i.v.) (Banks et al., 1993; Banks et al., 1996). PACAP is also protective in other neurodegenerative conditions, e.g., in association with Parkinson's (Reglodi et al., 2006; Reglodi et al., 2004) and Alzheimer's disease (Kojro et al., 2006; Rat et al., 2011). In cell culture, PACAP promotes the survival of cultured cortical neurons under hypoxic/ischemic and excitotoxic conditions (Frechilla et al., 2001; Morio et al., 1996; Nowak et al., 2007; Pellegri et al., 1998; Said et al., 1998; Shintani et al., 2005; Stumm et al., 2007). Multiple other cell types are protected by PACAP under a variety of conditions that promote apoptosis. Specifically, PACAP ameliorates cell death in hippocampal cultures exposed to the HIV envelope protein gp120 (Arimura et al., 1994) and in cerebellar granule cells incubated in low-potassium or serum-free medium, or exposed to ethanol or oxidative stress (Cavallaro et al., 1996; Kienlen Campard et al., 1997; Tabuchi et al., 2003; Vaudry et al., 2002b; Vaudry et al., 2002c; Villalba et al., 1997). Serum and NGF withdrawal-induced cell death in differentiated PC12 cells (Tanaka et al., 1997) and primary sympathetic neurons (May et al., 2010) is reduced by PACAP. PACAP also rescues motoneurons (Tomimatsu and Arakawa, 2008), retinal neurons (Shoge et al., 1999) and PC12 cells (Onoue et al., 2002b) from glutamate-induced excitotoxicity.

PACAP's neuroprotective effects during glutamate-induced excitotoxicity in cortical neurons (Morio et al., 1996; Said et al., 1998) and during K^+ and serum deprivation and hydrogen peroxide exposure of cerebellar granule neurons (Cavallaro et al., 1996; Vaudry et al., 2002b) are mimicked by intracellular cAMP production, suggesting that PACAP mediates its effects via a cAMP dependent signaling pathway. Activation of PKA (Kienlen Campard et al., 1997; Shoge et al., 1999; Tanaka et al., 1997; Tomimatsu and Arakawa, 2008; Vaudry et al., 1998b) and ERK1/2 (May et al., 2010; Pugh and Margiotta, 2006; Vaudry et al., 2002b; Villalba et al., 1997) have been implicated in mediating PACAP's neuroprotective effects in various cell types

under diverse conditions promoting apoptosis. PACAP-mediated neuritogenesis in PC12 cells can occur via PKA dependent (Hernandez et al., 1995) and PKA independent but cAMP and ERK1/2 dependent (Ravni et al., 2008) signaling pathways.

It has been suggested that PACAP mediates its neuroprotective effects via direct and indirect mechanisms (Dejda et al., 2011). The activation of PKA (Shoge et al., 1999; Stetler et al., 2010; Tanaka et al., 1997) and ERK1/2 (May et al., 2010; Pugh and Margiotta, 2006; Stumm et al., 2007; Villalba et al., 1997) and the inhibition of caspase-3 (Dejda et al., 2008; Onoue et al., 2002a; Vaudry et al., 2002b) have been shown to be directly involved in PACAP-mediated prevention of neuronal apoptosis induced by various insults. PACAP also suppresses cell death via BDNF gene induction in cortical neurons exposed to excitotoxicity (Frechilla et al., 2001). Indirectly, PACAP may mediate neuroprotection via modulation of glial cells, such as astroglia (Stumm et al., 2007) and microglia (Armstrong et al., 2008; Delgado et al., 2003b) to provide neurotrophic support and control of the inflammatory response, respectively. PACAP-induced astroglial IL-6 release (Gottschall et al., 1994) has been implicated in PACAP's neuroprotective effects during ischemia in vivo (Ohtaki et al., 2006).

1.4 Stanniocalcin 1 (STC1), a potential mediator of PACAP's cytoprotective effects

STC (previously called teleocalcin) was originally identified in bony fish, where it is secreted from the corpuscules of Stannius (a kidney-associated specialized gland) in response to increased extracellular calcium levels, regulating calcium and phosphate homeostasis and protecting against toxic hypercalcemia (Wagner et al., 1989; Wagner and Jaworski, 1994). Specifically, STC reduces plasma [Ca^{2+}] by inhibiting calcium uptake through gills (Wagner et al., 1986) and gut (Sundell et al., 1992) and stimulates renal phosphate (P_i) reabsorption through a cAMP and PKA dependent pathway (Lu et al., 1994). For almost ten years, STC was considered to be a polypeptide hormone with calcitonin- and parathyroid hormone (PTH)-like properties unique to fish (Gerritsen and Wagner, 2005). Its discovery in mammals and molecular cloning revealed that STC is highly conserved through vertebrate evolution, especially within its N-terminal and core region (Chang et al., 1996; Chang et al.,

1995; Wagner et al., 1995). A second STC protein with 30% sequence homology to STC1 has been identified, first in mammals and subsequently in fish (Chang and Reddel, 1998; Luo et al., 2005). STC1 is a ~28-kDa glycoprotein that exists as a 56-kDa homodimer; no significant homology to other known proteins or previously identified motifs has been detected to date. A notable feature of STC1 are its 11 cysteine residues, of which the first ten form five intramolecular disulfide bonds and the last one forms an interchain disulfide linkage creating a homodimer (Trindade et al., 2009; Wagner and Dimattia, 2006) (Fig. 5).

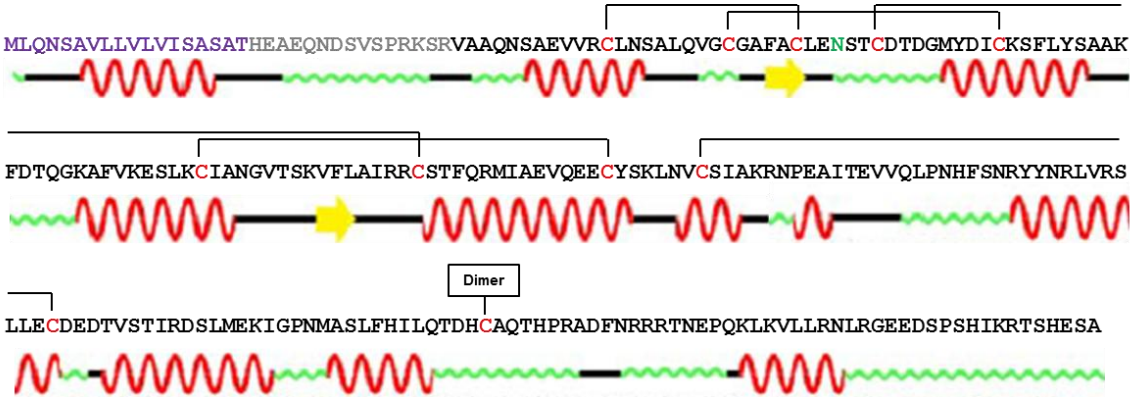


Figure 5: Amino acid sequence and predicted secondary structure of human STC1. The primary translation product is 247 residues and 25 kDa. Different regions from N- to C-terminus: signal peptide: 1-28 (purple), pro-peptide: 19-33 (gray), mature protein: 34-247 (black). 11 cysteine residues (red) give rise to five intramolecular disulfide bridges (indicated by black horizontal brackets) and one interchain disulfide linkage, creating a homodimer. N-linked glycosylation (green) at the site of the type N-X-T/S adds ~3 kDa. Below the sequence is a schematic representation of the predicted secondary structure: red: alpha helix, yellow: beta sheet, green: coil region, black: not assigned (modified from Trindade et al., 2009).

In mammals, STC1 is expressed in many tissues, in contrast to its localized glandular expression in fish, suggesting an autocrine/paracrine rather than an endocrine function extending beyond the regulation of mineral metabolism (De Niu et al., 2000). The highest level of expression is seen in adult ovaries, where STC1 exists as three higher molecular proteins of 84, 112 and 135 kDa. All three “big STCs” are chemically reduced to one 45 kDa protein (Paciga et al., 2002). STC1 of 56 kDa is, among other tissues, expressed in kidney, adrenal, heart, lung, thymus, brain and developing bone. STC1 protein can accumulate in tissues through receptor-mediated

sequestration. Subcellularly, it is targeted to mitochondria in nephron cells and liver hepatocytes, increasing respiration rate (Ellard et al., 2007; McCudden et al., 2002), and to other organelles such as lipid storage droplets (LSD) in adipocytes and steroidogenic cells (Paciga et al., 2003) or the nucleus in lactating mammary gland alveolar cells (Hasilo et al., 2005). Within the kidney, STC1 stimulates P_i reabsorption, consistent with its function in fish (Wagner et al., 1997).

STC1 is upregulated during neuronal differentiation (Zhang et al., 1998) and in neurons of the ischemic penumbra (i.e., ischemic but still viable cerebral tissue), presumably supporting neuronal survival (Zhang et al., 2000). In cell culture, STC1 increases cell resistance to hypoxic and hypercalcemic insult (Zhang et al., 2000). Induction of STC1 after neuronal and myocardial hypoxic preconditioning, conferring resistance to further ischemic damage, is dependent on IL-6 (Westberg et al., 2007a; Westberg et al., 2007b). STC1 is a PACAP-regulated gene, as shown in chromaffin cells of the adrenal medulla by our laboratory (Ait-Ali et al., 2010), potentially mediating PACAP's neurotrophic and cytoprotective effects. STC2 is also induced by various stressors, contributing to cell survival; however, its induction is, unlike that of STC1, dependent on the unfolded protein response (UPR) activated by endoplasmic reticulum (ER) stress (Ito et al., 2004).

STC1 and STC2 transgenic mice exhibit altered mineral homeostasis, higher rates of metabolism, reduced female fertility and growth retardation (Filvaroff et al., 2002; Gagliardi et al., 2005; Varghese et al., 2002). STC1-, STC2- and STC1/2-null mice, however, show no changes in mineral homeostasis, fertility or development. Increased postnatal growth in STC2- and STC1/2-null mice is the only detected phenotype so far (Chang et al., 2005; Chang et al., 2008).

1.5 Aims

As outlined above, PACAP's effects in the central and peripheral nervous system are mainly mediated by its cognate G protein-coupled receptor PAC1. The predominant variants are the receptors with the hop cassette (PAC1hop) or no cassette (PAC1null) in the third intracellular loop (ic3) and a full-length N-terminus. Receptors containing a hip cassette (PAC1hip) are less abundant. Although the different ic3 splice variants were first discovered almost 20 years ago, an understanding of the signaling pathways used by these different variants in neural and neuroendocrine cells is still lacking.

Moreover, whether these variants mediate different functional outcomes, specifically under paraphysiological conditions, such as prolonged stress, is not clear.

Therefore, the aim of the present study was to investigate the induction of the second messengers cAMP and Ca^{2+} by the PAC1hop, null and hip receptor variants with a full-length N-terminus in neural and neuroendocrine cell lines through over-expression of the respective receptor variants. Moreover, the signal transduction pathway mediating the induction of STC1, a gene potentially involved in PACAP's neuroprotective effects in vivo, was characterized. The biological significance of mammalian STC1 still remains elusive. Elucidation of its gene regulation will give important information towards understanding its function in vivo. Experiments from heterologous expression systems were extended to primary cortical neurons. The detailed aims were the following:

(1) Characterization of PAC1 splice variant-specific signaling mediating sustained catecholamine secretion from neuroendocrine cells

To determine PAC1 splice variant-specific signaling in neuroendocrine cells, the three main splice variants in the mature nervous system, the PAC1hop1, null and hip variant with a full-length N-terminus, were expressed in the rat pheochromocytoma PC12-G cell line. The intracellular Ca^{2+} response and catecholamine secretion, in particular sustained secretion was investigated to give new insight into the mechanisms by which PACAP induces long-term secretion from adrenomedullary chromaffin cells in vivo to restore homeostasis during periods of prolonged stress.

(2) Characterization of PAC1 splice variant-specific signaling in neural cells of the central nervous system and the signaling pathways mediating STC1 gene induction

The three PAC1 variants were expressed in the mouse neuroblastoma x rat glioma NG108-15 cell line and the intracellular cAMP and Ca^{2+} responses measured. Moreover, signaling pathways mediating ERK1/2 activation and STC1 induction were investigated to provide preliminary information about the regulation of the putative neuroprotectant STC1 in the central nervous system.

(3) Establishing the signaling pathways mediating STC1 gene induction in primary neurons, potentially mediating PACAP dependent ischemic stress responses in vivo

To extend results obtained from heterologous expression systems, signaling pathways mediating STC1 gene induction were investigated in primary cultures of differentiated rat cortical neurons. Elucidation of STC1 gene induction in primary neurons was used to provide important insights in its regulation in vivo, helping to identify how PACAP mediates its neuroprotective effects in neurodegenerative disorders, such as stroke.

(4) Effects of PACAP and the PACAP target gene STC1 in neuroprotection

Neuroprotective effects of PACAP in conditions associated with stroke were studied in primary rat cortical neurons. Moreover, STC1 was over-expressed in neurons to study its role in neuroprotection to provide mechanistic insights into how PACAP mediates its neuroprotective effects during stroke in vivo.

2. Material

2.1 Chemicals

(+)-MK-801 hydrogen maleate	Sigma, St. Louis, MO
0.25% Trypsin-EDTA	Gibco, Carlsbad, CA
2′5′-Dideoxyadenosine, 1 mg	CalBiochem, San Diego, CA
2′5′-Dideoxyadenosine, 5 mg	Sigma, St. Louis, MO
3(4,5-dimethylthiazolyl-2)2,5-diphenyl-tetrazoliumbromide	Sigma, St. Louis, MO
3-isobutyl-1-methylxanthine (IBMX)	Sigma, St. Louis, MO
Acetic acid glacial	Chempure, New York, NY
Agarose	Invitrogen, Carlsbad, CA
Ampicillin sodium salt	Sigma, St. Louis, MO
Ascomycin	Sigma, St. Louis, MO
B-27 serum-free supplement, 50X	Gibco, Carlsbad, CA
BAPTA, AM	Invitrogen, Carlsbad, CA
Bisbenzimide Hoechst 33342	Sigma, St. Louis, MO
Bisindolylmaleimide I	Sigma, St. Louis, MO
BSA	Santa Cruz Biotechnology, Santa Cruz, CA
Cocktail inhibitor tablet	Roche Applied Science, Mannheim, Germany
Cyclosporin A	Sigma, St. Louis, MO
DEPC treated water	Quality Biological Inc., Gaithersburg, MD
Dexamethasone	Sigma, St. Louis, MO
Dimethyl sulfoxide (DMSO)	Sigma, St. Louis, MO
Dimethylformamide (DMF)	Sigma, St. Louis, MO
DMEM 1X, high glucose	Gibco, Carlsbad, CA
EDTA, 0.5 M	Mediatech Inc., Herndon, VA
Ethidium bromide solution	Invitrogen, Carlsbad, CA
Fetal bovine serum (FBS)	Gibco, Carlsbad, CA
Filter Paper Sandwich (0.45µm pore size)	Invitrogen, Carlsbad, CA
Forskolin, Coleus forkohlii	CalBiochem, San Diego, CA
Fura-2 AM	Molecular Probes, Eugene, OR
G418, Geneticin	Gibco, Carlsbad, CA
Gene Amp 10 mM dNTP Mix with dTTP	Applied Biosystems, Carlsbad, CA
GeneRuler DNA ladders (100 bp and 1 kb)	Fermentas, Glen Burnie, MD
Glycerol	Sigma, St. Louis, MO
H-89, Dihydrochloride	CalBiochem, San Diego, CA

Halt phosphatase inhibitor cocktail	Thermo Scientific, Rockford, IL
HAT supplement, 50X	Gibco, Carlsbad, CA
HEPES, 1 M	Gibco, Carlsbad, CA
Hoechst 33342	Sigma, St. Louis, MO
Horse Serum	Cambrex, East Rutherford, NJ
iQ SYBR Green Supermix	Bio-Rad, Carlsbad, CA
Levo-7[^{3}H]-Norepinephrine	PerkinElmer, Waltham, MA
L-Glutamic acid	Sigma, St. Louis, MO
L-glutamine, 200 mM	Gibco, Carlsbad, CA
Magic Mark XP Western Standard	Invitrogen, Carlsbad, CA
Methanol	Fisher Scientific, Pittsburgh, PA
Neurobasal medium	Gibco, Carlsbad, CA
Neurobasal-A medium without glucose	Gibco, Carlsbad, CA
Neurobasal-A medium	Gibco, Carlsbad, CA
Nitric acid	Sigma, St. Louis, MO
NMDA	Sigma, St. Louis, MO
Norepinephrine, levo-[7-^{3}H]-	PerkinElmer, Waltham, MA
NuPAGE LDS sample buffer	Invitrogen, Carlsbad, CA
NuPAGE MOPS SDS Running Buffer	Invitrogen, Carlsbad, CA
NuPAGE Reducing agent, 10X	Invitrogen, Carlsbad, CA
NuPAGE transfer buffer	Invitrogen, Carlsbad, CA
NuPAGE transfer buffer, 20X	Invitrogen, Carlsbad, CA
PACAP(6-38)	Phoenix Pharmaceuticals, Mountain View, CA
PACAP38 (Human, Ovine, Rat)	Phoenix Pharmaceuticals, Mountain View, CA
Penicillin-Streptomycin	Gibco, Carlsbad, CA
Poly-L-lysine hydrobromide, mol wt >300,000	Sigma, St. Louis, MO
Ponceau S	Sigma, St. Louis, MO
Propidium Iodide	Sigma, St. Louis, MO
Puromycin, DiHCl, Cell Culture-T	CalBiochem, San Diego, CA
Restore Western Blot Stripping Buffer	Thermo Fisher Scientific, Rockford, IL
See blue Plus 2 Prestained Standard 1x	Invitrogen, Carlsbad, CA
SOC	Quality Biological Inc., Gaithersburg, MD
Sodium chloride, 5 M	Quality Biological Inc., Gaithersburg, MD
Sodium Dodecyl Sulfate (SDS)	Bio-Rad, Carlsbad, CA
Super Signal West Pico Chemiluminescence Substrate	Thermo Fisher Scientific, Rockford, IL
TAE	Quality Biological Inc., Gaithersburg, MD
TrackIt DNA ladder (100 bp)	Invitrogen, Carlsbad, CA
Tris-HCl	Quality Biological Inc., Gaithersburg, MD
Triton X-100	Sigma, St. Louis, MO
Tween 20	Sigma, St. Louis, MO

U0126	CalBiochem, San Diego, CA
U-73122	CalBiochem, San Diego, CA
U-73343	CalBiochem, San Diego, CA
Ultima Gold ^{TM} (high flash-point LSC cocktail)	PerkinElmer, Waltham, MA

2.2 Enzymes and inhibitors

Ampli Taq Gold DNA Polymerase (5U/µl)	Applied Biosystems, Carlsbad, CA
Calf intestinal alkaline phosphatase (CIP)	New England Biolabs, Ipswich, MA
DNase I (D4527)	Sigma, St. Louis, MO
DNase I recombinant, RNase-free (10U/µl)	Roche Applied Science, Mannheim, Germany
NEBuffer for Restriction Endonucleases	New England Biolabs, Ipswich, MA
Restriction Endonucleases	New England Biolabs, Ipswich, MA
Restriction Endonucleases	Roche Applied Science, Mannheim, Germany
RNase inhibitor	Invitrogen, Carlsbad, CA
Superscript II RT	Invitrogen, Carlsbad, CA
SuRE Cut Buffers for Restriction Endonucleases	Roche Applied Science, Mannheim, Germany
T4 DNA ligase	Roche Applied Science, Mannheim, Germany
T4 DNA Polymerase	Invitrogen, Carlsbad, CA

2.3 Kits

cAMP Biotrak Enzymeimmunoassay (EIA) System	Amersham Biosciences
DC protein assay	Bio-Rad, Hercules, CA
Plasmid Maxi Kit	Qiagen, Valencia, CA
ProFection Mammalian Transfection System Kit	Promega, Madison, WI
QIAprep Spin Miniprep Kit	Qiagen, Valencia, CA
QIAquick Gel Extraction Kit	Qiagen, Valencia, CA
Rapid DNA Ligation Kit	Roche Applied Science, Mannheim, Germany
RNeasy Mini Kit	Qiagen, Valencia, CA
TOPO TA Cloning Kit (pCR2.1-TOPO Vector)	Invitrogen, Carlsbad, CA
Wizard DNA clean-up system	Promega, Madison, WI

2.4 Antibodies

Goat Ant-Rabbit IgG, Peroxidase Conjugated	Thermo Scientific, Rockford, IL
p44/42 MAPK (Erk1/2)	Cell Signaling Technology, Beverly, MA
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology, Beverly, MA

2.5 Oligonucleotides

Oligonucleotides were selected based on sequences published in PrimerBank (http://pga.mgh.harvard.edu/primerbank/) or designed with Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and tested with the PCR primer stats feature of the sequence manipulation suite (http://www.bioinformatics.org/sms2/). Nucleotide sequences were tested for homologous sequences using BLAST (Basic Logical Alignment Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The design was based on length (18-26 bases), guanine and cytosine (GC) content (40-60%) and melting temperature (55-70°C). Moreover, the presence of more than one G or C in the last five bases was favored. Oligonucleotides that had more than three self-annealing bases in a row or lead to the formation of hairpins were excluded. All oligonucleotides were synthesized by Integrated DNA Technologies (IDT).

Primer	**Sequence (5′ → 3′)**	**Amplicon [bp]**	**GenBank Accession**
PAC1 F [b]	GGC CCC GTG GTT GGC TCT ATA ATG G		Z23279.1 and NM_001025372.1
PAC1 R [b]	GAG AGA AGG CGA ATA CTG TG	187 or 271	
PAC1hop R [b]	AGA GTA ATG GTG GAT AGT TCT GAC A	200	Z23274.1 and NM_007407.3
PAC1hip R [b]	TGG GGA CTC TCA GTC TTA AA	142	Z23273.1
VPAC1 F	GCA GCA ACA GAC CAA GTT CTA C		NM_012685.2 and NM_011703.4
VPAC1 R	TGA ACA GGC TCA AGA TAG CCA T	107	
VPAC2 F	AAG CAA AAA CTG CAC TAG TGA		NM_017238.1 and NM_009511.2
VPAC2 R	GCC CAA GGT ATA AAT GGC CTT CA	133	
GAPDH F	GTT ACC AGG GCT GCC TTC TC		NM_017008.3 and NM_008084.2
GAPDH R	GGG TTT CCC GTT GAT GAC C	168	
STC1 F	CTA CTT TCC AGA GGA TGA TCG C		NM_031123.2 and NM_009285.3
STC1 R	ACT TCA GTG ATG GCT TCC GG	100	
STC2 F	GTC ACC CGA AGC GTC CAG GCT		NM_022230.1 and NM_011491.3
STC2 R	TCC CTC GCT CAC CCT TGG CA	208	
BDNF F	TCA TAC TTC GGT TGC ATG AAG G		NM_012513.3 and NM_007540.4
BDNF R	AGA CCT CTC GAA CCT GCC C	137	

All primers were designed to match the respective rat and mouse sequences.

[b] The PAC1 forward primer was used in combination with all PAC1 reverse primers. PAC1 F matches the beginning of transmembrane region five (TM 5) corresponding to bases 1018 to 1037 of the rat PAC1null sequence Z23279. PAC1 R matches the end of TM 6 corresponding to bases 1185 to 1204 of the rat PAC1null sequence Z23279. PAC1hop R matches the end of the hop cassette corresponding to bases 1193-1217 of the rat PAC1hop1 sequence Z23274. PAC1hip R matches the beginning of the hip cassette corresponding to bases 1140-1159 of the rat PAC1hip sequence Z23273. PAC1 F/PAC1 R generates a 187- or 271-bp fragment, depending on whether or not a hip- or hop-insert is present.

2.6 Plasmids

pBluescript II KS (+)-STC1	Gopal Thinakaran, Univ. of Chicago
pLVX-IRES-ZsGreen1	Clontech, Mountain View, CA
pPRIG	Patrick Martin, Univ. of Nice
pPRIG-rPAC1null, hop1 and hip	Tomris Mustafa, NIH

2.7 Buffers and solutions

KRB	20 mM HEPES
	5.5 mM glucose
	125 mM NaCl
	5 mM KCl
	1 mM Na_2HPO_4
	1 mM $MgSO_4$
	1 mM $CaCl_2$
Ca^{2+}-free KRB	20 mM HEPES
	5.5 mM glucose
	125 mM NaCl
	5 mM KCl
	1 mM Na_2HPO_4
	1 mM $MgSO_4$
	100 µM EGTA

WB lysis buffer	1X NuPAGE LDS sample buffer 1X NuPAGE reducing agent 1X cocktail inhibitor tablet 1X Halt phosphatase inhibitor cocktail
Ponceau S solution	0.1% Ponceau S 5% acetic acid glacial
TBS	50 mM Tris-HCl (pH 8) 150 mM NaCl
TBST	50 mM Tris-HCl (pH 8) 150 mM NaCl 0.1% Tween 20
WB blocking buffer	TBST 5% skim milk
WB transfer buffer	1X NuPAGE transfer buffer 10% Methanol

2.8 Cell culture media

293T cells	DMEM 1X, high glucose supplemented with: 10% FBS, heat-inactivated 2 mM L-glutamine 100 U/ml penicillin and 100 µg/ml streptomycin
PC12-G cells	DMEM 1X, high glucose supplemented with: 7% FBS, heat-inactivated 7% Horse serum 2 mM L-glutamine 25mM HEPES 100 U/ml penicillin and 100 µg/ml streptomycin

NG108-15 cells	DMEM 1X, high glucose supplemented with: 10% FBS, heat-inactivated 2 mM L-glutamine 1X HAT 100 U/ml penicillin and 100 µg/ml streptomycin
Cortical cells	1st day: DMEM 1X, high glucose supplemented with: 10% FBS, heat-inactivated 2 mM L-glutamine After 1st day: Neurobasal medium supplemented with: 1X B-27 serum-free supplement 0.5 mM L-glutamine 25 µM L-glutamate
OGD medium	Glucose-free Neurobasal-A medium supplemented with: 1X B-27 serum-free supplement 0.5 mM L-glutamine

Heat-inactivation of FBS for 30 min at 56°C

2.9 Cells

HEK 293T cells	Cell Genesys Inc., Foster City, CA
MAX Efficiancy DH5α competent cells	Invitrogen, Carlsbad, CA
NG108-15 cells	ATCC, Manassas, VA
PC12-G cells	(Greene and Tischler, 1976)
PC12-bPAC1hop cells	(Mustafa et al., 2007)

2.10 Animals

Wistar rats (17 days pregnant) were purchased from Charles River, Wilmington, MA

2.11 Equipment

C24 incubator shaker	New Brunswick Scientific, Edison, NJ
Centrifuge 5415C	Eppendorf
Centrifuge 5810	Eppendorf
Centrifuge RC5C Plus	Sorvall
Cetus DNA Thermal Cycler	PerkinElmer, Waltham, MA
Dual-Intensity Transilluminator	UVP
FluorChem 8800 Imaging System	Alpha Innotech
iCycler iQ Real Time PCR System	Bio-Rad, Carlsbad, CA
Image Station 440CF	Kodak
Isotemp Waterbath	Thermo Fisher Scientific
LS 6500 Scintillation Counter	Beckman Coulter, Brea, CA
Microscope IX70	Olympus
NanoVue Spectrophotometer	GE Healthcare, Piscataway, NJ
Nikon Eclipe Ti inverted microscope	Nikon instruments
PTC-225 Peltier Thermal Cycler	MJ Research
Rotors SLA-1500/3000	Sorvall
SpectraMax 340 Microplate Reader	Molecular Devices
The Belly Dancer	Stovall Life Science Inc., Greensboro, NC
Ultrasonic processor GE 130PB	Hielscher, Ringwood, NJ
XCELLII Blot Module (E19051)	Invitrogen, Carlsbad, CA

2.12 Software

ImageJ	public domain
iVision	BioVision Technologies, Exton, PA
MetaFluor	Molecular Devices, Silicon Valley, CA
NIS Elements	Nikon Instruments, Melville, NY
Prism 4	GraphPad Software, La Jolla, CA

2.13 Other supplies

0.45 μm syringe filter	Millipore, Bedford, MA
10-cm dish	Becton Dickinson, Franklin Lakes, NJ
162 cm^2 cell culture flask	Corning Incorporated, Corning, NY
75 cm^2 cell culture flask	Becton Dickinson, Franklin Lakes, NJ
Amicon Ultra-15 Centrifugal Filter Units	Millipore, Bedford, MA
Cryotube vial	Thermo Scientific, Rockford, IL
Falcon tube	Becton Dickinson, Franklin Lakes, NJ

Glass cover slips (diameter 1.5 cm)	Assistent, Sondheim/Rhoen, Germany
Multiple well culture plates	Corning Incorporated, Corning, NY
NuPAGE Novex Pre-Cast 4-12% Bis-Tris Gel	Invitrogen, Carlsbad, CA
Skim milk	Bio-Rad, Hercules, CA
Sponge pads	Invitrogen, Carlsbad, CA

3. Methods

3.1 Cell culture

3.1.1 Culture and propagation of PC12-G cells

PC12-G cells, derived from a rat adrenal pheochromocytoma (Greene and Tischler, 1976) were obtained from the laboratory of Dr. Gordon Guroff at NIH (Rausch et al., 1988) and cultured in 1X Dulbecco's modified Eagle's medium (DMEM) High Glucose (4.5 g/L D-Glucose) supplemented with 7% heat-inactivated (56ºC for 30 min) fetal bovine serum, 7% horse serum, 25 mM HEPES, penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) and 2 mM L-glutamine under 90% air and 10% CO_2. Cells were grown in 75 or 162 cm^2 cell culture flasks and passaged at approximately 80% cell density. Cells were mechanically dislodged by vigorous tapping and centrifuged for 5 min at 300 x g. Cells were resuspended in 10 ml fresh medium and triturated extensively before counting and replating to avoid the formation of cell aggregates. The cell suspension was triturated up to 10 times by passage through a 10-ml pipette fitted with a yellow (200 µl) pipette tip.

For experiments, cells were plated at a density of 100,000 cells/ 0.95 cm^2 growth area on poly-L-lysine-coated (0.1 mg/ml) multiple well culture plates and allowed to adhere overnight.

3.1.2 Culture and propagation of NG108-15 cells

NG108-15 cells (mouse neuroblastoma x rat glioma hybrid), obtained from the American Type Culture Collection (ATCC) were cultured in 1X DMEM High Glucose (4.5 g/L D-Glucose) supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin), 2 mM L-glutamine and 1X HAT (hypoxanthine, aminopterine, thymidine; for nucleotide synthesis via the salvage pathway) to maintain hybrid selection under 95% air and 5% CO_2. Cells were grown in 75 or 162 cm^2 cell culture flasks and passaged at approximately 80% cell density. Cells were mechanically dislodged by vigorous tapping, centrifuged for 5 min at 150 x g and resuspended in 10 ml fresh medium.

For experiments, cells were plated at a density of 30,000 cells/ 0.95 cm^2 growth area on poly-L-lysine-coated (0.1 mg/ml) multiple well culture plates and allowed to adhere overnight.

3.1.3 Culture and propagation of 293T cells

The human embryonic kidney (HEK) 293T cell line was cultured in 1X DMEM High Glucose (4.5 g/L D-Glucose) supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) and 2 mM L-glutamine under 95% air and 5% CO_2. Cells were grown in 75 cm^2 cell culture flasks and passaged at approximately 80% cell density. Cells were washed once with 5 ml PBS and incubated in 1 ml 0.25% Trypsin-EDTA until cells were detached (after ~3 min). 9 ml medium was added and cells were resuspended.

For transfection experiments, 2 x 10^6 cells were plated in poly-L-lysine-coated (0.1 mg/ml) 10-cm dishes (in 10 ml) and allowed to adhere overnight.

3.1.4 Preparation of frozen cell stocks

Frozen stocks of low passage numbers were stored in liquid nitrogen and prepared by resuspending ~25 x 10^6 PC12-G cells, ~6 x 10^6 NG108-15 cells or ~20 x 10^6 293T cells in 1 ml cold complete medium containing 5% (PC12-G cells) or 7.5% (NG108-15 and 293T cells) dimethyl sulfoxide (DMSO). The cell suspension was transferred to a cryostat vial, stored overnight at -80°C in an insulated container before transfer to liquid nitrogen. Cells were thawed in a 37°C water bath (no more than 1 min), transferred to a 50 ml Falcon tube containing 9 ml complete medium and centrifuged for 5 min at 300 x g (PC12-G cells) or 150 x g (NG108-15 and 293T cells). Cells were resuspended in medium and transferred to a 75 cm^2 cell culture flask. Cells were passaged at least once before being used in experiments and used between passage seven and 25.

3.1.5 Preparation and culture of primary rat cortical neurons

Rat cortical neurons were prepared from cortices of embryos from 18-day-pregnant Wistar rats. Pregnant animals were killed by exposure to CO_2 and embryos were extracted. After extracting the embryonic brains, the cortices were dissected and placed in Neurobasal medium supplemented with 1X B27, 500 µM L-glutamine and

25 µM L-glutamate (complete Neurobasal medium). Cortices were triturated in complete Neurobasal medium containing 50 µg/ml DNase and 0.25% Trypsin. After a 15-min-incubation, 30% horse serum was added and cells were triturated again. Cells were washed twice with complete Neurobasal medium and resuspended in 1X DMEM High Glucose (4.5 g/L D-Glucose) supplemented with 10% FBS and 2 mM L-glutamine. Following filtration, cells were plated at a density of 125,000 cells/ 0.95 cm^2 growth area on poly-L-lysine-coated (0.1 mg/ml) plates. The medium was changed after one day in culture to complete Neurobasal medium. Cells were kept under 95% air and 5% CO_2 and used after 8-13 days in culture.

3.1.6 Coating of cell culture plates

Multiple well culture plates and 10-cm dishes were coated with 0.1 mg/ml poly-L-lysine (PLL). Dishes were incubated with PLL for 1 to 16 h, washed three times with PBS and dried overnight. For the viability of cortical cultures, it was crucial to incubate the dishes with PLL solution for at least 6 h.

3.1.7 Growth area of multiple well culture plates

The growth area per well is 9.5 cm^2 for 6-well, 3.8 cm^2 for 12-well, 1.9 cm^2 for 24-well, 0.95 cm^2 for 48-well and 0.32 cm^2 for 96-well plates.

3.2 Virus production and infection of cells

3.2.1 Production of gammaretroviral particles and infection of PC12-G and NG108-15 cells

Retroviral vectors were produced by a three-plasmid co-transfection of 293T cells with the calcium phosphate method (ProFection Mammalian Transfection System Kit, Promega) according to the manufacturer's instructions. The retroviral vector encoding self-inactivating (SIN) 3′-long terminal repeat (LTR) regions together with the transgene targeted for genomic integration was co-transfected with plasmids encoding transcripts for packaging proteins (gag/pol and env).

293T cells were plated in 10-cm dishes and incubated overnight (see 3.1.3). Three hours before transfection the medium was replaced with 10 ml fresh medium. 10 µg of the bicistronic retroviral vector pPRIG (plasmid Polylinker Retroviral IRES

GFP) (Martin et al., 2006) expressing the respective PAC1 receptor (rat PAC1null, hop1 and hip with a full-length N-terminus, which were subcloned from pRK8 vectors (Spengler et al., 1993) into the EcoRI/SalI sites of pPRIG) and the tracer enhanced green fluorescent protein (eGFP) from a unique cytomegalovirus (CMV) promoter, together with the Murine Leukemia Virus (MuLV)-based gag/pol plasmid, pIK6MuLVgp (2.5 µg), and a VSV-G envelope plasmid (5 µg) were co-transfected and incubated overnight. The medium was replaced with 5.5 ml fresh medium and retroviral vector-containing supernatant was harvested 48 h post-transfection. 5.5 ml fresh medium was added and the second supernatant was harvested 24 h later. The supernatant was filtered through a 0.45 µm syringe filter and used for infection of PC12-G and NG108-15 cells. Aliquots were stored at -80°C.

To generate cell lines stably expressing the PAC1 receptor variants null, hop1 and hip, PC12-G (3.1.1) or NG108-15 cells (3.1.2) were plated onto 12-well plates and incubated overnight. Before infection, the medium was removed and 0.5 ml fresh medium and 0.5 ml virus preparation was added. After overnight incubation, the medium was changed. Transduction efficiency of the PAC1 receptor variants was determined by direct visualization of eGFP, expressed from its IRES-dependent cistron.

3.2.2 Production of lentiviral particles and infection of primary rat cortical neurons

Lentiviral vectors can be used for transducing non-dividing mammalian cells, such as neurons and were generated according to the protocol used for production of gammaretroviral particles (3.2.1) with the following modifications: 10 µg of the bicistronic lentiviral expression vector pLVX-IRES-ZsGreen1 (Clontech) co-expressing stanniocalcin 1 (which was subcloned from pBluescript II KS (+)-STC1 (G. Thinakaran; XbaI/Asp718 blunted) into the XbaI/EcoRI blunted sites of pLVX) from a CMV promoter and simultaneously the tracer ZsGreen1 from its IRES dependent cistron were co-transfected with plasmids expressing gag/pol/rev (6.5 µg) and VSV-G envelope (3.5 µg). The combined 48- and 72-h supernatant was filtered and concentrated 50-fold through ultrafiltration (Amicon Ultra-15 Centrifugal Filter Units) for 15 min at 3,500 x g at 4°C. The concentrated virus preparation was used for

infection and stored at 4°C for up to one month. For long-term storage, aliquots were kept at -80°C.

Infection of primary rat cortical neurons was carried out in 24-well plates with 250,000 cells plated per well in 1 ml medium. At 4 days in vitro (div), 700 µl medium was removed and 75 µl concentrated virus was added per well. Cells were incubated overnight and the same medium was added back to the cells. Cells were cultured for another 6 days before being used in experiments.

3.3 RNA isolation

RNA extraction of PC12-G, NG108-15 and primary rat cortical neurons was carried out in 6-well plates with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of the RNA was determined by spectrophotometry.

3.4 DNase digestion

RNA samples were treated with 0.25 U DNase I (Roche) per 1 µg RNA and incubated at 37 °C for 20 min. The DNase was inactivated by addition of EDTA (final concentration of 8 mM) and incubation at 75 °C for 10 min. The RNA was stored at -80°C.

3.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

3.5.1 Complementary DNA (cDNA) synthesis

1 µg DNase-digested RNA was reverse-transcribed with the SuperScript II Reverse Transcriptase (RT) (Invitrogen). RNA, 50 ng random hexamers and 10 µmol dNTP-Mix were incubated at 65°C for 5 min in a total volume of 12 µl. The reaction was chilled to 8°C and 10 µl RT-Mix was added. The RT-Mix contained 2X RT buffer, 100 µmol $MgCl_2$, 0.2 µmol DTT, 20 U RNase inhibitor and 50 U RT. The reaction was incubated at 25°C for 10 min, 42°C for 50 min and at 70°C for 15 min to heat-inactivate the RT. As a negative control, the reaction was performed without the SuperScript II RT. The cDNA was diluted 1:2 and stored at -20°C.

3.5.2 Polymerase chain reaction (PCR)

2.5 µl cDNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 800 µM dNTP-Mix, 100 nM Primer-Mix and 0.5 U Taq-Polymerase were diluted in a total volume of 25 µl. The reaction was incubated for 5 min at 95°C, followed by 25-35 cycles at 95°C (denaturation), 55°C (annealing) and 72°C (elongation) for 30 sec, respectively. After a final incubation at 72°C for 7 min the reaction was cooled to 4°C and stored at -20°C. As negative controls, the reaction was performed with the RT reaction not containing any enzyme as well as with water instead of cDNA.

3.6 Quantitative real time polymerase chain reaction (qRT-PCR)

2 µl cDNA, 1X iQ SYBR Green Supermix (containing the fluorescent dye SYBR Green I) and 200 nM Primer-Mix were diluted in a total volume of 25 µl. The reaction was incubated for 3 min at 95°C, for 40 cycles at 95°C for 10 sec (denaturation) and 55°C for 45 sec (annealing and elongation), and at 95°C and 55°C for 1 min each. After real time PCR amplification, a melting curve was performed, for which the temperature was raised by 0.5°C from 55°C to 95°C for 10 sec each to ensure detection of the desired amplicon. As negative control, the reaction was performed with water instead of cDNA. PCR was performed for the gene of interest as well as for the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). For both genes, seven standards were run (2 pg – 0.128 fg; successive 5-fold dilution). cDNA levels of the gene of interest were normalized to GAPDH levels.

3.7 Cloning

Plasmids were cut with restriction enzymes in their respective buffers. 5-10 µg plasmid DNA was digested 2 h to overnight. Fragments were separated on an agarose gel and purified with the QIAquick Gel Extraction Kit. To prevent self-ligation, the vector DNA was dephosphorylated with calf intestinal alkaline phosphatase (CIP; 0.5 U/µg DNA) in 1X NEBuffer 3 for 1 h at 37°C. The treated DNA was cleaned up with the Wizard DNA clean-up system. Vector and insert DNA were diluted 1:3 in 1X DNA dilution buffer (maximal amount of DNA was 200 ng) and ligated with the T4 DNA ligase in 1X T4 ligation buffer. The reaction was incubated for 5 min at RT and transformed into E. coli DH5α competent cells. 40 µl cells were thawed on ice, 2 µl

cloning reaction was added and incubated for 5-30 min on ice. Cells were heat-shocked for 30 sec at 42°C. 250 µl SOC medium (Super Optimal broth medium + glucose to initiate Catabolite repression) was added and the cells were incubated for 1 h at 37°C with shaking. 50-150 µl were spread on an LB plate containing 100 µg/ml ampicillin, IPTG and X-Gal and incubated overnight at 37°C. 5-10 white colonies were picked and cultured in 4 ml selective LB medium. The plasmid DNA was isolated with the QIAprep Spin Miniprep Kit, digested with respective restriction enzymes and run on an agarose gel. 100-200 ml selective medium was inoculated with a positive clone and plasmid DNA was isolated with the QIAGEN Plasmid Maxi Kit. Glycerol stocks (20% glycerol) were made of the bacterial culture and stored at -80°C.

Plasmids were sequenced by the dideoxy chain-termination method (DNA Sequencing Facility, NINDS, National Institutes of Health).

3.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to their size with small fragments running faster than large ones. The concentration of the agarose was chosen depending on the size of the DNA fragments. 1.8% agarose was used to separate fragments ranging in size from 100-300 bp and 0.8% agarose was used for fragments larger than 500 bp. Agarose was dissolved in 1X TAE (Tris-Acetate-EDTA) buffer by gentle boiling in a microwave and 0.2 µg/ml ethidium bromide (EtBr) was added. 1X DNA gel loading solution was added to the DNA and loaded onto the gel. The gel was run at 100 V for 1 to 1.5 h. The EtBr stained bands were visualized on a UV transilluminator and imaged.

3.9 Immunoblotting

NG108-15 and primary rat cortical cells (in 12-well plates) were lysed in 100 µl of freshly prepared lysis buffer (1X NuPAGE LDS sample buffer, 1X NuPAGE reducing agent, 1X Roche cocktail inhibitor tablet and 1X Halt phosphatase inhibitor cocktail) and sonicated for 10 sec to reduce sample viscosity. Samples were heat-treated for 5 min at 95°C and micro-centrifuged. Standards (See Blue Plus 2 Prestained Standard and Magic Mark XP Western Standard) and samples were loaded

onto a NuPAGE Novex Pre-Cast 4-12% Bis-Tris Gel and run at 120 V for 1.5 h in 1X NuPAGE MOPS SDS Running Buffer. Separated proteins were electrotransferred to a nitrocellulose membrane (Filter Paper Sandwich, 0.45-µm pore size) at 30 V for 1.5 h in cold 1X NuPAGE transfer buffer. The transferred proteins were visualized with PonceauS staining. The membrane was blocked with TBST (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) + 5% skim milk for 1 h at room temperature before incubation with the primary antibody overnight at 4 °C. The membrane was washed three times for 15 min with TBST before incubating with the HRP-conjugated secondary antibody for 1 h at room temperature. Following three 15-min washing steps in TBST and one in TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), immunoreactive bands were visualized with the Super Signal West Pico Chemiluminescence Substrate, the FluorChem 8800 Imaging System (Alpha Innotech) and iVision software. Exposure times varied from 30 sec to 8 min depending on the intensity of the signal. To remove the bound antibody, the membrane was incubated in Restore Western Blot Stripping Buffer for 15 min and washed twice in TBS. Blocking, antibody incubation and washing was carried out under gentle agitation. The membrane was dried and stored at RT. Signals were quantified with the ImageJ software.

3.10 Single cell calcium measurements

Cells were plated onto 1.5-cm-diameter glass cover slips (in 12-well plates) coated with 0.5 mg/ml poly-L-lysine and incubated overnight. Before coating, cover-slips were soaked in alcohol for 5 min, washed, soaked in 0.1 M nitric acid for 5 min, washed again and air-dried. Cover slips were sterilized at 80°C for at least 30 h. Before the experiment, cells were washed once with Krebs-Ringer buffer (KRB: 20 mM HEPES, 125 mM NaCl, 5.5 mM glucose, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgSO_4$ and 1 mM $CaCl_2$; pH 7.3) and loaded with 4 µM fura-2 AM for 22 min under gentle agitation. Cells were washed once with KRB and incubated for an additional 22 min in KRB. Cover slips were mounted onto a custom-built perfusion chamber and placed on an inverted Olympus IX70 microscope. Cells were perfused with KRB in the presence or absence of drugs at a flow rate of 800 µl/min. Ca^{2+}-free measurements were carried out in KRB without $CaCl_2$ supplemented with 100 µM EGTA. Intracellular Ca^{2+} concentrations were measured using the 340/380 excitation ratio (R

340/380) and an emission wavelength of 510 nm. Images were captured every 2 sec and analyzed with the software MetaFluor. Cells simultaneously expressing the protein of interest and the easily detectable tracer GFP from a single bicistronic mRNA were selected based on fluorescence detection.

3.11 [^{3}H]-norepinephrine uptake and release assay

Catecholamine secretion from dense-core vesicles (DCVs) in intact PC12-G cells was assayed by the release of pre-loaded radioactive norepinephrine (NE). Cells were plated onto 48-well plates and labeled overnight at 37°C with 1 µCi/ml Levo-7[^{3}H]-Norepinephrine (1 mCi/ml, 12.0 Ci/mmol) in medium containing 0.5 mM ascorbic acid. Cells were washed once with medium and incubated in medium in the presence or absence of drugs for the indicated time period at 37°C. The supernatant was collected and cells were lysed in medium containing 1% Triton-X 100 for 30 min under constant agitation. 200 µl of the supernatant and cell lysate, respectively, were mixed with 5 ml LSC cocktail and the level of radioactivity was determined by liquid scintillation counting. The secreted [^{3}H]-NE was calculated as the percentage of the total radioactivity.

3.12 Cyclic AMP measurements

Intracellular cAMP levels were measured with the cAMP Biotrak Enzymeimmunoassay (EIA) System (Amersham Biosciences) using the non-acetylation EIA procedure with the provided lysis reagent according to the manufacturer's instructions. Cells were plated onto 48-well plates and incubated overnight. Where noted, cells were pre-treated with varying concentrations of the AC-inhibitor 2′5′-dideoxyadenosine for 30 min. Following pre-treatment, cells were stimulated with 100 nM PACAP-38 or 25 µM forskolin in medium containing 500 µM of the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) for 20 min at 37°C.

3.13 Induction of cell death in primary rat cortical neurons and assessment of cell viability

3.13.1 Induction of cell death by glutamate-induced excitotoxicity

Excitotoxicity was induced with 5 µM N-methyl-D-aspartic acid (NMDA) or 1 mM L-glutamic acid. After incubating the cells for various time periods, viability was assessed with the MTT (3.13.3) assay or PI staining (3.13.4).

3.13.2 Induction of cell death by oxygen-glucose-deprivation (OGD)

Neurobasal medium was changed to OGD medium (Neurobasal-A medium without glucose supplemented with 1X B-27 and 0.5 mM L-glutamine) and the cells were placed into a humidified OGD chamber. Cells were flushed for 30 min with 95% N_2 and 5% CO_2 (flow rate: 4 L/min) at RT. After flushing, the chamber was sealed and incubated for another 2.5 h at 37°C under 95% air and 5% CO_2 conditions. The medium was replaced with Neurobasal-A medium supplemented with 1X B-27 and 0.5 mM L-glutamine and after another 21-h-incubation (total incubation of 24 h) cell viability was assessed with the MTT assay (3.13.3).

3.13.3 MTT cell viability assay

Cell viability of primary rat cortical neurons (in 24-well plates) was determined with the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) assay. The yellow MTT solution is converted to water-insoluble MTT-formazan of blue color by mitochondrial dehydrogenases of living cells. MTT was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove residual insoluble residues and added to cells (60 µl per 500 µl medium). Cells were incubated at 37°C for 2 h and the blue crystals were solubilized with SDS-DMF solution (20% SDS in 50% DMF, pH 4.7; 0.5 ml per 0.5 ml medium). After overnight-incubation at 37°C, 200 µl were transferred to a 96-well plate and read on a microplate reader. The intensity was measured colorimetrically at a test wavelength of 570 nm and a reference wavelength of 650 nm.

3.13.4 Propidium iodide (PI) staining of dead cells

Primary rat cortical neurons were incubated with 2 µg/ml propidium iodide (PI) for 10 min at 37°C. PI intercalates into double-stranded DNA. It can penetrate cell membranes from dying or dead cells but is excluded from viable cells. For DNA staining of all cells, cultures were incubated with 10 µg/ml Hoechst 33342 20 min prior to PI staining. Pictures of the red-fluorescent PI- and the blue-fluorescent Hoechst-stained nuclei were taken on a Nikon eclipse Ti inverted microscope using NIS Elements software.

3.14 Statistical analysis

Statistical analysis was carried out in Prism 4 (GraphPad Software) by unpaired t-test, one-way ANOVA with Dunnett's or Tukey's Multiple Comparison Test or two-way ANOVA with Bonferroni post-tests. Significance was set at $p < 0.05$.

4. Results

4.1 Second messenger generation and catecholamine secretion by different splice variants of the PAC1 receptor in neuroendocrine and neural cells

To investigate the importance of the different insertions in the third intracellular loop (ic3) of PAC1 in PACAPergic neural signaling, the PAC1null, hop1 and hip receptor variants with a full-length N-terminus were expressed in pheochromocytoma PC12-G and neuroblastoma x glioma NG108-15 cells and intracellular cAMP and calcium elevation and catecholamine (CA) secretion were measured. Experiments were extended to primary rat cortical neurons.

4.1.1 Functional characterization of the bovine PAC1hop receptor (bPAC1hop) in PC12-G cells

The bovine PAC1hop1 receptor (bPAC1hop) was stably introduced into the rat pheochromocytoma-derived PC12-G cell line as previously described (Mustafa et al., 2007). Briefly, cells were transfected with pcDNA3 containing bPAC1hop using Lipofectamine and selected with G418. A stably transfected clone, expressing bPAC1hop at physiological levels found in bovine chromaffin cells (BCCs) was chosen (PC12-bPAC1hop).

Naive PC12-G cells express sufficient levels of PAC1 to provide a maximal cAMP response. Levels are too low, however, to drive a full Ca^{2+} response and CA secretion (Mustafa et al., 2007). To determine which PACAP receptors are driving a full cAMP and a low-level Ca^{2+} response in PC12-G cells, an analysis of VPAC/PAC receptor-mRNAs was performed by RT-PCR. Naïve PC12-G cells expressed low levels of the PAC1hop and very low levels of the PAC1hip receptor variant, but no expression of PAC1null or VPAC1 and 2 receptors was detected (Fig. 6A), suggesting that the PACAP-mediated response in PC12-G cells is mediated predominantly by PAC1hop with a minor contribution of PAC1hip.

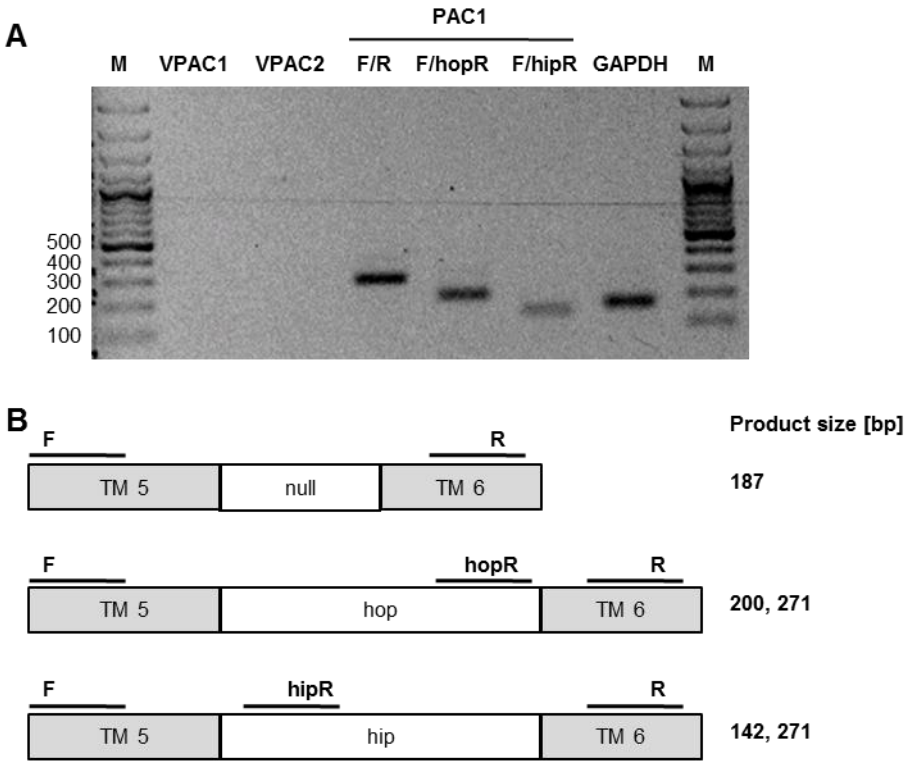


Figure 6: PC12-G cells express low levels of the PAC1hop and very low levels of the PAC1hip receptor variant. (A) RT-PCR analysis of endogenous VPAC1, VPAC2 and PAC1 receptors in PC12-G cells. Total RNA was reverse-transcribed and PCR-amplified (35 cycles) with different primer pairs for VPAC1, VPAC2 and PAC1. To differentiate between PAC1 splice variants of the third intracellular loop, different primer pairs within the transmembrane region 5 and 6 (TM 5 and 6) were used. The same forward primer (F) was used with different reverse primers (R): F/R generates a 187- or 271-bp fragment, depending on whether or not a hip- or hop-insert is present; the hop-specific primer pair F/hopR generates a 200-bp fragment; the hip-specific primer pair F/hipR generates a 142-bp fragment. Primers for VPAC1 generate a 107-bp fragment and for VPAC2 a 133-bp fragment. GAPDH product size is 168-bp. M: 100-bp DNA ladder. (B) Schematic representation of PAC1 primer pairs.

4.1.1.1 bPAC1hop increases the intracellular Ca^{2+} response, which consists of Ca^{2+} mobilization and influx

The expression of bPAC1hop in PC12-G cells reconstituted a Ca^{2+} response seen in primary BCCs as determined by single cell Ca^{2+} imaging experiments of naïve PC12 and PC12-bPAC1hop cells, confirming previously published data by T. Mustafa. Naive PC12 cells responded to 100 nM PACAP with a very small but prolonged increase of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), whereas PC12-bPAC1hop cells showed a large and rapid rise of $[Ca^{2+}]_i$ followed by a large persisting plateau.

Depolarization-induced Ca^{2+} influx was similar in both cell lines as determined by stimulation with 55 mM KCl following stimulation with PACAP (Fig. 7A). To differentiate between Ca^{2+} influx from the extracellular and Ca^{2+} release from intracellular stores (i.e., Ca^{2+} mobilization), cells were stimulated with PACAP in Ca^{2+}-free buffer. Both PC12 and PC12-bPAC1hop cells showed an initial Ca^{2+} mobilization, which was followed by a prolonged influx upon reintroduction of Ca^{2+}. Mobilization and influx were much greater in bPAC1hop-expressing cells, reflecting the limited coupling to Ca^{2+} of endogenous PAC1 receptors expressed in naïve PC12-G cells (Fig. 7B).

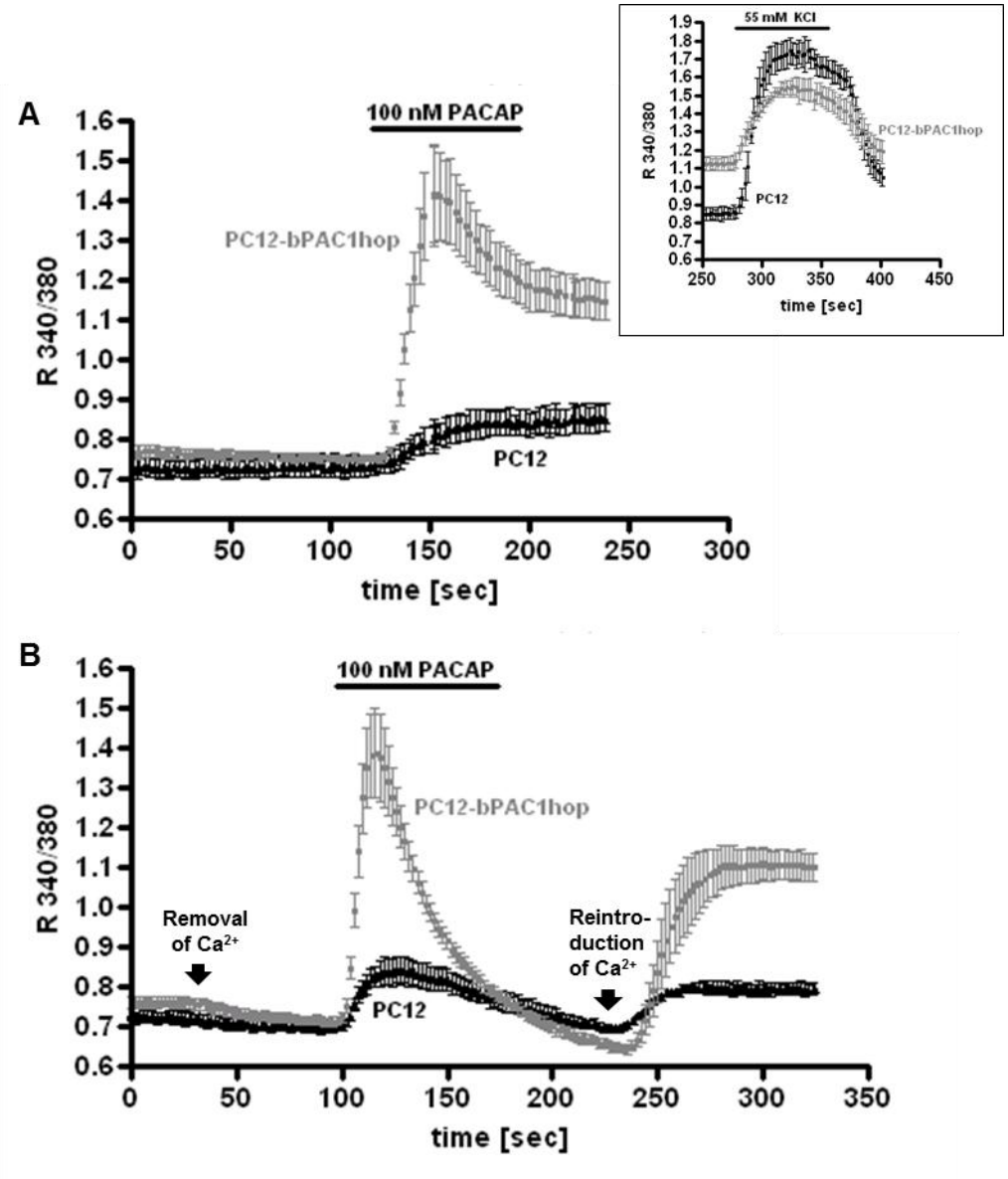


Figure 7: Expression of the bovine PAC1hop receptor (bPAC1hop) in PC12-G cells increases intracellular Ca^{2+} mobilization and influx upon PACAP treatment. PC12 cells (black line) and PC12-bPAC1hop cells (gray line) were loaded with 4 µM fura-2 AM and

stimulated with 100 nM PACAP-38 for 75 sec in Ca^{2+}-containing (A) or Ca^{2+}-free KRB (B). Insert in (A) shows depolarization-induced Ca^{2+} influx by 55 mM KCl following PACAP treatment. Intracellular Ca^{2+} concentrations were measured in single cells using the 340/380 excitation ratio (R 340/380) and an emission wavelength of 510 nm. Images were captured every 2 sec. Plots represent the average ± SEM of at least three independent experiments. Each experiment represents 20-50 cells (A: PC12: n=111, PC12-bPAC1hop: n=99; B: PC12: n=106, PC12-bPAC1hop: n=189).

4.1.1.2 bPAC1hop increases acute and confers prolonged catecholamine secretion

To determine whether the increased Ca^{2+} response of PC12-bPAC1hop cells is reflected in the secretory response, the release of catecholamines (CAs) was measured in PC12 and PC12-bPAC1hop cells upon treatment with 100 nM PACAP. PC12-G cells released low levels of pre-loaded [^{3}H]-norepinephrine (NE) after a 30-min-exposure to PACAP, which is in disagreement with previously published data by Mustafa et al., demonstrating a lack of CA release in naïve PC12-G cells. Experiments by Mustafa et al. were carried out in Krebs-Ringer buffer (KRB) and showed a high basal release (approximately 10% of total content). In this study, complete medium was used, which reduced basal release to about 5%, unmasking PACAP-mediated release. CA release was significantly enhanced in PC12-bPAC1hop cells to about 30% of total content, which is comparable to the release measured by Mustafa et al. and similar to membrane depolarization-mediated release by 55 mM KCl. KCl-mediated release was slightly higher in PC12-bPAC1hop cells compared to naïve PC12-G cells (~25% of total content), suggesting a larger readily releasable pool (RRP) size of DCVs in these cells (Fig. 8).

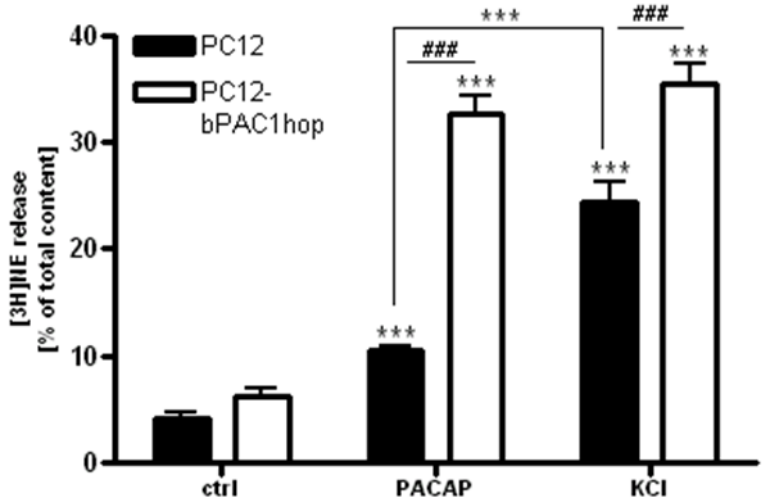
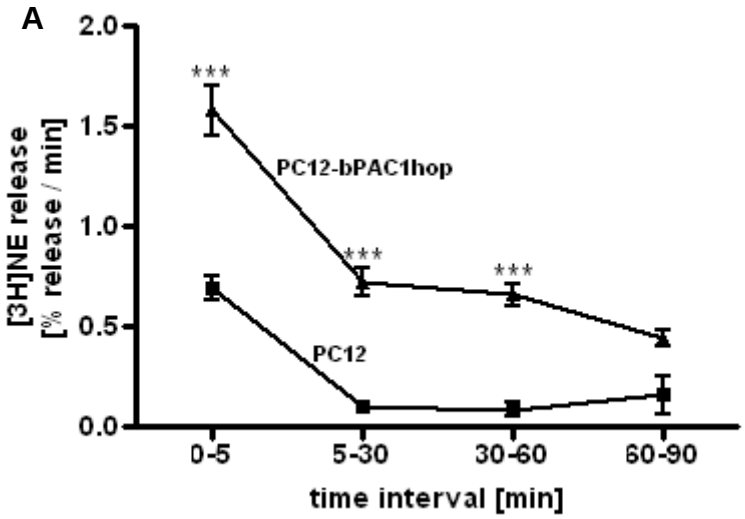


Figure 8: Expression of the bovine PAC1hop receptor (bPAC1hop) enhances catecholamine secretion in PC12-G cells. PC12 cells (black bars) and PC12-bPAC1hop cells (white bars) were labeled with 1 µCi/ml Levo-7[^{3}H]-Norepinephrine and stimulated with 100 nM PACAP-38 or 55 mM KCl for 30 min. The supernatant was collected, cells were lysed and the level of radioactivity was determined. The secreted [^{3}H]-NE in the supernatant was calculated as the percentage of total radioactivity. Values represent the grand mean ± SEM of at least four independent experiments performed in duplicates. *** P < 0.001 versus control or between treatments; one-way ANOVA, with Tukey's Multiple Comparison Test. ### P < 0.001 between cell lines; two-way ANOVA, with Bonferroni post-tests.

To differentiate between acute and sustained secretion, CA release was measured after different incubation times. Both PC12 and PC12-bPAC1hop cells responded to 100 nM PACAP with an acute release of pre-loaded [^{3}H]-NE within 5 min. Acute secretion was higher in PC12-bPAC1hop cells. Moreover, expression of the bPAC1hop receptor conferred sustained secretion (5-60 min), which was not seen in naïve PC12-G cells, suggesting that the low density of endogenous PAC1 receptors in PC12-G cells is not sufficient to drive a full acute secretory response and more importantly a prolonged secretory response (Fig. 9A). Membrane depolarization with 55 mM KCl induced acute but not prolonged CA secretion, which was also increased in PC12-bPAC1hop cells (Fig. 9B), in agreement with an increased KCl-mediated release after 30 min.



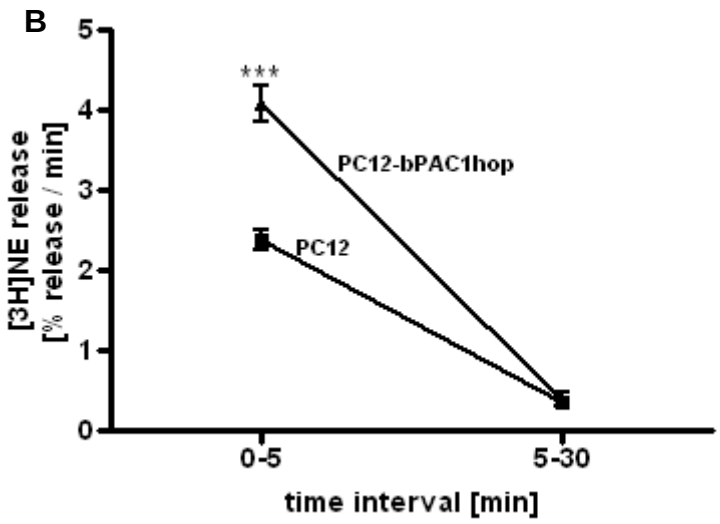


Figure 9: Expression of the bovine PAC1hop receptor (bPAC1hop) in PC12-G cells confers prolonged PACAP-evoked catecholamine secretion. Cells were labeled with 1 µCi/ml Levo-7[^{3}H]-Norepinephrine and stimulated with (A) 100 nM PACAP-38 for 5, 30, 60 or 90 min or (B) 55 mM KCl for 5 or 30 min. The supernatant was collected, cells were lysed and the level of radioactivity was determined. The secreted [^{3}H]-NE in the supernatant was calculated as the percentage of total radioactivity and is shown as % release/min. Basal release was subtracted from the respective time interval (see Table 1 for basal versus PACAP-stimulated release). Values represent the grand mean ± SEM of three to six independent experiments performed in duplicates. *** P < 0.001 versus PC12 cells; two-way ANOVA, with Bonferroni post-tests.

Time interval [min]		**0-5**	**5-30**	**30-60**	**60-90**
PC12	**basal**	0.42 ± 0.07	0.09 ± 0.02	0.06 ± 0.02	0.11 ± 0.02
	PACAP	1.11 ± 0.07	0.20 ± 0.01	0.14 ± 0.05	0.27 ± 0.11
PC12-bPAC1hop	**basal**	0.62 ± 0.09	0.11 ± 0.02	0.12 ± 0.01	0.14 ± 0.04
	PACAP	2.20 ± 0.12	0.83 ± 0.06	0.77 ± 0.06	0.58 ± 0.01

Table 1: Basal versus PACAP-stimulated CA release in % release / min in PC12 and PC12-bPAC1hop cells. Values represent the grand mean ± SEM of experiments shown in Figure 9A.

4.1.2 Functional characterization of the rat PAC1hop, null and hip receptor variants (rPAC1hop, null and hip) in PC12-G cells

The rat PAC1hop1, null and hip receptor variants were stably introduced into PC12-G cells through infection with gammaretroviral particles to characterize and compare the

three different PAC1 variants. Moreover, the expression of rat receptors in a cell line derived from a rat tumor abolishes inter-species differences that were present in the PC12-bPAC1hop cell line. The rat PAC1 receptors were expressed from a CMV promoter generating stable cell lines with similar expression levels. Viral particles were made with the bicistronic retroviral vector pPRIG and the expression of the receptor was monitored by the expression of enhanced green fluorescent protein (eGFP) from its IRES-dependent cistron. The transfection efficiency was between 45 and 80%. Specifically, 58% of pPRIG-rPAC1hop, 79% of pPRIG-rPAC1null, 45% of pPRIG-rPAC1hip and 56% of pPRIG-control infected PC12 cells were GFP-positive. Figure 10 shows representative pictures of PC12 cells over-expressing the rat PAC1hop receptor (PC12-rPAC1hop).

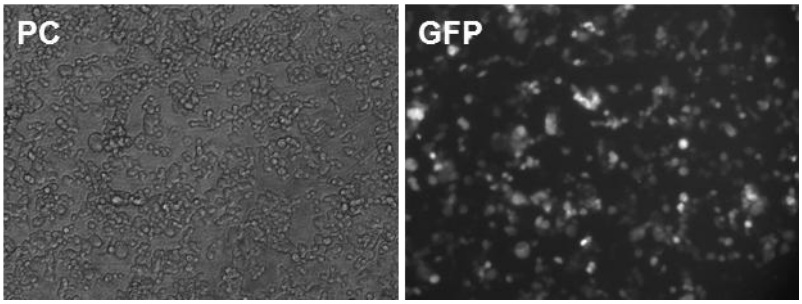


Figure 10: Photomicrographs of PC12 cells stably expressing rat PAC1hop-IRES-eGFP (PC12-rPAC1hop). Cells were infected with viral particles made with the bicistronic retroviral vector pPRIG. PC: phase contrast, GFP: green fluorescent protein (grayscale image).

4.1.2.1 rPAC1hop and null increase the intracellular Ca^{2+} response

Expression of rPAC1hop in PC12-G cells increased the intracellular Ca^{2+} response upon treatment with 100 nM PACAP, reminiscent of the Ca^{2+} response seen in PC12 cells expressing the bovine PAC1hop receptor as shown in Figure 7A. The initial rise in $[Ca^{2+}]_i$, which was attributable to Ca^{2+} release from intracellular stores was slightly higher in bPAC1hop- compared to rPAC1hop-expressing cells, whereas the prolonged Ca^{2+} influx plateau phase was similar in the bovine and rat receptor-expressing cell lines. Expression of rPAC1null also increased the intracellular Ca^{2+} response similar to rPAC1hop, but with a slightly smaller initial rise, suggesting that the hop cassette is not required for mediating an intracellular Ca^{2+} response. Expression of rPAC1hip, in contrast, did not elevate the intracellular Ca^{2+} response beyond the small rise observed in naïve PC12-G cells, suggesting that the hip cassette abolishes coupling to Ca^{2+}.

Expression of GFP alone did not affect the elevation of $[Ca^{2+}]_i$ elicited by PACAP, however, somewhat reduced basal $[Ca^{2+}]_i$. Depolarization-induced Ca^{2+} influx was similar in the five cell lines as determined by stimulation with 55 mM KCl following stimulation with PACAP (Fig. 11).

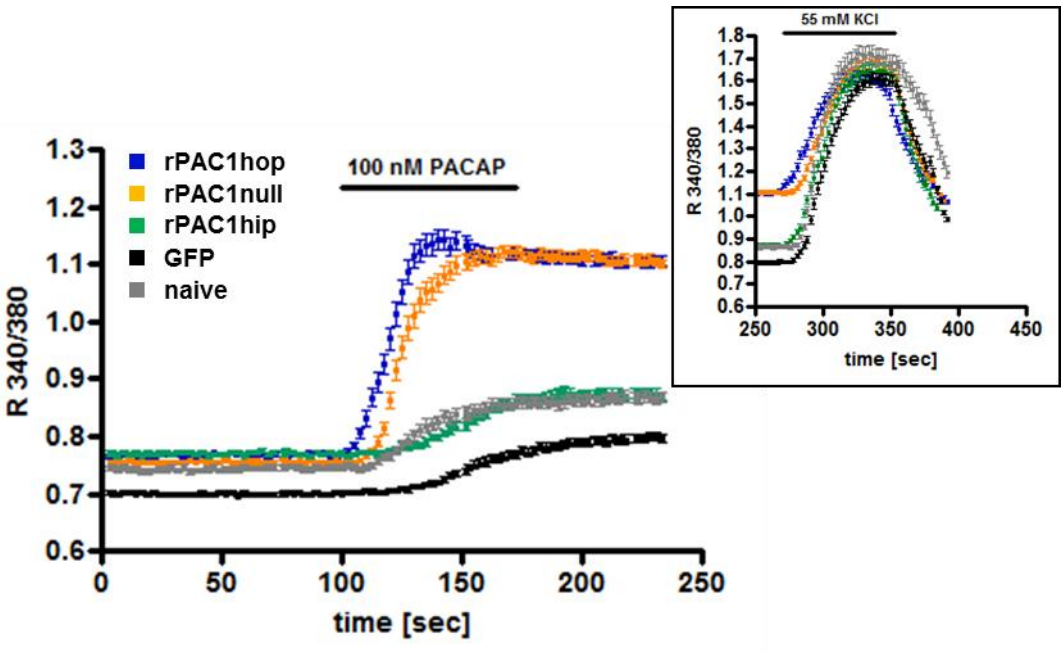


Figure 11: Expression of the rat PAC1hop and null but not the hip receptor in PC12-G cells increases the PACAP-induced Ca^{2+} response. Cells were loaded with 4 µM fura-2 AM and stimulated with 100 nM PACAP-38 for 75 sec in KRB. Insert shows depolarization-induced Ca^{2+} influx by 55 mM KCl following PACAP treatment. $[Ca^{2+}]_i$ were measured in single cells using the 340/380 excitation ratio (R 340/380) and an emission wavelength of 510 nm. Images were captured every 2 sec. Plots represent the average ± SEM of five (naïve), seven (GFP and hip) or eight (null and hop) independent experiments. Each experiment represents 10-50 cells (hop: n=236, null: n=269, hip: n=172, GFP: n=217, naïve: 167).

4.1.2.2 rPAC1hop and null increase acute catecholamine secretion but only rPAChop confers prolonged secretion

Next, the effect of the different rat PAC1 receptor variants on CA secretion was examined in PC12-G cells. Expression of rPAC1hop and null increased PACAP-induced acute release of pre-loaded [^{3}H]-NE within 5 min, whereas only rPAC1hop conferred sustained secretion (5-60 min), suggesting that PACAP-mediated long-term release of CAs requires the presence of the hop cassette in the third intracellular loop of the receptor. Expression of rPAC1hip did not increase the secretory response beyond the low-level acute release observed in naïve PC12 cells, in agreement with a

lack of coupling to Ca^{2+}. Expression of GFP alone did not affect the secretory behavior (Fig. 12).

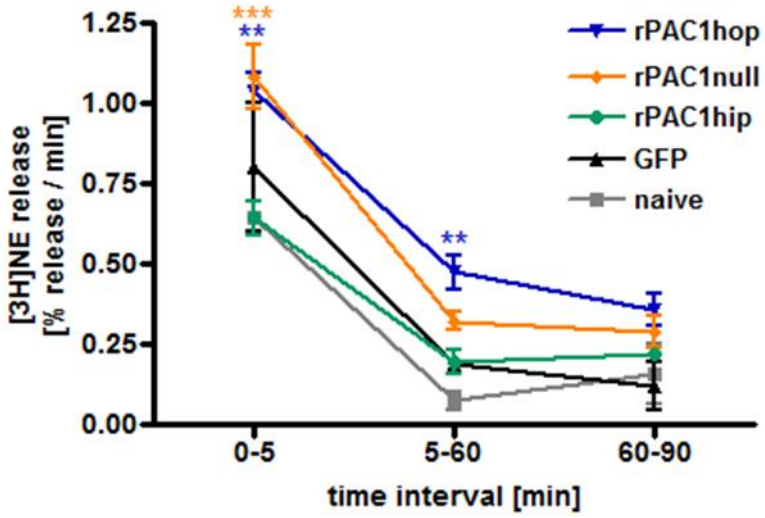


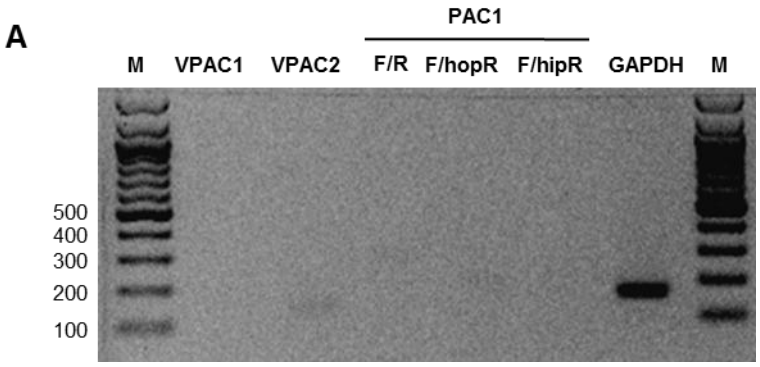
Figure 12: Expression of the rat PAC1hop and null receptor in PC12-G cells increases acute PACAP-evoked catecholamine secretion, but only PAC1hop confers prolonged secretion. Expression of PAC1hip is without effect. Cells were labeled with 1 µCi/ml Levo-7[^{3}H]-Norepinephrine and stimulated with 100 nM PACAP-38 for 5, 60 or 90 min. The supernatant was collected, cells were lysed and the level of radioactivity was determined. The secreted [^{3}H]-NE in the supernatant was calculated as the percentage of total radioactivity and is shown as % release/min. Basal release was subtracted from the respective time interval (see Table 2 for basal versus PACAP-stimulated release). Values represent the grand mean ± SEM of three to six independent experiments performed in duplicates. . *** P < 0.001, ** P < 0.01 versus naïve PC12 cells; two-way ANOVA, with Bonferroni post-tests.

Time interval [min]		**0-5**	**5-60**	**60-90**
Naïve PC12	**basal**	0.49 ± 0.10	0.07 ± 0.01	0.11 ± 0.02
	PACAP	1.13 ± 0.10	0.14 ± 0.03	0.27 ± 0.11
PC12-GFP	**basal**	0.52 ± 0.06	0.09 ± 0.01	0.09 ± 0.00
	PACAP	1.32 ± 0.26	0.28 ± 0.02	0.21 ± 0.07
PC12-rPAC1hop	**basal**	0.69 ± 0.10	0.08 ± 0.01	0.07 ± 0.02
	PACAP	1.73 ± 0.13	0.55 ± 0.05	0.43 ± 0.04
PC12-rPAC1null	**basal**	0.64 ± 0.05	0.07 ± 0.01	0.07 ± 0.01
	PACAP	1.72 ± 0.16	0.41 ± 0.04	0.36 ± 0.05
PC12-rPAC1hip	**basal**	0.53 ± 0.02	0.07 ± 0.01	0.07 ± 0.02
	PACAP	1.17 ± 0.05	0.26 ± 0.04	0.31 ± 0.02

Table 2: Basal versus PACAP-stimulated CA release in % release / min in naïve PC12, PC12-GFP and PC12-rPAC1hop, null and hip cells. Values represent the grand mean ± SEM of experiments shown in Figure 12.

4.1.3 Functional characterization of rPAC1hop, null and hip in NG108-15 cells

To characterize each individual PAC1 splice variant separately in neural cells, the mouse neuroblastoma x rat glioma cell line NG108-15 was transduced with the rat PAC1null, hop1 or hip receptor variants. NG108-15 cells expressed negligible levels of PACAP receptors endogenously. A very low expression of VPAC2 and PAC1hop receptors was observed (Fig. 13A). Levels were too low, however, to evoke a response upon PACAP treatment. NG108-15 cells are capable of G protein signaling through activation of endogenous GPCRs for other neuropeptide ligands (Ghadessy and Kelly, 2002; Hamprecht et al., 1985; Ishihara et al., 1991; Propst et al., 1979) without having any "background" PACAP response as observed in PC12-G cells and were therefore chosen to characterize PAC1 splice variant-specific signaling. NG108-15 cells stably over-expressing the rat PAC1 receptor splice variants hop1, null and hip, respectively, were generated through infection with gammaretroviral particles as described for PC12-G cells (4.1.2). The transfection efficiency was between 75 and 90%. Specifically, 89% of pPRIG-rPAC1hop, 81% of pPRIG-rPAC1null, 76% of pPRIG-rPAC1hip and 86% of pPRIG-control infected NG108-15 cells were GFP-positive. Fig. 13B and C show RT-PCR analysis of reverse-transcribed mRNAs from the NG108-15 cell lines PAC1null, PAC1hop and PAC1hip and representative pictures of NG108-15-rPAC1hop cells.



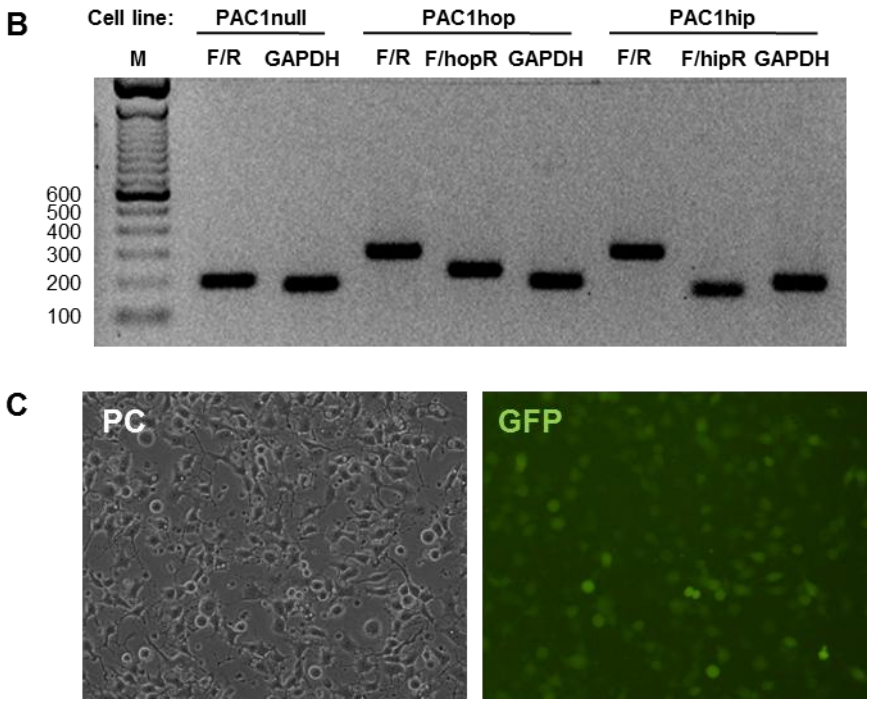


Figure 13: Expression of rat PAC1 receptor variants in NG108-15 cells before and after retroviral transduction. RT-PCR analysis of (A) endogenous VPAC1, VPAC2 and PAC1 receptors in NG108-15 cells and (B) PAC1 receptors in cells that were infected with viral particles made with the bicistronic retroviral vector pPRIG to generate stably expressing cell lines (PAC1null, PAC1hop and PAC1hip). Total RNA was reverse-transcribed and PCR-amplified (35 cycles) using different primers within the transmembrane region 5 and 6 (TM 5 and 6). The same forward primer (F) was used with different reverse primers (R): F/R generates a 187- or 271-bp fragment, depending on whether or not a hip- or hop-insert is present; the hop-specific primer pair F/hopR generates a 200-bp fragment; the hip-specific primer pair F/hipR generates a 142-bp fragment (schematic representation see Fig. 6B). Primers for VPAC1 generate a 107-bp fragment and for VPAC2 a 133-bp fragment. GAPDH product size is 168-bp. M: 100-bp DNA ladder. (C) Photomicrographs of NG108-15 cells stably expressing rat PAC1hop-IRES-eGFP (NG108-15-rPAC1hop). PC: phase contrast, GFP: green fluorescent protein.

4.1.3.1 rPAC1hop, null and hip confer intracellular cAMP generation

PAC1 receptors potently stimulate adenylate cyclases (ACs) to increase intracellular cAMP levels. Expression of all three rat PAC1 receptor splice variants hop, null and hip in NG108-15 cells conferred an increase in intracellular cAMP generation upon treatment with 100 nM PACAP, indicating that coupling to ACs, presumably through Gs, was reconstituted in these cells. Cyclic AMP production was greater in PAC1hop- and null- compared to hip-expressing cells, which is in agreement with the first report

published on the differential signaling properties of the different rat PAC1 splice variants in non-neural cells (Spengler et al., 1993). The expression of GFP alone was without effect. The amount of cAMP generated upon stimulation with 25 µM of the AC activator forskolin was, as expected, similar in all cell lines and represents the maximal amount of intracellular cAMP generation. PACAP-induced cAMP generation was calculated as the percentage of maximal cAMP generation (Fig. 14). The absolute amounts of cAMP in untreated cells as well as those generated upon PACAP or forskolin treatment are shown in Table 3.

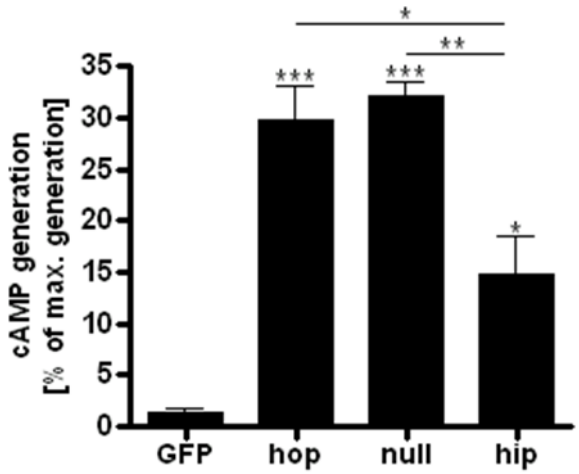


Figure 14: Expression of the rat PAC1 receptor splice variants hop, null and hip in NG108-15 cells confers intracellular cAMP production upon PACAP treatment. Cyclic AMP generation is greater in PAC1hop- and null- than in PAC1hip-expressing cells. Cells were treated with 100 nM PACAP-38 for 20 min and intracellular cAMP levels were measured. Plot represents the grand mean ± SEM of three independent experiments performed in triplicates. Values are expressed as percentage of maximal intracellular cAMP generation, which was determined by stimulation with 25 µM forskolin. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus GFP- and between PAC1 variant-expressing cell lines; one-way ANOVA, with Tukey's Multiple Comparison Test.

	GFP	**PAC1hop**	**PAC1null**	**PAC1hip**
control	0.11 ± 0.02	0.15 ± 0.03	0.13 ± 0.004	0.11 ± 0.02
PACAP	0.17 ± 0.04	4.60 ± 1.07	4.99 ± 1.30	1.86 ± 0.34
Forskolin	13.72 ± 4.83	15.14 ± 2.07	15.52 ± 4.04	15.19 ± 5.29

Table 3: Absolute values of intracellular cAMP levels in pmol/well in NG108-15 cells expressing the rat PAC1 receptor splice variants or GFP. Values represent the grand mean ± SEM of the three experiments shown in Figure 14.

To test whether intracellular cAMP generation can be blocked pharmacologically, NG108-15-rPAC1hop cells were incubated with various concentrations of the AC inhibitor 2′5′-dideoxyadenosine (ddAd) (Tesmer et al., 2000) prior to PACAP treatment. ddAd reduced PACAP-induced intracellular cAMP generation in a concentration-dependent manner. However, even the highest concentration of ddAd used did not block cAMP generation by PACAP completely (control and 600 µM ddAd + PACAP: $P < 0.001$ in unpaired t-test). The amount of cAMP generated upon PACAP treatment with or without pre-treatment with ddAd was calculated as the percentage of cAMP generated upon treatment with 25 µM forskolin which represents the maximal amount of intracellular cAMP generation (Fig. 15). The absolute amounts of cAMP in untreated and treated cells are shown in Table 4.

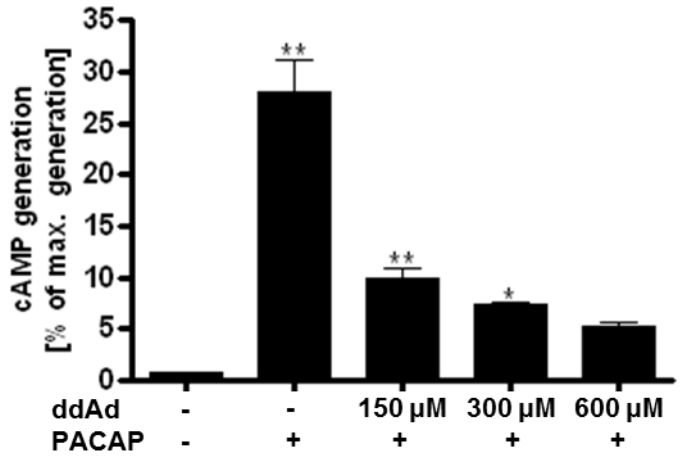


Figure 15: The AC inhibitor 2'5'-dideoxyadenosine reduces PACAP-induced intracellular cAMP generation in a concentration-dependent manner in NG108-15-rPAC1hop cells. Cells were pre-treated for 30 min with 150, 300 or 600 µM 2'5'-dideoxyadenosine (ddAd) before treatment with 100 nM PACAP-38 for 20 min and measurement of intracellular cAMP levels. Plot represents the grand mean ± SEM of three independent experiments performed in triplicates. Values are expressed as percentage of maximal intracellular cAMP generation, which was determined by stimulation with 25 µM forskolin. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

control	PACAP	150 µM ddAd + PACAP	300 µM ddAd + PACAP	600 µM ddAd + PACAP	Forskolin
0.13 ± 0.01	5.71 ± 1.56	2.00 ± 0.43	1.47 ± 0.26	1.05 ± 0.18	19.89 ± 3.65

Table 4: Absolute values of intracellular cAMP levels in pmol/well in NG108-15-rPAC1hop cells with or without 2'5'-dideoxyadenosine (ddAd) pre-treatment. Values represent the grand mean ± SEM of the three experiments shown in Figure 15.

4.1.3.2 rPAC1hop and null confer an intracellular Ca^{2+} response

Expression of rPAC1hop and null in NG108-15 cells conferred a rise in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) upon treatment with 100 nM PACAP, whereas expression of rPAC1hip was without effect. In both cell lines the response consisted of a rapid and transient rise of $[Ca^{2+}]_i$ reminiscent of IP_3 receptor-mediated Ca^{2+} release from intracellular stores, with no obvious Ca^{2+} influx "plateau phase" as observed in PC12-G cells. $[Ca^{2+}]_i$ elevation was greater in PAC1hop- than in PAC1null-expressing cells, suggesting that the efficacy of coupling to Ca^{2+} is increased by expression of the hop cassette in the third intracellular loop of the receptor, whereas coupling to Ca^{2+} is abolished by expression of the hip cassette. Expression of GFP alone was without effect. Depolarization-induced Ca^{2+} influx was similar in the five cell lines as determined by stimulation with 55 mM KCl following stimulation with PACAP (Fig. 16).

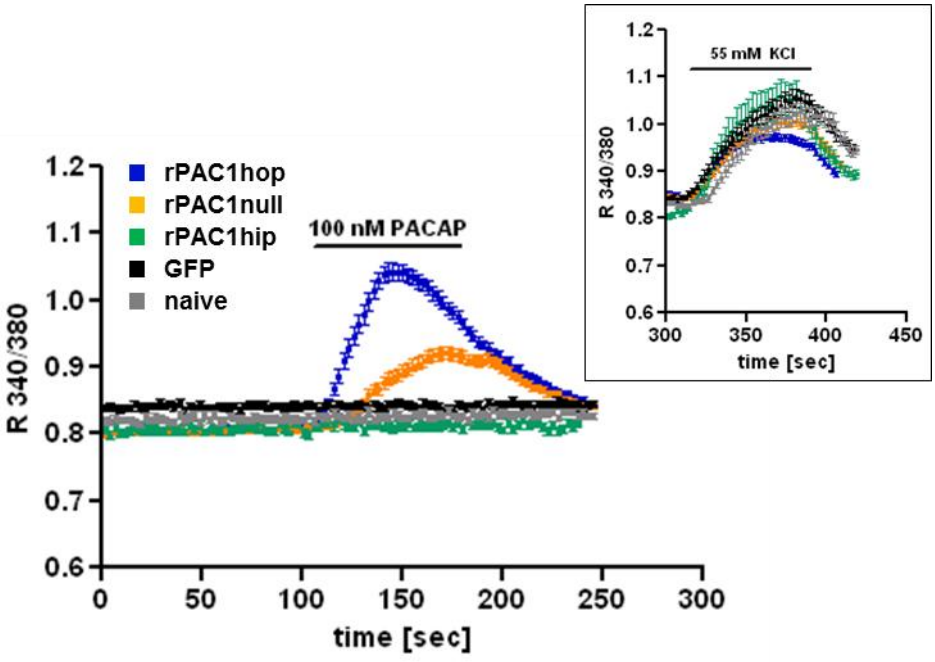


Figure 16: Expression of the rat PAC1hop and null but not the hip receptor in NG108-15 cells confers an increase in intracellular Ca^{2+} upon PACAP treatment. PAC1hop is more efficacious in increasing $[Ca^{2+}]_i$ than PAC1null. Cells were loaded with 4 µM fura-2 AM and stimulated with 100 nM PACAP-38 for 75 sec in KRB. Insert shows depolarization-induced Ca^{2+} influx by 55 mM KCl following PACAP treatment. Intracellular Ca^{2+} concentrations were measured in single cells using the 340/380 excitation ratio (R 340/380) and an emission wavelength of 510 nm. Images were captured every 2 sec. Plots represent the average ± SEM of five (naïve), six (rPAC1hip and GFP) or 19 (rPAC1hop and null) independent experiments. Each experiment represents 10-25 cells (hop: n=376, null: n=302, hip: n=85, GFP: n=94, naïve: n=94).

4.1.4 Functional characterization of PACAP-mediated signaling in cultured rat cortical neurons

4.1.4.1 Rat cortical neurons mainly express PAC1hop and null

PACAP signaling was next examined in primary cultures of rat cortical neurons. To determine which PACAP receptors are responsible for mediating PACAP's effects, an analysis of VPAC/PAC receptor-mRNAs was performed by RT-PCR. Cortical neurons predominantly expressed the PAC1hop and null receptor variants. A low expression of PAC1hip receptors and a very low expression of VPAC1 and VPAC2 receptors was also detected, suggesting that PACAP's action in primary cortical neurons is mainly mediated by the PAC1hop and null receptor variants with a minor contribution of PAC1hip and VPACs (Fig. 17).

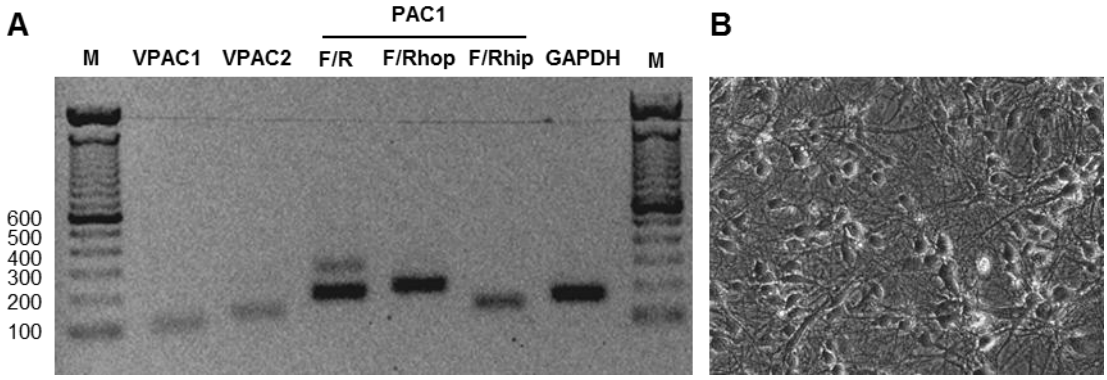


Figure 17: Rat cortical neurons predominantly express the PAC1null and hop receptor variants. (A) RT-PCR analysis of endogenous VPAC1, VPAC2 and PAC1 receptors in primary cultures of rat cortical neurons. Total RNA was reverse-transcribed and PCR-amplified (35 cycles) with different primer pairs for rat VPAC1, VPAC2 and PAC1. To differentiate between PAC1 splice variants of the third intracellular loop different primer pairs within the transmembrane region 5 and 6 (TM 5 and 6) were used. The same forward primer

(F) was used with different reverse primers (R): F/R generates a 187- or 271-bp fragment, depending on whether or not a hip- or hop-insert is present; the hop-specific primer pair F/hopR generates a 200-bp fragment; the hip-specific primer pair F/hipR generates a 142-bp fragment (schematic representation see Fig. 6B). Primers for VPAC1 generate a 107-bp fragment and for VPAC2 a 133-bp fragment. GAPDH product size is 168-bp. M: 100-bp DNA ladder. (B) Photomicrograph of rat cortical neurons.

4.1.4.2 PACAP increases intracellular cAMP generation, which can be pharmacologically blocked by 2′5′-dideoxyadenosine

Treatment of rat cortical neurons with 100 nM PACAP resulted in a robust increase of intracellular cAMP. The amount generated was approximately 60% of the amount generated by supramaximal stimulation of adenylate cyclases (ACs) with 25 µM forskolin. The AC inhibitor 2′5′-dideoxyadenosine (ddAd) reduced PACAP-stimulated cAMP levels in a concentration-dependent manner. At a concentration of 600 µM, ddAd completely blocked cAMP production. Cyclic AMP generation by PACAP with or without ddAd pre-treatment is shown as percentage of maximal cAMP generation determined by stimulation with 25 µM forskolin (Fig. 18). The absolute amounts of cAMP in untreated and treated cells are shown in Table 5.

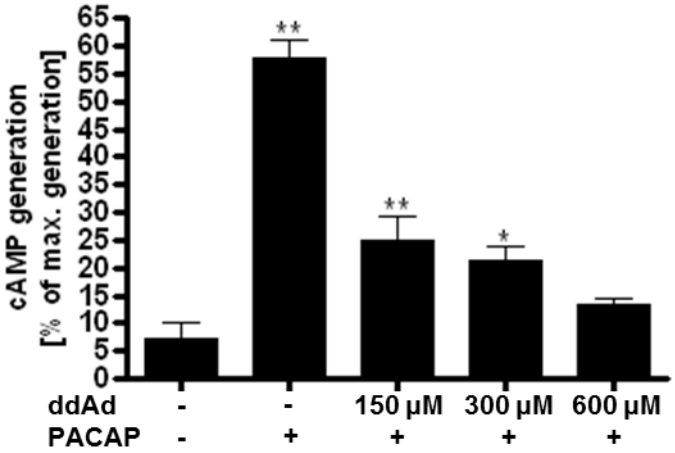


Figure 18: PACAP stimulation triggers intracellular cAMP generation in rat cortical neurons, which can be blocked by high concentrations of the AC inhibitor 2'5'-dideoxyadenosine. Cells were treated with 100 nM PACAP-38 for 20 min with or without pre-treatment with 150, 300 or 600 µM 2'5'-dideoxyadenosine (ddAd) and intracellular cAMP levels were measured. Plot represents the grand mean ± SEM of three independent experiments performed in triplicates. Values are expressed as percentage of maximal intracellular cAMP generation, which was determined by stimulation with 25 µM forskolin. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

control	PACAP	150 µM ddAd + PACAP	300 µM ddAd + PACAP	600 µM ddAd + PACAP	Forskolin
0.12 ± 0.04	1.15 ± 0.41	0.54 ± 0.28	0.43 ± 0.17	0.26 ± 0.10	2.02 ± 0.73

Table 5: Absolute values of intracellular cAMP levels in pmol/well in rat cortical neurons with or without 2'5'-dideoxyadenosine (ddAd) pre-treatment. Values represent the grand mean ± SEM of the three experiments shown in Figure 18.

4.1.4.3 PACAP induces Ca^{2+} mobilization and influx

Exposure of rat cortical neurons to 100 nM PACAP also resulted in a rapid elevation of $[Ca^{2+}]_i$ followed by a persisting plateau. To differentiate between Ca^{2+} mobilization and influx, cells were stimulated in Ca^{2+}-free buffer. PACAP induced a rapid and temporally restricted rise in intracellular Ca^{2+}, representing Ca^{2+} release from intracellular stores (Ca^{2+} mobilization). Upon reintroduction of Ca^{2+}, Ca^{2+} entered the cell leading to prolonged Ca^{2+} influx (Fig. 19). The PACAP-induced Ca^{2+} response was reminiscent of the Ca^{2+} response observed in PAC1hop- or null-over-expressing PC12-G cells, which is in agreement with a predominant expression of PAC1hop and null receptors in cortical neurons. Approximately 25% of all cells showed no response or a very weak response to PACAP. These PACAP-non-responders were excluded from Figure 19. KCl stimulation following PACAP treatment induced Ca^{2+} influx in only 15% of PACAP-responding cells (left insert in Fig. 19); these cells did not show a prolonged Ca^{2+} influx plateau phase upon PACAP treatment. In the majority of PACAP-responders, where PACAP elicited both Ca^{2+} mobilization and influx, KCl did not increase intracellular Ca^{2+} levels and instead lead to a slight decrease (right insert in Fig. 19; PACAP response shown in Fig. 19 includes KCl-responders and -non-responders). When cells were stimulated with KCl without previous PACAP stimulation, all cells showed a depolarization-induced Ca^{2+} influx (not shown), suggesting that PACAP receptor activation modulates voltage-gated calcium channels (VGCCs). Moreover, all PACAP-non-responders showed a robust rise in $[Ca^{2+}]_i$ upon depolarization (not shown).

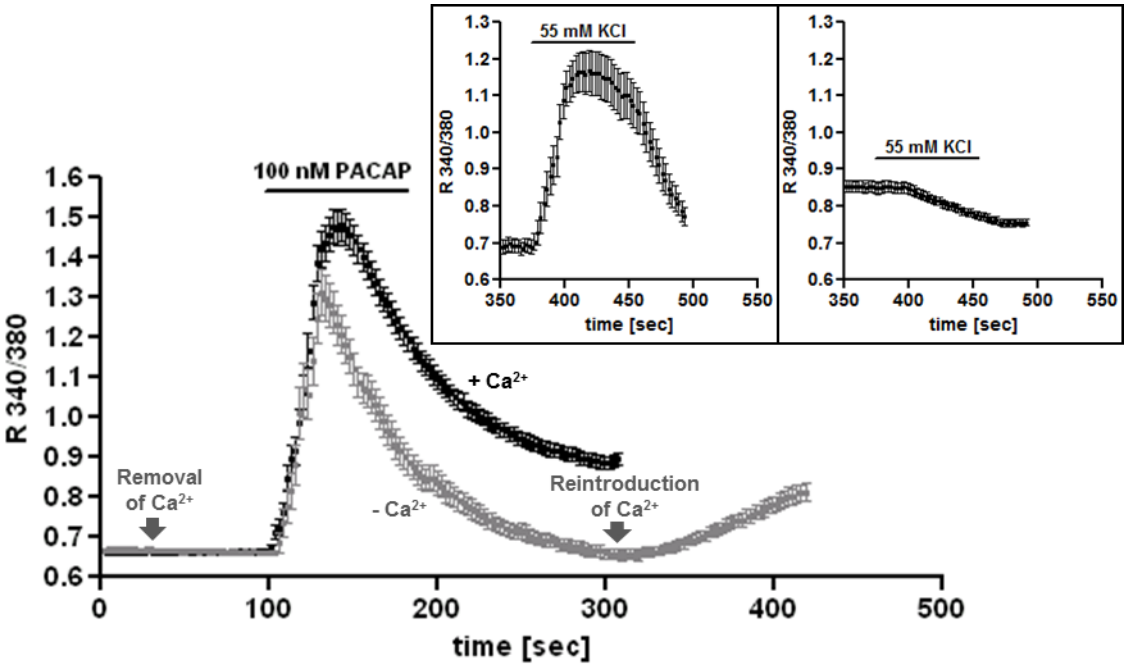


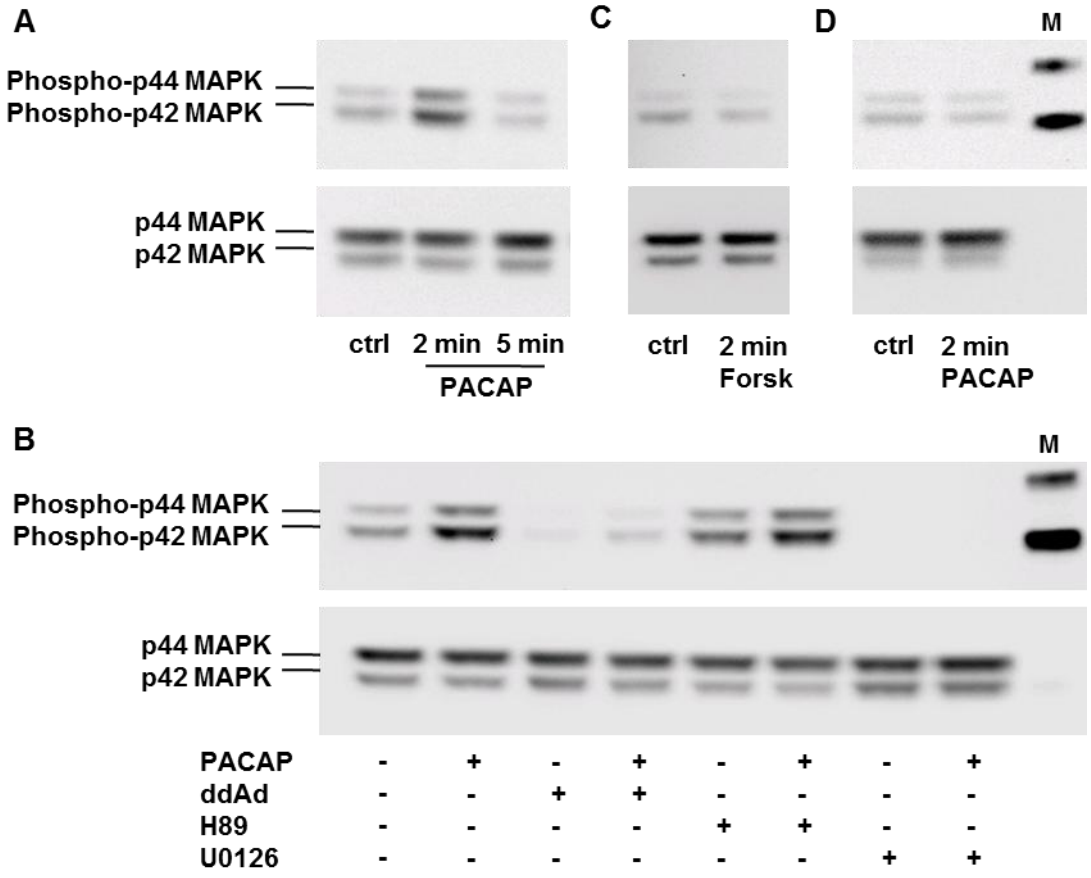
Figure 19: PACAP stimulation triggers intracellular Ca^{2+} mobilization and influx in the majority of rat cortical neurons. Cells were loaded with 4 µM fura-2 AM and stimulated with 100 nM PACAP-38 for 75 sec in Ca^{2+}-containing (black line) or Ca^{2+}-free KRB (gray line). Inserts show depolarization-induced Ca^{2+} influx by 55 mM KCl following PACAP treatment in Ca^{2+}-containing buffer. Intracellular Ca^{2+} concentrations were measured in single cells using the 340/380 excitation ratio (R 340/380) and an emission wavelength of 510 nm. Images were captured every 2 sec. ~75% of all cells respond to PACAP with a robust increase in $[Ca^{2+}]_i$, whereas the remaining 25% show no or a very weak response and are excluded from this graph. ~15% of PACAP-responding cells show a depolarization-induced Ca^{2+} influx following PACAP treatment (left insert), whereas 85% do not (right insert). Plots represent the average ± SEM of four (- Ca^{2+}) or 11 (+ Ca^{2+}) independent experiments. Each experiment represents 6-22 cells (- Ca^{2+}: n=59, + Ca^{2+}: n=155).

4.2 Signaling to MAPK activation in neural cells

4.2.1 PACAP activates the MAPK ERK1/2 through cAMP but not PKA in NG108-15-rPAC1hop cells

The importance of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway in mediating PACAP's neurotrophic effects has been extensively studied in PC12 cells. Here, the activation of ERK was characterized in NG108-15 cells. Exposure of NG108-15 cells stably expressing the rat PAC1hop receptor to 100 nM PACAP resulted in a rapid activation of ERK as determined by

the phosphorylation of the p44/42 MAPK (ERK1/2). The activation was transient with its maximum at 2 min and subsiding by 5 min (Fig. 20A+E). The AC activator forskolin failed to activate ERK (Fig. 20C). However, PACAP's stimulatory effects on ERK were blocked by the AC inhibitor 2′5′-dideoxyadenosine (ddAd), suggesting that cAMP generation by PACAP is necessary but not sufficient to activate ERK in NG108-15 cells. Moreover, the PKA inhibitor H89 did not block ERK activation, suggesting a cAMP dependent but PKA independent activation of ERK by PACAP. The MEK1/2 inhibitor U0126 completely blocked ERK activation, as expected (Fig. 20B+F). No treatment affected total ERK levels. PACAP did not activate ERK in NG108-15 cells expressing GFP only (Fig. 20D).



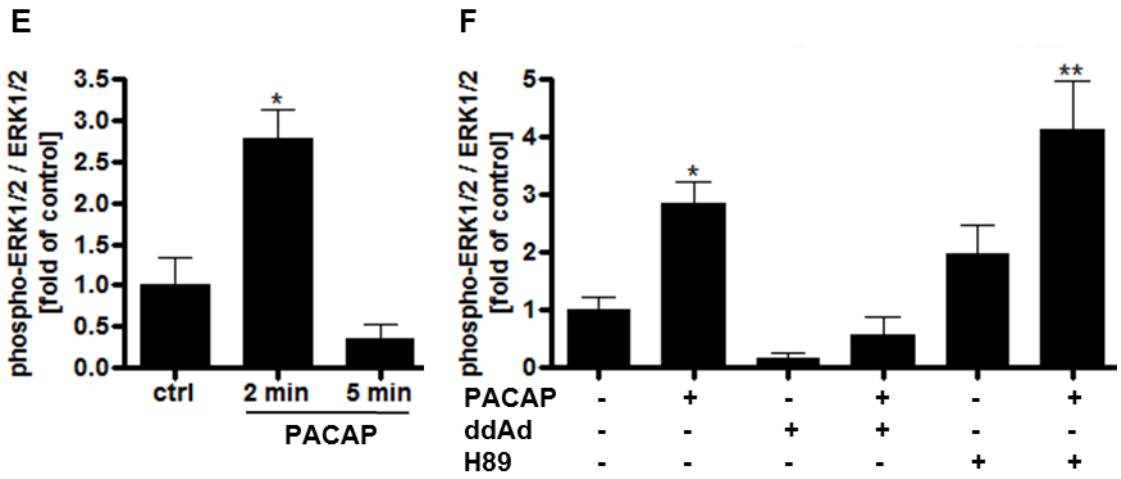
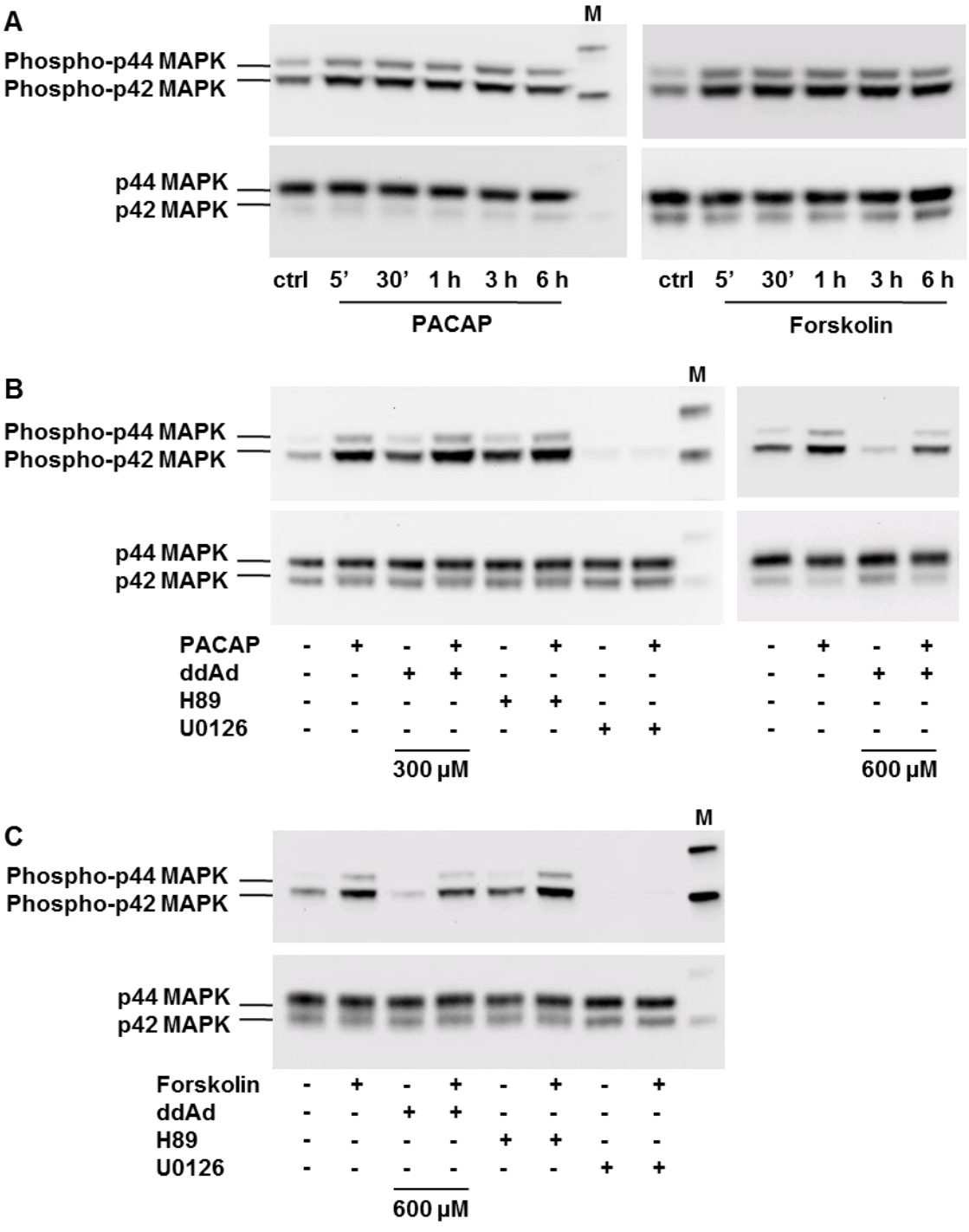


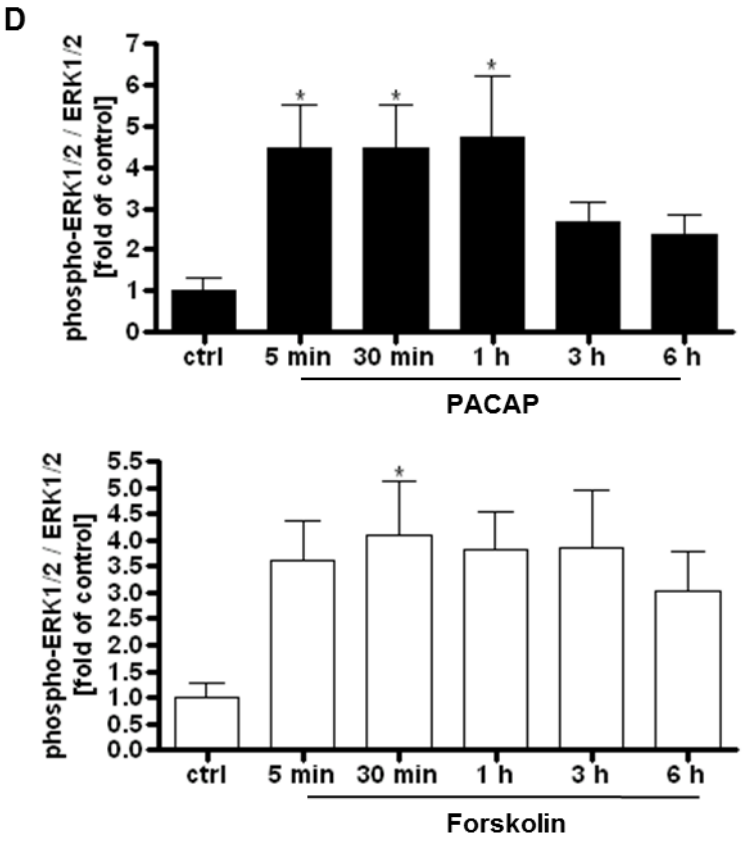
Figure 20: PACAP rapidly activates ERK1/2 (p44/42 MAPK) in NG108-15-rPAC1hop cells. Activation is blocked by the AC inhibitor 2'5'-dideoxyadenosine but not blocked by the PKA inhibitor H89. NG108-15-rPAC1hop cells (A-C) and NG108-15-GFP cells (D) were stimulated with 100 nM PACAP-38 or 25 µM forskolin in the presence or absence of the AC inhibitor 2'5'-dideoxyadenosine (ddAd, 300 µM), the PKA inhibitor H89 (10 µM) or the MEK1/2 inhibitor U0126 (10 µM) (30 min pre-treatment with inhibitors). Whole cell extracts were harvested after 2 or 5 min. Expression of the rat PAC1hop receptor in NG108-15 cells confers a rapid and transient activation of ERK1/2 (A), which is not seen in control cells expressing GFP only (D). ERK1/2 activation is unaffected by H89, but blocked by ddAd and U0126 (B). Forskolin fails to activate ERK1/2 (C). No treatment affects total ERK1/2 levels. M: 40 and 50 kDa band. (E+F) Quantification of A and B. Values are expressed as the ratio of phospho-ERK divided by total ERK and represent the mean ± SEM of three independent experiments performed in singlicates. The control value represents the mean of the fold of average control, which was determined by calculating the p-ERK/ERK mean of the three control values and dividing each p-ERK/ERK control value by this mean. U0126 is not shown in the plot. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

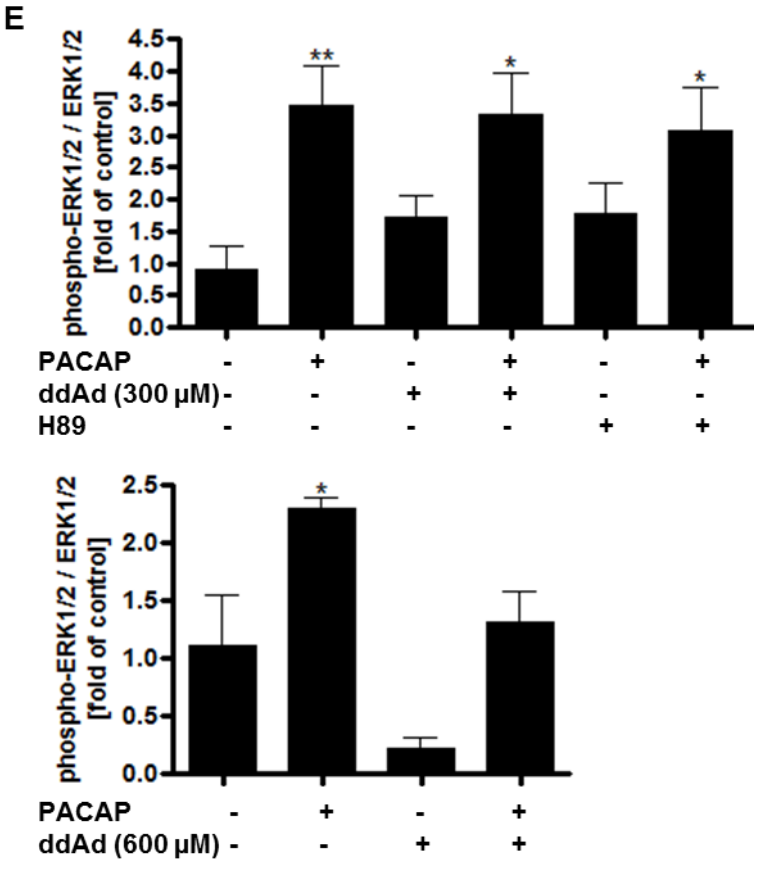
4.2.2 PACAP and forskolin activate the MAPK ERK1/2 through cAMP but not PKA in cultured rat cortical neurons

Primary cultures of rat cortical neurons responded to 100 nM PACAP and 25 µM of the AC activator forskolin with a rapid and prolonged activation of the MAPK ERK1/2 (p44/42 MAPK) as determined by phosphorylation of the two proteins (Fig. 21A+D). ERK1/2 activation was blocked by 600 µM of the AC inhibitor 2′5′-dideoxyadenosine (ddAd). 300 µM ddAd, however, failed to block PACAP-mediated ERK activation, indicating that very low levels of cAMP are sufficient to activate

ERK (see Fig. 18 for effects of ddAd on PACAP-stimulated cAMP generation). The PKA inhibitor H89 failed to block PACAP- as well as forskolin-induced ERK activation, suggesting that ERK activation proceeds through cAMP but not PKA in cortical neurons. The MEK1/2 inhibitor U0126 completely blocked ERK activation, as expected (Fig. 21B, C, E, F). No treatment affected total ERK levels.




D
phospho-ERK1/2 / ERK1/2 [fold of control]
ctrl
5 min
30 min
1 h
3 h
6 h
PACAP
Forskolin


E
phospho-ERK1/2 / ERK1/2 [fold of control]
PACAP
ddAd (300 µM)
H89
ddAd (600 µM)

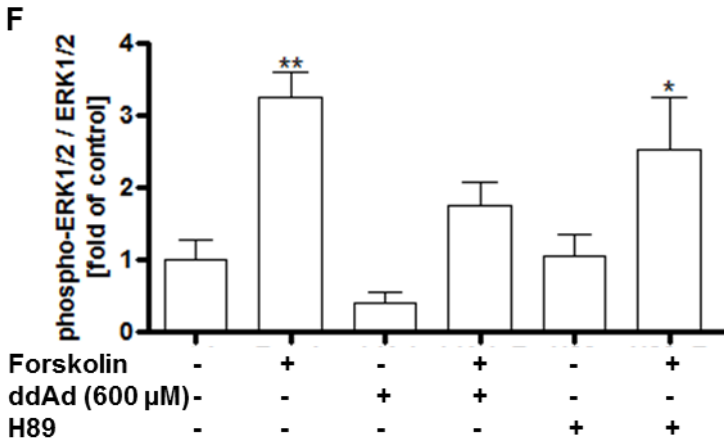


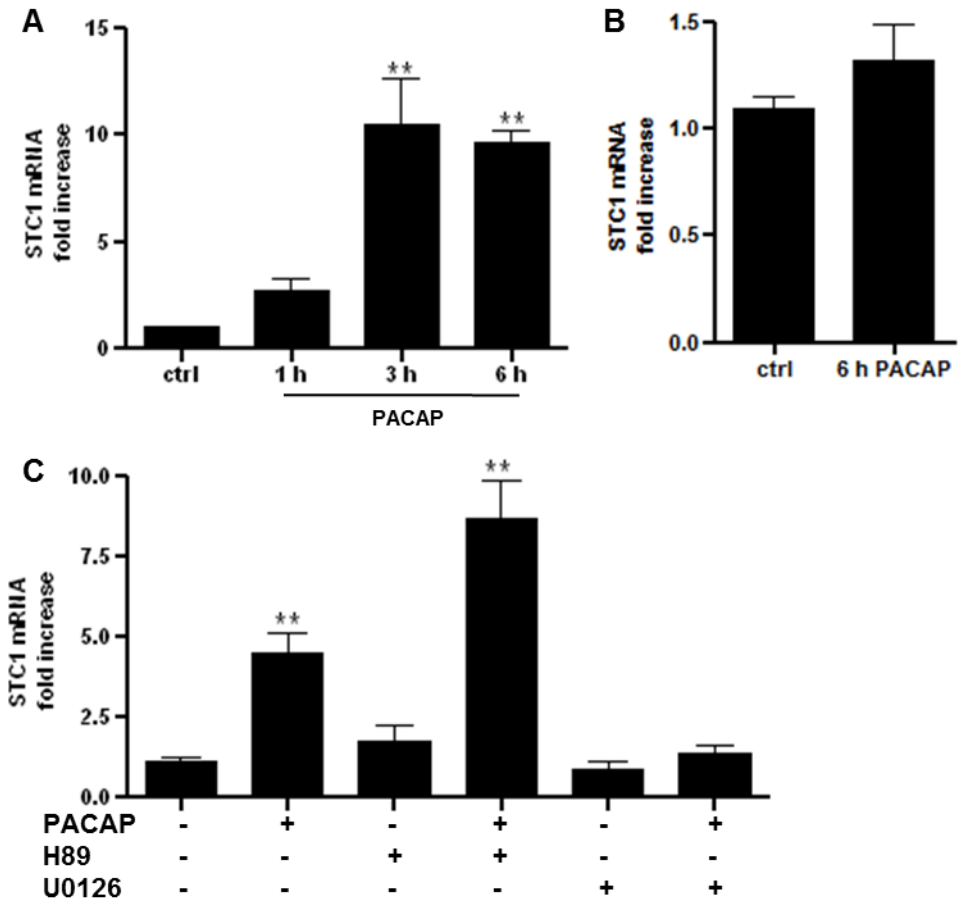
Figure 21: PACAP and forskolin activation of the p44/42 MAPK (ERK1/2) is rapid and prolonged in rat cortical neurons (A). Activation is blocked by the AC inhibitor 2'5'-dideoxyadenosine but not blocked by the PKA inhibitor H89 (B+C). Cells were stimulated with 100 nM PACAP-38 or 25 µM forskolin in the presence or absence of the AC inhibitor 2'5'-dideoxyadenosine (ddAd, 300 and 600 µM), the PKA inhibitor H89 (10 µM) or the MEK1/2 inhibitor U0126 (10 µM) (30 min pre-treatment with inhibitors). Whole cell extracts were harvested after 5 min, 30 min, 1, 3 or 6 h (A) or after 5 min (B+C). ERK activation by PACAP and forskolin is rapid and prolonged (A) and unaffected by H89, but blocked by 600 µM ddAd and U0126 (B+C). No treatment affects total ERK1/2 levels. M: 40 and 50 kDa band. (D-F) Quantification of A-C. Values are expressed as the ratio of phospho-ERK divided by total ERK and represent the mean ± SEM of three to seven independent experiments performed in singlicates. The control value represents the mean of the fold of average control, which was determined by calculating the p-ERK/ERK mean of the control values and dividing each p-ERK/ERK control value by this mean. U0126 is not shown in the plot. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

4.3 Signaling pathways mediating gene induction in neural cells

4.3.1 PACAP induces STC1 through ERK1/2 but not PKA in NG108-15-rPAC1hop cells

In a microarray analysis we identified stanniocalcin 1 (STC1) up-regulated upon 6 h of PACAP treatment in PC12-G cells expressing physiological levels of the bovine PAC1hop receptor (PC12-bPAC1hop) and in primary bovine chromaffin cells (BCCs) (Ait-Ali et al., 2010). In this study the induction of STC1 was examined in cells of the central nervous system, where STC1 could be a mediator of PACAP's neuroprotective effects in e.g., stroke. NG108-15 cells stably over-expressing the rat

PAC1hop receptor (NG108-15-rPAC1hop) responded to 100 nM PACAP with an induction of STC1 mRNA after 3 and 6 h (Fig. 22A), which was not seen in control cells expressing GFP only (Fig. 22B) as measured by qRT-PCR. PACAP-mediated STC1 induction after 6 h was completely blocked by the MEK1/2 inhibitor U0126, whereas the PKA inhibitor H89 was without effect (Fig. 22C), suggesting that PACAP signaling to the STC1 gene proceeds through the MAPK ERK1/2 independently of PKA activation, which is in agreement with a PKA independent but cAMP dependent activation of ERK itself (see Fig. 20). The AC activator forskolin, in contrast, induced STC1 mRNA in an H89 sensitive manner (Fig. 22D). Treatment with inhibitors only was without effect. These results suggest that two pathways for STC1 gene induction co-exist in NG108-15 cells, one through PACAP, where cAMP is necessary but not sufficient to activate ERK1/2 independently of PKA and another through intracellular cAMP generation only (by forskolin) and activation of PKA.



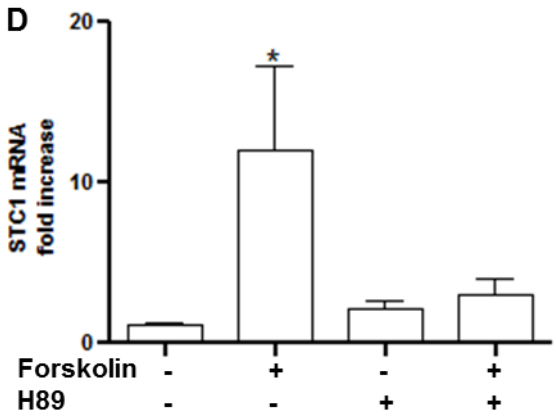


Figure 22: PACAP induces STC1 mRNA after 3 and 6 h in NG108-15-rPAC1hop cells (A). Induction after 6 h is blocked by the MEK1/2 inhibitor U0126 but not blocked by the PKA inhibitor H89 (C). STC1 induction by forskolin, in contrast, is blocked by H89 (D). NG108-15-rPAC1hop cells (A, C, D) and NG108-15-GFP cells (B) were stimulated with 100 nM PACAP-38 (black bars) or 25 µM forskolin (white bars) in the presence or absence of the PKA inhibitor H89 (10 µM) or the MEK1/2 inhibitor U0126 (10 µM) (30 min pre-treatment with inhibitors) and lysed after 1, 3 or 6 h (A) or after 6 h (B-D). Total RNA was extracted and reverse-transcribed. Transcript levels were measured by qRT-PCR. Values represent the grand mean ± SEM of three to four independent experiments performed in triplicates and are expressed as fold change of STC1 versus GAPDH mRNA. * $P < 0.05$, ** $P < 0.01$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test (A, C, D); not significantly different; unpaired t-test (B).

4.3.2 PACAP and forskolin induce STC1 through ERK1/2 but not PKA in cultured rat cortical neurons

Next, the induction of STC1 in primary cultures of rat cortical neurons was examined. Exposure of cortical neurons to 100 nM PACAP for 3 or 6 h resulted in a robust increase of STC1 mRNA (Fig. 23A), similar to the level of induction observed in NG108-15-rPAC1hop cells. The MEK1/2 inhibitor U0126 blocked the induction of STC1 after 6 h, whereas the PKA inhibitor H89 was without effect (Fig. 23B), which is in agreement with results obtained from NG108-15 cells. The AC activator forskolin also induced STC1 gene transcription, but unlike in NG108-15 cells, via the same signaling pathway used by PACAP; i.e., induction was blocked by U0126 but not blocked by H89 (Fig. 23C). Treatment with inhibitors only was without effect. These results suggest that a single signaling pathway to the STC1 gene exists in differentiated cortical neurons, proceeding through cAMP and the MAPK ERK1/2 but

not through PKA, in agreement with a cAMP dependent but PKA independent activation of ERK itself (see Fig. 21).

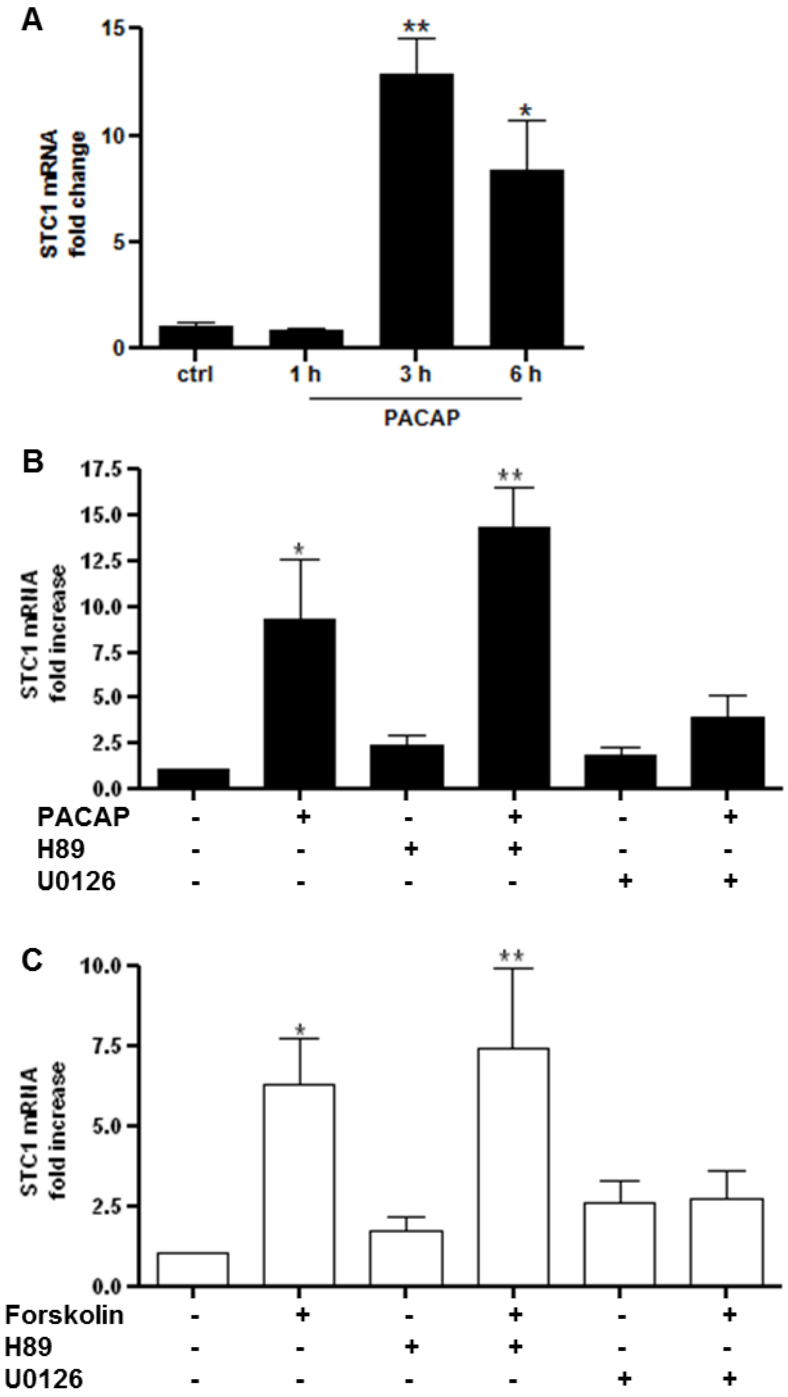


Fig. 23: PACAP induces STC1 mRNA after 3 and 6 h in rat cortical neurons (A). Induction by PACAP or forskolin after 6 h is blocked by the MEK1/2 inhibitor U0126 but not blocked by the PKA inhibitor H89 (B+C). Cells were stimulated with 100 nM PACAP-38 (black bars) or 25 µM forskolin (white bars) in the presence or absence of the PKA inhibitor H89 (10 µM) or the MEK1/2 inhibitor U0126 (10 µM) (30 min pre-treatment with inhibitors). Cells were lysed after 1, 3 or 6 h (A) or after 6 h (B+C). Total RNA was extracted and reverse-

transcribed. Transcript levels were measured by qRT-PCR. Values in A represent the mean ± SEM of one experiment performed in triplicates. Experiment was repeated once with similar results. In B and C, values represent the grand mean ± SEM of five to nine independent experiments performed in triplicates. Values are expressed as fold change of STC1 versus GAPDH mRNA. * $P < 0.05$, ** $P < 0.01$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

4.3.3 PACAP does not induce STC2 in cultured rat cortical neurons

Next, the induction of the second STC paralog, STC2, was examined by qRT-PCR in primary cultures of rat cortical neurons. PACAP, however, failed to induce STC2 mRNA significantly at each time point measured (1, 3 and 6 h) (Fig. 24), suggesting that only STC1 but not STC2 is regulated by PACAP in cortical neurons.

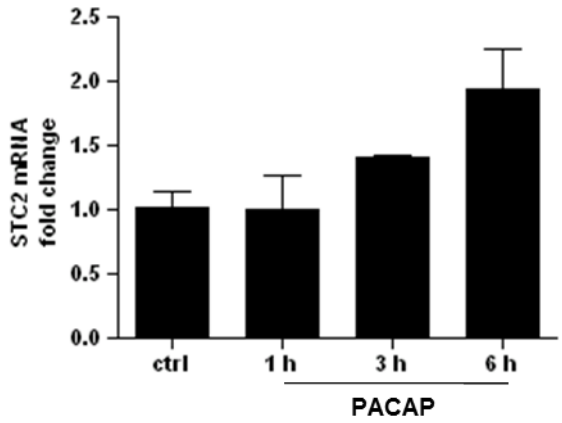


Figure 24: PACAP does not induce STC2 mRNA in rat cortical neurons. Cells were stimulated with 100 nM PACAP-38 and lysed after 1, 3 or 6 h. Total RNA was extracted and reverse-transcribed. Transcript levels were measured by qRT-PCR. Values represent the mean ± SEM of one experiment performed in triplicates. Experiment was repeated once with similar results. Values are expressed as fold change of STC2 versus GAPDH mRNA. Not significantly different from control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

4.3.4 PACAP induces BDNF through PKA in cultured rat cortical neurons

Neurotrophic factors like brain-derived neurotrophic factor (BDNF) are important mediators of neuronal survival after brain injury. PACAP has been shown to induce BDNF in cultures of cortical neurons, mediating PACAP's protective effects against excitotoxic insult (Frechilla et al., 2001; Pellegri et al., 1998). Exposure to 100 nM PACAP induced BDNF mRNA significantly after 3 h (Fig. 25A). This induction was,

unlike the induction of STC1, blocked by the PKA inhibitor H89 (Fig. 25B), suggesting that two potentially neuroprotective pathways co-exist in cortical neurons, one being PKA dependent, and another being PKA independent but ERK1/2 dependent.

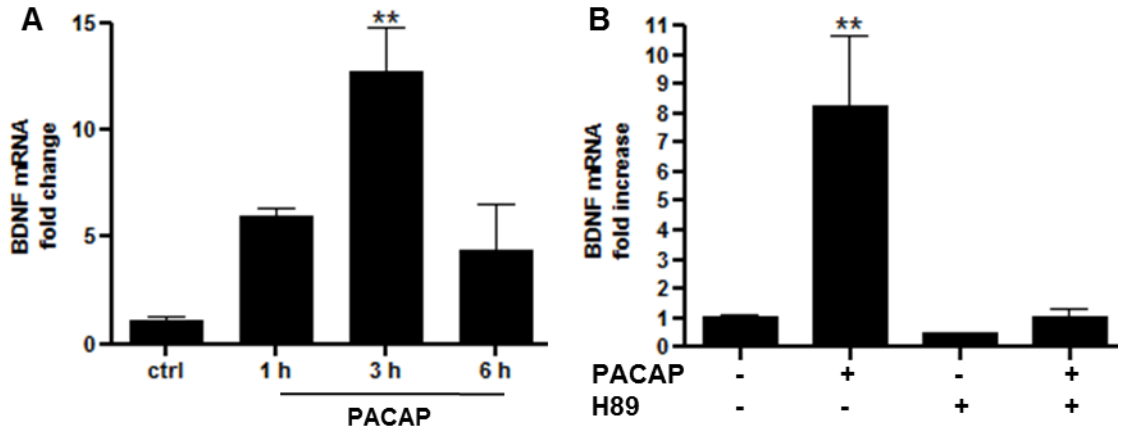


Fig. 25: Induction of BDNF mRNA by PACAP is blocked by the PKA inhibitor H89 in rat cortical neurons. Cells were stimulated with 100 nM PACAP-38 in the presence or absence of the PKA inhibitor H89 (10 µM, 30 min pre-treatment). Cells were lysed after 1, 3 or 6 h (A) or after 3 h (B). Total RNA was extracted and reverse-transcribed. Transcript levels were measured by qRT-PCR. Values in A represent the mean ± SEM of one experiment performed in triplicates. Experiment was repeated once with similar results. In B, values represent the grand mean ± SEM of four independent experiments performed in triplicates. Values are expressed as fold change of BDNF versus GAPDH mRNA. ** $P < 0.01$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

4.4 Effects of PACAP and STC1 on neuronal survival during glutamate-induced excitotoxicity and oxygen-glucose-deprivation (OGD)

To investigate the neuroprotective effects of PACAP in the neuronal cell culture system used in this study and to determine whether the PACAP target gene STC1 supports cell survival potentially mediating PACAP's neuroprotective effect, primary cultures of rat cortical neurons were subjected to glutamate-induced excitotoxicity and oxygen-glucose-deprivation (OGD), two cell culture models for ischemia and stroke. Excitotoxicity refers to the damaging of neurons and induction of apoptosis through an over-activation of excitatory glutamate receptors, specifically NMDA receptors, occurring after brain injury, such as stroke.

4.4.1 PACAP fails to prevent cell death of cultured rat cortical neurons during OGD and excitotoxicity

Primary cultures of rat cortical neurons were subjected to 3 h of oxygen-glucose-deprivation (OGD) followed by 21 h of restoration of oxygen/glucose (ROD) in the presence or absence of various concentrations of PACAP. The level of cell viability was determined by conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazoliumbromide (MTT), which occurs only in living cells. OGD reduced cell survival by approximately 40%. Unexpectedly, neither dose of PACAP tested (1, 10 and 100 nM) reduced OGD-induced neuronal cell death (Fig. 26).

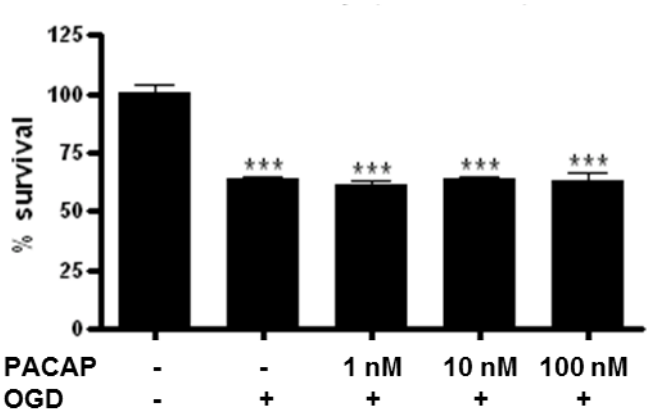
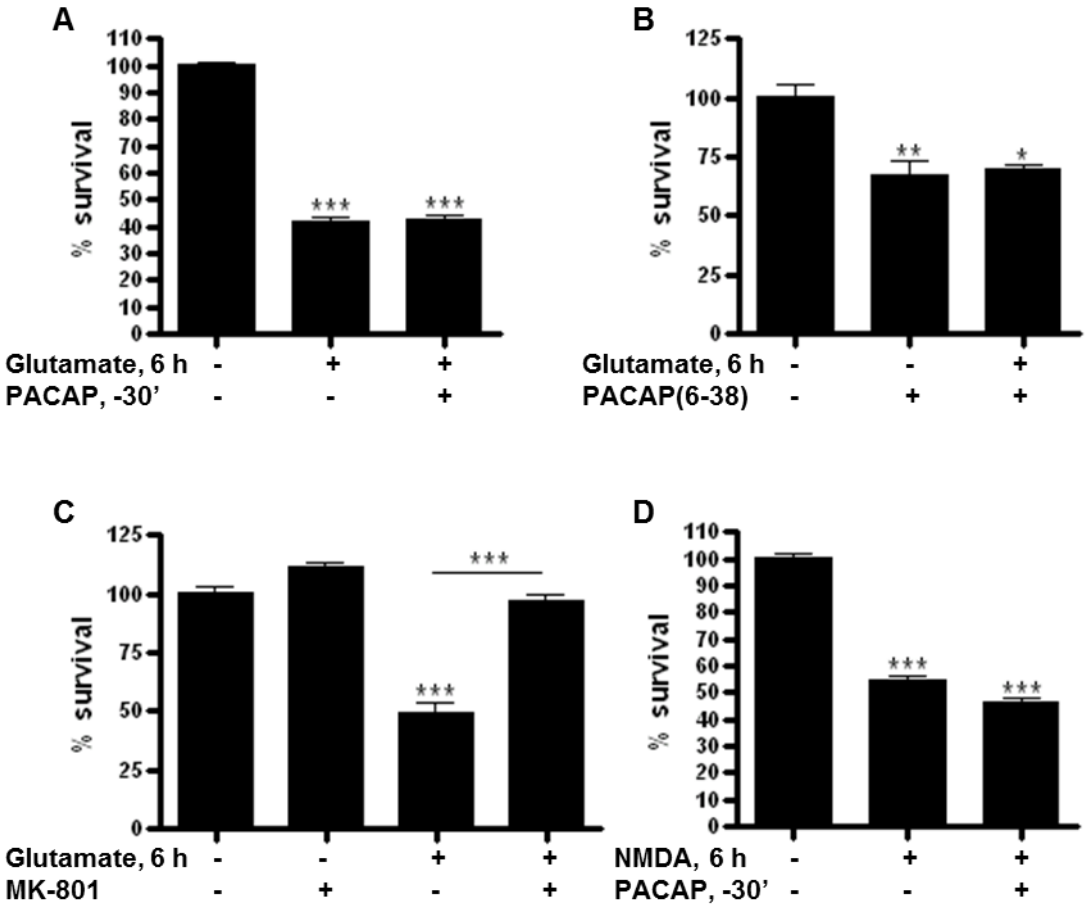


Fig. 26: Oxygen-glucose-deprivation (OGD) causes cell death in rat cortical neurons, which is not prevented by PACAP treatment. Cells were subjected to 3 h of OGD in the presence or absence of various concentrations of PACAP-38. After 24 h neuronal viability was assessed by measurement of MTT conversion. Plot represents the mean ± SEM of one experiment performed in triplicates. Values are expressed as % survival compared to control. *** $P < 0.001$ versus control and between treatments; one-way ANOVA, with Tukey's Multiple Comparison Test.

Exposure of rat cortical neurons to 1 mM L-glutamate or 5 µM N-methyl-D-aspartate (NMDA) for 6 or 24 h reduced cell survival by approximately 50% as determined by MTT conversion. Unexpectedly, 100 nM PACAP, when present 30 min or 6 h prior to the induction of excitotoxicity and throughout the experiment, failed to reduce cell death (Fig. 27 A, D, E). PACAP was also without effect when applied simultaneously with NMDA for 6 or 24 h or when given 24 h, 2 or 6 days prior to a 6 h glutamate treatment (not shown). To determine whether endogenous, in contrast to exogenous PACAP contributes to cell survival during excitotoxicity,

cortical neurons were treated with NMDA for 6 or 24 h in the presence or absence of 100 nM of the PACAP antagonist PACAP(6-38). However, PACAP(6-38) did not exacerbate cell death induced by excitotoxicity, suggesting that endogenous PACAP does not play a role in neuronal survival during excitotoxicity (Fig. 27B+F). As expected, the NMDA receptor antagonist (+)-MK-801 hydrogen maleate (100 nM) completely reversed cell death by glutamate (Fig. 27C), suggesting that glutamate-induced cell death is mediated by activation of the NMDA receptor subfamily of glutamate receptors, which is in agreement with a similar effect of glutamate and NMDA on the induction of cell death in rat cortical neurons. These results suggest that, in primary cultures of cortical neurons, neither endogenous nor exogenous PACAP supports cell survival during short- (6 h) or long (24 h)-term excitotoxicity, mediated by over-activation of NMDA receptors, which is associated with stroke as part of the ischemic cascade and other neurodegenerative disorders.



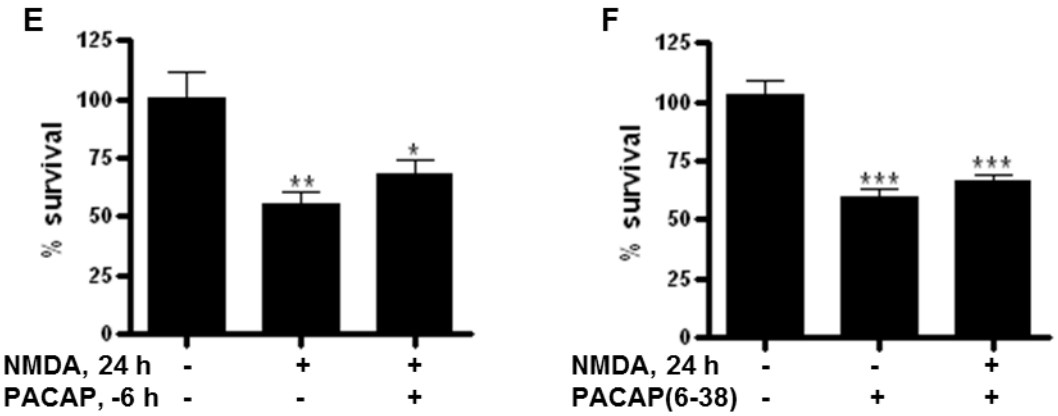


Figure 27: Excitotoxicity induces cell death in rat cortical neurons through activation of NMDA receptors. PACAP is without neuroprotective effect. Cells were treated with 1 mM glutamate (A-C) or 5 µM NMDA (D-F) for 6 or 24 h in the presence or absence of 100 nM PACAP-38 (pre-treated for 30 min or 6 h), 100 nM PACAP(6-38) or 100 nM (+)-MK-801 hydrogen maleate. Neuronal viability was assessed by measurement of MTT conversion. Values represent the mean ± SEM of one experiment performed in triplicates (A-D) or quadruplicates (F) or the grand mean of five independent experiments performed in triplicates (E) and are expressed as % survival compared to control. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus control and between treatments; one-way ANOVA, with Tukey's Multiple Comparison Test.

4.4.2 STC1 over-expression does not prevent cell death of cultured rat cortical neurons during excitotoxicity

To determine whether stanniocalcin 1 (STC1) can support neuronal survival during excitotoxic insult, primary cultures of rat cortical neurons over-expressing STC1 were generated through infection with lentiviral particles. Lentivirus was made with the pLVX-IRES-ZsGreen1 vector, a bicistronic lentiviral expression vector containing an internal ribosomal entry site (IRES) to simultaneously co-express the gene-of-interest (i.e., STC1) and the ZsGreen1 fluorescent protein from a single mRNA transcript. Neurons were infected at 4 days in vitro (div) and the expression of STC1 was monitored by co-expression of ZsGreen1. Six days later, cell death was induced by 1 mM glutamate for 6 h in STC1- and ZsGreen1 only-expressing neurons and measured by MTT conversion. Glutamate-induced excitotoxicity reduced cell survival by approximately 40% in both ZsGreen1 only- and STC1-expressing neurons (Fig. 28), suggesting that over-expression of the putative neuroprotectant STC1 is without neuroprotective effect during short-term excitotoxicity in primary cultures of cortical neurons.

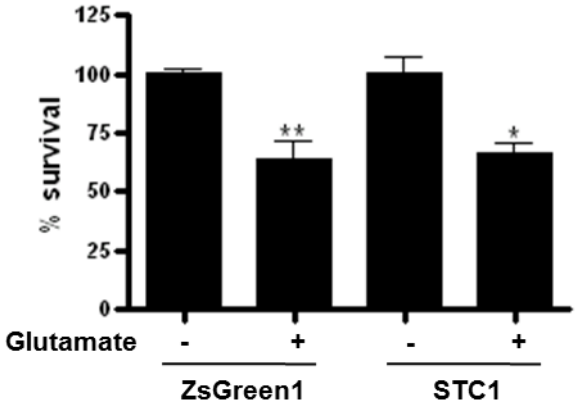


Figure 28: Excitotoxicity-induced cell death in rat cortical neurons is not reduced by STC1 over-expression. Cells were infected with lentivirus expressing the ZsGreen1 fluorescent protein only or ZsGreen1-IRES-STC1. Six days later, cells were treated with 1 mM glutamate for 6 h and neuronal viability was assessed by measurement of MTT conversion. Values represent the mean ± SEM of three independent experiments performed in singlicates. Values are expressed as % survival compared to respective control. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Tukey's Multiple Comparison Test.

Propidium iodide (PI) staining was further used to assess cell death of neuronal cultures. PI stains nuclei of cells with disrupted plasma membranes, therefore staining dead or dying cells. Cells were counterstained with Hoechst 33342, which stains all nuclei. Neurons expressing ZsGreen1 only or STC1-IRES-ZsGreen1 were subjected to excitotoxicity for 6 h and stained with Hoechst and PI. Cell death was assessed by fluorescence microscopy. Figure 29 shows the respective ZsGreen1, PI, Hoechst and phase contrast (PC) pictures. Treatment with 1 mM glutamate increased the amount of PI positive cells in both ZsGreen1 only- and STC1-IRES-ZsGreen1-expressing neurons to about the same level. This suggests that the induction of cell death is not prevented by STC1 over-expression, in agreement with results obtained with the MTT assay.

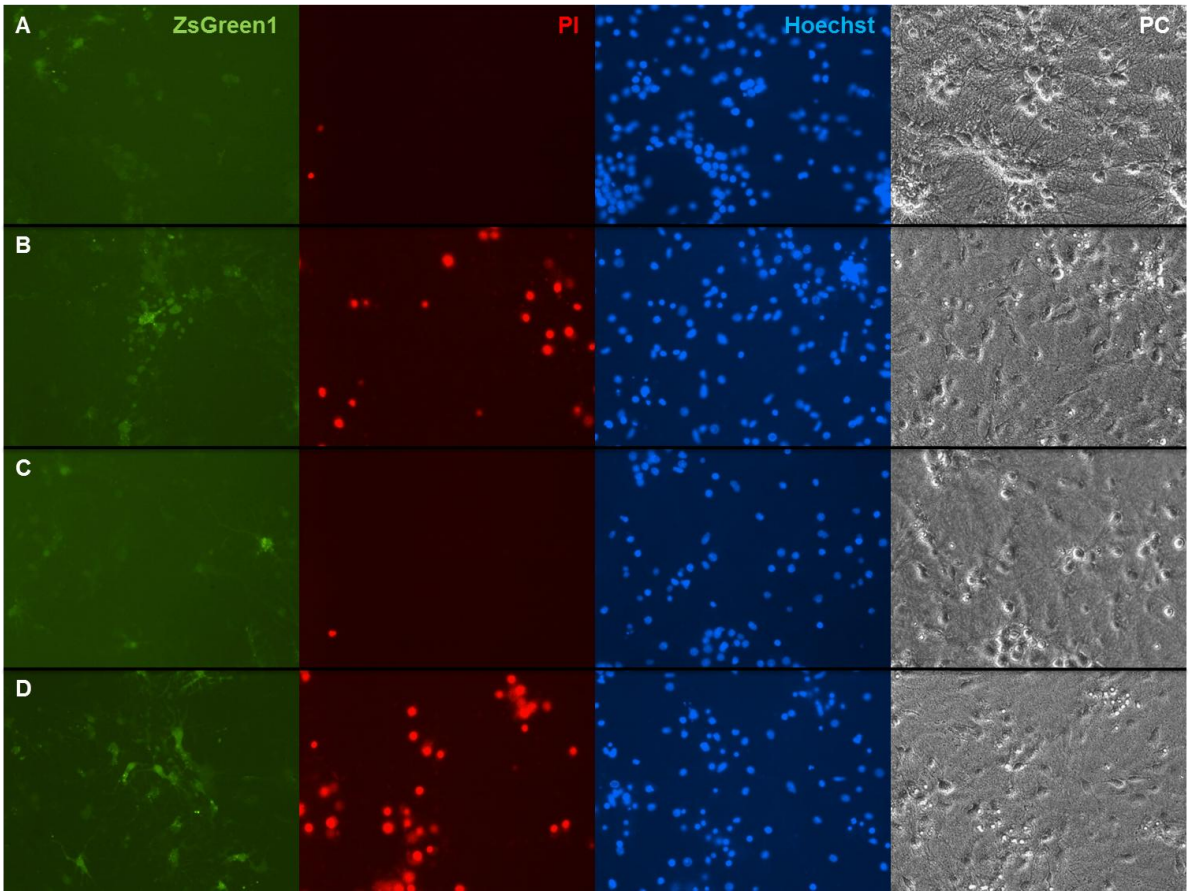


Figure 29: Excitotoxicity-induced cell death in rat cortical neurons is not reduced by STC1 over-expression. Cells were infected with lentiviruses expressing the ZsGreen1 fluorescent protein only (A+B) or ZsGreen1-IRES-STC1 (C+D). Six days later, cells were treated with 1 mM glutamate for 6 h (B+D) and stained with propidium iodide (PI) and Hoechst 33342. At least three fluorescent pictures were taken per group and experiment was repeated twice with similar results. A: ZsGreen1 only, no treatment, B: ZsGreen1 only, glutamate treatment, C: ZsGreen1-IRES-STC1, no treatment, D: ZsGreen1-IRES-STC1, glutamate treatment.

5. Discussion

In the present study signal transduction pathways mediated by PAC1 splice variants of the third intracellular loop (ic3) have been characterized in neural and neuroendocrine cell lines. Investigations were extended to primary neurons. The importance of PACAP under paraphysiological and pathophysiological conditions, in particular its role in prolonged catecholamine (CA) secretion from adrenomedullary chromaffin cells and in the induction of genes in neural cells of the central nervous system potentially mediating PACAP's neuroprotective effects during ischemic stress has been investigated.

The main new findings of this thesis are the following:

(1) The hop cassette in ic3 of PAC1 is required for the induction of a maximal intracellular Ca^{2+} response and prolonged CA secretion from neuroendocrine PC12-G cells. PAC1null increases $[Ca^{2+}]_i$ somewhat less and does not induce prolonged CA release. PAC1hip neither increases $[Ca^{2+}]_i$ nor CA secretion.

(2) PAC1hop, null and hip mediate an intracellular cAMP response in neural NG108-15 cells, while elevation of $[Ca^{2+}]_i$ occurs most efficiently in PAC1hop-, to a lesser extent in PAC1null-, and is absent in PAC1hip-expressing cells.

(3) Activation of ERK1/2 by PACAP is mediated through non-canonical (PKA independent) cAMP dependent signaling in neural NG108-15 cells and primary cortical neurons.

(4) Induction of STC1 by PACAP is dependent on ERK1/2 activation proceeding independently of PKA in neural NG108-15 cells and primary cortical neurons.

(5) Neither PACAP treatment nor STC1 over-expression increases cell viability in neuronal cell culture models of stroke. PACAP may mediate its neuroprotective effects in vivo in concert with cells absent in cultures of cortical neurons, such as glial cells.

5.1 Structural basis for PAC1 receptor coupling to Ca^{2+} and release of catecholamines from neuroendocrine cells

The neuroendocrine pheochromocytoma PC12 cell line, derived from a rat adrenomedullary tumor (Greene and Tischler, 1976), has long been used as a convenient model system for studies of the regulated dense core vesicle (DCV) secretory pathway (Martin and Grishanin, 2003). Elevation of intracellular Ca^{2+} triggers release of small and large DCVs containing biogenic amines and neuropeptides as well as of small synaptic vesicles containing acetylcholine (ACh) (Bauerfeind et al., 1993; Greene and Rein, 1977; Rebois et al., 1980).

PACAP is expressed in all cholinergic adrenomedullary nerve terminals (coextensive with VAChT) (Hamelink et al., 2002b). Release of ACh and PACAP from splanchnic nerve endings mediates short- and long-term catecholamine (CA) release from chromaffin cells (CCs), respectively (Watanabe et al., 1992; Watanabe et al., 1995). In mice lacking the PACAP gene, plasma epinephrine levels are only modestly reduced during the initial phase of metabolic stress (hypoglycemia) but more profoundly during later phases, leading to longer-lasting insulin-induced hypoglycemia with dose-dependent lethality. Thus, acute responses to splanchnic nerve activation are mediated mainly by ACh, whereas PACAP is needed for prolonged secretion of CAs from chromaffin cells, required for compensatory gluconeogenesis during periods of prolonged metabolic stress (Hamelink et al., 2002b; Haycock, 1996). This is in agreement with studies in cultured CCs, showing that cholinergic activation of CA release desensitizes within a few minutes (Boksa and Livett, 1984), whereas PACAP-mediated release persists over longer periods of time (Babinski et al., 1996; Watanabe et al., 1992) and occurs under stress-mediated elevated splanchnic firing (Kuri et al., 2009).

The PC12-G cell line used in this study expressed low levels of the PACAP receptor variants PAC1hop and PAC1hip and stimulation with PACAP triggered a moderate intracellular Ca^{2+} response, which is in agreement with previously published results (Mustafa et al., 2007; Ravni et al., 2006). The Ca^{2+} response consisted of a rapid and transient rise in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) through Ca^{2+} release from intracellular stores, presumably through Gq-mediated activation of phospholipase Cβ (PLCβ) and IP_3 receptor-mediated Ca^{2+} mobilization, which was followed by a prolonged Ca^{2+} influx plateau phase. The PACAP-induced Ca^{2+}

response in PC12-G cells, however, was much smaller compared to the response seen in primary bovine chromaffin cells (BCCs) (Hamelink et al., 2002a), suggesting that the low expression level of PAC1 receptors in PC12-G cells is not sufficient to drive a full Ca^{2+} response. BCCs express only one PACAP receptor, the PAC1hop variant with a full length N-terminus. Therefore, we transfected PC12-G cells with this bovine PAC1hop receptor (bPAC1hop) and selected a stable cell line that expressed bPAC1hop at physiological levels. This gave us the opportunity to study the Ca^{2+} and secretory pathway in cells in continuous culture with a well-defined secretory phenotype. We found that increasing the receptor level to physiological levels reconstituted a Ca^{2+} response similar to the one observed in BCCs with an increase in both Ca^{2+} mobilization and influx, confirming previously published data (Mustafa et al., 2007). The increase in the intracellular Ca^{2+} response was reflected in an increased secretory response. Naïve PC12-G cells responded to PACAP treatment with a low-level release of CAs, which did not exceed the phase of acute secretion (0-5 min). Expression of bPAC1hop increased acute secretion and, more importantly, conferred prolonged secretion, which lasted for more than one hour. These data suggest that physiological levels of the PAC1hop receptor are needed for PACAP's long-term effects on CA secretion from adrenomedullary chromaffin cells, required for PACAP's homeostatic effects in vivo during prolonged periods of stress. Previous studies have demonstrated the ability of PACAP to induce a robust Ca^{2+} response and prolonged CA secretion in naïve PC12 cells (Taupenot et al., 1999), which is likely due to a higher expression of PAC1hop than in PC12-G cells (Cavallaro et al., 1995). The PACAP-mediated acute and prolonged secretory response are mediated via distinct mechanisms in PC12 cells; acute CA release is mediated by Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs), whereas sustained secretion involves a Gq/PLCß/store-operated Ca^{2+} channel (SOCC) pathway (Mustafa et al., 2010; Taupenot et al., 1999). The importance of PLCß activation in sustained release is in agreement with a predominant role of the PAC1hop receptor which engages dual signaling through adenylate cyclases (ACs) and PLCß.

All PACAP receptors couple to ACs through Gs and endogenous PAC1 receptors in PC12-G cells are maximally coupled to ACs so that expression of bPAC1hop does not further increase intracellular cAMP generation (Mustafa et al., 2007). PACAP-mediated signaling through cAMP and the cAMP dependent protein

kinase (PKA) is important in the sustained activation and mRNA elevation of tyrosine hydroxylase (TH), the rate-limiting enzyme in CA biosynthesis. PKA regulates the activity of TH by phosphorylation at Ser40 and by increasing its transcription to maintain CA stores for subsequent release (Bobrovskaya et al., 2007; Corbitt et al., 1998; Haycock, 1996). The PKA-mediated effect on TH activity is modulated by PKC and Ca^{2+} (Corbitt et al., 2002; Marley et al., 1996; Rius et al., 1994), indicating a dual role for Gq-mediated signaling by PACAP in sustained secretion: modulation of TH levels and activity to maintain CA stores and mediating their subsequent release.

PKC activation has also been shown to increase the size of the readily releasable pool (RRP) in CCs leading to enhanced exocytosis upon depolarization (Gillis et al., 1996; Vitale et al., 1995). This could account for the increased depolarization-evoked secretion in PC12-G cells expressing the bPAC1hop receptor observed in the present study.

Chromaffin cells of the rodent adrenal gland express three isoforms of the PAC1 receptor, PAC1hop, null and hip, with PAC1hop being the most abundant variant (Nogi et al., 1997; Stroth, 2010; Ushiyama et al., 2007). Thus, experiments were extended to PC12-G cells expressing the three rat PAC1 variants, respectively. We generated PC12-G cells, expressing the rat PAC1hop, null or hip variant with a full-length N-terminus through infection with gammaretroviral vectors to generate cell lines stably expressing the respective receptor variant at similar densities. Increasing the expression of the rPAC1hop receptor increased the PACAP-mediated Ca^{2+} response to a level similar to the response seen in bPAC1hop-expressing PC12-G cells. The receptor not expressing any insert in the third intracellular loop (ic3), PAC1null, evoked a similar Ca^{2+} response, with an only somewhat smaller initial rise in Ca^{2+}. The receptor expressing the hip cassette in ic3, PAC1hip, did not increase the low-level Ca^{2+} response observed in naive PC12-G cells, suggesting that expression of the hip cassette abolishes coupling to Ca^{2+}. Expression of either PAC1hop or null increased the acute secretory response, whereas only PAC1hop conferred sustained CA release. PAC1hip was without effect, in agreement with a lack of increasing the intracellular Ca^{2+} response. These results suggest that the PAC1hop receptor is the only variant mediating prolonged CA release. The slightly higher rise in the initial Ca^{2+} response, presumably Ca^{2+} mobilization, by PAC1hop compared to PAC1null may be important in mediating a prolonged secretory response which is, as mentioned

above, dependent on PLCß-mediated signaling. It will be of particular interest to investigate whether the Ca^{2+} influx plateau phase in PAC1hop- and PAC1null-expressing PC12-G cells is mediated by different Ca^{2+} channels. Perhaps PAC1null fails to activate store-operated Ca^{2+} entry (SOCE) and therefore fails to mediate prolonged release of DCVs. Taken together we provide evidence that PACAP-mediated signaling through PAC1hop, the predominant PACAP receptor in CCs of the adrenal gland in vivo, mediates a Ca^{2+} and secretory response, required for sustained CA secretion to restore homeostasis during prolonged periods of stress.

5.2 Second messenger generation and MAPK activation in differentiated and undifferentiated cells of the central nervous system

5.2.1 Structural basis for PAC1 receptor coupling to second messenger generation in undifferentiated NG108-15 cells

In addition to PACAP's function as a neuropeptide slow transmitter at the peripheral splanchnico-adrenomedullary synapse regulating prolonged DCV release from CCs during stress-responding, PACAP acts as an important neurotrophic factor during brain development and as a neuroprotective factor during brain injury (Arimura, 1998; Mustafa and Eiden, 2006; Ohtaki et al., 2008; Shioda et al., 2006). As mentioned above, activation of its cognate receptor PAC1 results in induction of combinatorial signaling through coupling to Gαs and Gαq and insertions in the third intracellular loop of the receptor modulate the efficacy of G protein coupling. Studies in heterologous non-neural cells show that the presence of the hip cassette in PAC1 interferes with G protein coupling (Lutz et al., 2006; Pisegna and Wank, 1996; Spengler et al., 1993), in agreement with results obtained from heterologous PC12-G cells in the present study. Multiple signaling pathways regulate neural differentiation and survival (Ohtaki et al., 2008; Shioda et al., 2006; Vaudry et al., 2002a), however, the functional importance of different PAC1 variants in engaging different signaling pathways in neural cells has not been previously delineated.

We chose the mouse neuroblastoma x rat glioma NG108-15 cell line to investigate PAC1-mediated signaling, because these cells, unlike PC12 cells, do not respond to PACAP endogenously but respond to other GPCR ligands (Hamprecht et

al., 1985), therefore providing an appropriate model system to study the different PAC1 splice variants separately introduced into a neural cell line. NG108-15 cells were transduced with the three major PAC1 splice variants of the adult nervous system, the PAC1null, hop1 and hip variant with a full-length N-terminus, by gammaretroviral infection and the PACAP-induced cAMP and Ca^{2+} response was measured. We found that PACAP triggered intracellular cAMP generation in all three cell lines. Maximal cAMP generation by 100 nM PACAP-38 was greater in PAC1hop- and null- compared to hip-expressing cells, which supports results generated from rat and human PAC1 splice variants expressed in non-neural cell lines (Lutz et al., 2006; Spengler et al., 1993). The PACAP-induced Ca^{2+} response consisted of a rapid and transient rise in $[Ca^{2+}]_i$ indicative of Ca^{2+} release from intracellular stores, which was not followed by a prolonged Ca^{2+} influx plateau phase observed in BCCs (Hamelink et al., 2002a), cortical neurons (Grimaldi and Cavallaro, 1999) and PC12 cells (this study). The intracellular Ca^{2+} response in NG108-15 cells expressing the bovine PAC1hop receptor has been shown to be mainly mediated through IP_3 receptor-activated Ca^{2+} mobilization (Mustafa et al., 2007), which is likely to also apply for the PAC1-mediated Ca^{2+} response measured here. Our results show that PAC1 receptor expression in NG108-15 cells reconstitutes cAMP and Ca^{2+} signaling, presumably through coupling to Gs and Gq, respectively. A Ca^{2+} response was induced by PAC1hop and null but not hip and the increase in $[Ca^{2+}]_i$ was greater in PAC1hop- than in PAC1null-expressing cells, in agreement with results obtained from heterologous PC12-G cells. This suggests that expression of the hop cassette in the third intracellular loop is required for the maximal induction of cAMP and Ca^{2+} combinatorial signaling. Expression of no cassette in ic3 as in PAC1null supports a reduced coupling to Ca^{2+} relative to PAC1hop, while the receptor with the hip cassette supports no measurable coupling to Ca^{2+} at all.

It has previously been shown that cAMP and Ca^{2+} combinatorial signaling is required for PACAP target gene induction (Girard et al., 2004; Hamelink et al., 2002a; Hashimoto et al., 2000) and involved in PACAP's neurotrophic and neuroprotective effects (Onoue et al., 2002b; Pugh and Margiotta, 2006; Tanaka et al., 1997; Vaudry et al., 2000a) in neuroendocrine and neural cells of the peripheral and central nervous system. PAC1 splice variant-specific immediate-early gene induction occurs in heterologous non-neural cells (Pisegna et al., 1996). Our results indicate that

PAC1 splice variant-specific expression, resulting in differential second messenger generation, is a regulatory mechanism by which PACAP mediates its pleiotropic effects in the nervous system, such as during development and during stress-responding throughout adulthood.

5.2.2 PACAP-mediated second messenger production in differentiated neurons

PACAP's neuroprotective effects in the central nervous system in vivo, specifically after middle cerebral artery occlusion (MCAO) producing cortical infarction (Chen et al., 2006; Ohtaki et al., 2006; Reglodi et al., 2000; Reglodi et al., 2002; Tamas et al., 2002) suggests that activation of PAC1 and respective signal transduction pathways mediate this effect. We found that cultures of rat cortical neurons predominantly expressed the PAC1null and hop receptor variants and low levels of PAC1hip, VPAC1 and VPAC2 receptors, in agreement with previously published reports showing a predominant PAC1hop and null expression in the human and rat brain and neuronal cells of various brain regions including the cerebral cortex (Cavallaro et al., 1996; Lutz et al., 2006; Nogi et al., 1997; Pisegna and Wank, 1996; Zhou et al., 2000). PACAP stimulation of cortical cultures triggered a robust intracellular cAMP production that was ~60% of the maximal cAMP production generated by direct supramaximal stimulation of adenylate cyclases (ACs) with 25 μM forskolin, suggesting that PAC1 receptors in differentiated cortical neurons are efficiently coupled to ACs, in agreement with a high endogenous expression level of PAC1hop and null receptors in these cells. The PACAP-evoked Ca^{2+} response consisted of a robust initial burst of Ca^{2+} mobilization, presumably Ca^{2+} release from IP_3 sensitive intracellular stores, followed by prolonged Ca^{2+} influx, in agreement with previously published results (Grimaldi and Cavallaro, 1999). Only ~75% of all cells responded to PACAP with a robust increase in $[Ca^{2+}]_i$, whereas the remaining 25% showed only a weak or no response. This indicates that a fraction of the cell population expresses no PACAP receptors or expresses PACAP receptors that either fail to couple to Ca^{2+} or couple to Ca^{2+} inefficiently, such as the PAC1hip or PAC1null receptor variant, respectively. It has been shown that PACAP fails to stimulate an intracellular Ca^{2+} response in cortical precursors endogenously expressing the PAC1null receptor, whereas ectopic expression of PAC1hop confers a PACAP-mediated rise in $[Ca^{2+}]_i$ (Lu et al., 1998; Nicot and DiCicco-Bloom, 2001). Only ~15% of the cells that

responded to PACAP with a rise in $[Ca^{2+}]_i$ responded to depolarizing concentrations of KCl with Ca^{2+} entry; this cell population seemed to respond to PACAP with Ca^{2+} mobilization only, lacking an obvious Ca^{2+} influx plateau phase. In the majority of cells, KCl did not stimulate Ca^{2+} influx. It is noteworthy that, without previous PACAP treatment, the majority of cells responded to KCl with Ca^{2+} entry (not shown), suggesting that PACAP receptor activation inhibits VGCCs. In the 25% of cells not showing any Ca^{2+} response to PACAP, subsequent stimulation resulted in a robust Ca^{2+} influx upon depolarization (not shown). These results suggest, that PACAP-mediated dual cAMP and Ca^{2+} signaling (Ca^{2+} influx) inhibits subsequent KCl-mediated Ca^{2+} influx through VGCCs in the majority of cortical neurons. Alternatively, specific βγ subunits coupled to PAC1hop may have the same effect (Garcia et al., 1998). Inhibition of N-type VGCCs by VIP has been previously shown in sympathetic neurons via a pertussis toxin-insensitive G protein-mediated pathway (Zhu and Ikeda, 1994; Zhu and Yakel, 1997). It will be interesting to investigate whether sub-populations of cortical neurons express different variants of the PAC1 receptor and whether PACAP-mediated signaling specifically regulates a certain type of VGCCs expressed in the majority of cortical neurons, such as the L-, N- or P/Q-type (Hofmann et al., 1994).

5.2.3 Signaling to ERK activation in undifferentiated NG108-15 cells and differentiated neurons

PACAP's neurotrophic effects in PC12 cells are mediated through activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway (Vaudry et al., 2002d). ERK activation regulates diverse cellular processes such as proliferation, differentiation and apoptosis. Intensity and spatio-temporal activation are key determinants in generating precise biological responses (Marshall, 1995; Pouyssegur and Lenormand, 2003; Sebolt-Leopold and Herrera, 2004). Cyclic AMP signaling is implicated in the regulation of ERK activation both positively and negatively depending on the cell type (Dumaz and Marais, 2005).

In undifferentiated NG108-15 cells expressing the PAC1hop receptor variant, PACAP treatment resulted in the activation of both cAMP and Ca^{2+} signaling, and a rapid and transient activation of ERK. ERK activation was blocked by pharmacological inhibition of ACs with 2′5′-dideoxyadenosine (ddAd) but was not

induced by cAMP generation through AC activation by forskolin, indicating that cAMP generation is required but not sufficient to activate ERK. These results suggest that maximal activation of ERK requires combinatorial signaling in this cell type and can therefore only be supported by the PAC1hop receptor variant. Preliminary data support this conclusion insofar that ERK activation in NG108-15 cells was greatest when the PAC1hop receptor was expressed, moderate in PAC1null- and suppressed in PAC1hip-expressing cells; also, inhibitors of the Ca^{2+} pathway, such as calcineurin and PKC inhibitors, blocked PAC1hop-mediated ERK activation. Activation of ERK upon PAC1hop activation was not blocked by PKA inhibition with H89, suggesting a cAMP-mediated pathway independently of the canonical cAMP effector PKA. This non-canonical cAMP pathway was previously described in pheochromocytoma PC12 (Ravni et al., 2008) and neuroblastoma SH-SY5Y cells (Monaghan et al., 2008), mediating PACAP's neurotrophic effects leading to neurite extension.

In differentiated cortical neurons, PACAP activated ERK1/2 in a rapid and sustained fashion, which was also blocked by AC but not PKA inhibition, therefore proceeding through this non-canonical cAMP pathway (i.e., independently of PKA) as well. High concentrations of the AC inhibitor ddAd were required to block ERK activation in cortical neurons, suggesting that very low concentrations of cAMP are sufficient to signal to ERK in these cells. PACAP-mediated ERK activation was, in contrast to NG108-15 cells, mimicked by the AC activator forskolin, which was also not blocked by inhibition of PKA with H89. These results suggest that cAMP is required and sufficient to engage a non-canonical (PKA independent) signaling pathway in cortical neurons to activate ERK. In differentiated CNS neurons, cAMP signaling seems to be efficiently coupled to this novel PKA independent pathway, whereas in less differentiated neural cells, as shown for NG108-15 cells, a co-factor, presumably Ca^{2+}, is required to engage this pathway.

5.3 Signaling to neuroprotective target genes in undifferentiated NG108-15 cells and differentiated neurons

In differentiated neurons, the MAPK/ERK pathway has been implicated in cell survival, e.g., following excitotoxic or hypoxic/ischemic injury (Elliott-Hunt et al., 2002; Gonzalez-Zulueta et al., 2000; Hetman and Gozdz, 2004; Hetman et al., 1999; Irving et al., 2000; Jin et al., 2002). The importance of ERK in mediating PACAP's

neuroprotective effects has been shown in cerebellar granule cells during K^+ deprivation (Villalba et al., 1997), and in parasympathetic (Pugh and Margiotta, 2006) and sympathetic neurons during growth factor withdrawal (May et al., 2010). The importance of ERK in the activation of PACAP target genes, potentially mediating PACAP's neuroprotective effects in the CNS, still remains to be delineated.

Here we show that PACAP induced stanniocalcin 1 (STC1) mRNA in NG108-15-rPAC1hop cells and rat cortical neurons. We previously identified STC1 as a PACAP target gene in PC12-G cells expressing physiological levels of the bovine PAC1hop receptor (PC12-bPAC1hop) and in primary BCCs (Ait-Ali et al., 2010). STC1 is upregulated during neuronal differentiation and the pathological condition of hypoxia/ischemia (Westberg et al., 2007a; Westberg et al., 2007b; Zhang et al., 2000; Zhang et al., 1998), therefore being a candidate target gene for mediating PACAP's neurotrophic effects in the central nervous system. PACAP-mediated STC1 induction was blocked by the MEK1/2 inhibitor U0126 in both NG108-15-rPAC1hop cells and cortical neurons, suggesting that the activation of ERK1/2 is required for STC1 induction by PACAP. The PKA inhibitor H89 failed to block STC1 induction by PACAP, indicating a PKA independent signaling pathway to the STC1 gene, in agreement with a PKA independent activation of ERK. In NG108-15-rPAC1hop cells, the AC activator forskolin also induced STC1 gene transcription, although it failed to activate ERK. Induction was blocked by H89, suggesting that two cAMP pathways for STC1 induction co-exist in this undifferentiated cell line. Non-canonical cAMP signaling to STC1 induction via ERK seems to require combinatorial signaling through cAMP and Ca^{2+} in these cells, which is mediated by PAC1hop and to a lesser extent by PAC1null, whereas canonical cAMP signaling to STC1 induction is activated through cAMP generation only, as mediated by pharmacological activation of ACs or activation of the PAC1hip variant. In cortical neurons, the AC activator forskolin activated the same signaling pathway as PACAP and induced STC1 via ERK but not PKA, suggesting that STC1 gene transcription is restricted to a single (non-canonical) cAMP signaling pathway in these differentiated cells. Activation of this non-canonical pathway and induction of certain genes may be involved in PACAP's cAMP dependent neuroprotective effects during excitotoxicity (Morio et al., 1996; Said et al., 1998; Shintani et al., 2005) and hypoxia/ischemia in neuronal cell culture (Stumm et al., 2007) and during stroke in vivo (Chen et al., 2006).

PACAP also activated the canonical cAMP/PKA pathway to induce the neurotrophin BDNF. BDNF has been implicated in PACAP's neuroprotective effects (Frechilla et al., 2001), suggesting that non-canonical and canonical cAMP signaling co-exist in CNS neurons mediating PACAP's neuroprotective effects in vivo. However, the selective pharmacological activation of a cAMP pathway not involved in physiological processes such as learning and memory might reduce the detrimental side effects of drugs targeting the cAMP/PKA pathway (Arnsten et al., 2005; Kandel, 2001; Kuo and Greengard, 1969).

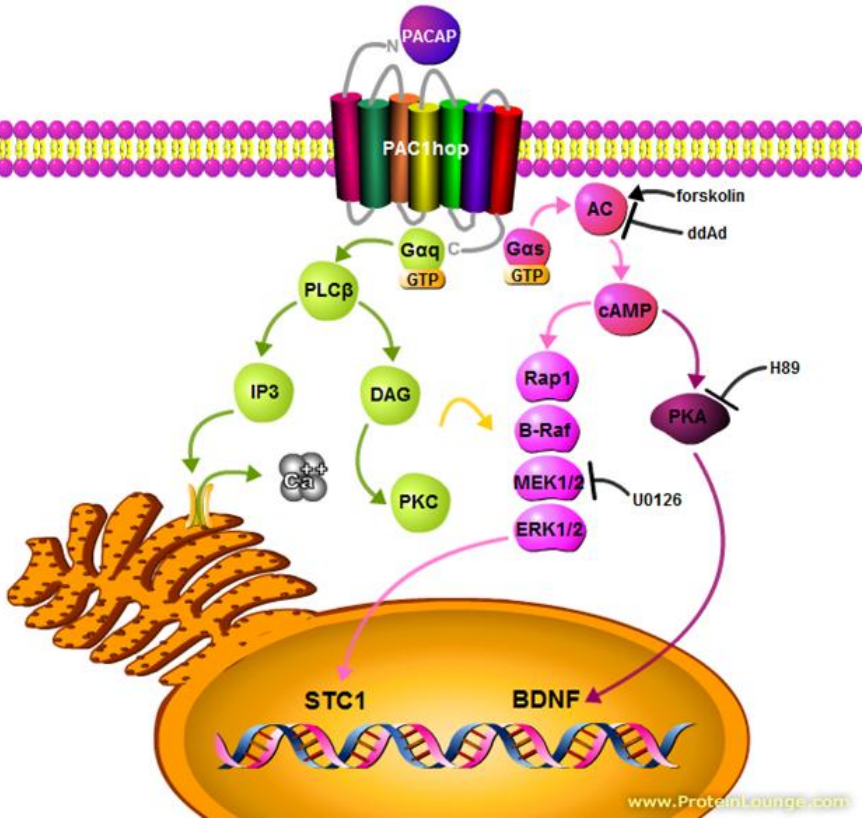


Figure 30: Schematic representation of PAC1hop-mediated signaling in cells of the central nervous system. PACAP-mediated activation of its receptor PAC1 expressing the hop cassette in the third intracellular loop results in activation of the two G proteins Gq and Gs, thereby inducing combinatorial signaling through cAMP and calcium. In less differentiated NG108-15 cells, combinatorial signaling is required for ERK activation (indicated by the yellow arrow), whereas in differentiated CNS neurons, activation of the cAMP pathway is sufficient to activate ERK. ERK activation is independent of the canonical cAMP effector PKA. Non-canonical cAMP signaling activates the STC1 gene, whereas cAMP/PKA signaling induces BDNF.

5.4 PACAP and neuroprotection

PACAP's neuroprotective effects during stroke and other neurodegenerative diseases in vivo and under various conditions promoting apoptosis in cell culture (vide supra) were sought to be confirmed in cell culture models of stroke. Brain injury caused by transient or permanent focal brain ischemia (stroke) develops from a complex sequence of pathophysiological events. Reduced supply of oxygen and glucose through inadequate blood supply triggers energy deficits and membrane potential changes, eventually causing neuronal depolarization. Increasing $[Ca^{2+}]_i$ activate the release of excitatory amino acids like L-glutamate, which accumulate in the extracellular space causing over-activation of ionotropic and metabotropic glutamate receptors. Ionic imbalances, especially Ca^{2+} overload through activation of ionotropic NMDA receptors, cause neuronal damage (Dirnagl et al., 1999; Martin et al., 1994; Sattler and Tymianski, 2001).

We subjected primary cultures of rat cortical neurons to oxygen-glucose-deprivation (OGD) and glutamate-induced excitotoxicity in the presence or absence of PACAP and measured neuronal viability through MTT conversion. Both OGD and excitotoxicity caused neuronal cell death. However, PACAP treatment failed to increase cell survival under both conditions. These results were very unexpected because PACAP has been previously shown to rescue neurons from OGD- (Stumm et al., 2007) and excitotoxicity-induced cell death (Frechilla et al., 2001; Morio et al., 1996; Shintani et al., 2005). The biological significance of STC1 and the importance of the non-canonical cAMP pathway in neuroprotection are still awaiting clarification; however, PACAP also induced the neurotrophic factor BDNF through activation of the canonical cAMP/PKA pathway. PACAP has been previously shown to induce the expression of BDNF during excitotoxicity in cortical neurons, increasing cell survival (Frechilla et al., 2001; Pellegri et al., 1998; Shintani et al., 2005) and to activate PKA in various cell types, mediating PACAP's neurotrophic and neuroprotective effects (Kienlen Campard et al., 1997; May et al., 2010; Onoue et al., 2002b; Pugh and Margiotta, 2006; Shoge et al., 1999; Stetler et al., 2010; Tanaka et al., 1997; Tomimatsu and Arakawa, 2008; Vaudry et al., 1998a; Villalba et al., 1997). The results of the present study indicate that activation of PACAP-mediated signaling pathways and induction of neurotrophic target genes do not increase the survival of cultured cortical neurons exposed to excitotoxicity or OGD.

It has been shown that PACAP increases neuronal survival during mild (90 min) OGD at concentrations below 20 nM, whereas it exacerbates cell death at 100 nM. Both low and high concentrations of PACAP activate ERK and PACAP's protective effect is dependent on the activation of ERK (Stumm et al., 2007), suggesting that high levels of cAMP or the activation of a second signaling pathway by high concentrations of PACAP exacerbate cell death, e.g., via activation of the p38 MAPK pathway (Stumm et al., 2007). PACAP has a high potency for cAMP generation (EC_{50} value near 1 nM) but a lower potency for inositol phosphate (InsP) production (approximately 1 log less) (Deutsch and Sun, 1992; Grimaldi and Cavallaro, 1999; Spengler et al., 1993); co-activation of PLCβ-mediated signaling at higher concentrations of PACAP might interfere with cAMP-mediated protection of CNS neurons. We therefore subjected cortical neurons to 3 h of OGD, causing approximately 40% cell death after 24 h, in the presence or absence of various concentrations of PACAP. However, every concentration tested (1, 10 and 100 nM PACAP) failed to either increase cell survival or exacerbate cell death caused by OGD.

PACAP has also been shown to promote neuronal survival during glutamate-induced excitotoxicity. In cultures of rat cortical neurons, exogenous PACAP is protective during short-term excitotoxicity (6 h) between 0.1 pM and 1 nM (Morio et al., 1996) and during long-term excitotoxicity (18 h) between 1 pM and most efficiently 100 nM (Frechilla et al., 2001). The PACAP antagonist PACAP(6-38) exacerbates cell death induced by acute glutamate exposure after 24 and more prominently after 48 h (Shintani et al., 2005). Cyclic AMP and BDNF have both been implicated in PACAP's neuroprotective effects during excitotoxicity. We subjected cultures of rat cortical neurons to short-term (6 h) and long-term (24 h) excitotoxicity in the presence or absence of PACAP (100 nM) or the PACAP antagonist PACAP(6-38) and found that neither PACAP increased nor PACAP(6-38) decreased cell survival, suggesting that neither exogenous nor endogenous PACAP is protective during short- or long-term excitotoxicity.

Both endogenous and exogenous PACAP have been shown to be protective during stroke in vivo and PACAP is even effective when administered after the onset of ischemia (Chen et al., 2006; Reglodi et al., 2000), suggesting that PACAP-activated signaling molecules and target genes represent promising targets for

therapeutic intervention after brain damage. However, our results indicating that PACAP does not increase survival in neuronal cell culture models of stroke must first be reconciled both with the demonstrable neuroprotective effect of PACAP during stroke in vivo and the reports in the literature showing that PACAP treatment does in fact ameliorate excitotoxic and hypoxic/ischemic cell death in cultured rodent cortical neurons. One possibility is that PACAP may mediate its neuroprotective effect in vivo via cells absent in cultures of cortical neurons, dependent on the type of preparation employed. In astrocytes, picomolar concentrations of PACAP trigger cAMP dependent ERK activation stimulating proliferation (Hashimoto et al., 2003; Moroo et al., 1998). Neuron-derived PACAP stimulates glutamate uptake in astrocytes (Figiel and Engele, 2000), suggesting that glial regulation of excess extracellular glutamate decreases neurodegeneration caused by excitotoxicity. PACAP also stimulates production of the neurotrophic cytokine IL-6 in astrocytes (Gottschall et al., 1994; Tatsuno et al., 1996; Van Wagoner and Benveniste, 1999), suggesting that PACAP release during injury mediates astrocyte proliferation (gliosis) and IL-6 secretion, thereby increasing neuronal survival. This conclusion is supported by in vivo data showing that PACAP decreases ischemic cell death in association with IL-6 (Ohtaki et al., 2006). PACAP has also been shown to be involved in controlling the immune response required for peripheral nerve regeneration after injury (Armstrong et al., 2008), which likely also applies for CNS injury. PACAP inhibits the production of pro-inflammatory cytokines and stimulates the production of anti-inflammatory cytokines in activated macrophages and microglia, and promotes differentiation of anti-inflammatory T helper 2 cells (Th2), thereby deactivating and eliminating stimulated immune cells to limit tissue damage associated with the immune response after injury (Delgado et al., 2003a). Inflammation accompanies and exacerbates most brain pathologies and PACAP's anti-inflammatory action in the central nervous system (Kong et al., 1999) most likely promotes regeneration after CNS damage induced by traumatic brain injury (TBI), stroke and other neurodegenerative diseases (Delgado et al., 2003b). It is therefore highly relevant to future work in this area to determine whether PACAP supports neuronal survival in cortical neuron/glia co-cultures subjected to OGD or glutamate-induced excitotoxicity and whether cell death in neuronal cultures is reduced by treatment with conditioned medium from PACAP-treated astrocyte cultures.

5.5 Stanniocalcin 1 and neuroprotection

Stanniocalcin 1 (STC1) is abundantly expressed in differentiated brain neurons (Zhang et al., 1998). It is upregulated in vivo following ischemia and it increases neural cell resistance to hypoxic insult upon over-expression in cell culture (Zhang et al., 2000). STC1, as a PACAP target gene, was therefore an important candidate as a mediator of PACAP's neuroprotective effects during stroke in vivo. To determine whether STC1 has neuroprotective properties, we over-expressed STC1 in primary cultures of rat cortical neurons and determined cell survival after excitotoxic insult. STC1 over-expression in neurons was achieved by infection with lentiviruses, which efficiently infect non-dividing cells. Excitotoxicity was induced with high concentrations of glutamate for 6 h, reducing cell survival by approximately 40% in both STC1- and mock-transduced neurons. These results indicate that over-expression of STC1 does not confer increased resistance to excitotoxic insult. STC1 over-expression has previously been shown to protect against hypoxic insult, induced by $CoCl_2$ treatment leading to Ca^{2+} influx, in human neural crest-derived Paju cells. Moreover, STC1 expression is increased in the penumbra zone following ischemic insult in the rat brain (Zhang et al., 2000), suggesting that increased STC1 expression confers neuronal survival during excitotoxicity. Our results were therefore unexpected and reinforce the notion that the excitotoxic cell culture model employed by our laboratory and others may not be appropriate to examination of neuroprotective effects of either STC1 or PACAP observed in vivo.

It is possible that STC1 is released from neurons to regulate intermediate cells in a paracrine fashion such as macrophages and endothelial cells, reducing inflammatory responses (Chakraborty et al., 2007; Chen et al., 2008; Sheikh-Hamad, 2010; Wang et al., 2009) and thereby ameliorating brain damage associated with inflammation in brain pathologies such as TBI (Long et al., 2003) and stroke (Zhang et al., 2000). The STC1-related protein STC2 has been shown to reduce endotoxin-mediated microglial activation and neuronal loss following excitotoxicity associated with ER stress (Byun et al., 2010). STC2, but not STC1, is induced by the unfolded protein response (UPR), a critical component of cell survival during ER stress (Ito et al., 2004). STC2 was not induced by PACAP in cortical neurons, as shown in the present study. It is possible that STC1 is up-regulated during brain injury

independently of the UPR to reduce inflammatory responses and thus increase neuronal survival analogous to the function of its paralog during ER stress.

5.6 Limitations of the NMDA cell culture model for neurodegenerative diseases

The theory that high concentrations of glutamate destroy neurons was established more than five decades ago (Lucas and Newhouse, 1957; Olney and Sharpe, 1969). Ca^{2+} influx through activation of the NMDA subtype of glutamate receptors was suggested as the main mediator of ischemia-induced excitotoxicity in the 1980s, based on a plethora of pharmacological and cell biological data obtained both in culture and in vivo (Choi and Rothman, 1990; Simon et al., 1984). However, in 2001, evaluation of all of the many clinical trials for stroke involving NMDA receptor antagonists led to the conclusion that they were uniformly ineffective for this therapeutic application (Albers et al., 2001; Sacco et al., 2001). With the approval of tissue plasminogen activator (tPA) for the treatment of acute ischemic stroke in 1996, treatment still remains limited to thrombolysis (Lee et al., 1999). Effective neuroprotective and restorative drug therapies for acute stroke and other neurodegenerative disorders are still lacking.

It was subsequently suggested that one of the main difficulties with the use of NMDA receptor antagonists as neuroprotectants in clinical trials of stroke was due to the short neuroprotective time window (Ikonomidou and Turski, 2002), as previously shown in rodent stroke models (Ma et al., 1998). It was hypothesized that acutely increased extracellular glutamate concentrations after brain injury might be neurotoxic, whereas moderately sustained glutamate elevation could actually be physiologically important in sustaining neuronal survival. NMDA receptor antagonists might therefore only be effective when administered prior to or shortly after the insult which is unlikely in a clinical emergency setting. In addition, NMDA receptor antagonists might actually exacerbate neuronal damage if administered during an inappropriate time window following stroke. NMDA receptors are not only localized synaptically but also extrasynaptically. Activation of synaptic NMDA receptors promotes neuronal survival via activation of the transcription factor cAMP response element binding protein (CREB) and induction of neuroprotective genes such as BDNF and bcl-2, whereas anti-survival signaling mediated by activation of

extrasynaptic NMDA receptors antagonizes this effect. Neuroprotective therapies for the treatment of pathological conditions associated with glutamate toxicity should therefore enhance synaptic activity and disrupt extrasynaptic NMDA receptor-mediated signaling. The low-affinity NMDA receptor blocker memantine has been shown to preferentially antagonize extrasynaptic NMDA receptors (Leveille et al., 2008) and to reduce clinical deterioration in moderate to severe Alzheimer's disease (Reisberg et al., 2003; Tariot et al., 2004). Drugs acting on NMDA receptors are still potential therapeutic agents for neurodegeneration. Synaptic versus extrasynaptic NMDA receptor activation, receptor composition of different subunits and activation of different downstream signaling molecules provide the basis for future developments of NMDA receptor-mediated neuroprotective therapies (Hardingham and Bading, 2010; Kemp and McKernan, 2002).

The complexity of NMDA receptor-activated signaling in neuronal survival and death clearly shows the problems one faces in modeling complex brain pathologies such as stroke in cell culture. Moreover, the outcome of therapeutic intervention in stroke patients and animal models of stroke are often inconsistent, successful in animal models but not in clinical trials, which might be due to high variability in clinical stroke patients. Rigorous preclinical testing is required for novel neuroprotective drugs to have the potential to succeed in a clinical trial (1999).

5.7 Concluding remarks and future directions

Evidence from numerous mouse models of permanent and transient ischemia, and various cell culture models of excitotoxicity and hypoxia/ischemia by several independent laboratories indicate a clinical importance of PACAP in stroke. PACAP has a reasonable chance to succeed in an appropriately designed clinical trial (1999). PACAP prevents neuronal death at picomolar to nanomolar concentrations; it is transported across the blood-brain barrier (BBB) (Banks et al., 1993; Banks et al., 1996) and is neuroprotective after ischemic injury when administered intravenously (i.v.), indicating that its transport across the BBB is sufficient to provide enough peptide to the brain, in particular to the tissue at risk, to prevent cell death. Moreover, PACAP has a wide therapeutic time window, supporting neuronal survival when administered immediately prior to until up to several hours after the onset of ischemia,

indicating its effectiveness during the acute phase of stroke as well as during secondary neuronal damage following stroke.

PACAP's widespread expression in the CNS and involvement in a plethora of physiological functions indicates that pharmacological modulation of downstream signaling molecules results in more precise control over neurodegenerative cell loss with a reduction in adverse effects. PACAP activates different GPCRs resulting in the activation of different signaling pathways such as Gs- and Gq-mediated signaling. Canonical cAMP signaling links cAMP through PKA to CREB activation and gene induction, inducing long-lasting changes in the cell. This cAMP/PKA signaling pathway, activated by Gαs coupled to ACs, is involved in many physiological processes such as learning and memory. In addition to activating this cAMP pathway, PACAP also activates so called non-canonical cAMP signaling (independent of PKA). We provide evidence for the existence of non-canonical cAMP signaling in CNS neurons to ERK1/2 activation and STC1 gene induction. Both canonical and non-canonical cAMP signaling may work together during brain injury. However, the selective pharmacological activation of a cAMP pathway not involved in physiological processes such as learning and memory might reduce adverse effects of drugs targeting the cAMP/PKA pathway.

Activation of this novel cAMP pathway in less differentiated cells of the CNS is supported by combinatorial signaling through cAMP and Ca^{2+} uniquely mediated by the PAC1hop receptor variant. PAC1hop-mediated combinatorial signaling is also required for prolonged CA secretion from adrenomedullary chromaffin cells, thereby mediating PACAP's homeostatic responses to prolonged stress in the PNS and determining whether an animal survives or not under these paraphysiological conditions (Hamelink et al., 2002b). Taken together, the results of the present study clearly show the importance of the neuropeptide slow transmitter PACAP and its cognate GPCR PAC1hop in mediating combinatorial signaling through cAMP and Ca^{2+} important during stress-responding in the peripheral and central nervous system.

Further studies are required to reconfirm the neuroprotective effects of PACAP and to specifically elucidate the mechanisms of its neuroprotective actions. It will be of paramount importance to understand whether PACAP has a direct neuroprotective effect or regulates intermediary cells such as glial cells in a paracrine fashion to confer neuroprotection indirectly. The results of the present study favor at

this time an indirect neuroprotective pathway for PACAP. Highly relevant future experiments include the direct comparison of PACAP's neuroprotective potential in cortical neuron-enriched and mixed cortical neuron/glia co-cultures subjected to excitotoxic or hypoxic/ischemic insult. Together with investigating the effects of conditioned medium from PACAP-treated astrocytes on the survival of challenged cortical neurons in culture, these experiments will give important insight into whether PACAP directly inhibits neuronal apoptosis, supports neuronal survival via astroglial production of a neurotrophic factor, or modulates glial cells to reduce acute glutamate overload and minimize chronic inflammatory processes.

It will also be of particular importance to further elucidate the biological significance of the PACAP target gene STC1 and to answer the question whether STC1 and non-canonical cAMP dependent signaling are implicated in PACAP's neuroprotective effects during ischemia. In cell culture, STC1 gene silencing with small interfering RNA (siRNA) could be employed to test whether STC1 is an essential mediator of PACAP's neuroprotective action. In vivo, the use of STC1-deficient mice will give new insight into whether STC1 is involved in mediating PACAP's neuroprotective effects during ischemia (MCAO). Moreover, further studies are required to show whether STC1 has a direct neuroprotective effect or regulates neuroprotection indirectly, as also proposed for PACAP. STC1 might be released from neurons to regulate intermediary cells such as microglia to reduce inflammatory processes associated with tissue damage.

PAC1 receptor coupling to second messenger production depends on the respective splice variant expressed. It will be interesting to investigate whether PAC1 splice variants are dynamically regulated during prolonged stress and ischemic injury to provide differential second messenger production and target gene induction. Differential PACAP signaling might be an important regulatory mechanism implicated in the pleiotropic actions of PACAP during brain injury and stress-responding in general. The engagement of non-canonical cAMP dependent signaling, in addition to the canonical pathway, which seems to be differentially regulated depending on cell type and maturation state, implies even more diversity in PACAP-mediated signaling. A more complete understanding of the complex signaling pathways induced by PACAP for the induction of target genes in a variety of cell types will be crucial for the development of therapeutic strategies for the treatment of

stroke and other neurodegenerative and stress-associated diseases, based on PACAP's pharmacological and physiological effects in vivo.

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Appendix

List of academic teachers

The following professors were my teachers at Philipps-University Marburg:
Aumüller, Berndt, Besedovsky, Czubayko, Daut, del Rey, Eilers, Elsässer, Feuser, Frenking, Garten, Gromes, Gudermann, Hasilik, Heeg, Heimbrodt, Jacob, Jungclas, Kirchner, Koolman, Kunz, Lang, Lill, Löffler, Moll, Müller, Petz, Renkawitz-Pohl, Renz, Röhm, Röper, Schäfer, Seitz, Steiniger, Suske, Voigt, Weihe.

Acknowledgments

Prof. Weihe - thank you very much for making this work possible, for invaluable discussions and constructive and inspiring comments. Thank you for your excellent mentorship since the beginning of my studies at Philipps-University Marburg and your greatly appreciated continuous support.

Lee Eiden - thank you very much for providing me with the opportunity to conduct my thesis project in your laboratory at the National Institutes of Health. Thank you for your support and advice throughout my studies.

All members of the Section on Molecular Neuroscience – Tomris, Djida, Xiuhuai, Chang-Mei, Charisse, Matt, Babru, David, Melanie and Niko, thanks for being such great colleagues and for your support. A special thanks to Niko, friend and colleague since the early days at Philipps-University, it's been a great pleasure spending this time with you.

All colleagues on the 5th floor of building 49 and all members of the Laboratory of Molecular and Cellular Regulation – Thank you for the pleasant atmosphere. A special thanks to Maribeth Eiden and the members of her lab for continuous support and advice.

Thank you everyone, whose path I crossed during these past five years; thanks to all the friends I made and thanks to all the friends who stayed.

The student learns by daily increment.
The way is gained by daily loss,
Loss upon loss until
At last comes rest.

By letting go, it all gets done;
The world is won by those who let it go!
But when you try and try,
The world is then beyond the winning.

Tao Té Ching

XXXVI