Nerve Growth Factor Signaling from Membrane Microdomain to Nucleus : Differential Regulation by Caveolins

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Nerve Growth Factor Signaling from Membrane Microdomain to Nucleus : Differential Regulation by Caveolins

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ABBREVIATIONS

AKTA	Also known as Protein Kinase B (PKB) serine/threonine protein kinase
ARMS	An ankyrin-rich membrane spanning protein
ATP	Adenosine-5'-triphosphate
B-Raf	Murine sarcoma viral oncogene homolog B1
BAD	Bcl-2-associated death promoter
Bax	Bcl-2–associated X protein
Bcl-XL	B-cell lymphoma-extra large
BDNF	Brian-derived neurotrophic factor
Bim	Bcl-2-like protein 11
c-fos	FBJ murine osteosarcoma viral oncogene homolog
c-jun	Jun proto-oncogene
C-Raf	RAF proto-oncogene serine-threonine protein kinase
CAP	Adenylate Cyclase Associated Protein
CRD	Cysteine rich domains
CREB	cAMP regulatory element binding protein
CrkII/Crk	Sarcoma virus CT10 oncogene homolog
CSD	Caveolin scaffolding domain
DAG	Diglyceride
DD	Death domain
DRG	Dorsal root ganglia
DRMs	Detergent resistant membranes
EGF	Epidermal growth factor
Elk-1	E twenty-six-like transcription factor 1
Erk 1/2	Extracellular-signal-regulated kinases 1/2
FRS2	Fibroblast growth factor receptor substrate 2
Grb2	Growth factor bound protein 2
GSK3B	Glycogen synthase kinase 3 beta
GTP	Guanosine-5'-triphosphate
IP3	Inositol trisphosphate
MAPK	Mitogen-activated protein kinase
MEK1	Dual specificity mitogen-activated protein kinase kinase 1
MURC	Muscle restricted coiled-coiled protein
NADE	*p75* NTR-associated Cell Death Executor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-aspartic acid
NRAGE	*p75* NTR-interacting MAGE homolog
NRIF	Neurotrophin receptor interacting factor
NT-3	Neurotrophin-3
NT-4	Nerotrophins-4
P53	Tumor protein 53
PDGF	Platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase

PI3K	Phosphatidylinositol 3-kinases
PI	Phosphatidylinositol
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLCγ	Phospholipase C-γ
PTRF	RNA Pol I transcription factor
Rap-1	Ras-proximate-1 or Ras-related protein 1
Ras	Rat sarcoma protein
Rsk	Ribosomal protein S6
SDPR	Serum deprivation protein response
SH2	Src Homology 2
Shc	SH2 containing sequence
Shp2	Src homology protein tyrosine phosphatase 2
Sos	Son of sevenless protein
SRBC	Sdr-related gene produce that binds to-c-kinase
SRE	The serum response element
SRF	Transcription factor serum response factor
TNFR	Tumor necrosis factor receptor
TRAFs	TNF receptor associated factors
TrkA	Neurotrophic tyrosine kinase receptor type 1 or TRK1

THESIS SUMMARY

Nerve Growth Factor (NGF) was the first discovered and the best characterized neurotrophic polypeptide. It has been shown to exert a variety of functions in the nervous system, which includes regulating neuronal specification, differentiation, survival, axon growth guidance, and neuronal plasticity. In addition to the above, NGF has also been shown to participate in other physiological processes, for example, in breast and prostate cancers, it is highly expressed and to be mitogenic for the tumor cells. Studies on NGF trafficking and signaling have continued to flourish underlining the importance and pertinence of further dissecting and understanding pathways for NGF signalling.

NGF binds to and signals *via* two receptors: a receptor common to all neurotrophins, p75 Neurotrophin Receptor ($p75^{NTR}$), member of the tumor necrosis factor receptor superfamily, and a receptor more specific to NGF, with a tyrosine kinase activity, Tropomyosine receptor kinase A (TrkA). At the plasma membrane, both TrkA and p75 receptors have been shown to localized to caveolae, specific subdomains that are enriched in cholesterol, sphingolipids and the presence of caveolin proteins. Caveolae has also been documented in the receptor endocytosis, cell signaling and breast cancer tumorigenesis. Cav-1 is the main component of caveolae, it can interact with numerous proteins involved in signal transduction, which results in compartmentalization of signaling molecules and their maintenance in an inactive conformation. Cav-2 is the other major component of caveolae, while being much less studied.

The focus of this work is on this membrane microenvironment mediated modulation of NGF signaling. The signaling endosome hypothesis proposes that TrkA internalized into endosomal compartments would promote a sustained signalling from the growth cone along the axon, all the way to the cell body. In the present work we found that overexpression of Cav-1 in mouse dorsal root ganglia neurons significantly impacted neurite extension. Similarly, overexpression of Cav-1 in PC12 cells strongly inhibits their ability to grow neurites in response to NGF. It inhibits NGF signaling without, impairing transient MAPK pathway activation. Rather, it does so by sequestering NGF receptors in lipid rafts, which correlates with the cell surface localization of downstream effectors, and phosphorylated-Rsk2, resulting in the prevention of the phosphorylation of CREB. By contrast, overexpression of Cav-2 potentiates NGF induced differentiation, which is accompanied by sustained activation

of downstream effectors, and standard internalization of the receptors. This differential effect could be due to the different localization of Caveolins, that results perturbing microenvironment, thereby affects the NGF signaling. Furthermore, PC12 cells expressing the non-phosphorylatable Cav-1 mutant (S80V), no longer present inhibited TrkA trafficking or CREB phosphorylation, but, on the contrary, behave much like Cav-2 PC12 cells.

These results offer novel insight into the relationship between membrane microdomains, receptors, and caveolins, specific scaffolding proteins important for regulating the NGF signaling pathway. Finally, our results open the way for investigation of the impact of TrkA and Cav-1 interplay in other models, such as tumorigenesis.

Key words: NGF signaling, NGF receptors, endocytosis, Caveolin-1, Caveolin-2, lipid raft/ Caveolae

Résumé

Le facteur de croissance neuronal (NGF) est le premier facteur de croissance à avoir été identifié et le mieux caractérisé. Il lui a été attribué une grande variété de fonctions dans le système nerveux, comme la régulation de la spécification neuronale, la différenciation, la survie, l'orientation de la croissance axonale et la plasticité neuronale. De plus, le NGF a été également démontré comme ayant un rôle dans d'autres systèmes physiologiques, par exemple, dans les cancers du sein et de la prostate il est fortement exprimé et serait donc mitogène pour les cellules tumorales. Bien que les voies de signalisation du NGF sont bien connues, les études sur le trafic et la signalisation du NGF ont continué à prospérer.

Le NGF est reconnu, et le signal qu'il véhicule est donc médié, par deux récepteurs membranaires : $p75^{NTR}$, un récepteur commun à toutes les neurotrophines, membre de la superfamille des récepteurs des facteurs de nécrose tumorale ; et TrkA (tropomyosine related kinase A), le récepteur spécifique au NGF. Il a été démontré que au niveau de la membrane, $p75^{NTR}$ et TrkA sont localisées dans les cavéoles, des microdomaines caractérisés par la présence de protéines cavéolines et enrichis en cholestérol et glycosphingolipides. Les cavéoles sont associées à la signalisation et l'endocytose du récepteur et impliqués dans le processus de tumorigenèse de cancer du sein. Cav-1 est le composant principal des cavéoles, il peut interagir avec de nombreuses protéines impliquées dans la transduction du signal, qui se traduit par un cloisonnement des molécules dans les cavéoles et de leur maintenance dans une conformation inactive. Cav-2 est l'autre composante majeure des cavéoles, mais il est encore moins connue et moins étudiée.

L'objectif de ce travail est d'étudier le rôle de ces micro-environnements membranaires dans la régulation du signal du NGF. L'hypothèse des ''endosomes de signalisation'' propose que l'internalisation de TrkA dans les endosomes induit une activation prolongée, Rap1-dépendante, de MAPK, nécessaire à la différenciation cellulaire. Dans le présent travail, nous avons constaté que la surexpression de Cav-1 dans les neurones des ganglions de la racine dorsale, diminue l'extension des neurites. De la même manière, la surexpression de Cav-1 dans les cellules PC12 inhibe les réponses cellulaires habituellement déclenchées par l'exposition au NGF. L'activation des effecteurs situés en aval de TrkA n'est pas inhibée.
L'expression de Cav-1 a pour résultat une inhibition de la sortie du récepteur des radeaux accompagné par la rétention au niveau de la surface cellulaire, des effecteurs situés en aval incluant Rsk2 phosphorylé. Dans le même temps, la présence de formes phosphorylées de CREB n'est plus détectable. En revanche, la surexpression de Cav-2 potentialise la différenciation des cellules induite par le NGF, ce qui est associé à une activation prolongée des effecteurs situés en aval et à une internalisation des récepteurs. Ces différents effets pourraient être dû à la localisation des cavéolines, qui résulte en une perturbation du microenvironnement des cellules et donc de la signalisation du NGF. En outre, l'expression d'une Cav-1 mutée sur la sérine 80 (S80V) dans des cellules PC12, ne gêne ni le trafic ni la signalisation de TrkA. Au contraire elles se comportent de façon semblable à des cellules Cav-2.

Mots clés: NGF signqlisation, NGF récepteurs, endocytose, Cavéolin-1, Cavéolin-2, Radeau/ Cavéoles

论文摘要

神经营养因子(NGF)是生长因子家族中最先被发现并且被深入研究的一类生长因子。它对神经系统中神经元的发育、分化、再生、功能特性的表达、突触定向生长以及可塑性方面均具有重要的调控作用。此外，它们在非神经系统尤其是肿瘤方面也发挥重要作用。例如，NGF 在乳腺癌和前列腺癌样本中的高表达能够促进细胞的有丝分裂。 尽管经历了大半个世纪的研究，仍有大量的学者投入到这一课题， 旨在逐步揭开其极复杂的信号转导机制。

NGF 通过与两种受体蛋白结合传导信号，一种是对 NGF 有低亲和力的受体 $p75^{NTR}$，它是肿瘤坏死因子受体家族中的一员，能以相同的亲和力与神经营养因子家族的其他成员结合，另一种是对 NGF 具有高亲和力的酪氨酸激酶受体 TrkA。有研究报道，在细胞膜表面， $p75^{NTR}$ 和 TrkA 在膜窖(Caveolae)这一富含胆固醇，鞘脂类和窖蛋白的区域富集。Caveolae 被证实与受体内吞，信号转导以及肿瘤发生等多种细胞生命活动相关。窖蛋白-1(Cav-1)是 Caveolae 的主要组成蛋白，它们可以在 Caveolae 中富集多种信号分子，抑制或者影响它们的活化，从而起到调控信号通路的作用。窖蛋白-2(Cav-2)，同样是 Caveolae 的主要组成蛋白，但人们对它在信号通路中的作用却知之甚少。

本论文主要研究 Cav-1 和 Cav-2 对 NGF 信号通路的影响，从而揭示可能的膜微环境对信号通路的调控机制。信号内吞体假说提出，NGF 诱导的细胞分化需要 TrkA 内化到内吞囊泡，并在那里经由小 G 蛋白 Rap-1 介导引发 MAPK 的持续活化。我们研究发现利用电转技术在小鼠背根神经节细胞瞬时表达 Cav-1 能显著抑制神经纤维生长。 在 PC12 细胞内，我们建立稳定过表达 Cav-1 或者 Cav-2 的单克隆细胞系，利用免疫荧光标记显示，过表达的 Cav-1 和 Cav-2 分别主要聚集在细胞膜表面以及高尔基体。 在 NGF 作用下，Cav-1 PC12 细胞不表现明显分化，并且与细胞周期相关的抗有丝分裂的 $p21^{waf1/CIP1}$ 蛋白表达受到抑制，但这些反应并不通过直接阻断丝裂原蛋白活化激酶(MAPK)磷酸化实现。利用受体荧光标记及脂筏抽提等技术，我们观察到在 NGF 作用下，野生型 PC12 细胞的 NGF 受体会脱离脂筏发生内化，但是在 Cav-1 PC12 细胞中，这一现象发生抑制，大部分 NGF 受体被钳制于脂筏内，其下游信号因子直至核糖体 S6 蛋白激酶-2(Rsk2)

也被锚钉于细胞膜上，导致其无法内化至细胞核活化重要的转录因子 cAMP 反应原件结合蛋白(CREB)，由此最终抑制了与生长分化相关基因的表达。与此相反，过表达 Cav-2 却能加强 NGF 的诱导分化作用，同时保持正常的受体内化过程，并能维持更长时间的下游信号的活化。综合以上结果，我们认为 Cav-1 和 Cav-2 蛋白在细胞内的不同定位，创造并改变了受体的微环境，从而使 Cav-1 和 Cav-2 PC12 细胞在 NGF 作用下表现出完全不同的应答。此外，有研究显示，Cav-1 蛋白氨基酸 80 位上的丝氨酸突变与肿瘤发生相关，过表达 Cav-1 S80V(蛋白丝氨酸 80 位点上缬氨酸突变)与 Cav-1 不同，并不抑制 NGF 引发的 TrkA 的转运和信号通路，而是表现了与 Cav-2 PC12 类似的应答反应。

以上的这些结果强调了细胞膜微环境对于招募活化信号分子从而调控信号通路所起的重要作用。我们的研究将为 TrkA 和 Cav-1 交互作用在其他的模型，例如肿瘤形成，中提供更好的理论基础。

关键词：神经营养因子信号通路，神经营养因子受体，受体内化，窖蛋白-1，窖蛋白-2，脂筏/窖膜

INTRODUCTION

This dissertation focuses on the signaling pathways and biological functions regulated by Caveolin-1 (Cav-1), which has been reported to act as a negative regulator of NGF induced differentiation in PC12 cells (Bilderback et al 1999). Here we present a further investigation, on Cav-1 and Cav-2, providing novel insights into the mechanism underlying their effect on NGF signaling. This introductory chapter provides background information about NGF signaling and provides the rationale behind my dissertation research.

1 NEUROTROPHIN OVERVIEW

The observation that mouse sarcoma tumors implanted within the body wall of a three day old embryo promoted the growth of spinal and sympathetic ganglia (Bueker 1948; Levi-Montalcini & Hamburger 1951), provided the initial clue that neuronal growth is a regulated process. Research into this process led to the discovery of Nerve Growth Factor (NGF), the best characterized member of neurotrophin family. Neurotrophins are a family of proteins that include Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerotrophin-4 (NT-4), having approximately 50% identity (Fig. 1) in their sequences. Many of the conserved residues are involved in the formation of dimers. While all of these neurotrophins can bind with low affinity to the p75 neurotrophin receptor ($p75^{NTR}$), NGF preferentially binds and activates TrkA (Neurotrophic tyrosine kinase receptor type 1 or TRK1), BDNF and NT4 binds to TrkB (Neurotrophic tyrosine kinase receptor type 2 or TRK2) receptor, and NT3 predominantly binds to TrkC receptor (Neurophic tyrosine kinase receptor type 3 or TRK3) (Fig. 2) (Tessarollo 1998). These signaling molecules are critical for the development and maintenance of the peripheral and central nervous system as they can promote survival, differentiation and apoptosis of neurons (Chao 2003; Harada et al 2011).

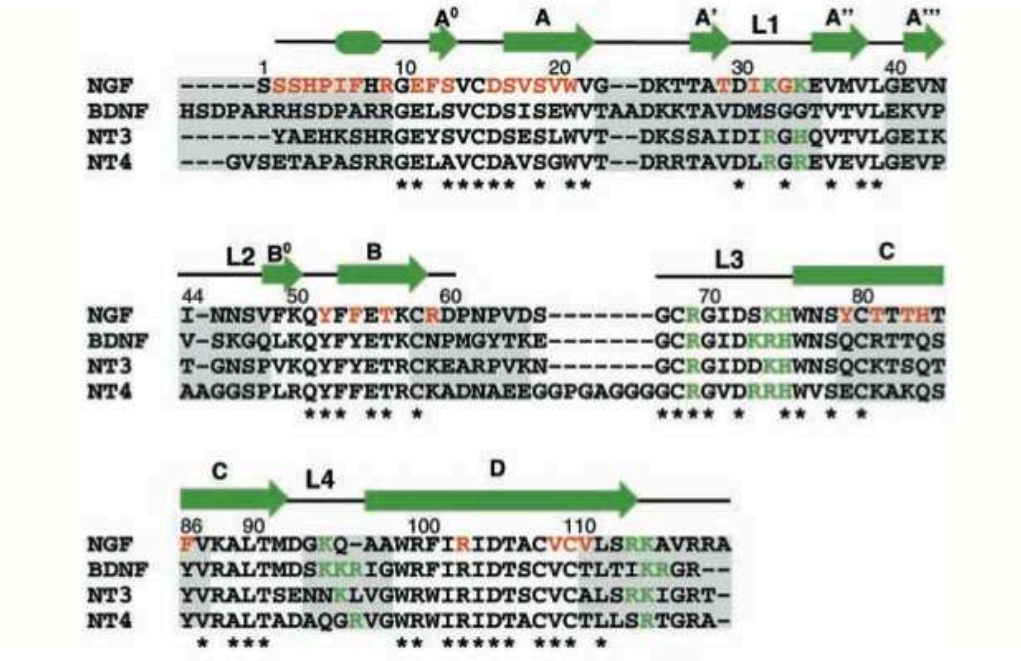

Figure 1. ***Sequence alignment of human neurotrophins.*** Conserved residues are marked with asterisk. NGF residues in contact with TrkA are shown in red, and residues important for $p75^{NTR}$ binding are shown in green. (Wiesmann & de Vos 2001)

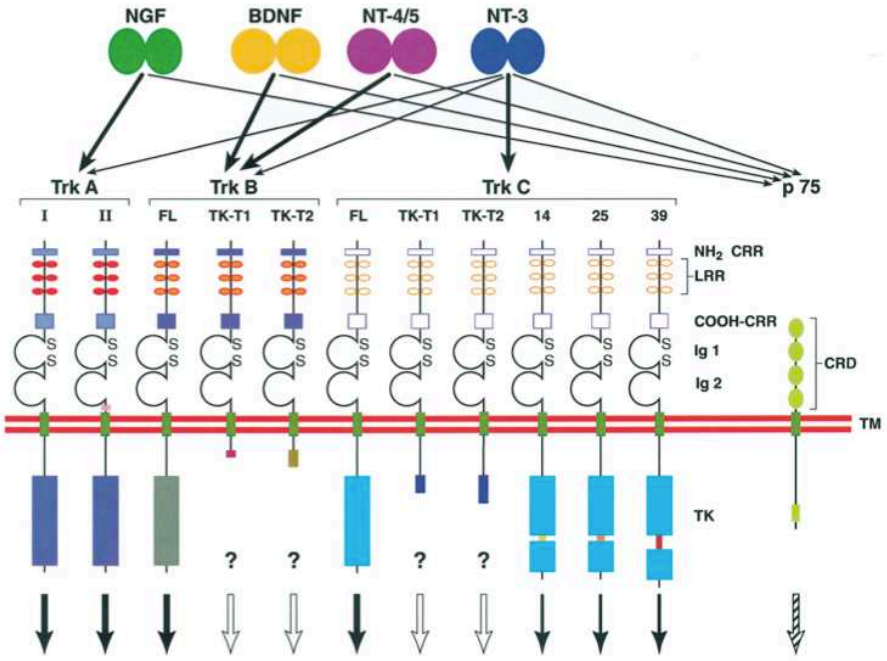

Figure 2. ***Schematic representation of neurotrophins and their receptors.*** Dimeric NGF, BDNF, NT3, NT4/5 are represented by two circles of ovals. Neurotrophins bind to their receptors with high affinity (bold arrows) or low affinity (thin arrows). CRR: Cysteine Rich Region; LRR: Leucine Repeat Region; Ig: Immunoglobulin; TK: Tyrosine Kinase; CRD: Cysteine Rich Domain; FL: Full length. (Tessarollo 1998)

1.1 NERVE GROWTH FACTOR (NGF)

The existence of a soluble freely-diffusing factor called "Nerve growth factor" was hypothesized over 60 years ago by Rita Levi-Montalcini (Levi-Montalcini & Hamburger 1951). They observed that a fragment of mouse sarcoma grafted onto chick caused a great ramification of nerve fibers into the mass of tumor cells. This effect was also observed when the graft was in agar, which only allows the diffusion of secreted substances from tumor cells, but not direct interaction. Further experiments revealed that only sympathetic neurons and some motor neurons were responsive to this factor. It was the first identified member of neurotrophin family found largely present in snake venom (Cohen 1959), guinea pig prostate (Harper et al 1979) and male mouse salivary glands (Bocchini & Angeletti 1969)

Studies on the physical and chemical properties of NGF were first carried out by Cohen in 1960 (Cohen 1960). There are two forms of NGF identified from different purification techniques - a high molecular weight and a low molecular weight form (which was known as 7S and 2.5S NGF on the basis of their sedimentation). 2.5S NGF is a 26 kDa, β subunit of the 7S NGF complex, which is composed of α-, β-, and γ-NGF (Fig. 3B). The high molecular weight form of NGF does not differ from the 2.5 S NGF in its biological activity. NGF is synthesized as a precursor, pro-NGF that undergoes post-translational processing to generate active mature NGF. In contract to the function of its mature form, whose interaction with TrkA and $p75^{NTR}$ leads to survival and differentiation, pro-NGF, when selectively bound to $p75^{NTR}$ and sortillin (not TrkA), provokes apoptosis and cell death (Nykjaer et al 2004). Crystallographic studies revealed that the NGF monomer has two pairs of antiparallel β-strands, with 3 hairpin loops on one end, and a cysteine-knot motif that stabilizes the fold on the other end (Fig. 3A). Two monomers are arranged in parallel to form a homodimer (Fig. 3C) thus conferring biological function (Wiesmann & de Vos 2001).

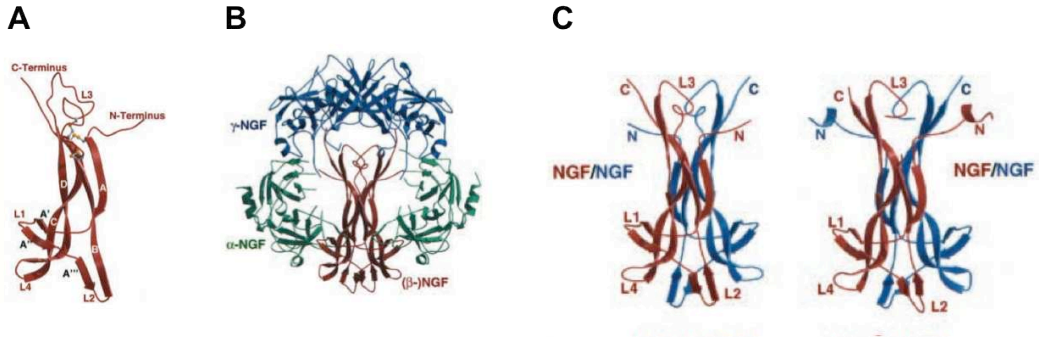

Figure 3. ***The composition and structure of NGF.*** **A**. Ribbon diagram depicting the structure of the NGF monomer (PDB code 1BFT). The cysteine-know motifs near the top of the molecule are shown in gray and yellow in ball-and stick rendering. **B**. Ribbon diagram showing the structure of the dimer of 7S NGF complex (PDB code 1SGF). **C**. Ribbon diagram depicting NGF dimers, left: unbound NGF (PDB code 1bet), right: NGF from the complex with second Ig-like domain of TrkA receptor (PCB code 1WWW). (Wiesmann & de Vos 2001)

1.1.1 NGF FUNCTION

The most fundamental role of NGF is as a key player in the regulation of peripheral and central nervous system development and maintenance. In the peripheral nervous system, it promotes survival of sympathetic and sensory neurons involved in nociception and temperature sensitivity. In the central nervous system, it promotes survival of cholinergic neurons in the basal forebrain (Chen et al 1997). NGF also affects other processes in neuronal cells such as synaptic rearrangement (Miyata et al 1986; Sharma et al 2010), dendritic arborization (Snider 1994), dendritic sprouting (Diamond et al 1992). Besides its role as a classical growth factor regulating neuronal cells, it also acts on endocrine, cardiovascular (Caporali & Emanueli 2009; Lazarovici et al 2006) and immune systems (Aloe et al 1994; Fiore et al 2009; Indo 2010).

Moreover, given the fact that NGF was originally found secreted from mouse sarcoma tissue, as a corollary, NGF is involved in the development of cancer. In fact, it has gained significant attention for its role in the pathogenesis of many cancers in recent years, which include neuroblastoma, meduloblastoma, glioma, pancreatic cancer, lung cancer, prostate cancer and breast cancer (Davidson et al 2004; Hondermarck 2012; Kruttgen et al 2006).

In PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla (Greene & Tischler 1976), NGF can evoke a rapid tyrosine phosphorylation of endogenous TrkA, eliciting signaling cascades necessary for the biological responses

which induces their differentiation into sympathetic neuron-like cells, expressing tyrosine hydroxylase, and becoming electrically excitable (Fujita et al 1989). As a result, PC12 cells are extensively used to study NGF signaling.

2 NGF RECEPTORS

Two types of NGF receptors have been identified, $p75^{NTR}$, which is the member of tumor necrosis factor receptor family, and TrkA, member of the receptor tyrosine kinase family. These receptors share no sequence similarity in either ligand-binding or cytoplasmic domains.

2.1 $p75^{NTR}$ receptor

$P75^{NTR}$ was cloned then initially characterized as a low-affinity NGF receptor (Chao et al 1986), and was later found to bind all the neurotrophins (Rodriguez-Tebar et al 1990). It is a 427 amino acid trans-membrane protein. The extracellular domain of $p75^{NTR}$ contains four highly conserved cysteine-rich domains (CRD) that are negatively charged. The modification at N- and O-glycosylation sites allow their targeting to the apical and axonal membrane respectively (Johnson et al 1986). The intracellular domain $p75^{NTR}$ does not show evidence of catalytic activity. It is composed of juxtamembrane domain (Chopper domain), the death-like domain (DD) that bears homology to the “death domain” sequence present in other TNF receptor superfamily members, and a Ser-Pro-Val (SPV) domain, all of which are involved in the recruitment of adaptor proteins. The juxtamembrane domain also contains a palmitoylation site (cysteine 279) that is important for the receptor membrane localization (Underwood & Coulson 2008) (Fig. 4).

The physiological roles of $p75^{NTR}$ are complex and remain elusive as they can collaborate with Trk receptors to either enhance (Barker & Shooter 1994; Verdi et al 1994) or reduce neurotrophin-mediated Trk receptor activation (Benedetti et al 1993; MacPhee & Barker 1997), they may also activate signaling cascades involved in apoptosis.

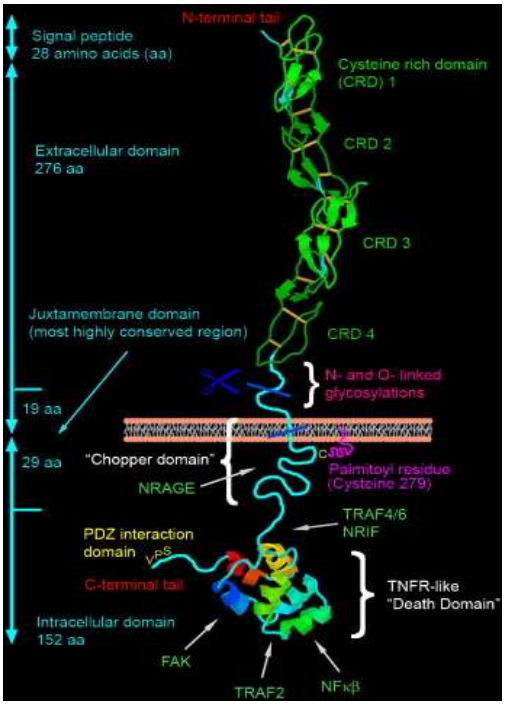

Figure 4. ***The Schematic representation of p75^{NTR}.*** The extracellular domain of P75^{NTR} is composed of four highly conserved cysteine rich domains. The intracellular part of p75^{NTR} contains a death domain and a Ser-Pro-Val (SPV) motif. (Underwood & Coulson 2008)

2.2 TrkA receptor

TrkA, initially characterized as a high affinity receptor for NGF, is a 790 amino acid (83 kDa) type 1 trans-membrane glycosylated protein. It's comprised of a large extracellular domain, a single transmembrane domain, and a cytoplasmic domain with kinase activity (Fig. 5). The extracellular domain contains two cysteine-rich clusters followed by three leucine repeats, another cysteine-rich cluster and two immunoglobulin-like (IgG) domains (Barbacid 1995). The extracellular domain of TrkA also contains N-glycosylation sites that can be further modified by the addition of sialic acid. Fully sialyated mature receptor is approximately 140 kDa while the non-sialylated, glycosylated form is approximately 110 kDa *cf.* (Jullien et al 2002). NGF binds to the IgG domain closest to the plasma membrane, causing receptor dimerization and activation (Lemmon & Schlessinger 2010). The intracellular part of TrkA contains a palmitoylation site (cysteine 436), that is essential for its targeting to lipid raft (Huang et al 1999). There are mainly five important residues involved during this process. Among these residues, Y490 and Y785 serve as the major docking sites for adaptor proteins allowing the down stream signaling. Y670, Y674 and Y675 are

present in the catalytic domain that are necessary for the kinase activity, whereas they also serve as docking sites for adaptor proteins, including SH2B, adaptor protein containing PH and SH domains, fibroblast growth factor receptor substrate 2 (Frs2), and growth factor receptor-bound protein 2 (Grb2) (Arevalo & Wu 2006; MacDonald et al 2000; Qian & Ginty 2001; Stephens et al 1994).

Alternative splicing of TrkA mRNA leads to the generation of three different forms of TrkA. TrkA-I is a ubiquitously expressed form and is preferentially expressed in non-neuronal tissues. TrkA-II is the longest form of TrkA (796 amino acid), whose biological activity is the same as TrkA-I, it is mainly expressed in neuronal cells (Barker et al 1993; Clary & Reichardt 1994). TrkA-III is an oncogene able to trigger signaling in an NGF-independent manner and promotes tumorigenic behavior in neuroblastoma cells (Tacconelli et al 2004).

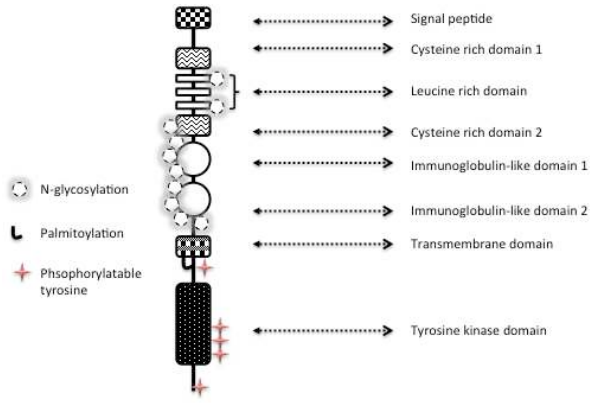

Figure 5. ***The Schematic representation of TrkA.*** The extracellular domain of TrkA is composed of two cysteine rich domains, three leucine rich domains and two Immunoglobulin-like domains. The intracellular part of TrkA contains a catalytic tyrosine kinase domain, a palmitoylation site and five phosphorylatable tyrosines. Adapted and modified (Roux & Barker 2002)

3 NGF SIGNALING PATHWAYS

3.1 TRKA SIGNALING PATHWAYS

Receptor tyrosine kinases are activated by the binding of their cognate ligands, resulting in phosphorylation of specific residues within the receptor intracellular domain. These phosphorylated residues, then initiate specific signaling pathways responsible for triggering numerous cellular processes such as cell proliferation, differentiation and migration (Fig. 6). The intracellular portion of TrkA contains a binding site for Adenosine-5'-triphosphate (ATP), lysine 538, the mutation of which

generates an inactive receptor (Kaplan et al 1991). Binding of NGF to TrkA triggers its trans-autophosphorylation. During this process five important tyrosine residues are phosphorylated (670, 674, 675, 490, 785). Tyrosine 490 within the juxtamembrane region, which is involved in SH2 containing sequence (Shc) adaptor protein recruitment (Stephens et al 1994); and tyrosine 785, which is responsible for the binding of phospholipase C-γ (PLCγ) (Loeb et al 1994; Stephens et al 1994). The phosphorylation of these tyrosine residues is the initial step of TrkA activation.

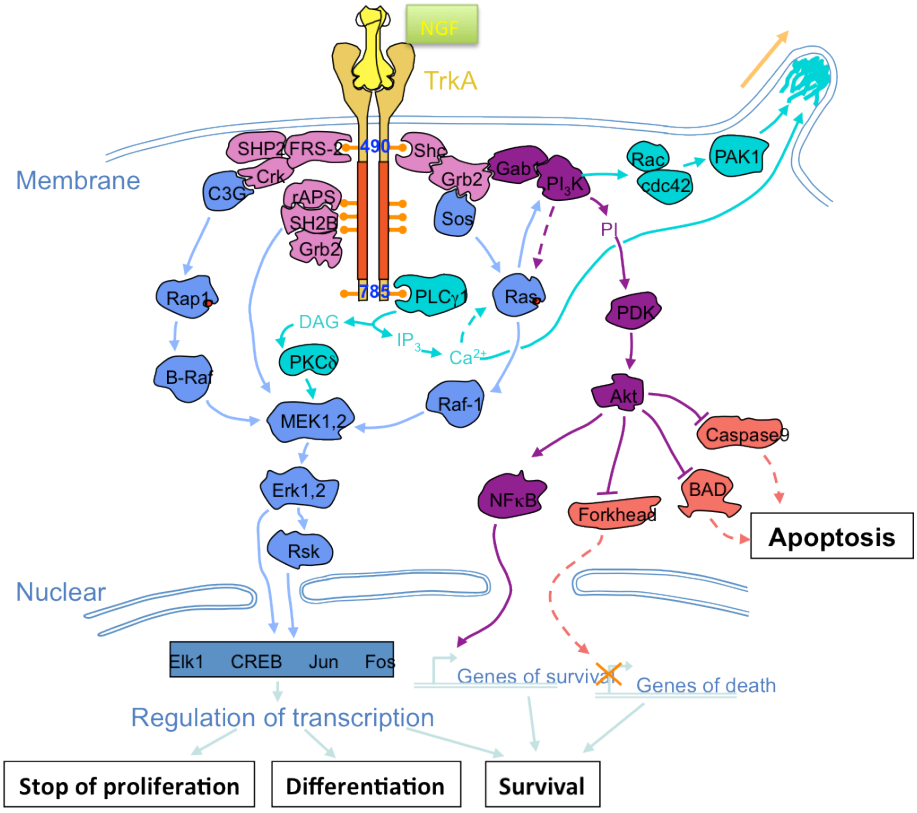

Figure 6. ***The signaling cascade downstream from TrkA.*** Schematic representation of NGF induced TrkA activation to a cascade of intracellular events, including MAPK (blue), PI3K/Akt (red) AND PLCγ (green) signaling pathways. Ultimately result in activation of gene expression, neuronal survival and neurite outgrowth. (Adapted from Guili thesis 2003)

3.1.1 RAS-MAPK SIGNALING

TrkA receptor stimulation by NGF engages transient Mitogen-activated protein kinase (MAPK) activation through the binding at Y490 of Shc, an adaptor protein that is critical to the activation of Rat sarcoma protein (Ras) (Basu et al 1994).

Phosphorylated Shc then recruits the Grb2 and Sos[1] complex *via* an SH2[2] interaction (Rozakis-Adcock et al 1992), thereby bringing Sos to the membrane where it behaves as a Ras GTP[3] exchange factor that promotes the transition from inactive Ras-GDP to active Ras-GTP (McCormick 1994). Activated Ras then recruits C-Raf[4] (Raf-1) (Van Aelst et al 1993), subsequently triggering activation of the MAPK cascade that includes MEK1[5] and Erk 1/2[6] (Jaiswal et al 1994). Activated Erk 1/2 translocate into the nucleus (Chen et al 1992) to stimulate Elk-1[7], leading to the interaction with the transcription factor serum response factor (SRF) and with CAGGAT binding site of the serum response element (SRE) within c-fos[8] gene that stimulate the initiation and maintenance of differentiation (Gille et al 1995; Hill et al 1993). In addition, CREB[9] that binds to a site called CRE within the c-fos promoter (Berkowitz et al 1989), also contributes to the regulation of c-fos transcription in response to NGF. In this case, activated Erk1/2, downstream in the Ras pathway, phosphorylates Rsk2 leading to the phosphorylation of CREB at serine 133 (Ginty et al 1994). Hence, the Ras pathway is able to regulate c-fos by parallel and cooperative pathways.

3.1.2 MAPK SIGNALING

NGF and EGF (epidermal growth factor) both activate the Raf MAPK pathway, however, EGF transiently stimulates the MAPK, ERK1 and ERK2, and provokes cellular proliferation while NGF leads to sustained activation and subsequently to neuronal differentiation. NGF promotes sustained activation of Erk1/2 through a set of signaling proteins that involves FRS2[10], CrkII[11]/Crk[12], guanine nucleotide exchange factor C3G, the small GTPase Rap-1[13], the protein tyrosine phosphatase Shp2[14] and serine threonine kinase B-Raf[15], resulting in sustained activation of MAPK (Marshall 1995; Wu et al 2001). The persistent Erk activation via Rap1 may induce expression of immediate early genes and their cognate proteins that interact with activated CREB, leading to the transcription of novel delayed response genes. Of the note, receptor

[1]Son of sevenless, [2]Src Homology, [3]Guanosine-5'-triphosphate, [4]RAF proto-oncogene serine-threonine protein kinase, [5]Dual specificity mitogen-activated protein kinase kinase 1, [6]Extracellular-signal-regulated kinases 1/2, [7]E twenty-six-like transcription factor 1, [8]FBJ murine osteosarcoma viral oncogene homolog, [9]cAMP regulatory element binding protein, [10]Fibroblast growth factor receptor substrate 2, [11/12]Adapter molecule crk also known as proto-oncogene c-Crk or p38 or v-crk sarcoma virus CT10 oncogene homolog, [13]Ras-proximate-1 or Ras-related protein 1, [14]Src homology protein tyrosine phosphatase 2, [15]Murine sarcoma viral oncogene homolog B1

internalization is required for this pathway (Wu et al 2001; York et al 2000). And it is likely that the early events triggered by Ras and C-Raf activation are necessary for the cells to respond to the later and sustained activation of Erk by Rap1 and B-Raf pathway (Sofroniew et al 2001).

3.1.3 PI3K/AKT SIGNALING

The PI3K[1]/AKT[2] pathway is essential for survival and neuronal development. Shc participates in this pathway by associating with Grb2 and Gab1 to recruit the catalytic subunit of PI3K to the plasma membrane, where it can phosphorylate membrane phosphoinositides (Holgado-Madruga et al 1997), resulting in the production of PI[3], which binds to and activates PDK1[4]. PDK1 associates with and phosphorylates the serine threonine kinase Akt (Alessi et al 1997), which is also known as PKB, allowing the phosphorylation of several downstream proteins important for controlling the cell survival (Crowder & Freeman 1998). These include BAD[5], the forkhead transcription factor, GSK3B[6], NFκB[7], and human caspase-9 (Brunet et al 1999). Taking BAD as an example, activated Akt phosphorylates BAD, leading to its association with the 14-3-3 protein and sequestering it from hetero-dimerization with Bcl-X_L[8], as a result, Bcl-X_L is able to hetero-dimerize with BAX, preventing BAX homo-dimerization thereby supporting cell survival (Zha et al 1996). Apart from its pro-survival function, PI3K/AKT pathway is also implicated in endocytosis and local axonal growth (Zhou et al 2004).

3.1.4 PLC-γ SIGNALING

Upon TrkA activation, phosphorylation of Y785 leads to the recruitment and activation of PLCγ (Vetter et al 1991), which mediates the hydrolysis of PIP2[9], yielding two products that each function as intracellular second messengers: IP3[10] and DAG[11] (Obermeier et al 1993). IP3 interacts with its specific receptor on the endoplasmic reticulum to induce the release of intracellular calcium. DAG activates various PKC isoforms, leading to subsequent Erk signaling *via* C-Raf (Corbit et al 1999).

[1]Phosphatidylinositol 3-kinases, [2]Also known as Protein Kinase B (PKB) serine/threonine protein kinase, [3]Phosphatidylinositol, [4]3-phosphoinositide-dependent protein kinase, [5]Bcl-2-associated death promoter, [6]Glycogen synthase kinase 3 beta, [7]Nuclear factor kappa-light-chain-enhancer of activated B cells, [8]B-cell lymphoma-extra large, [9]Phosphatidylinositol 4,5-bisphosphate, [10]Inositol trisphosphate, [11]Diglyceride

It is clear that this simplified vision does not reflect the whole scheme of TrkA signaling pathway. And certainly, this network involves interplay with $p75^{NTR}$ and downstream effectors.

3.2 $p75^{NTR}$ SIGNALING PATHWAY

$P75^{NTR}$ is a transmembrane glycoprotein and a member of the tumor necrosis factor (TNF) superfamily, it has been shown to bind both the mature and the precursor form of neurotrophins (Lu et al 2005). It mediates a very broad rang of cellular functions that resembling "yin and yang"; *e.g*. it can increase or inhibit axon growth (Fujita et al 2011; Yamashita et al 1999), reduce or promote neuronal cell survival (DeFreitas et al 2001; Kenchappa et al 2010), and is crucial or not required for inhibition of neuronal regeneration (Boyd & Gordon 2001; Song et al 2004). The crystal structure of NGF complexed with the extracellular domain of $p75^{NTR}$ reveals an NGF homodimer asymmetrically bound to a $p75^{NTR}$ monomer, suggesting that the outcome of $p75^{NTR}$ signaling might depend on the type of co-receptor (Bronfman & Fainzilber 2004).

3.2.1 SIGNALING CELL SURVIVAL

An important pathway of $p75^{NTR}$ to promote cell survival is thought to relay on Akt and NFκB pathway. Neutrophins promote the association of TRAF6 to the cytoplasmic domain of $p75^{NTR}$, activating several intermediate proteins that ultimately cause activation of NFκB and degradation of the NFκB inhibitory protein IκB. (Fig. 7) (Khursigara et al 1999; Lu et al 2005; Reichardt 2006). $P75^{NTR}$ associates with ARMS[1], which serves as a bridge binding protein to Trk receptors is important for the sustained MAPK activation (Arevalo et al 2004). Recently studies reveal that, in PC12 cells, NGF induces rapid and robust α-secretase and γ-secretase-dependent cleavage of $p75^{NTR}$, releasing its intracellular domain into the cytosol, which plays important role in Akt phosphorylation (Ceni et al 2010).

[1] An ankyrin-rich membrane spanning protein

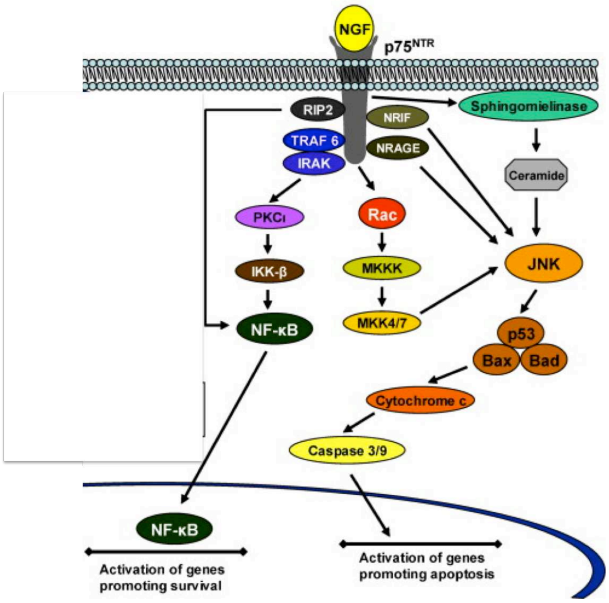

Figure 7. ***The signaling cascade downstream from p75^{NTR}.*** Schematic representation of p75^{NTR} mediated signal pathway. Binding of neurotrophins regulates gene expression, cell cycle, apoptosis, mitogenic responses, and growth cone motility. (Niewiadomska et al 2011)

3.2.2 SIGNALING APOPTOSIS

p75^{NTR} is abundantly expressed in the developing nervous system. At adult stages, p75^{NTR} expression is switched off or retained at lower levels (Ernfors et al 1989; Friedman et al 1991). When peripheral nerve crush or injury occurs, p75^{NTR} expression is strongly induced, leading to apoptotic cell death. These apoptotic pathways have been shown to involve the activation of JNK. Recruitment of NRIF[1], NADE[2], NRAGE[3] and TRAFs[4] to the p75^{NTR} death domain will lead to the activation of JNK and promote p75^{NTR}-dependent apoptosis. Further downstream events include phosphorylation of c-jun[5], activation of p53[6], Bad, Bim[7], Bax[8] and caspase 9, 6 and 3 (Barker 2004; Becker et al 2004; Bhakar et al 2003; Okuno et al 2004). Ligand engagement of p75^{NTR} can also induce ceramide production, which will act as second messengers ultimately activate the JNK kinase (Arevalo & Wu 2006; Blochl & Blochl 2007).

[1]Neurotrophin receptor interacting factor, [2]p75 NTR-associated Cell Death Executor, [3]p75 NTR-interacting MAGE homolog, [4]TNF receptor associated factors, [5]Jun proto-oncogene, [6]Tumor protein 53, [7]Bcl-2-like protein 11, [8]Bcl-2–associated X protein

3.3 INTERPLAY BETWEEN TRKA AND $p75^{NTR}$

Neurotrophins are much more effective at inducing apoptosis through $p75^{NTR}$ in the absence of TrkA receptor activation (Yoon et al 1998) since activated TrkA completely abolishes Jun kinase, but not NFκB cascade mediated by $p75^{NTR}$.

In vitro studies have documented that $p75^{NTR}$ enhances the response of Trk to neurotrophins. When both TrkA and $p75^{NTR}$ are present, NGF binds TrkA with high affinity (10^{-11}M) (Chao & Hempstead 1995). To define a structural basis for this high-affinity site, crystallographic studies and molecular modeling were performed. It is revealed that TrkA and $p75^{NTR}$ will be in an antiparallel orientation when simultaneously binding NGF. Additionally, TrkA and $p75^{NTR}$ interact with NGF through non-overlapping binding sites on the same face of NGF, which disables the formation of TrkA/ $p75^{NTR}$/ NGF at 2:2:2 symmetric tri-complexes (Wehrman et al 2007) (Fig. 8). $p75^{NTR}$ is capable of promoting the retrograde transport of neurotrophins (Curtis et al 1995), while others have reported that $p75^{NTR}$ reduced TrkA ubiquitination and delayed Trk internalization and degradation (Makkerh et al 2005).

In summary, NGF can either promote neuronal survival or death depending on the outcome of the interplay between these two receptors. Although there remain some areas to be clarified regarding the regulation of this process, the regulated recruitment of specific partners to the signaling complex is one possible explanation. The receptor microenvironment, which partly determines the partners available for activating the intracellular pathways, is of an extreme importance for the outcome of NGF binding. Since both receptors are membrane proteins and have been reported to locate in lipid raft micro-domains, it is thus important to address the role of this "functional structure" in NGF signaling.

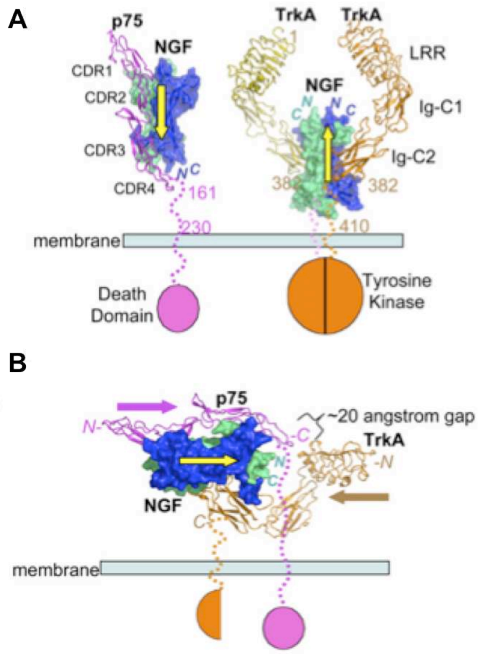

Figure 8. ***Structural analysis of NGF/p75 and NGF/TrkA complexes.*** **A**. On the left is the structure of NGF complex to p75, and the neighboring complex of NGF with TrkA. The polarity of NGF is indicated with yellow arrows. **B**. A ternary complex of a p75/NGF/TrkA 1:2:1 heterotrimer, the receptors lie parallel to the membrane so as to sandwich NGF in antiparallel orientations. (Wehrman et al 2007)

3.4 NGF SIGNALING AND BREAST CANCER

NGF was first shown to be secreted from mouse sarcoma tissue. In recent years, more attention has been given to its non-neuronal functions.

In breast cancers, it has been shown that NGF and TrkA are overexpressed compared to normal breast tissues (Adriaenssens et al 2008; Lagadec et al 2009). NGF secreted by breast cancer cells could stimulate tumor angiogenesis and increase growth, migration, invasion and permeability of endothelial cells (Romon et al 2010). Moreover, inhibition of NGF with neutralizing antibodies, or small interfering RNA, strongly reduces angiogenesis in immunodeficient mice (Adriaenssens et al 2008). Together, it indicates that NGF and it's signaling pathway(s) offer potential therapeutic targets in breast cancer.

4 LIPID RAFTS AND CAVEOLAE

4.1 LIPID RAFTS

According to the Singer-Nicolson fluidic mosaic model of cell membranes, proteins were embedded in a phospholipid bilayer evenly distributed in the cell membrane (Singer & Nicolson 1972). This model has later been enhanced with the discovery of microdomains of membranes that are enriched in cholesterol and glycosphingolipids, now referred to as lipid rafts (Simons & Ikonen 1997). Although the contention on lipid rafts, with regard to their existence or biological relevance, has kept researchers debating for more than a decade (Leslie 2011). There is compelling evidence that lipid rafts are microdomains in the plasma membrane and intracellular organelles, approximately 50-100 nm in diameter, that are important for signal transduction and membrane trafficking (Simons & Ikonen 1997).

4.1.1 COMPOSITION OF LIPID RAFTS

The common feature of lipid rafts is their insolubility in cold non-ionic detergents, and they are therefore referred to detergent-resistant membranes (DRMs). Analysis of lipid rafts shows that most of the typical raft lipids (e.g., cholesterol, sphingomyelin, and glycosphingolipids) tend to be found in the outer leaflet of the membrane, with ethanolamine-containing glycerophospholipids preferentially localized to the inner leaflet of the membrane. This implies that rafts are probably asymmetric bilayer structures (Pike 2003). GM1 ganglioside is an important component of plasma membranes and particularly enriched in lipid rafts, it has been shown to aggregate with cholesterol and sphingomyelins and is widely accepted now as a marker of lipid raft (Guan 2004; Lingwood & Simons 2010).

A variety of proteins have been found enriched in lipid rafts and/or caveolae (flask-shaped invaginations of plasma membrane), which includes caveolins; flotillins; GPI-linked proteins Src family kinases; receptors for EGF[1], NGF, and PDGF[2]; Grb2; Shc;

[1]Epidermal growth factor, [2]Platelet-derived growth factor

MAPK[1]; PKC; PI3K (Bickel et al 1997; Brown & Rose 1992; Chun et al 1994; Huang et al 1999; Liu et al 1996; Smart et al 1999; Song et al 1996a). The mechanisms employed for their localization into lipid rafts are numerous, which include the presence of specific binding motifs (such as GPI-anchors) in their structure, or the palmitoylation, acylation or myristoylation (Brown & Rose 1992; Wang et al 2001).

4.1.2 LIPID RAFTS AND SIGNALING

Lipid rafts can be viewed as signaling platforms that serve to co-localize requisite components, facilitating their interaction and supporting signaling (Pike 2003; Simons & Toomre 2000). Since they are very dynamic structures, the aggregation and/or the segregation of signaling components can occur over very short period of time (Golub et al 2004; Pike 2003). Due to the specific lipid environment or the result of the close proximity of a signaling protein with a regulatory molecule, lipid rafts can modulate the activity of proteins located within them. In a more complex model, complementary components of a signaling pathway can be segregated into different lipid rafts, and when stimulated with a hormone or growth factor, transient fusion of lipid rafts occurs, consequently leading to the activation of multiple signaling pathways (Pike 2003).

Some raft proteins partition to various degrees between the raft and non-raft domains of the membrane. This gives rise to the hypothesis that the same receptor could activate different signaling pathways depending on where it is located. Changes in the partitioning of molecules between the raft and non-raft compartment could alter the signaling. It has been reported that NMDA[2] receptors located in lipid rafts mediate neurotoxicity (Abulrob et al 2005; Frank et al 2004), whereas outside of lipid rafts they are responsible for glutamate-mediated growth cone guidance (Zweifel et al 2005).

Many receptor tyrosine kinases are localized in lipid rafts (summarized in Table. 1) (Note that the list may not be complete) and the effect of ligand binding on receptor localization is variable. A receptor can either move into or out of the lipid raft compartment. For example, EGF receptors rapidly move ***out*** of lipid rafts upon

[1]Mitogen-activated protein kinases, [2]*N*-Methyl-D-aspartic acid, [3]Adenylate Cyclase Associated Protein

activation by ligand (Mineo et al 1999). In the cells lacking caveolae, insulin receptors are recruited ***into*** the rafts by ligand binding.

In the case of NGF signalling, it was reported that following NGF exposure, TrkA and $p75^{NTR}$ move ***into*** lipid raft fraction, possibly with the help of the adapter protein CAP^{3}, which links TrkA complexes to foltillin (Limpert et al 2007). Proteins implicated in NGF signaling pathway, *e.g.*Ras, Src, Grb2, Erk, PLC and Shc are also found in this fraction (de Laurentiis et al 2007; Huang et al 1999; Tsui-Pierchala et al 2002). Of note, 90% of high affinity binding between TrkA and $p75^{NTR}$ takes place in lipid rafts. And modulation of the lipid raft composition by depletion of cholesterol or over-expression of GM-1 ganglioside has been shown to inhibit NGF signaling (Nishio et al 2004; Peiro et al 2000). These observations indicate that lipid rafts are vital for NGF signaling, and to a major extent, that the onset of NGF signaling may take place in lipid rafts.

4.2 CAVEOLAE

Caveolae, first identified by electron microscopy in 1950s (Palade 1953; Yamada 1955), are a subgroup of lipid rafts, which are flask-shaped invaginations of plasma membrane (Fig. 9). They are distinguished from other lipid rafts by the presence of cholesterol-binding protein Caveolin-1.

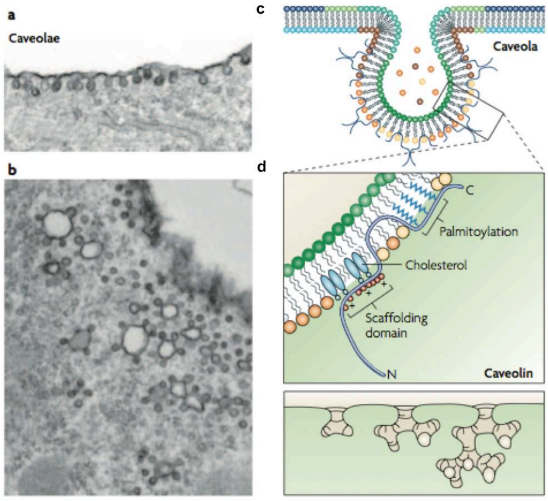

Figure 9. ***Structure of Caveolae.*** (a, b) The electron micrographs show caveolae in adipocytes (surface connected and intracellular). (c, d) Indication of how caveolin is inserted into the caveolar membrane, with the N and C termini facing the cytoplasm and a putative "hairpin" intramembrane domain embedded within the membrane bilayer. The scaffolding domain, might have a role in cholesterol interaction through conserved basic and bulky hydrophobic residues. (Parton & Simons 2007)

4.2.1 THE PROTEINS OF CAVEOLAE

4.2.1.1 CAVEOLIN FAMILY

The caveolin gene family has three members in vertebrates: Caveolin-1 (Cav-1), Caveolin-2 (Cav-2) and Caveolin-3 (Cav-3), 21-24 kDa integral membrane proteins, serving as the protein markers of caveolae (Fig. 10). Cav-1 is found predominantly at the plasma membrane but also in the Golgi, the endoplasmic reticulum, and at cytosolic locations (Schlegel et al 2001; Tagawa et al 2005), Cav-2 and Cav-1 are ubiquitously expressed, while Cav-3 is present in skeletal, cardiac and smooth muscle cells (Song et al 1996b). Cav-2 is generally co-expressed with Cav-1 and requires Cav-1 for its' stabilization and plasma membrane localization (Parolini et al 1999). Cav-1 and Cav-3, but not Cav-2, are necessary and sufficient for caveolae formation (Drab et al 2001; Galbiati et al 2001; Razani et al 2001).

The human Cav-2 gene is approximately 38% identical and 58% similar to human Cav-1 at the level of amino acid sequence, while Cav-3 shares more significant homology with Cav-1, approximately 65% identical and 85% similar (Cohen et al 2004). Multiple sequence alignment has identified a signature sequence (FEDVIAEP), an eight amino acid motif that is identical between all three caveolin proteins (Fig. 11).

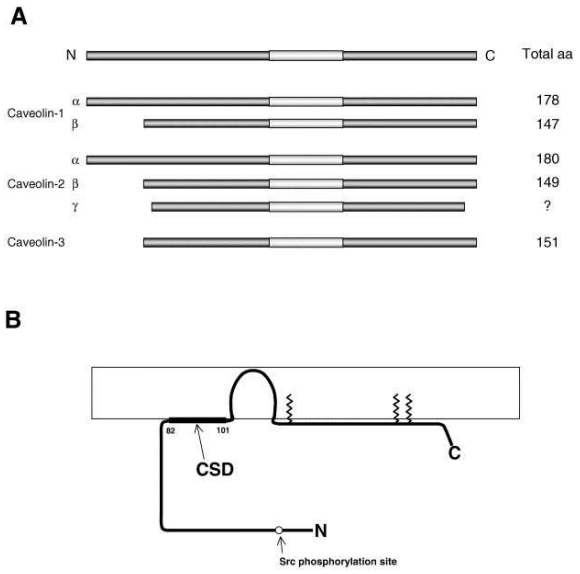

Figure 10. ***Mammalian Caveolins: diversity and stucture.*** **A**. Schematic representation of the different Caveolin proteins. The membrane-spanning region is shown in light grey. **B**. Diagram summarizing the membrane topology of Caveolin-1α. The CSD (Caveolin Scaffolding domain), three palmitoylation sites and a Src phosphorylation site (Tyr14) are indicated (Couet et al 2001).

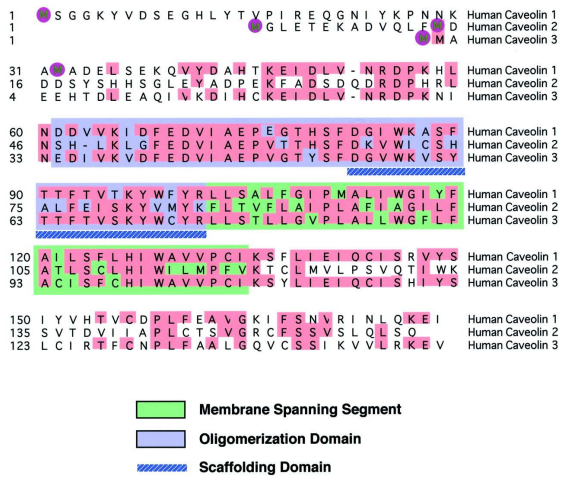

Figure 11. ***Sequence alignment of the human caveolin gene family.*** An alignment of the protein sequences of human caveolin-1, -2, and -3 is shown. Identical residues are boxed and highlighted in red. Translation initiation sites are circled. In addition, the positions of the membrane-spanning segment (green), the oligomerization domain (blue), and the scaffolding domain (a subregion of the oligomerization domain—hashed blue) are indicated. (Razani et al 2002)

4.2.1.1.1 CAVEOLIN-1

Caveolin-1 (Cav-1), also known as VIP21 protein, was initially identified as a tyrosine-phosphorylated protein in Rous sarcoma virus-transformed fibroblasts (Glenney & Soppet 1992). Cav-1 is found abundantly expressed in terminally differentiated cell types, such as adipocytes, endothelia cells, pneumocytes, smooth and striated muscle cells. It is a 178 amino acid integral membrane protein, with both N- and C-termini facing the cytoplasm, forming hairpin like structure. Cav-1 has two isoforms (α and β), the α-isoform has an additional 31 amino acids at the N-terminus (Scherer et al 1995), and both isoforms are capable of driving caveolae formation. Several domains have been identified in Cav-1, including two membrane-spanning domains (residue 82-101, also called Scaffolding Domain, 135-150), an oligomerization domain (61-101), and a caveolin scaffolding domain (CSD) (82-101) (Sargiacomo et al 1995; Schlegel et al 1999; Song et al 1997; Woodman et al 2002). Of note, the CSD is a domain that binds and regulates the activity of numerous signaling molecules (Byrne et al 2012). Cav-1 can be phosphorylated at tyrosine 14 or at serine 80 and 168 in response to various stimuli. Cav-1 is palmitoylated on cysteine 133, 143 and 156 for cholesterol binding and interaction (Fig. 10, 12). Caveolin-1 exists as either a homo-oligomer of approximately 14 to 16 monomers or as a hetero-oligomer with Caveolin-2.

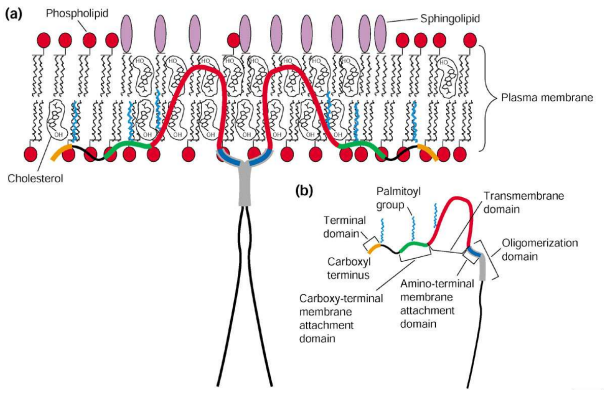

Figure 12. ***The view of Caveolin-1 membrane topology.*** Caveolin-1 exists as either a homo-oligomer of ~14 to 16 monomers (shown as a dimer for simplicity) or as a hetero-oligomer with Caveolin-2. (a) To Caveolin-1 monomers are shown forming a dimer. (b) The domains in Caveolin-1 (Williams & Lisanti 2004).

4.2.1.1.2 CAVEOLIN-2

Caveolin-2 (Cav-2) was identified through micro sequencing of caveolin-rich domains purified from adipocyte derived caveolae (Scherer et al 1996). It is a 162 amino acid integral membrane protein. It has three isoforms (α, β, γ). Cav-2 interacts with Cav-1 to form hetero-oligomeric complex, this interaction is necessary for the transport of Cav-2 to the cell surface. In the absence of Cav-1, Cav-2 is retained in the Golgi complex and undergoes degradation (Parolini et al 1999). In epithelial and endothelial cells, when phosphorylated at Serine 23, Cav-2 localized mostly to plasma membrane caveolae. Cav-2 can undergo Src-induced phosphorylation on tyrosine 19 and 27 to serve as a docking site for SH2 domain containing proteins (Lee et al 2002). Although relative to Cav-1, the role of Cav-2 remains less clear.

4.2.1.1.3 CAVEOLIN-3

Caveolin-3 (Cav-3) was found by cDNA library screening for Cav-1 homologous genes. It is a 151 amino acid, integral membrane protein (Razani et al 2002). In skeletal muscle, Cav-3 is localized to the sarcolemma, where the caveolae function as platforms that concentrate ion channels, kinases, and signaling molecules (Ohsawa et al 2008). Cav-3 plays critical role in muscle cell development and physiology. The mutations in Cav-3 have been shown to cause skeletal muscle pathology and hypertrophic cardiomyopathy (Gazzerro et al 2010). Recently, it was shown to be

involved in the TGF-β-dependent satellite cell proliferation and myoblast differentiation (Ohsawa et al 2012).

4.2.1.1.4 CAVEOLIN AND BREAST CANCER

Previous studies on Caveolins indicate that Cav-1 is downregulated in breast cancer samples and initially regarded as a tumor suppressor (Williams et al 2003). Expression of Cav-1 in MCF7 and T47D breast cancer cell lines slowed cell proliferation and growth (Fiucci et al 2002; Lee et al 1998). Recent studies suggest the mechanism by which the loss of stromal Cav-1 induces the extracellular matrix remodeling, or through the regulation on signaling transduction, to promote tumor progression (Sotgia et al 2012). A point mutation in codon P132 of Cav-1, (P132L) reported to occur in 16% of human breast cancers (Hayashi et al 2001), has been shown to cause significant increase in tumor migration, invasion and metastasis (Bonuccelli et al 2009). Several other Cav-1 mutations were then identified (Li et al 2006), all together indicating the possible role of Cav-1 in human breast cancer pathogenesis.

4.2.1.2 CAVIN FAMILY

Recent publications have shed light on the newly identified family of proteins, termed cavins, whose study is expected to lead to significant advances in our understanding of caveolar biogenesis and function. The cavin family has four members, and in fact each of them has been given several different names: Cavin-1 as RNA Pol I transcription factor (PTRF) (Hill et al 2008), Cavin-2 as serum deprivation protein response (SDPR) (Hansen et al 2009), Cavin-3 as sdr-related gene product that binds to-c-kinase (SRBC) (McMahon et al 2009), Cavin-4 as muscle restricted coiled-coiled protein (MURC) (Bastiani et al 2009), all sharing homology with Cavin-1 (Fig. 13). These proteins all contain leucine zipper-like domains that are involved in protein-protein interactions and proline, glutamic acid, serine and threonine-rich domains (PEST domains), which play a role in targeting proteins towards proteolytic degradation (Aboulaich et al 2004).

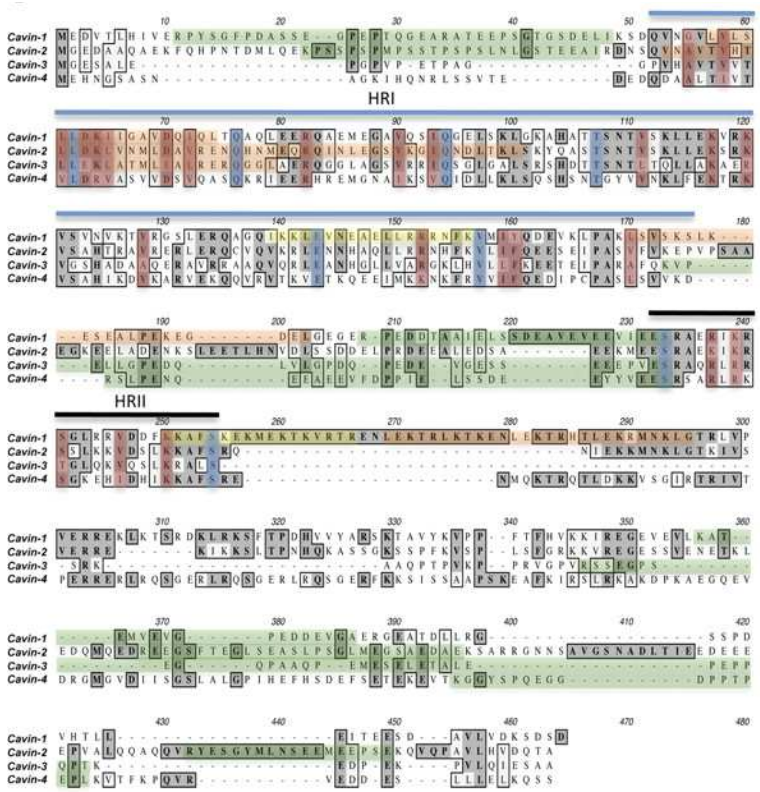

Figure 13. ***Sequence alignment of the murin caveolin PTRF/Cavin family.*** Dark gray denotes identity, light gray denotes similarity. Proposed cavin homology regions (HR) are marked with lines. Blue indicates identical amino acids, and red indicates conserved amino acids in evolution across all family members. Green indicates putative PEST domains, orange indicates leucine-rich regions, and yellow indicates nuclear localization sequences. (Bastiani et al 2009)

4.2.1.2.1 CAVIN-1

Cavin-1 was first identified as a soluble nuclear factor that regulates transcription by yeast two-hybrid systems (Jansa et al 1998). Until recently, this protein was found abundantly expressed in caveolae membranes derived from adipocytes (Vinten et al 2005). Further study revealed that Cavin-1 is recruited by caveolins to plasma membrane caveolae and is necessary for caveolae formation, absence of Cavin-1 leads to the loss of caveolae and accelerated mobility and lysosomal degradation of caveolins (Hill et al 2008).

4.2.1.2.2 CAVIN-2

Cavin-2, the second member of the cavin family was found to be rich in caveolae and to co-localize with Caveolin-1 (Mineo et al 1998). Cavin-2 shares more than 20% similarities with Cavin-1. Cavin-2 down-regulation leads to the loss of Cavin-1 and

Caveolins, and thus limits caveolae formation suggesting that Caveolin-1, Cavin-1 and Cavin-2 are functionally interdependent (Hansen et al 2009). Unlike Caveolin-1 or Cavin-1, Cavin-2 alone does not increase caveolae number.

4.2.1.2.3 CAVIN-3/CAVIN-4

Cavin-3 was identified in screens identifying PKC delta binding proteins (Izumi et al 1997), while previous studies showed that it was enriched in detergent-free caveolae extracts. In the absence of Cavin-3, traffic of intracellular Caveolin-1 is markedly impaired, suggesting its role in the regulation of caveolar endocytic pathway.

Cavin-4 was found specifically expressed in cardiac and muscle tissues, which is in agreement with Caveolin-3 expression. Cavin-4 was also shown to be associated with cardiac dysfunction and muscle biogenesis (Tagawa et al 2008). Together, these findings point to the role of Cavin-4 as a new candidate for muscle-related caveolinpathies.

4.2.2 CAVEOLAE AND SIGNALING

Caveolae are abundantly found in quiescent cells, such as adipocytes, endothelial cells, fibroblasts and smooth muscle cells. Being the protein marker for caveolae, Cav-1 has been shown to bind and inhibit a large number of proteins that are involved in signaling (Couet et al 1997; Patel et al 2008). It raises the hypothesis that Cav-1 can serve to compartmentalize certain signaling molecules within caveolae, thereby allowing rapid and selective modulation of cell signaling events. For instance, EGFR and NGF receptors are both negatively regulated by Cav-1 (Bilderback et al 1999; Han et al 2009; Park et al 2001). In PC12 cells, it has been shown that Cav-1 impairs TrkA activation, leading to inhibition of cell differentiation (Bilderback et al 1997). Peiro and his colleagues demonstrated that a fraction of TrkA co-localized with Cav-1 and was present in the caveolae of PC12 cells (Peiro et al 2000). Whereas others could not detect Cav-1 in these cells (Bilderback et al 1997; Huang et al 1999). Nevertheless, both reported that TrkA and $p75^{NTR}$ were present in low buoyant density fractions (lipid raft/cavolae). Further, high affinity binding of NGF to receptors, and association of TrkA with Shc and PLCγ also occur in this domain (Huang et al 1999). All of these

observations suggest that TrkA and $p75^{NTR}$ are present in both rafts and caveolae, and activation of the NGF pathway occurs in either subdomain.

Cav-1 also directly binds EGFR to negatively regulate its signaling and EGF-induced cell migration (Couet et al 1997; Zhang et al 2000a). Inactive EGFR is found in raft fractions and co-immunoprecipitates with Cav-1, whereas activated EGFR is lost in these factions. However, it has also been reported that activated EGFR is detected in detergent resistant membranes and immunoprecipitates with Cav-1 and PLCγ (Jang et al 2001; Mineo et al 1996), however, this difference in results may be attributable to the different approaches in lipid raft preparation. Based on these seemingly opposite results, it is proposed that inactivated EGFR is clustered in caveolae and upon activation taking place in this domain, the EGFR exits from it, thus enabling the downstream signaling. Cav-1 ***overexpression*** has also been observed to promote EGFR activation. Cav-1 ***knockdown*** decreases EGF signaling, resulting in increased tumor cell migration and proliferation (Agelaki et al 2009; Park & Han 2009). It is clear that the EGF-induced Grb2-Sos-Ras pathway leading to MAPK activation is negatively regulated by Cav-1, but activation of PI3K may require expression of Cav-1. Thus, depending on the pathway, Cav-1 can either positively or negatively regulate EGFR.

Receptor tyrosine kinase	Approach for lipid raft/caveolae preparation	Enrichment in lipid rafts/in Caveolae	Change upon agonist application	CO-IP with Cav-1	References
EGF	non-detergent	Caveolae	EGFR moves out of caveolae fraction	yes	(Mineo et al 1999)
	non-detergent	lipid rafts /Caveolae	In the absence of ligand, EGFR is located in raft; in the presence of ligand, EGFR transiently located in Caveolae	yes	(Matveev & Smart 2002)
FGF	1% Triton X-100	lipid rafts	N.D.	N.D.	(Bryant et al 2009)
IGF	non-detergent	lipid rafts /Caveolae	N.D.	yes	(Hong et al 2004; Huo et al 2003)
Insulin	0.1% Triton X-100	lipid rafts	IR moves into lipid raft/fraction (in the cells that do not express caveolin)	N.D.	(Gustavsson et al 1999)
	non-detergent	Caveolae	N.D.	yes	(Vainio et al 2002)
PDGF	non-detergent	Caveolae	PDGFR moves out of caveolae/ fraction	yes	(Liu et al 1997; Liu et al 1996)
	non-detergent	lipid rafts/ Caveolae	In the absence of ligand, PDGFR is located in raft; in the presence of ligand, PDGFR is located in Caveolae	yes	(Matveev & Smart 2002)
VEGF	non-detergent	Caveolae	N.D.	N.D.	(Cho et al 2004)
ErbB2	non-detergent	Caveolae	Relatively unchanged	N.D.	(Mineo et al 1999)
NGF	non-detergent	Lipid rafts/ Caveolae	N.D.	yes	(Bilderback et al 1997; Peiro et al 2000)
	0.5% Triton X-100		TrkA moves into lipid raft/ fraction	N.D.	(Limpert et al 2007)
BDNF	1% Triton X-100		TrkB moves into lipid raft/fraction	N.D.	(Suzuki et al 2004)
GDNF	1% Triton X-100		c-RET moves into lipid raft/fraction	N.D.	(Tansey et al 2000)
Tie2	1% Triton X-100		A minor fraction of Tie2R moves into lipid raft/ fraction	N.D.	(Katoh et al 2009)

Table 1 ***Ligand impact on raft localization of Receptor Tyrosine kinases.*** **CO-IP (CO-Immunoprecipitation);** ***N.D. (not described).***

5 TRKA TRAFFICKING AND INTERNALIZATION

5.1 RETROGRADE SIGNALING

In sensory neurons, the distance between neuronal terminations and neuron cell body could easily reach one meter. Therefore, the ligand receptor complexes recruit the cellular machinery responsible for endocytosis, trafficking and finally retrograde long distances, through dynein based microtubule mechanism, to the cell nuclei.

Retrograde endosomal signaling is a complex process comprising the internalization of ligand-receptor complexes in axon terminals, the sorting of the complexes into active signaling vesicles, translocation of the endosomes along the axonal microtubule network to cells bodies, and ultimately, destruction of the retrograde signaling complex (Zweifel et al 2005). Several mechanisms of retrograde transport have been proposed, among them, the "signaling-endosome model" is the most accepted. In this model, NGF-TrkA containing vesicles have characteristics of early endosomes, *i.e.* coupled to Rab5 and EEA1, and are associated with signaling components of the MAPK and PI3K pathways (Delcroix et al 2003). It is now appreciated that TrkA signaling endosomes mediated retrograde control of neuronal survival, growth, gene expression and synaptogenic signaling events (Howe & Mobley 2005; Pazyra-Murphy et al 2009; Sharma et al 2010). Recently, it has been reported that actin depolymerzation, which enables endosome maturation, is essential for initiation of NGF/TrkA endosome trafficking and activation of retrograde signaling to support neuronal survival (Harrington et al 2011).

5.2 TRKA INTERNALIZATION

The addition of NGF decreases the half-life of cell surface $gp140^{TrkA}$ from 100 to 35 min and leads to enhanced lysosomal degradation of the receptor (Jullien et al 2002). After ligand-induced activation, receptors usually are transfered into endocytic vesicles and are targeted for lysosomal or proteasomal degradation in PC12 cells. At present, the main endocytic routes for membrane receptors include at least 4 pathways. However, there's still some debate as to which, if any, endocytic route plays the dominant role.

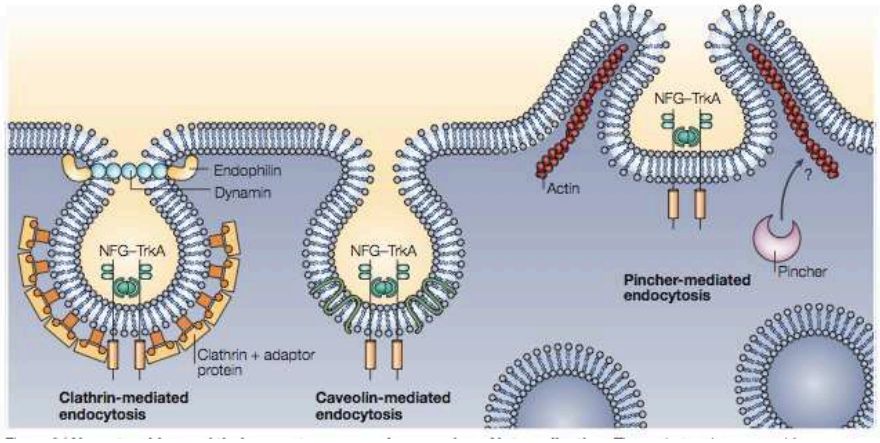

Figure 14. ***Models of NGF and TrkA internalization.*** NGF-TrkA complex, uses three distinct internalization pathways. There is considerable evidence that NGF-TrkA complexes are internalized through a clathrin/dynamin-dependent process. The observation that TrkA is localized in caveolin-like domain in PC12 and primary sympathetic cultures makes it a particularly interesting candidate. (Zweifel et al 2005)

5.2.1 CLATHRIN-MEDIATED ENDOCYTOSIS

Clathrin-mediated endocytosis (Fig. 14) is the most studied endocytic pathway of membrane receptors. NGF increases the association of clathrin with membranes, and induces the formation of complexes of activated TrkA, the clathrin heavy chain and the clathrin adaptor protein AP2 (Beattie et al 2000; Howe et al 2001). However, inhibition of clathrin-dependent internalization does not totally prevent TrkA internalization, suggesting there also exists other pathways. Dynamin, a GTPase involved in the scission of clathrin-coated vesicles from cell membranes is also required in this process.

5.2.2 CAVEOLAE-MEDIATED ENDOCYTOSIS

Caveolae (Fig. 14), one of the major structures mediating clathrin-independent endocytosis, have been well-characterized (Doherty & McMahon 2009; Nabi & Le 2003). To date, there is no direct proof of caveolae-mediated internalization of TrkA. However, it has been shown that NGF binding to both TrkA and $p75^{NTR}$ were enriched in caveolae. It was only in caveolae membrane preparations that activated TrkA was coimmunoprecipitated with phosphorylated Shc and PLCγ. These results suggest that NGF signaling might be initiated frpm these membranes (Huang et al 1999). Similar studies of EGFR, indeed have shown that migration of receptor out of

the caveolae fraction is necessary for their subsequent internalization and trafficking by clathrin-dependent endocytosis (Mineo et al 1999). Similar patterns could be expected for TrkA.

5.2.3 MACROPINOCYTOSIS AND MEMBRANE RUFFLING

Another TrkA internalization model involves the participation of a protein called Pincher (Fig. 14) (Philippidou et al 2011; Shao et al 2002). When overexpressed in PC12 cells and sympathetic neurons, the ligand-dependent endocytosis of TrkA at plasma membrane ruffles is enhanced (Shao et al 2002; Valdez et al 2005). Interestingly, Pincher is not localized to clathrin-coated invaginations. Finally, pincher coupled endosomes are associated with Erk5 and are not easily degraded by lysosomes.

5.3 MODULATION OF NGF SIGNALING BY TRKA INTERNALIZATION

Two different lines of investigation have provided evidence that cytolocalization of NGF receptor/NGF complexes determines the type of cellular response. It has been shown that a temperature-sensitive mutant of dynamin inhibits TrkA internalization, allows NGF-induced cell survival but not cell differentiation. (Zhang et al 2000b). Similar results were obtained when cells were exposed to cross-linked to beads NGF (MacInnis & Campenot 2002). Thus, activation of cell surface TrkA, which leads to transient MAPK signaling, is able to maintain cell survival via PI3K/AKT pathway, while internalization of the receptor, which leads to differentiation, is necessary for sustained MAPK activation. Moreover, it has been shown that Ras/MAPK is activated from the lipid raft at the cell surface, whereas the Rap/MAPK activation occurs at the level of the endosome (Wu et al 2001). From there derives the hypothesis that NGF signaling is different depending on the cytolocalization of the activated receptor complex. From there derives the hypothesis that NGF signaling is different depending on the cytolocalization of the activated receptor complex. Based on the above, one may ask how the NGF receptors are behaving with regard to their localization in lipid raft.

CONTEXT OF THESIS RESEARCH

Membrane domains are essential for the regulation of growth factor receptor mediated responses. NGF is the founding member of the neurotrophic factor family and has been shown to have multiple functions in the nervous system but also other tissues. Presence of NGF, its' receptors and perturbation of NGF signaling is observed in various cancers.

In PC12 cells, NGF receptors (TrkA and $p75^{NTR}$) are located in caveolae, a subset of lipid rafts containing caveolin proteins, that serves as organizing center for cellular signal transduction (Patel et al 2008). Interestingly, it has been shown that Caveolin-1 expression results in inhibition of NGF-induced differentiation in PC12 cells (Bilderback et al 1999). In breast cancers, NGF and TrkA are overexpressed, which results in increased growth and migration. Also, Cav-1 is found to be downregulated or mutated in breast cancer tissues.

The research presented herein was aimed at investigating the potential impact of Caveolins on NGF signaling and obtaining a better understanding of their relation to NGF receptor trafficking in the context of cancer and neuronal differentiation and function.

METHODOLOGY

The methods used in this work are described in the accompanying papers. Several major parts involving gradient centrifugation for purification of lipid rafts. Dorsal root ganglia (DRG) neuron dissociation, and Cav-1 silencing will be discussed in more detail.

1.1 SUCROSE DENSITY GRADIENT CENTRIFUGATION

There are some considerations when choosing the approach for lipid raft extraction. Although Triton X-100 has been commonly used, the detergent technique has several pitfalls, as it is prone to inconsistent results, and can solubilize proteins that are only weakly associated with lipid rafts (Allen et al 2007). Therefore, a non-detergent-based procedure for the isolation of lipid rafts based on pH and carbonate resistance was developed. Sodium carbonate, at high pH (pH 11.0), separates proteins that are firmly attached to membranes from those that are more peripherally associated. Rafts are isolated by centrifugation and fractions are harvested. In this work, we utilized the method of sodium carbonate extraction according to previously published studies (Song et al 1996a).

Sub-confluent PC12 cells growing in one 175-cm^2 flask were harvested and re-suspended in 1.5ml of 500mM sodium carbonate, pH 11.0. Homogenization was carried out using sonicator (thirty 3-s bursts at 3-s intervals). The homogenate was then adjusted to 45% sucrose by the addition of 1.5ml of 90% sucrose prepared in H_2O and placed at the bottom of an ultracentrifuge tube. A 5-35% discontinuous sucrose gradient was formed above (3ml of 5% sucrose/6ml 35% sucrose; both in 250mM Na_2CO_3, pH 11.0) and centrifuged at 39,000rpm for 14h in a SW41 rotor (BECKMAN). A light-scattering band confined to the 5-35% sucrose interface was observed after centrifugation. From the top of each gradient, 12 fractions (1ml for each fraction) were collected, and subjected to SDS-PAGE for western blot detection as shown in Fig. 15.

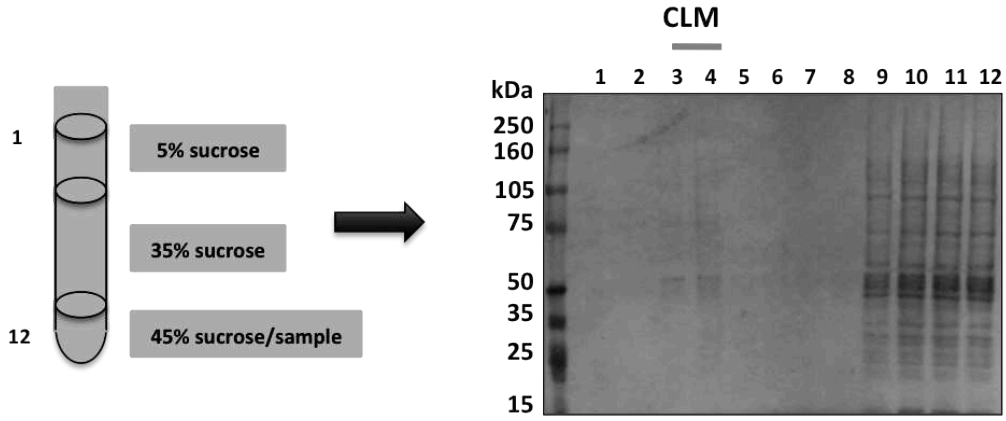

Figure 15. ***A schematic overview of the procedure for analysis of lipid rafts.*** Cells are homogenized, the discontinuous sucrose gradient is formed above the sample, and then subjected to ultra-centrifugation. Fractions are taken from the top of the gradient and subjected to SDS-PAGE.

1.2 DRG NEURON DISSECTION AND ELECTROPORATION

Dorsal root ganglion (DRG) is a nodule on a dorsal root that contains cell bodies of primary sensory neurons in afferent spinal nerves. It was widely utilized as a model system in which to examine the mechanisms of neurotrophins as many DRG neurons express neurotrophic factor receptors and require specific neurotrophins for survival and differentiation (Carroll et al 1992; Riccio et al 1997; White et al 1996). It was reported that, at embryonic day (E) 13 and 15, the proportion of DRG neurons expressing TrkA increases to 80% (Farinas et al 1998; White et al 1996). In our work, cultures of E14.5 DRG neurons were prepared to study effect of Cav-1 on the response to NGF in a primary system (Fig. 16). Please refer to material and method of Manuscript 1 for detailed cell culture approach.

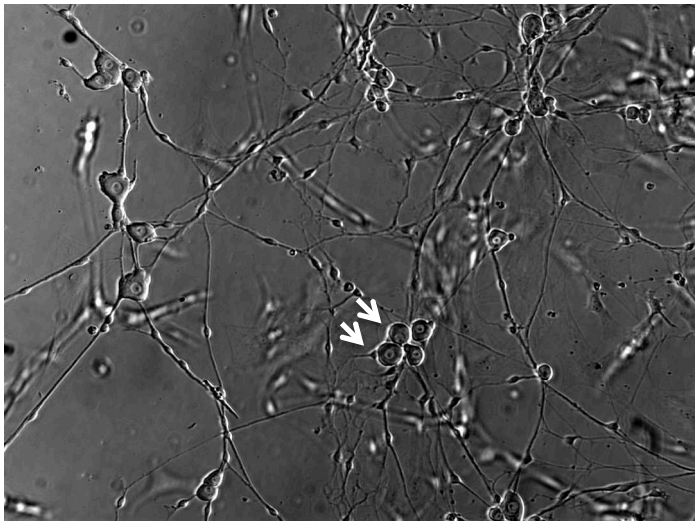

Figure 16. ***Representative image of DRG culture.*** DRG neurons were dissected from embryonic day 14.5 mouse and maintained for 48 hours with 50 ng/ml NGF. Arrows indicates bodies of DRG neurons.

1.3 TRANSFECTION OF PC12 CELLS WITH siRNA AGAINST CAV-1

Small interfering RNAs (siRNAs) are double-stranded RNA molecules that are able to silence a single gene. In recent years, it has become a useful tool in the studies of protein function (Scherer & Rossi 2003). The siRNA (usually 19-21 base pairs), after being introduced into the cell, can bind to the target mRNA and then be recognized by the RNA induced silencing complex (RISC) complex, leading to targeted degradation. In this work, we used commercialized siRNA targeting two parts of Cav-1 mRNA (Santa Cruz, sc-106996). PC12 cells were transfected with 60 nM siRNA (Cav-1 targeted or scrambled siRNA) and were allowed to recover for 48 h before assay.

RESULTS

1. MANUSCRIPT1

Highly efficient method for gene delivery in mouse dorsal root ganglia neurons

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Keywords : DRG neuron, Neon transfection, electroporation efficacy

Characters

Abstract

The development of gene transfection technologies has greatly advanced our understanding of life sciences. Electroporation has become as an effective and commonly used method for introducing DNA into neurons and in intact brain tissue. In our study, the Neon electroporation system was first refined to transfect genes into embryonic mouse Day 13.5 to 16 dorsal root ganglia neurons (DRGn). By optimizing the culture condition and parameters including voltage and duration for this specific electroporation, surprisingly high efficiency and low toxicity for targeting DNA into DRGn were achieved. Moreover, we are able to manipulate on rather small amount of cells compared with other tradition electroporation methods. This approach is expected to greatly facilitate the study of gene function in DRG cultures.

Introduction

Culture of primary cells has been extensively used to study neuronal survival, signal transduction, development, and neurite outgrowth. Gene transfer, through both viral and non-viral methods, has become a powerful technique to assess the effects of expression of selected genes. Adenovirus, herpes-simplex virus (HSV), lentivirus, and adeno-associated virus (AAV) have been reported to deliver transgenes both *in vivo* and *in vitro* (Chattopadhyay et al., 2005; Glatzel et al., 2000; Towne et al., 2009; Yu et al., 2011). While effective, these approaches are time-consuming, labour-intensive and carry some potential biohazard risk. Non-viral methods, which mainly include microinjection of DNA, biolistic, sonoporation, lipid-, chemical-based transfer and electroporation, offer faster and safer means for gene delivery (Table 1). Electroporation, particularly because of its ease of use, combined with efficient and precise targeting in space and time, has become an effective method for introducing DNA into neurons in culture, slices and in intact neural tissue of Xenopus, chick and mouse (Falk et al., 2007; Kawabata et al., 2004; Saijilafu et al., 2011). Dorsal root ganglia (DRG) derived sensory neurons that are selectively sensitive to NGF, BDNF and NT3, provide an excellent model in which to study the mechanisms of axonal regeneration, neurotrophin signaling, peripheral nervous system development and peripheral neuron disease (Melli and Hoke, 2009; Newbern et al., 2011). The previous electroporation methods of dissociated DRG neurons always require large amount of cells, for instance, by using the regular Amaxa system, one of the best known and commonly performed transfection methods now in the labs, requires 1×10^6 DRG cells for each electroporation (Chadborn et al., 2006). This is a major

obstacle when working with embryos. Additionally, the low survival and transfection rate with the standard Amaxa system are of major concern. By defining the Neon system on dissociated DRG neurons, which is the first reported protocol by our study, allows us to work with young embryos whose cell number is limiting and furthermore, to perform 6 electroporation conditions from the same batch of dissociated cells thereby facilitating comparisons.

Here, we describe an optimized electroporation procedure to introduce DNA into mouse embryonic DRG neurons, in a rapid and highly effective manner with efficiencies comparable to those reported for viral infection, while maintaining high viability and transgene expression.

Materials & Methods

Reagents

Poly-L-Lysine (S1399, Sigma), Laminin (L2020, Sigma), calcium/magnesium free HBSS (Life technologies), Trypsin (T5266, Sigma, pH=7.2), DNAse Ⅰ (DN25, Sigma), Nutrient Mixture F-12 (Life technologies), Fetal calf serum (FCS) (Life technologies), penicillin/streptomycin (P/S) (15140, Life technologies), Nerve growth factor (NGF) (mouse 2.5S; N-100, Alomone Labs), Cytosine-arabinoside "Cyara" (C1768, Sigma), anti- β Ⅲ tubulin antibody (Santa Cruz, SC-53140), secondary antibodies conjugated to Alexa 488 (Jackson ImmunoResearch, 115-485-003), Vectashield (Vector, H-1000).

Preparation of coated coverslips

Coverslips were dipped in 96% ethanol for 30min, then washed with distilled water and air dried. Poly-L-lysine (50ug/ml) was spread equally over the surface of the coverslip, followed by incubation for 3h at room temperature or overnight at 4°C. They were then rinsed with distilled water then 50ul Laminin (10ug/ul) in HBSS was applied on a coverslip. A second coverslip was placed, coated side down, on the first one, then allowed to sit overnight at 37°C. After two washes with HBSS, the coverslips were placed one per well into a 24-well tissue culture plate. Wells were then filled with 500ul of culture medium without antibiotics and plates pre-incubated in a humidified 37°C/5% CO2 incubator prior to use.

Isolation and dissociation of Dorsal Root Ganglia from mouse embryons

We used a modified protocol, adapted from a previously described procedure for isolation and culture of dorsal root ganglia neuons (Castellani et al., 2004) . Time pregnant mice at E14.5 were sacrificed by cervical dislocation and embryos were removed from the uterus kept on ice. Heads were removed from the embryos in ice cold HBSS (Ca^{++}/Mg^{++} free), pin down the embryo on the silicon coated dish with dorsal side up in cold HBSS with 2% glucose, cut the skin of the embryo along the dorsal midline, cut the cartilage along the midline and open the spinal cord with forceps tips, slide forceps along the spinal cord to take it out, remove the meninge to visualize the DRG along the vertebral canal, take them out one by one by forceps into 300ul cold HBSS in 1.5ml ependorff tube kept on ice. Change buffer for every embryos.

Dissected DRGs were digested by adding 30ul of trypsin (25mg/ml) with final concentration at 2.5 mg/mL in Ca^{++}/Mg^{++}-free HBSS and incubated for 10 min at 37°C. Followed by adding 10ul of DNAse Ⅰ (0.1mg/ml) with final concentration at 0.033 mg/ml in to cells, and returned to 37°C for an additional 10 min's incubation, gently shaking the tube every 10 min to equally distribute the DRGs. Neutralization was performed by adding 30ul of serum and 500ul of culture medium (F12/10% FBS), trypsin-containing medium was removed, mechanical trituration was carried out using a fire polished glass Pasteur pipette by triturating approximately 15 times in 200ul F12+10%FCS without antibiotics and counted to evaluate the number of condition (6-9 × 10^4 cells per condition) before re-suspended into electroporation buffer.

In order to maintain good survival rate of the cells, it is essential to keep the dissection within one hour after sacrifice of the mother

Transfection

After counting the cells, add 1.3ml Ca^{++}/Mg^{++}-free HBSS and centrifuge for 3min at 900 rcf, resuspend the cells with half of the electroporation buffer R (Invitrogen) that would be needed for the number of condition estimated, count the cells again before adjusting the amount to 6-9 × 10^4 cells per condition with electroporation buffer R (10ul per electroporation). 10ul of cells were gently pipetted into each microfuge tube , which was then mixed with the appropriate endofree plasmids, *i.e.* pEGFP (Lonza) (0.5ug) and pCDNA3.1-Cav-1 fused to RFP (pCDNA3.1-Cav-1-RFP)(gift from R.E Pagano, Addgene Plasmid 14434) (Sharma et al., 2004) (Sharma et al., 2004) or pCDNA3.1-RFP (modified by removing the Cav1 coding sequence from the

addgene plasmid 14434) (1 or 2ug). Cells were transfected using the Neon® transfection system. Electrical parameters *i.e.* voltage and pulse length, were varied in order to find the optimal conditions. Cells should not be kept in the electroporation buffer for longer than 10 min. After electroporation, cells were split and immediately transferred into two wells with the poly-L-Lysine- and laminin-coated coverslips, containing pre-warmed medium, and gently rocked to ensure even distribution of the cells which were then left to settle for 1 h. NGF (50ng/ml) and antibiotics (1% final) were added to the medium and 12 h later, Cyara (10uM) was added to remove contaminating glial cells.

Imaging and Data analysis

Control and caveolin-1 cultures from the same dissociation batch were fixed at different times points in 2% paraformaldehyde overnight at 4°C and neurite growth was analyzed. (Zeiss AxioImager, Z1 upright). 4 montages of 9 images were collected in different regions of the coverslips to minimize bias due to different cell density. Number of transfected neurons per image was calculated and quantified from the GFP and RFP channel in ImageJ. For staining of neurons, cells were fixed in 2% paraformaldehyde overnight at 4°C and permeabilized for 5 min with 0.1% Triton X-100 in PBS. After washing with PBS, cells were blocked for 1 hour at room temperature with 10% normal goat serum/1% BSA/0.1% Triton in PBS and then incubated with anti-β III tubulin antibody overnight 4°C, and visualized with secondary antibodies conjugated to Alexa 488. Coverslips were mounted in

Vectashield. Images were acquired using Olympus FluoView FV10i. Data acquisition was performed with Olympus FluoView software.

Statistics

Statistical significance was tested with unpaired Student's two-tailed t-test.

Results

Optimization of transfection conditions for dissociated DRG neurons

To determine the protocol which would produce the highest possible transfection efficiency and greatest survival, the DRG neurons were electroporated with 1ug pEGFP, with electroporation conditions optimized for voltage, duration and number of pulses. The three pulse parameters listed in the table were initially tested in this work as they have been successfully applied to hippocampal, cortical and neuronal stem cells from embryos with similar stage. After 24 hour, cell viability and transfection efficiency were assessed by visual inspection. As shown in Table 2, electroporation with two pulses at 1300V with a pulse width of 20 ms, produced high transfection efficiencies and cell viability. Therefore, this setting was utilized for subsequent experiments.

The amount of pCDNA3.1-Cav-1-RFP or pCDNA3.1-RFP DNA was then varied between 1ug, 2ug for which the volume was normalized to 10% of the total reaction volume in distilled water; 0.5ug pEGFP was co-transfected for neurite tracing. 24 h and 48 h post transfection, the cells were fixed and processed for microscopic analysis. As shown in Table 2, over 50% of the neurons were positive for GFP, while

169 increaseing RFP or Cav1-RFP plasmid concentration did not result in significantly
170 higher transfection efficiency. After 48 hours, a slight increase in GFP- and RFP-
171 positive neurons was observed, indicating the survival of most transfected cells.
172 Essentially all of the cells carrying GFP were also transfected with RFP or Cav1-RFP
173 (Fig. 1A, B). Both EGFP and RFP were sufficiently visualized over the entire length of
174 the axons, whereas Cav1-RPF was mainly expressed in the cell body (Fig. 2)

175 Other parameters, such as the cell density and plasmid purity are also important
176 issues. We have tried different amount of cells raning from 2×10^4 to 9×10^4 cells, it
177 turned that lower cell density caused decreased transfect effency. Also, avoiding
178 multiple centirfugations during the procedure can help to minimize the loss of the
179 cells.

180 In the case of plasmid preparation, we strongly recommend the use of endo-free
181 methods as they result in high cell viability and transfection efficiency.

182

183 ***The survival rate of DRG neurons after electroporation***

184 Since high electroporation efficiencies are often achieved at the expense of cell
185 survival, we evaluated survival subsequent to Neon electroporation. Under phase-
186 contrast, healthy neuronal cells are easily distinguished as their cell bodies are round
187 and phase bright and display extensive neurite outgrowth, as shown in the figure 3.
188 Electroporation of cells does not alter differentiation and outgrowth of DRG
189 neurons.(Fig. 3). 24 h and 48 h post electroporation, neuronal survival rates were

64.6±5.6% (40 images representative of 692 non transfected neurons and 44 images representative of 507 GFP/RFP expressing neurons) and 63.1±13.8% (29 images representative of 517 non transfected neurons and 31 images representative of 360 GFP/RFP expressing neurons) of naïve, untransfected controls (Fig. 4).

Discussion

Electroporation, because of its ease of use, reproducibility, high efficiency, has been developed and wildly used for introducing various molecules into cells. In this study, we describe an optimized method for gene delivery into embryonic DRG neurons, with high transfection efficiency of ≥ 50%, and low cytotoxicity.

As neurons present a particular challenge to successful gene transfer, adenovirus (Ad), adno-associated virus (AAV) (Glatzel et al., 2000), herpes simplex virus (HSV) (Storey et al., 2002), and lentivirus (Yu et al., 2011) have been applied in DRG cells. Although they effectively deliver genes into DRG cells, several viruses can affect sensory neuron physiology (Farkas et al., 1994; Fukuda and Kurata, 1981; Maehlen et al., 1991) and thus limit their use in some experiments.

The Neon® transfection system resulted in a mean transfection efficiency over 50% in DRG neurons, which to our knowledge, is the highest reported for a nonviral transfection method, and can be compatible with viral infection. Usually, 5×10^6 cells are required for each electroporation to achieve reasonable number of transfectants and good cell survival. However, using the approach presented herein 6-9 $\times 10^4$ cells were sufficient for each transfection. Thus, with the cells obtained, for

example, from one E14.5 embryo, four electroporations can be performed, which largely expands the experimental conditions, while reducing the total number of embryos needed for certain types of experiments. The other main advantage of our method is that, when electroporated with mixed plasmids comprising EGFP and RFP at a ratio of 1:2 to 1:4, we found that almost all of the neurons expressed both of these fluorescent proteins. As EGFP was sufficiently detectable the entire length of the axons, one can easily analyze the effect of transfected genes with a GFP-based imaging system. By combining other plasmid constructs, such as TET-ON/OFF system (Shaikh and Nicholson, 2006) and using cell type-specific promoters (Boulaire et al., 2009; Gulick and Robbins, 2009; Hitoshi et al., 1999), it should be possible to express transgenes in a spatiotemporal manner. In summary, the transfection protocol presented herein for DRG neurons, employed the Neon® transfection system and effectively enables heterologous gene expression in DRG neurons.

While viral transduction / infection has yielded significant advances, it remains more labor intensive than electroporation of purified plasmids. Furthermore, there are safety issues regarding the types of genes that can be introduced under standard laboratory conditions *e.g.* oncogenes or genes that inhibit apoptosis or autophagy. Clearly, implementation of the protocol proposed herein alleviates both issues, enabling investigation of the impact of such genes or modulators thereof on DRG survival, differentiation and function.

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334
335

NON-VIRUAL METHOD	MODEL		MAXIMAL EFFICIENCY		CELL SURVIVAL	REF
	precursors	postmitotic cells	DNA plasmids	RNAi		
Ca^{2+}-phosphate/DNA coprecipitation			typically between 1-5%, rarely reaches 30%	efficient	good	(Goetze et al., 2004) (Dahm et al., 2008)
Lipofection	cell lines	CGNs	up to 85%, but vary between cell lines	efficient, up to 83%	good	(Butcher et al., 2009) (Jacobsen et al., 2006)
		Rat hippocampal neurons	typically between 1-5%, rarely reaches 30%			(Dalby et al., 2004) (Tonges et al., 2006)
Biolistics			typically around 2%, rarely reaches 10% in clutured neurons, up to 34% in slice culture			(Karra and Dahm, 2010)
		DRGn/SCGn	5-20%			(Dib-Hajj et al., 2009)
Sonoporation		DRGn	31%		35%	(Lin et al., 2010)
Nucleofection	NSC		77%	efficient	37%	(Bertram et al., 2012)
	NPCs		50-60%			(Dieterlen et al., 2009)
Electroporation		DRGn	39-42%	efficient		(McCall et al., 2012)
		DRGn/SCGn	5-20%			(Dib-Hajj et al., 2009)

*NPCs: neural progenitor cells;CGNs: cerebellar granule neurons; SCGn: superior cervical ganglion neurons

336 **Table 1** ***Comparison of non-viral methods commonly used to transfect mammalian neurons.***

337

Conditions	Survival	Efficiency
1. 1500V 20ms 1 pulses	++	+
2. 1600V 10ms 3 pulses	+	++
3. 1300V 20ms 2 pulses	++	++

338

339 **Table 2** ***Optimization of electroporation.*** DRG neurons were electroporated with
340 pEGFP (1ug, Lonza). Electroporation conditions optimized for voltage, duration and
341 number of pulses. After 24 hr, cell viability and transfection efficiency were assessed
342 by visual inspection.

Time point	Construct	Percentage of GFP(+) neurons/total neurons	Percentage of RFP(+) neurons/total neurons	Percentage of Cav1-RFP(+) neurons/total neurons
24hr	0.5ug GFP/1ug RFP	58.1%±12.8%	57.9%±12.5%	
	0.5ug GFP/1ug Cav1-RFP	51.5%±7.0%		53.9%±6.0%
	0.5ug GFP/2ug RFP	55.0%±10.8%	55.2%±10.7%	
	0.5ug GFP/2ug Cav1-RFP	50.5±10.8%		52.6%±12.4%
48hr	0.5ug GFP/1ug RFP	62.6%±8.6%	62.6%±8.6%	
	0.5ug GFP/1ug Cav1-RFP	67%±12.8%		80.3%±9.5%
	0.5ug GFP/2ug RFP	68.6%±11.3%	68.0%±10.7%	
	0.5ug GFP/2ug Cav1-RFP	60.1%±18.1%		64.5%±16.7%

Table 3 ***Percentage of RFP and Cav1-RFP positive cells with different expression constructs.*** Cells were transfected with pEGFP and each expression construct for RFP at two different concentrations and the number of transfected cells was determined by visual inspection. Results were obtained from three independent experiments. Data represent Mean ± SEM.

348

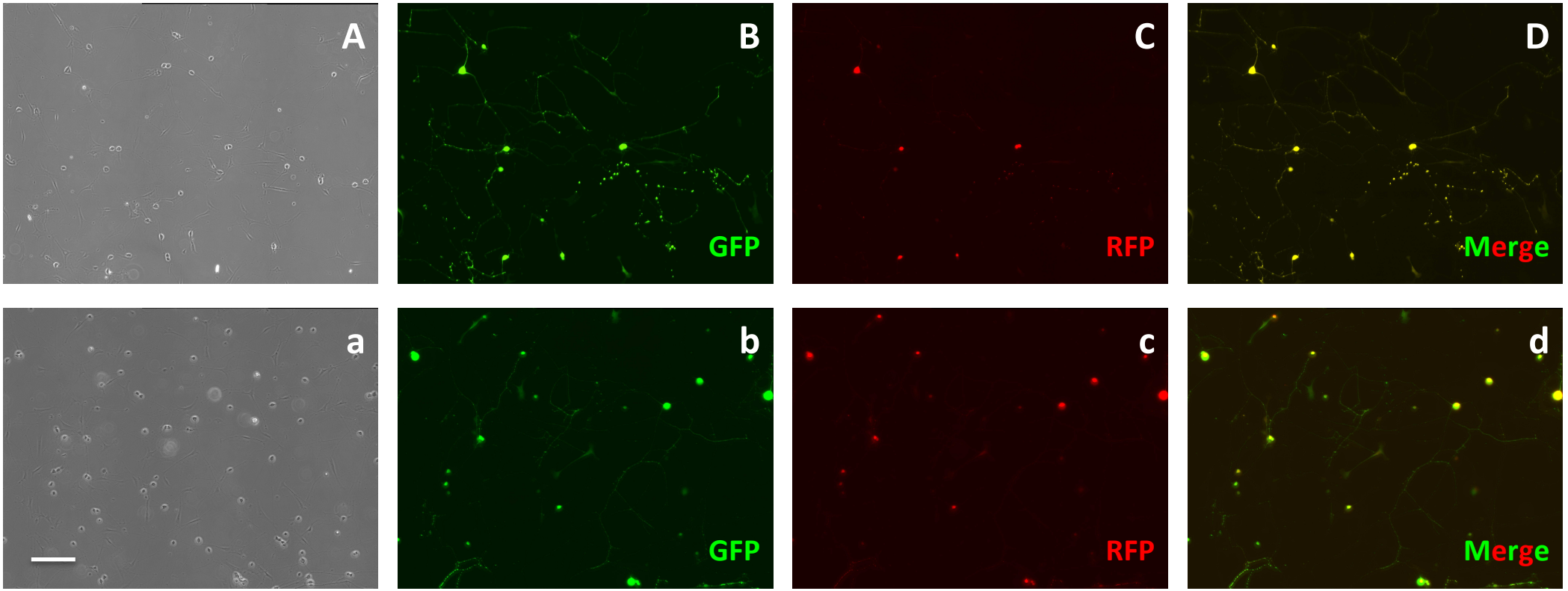

Figure 1A ***Expression of fluorescent proteins in DRG neurons.*** Neon transfection leads to efficient electroporation of E14.5 DRG neurons. Neurons were transfected with 1ug or 2ug RFP(C,c) combined with 0.5ug EGFP (B,b). 24 hr after electroporation, cells were fixed and observed under the microscope. Scale Bars represent 200µm.

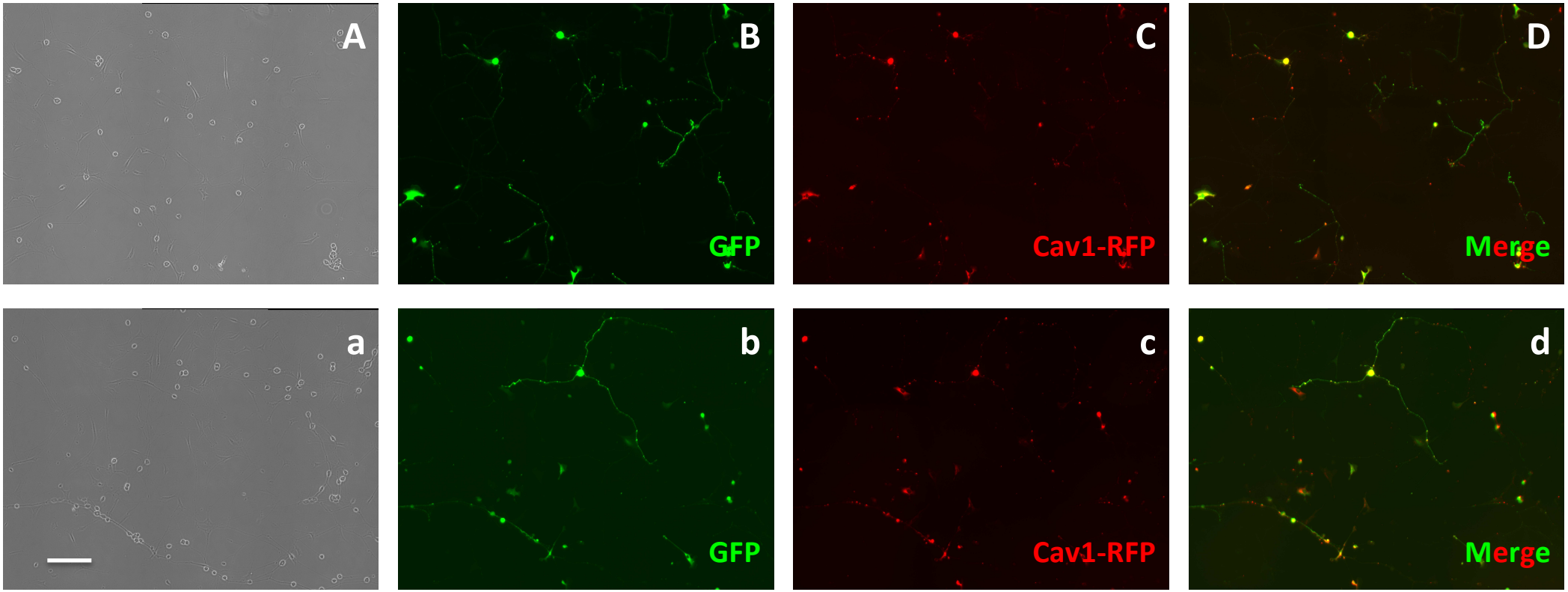

Figure 1B ***Expression of fluorescent proteins in DRG neurons.*** Neon transfection leads to efficient electroporation of E14.5 DRG neurons. Neurons were transfected with 1ug or 2ug Cav1-RFP (C,c) combined with 0.5ug eGFP (B,b). 24 hr after electroporation, cells were fixed and observed under the microscope. Scale Bars represent 200µm.

351

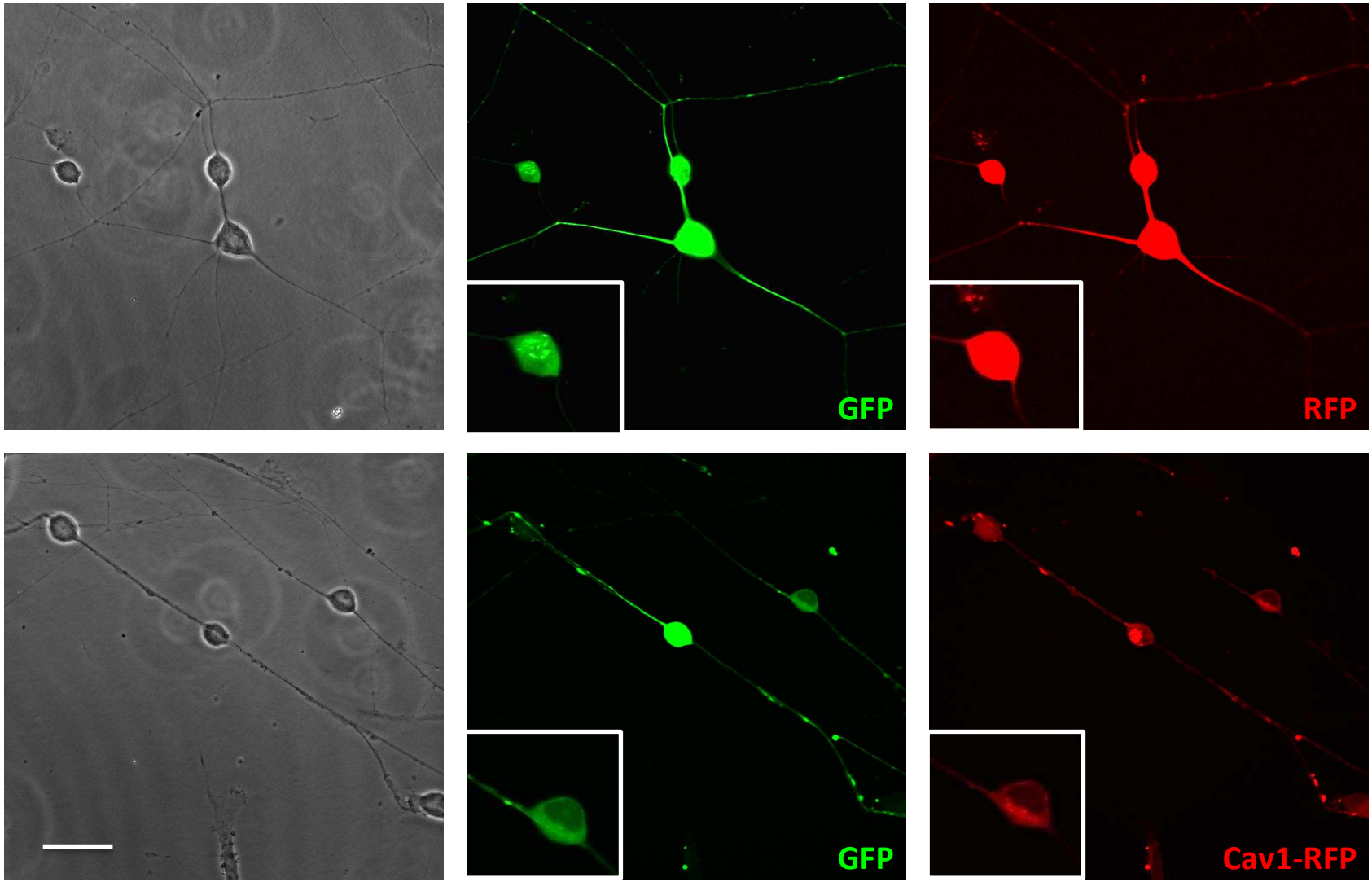

Figure 2 ***Expression of fluorescent proteins in DRG neurons.*** Neurons were transfected with 1ug RFP or Cav1-RFP combined with 0.5ug eGFP (F,f). 24 hr after electroporation, cells were fixed and observed under the microscope. White boxes show zoomed cells. Scale Bars represent 50µm.

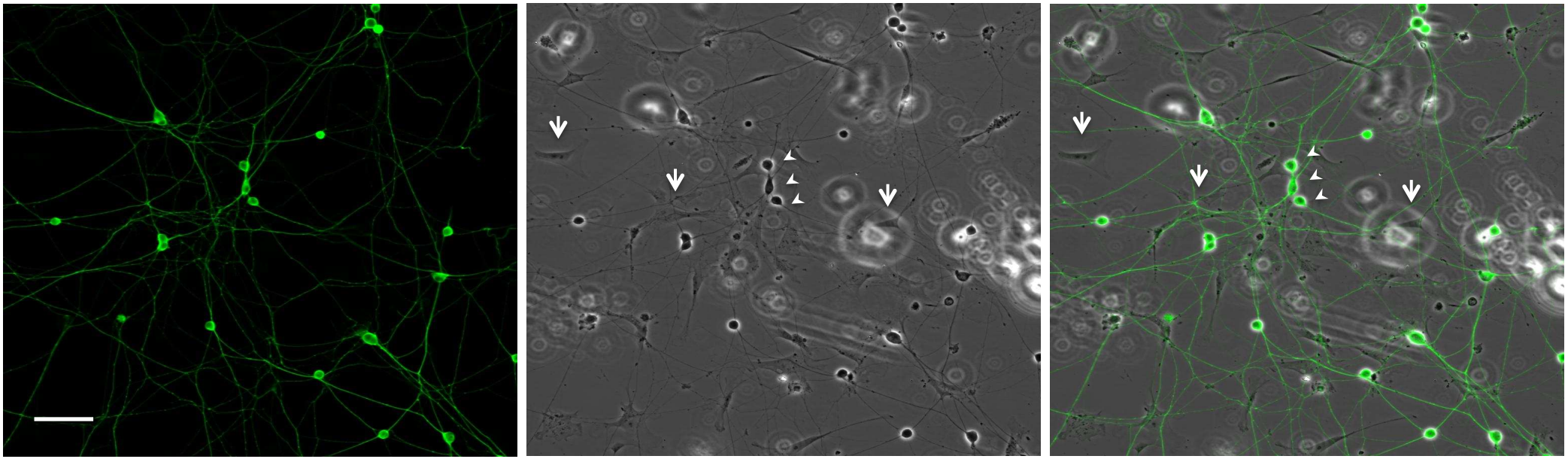

Figure 3 ***Representative DRG neurons in culture.*** DRG neurons were dissected and maintained in culture. 24 h later, cells were fixed and labeled with anti-*βIII* tubulin antibody. Arrows and arrowheads point to glial and neuronal cells. Scale Bars represent 100µm.

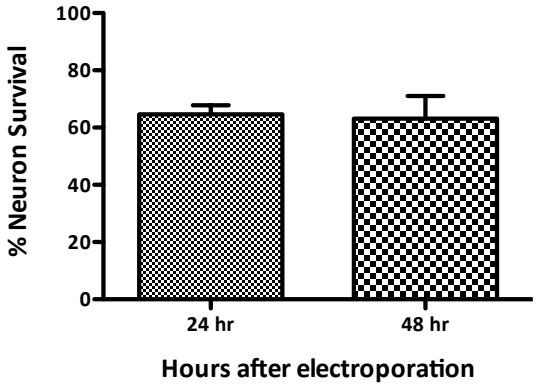

Figure 4 ***The viability of DRG neurons after electroporation.*** Neurons were transfected with 1ug RFP combined with 0.5ug EGFP. 24h and 48h after electroporation, cells were fixed and neuron survival was calculated compared to naïve, untransfected neurons. Results were obtained from three independent experiments. Data represent mean ± SEM.

Sensory neurons from Dorsal Root Ganglia (DRG) express different set of Trk receptors. At embryonic day 14.5, nearly 80% of DRG neurons express TrkA and responsive to NGF exposure, that makes them a very good model for studying NGF signaling. Gene transfer, has become a powerful technique for biological research. However, low transfection efficiency still remains an obstacle in some primary cells.
By optimizing Neon transfection system, we succeeded in transfecting DRG neurons at high efficiency (mean transfection efficiency over 50%) and with little loss of cell viability (mean survival rate over 60%). This work, as has been described in Manuscript 1, gives us the opportunity to evaluate the impact of Caveolin-1 overexpression in the primary DRG cultures.

In the following manuscript, we will further investigate the role of caveolins in NGF signaling.

2. MANUSCRIPT2

Nerve Growth Factor Signaling from Membrane Microdomains to Nucleus : Differential Regulation by Caveolins

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Running title : Modulation of NGF Signaling by Cav-1 and Cav-2.

Keywords : Lipid rafts, membrane microdomains, Growth factor signalling, NGF, Caveolin, Trk, trafficking, PC12, Dorsal root ganglion neurons

40424 Characters

Abstract

Membrane microdomains have emerged as essential functional modules of the cell, critical for the regulation of growth factor receptor-mediated responses. Herein we describe the dichotomy between caveolin-1 and caveolin-2, structural and regulatory components of microdomains, in modulating proliferation and differentiation. Caveolin-2 *potentiates* while caveolin-1 *inhibits* Nerve Growth Factor (NGF) signaling and subsequent cell differentiation. Caveolin-2 does not appear to impair NGF receptor trafficking but elicits prolonged and stronger activation of MAPK, Rsk2 and CREB. In contrast, caveolin-1 does not alter *initiation* of the NGF signaling pathway activation, rather, it acts at least in part, by *sequestering* the cognate receptors, TrkA and $p75^{NTR}$, at the plasma membrane, together with the phosphorylated form of the downstream effector Rsk2, which ultimately prevents CREB phosphorylation. The non-phosphorylatable caveolin-1 Serine 80 mutant (S80V), no longer inhibits TrkA trafficking or CREB phosphorylation. The results presented herein underline the role of caveolin and receptor signalling complex interplay in the context of tumorigenesis and neuronal development.

Introduction

Membrane microdomains, rich in cholesterol, sphingomyelins, and glycolipids, called lipid rafts, are functional modules of the cell membrane that play a key role in regulating cellular responses to environmental stimuli – *e.g.* the presence or absence of growth factors or infectious agents – through their capacity to attract or deploy select cellular components (Simons and Toomre, 2000). Some domains are characterized by the presence of specific structural and regulatory proteins called caveolins which, when present in appropriate amounts and with specific post-translational modification, can form caveolae (de Laurentiis et al., 2007; Parton and Simons, 2007). The caveolin family consists of two ubiquitously-expressed genes, caveolin-1 (Cav-1) and Cav-2, and one specifically expressed in smooth and skeletal muscles, Cav-3 (Tang et al., 1996; Way and Parton, 1995). While expression of Cav-1 or Cav-3 is sufficient for the formation of caveolae, expression of Cav-2 is not (Fra et al., 1995; Lipardi et al., 1998; Sowa et al., 2008). Cav-1, which has been particularly well studied, interacts with numerous proteins involved in signal transduction, which could result in compartmentalization of signaling molecules and their maintenance in an inactive conformation (de Laurentiis et al., 2007; Okamoto, 1998). Once internalized, caveolae domains fuse into structures called “Caveosomes” which offer a unique environment or to early endosome membranes, offering therein a stable structure from which certain components are released while others are retained (Pelkmans et al., 2004).

Initially difficult to detect *e.g.* (Wu et al., 1997), caveolins have been observed in brain and several neuronal cell types, playing key roles in neuronal signalling, underlined by correlative and functional observation that certain neurodegenerative diseases may have links to

microdomain components including Cav-1 (Gaudreault et al., 2004; Head et al., 2010; Stern and Mermelstein, 2010). Similarly, mutations, loss of or over expression of Cav-1 have been associated with numerous cancers (Burgermeister et al., 2008; Galbiati et al., 1998a; Sainz-Jaspeado et al., 2011; Williams and Lisanti, 2005) resulting in it being considered as a bio-marker and potential therapeutic target (Patani et al., 2012; Sotgia et al., 2011; Tahir et al., 2008).

Nerve Growth Factor (NGF) is the founding member of a family of polypeptide neurotrophic factors. Originally named because of their ability to sustain neuronal survival, neurotrophins have been shown to have multiple functions in the nervous system but also other tissues (Bibel and Barde, 2000). NGF, for example, regulates proliferation, migration and/or differentiation of fibroblasts, endothelial cells and a subset of immune cells (Auffray et al., 1996; Chevalier et al., 1994; Kohyama et al., 2002; Palazzo et al.; Park et al., 2007). As result, it controls angiogenesis and inflammation under both physiological and pathological conditions (Frossard et al., 2004; Hoyle, 2003; Nico et al., 2008). Perturbation of NGF signaling is observed in various cancers including breast, prostate and lung cancers (Dolle et al., 2004; Papatsoris et al., 2007; Perez-Pinera et al., 2007; Ricci et al., 2001). In neurons, NGF regulates subtype specification, survival and axon growth and guidance during development (Campenot, 1994; Dontchev and Letourneau, 2002; Ernsberger, 2009; Kuruvilla et al., 2004; Patel et al., 2000; Tucker et al., 2001; Zhou et al., 2004). NGF also contributes to myelination and to the modulation of neuronal excitability, plasticity and regeneration (Chan et al., 2004; Ernsberger, 2009; Lykissas et al., 2007). Deregulation of NGF signaling participates in neurodegeneration during Alzheimer disease (Capsoni and Cattaneo, 2006; Hennigan et al., 2007; Woolf, 1996). Moreover, studies of NGF signaling have provided important conceptual insights on how growth factor signaling could be regulated. Notably, regarding the concept of

signaling endosomes which provided important indications on how certain stimuli can be propagated in the cell (Sadowski et al., 2009).

The PC12 rat pheochromocytoma cells, which stop proliferating and form long neurites in presence of NGF, have been a model of choice to study NGF signaling (Greene and Tischler, 1976). As a result, studies on PC12 cells have provided many seminal contributions to our current understanding of the NGF signaling pathways. Of particular import, many of the signaling molecules and the interactions initially described in PC12 cells as part of these pathways, are also relevant for neurons.

NGF signaling pathways are initiated by its binding to membrane receptors: a receptor common to all neurotrophins, p75 Neurotrophin Receptor ($p75^{NTR}$), member of the tumor necrosis factor receptor superfamily (Chao et al., 1986) and a receptor more specific to NGF, with a tyrosine kinase activity, Tropomyosine receptor kinase A (TrkA) (Klein et al., 1991) (Martin-Zanca et al., 1989). NGF binding to TrkA triggers kinase activation and trans-autophosphorylation (Reichardt, 2006). Internalization of the activated receptors terminates this signaling from the membrane and initiates signaling from endosomes. TrkA internalized into endosomal compartments would promote a sustained, Rap1-dependent MAPK activation necessary for cell differentiation (Meakin et al., 1999; Wu et al., 2001; York et al., 2000; Zhang et al., 2000). According to this sequential model of NGF signaling, molecules that regulate the recruitment of the activated receptors in specific sub-domains of the plasma membrane and receptor internalization are likely to provide important insight in the ways NGF signaling is spatio-temporally controlled.

121 At the cell membrane, TrkA and $p75^{NTR}$ are concentrated in different subtypes of lipid rafts
122 including caveolae (Huang et al., 1999; Peiro et al., 2000). In addition to its localization in
123 caveolae, the NGF-receptor TrkA was shown to interact with Cav-1 (Bilderback et al., 1999).
124 Consistently, perturbation of caveolae and modification of Cav-1 expression, alters NGF
125 signaling in different cell types (Bilderback et al., 1999; Peiro et al., 2000; Schmitz et al.). Over-
126 expression of Cav-1 in PC12 cells strongly inhibits their ability grow neurites in response to
127 NGF (Bilderback et al., 1999). Furthermore, its over-expression impairs re-growth of severed
128 neurites from differentiated PC12 (Gaudreault et al., 2005). How this is relevant to neurons
129 remains to be determined since this effect in PC12 cells correlated with dynamic change of
130 expression of caveolins in response to NGF (Galbiati et al., 1998b). More generally, how
131 caveolins impact NGF signaling from TrkA remains to be clarified. The effects of Cav-1
132 overexpression on TrkA trafficking and downstream signaling have not yet been
133 assessed. Finally, whether this effect is specific to Cav-1 is not known. This is particularly
134 interesting considering the different role of Cav-1 and Cav-2 in Caveolae formation and their
135 differential regulation during NGF-induced PC12 differentiation (Galbiati et al., 1998b).
136
137 The present article offers more insight into these issues while investigating the effect of the
138 much less studied Cav-2. We demonstrate that Cav-1 overexpression similarly inhibits neurite
139 growth from neurons of the dorsal root ganglia. We find differential modulation of NGF
140 signaling events by Cav-1 and Cav-2. Cav-2 *potentiates* NGF signaling and the resulting
141 physiological response. By contrast, Cav-1 *inhibits* NGF signaling without, however, impairing
142 transient MAPK pathway activation, but rather, by sequestering NGF receptors and
143 downstream effector, phosphorylated-Rsk2, at the plasma membrane, resulting in the
144 prevention of the phosphorylation of CREB. Some insight into the mechanism of action of Cav-
145 1 is afforded by the study of the non-phosphorylatable Cav-1 mutant (S80V), which no longer

146 inhibits TrkA trafficking or CREB phosphorylation. In summary, our results contribute to
147 understanding of the impact of TrkA and caveolin interplay in tumorigenesis and neuronal
148 differentiation.
149

Results

Caveolin-1 overexpression impairs neurite growth of embryonic sensory neurons

Sensory neurons from dorsal root ganglia (DRG) expressed different set of Trk receptors and largely contributed to establish the role of neurotrophins (Ernsberger, 2009). NGF was shown to regulate morphology and growth of DRG axons *in vitro* (Ben-Zvi et al., 2008; Lentz et al., 1999; Liu et al., 2002). We chose to perform the overexpression in embryonic DRG neurons from stage E14.5 cultured on laminin substratum. At this stage, TrkA is expressed in nearly 80% of the DRG neurons and NGF was shown to enhance laminin-induced outgrowth (Liu et al., 2002; White et al., 1996). Consistently with previous reports in rat neurons, we found that Cav-1 is expressed by TrkA-positive DRG neurons and found both in the soma and the neurites (Galbiati et al., 1998b) (Fig.1A). To get efficient transfection with low amount of cells, we optimized electroporation of DRG neurons using Neon™ transfection system. Using this procedure, we transfected more than 40% of the neurons (Fig. 1B and C). We found that many DRG neurons transfected with Cav-1-RFP (Sharma et al., 2004) and GFP, differentiate (Fig. 1D-E). Quantification of the total neurite length of transfected neurons showed that neurons co-expressing Cav-1-RFP and GFP grew shorter neurites than RFP- or GFP-expressing ones. Indeed, average length decreased by 18% in Cav-1 over-expressing neurons after one day *in vitro* (12 montages representative of 339 RFP-positive neurons and 293 Cav-1-RFP-expressing neurons, p=0,018). In parallel with a progressive accumulation of Cav-1, this decrease reached 33% after two days (434 RFP-positive and 308 RFP-positive neurons, p=0,00009) (Fig. 1H). This effect was even stronger when the amount of plasmid is doubled, further supporting a dose-dependent effect. Average total neurite length dropped by 34% (361 RFP-positive and 416 RFP-positive neurons, p=0,002) after the first day and 41% on the second day (296 RFP-positive and 351 RFP-positive neurons, p=0,000002). Thus,

Fig. 1

overexpression of Cav-1 appears to impair neurite growth of DRG neurons as it does in PC12 cells.

Effect of Cav-1 and Cav-2 on NGF-induced neurite formation in PC12 cells

Having demonstrated that Cav-1 overexpression in PC12 cells could mirror what is happening in DRG neurons, we decided to study in detail how caveolins act upon NGF signaling in the PC12 line, which are more amenable to manipulations.

Interestingly, Cav-1 and Cav-2 exhibit different kinetic of expression during NGF differentiation. In agreement with previously-published studies (Galbiati et al., 1998b), Cav-1 expression is almost undetectable in sparse populations and progressively increases in PC12 cells in response to NGF. In contrast, Cav-2 expression increases to a maximum after 2 days of treatment with NGF then drops precipitously (Fig. S1). Since, Cav-1 and Cav-2 respectively have an expression peak during late and early phases of neuritogenesis, we wondered whether they could have different effects on NGF-induced PC12 cell morphological differentiation.

In order to evaluate the impact of caveolin expression on global NGF signalling, wild-type PC12 cells were stably-transfected and assayed for transgene expression. Clones expressing Cav-1 or Cav-2 were selected for subsequent studies with most experiments being performed on two or more clones (Table S1). NGF induces PC12 cells to stop proliferating and to differentiate into a sympathetic neuron-like phenotype (Greene and Tischler, 1976). Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were plated at low density and exposed for three days to NGF concentrations ranging from 5 ng/ml to 50 ng/ml. In response to NGF, wild-type PC12 cells extended a characteristic neurite network in a dose-dependent manner whereas Cav-1 PC12 cells did not elicit any significant process extension at all NGF concentrations evaluated (Fig. 2A), a result consistent with previous studies (Bilderback et al., **Fig. 2**

1999; Galbiati et al., 1998b). In contrast, Cav-2 PC12 cells exposed to NGF developed a more extensive and denser neurite network than wild-type PC12 cells (Fig. 2A). NGF-induced morphological differentiation of Cav-2 PC12 cells thus appears to be potentiated in comparison to the response of wild-type PC12 cells. The global evaluation of differentiation of each cell line at the different NGF concentrations is represented in Fig. 2B. These observations suggest that Cav-1 and Cav-2 have opposite effects on NGF-induced PC12 cell morphological differentiation.

Effect of Cav-1 and Cav-2 on the anti-mitogenic response to NGF

PC12 cells respond to NGF by induction of an anti-mitogenic response (Greene and Tischler, 1976) elicited by cell cycle arrest in the G1 phase (Rudkin et al., 1989), and the triggering of differentiation. Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were plated at low density in the presence of serum and grown for two days prior the addition of 20ng/ml NGF. In the presence of NGF, wild-type PC12 cells and Cav-2 PC12 cells undergo a final round of division and stop proliferating with similar kinetics (Fig. 3A). It would appear that the potentiation of NGF-induced differentiation, observed with Cav-2 over-expression, is not a consequence of an enhanced anti-mitogenic response. In contrast, Cav-1 PC12 cells did not stop proliferating after NGF addition and showed a growth rate similar to that of exponentially-growing wild-type PC12 cells in absence of NGF (Fig. 3A). The anti-mitogenic effect of NGF is propagated *via* the induction of the Cyclin-Dependent Kinase inhibitor $p21^{WAF/Cip1}$ (van Grunsven et al., 1996; Yan and Ziff, 1995). To test if Cav-1 or Cav-2 expression alters $p21^{WAF/Cip1}$ levels, equal numbers of cells exposed to 20ng /ml of NGF for 0, 1, or 3 days were lysed and protein expression ascertained by western analysis. Fig. 3B shows that $p21^{WAF/Cip1}$ levels in wild-type PC12 cells and in Cav-2 PC12 cells markedly increase during the

Fig. 3

time course of NGF treatment. No change in $p21^{WAF/Cip1}$ level was detectable in Cav-1 PC12 cells exposed to NGF.

The minimal $p21^{WAF/Cip1}$–promoter luciferase construct (p2193S-Luc) was used as reporter gene for the NGF signaling pathway (Billon et al., 1999; Billon et al., 1996; Datto et al., 1995). In wild-type PC12 cells treated with NGF for 48 hours, the promoter is activated as ascertained by an increase in Firefly luciferase activity. This activation of the p21 promoter is also found in Cav-2 PC12 cells. In contrast, NGF-induced p21 promoter activation is significantly reduced in Cav-1 PC12 cells (Fig. 3C).

These results suggest that Cav-1, but not Cav-2 expression results in inhibition of the anti-mitogenic effect of NGF, at least in part, by impairing activation of transcription of $p21^{WAF/Cip1}$.

Effect of Cav-1 and Cav-2 on NGF-induced TrkA and $p75^{NTR}$ internalization

NGF receptor trafficking is essential for regulating many of the subsequent cellular responses (Bhattacharyya et al., 1997; Grimes et al., 1997; Grimes et al., 1996; Harrington et al., 2011; Jullien et al., 2003; MacInnis et al., 2003; Perrone et al., 2005; Senger and Campenot, 1997; Urdiales et al., 1998; Xu et al., 2000). The effect of Cav-1 and Cav-2 expression on TrkA was monitored in clones of PC12 cells stably-expressing these proteins. Following NGF treatment, Fig. 4A shows that TrkA and $p75^{NTR}$ exit from lipid rafts in wild-type PC12 and Cav-2 PC12 cells. **Fig. 4** In contrast, TrkA and $p75^{NTR}$ remain in lipid rafts in Cav-1 expressing cells, indicating that Cav-1 is retaining NGF receptors in lipid rafts. Quantification of several independent experiments (Fig. 4B) shows that Cav-1 almost totally inhibits exit of TrkA and $p75^{NTR}$ from lipid rafts whereas Cav-2 does not.

Ligand-induced internalization of TrkA and subcellular localization of TrkA were evaluated using confocal microscopy (Fig. 5). Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were transiently transfected with a chimeric TrkA-EGFP receptor (Jullien et al., 2003). Expression of this receptor, together with the use of RTA antibody as an NGF agonist (Clary et al., 1994; Jullien et al., 2003) allow simultaneous monitoring of two different pools of TrkA in a single cell. (i) "TrkA-EGFP" reflects EGFP fluorescence, which corresponds to all of the cellular localizations of TrkA-EGFP. (ii) "Cell surface TrkA at t=0 min" is identified by incubation of cells at 4°C for 30 min in presence of RTA, followed by fixation, permeabilization and labeling of cells with rhodamine-conjugated anti-rabbit antibody. This allows the detection of TrkA-RTA complexes, which are initially at the cell surface. Following the shift to 37°C, it is possible to monitor the fate of the receptors that have moved from the cell surface to intracellular locations during the incubation time. As shown in Fig. 5, first row, incubation of PC12 cells in the presence of RTA at 4°C does not allow receptor internalization, reflected by the presence of RTA-TrkA complexes exclusively at the cell surface. Incubation of PC12 cells and of Cav-2 PC12 cells for 20 min at 37°C in the presence of RTA leads to partial redistribution of the TrkA-antibody complex inside the cells. Intracellular RTA-TrkA complexes correspond to internalized receptors. By contrast, following the shift to 37°C of Cav-1 PC12 cells treated with RTA, little or no intracellular RTA-TrkA complexes are detected, indicating that TrkA internalization is impaired in Cav-1 over-expressing cells.

Fig. 5

Thus, the differential internalization of TrkA in presence of increased levels of Cav-1 and Cav-2 appears to mirror the functional outcome of Cav-1 and Cav-2 over-expression on PC12 differentiation.

Interplay of p75^{NTR} and TrkA

When PC12 cells are treated with MC192 monoclonal antibody, specific for the extracellular domain of $p75^{NTR}$, (Barker and Shooter, 1994) TrkA internalization is inhibited, even in presence of high NGF concentrations (Jullien et al., 2002). Fig. 6 illustrates that exposure of **Fig. 6** PC12 cells to this anti-$p75^{NTR}$ antibody almost completely abolishes NGF-induced TrkA and $p75^{NTR}$ exit from lipid rafts, much like Cav-1 expression. This suggests that part of the role of $p75^{NTR}$ in TrkA internalization may be to participate in, or regulate, the exit from lipid rafts in response to NGF.

Effect of Cav-1 and Cav-2 on activation of TrkA and downstream signaling effectors

NGF signaling and PC12 cell differentiation are mediated *via* TrkA activation (Loeb et al., 1991). Since receptor endocytosis is important to orchestrate NGF signaling, we tested whether Cav-1 and Cav-2 differentially regulate the canonical NGF signaling cascade *via* Ras/Raf/MAPK. Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were exposed to 20ng/ml NGF for 10 min. In absence of NGF, no TrkA phosphorylation was detectable in any cell line. However 10 min exposure to NGF resulted in marked tyrosine phosphorylation in all cell lines, with an increased phosphorylation of TrkA in Cav-1 and Cav-2 PC12 cells compared to TrkA phosphorylation in wild-type PC12 cells (Fig. 7). **Fig. 7**

Thus, Cav-1 expression does not appear to inhibit phosphorylation of TrkA under the experimental conditions used herein. To further characterize the impact of Cav-1 and Cav-2, downstream effectors of the NGF signaling pathway were monitored. Phosphorylated TrkA leads to MAPK activation that induces Rsk phosphorylation. Rsk will ultimately phosphorylate CREB. Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were treated with 20ng/ml NGF for 0 to 6h. Activation status of several effectors of the Ras-MAPK pathway was analysed by immunoblot using antibodies directed towards their phosphorylated forms. NGF treatment

of wild-type PC12 cells results in a prolonged activation of MAP kinases ERK1 and ERK2, with phosphorylation peaking at 30 min treatment, and detectable up to 2h after NGF addition (Fig. 8A). Rsk2 activation by phosphorylation is also readily detected and lasts up to 6h after addition of NGF, while CREB phosphorylation on Serine 133 peaks at 30 min, decreasing to the limit of detection after 2h. **Fig. 8**

Cav-2 PC12 cells exhibit an even longer duration of MAPK, Rsk2 and CREB activation. The phosphorylated forms of the proteins are easily detectable up to 6h after NGF addition (Fig. 8 C). These results show that TrkA activation in Cav-2 PC12 cells is accompanied by the lengthening of the duration of MAPK pathway activation. Extended MAPK activation has been proposed to be critical in mediating the differentiation effects of NGF in PC12 cells (Marshall, 1995), this could provide an explanation for the potentiating effects of Cav-2 expression on NGF-induced differentiation. P-JNK and P-Akt activation were also increased in response to NGF exposure to all three cell lines, with slightly prolonged activation in Cav-2-PC12 compared to Cav-1 and wild-type PC12 (Figure S2).

In Cav-1 PC12 cells, Erk1 and Erk2 are activated with kinetics very similar to that observed in wild-type PC12 cells (Fig. 8B). RSK2 is also activated, although phosphorylation does not last as long as that observed in wild-type PC12 cells. CREB phosphorylation on Serine 133 was barely detectable. This result shows that the activation of CREB is *inhibited* in Cav-1 PC12 cells. Thus, it appears that Cav-1 expression blocks CREB activation while Cav-2 expression sustains it.

Effect of Cav-1 and Cav-2 expression on TrkA effector localization

Normal activation of CREB in response to NGF is achieved *via* phosphorylation of Serine 133 by Rsk2 (Ginty et al., 1994; Xing et al., 1996). Following activation, pRsk2 is translocated into

the nucleus where it phosphorylates CREB (Xing et al., 1996). Since Rsk2 gets phosphorylated in Cav-1 over-expressing PC12, we investigated if the lack of CREB phosphorylation was due to impaired pRsk translocation. Subcellular localization of pRsk2, CREB and pCREB was studied by confocal immunofluorescence microscopy in the Caveolin-expressing cells. Fig. 8 A and B show that pCREB was detected in the nucleus of wild-type PC12 cells and Cav-2 PC12 cells with a greater level of activation in Cav-2 PC12 cells. In the nucleus of Cav-1 PC12 cells, the level of CREB activation was essentially completely inhibited. Furthermore, in wild-type PC12 cells and in Cav-2 PC12 cells exposed to 20ng/ml NGF for 30 min, pRsk2 is mainly located in the nucleus. By contrast, in Cav-1 PC12 cells exposed to NGF, the level of pRsk2 in the nuclear compartment is drastically diminished. Moreover, accumulation of pRsk2 can be seen in isolated foci at the periphery of the cell. Thus the absence of enhanced nuclear translocation of pRsk2 in Cav-1 PC12 cells may explain why CREB is not phosphorylated in the nucleus of these cells.

Fig. 9 shows that pRsk2, accumulated at the periphery of the cell, is distributed in a pattern closely resembling that of Cav-1. Subcellular localization of Cav-1 was also analyzed in Cav-1 PC12 cells transiently-transfected with a TrkA-EGFP (Jullien et al., 2003). Cav-1 and TrkA are co-distributed at the cell surface. These results suggest that Cav-1 sequesters TrkA and downstream effectors of the NGF signaling pathway at the cell membrane. **Fig. 9**

Effect of Cav-1 point mutations on the NGF signaling pathway

Paracrine/autocrine activation of NGF signaling has been implicated in tumour progression, cell survival and metastasis and deletion or mutation of Cav-1 have been reported for certain cancers, notably breast cancer (Descamps et al., 2001; Dolle et al., 2004; Li et al., 2006; Nico et al., 2008; Zhu et al., 2002) while overexpression has been observed in certain prostate cancer

346 lines (Tahir et al., 2001; Tahir et al., 2008). Mutations of specific codons, *e.g.* those coding for
347 Pro132 and Serine 80, have been reported to potentially be able to override or inactivate the
348 growth inhibitory activity of Cav-1 (Hayashi et al., 2001; Schlegel et al., 2001; Williams and
349 Lisanti, 2005).
350
351 PC12 cells were stably-transfected with Cav-1 S80V (non-phosphorylatable form) and assayed
352 for transgene expression. Individual clones expressing Cav-1 S80V were selected, and the
353 impact on specific parameters evaluated as described above with cells expressing wild-type
354 caveolins. Fig. 1 A and B show that, Cav-1 S80V PC12 cells exposed to NGF developed a more
355 extensive and denser neurite network than wild-type PC12 cells. This enhanced morphological
356 differentiation was observed even at low concentrations of NGF. The NGF-induced
357 morphological differentiation of Cav-1 S80V PC12 cells appears to be potentiated in
358 comparison to wild-type PC12 cell response, as was observed with the differentiation of Cav-2
359 PC12 cells (Fig. 1). Fig. 2 A shows that, following NGF exposure, Cav-1 S80V PC12 cells and
360 wild-type PC12 cells stop proliferating with similar kinetics. Thus, expression of Cav-1 S80V
361 does not result in the inhibition of the anti-mitogenic effect nor of the differentiation
362 response that are observed with Cav-1 expression. Furthermore TrkA internalization is not
363 affected by expression of Cav-1 S80V, in contrast to that of Cav-1 (Fig. 4). Finally, in Cav-1
364 PC12 cells, the reduction of nuclear pRsk2, concomitant with the accumulation of pRsk2 at the
365 cell periphery, is associated with an almost total inhibition of CREB phosphorylation. In
366 contrast, NGF treatment of Cav-1 S80V PC12 cells results in nuclear localization of pRsk2 along
367 with a high level of pCREB, superior to that observed in NGF treated wild-type PC12 cells (Fig.
368 8 A and B).
369

370 Taken together, these results show, that while expression of Cav-1 inhibits TrkA trafficking and

371 NGF signaling, the Cav-1 S80V mutant does not and actually *potentiates* the NGF response.

372

373

Discussion

The aim of this study was to gain insight into the mechanisms underlying the effects of membrane microdomain components Cav-1 and Cav-2 on growth factor signaling using the NGF receptors TrkA and p75NTR as model.

A “ying and yang” dichotomy between Cav-1 and Cav-2 was observed, with Cav-1 inhibiting and Cav-2 potentiating the response to NGF. In PC12 cells, Cav-1 expression inhibited NGF-induced cell cycle arrest and morphological differentiation, coincident with a reduced expression of CDK inhibitor $p21^{WAF/Cip1}$. Cav-1 expression impaired exit from lipid rafts and internalization of NGF receptors, TrkA and $p75^{NTR}$, without, however, abrogating TrkA short-term activation, nor downstream effector activation up to and including Rsk2. Remarkably, Cav-1 expression was associated with accumulation of activated Rsk2 at the plasma membrane and subsequent inhibition of CREB phosphorylation.

By contrast, PC12 cells expressing Cav-2, displayed normal kinetics of cell-cycle arrest when exposed to NGF, while morphological differentiation was potentiated. In Cav-2 PC12 cells, NGF receptor exit from lipid rafts and internalization, as well as nuclear localization of pRsk2, were similar to that observed in wild-type PC12 cells. However, TrkA effectors MAPK, Rsk2 and CREB exhibited higher levels and/or longer activation kinetics as did JNK and Akt. Taken together, these results demonstrate differential modulation of NGF signaling events by Cav-1 and Cav-2.

Phosphorylation of Cav-1 on Serine 80 has been shown to stimulate cell survival, clonal growth and metastasis (Tahir et al., 2001; Tahir et al., 2008), possibly through modulation of Cav-1 aggregation and sequestration of cholesterol (Bauer and Pelkmans, 2006). Several experiments were performed using the S80V mutant of Cav-1 to assess the importance of Serine 80 phosphorylation on TrkA trafficking and signaling. Expression of the Cav-1 S80V mutant in PC12 cells did not result in the inhibition of NGF-induced cell differentiation observed in Cav-1 PC12 cells. The inhibitory effect of Cav-1 on the anti-mitogenic response to NGF was also abrogated. In agreement with this, Cav-1 S80V PC12 cells did not show impaired internalization of TrkA, nor accumulation of activated Rsk2 at the plasma membrane, nor reduced CREB phosphorylation. These results offer clear insight into the potential mechanism of action of caveolins in signaling pathways involving p75NTR or TrkA, such as during neuronal development and in certain cancers.

Cav-1 overexpression in embryonic sensory neurons

Increase in Cav-1 expression is observed subsequent to neuronal damage in the central nervous system (Gaudreault et al., 2005). It is correlated with post injury reactive synaptogenesis in the adult hippocampus. Using neuronal damage in PC12 cultures as a model for neuronal plasticity during compensatory synaptic remodeling, these authors observed that adenovirus mediated overexpression of Cav-1 in damaged cultures resulted in a reduction in reactive neurite outgrowth and significantly shorter neurites. The overexpression of Cav-1 in primary DRG resulted in a significant decrease in overall neurite length in agreement with those in the PC12 model as described. The mechanism *via* which Cav-1 exerts this effect remains to be elucidated. Among several potential mechanisms, it was suggested that inhibition of TrkA activation could be responsible for these observations.

NGF-induced exit of TrkA and p75^{NTR} from lipid rafts in wild-type PC12 cells

Numerous growth factor receptors have been observed to move in to or out of membrane microdomains. Here we show that NGF induces TrkA and p75^{NTR} exit from lipid rafts in PC12 cells. It has been reported that TrkA and TrkB reside outside lipid rafts prior to neurotrophic factor stimulation, then concentrate in lipid rafts consecutive to treatment (Limpert et al., 2007; Pereira and Chao, 2007). In contrast, a number of studies have provided evidence that in absence of treatment, TrkA is enriched in lipid rafts (Bilderback et al., 1999; Huang et al., 1999; Nishio et al., 2004; Peiro et al., 2000; Schmitz et al., 2009; Wu et al., 1997) as observed herein.

The use of different methods for lipid rafts isolation could explain the discrepancies. While we, and others (Bilderback et al., 1999; Huang et al., 1999; Nishio et al., 2004; Peiro et al., 2000; Wu et al., 1997) used a detergent-*independent* method, either *in vivo* or in cultured cells, Limpert et al., 2007 and Pereira et al., 2007 used a Triton X100-dependent method, which has been shown to induce the formation of non-physiological structures (Ingelmo-Torres et al., 2009). Nevertheless, there appears to be an interesting paradox that merits clarification, which would shed insight on the microenvironment of the receptors in the different cellular membranes.

Effect of Cav-1 on NGF-induced TrkA and p75^{NTR} exit from lipid rafts and internalization

The results presented herein confirm that, in PC12 cells, expression of Cav-1 inhibits NGF-induced morphological differentiation (Bilderback et al., 1999; Galbiati et al., 1998b). This is complemented by the observation in the present study, that Cav-1 PC12 cells continue to divide in the presence of NGF and the finding that the NGF-induced expression of an essential CDK inhibitor, p21$^{WAF/Cip1}$, responsible in part for the cell cycle arrest, (Billon et al., 1996; van

Grunsven et al., 1996; Yan and Ziff, 1997) is prevented. This is strong evidence that expression of Cav-1 is associated with in depth modifications of NGF receptor signaling and trafficking.

Cav-1 expression impacts upon TrkA at the plasma membrane, where it can interact with the receptor, in lipid rafts (Bilderback et al., 1997; Huang et al., 1999; Limpert et al., 2007; Peiro et al., 2000; Schmitz et al., 2009). Expression of Cav-1 inhibits NGF-induced TrkA and $p75^{NTR}$ exit from lipid rafts and TrkA internalization is equally abrogated. The fact that the Δ8 TrkA-EGFP construct lacking specific internalization motifs and showing a significantly inhibited internalization (Jullien et al., 2003), exits the lipid rafts in the presence of NGF (Figure S3), suggests that TrkA exit from lipid rafts precedes its internalization and is independent of its "activation" *via* the classic tyrosine phosphorylation sites. Exit from lipid rafts is a necessary preliminary step for clathrin-dependent endocytosis of epidermal growth factor (EGFR), B cell antigen receptor and anthrax toxin receptor (Abrami et al., 2003; Lajoie and Nabi, 2007; Puri et al., 2005; Stoddart et al., 2002). This leads us to the conclusion that expression of Cav-1 prevents TrkA internalization by inhibiting TrkA exit from lipid rafts.

Effect of Cav-1 on activation of TrkA and downstream effectors

TrkA internalization plays a key role in activation of signaling pathways leading to full differentiation (Marshall, 1995; Meakin et al., 1999; Wu et al., 2001; Xu et al., 2000; York et al., 2000). The impact of Cav-1 sequestration of TrkA in lipid rafts, on the activation of TrkA and its downstream effectors was evaluated. We show that in Cav-1-expressing cells TrkA phosphorylation is not inhibited and is even enhanced, after 10 minutes of NGF treatment. The same trend is seen after 10 minutes of treatment in dynamin temperature sensitive (TS) cells where TrkA internalization is inhibited (Zhang et al., 2000). It is conceivable that the short term activation of TrkA seen in Cav-1 cells results from its physical sequestration at the plasma

membrane. However, it has been shown that Cav-1 expression, just as inhibition of internalization in dynamin TS cells, results in reduced phosphorylation of TrkA after 30 minutes of NGF treatment (Bilderback et al., 1999; Zhang et al., 2000). Therefore, in the light of our results, we conclude that Cav-1 inhibits the long-term signaling response *via* inhibition of its internalization and not *via* inhibition of it's kinase activity.

We observed that the ERK1/2 downstream kinase Rsk2 was activated in a non-sustained manner. This is in good agreement with findings showing that inhibition of TrkA internalization, whether using NGF covalently crosslinked to beads or in dynamin TS cells, does not allow prolonged activation of ERK1/2 (MacInnis and Campenot, 2002; Wu et al., 2001; Zhang et al., 2000). Furthermore, we observed that the activated Rsk2 was maintained at the cell surface. This was associated with the prevention of NGF-induced CREB activation. CREB is a transcription factor acting as a dimer to induce numerous genes, including genes involved in transcription control, cell cycle and cell survival including $p21^{WAF1/Cip1}$ (Alberini, 2009; Lonze and Ginty, 2002; Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999). NGF triggers the phosphorylation of CREB at a critical regulatory site, Ser 133 (Ginty et al., 1994). Once phosphorylated at this site, CREB induces transcription of immediate early genes, by cooperating with other transcription factors (Alberini, 2009; Lonze and Ginty, 2002). While several kinases have been shown to be able to phosphorylate CREB on Ser 133, it appears that, in NGF-treated PC12 cells, Rsk2 is key to this process (Alberini, 2009; Ginty et al., 1994; Lonze and Ginty, 2002; Xing et al., 1996). That CREB is not phosphorylated in cells expressing Cav-1 is, thus, due to the sequestration of pRsk2 at the plasma membrane, which prevents it from translocating to the nucleus.

Cav-1 mode of action

497 It has been reported that Cav-1 modulates the activity of several growth factor receptors
498 notably, interacting with EGFR, and that this interaction translates into an inhibition of EGFR
499 kinase activity. Cav-1 is also able to directly inhibit MEK-1 and ERK-2 kinase activity *in vitro*
500 (Couet et al., 1997; Engelman et al., 1998a). Clearly, In the light of our results, it would appear
501 that, at least in the early steps of NGF signaling pathway activation, Cav-1 does not act *directly*
502 on the kinase activity of TrkA nor its down-stream effectors up to and including Rsk2, since all
503 are *activated* in Cav-1-expressing cells. The inhibition of the NGF response is due to
504 *sequestration* at the cell membrane, of TrkA *and* its signaling complex. It has been proposed
505 by Lajoie and Nabi 2007 that Cav-1 negatively regulates endocytosis either by stabilizing raft
506 invagination at the cell surface or by *sequestering* key structural components, notably
507 including cholesterol and dynamin (Kim and Bertics, 2002; Pike, 2006), independently from
508 the presence of caveolae (Lajoie et al., 2009). The fact that pRsk2 accumulates at the
509 periphery of the cell following a pattern closely resembling that of Cav-1 suggests that Cav-1 is
510 sequestering the TrkA signaling complex by direct-interaction with TrkA (Bilderback et al.,
511 1999; Schmitz et al., 2009) and/or other members of its signaling complex (Hua et al., 2003; Li
512 et al., 1996; Zhang et al., 2007). Such differential action has been observed in the case of
513 EGFR. Notably, *Spry* proteins interact with Cav-1 and depending on the isoform of *Spry*, the
514 EGF-induced proliferation is attenuated without inhibiting ERK1/2 activation (Cabrita et al.,
515 2006; Impagnatiello et al., 2001). Such a mechanism could explain Cav-1 dependent inhibition
516 of differentiation in NGF-treated PC12 cells, with partners remaining to be identified.
517 Overexpression of Cav-1 is associated with inhibition of proliferation, *e.g.* in mouse embryo
518 fibroblasts and premature senescence *via* a p53/p21$^{CIP1/Waf\ 1}$ mechanism (Galbiati et al., 2001;
519 Volonte et al., 2002). The current understanding of Cav-1 action suggests that it exerts a
520 number of its effects *via* its direct binding to key signalling molecules, sequestering them
521 within lipid rafts (Zou et al., 2011). This, in turn, modulates signalling *via* the downstream

pathways. The results presented herein indicate that the overexpression of Cav-1 in PC12 cells results in the apparent stimulation of proliferation (actually prevention of the anti-proliferative response), at least in part *via* prevention of the induction of $p21^{Cip1/Waf1}$, consistent with this possibility. In contrast, however, the NGF-TrkA, Ras, Raf, MAPK dependent signalling pathway is not inhibited *per se* since MAPK, and Rsk2 are indeed activated with similar kinetics in PC12 clonal lines stably-expressing Cav-1 as compared to wild-type PC12 cells in response to NGF. What has been clearly shown is that CREB phosphorylation is significantly diminished due to the physical sequestration of activated Rsk2 at the cell membrane. Thus, it would appear that, while some of the endogenous inactivated Ras may be sequestered by the additional Cav-1, there is clear and reproducible activation of the pathway. The "final" triggering event essential for propagation of the anti-proliferative and differentiation responses cannot occur since the penultimate "trigger" of this branch, activated Rks2, cannot reach its target – CREB.

These results are supported by p21-luciferase assays in which Cav-1 PC12 cells, transfected with constitutively-active H-RasV12, showed similar activation of the p21-promoter as compared to transfected wild-type PC12 cells (Figure S4). Indeed, H-RasV12 was reported to have significantly reduced binding to Cav-1, in contrast to Ras wild-type (Song et al. 1996).

Another possible mechanism of action of Cav-1 could be *via* its interaction with Nitric Oxide Synthase (NOS). Indeed, Cav-1 has been reported to bind to and *inhibit* the activity of NOS and the production in Nitric Oxide (NO) in several different cell types (Michel et al., 1997; Michel and Feron, 1997; Shen et al., 2008; Zhao et al., 2009). NGF treatment of PC12 cells induces NOS and production of NO, essential for stimulating cell cycle arrest *via* $p21^{cip1}$ induction and neurite outgrowth (Meini et al., 2006; Peunova and Enikolopov, 1995). Chemical inhibition of

NOS inhibits sustained MAPK activation, the anti-mitogenic response and neuronal differentiation (Kalisch et al., 2003). It follows that, in Cav-1 expressing PC12 cells, the inhibition of NGF signaling could be a direct result of its impact on NOS. This, notwithstanding, the expression of Cav-1 does *not* prevent NGF-induced prolonged activation of MAPK nor that of Rsk2. It follows that the most predominant initial effect of Cav-1 expression is the immobilization of the NGF receptor complex in lipid rafts preventing the nuclear translocation of Rsk2. This results in the prevention of the phosphorylation of selected targets, of which CREB.

It is also interesting to note that MC192 treatment mimics Cav-1 effects on internalization of TrkA (Jullien et al., 2002). MC192 binds to the extracellular domain of $p75^{NTR}$, and without preventing the binding of NGF, attenuates NGF signaling *via* TrkA (Barker and Shooter, 1994; Bilderback et al., 1997). Application of MC192 to adult DRG resulted in a slight decrease in TrkA phosphorylation (30 min and 24h) and yet was shown to significantly attenuate, if not block, neurite outgrowth (Kimpinski et al., 1999). The results presented herein offer novel insight into the mechanism *via* which MC192 is acting on NGF signalling. Namely, they indicate that MC192 prevents NGF-induced exit of both $p75^{NTR}$ *and* TrkA from lipid rafts. This result suggests that the conformational change provoked (or prevented) by the binding of MC192 to p75NTR, directly impacts upon TrkA conformation, preventing it's and p75NTR's NGF-dependent exit from lipid rafts and thus, internalization. The sequestration in lipid rafts would therefore afford an initial explanation of the previously-observed reduction of internalization of TrkA (Jullien et al., 2002). Further, this observation offers a novel means with which to study the intimate interactions between these two receptors that have been so elusive to the field.

572 ***Effect of Cav-1 mutations on NGF signaling pathways***

573 Cav-1 has been proposed to be a tumour suppressor or promoter, depending on the type of
574 cancer, *e.g.* (Engelman et al., 1998b; Patani et al., 2012). The ability of Cav-1 to inhibit NGF
575 signaling corroborates the tumor supressor functionality. Indeed some cancers express or
576 over-express NGF. In this case, NGF can act as a paracrine/autocrine factor allowing tumor
577 progression and cell survival *via* activation of its receptors, TrkA and $p75^{NTR}$ (Descamps et al.,
578 2001; Dolle et al., 2004; Nico et al., 2008; Zhu et al., 2002). Furthermore many Cav-1 deletions
579 and mutations have been associated with the tumorigenic process (de Laurentiis et al., 2007;
580 Williams and Lisanti, 2005). Here we have investigated the potential impact of
581 phosphorylation on Serine 80, an event proposed to override Cav-1 tumor-suppressor effects,
582 using a non-phosphorylatable form Cav-1 S80V.

583

584 Cav-1 S80V-expressing PC12 cells essentially behaved as wild-type, and to a certain extent, like
585 Cav-2-expressing PC12 cells, exhibiting enhanced CREB phosphorylation and differentiation.
586 This suggests that Serine 80 phosphorylation is necessary for maintaining TrkA at the
587 membrane. Phosphorylation of Serine 80 is possibly necessary for Cav-1 secretion (Schlegel et
588 al., 2001) which has been shown to act in an autocrine/paracrine manner, directly stimulating
589 cell growth and survival in prostate tumors and related cell lines (Tahir et al., 2001; Tahir et
590 al., 2008). In apparent contrast, results herein indicate that prevention of Serine 80
591 phosphorylation *enhances* the NGF-induced MAPK pathway as reflected by the increased
592 CREB phosphorylation and neurite outgrowth. It is to be noted, that NGF-induced activation of
593 the MAPK pathway in PC12 cells leads to an opposite cellular response – *i.e.* differentiation -
594 as compared with other tumoral types – *i.e.* potentiation of cell proliferation. These results
595 therefore highlight another function of Serine 80 phosphorylation in addition to its action on
596 Cav-1 secretion *i.e* the immobilization of selected signaling molecules at the cell membrane.

This also provides example that Cav-1 S80 mutation can lead to significant deviation from normal cell fate through its effects on TrkA trafficking and signaling and thus, presumably, to tumorigenesis.

Effect of Cav-2 on the NGF signaling pathway

Cav-2 expression in PC12 cells resulted in enhanced morphological differentiation. The potentiating effect of Cav-2 is associated with an increase and/or a longer duration of downstream effector activation as compared to wild-type PC12 cells. Cav-2 is up-regulated in oesophageal and bladder carcinomas (Fong et al., 2003; Hu et al., 2001), which is consistent with the idea that Cav-2 could potentiate the MAPK pathway. Along the same lines, Cav-2 has been suggested to modulate mitosis (Sowa et al., 2008).

Very little is known about Cav-2 which has been often been considered as an accessory protein (Lahtinen et al., 2003; Razani et al., 2002; Scherer et al., 1997). However Cav-2 has been shown to be directly involved in endocytosis of bacteria and apical trafficking of lipids (Chan et al., 2009; Parker et al., 2009). One possible mechanism by which Cav-2 could trigger an apparent enhancement of TrkA signaling pathway activation, could thus be to regulate TrkA cell surface levels by enhancing TrkA targeting to the plasma membrane from the trans-golgi network. Cell surface levels of TrkA, and in particular the ratio to $p75^{NTR}$, have been proposed to be critical in modulating NGF signaling (Esposito et al., 2001; Twiss et al., 1998; Urdiales et al., 1998; Xu et al., 2000). Alternatively Cav-2 could promote TrkA signaling in manner similar to that which has been observed for the Insulin Receptor (IR). In that case, Cav-2 phosphorylation prolonged IR activation, by preventing its interaction with an inhibitory protein, SOCS-3 (Kwon and Pak, 2009).

However the bulk of Cav-2 is not targeted to the plasma membrane and is localized in the Golgi apparatus (Mora et al., 1999). This observation suggests that Cav-2 modulation of the NGF signaling pathway, may not be mediated by a *direct* interaction between Cav-2 and TrkA. One possible mechanism by which Cav-2, located in the Golgi apparatus, could trigger its effects could rely on another mechanism involving sequestration of a negative regulator of TrkA signaling in the Golgi apparatus, such as a phosphatase. Of import to this possibility, Leucocyte common Antigen Related phosphatase (LAR) is preferentially located in lipid rafts and has been reported to interact directly with Cav-1 (Caselli et al., 2002). Downregulation of LAR expression resulted in potentiation of NGF-induced morphological differentiation of PC12 cells (Tisi et al., 2000), a phenotype very similar to that observed in Cav-2-PC12 cells.

Conclusion

In summary, the data presented herein provide evidence for a differential role of Cav-1 and Cav-2 on NGF signaling. Cav-1 inhibits NGF-induced cell responses acting on the trafficking of p75 ^{NTR}, TrkA and its downstream effectors in a heretofore novel manner – by sequestration of key signalling molecules at the cell membrane resulting in the prevention of their nuclear translocation and phosphorylation of a key transcription factor CREB. Cav-1 S80V, a non-phosphorylatable form of Cav-1, does not present the same inhibitory effects as Cav-1 on TrkA trafficking and downstream signaling, suggesting that phosphorylation of this residue is key to this effect. It contributes to understanding the molecular impact of specific Cav-1 mutations in certain cancers where TrkA, p75NTR and NGF are involved in the tumorigenic response. Cav-2 has the opposite effect to Cav-1, enhancing the cellular response to NGF. Caveolins are key components of NGF receptor microenvironment that play an important role in the outcome of NGF signaling. This highlights the need for a better understanding of the impact of major lipid

raft components on NGF receptor trafficking and subsequent NGF signaling in the context of cancer and neuronal differentiation and function.

650 ## Materials and methods

651 ***Reagents***

652 Cell culture media is from Invitrogen. Fetal bovine serum and horse serum were from Sigma
653 and PAA respectively. Transfection reagent lipofectamine 2000 is from Invitrogen. Dual
654 luciferase kits are from Promega. ECL chemiluminescence system detection system is from
655 Amersham Pharmacia. ProLong Gold antifade reagent is from Invitrogen and Vectashield with
656 DAPI is from Abcys. NGF 2.5S Grade II from mouse submaxillary glands was purchased from
657 Alomone Labs. Anti-Histone H1 antibody is from Upstate Biotechnology. Anti-TrkA
658 extracellular domain (RTA) (Clary et al., 1994) was a kind gift of Dr L. Reichardt (University of
659 California, San Francisco, USA). Anti-$p75^{NTR}$ carboxy-terminus serum is a kind gift of Dr M. V.
660 Chao (New York University Medical Center, New York, USA). Anti-$p75^{NTR}$ extracellular domain
661 mAb MC192 is from Chemicon International, France. Anti p21 mAb CP36 is a kind gift of Pr W.
662 Harper (Baylor College of Medicine, Houston, USA). Anti-Trk C-14, anti-Phosphotyrosine
663 (PY99), anti-pRsk2 and anti-Cav-1 antibodies are from Santa Cruz Biotechnology, USA. Anti-
664 phospho-MAPK antibodies are from Promega, USA. Anti-pCREB antibody is from Cell Signalling
665 Technology. Anti-Cav-2 mAb is from Transduction Laboratories. Anti-ß-tubulin mAb is from
666 Sigma. Protein A Sepharose 4 Fast Flow is from Amersham Pharmacia Biotech, Sweden. HRPO-
667 linked anti-rabbit or anti-mouse IgG secondary antibody where purchased from GE
668 Healthcare. Dylight Fluo-linked anti-rabbit or anti-mouse IgG secondary antibodies are from
669 ThermoFisher Scientist. Alexa-Fluo linked anti-rabbit, anti-mouse and anti-goat antibodies are
670 from Invitrogen. K252a is from Calbiochem.

671

672 ***Cell culture and transfection***

673 PC12 cells were grown as previously described (van Grunsven et al., 1996). cDNAs of human
674 Caveolin 1 (IMAGE #488533) and human Caveolin 2 (IMAGE #491497) were purchased from

675 Research Genetics, USA. Caveolin 1 and 2 cDNAs were subcloned in the pcDNA3.1 (+) vector
676 (Clontech) and correct cloning was verified by sequencing. PC12-Cav-1 and PC12-Cav-2
677 monoclonal populations were obtained by calcium-phosphate transfection of PC12 cells with
678 pcDNA3.1-Cav-1 or pcDNA3.1-Cav-2 constructs, followed by an initial selection of 4 weeks in
679 the presence of 0.4 mg/ml of G418 (Life Technologies, France). Antibiotic-resistant colonies
680 were assayed for transgene expression by immunoblot analysis. Taking pcDNA3.1-Cav-1
681 plasmid as a template pcDNA3.1-Cav-1 S80V mutant was generated using QuickChange XL Site
682 directed Mutagenesis Kit (Stratagene) according to the manufacturer's instruction. Primers
683 were purchased from MWG-Biotech AG: S80V mutant: sense: 5'-CCA GAA GGG ACA CAC **GTG**
684 TTT GAC GGC ATT TGG AAG GCC AGC-3', anti-sense: 5'-GCT GGC CTT CCA AAT GCC GTC AAA
685 **CAC** GTG TGT CCC TTC TGG-3'. PC12-Cav-1 S80V monoclonal populations were obtained by
686 lipofectamine 2000 transfection of PC12 cells with pcDNA3.1-Cav-1 S80V constructs, followed
687 by an initial selection of 4 weeks in the presence of 0.8 mg/ml of G418 (Life Technologies,
688 France). Antibiotic-resistant colonies were assayed for transgene expression by immunoblot
689 analysis.
690
691 ***DRG Neuron culture, transfection and quantification***
692 All procedures were performed in accordance with French and European legislation on animal
693 experimentation. Primary DRG neurons were prepared from E14.5 mouse embryos. DRG were
694 dissected in ice cold HBSS (Gibco). Dissected DRG were incubated with 2.5 mg/mL trypsin
695 (T5266, Sigma) in ca++/Mg++ free HBSS for 10 min at 37°c. 0.033 mg/ml DNAse 1 (DN25,
696 Sigma) was added and DRG were return at 37°c for additional 10 min. After neutralization
697 with serum, trypsin-containing medium was removed and DRG were mechanically dissociated
698 with fire polished pipette in F12+10%FCS without antibiotics. Dissociated cells were rinsed in
699 ca++/Mg++ free PBS and resuspend in the electroporation buffer provided (Invitrogen). Cells

700 (0.7x105 cells per condition) were electroporated in 10µl Neon™ tips using Neon™
701 transfection device according to manufacturer's protocol (Invitrogen). Optimal electrical
702 parameters were 2 pulses of 20ms and 1300V. Cells were electroporated with pEGFP
703 (Clontech) (0.5µg) and pCDNA3.1+Cav-1 fused to RFP (gift from R.E Pagano, Addgene Plasmid
704 14434) (Sharma et al., 2004) or pCDNA3.1+RFP (1 or 2µg) endotoxin free plasmids (XtraMaxi,
705 Nucleobond). Control RFP was derived from the Cav-1-RFP plasmid. The Cav-1 coding
706 sequence was removed by BamHI and Not1 digestion and plasmid ligation was performed
707 after Kleenow fill-in. Because of the low number of cells required, both control and Cav-1
708 electroporations could be performed on the same batch of dissociated cells.
709 Electroporated cells were plated on poly-L-Polylysine and laminin coated coverslips (PLL: 50
710 µg/ml; laminin: 10 µg/µL, Sigma). 1 hour after plating medium (F12+10%FCS) was
711 complement with 50ng/ml NGF (N0513, Sigma) and antibiotics (1% final, 15140,Gibco). 10µM
712 of AraC was added the next morning (C1768, Sigma).
713 Control and caveolin-1 cultures from the same dissociation batch were fixed at different times
714 points in 2% paraformaldehyde at 4°c and neurite growth was analyzed. 4 montages of 9
715 images were collected in different regions of the coverslips to minimize bias due to different
716 cell density. Number of transfected neurons per image was calculated and neurite length
717 were quantified from the GFP channel in ImageJ using NeuronJ plugin (Meijering et al., 2004).
718 Statistical significance was tested with two-tail unpaired t-test.
719
720 ***Western Blot Analysis***
721 To obtain lysates, cells were washed once and collected in ice cold PBS. Pelleted cells were
722 resuspended in lysis buffer (20 mM tris-HCl, pH 8.0; 137 mM NaCl; 2 mM EDTA; 10% glycerol;
723 1% Nonidet P-40; 20 µM leupeptin; 1mM sodium vanadate; 1 mM Pefabloc; 0.15 U/ml
724 aprotinin; 1 mM ß -glycerophosphate; 3 mM sodium-fluoride). After 30 min incubation on a

rotating wheel at 4 °C, the extracts were clarified by centrifugation at 12000 xg for 20 min. Protein concentration of the supernatants was quantitated using the DC protein assay (Bio-Rad). 5 X SDS-PAGE sample buffer was added to the lysates prior to boiling for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TBST (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 3 mM KCl, 0.1% Tween-20) containing 5 % (w/v) dried milk or 5 % (w/v) Bovine Serum Albumin (BSA)(sigma) depending on the primary antibody, incubated 1 h at room temperature with primary antibody diluted in TBST with 5 % BSA. After two washes with TBST, membranes were incubated 1 h with horseradish peroxidase-conjugated secondary antibodies, washed twice with TBST and immunoreactive proteins were visualized using chemiluminescence. Alternatively, to obtain quantitative results, protein expression was evaluated by SDS PAGE followed with a western blot analysis using the Odyssey. In this case, membranes were blocked in TBS (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 3 mM KCl) containing 5 % (w/v) BSA, incubated 1 h at room temperature with primary antibody diluted in TBST with 5 % BSA. After four washes with TBST, membranes were incubated 1 h with Dylight Fluo-linked secondary antibody allowing quantitative infrared fluorescence detection using Odyssey imaging system Odyssey (ODY-1092, ScienceTec).

Luciferase assays

Wild type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were spread on Collagen/poly L-Lysine-coated 6 well dishes (Jullien et al., 2002) and transfected with lipofectamine 2000 with 2 ug of p21P93SLuc reporter as previously described (Billon et al., 1996; Yan and Ziff, 1997) and 0,1 ug of the reporter pEGFP-C2 or alternatively with 0,1 ug of pCMV H-RasV12. Twenty-four hours after transfection cells were transferred to a 96-plates, and treated or not with 50 ng/ml of NGF. Forty-eight hours later, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega).

750

751 ***Immunoprecipitation***

752 Following NGF treatment, cells were collected and lysed. Cleared lysates (1 mg protein per
753 immunoprecipitate) were then incubated on a rotating wheel for 2 h at 4 °C with 2 µg of Trk C-
754 14 antibody. Protein A Sepharose beads were added to bind the antibodies ; after 2 h at 4°C
755 beads were washed for times with lysis buffer and proteins were eluted by boiling for 5 min in
756 sample buffer.

757

758 ***Isolation of lipid raft***

759 Lipid rafts (LR) were isolated essentially as described (Song et al., 1996). Briefly, PC12 cells
760 from a 162 cm^2 cell culture flask grown to 60-70% confluence were collected in ice-cold PBS
761 and resuspended in 1.5 ml of Na_2CO_3 0.5 M pH 11.0. Homogenization was carried out using a
762 sonicator (thirty 3-sec bursts on ice ; Vibra Cells, Sonics & Materials, USA). The homogenate (6
763 mg protein/1.5 ml) was then adjusted to 45% sucrose by addition of 1.5 ml of 90% sucrose
764 prepared in H_2O and loaded in a Beckman ultracentrifuge tube under a 5%-35% discontinuous
765 sucrose gradient (3 ml of 5% sucrose / 6 ml of 35% sucrose ; both in 250 mM Na_2CO_3 pH 11.0).
766 After centrifugation at 39,000 rpm for 14h in a SW41 rotor (Beckman), lipid rafts were
767 apparent as a light-scattering band confined to the 5%-35% sucrose interface. Twelve fraction
768 of 1 ml were collected by pipetting from the top of the gradient 20 µl were used for SDS-
769 PAGE. The lipid rafts were present in fraction 3 and 4.

770

771 ***Confocal immunofluorescence microscopy***

772 PC12 cells were spread on Collagen/poly L-Lysine-coated cover slips (Jullien et al., 2002). Cells
773 were rinsed twice with PBS, fixed 30 min in PBS 3.7% formaldehyde and permeabilized one
774 minute in PBS 0,5% Triton X-100. After washing with PBS, cells were blocked with PBS 0.5%

BSA for 30 minutes and incubated 1 h with primary antibodies diluted in blocking buffer. Cells were washed three times in PBS and incubated 1 h with secondary antibodies conjugated to Alexa 488, Alexa 555 or Alexa 647. Cells were washed again three times in PBS and coverslips were mounted with ProLong Gold antifade or Vectashield with DAPI reagent. Scanning fluorescence images were acquired using the DM6000-confocal unit coupled to a Leica spectral confocal TCS SP5 AOBS with a HCX Plan APO 63/1.2 W Corr oil-immersion objective. Data acquisition was performed with Leica LAS AF SP5 software. Quantitation of the fluorescence was assessed using Image J software. For the study of TrkA internalization, cells were transiently transfected with a TrkA-EGFP fusion protein expression vector (Jullien et al., 2003) using lipofectamine 2000, and immunostaining was performed 48h post transfection. cells were preincubated for 15 min at 4°C with RTA antibody (1/500 dilution). Cells were then replaced at 37°C for 20 min prior to standard fixation and immunostaining procedure as described above. Scanning fluorescence images were acquired using the MRC1000-confocal laser unit (Bio-Rad Labs, Hercules, CA) coupled to a Zeiss Axioplan Microscope equiped with a Zeiss Plan-Apochromat 63X/1.4 oil-immersion objective, an LSM 510 camera. Data acquisition was performed with LSM510 software.

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Figures and legends

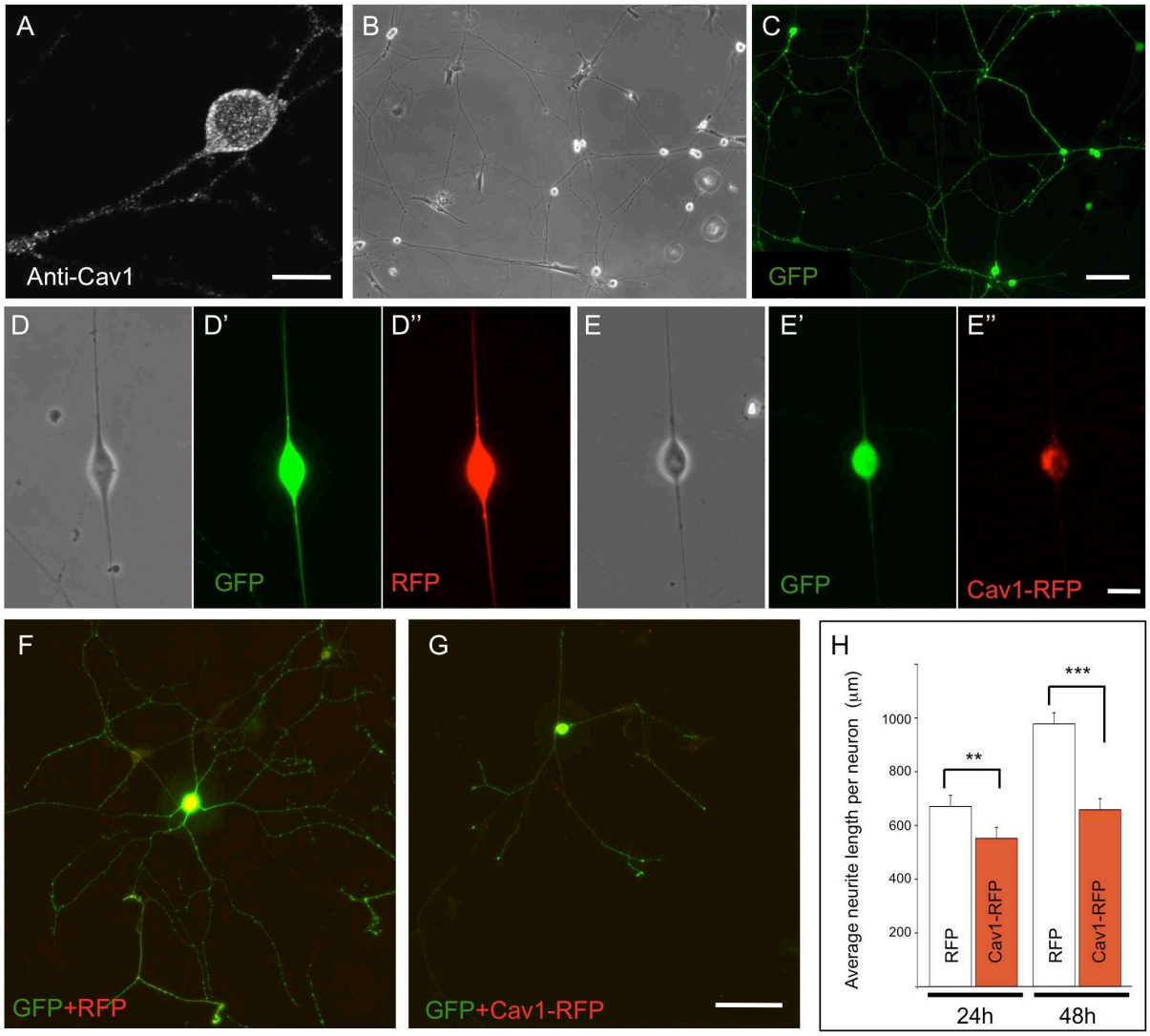


Figure 1 : Cav-1 expression inhibits neurite outgrowth from mice Dorsal root ganglia in culture.

Cav-1 is detected in both the soma and the neuritic processes of E14.5 DRG neurons (A). Neon transfection leads to efficient electroporation of E14.5 DRG neurons with little adverse effects (B and C). Neurons co-expressing GFP and RFP or GFP and Cav-1-RFP can differentiate *in vitro* (D-E''). Nevertheless, neurons expressing Cav-1-RFP grew shorter processes than neurons expressing RFP (F and G). The length of GFP positive neurites measured and divided by the number of transfected neurons. Results are pooled from three sets of cultures, each culture included four mosaic fields containing >250 transfected cells. Mean ± SEM; (**p<0.01; ***p<0.00001). Scale Bars represent 100µm in C and G and 10 in A and E ''.

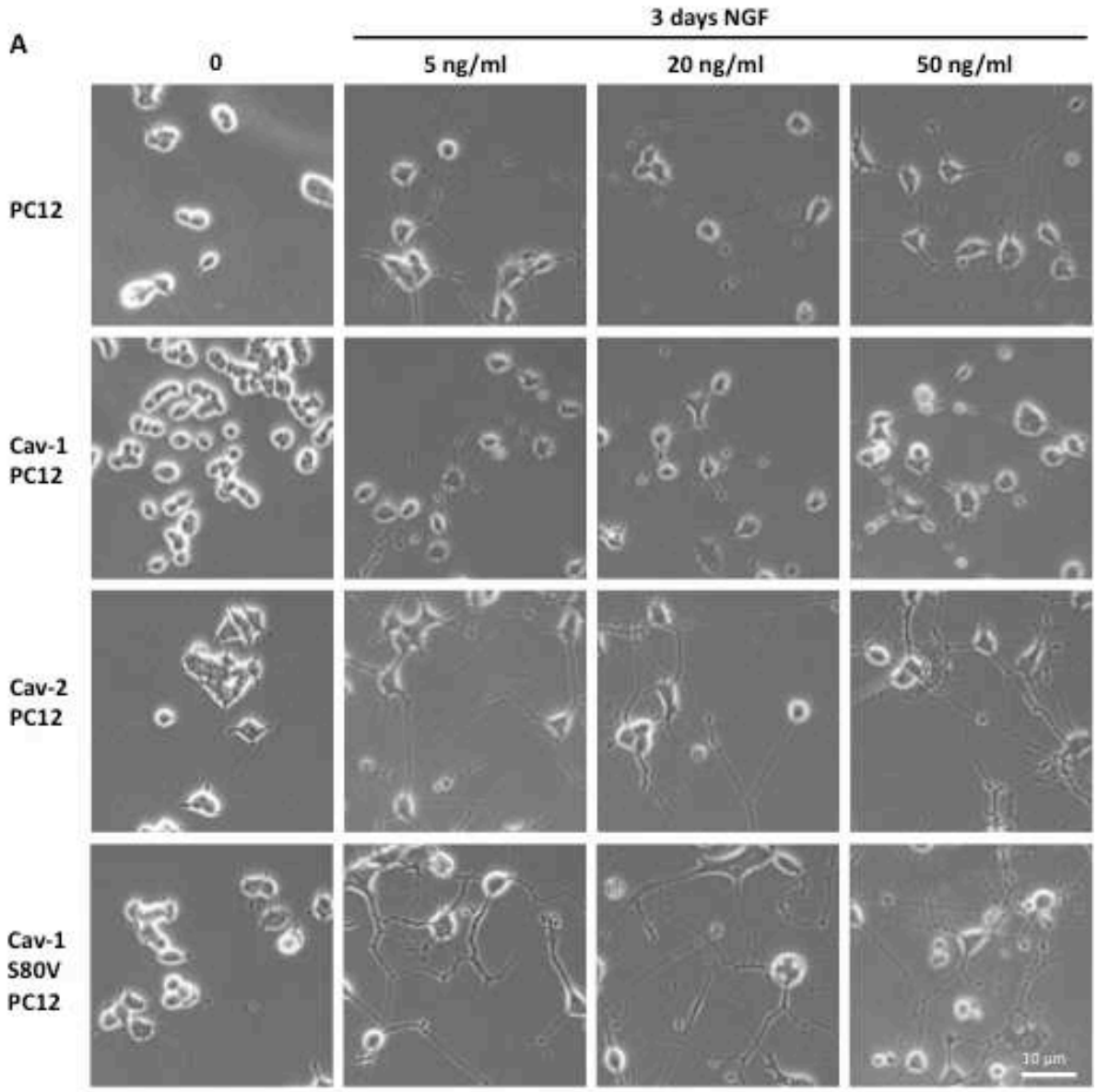

A
3 days NGF
0
5 ng/ml
20 ng/ml
50 ng/ml
PC12
Cav-1 PC12
Cav-2 PC12
Cav-1 S80V PC12
10 µm
B
% of cells with dendrites
NGF (ng/ml)
PC12
Cav-1 PC12
Cav-2 PC12
Cav-1 S80V
0
10
20
30
40
50
60
70
80
90
100
0
5
50

Figure 2: Effect of Cav-1 and Cav-2 expression on NGF-induced PC12 cell differentiation. Wild-type PC12 cells, stably transfected Cav-1-PC12 cells Cav-2-PC12 or Cav-1 S80V-PC12 cells were plated at density of 100 000 cells/well on collagen/poly-lysine coated 6 wells dishes. Cells were maintained in DMEM medium supplemented with serum for 24 hours. After 17 hours of serum deprivation NGF was added at increasing concentrations (0, 5, 50ng/ml NGF). After 3 days in culture, cells were photographed. (B) Analysis of neurite outgrowth. Quantification of the percentage of cells exhibiting neurites longer than three cell body diameters after 72 hours of NGF treatment at 0, 5, and 50 ng/ml (Approximately 100 cells per condition from 5 independent experiments were taken into account. Values are mean percentage ± SEM).

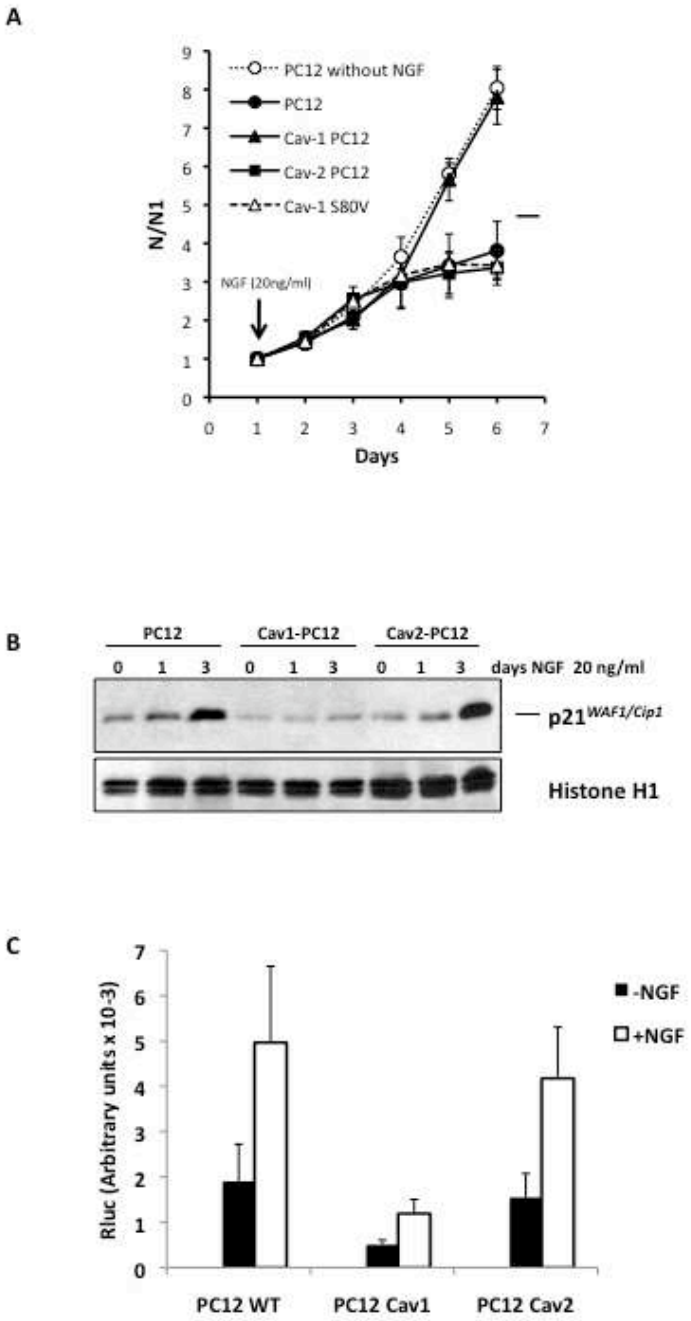


Figure 3: Effect of Cav-1 and Cav-2 expression on the anti-mitogenic effect of NGF. (A) Wild-type PC12 cells, Cav-1 PC12 cells, Cav-2 PC12 and Cav-1 S80V PC12 cells were plated at low density in 25 cm2 dishes containing medium supplemented with serum. NGF 20 ng/ml was added to the medium on day 2, except for one series of dishes that was left untreated. Cell number was counted every day for 6 days. The experiment was performed three times, with two different clones of Cav-1-PC12 and Cav-2-PC12 cells. Cell number is normalized to cell number at day 1 (N1). Values are mean ± SEM. (B) Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were exposed to 20 ng/ml NGF for 0, 1 day or 3 days. Proteins were extracted and resolved by SDS-PAGE and immunoblotted with CP36 monoclonal antibody directed against $p21^{WAF1/Cip1}$. Equal loading was verified by reprobing the same blot with anti-Histone H1 antibody. (C) The NGF-dependent induction of $p21^{WAF1/Cip1}$ was

analyzed in wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells. Cells were transiently-transfected with the minimal p21 promoter-Luciferase reporter (p21-Luc) and treated or not with NGF (50 ng/ml for 48 h) as described in the Materials and methods section. (Mean ± SEM of three independent experiments).

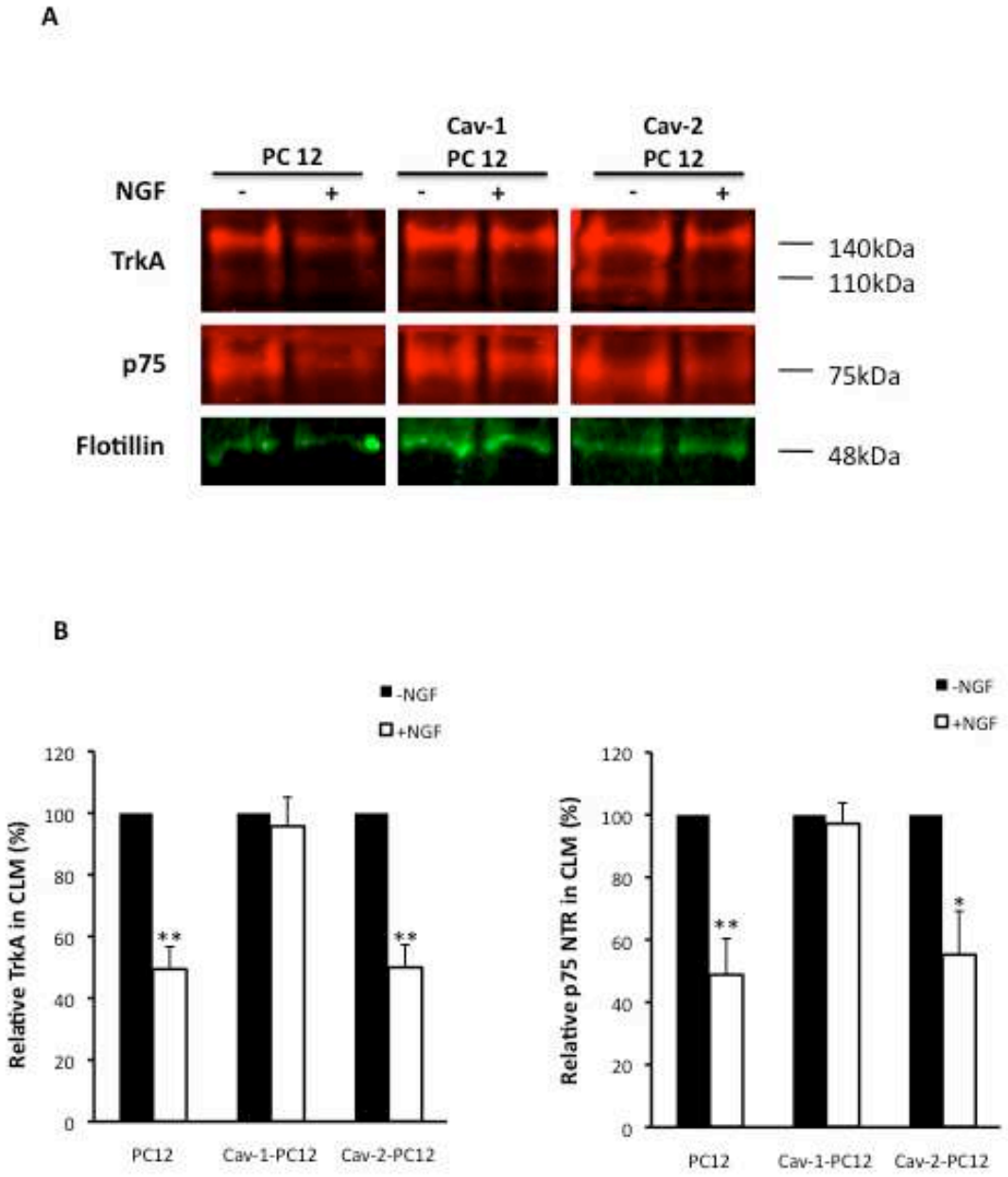


Figure 4

Figure 4: Effect of Cav-1 and Cav-2 expression on NGF receptor exit from lipid rafts. (A) TrkA and $p75^{NTR}$ levels in the lipid raft fraction before and after addition of NGF (20 ng/ml for 45 min) to cultures of wild-type PC12, Cav-1-PC12 and Cav-2-PC12 cells isolated as described under Materials and methods. Lipid raft fractions were then subjected to Western analysis. Nitrocellulose membranes were probed with RTA, anti-$p75^{NTR}$ and anti-flotillin-1 antibodies. Flotillin-1 was used as a loading control. The Odyssey imaging system was used for quantitative

infrared fluorescence detection of the relative amount of proteins. (B) Analysis of TrkA and $p75^{NTR}$ exit from lipid rafts. Level of TrkA and $p75^{NTR}$ in lipid rafts was normalized for flotillin-1 level for each sample. Systematic comparison of data with and without flotillin correction gave identical results. TrkA and $p75^{NTR}$ exit from the lipid raft fraction was then analyzed and represented as percent of the TrkA in lipid rafts compared to the amount observed in the absence of NGF, considered as 100 percent (Mean ± SEM of three independent experiments; *P<0.05, **P<0.01).

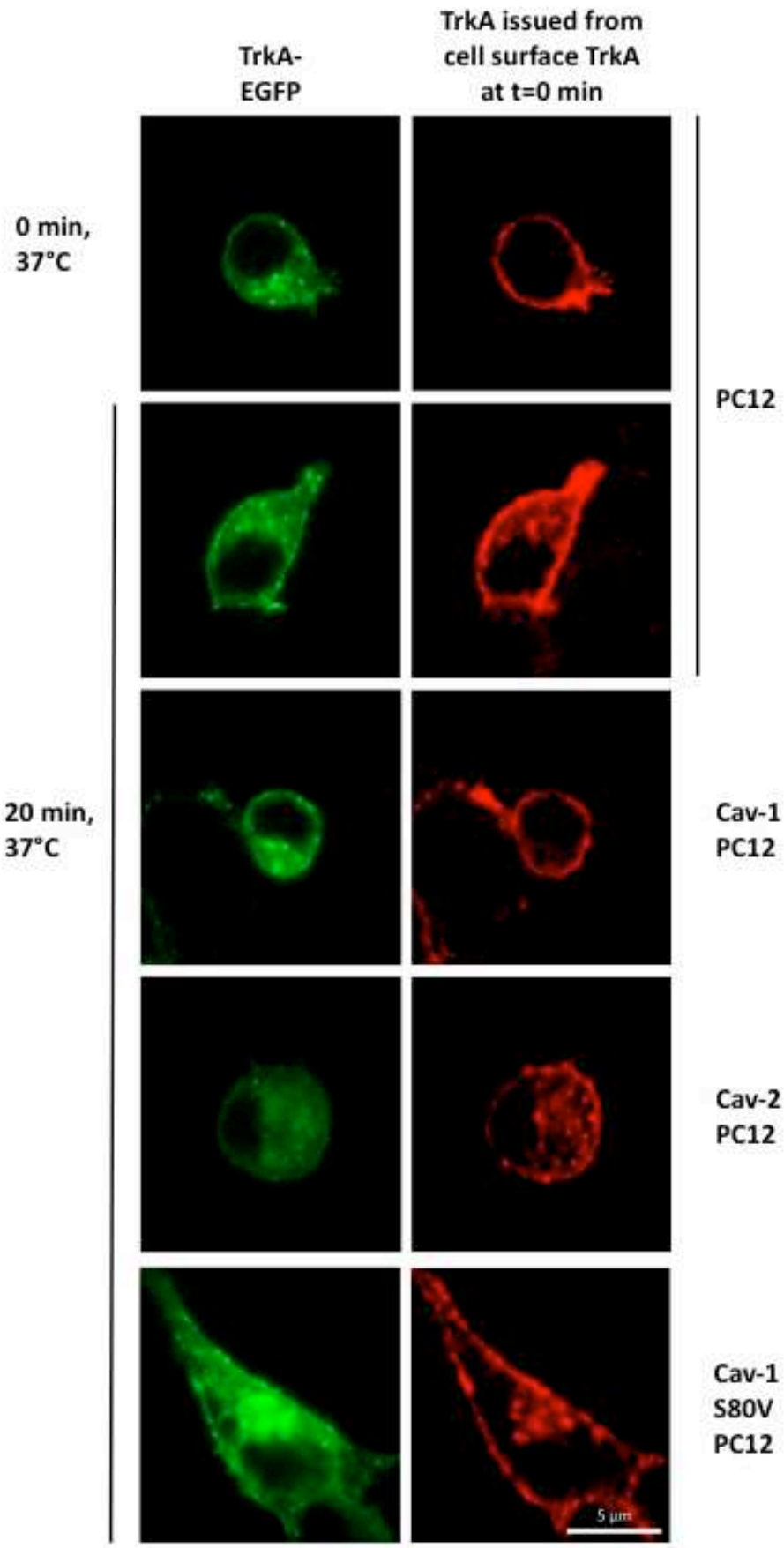

TrkA-
EGFP
TrkA issued from
cell surface TrkA
at t=0 min
0 min,
37°C
PC12
20 min,
37°C
Cav-1
PC12
Cav-2
PC12
Cav-1
S80V
PC12
5 µm

Figure 5: Effect of Cav-1 and Cav-2 expression on TrkA trafficking. Internalization of TrkA was provoked by RTA addition to wild-type PC12, Cav-1 PC12, Cav-2 PC12 and Cav-1 S80V PC12 cells transiently expressing TrkA-EGFP chimerae (green), as described under Materials and methods. After fixation and permeabilization of the cell, RTA localization was determined using a secondary antibody labeled with rhodamine (red) at the indicated times. Rhodamine fluorescence indicates the location of TrkA-RTA and TrkA-EGFP-RTA complexes that were at the cell surface at the beginning of the experiment (Cell Surface TrkA at t=0 min). Distribution of cell surface TrkA (at t=0) in Cav-1-PC12, Cav-2-PC12 and Cav-1 S80V-PC12 cells after 0 min at 37°C is identical to that observed in PC12 cells.

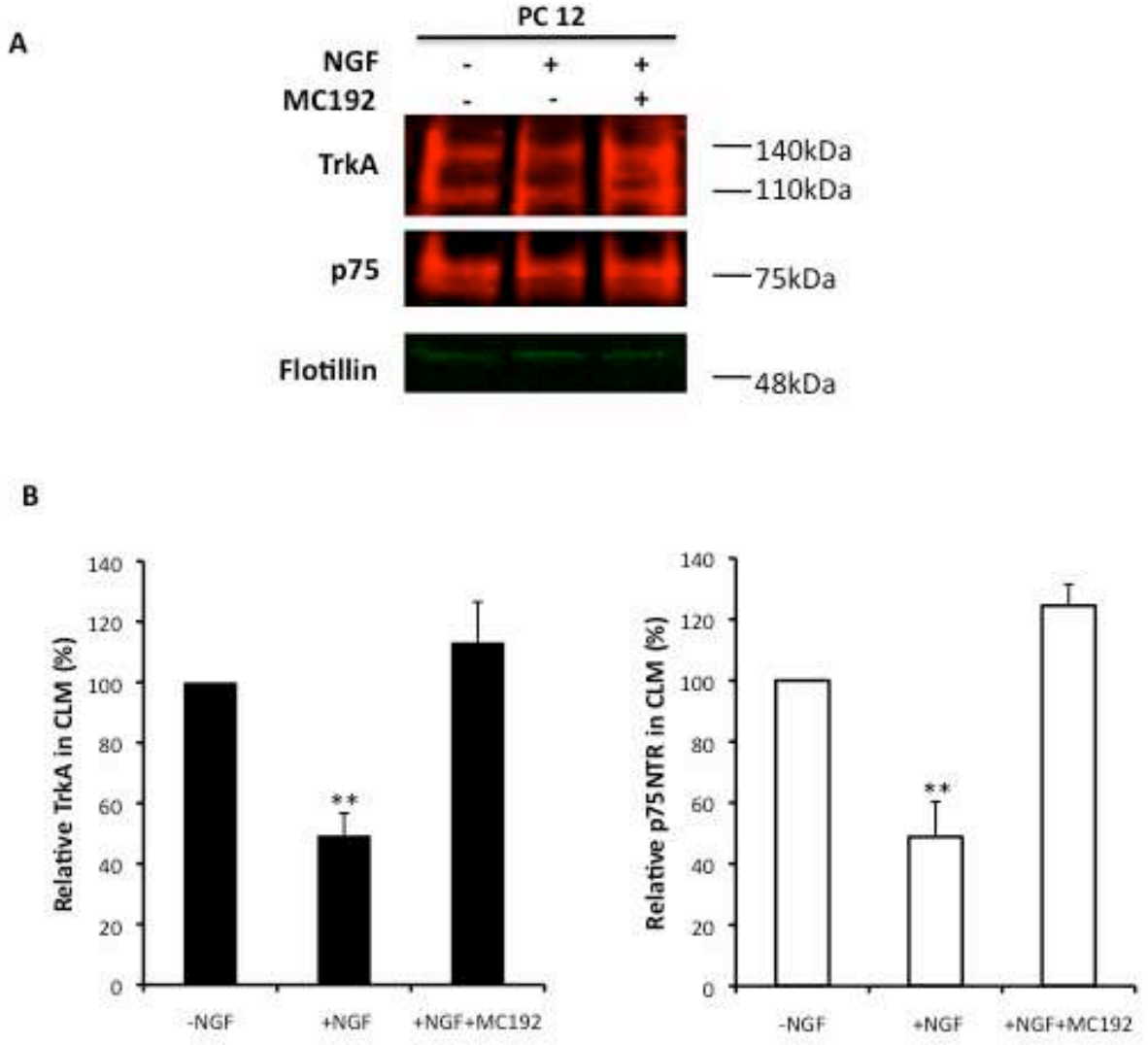
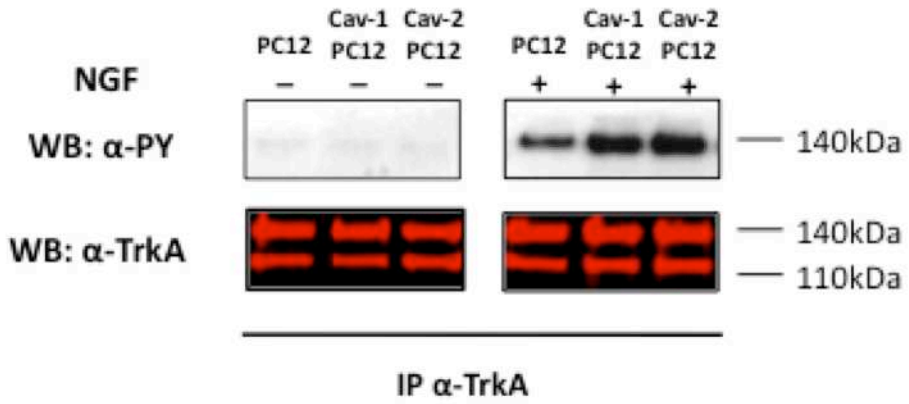


Figure 6

Figure 6: Effect of MC192 on TrkA exit from lipid rafts before and after addition of NGF. Lipid rafts were isolated from wild-type PC12 cells treated with NGF (20 ng/ml for 45 min), treated with $p75^{NTR}$ MC192 monoclonal antibody (8 ng/ml) for 30 min prior to and during NGF exposure (20 ng/ml for 45 min) and from untreated wild-type PC12 cells were isolated as described under Materials and methods. Lipid rafts were then subjected to Western analysis. Nitrocellulose membranes were probed with RTA and anti-flotillin-1 antibody to detect TrkA expression and flotillin-1 was used as a loading control. Revelation was achieved using quantitative infrared fluorescence detection using the Odyssey imaging system. (B) Analysis of TrkA exit from lipid rafts. Level

of TrkA in the lipid rafts was normalized to the Flotillin-1 level for each sample. TrkA exit from the lipid raft fraction was then analyzed and represented as percent of the TrkA in lipid rafts *vs* TrkA in wild-type PC12 cells considered as 100 percent. The data shown are an mean ± SEM of four independent experiments (**P<0.01).



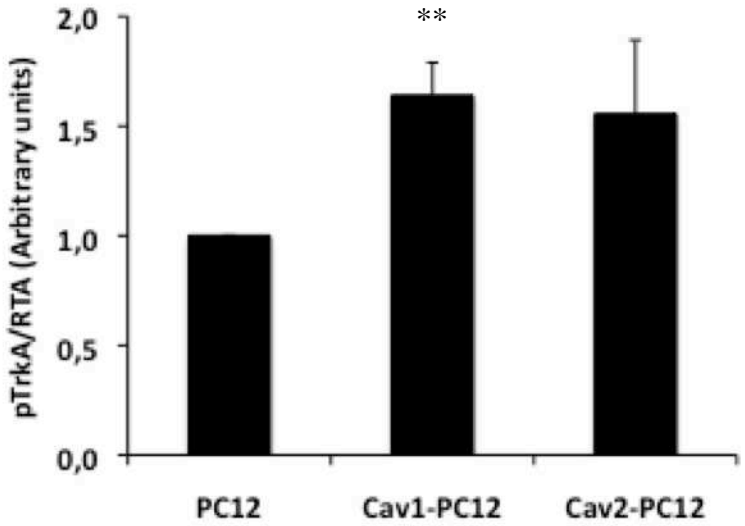


Figure 7: Effect of Cav-1 and Cav-2 expression on TrkA activation. Wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells were left untreated or treated with NGF 20 ng/ml for 10 min after 17 hours of serum deprivation. One mg protein of cell lysate was immunoprecipitated with a polyclonal antibody directed against TrkA C-terminal domain (anti-Trk C-14), and immunoprecipitates were subjected to Western analysis. Membrane was probed with an antibody to phosphotyrosine followed by a secondary antibody conjugated to horseradish

peroxidase that allows chemiluminescence detection. The same blot was re-probed with RTA polyclonal antibody to TrkA followed by a secondary antibody conjugated to a fluorochrome, which allows infrared fluorescence detection using the Odyssey imaging system without cross-reacting with the first antibodies. (Mean ± SEM from three independent experiments; (**P<0.01).

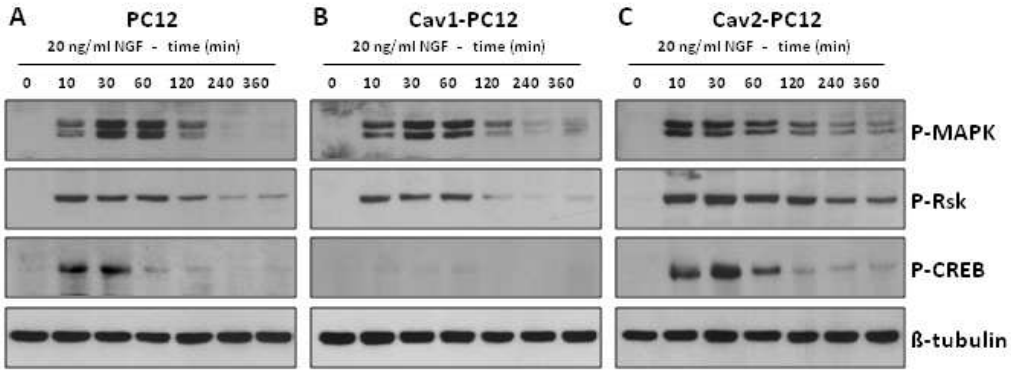


Figure 8: Kinetics of activation of the MAPK pathway in PC12 cells stably-transfected with Cav-1 or Cav-2

Wild-type PC12 cells (A), Cav-1 PC12 cells (B) and Cav-2 PC12 cells (C) were exposed up to 360 min to NGF 20 ng/ml. Cells were collected and proteins were extracted in lysis buffer. Protein concentrations in the lysates were determined and 40 µg were used for Western blot analysis. Blots were probed with phospho-specific antibodies to MAPK, Rsk2 and CREB. Equal loading was controlled using a β-tubulin antibody.

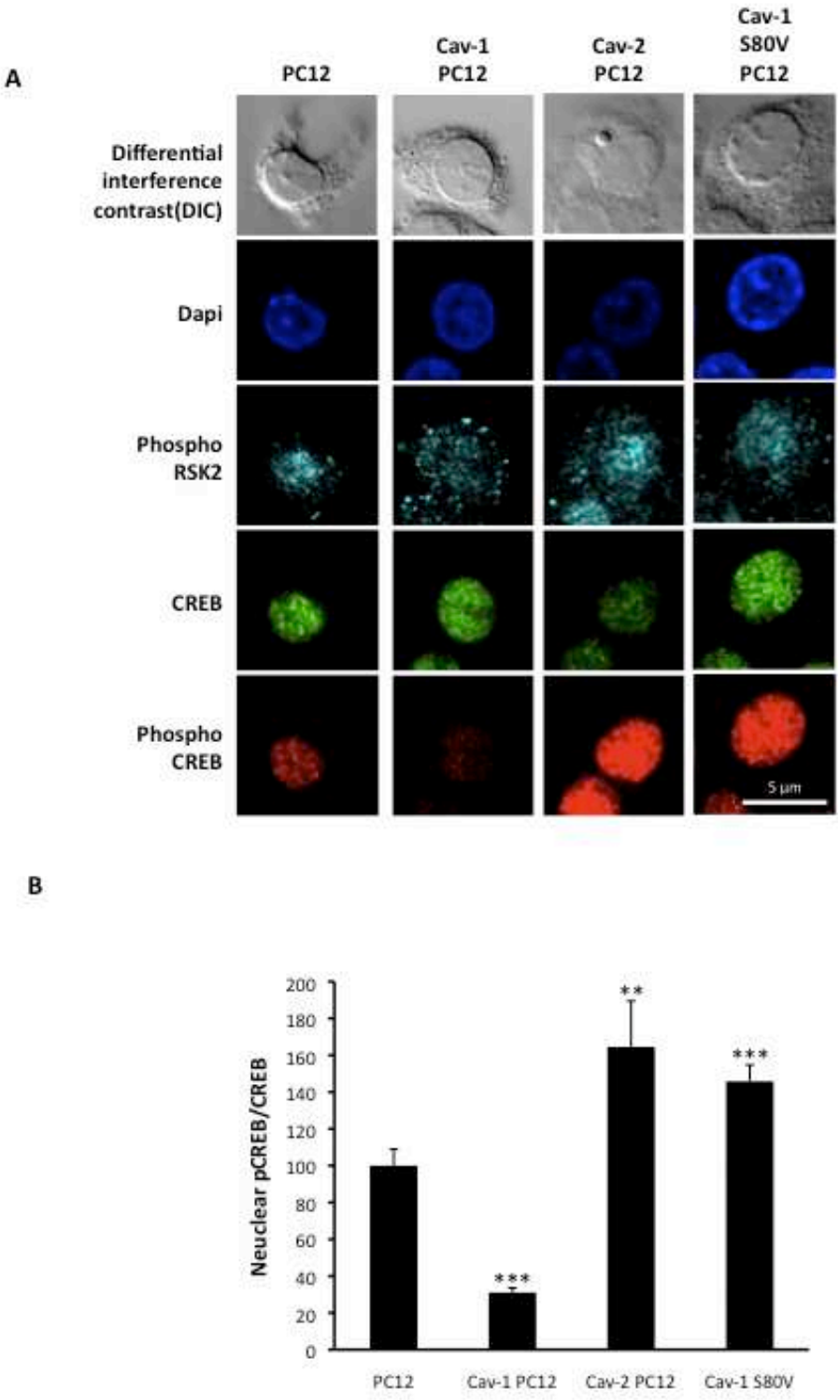


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Figure 9: Effect of Cav-1, Cav-2 and Cav-1 S80V expression on TrkA effector localization. (A) Wild-type PC12 cells, Cav-1 PC12 cells Cav-2 PC12 and Cav-1 S80V PC12 cells were plated on collagen/poly-lysine coated coverslips and exposed to 20 ng/ml NGF for 30 min after 17 hours of serum deprivation. Cells were fixed and simultaneously stained with an anti-pRsk2 antibody (cyan), an anti-CREB antibody (green) and an anti-pCREB antibody (red). Cells were mounted in mounting medium containing DAPI to visualize the nucleus. (B) Quantification of CREB phosphorylation level in the nucleus. For each single cell, quantitation of fluorescence representative of pCREB and CREB in the nucleus was assessed using Image J software. Level of CREB phosphorylation was evaluated by dividing pCREB fluorescence by CREB fluorescence. Results are represented as percent of CREB phosphorylation observed with CREB phosphorylation in wild-type PC12 cells considered as 100 percent. (Mean of 20 to 70 cells per conditions ± SEM; (**P<0.01 ; (***P<0.001).

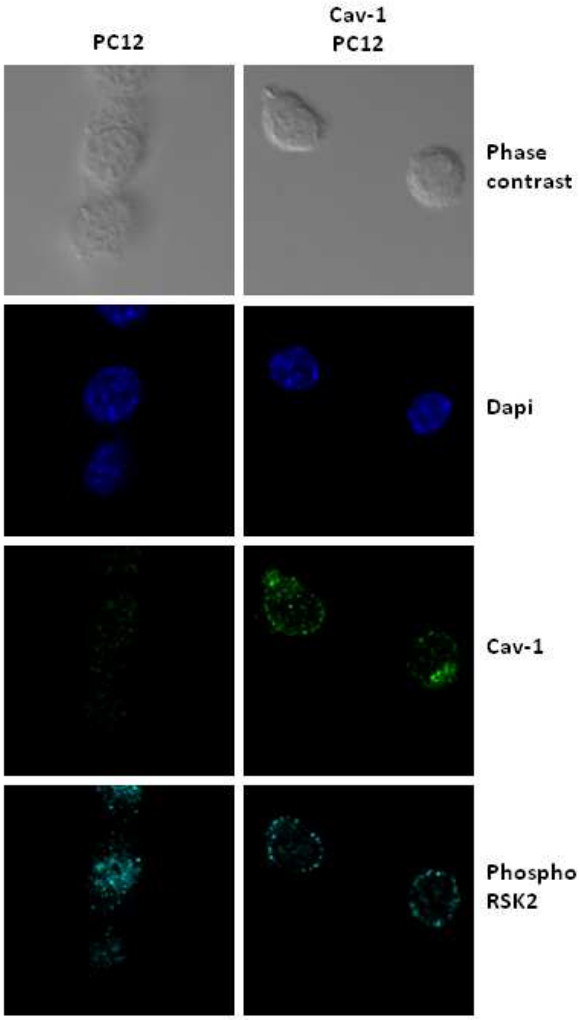

Figure 10: Cav-1 and phospo-Rsk2 localization in wild-type PC12 cells and Cav-1 PC12 cells

Wild-type PC12 cells and Cav-1-PC12 cells were plated on collagen/poly-lysine coated coverslips and exposed to 20 ng/ml NGF for 30 min after 17 hours of serum deprivation. Cells were fixed and simultaneously stained with an anti-pRsk2 antibody (cyan) and an anti-Cav-1 antibody (green).

Supplementary Material

	CAV-1							CAV-2				
Clone	3	11	12	16	18	20	Clone	5	11	13	14	23
Proliferation			+	+			Proliferation	+				+
Differentiation	+	+	+	+	+	+	Differentiation	+	+	+	+	+
WESTERN							**Western**					
Cav-1	++	+++	+	++	+++	+++/ +/-	Cav-2	++	++	+++	++	+
TrkA PO4			+	+	+	+	TrkA PO4	+		+	+	+
pMAPK 42/44	+	+	+	+	+		pMAPK 42/44	+	+			+
pRSK	+	+		+	+	+	pRSK	+	+			
pCREB/CREB	+	+		+	+	+	pCREB/CREB	+	+			
p38				+			p38	+				
pAkt	+			+			pAkt	+	+			
pJNK	+			+			pJNK	+	+			
CLM		+		+		+	**CLM**	+	+			+
IF		+		+		+	**IF**	+	+			

Table S1: Summary of the experiments performed on different clones of PC12 Cells stably-expressing Cav-1 or Cav-2. Analyses of the different clones, performed as described in the Materials & Methods section, yielded comparable results.

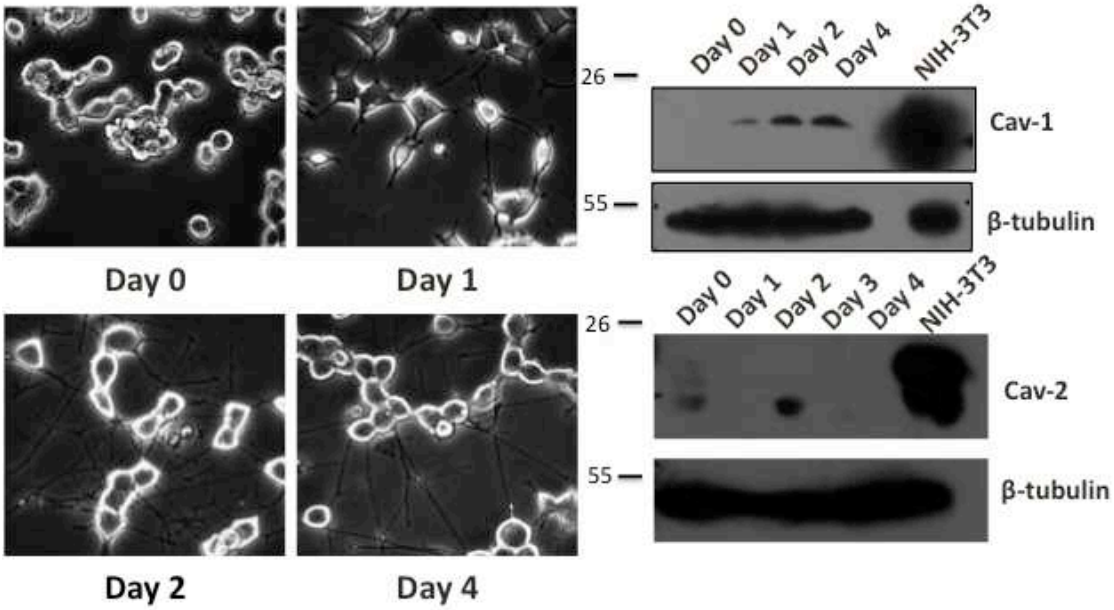

Figure S1: Expression of endogenous Cav-1 and Cav-2 in response to NGF

PC12 cells were exposed to NGF (50ng/ml), harvested and extracted for western blot analysis for Cav-1 and Cav-2 as described in the Materials and methods section. Exponentially-growing NIH-3T3 cells are used as positive control. Beta-tubulin is used as loading control.

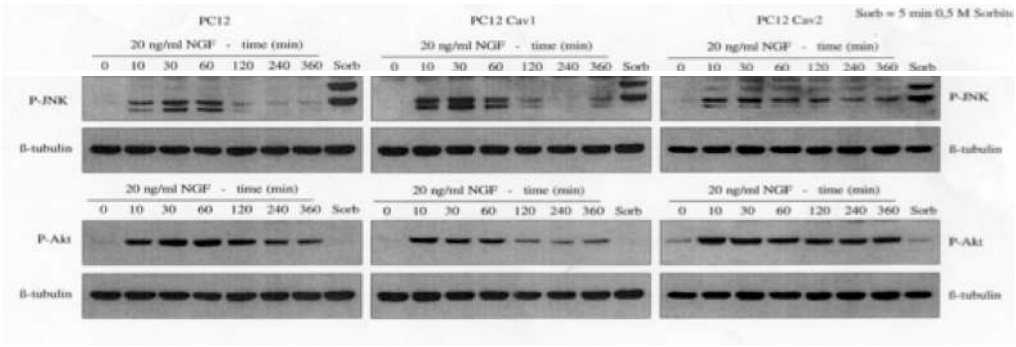

Figure S2: Kinetics of activation of the JNK & Akt in PC12 cells stably-transfected with Cav-1 or Cav-2

Wild-type PC12 cells (A), Cav-1 PC12 cells (B) and Cav-2 PC12 cells (C) were exposed up to 360 min to NGF 20 ng/ml. Cells were collected and proteins were extracted in lysis buffer. Protein concentrations in the lysates were determined and 40 µg were used for Western blot analysis. Blots were probed with phospho-specific antibodies to JNK and Akt. Equal loading was controlled using a β-tubulin antibody.

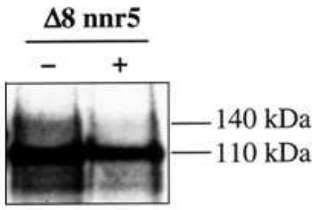

Figure S3: Effect of TrkA deletion on its exit from lipid rafts

Δ8 TrkA-EGFP level in the lipid raft fraction before and after addition of NGF in Δ8-nnr5 PC12 cells (NGF non responsive PC12 cells due to the absence of endogenous TrkA expression, stably expressing Δ8 TrkA-EGFP (Jullien et al., 2003))**.** Lipid rafts of Δ8-nnr5 cells exposed or not to NGF (50 ng/ml for 30 min) were isolated as described under "Materials and methods". Lipid raft fractions were then subjected to Western analysis and membranes were probed with RTA.

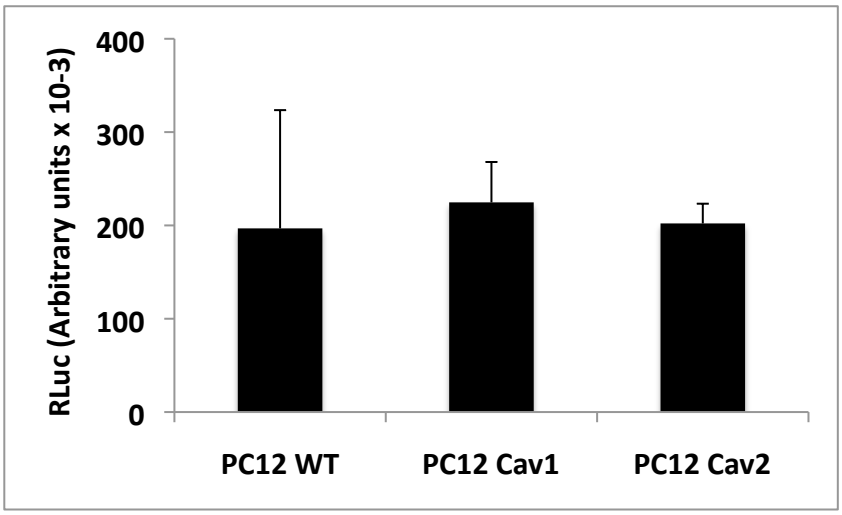

Figure S4: Effect of Cav-1 and Cav-2 expression on H-RasV12 induced p21 induction

The NGF-independent induction of $p21^{WAF1/Cip1}$ was analyzed in wild-type PC12 cells, Cav-1 PC12 cells and Cav-2 PC12 cells. Cells were transiently-transfected with the minimal p21 promoter-Luciferase reporter (p21-Luc) and the constitutively active H-RASV12 as described in Materials and Methods section. The promoter activity then measured in each experiment was normalized to transfection efficiency (average of 2 experiments ± SD).

Membrane microdomains have emerged as essential functional modules of the cells. In Manuscript 2, we have shown that Caveolin-1 and Caveolin-2, the main components of caveolae, differentially regulate NGF mediated cell responses. Caveolin-1 inhibits while Caveolin-2 potentiates NGF signaling and the subsequent celluar response. The non-phosphorylatable Caveolin-1 S80V, no longer inhibits TrkA trafficking and signaling but behaves much like that which is observed with Caveolin-2 PC12 cells. It is conceivable that the loss of its inhibitory effect could be due to the reduction of its targeting to the cell membrane. This is supported by the observation that Cav-1 S80V appears to be mainly located in the nucleus. It is noteworthy that pRSK2 is also found in the nucleus subsequent to activation of these cells with NGF (Fig. 18), in contrast to the membrane retention in Cav-1 clones.

In our study, the "Gain of function" approach has been routinely utilized. Nevertheless, we are also interested in the impact of "Loss of function" of Caveolins on NGF induced cell response. Thus, we downregulated endogenous Caveolin-1 in PC12 cells by transient transfection of siRNA, and we observed a more robust differentiation potential in these cells following NGF activation (Fig. 17). This indicates that the increase in Cav-1 in response to NGF acts to attenuate the rate of differentiation.

Finally, we found that Cav-1 levels are very low in exponentially-growing PC12 cells, but increase on approach to high density or in the presence of NGF (Fig. 20), which is in accordance with the work of Galbiati. In PC12 cells, we observe that Cav-1 gets phosphorylated at tyrosine 14 following NGF activation, which has not yet been reported. Interestingly, there seems to be tendency in nuclear-cyto translocation of PTRF (also known as Cavin-1; Polymerase I and transcript release factor), a latest characterized protein necessary for formation of caveolae (Fig. 19). Very recently, it has been reported that Cav-1 phosphorylation on tyrosine 14 can act via PKC to relieve Egr-1 (early growth response-1) transcriptional inhibition of Cav-1 and PTRF, thereafter, inducing caveolae biogenesis (Joshi et al 2012). We have evidence to suggest that the changes observed regarding the cytolocalisation of Cav-1 and PTRF in PC12 cells following NGF activation, may contribute to the formation of caveolae.

Taken together, our observations have shed light on the impact of selected components of lipid rafts on TrkA trafficking and subsequent signaling in response to NGF.

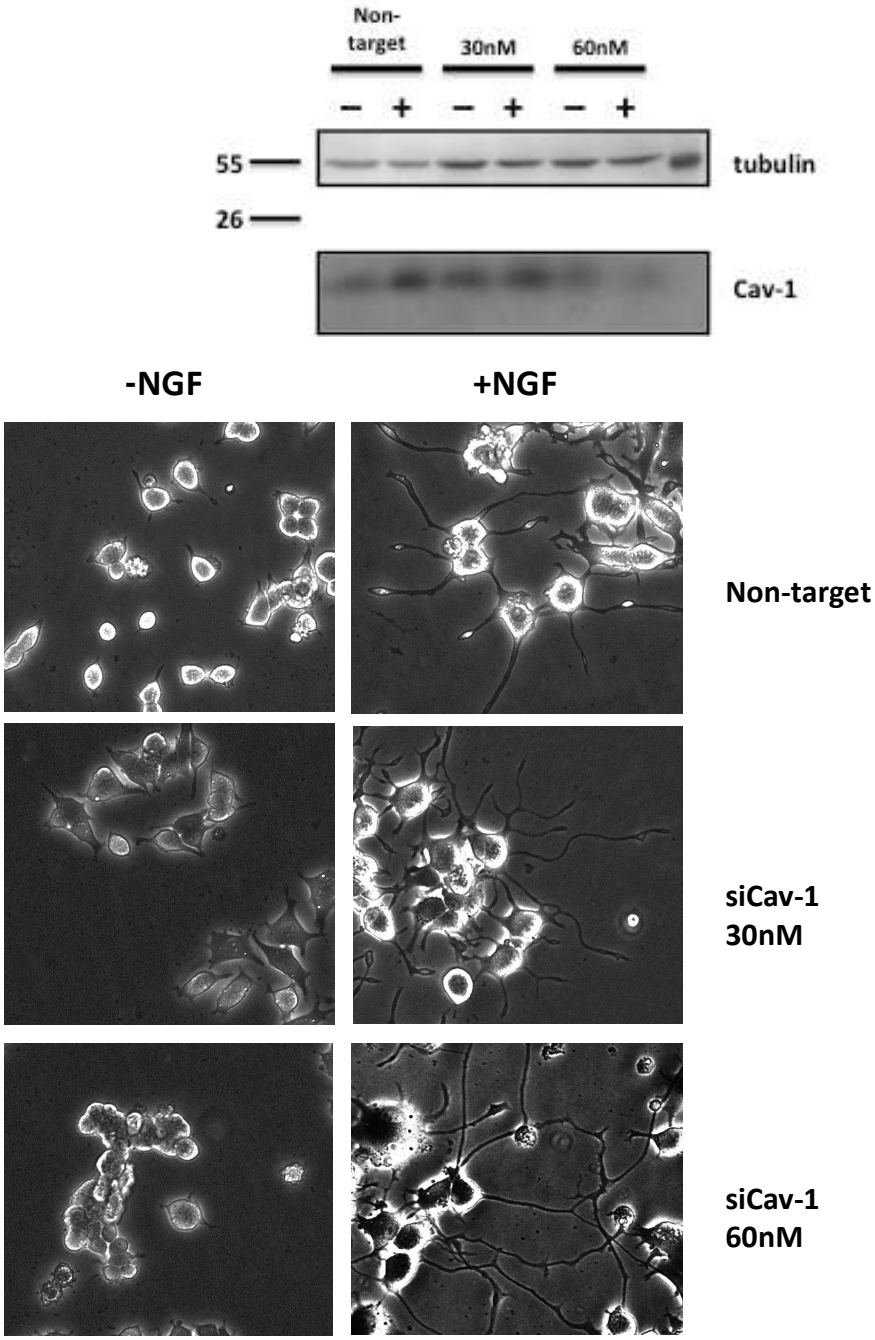

Figure 17. ***Effect of Cav-1 down regulation on NGF induced cell differentiation.*** Wild-type PC12 cells, plated on collagen/poly-lysine coated 6 wells dishes, until cells reached 80% confluent, were transiently transfected with siRNA against caveolin-1 (sc-106996) or scrambled siRNA (sc-36869) using lipofectamin 2000 according to the manufacture's instruction. Cells were maintained in culture medium supplemented with 0 or 50ng/ml NGF. After 3 days, cells were photographed and then subject to immunoblot for the analysis of knockdown efficiency.

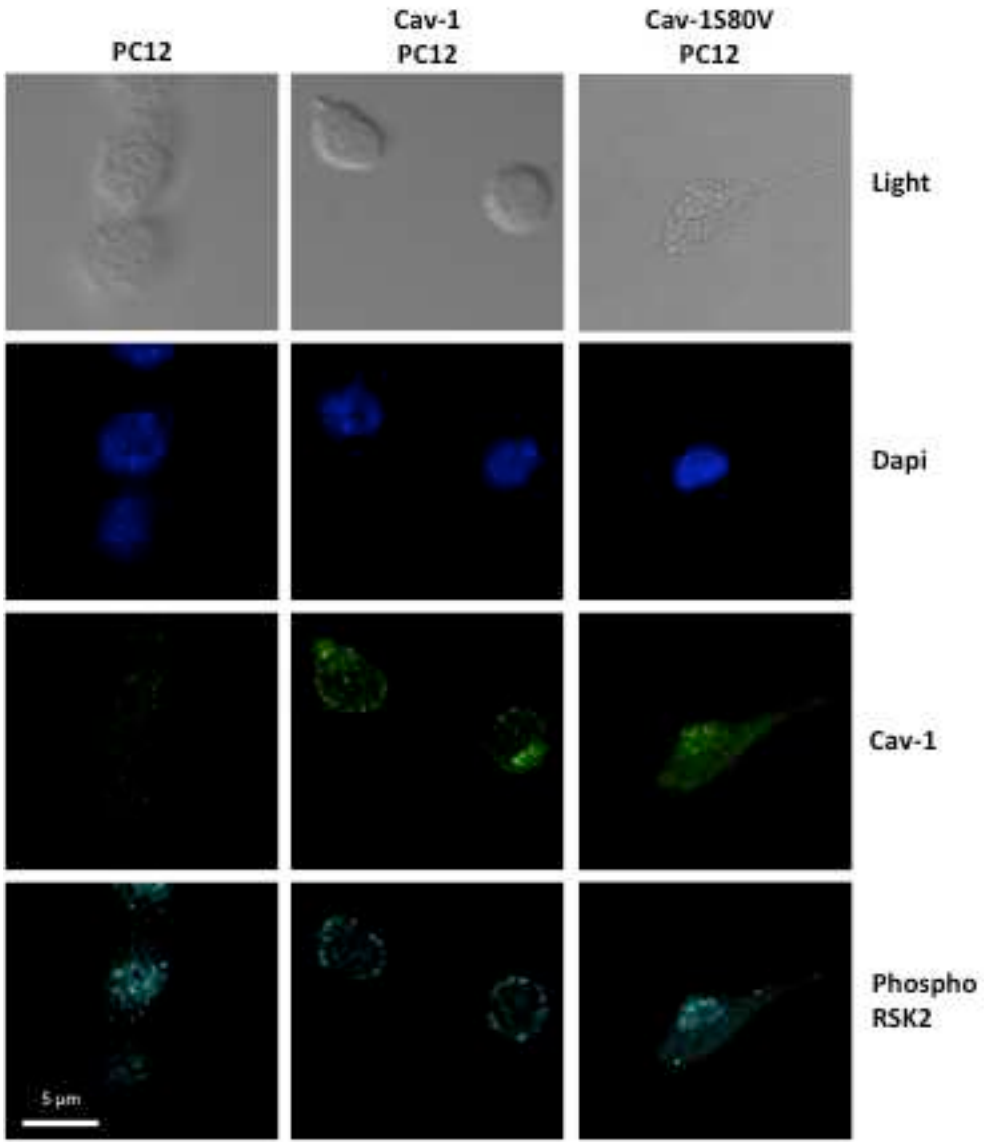

Figure 18. ***Cav-1 and phospo-Rsk2 localization in wild-type PC12 cells, Cav-1 PC12 cells, and Cav-1-S80V cells.*** Wild-type PC12 cells, Cav-1-PC12 cells and Cav-1-S80V-PC12 cells were plated on collagen/poly-lysine coated coverslips and exposed to 20 ng/ml NGF for 30 min after 17 hours of serum deprivation. Cells were fixed and simultaneously stained with an anti-pRsk2 antibody (cyan) and an anti-Cav-1 antibody (green).

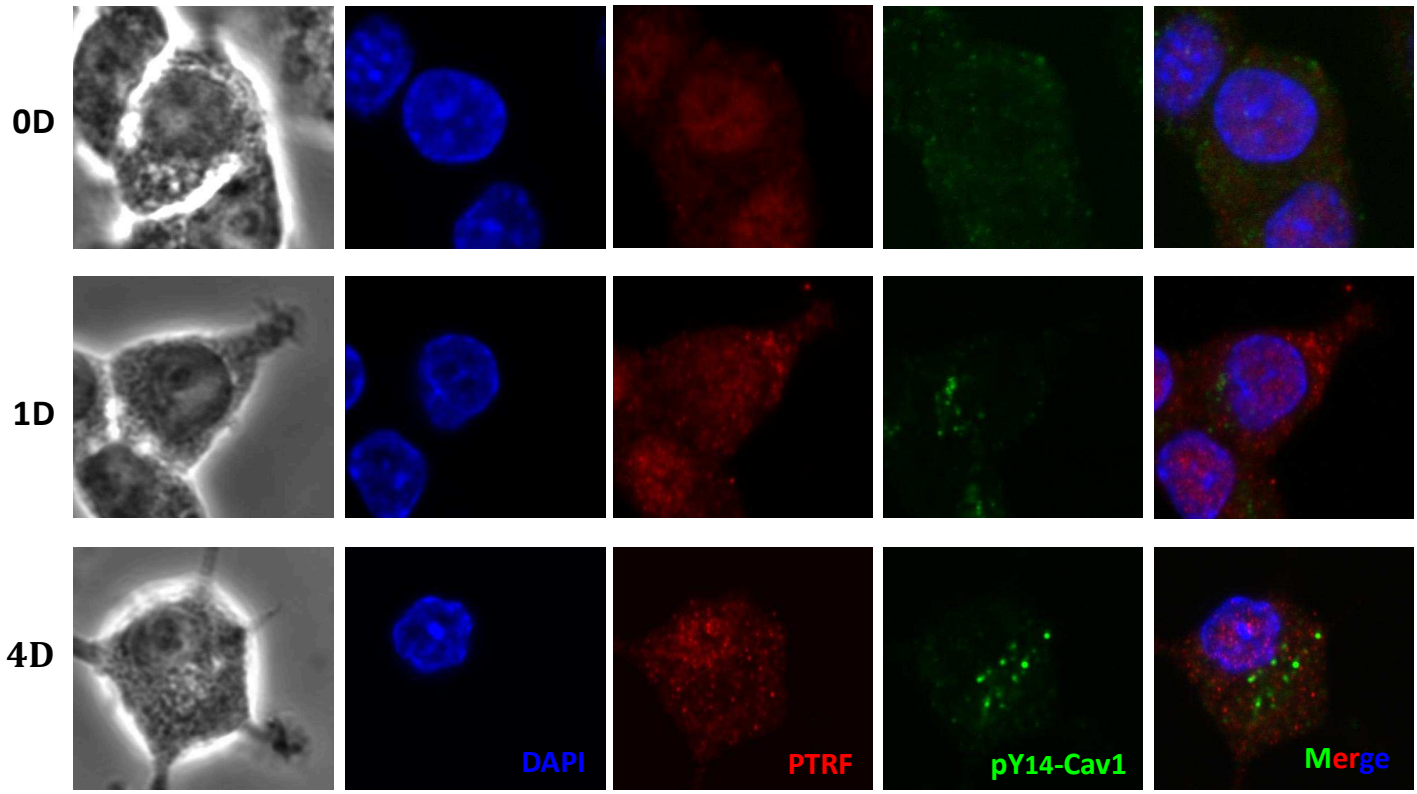

Figure 19. ***Phosporylation of Cav-1 and trans-location of PTRF in the presence of NGF***. Wild-type PC12 cells were plated on collagen/poly-lysine coated coverslips and exposed to 50 ng/ml NGF for 1 day or 4 days in the presence of serum. Cells were fixed and simultaneously stained with an anti-PTRF (abcam-48824) antibody (red) and an anti-pY14Cav-1 (BD Transduction Laboratories, 611338) antibody (green).

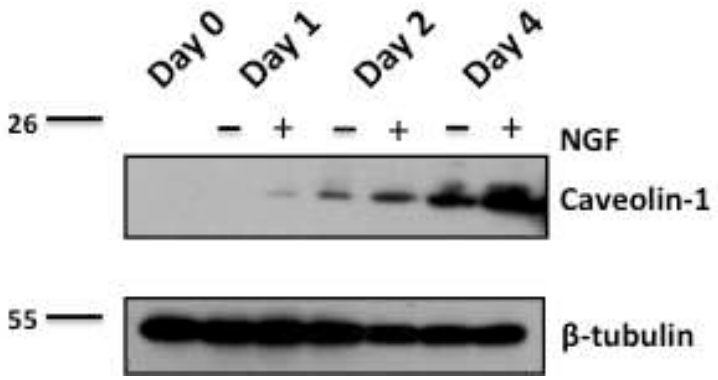

Figure 20. ***Expression of endogenous Cav-1 in the exponentially and NGF-exposed PC12 cells*****.** PC12 cells were exposed to NGF (50ng/ml) or not in the presence of serum. Lysates were prepared from day 0 to day 4, and then subjected to immunoblot analysis with caveolin-1 (Santa Cruz, pAb N-20) specific antibody probes. Equal loading was controlled using a β-tubulin antibody.

CONCLUSIONS AND PERSPECTIVES

In our study, we have shown that caveolins have the potential to influence NGF induced receptor internalization and subsequent signaling. From the data presented in this thesis, we can draw some general conclusions:

- Cav-1 inhibits NGF signaling and subsequent cell differentiation, without impairing transient MAPK pathway activation. Rather, it sequesters NGF receptors and downstream effectors, and phosphorylated-Rsk2 at the plasma membrane, resulting in the prevention of phosphorylation of CREB.
- Cav-2 potentiates NGF signaling and subsequent cell differentiation, which is accompanied by sustained activation of downstream effectors, and standard internalization of the receptors.
- Cav-1-S80V, the non-phosphorylatable Cav-1 mutant, does not inhibit NGF signaling and subsequent cell differentiation. On the contrary, it behaves much like with Cav-2 when expressed in PC12 cells.
- Cav-1 levels are very low in exponentially-growing PC12 cells, but increase on approach to high density or in the presence of NGF which seems to be accompanied with the nucleo-cytoplasmic translocation of PTRF. In light of the observed nucleo-cytoplasmic translocation of PTRF. These observations raise the question of how these proteins might be regulated, and how they cooperate in NGF signaling.

In this work, we have made several significant observations and from those, many questions arose. The understating processes of these questions help us gain more insights, and meanwhile quest deeper questions, the most prominent of which are discussed below:

Characterization of other pathways: In our study, we were focusing on the impact of caveolins in the NGF signaling leading to cell differentiation and proliferation. However, NGF also stimulates cell survival, so it would thus be of great interest to study the impact of their expression on localization and activation of other effectors

involved in cell survival, such as PI3K, Akt and NFκB. And some preliminary experiments, for instance, to look at the survival of various caveolin PC12 clones when deprived of NGF, have been initiated.

Monitoring protein-protein interactions: The immunoprecipitation studies have already shown that Cav-1 can interact both with TrkA and $p75^{NTR}$, however, the respective binding motifs of Cav-1 for and TrkA and $p75^{NTR}$ remain unknown. To accomplish that, various truncations of above mentioned proteins have been constructed in our lab, and will be tested in either yeast two hybrid or co-immunoprecipitation assays. Since PTRF has been found to be directly associated with Cav-1, it will be very interesting to appreciate if, when and where they interact with regard to the NGF receptors. It is also to note that, PTRF, which has not yet been characterized for its interaction with TrkA and $p75^{NTR}$, could be a very interesting candidate in this study so as to broaden our knowledge on the interplay between NGF receptors and various of caveolae proteins.

Impact of other ligands on receptor exit from lipid raft: In our study, we have shown that when the cells are exposed to NGF, both TrkA and $p75^{NTR}$ leave the lipid rafts. It would be of particular important to appreciate the impact of other ligands, not able to bind TrkA, for example, on the movement of the NGF receptors from lipid rafts. Further to study the behavior of another partner of p75NTR, sortillin, would add another dimension to our understanding, were it to behave in a similar manner or not to TrkA when exposed to proNGF. The objective is to appreciate if there is a differential regulation of raft exit depending on ligand receptor pairs, and thus, the corresponding physiological responses.

Real time monitoring of receptor trafficking: Nowadays**,** the time-lapse microscopy and rapid spinning disk confocal microscopy provide very powerful tools in monitoring receptors trafficking in real-time and space. For instance, formation and regulation of lipid rafts could be tracked in living cells with Cholera toxic subunit B (CT-B), which specifically binds to the glycosphingolipid GM1. With the combination

of TrkA, $p75^{NTR}$ or other receptors of interest fused to fluorescent proteins, a real-time and dynamic visualization of various signaling events in the specific intracellular organelles can be obtained. Moreover, various ligands such as BDNF or pro-NGF could be tested, more quickly and efficiently, under this frame.

Impact of endogenous Caveolin/PTRF proteins in PC12 cells: In this work, we have found that Cav-1 levels are very low in exponentially-growing PC12 cells, but increase on approach to high density or in the presence of NGF, which seems to be accompanied with the nucleo-cytoplasmic translocation of PTRF. These observations raise the questions of the how these proteins might be regulated to the stimulus, and how they cooperate in NGF signaling.

Impact of mutated Cav-1 on TrkA trafficking and signaling: In this study, we have shown the potentiation of the NGF response by Cav-2 and the S80V Cav-1 mutant. Why this single amino acid substitution would be enough to transform Cav-1 into a protein functionally resembling Cav-2 is very interesting, and requires further clarification. In particular, if and how Cav-1 gets phosphorylated at this site following NGF treatment would be of import to understanding the underlying mechanism of action. Moreover, clonal PC12 cells expressing S80E (sustained phosphorylation), S80A (non-phosphorylatable) and P132L have already been established in our lab for the purpose of this study. It would be also very interesting to study the impact of other Cav-1 mutations found in breast cancer, on TrkA trafficking and signaling.

Overall, the studies performed in the context of this thesis, and the questions they raise for the future, offer new insights in the understanding of NGF signaling pathways in relation to lipid rafts and raft components. It is expected that their extension to other physiological processes, such as cancer may offer opportunities for diagnosis and or treatment

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APPENDIX

YU Lingli PUBLICATIONS&CONFERENCES

YU Lingli Publications:

PhD Degree

1. **Yu L***, Ding Y*, Spencer A, Ma J, Lu R, Rudkin BB[#], Yuan C.[#] (2012**).** Dorsal root ganglion progenitors differentiate to gamma-aminobutyric acid-and choline acetyltransferase-positive neurons***. Neural Regeneration Research***. 7:485-491.

2. Lu R, Ma J, Zhang Y, **Yu L**, Spencer A, Cluet D, Rudkin BB[#], Yuan CG[#] (2011). Effect of Caveolin-1 on Proliferation and Survival of MCF-7 Breast Cancer Cells. ***Ch J Cell Biol*** 33:983-987.

Master's Degree

3. Zhu Q, MA J, **Yu L**, Yuan C (2009). Grafted neural stem cells migrate to substantia nigra and improve behavior in Parkinsonian rats. ***Neuroscience Letters***. 462(3): 213-218.

4. Ma Ji, Shan L, **Yu L**, Yuan CG. (2008). Expression of galanin and galanin receptors in neurogenesis regions of adult mouse brain and effect of galanin on the neural stem cell's differentiation. ***Journal of Molecular Cell Biology***. 41:359-366.

Submitted or in preparation:
PhD Degree

1. Spencer A*, Guili V*, **Yu L**, Ding Y, Reynaud F, Falk J, Ma J, Jullien J, Koubi D, Gautier E, Cluet D, Yuan CG[#], Rudkin BB[#]. Nerve Growth Factor Signaling from Membrane Microdomains to Nucleus : Differential Regulation by Caveolins. (Submitted)

2. **Yu L**, Reynaud F, Falk J, Yuan C[#], Rudkin BB[#]. Highly efficient non–viral protocol for gene delivery in mouse dorsal root ganglia neurons.

3. Cluet D, Dibenedetto S, Drouin E, Spichty M, **Yu L**, Léault J, Thomas JL, Zhang Q, Ding S, Gangloff Y G, Yuan CG, Schaeffer L, Zoli M, Rudkin BB. Computer assisted global image analysis of skeletal muscle cross sections: from fiber size to central nuclei.

*Equal contribution #Co-corresponding authors

Participation in international conferences:

1. ***NGF 2010***. Neutrophic factors in health and disease. June 10-13, 2010. Helsinki, Finland. (Poster presented by lingli YU)

2. ***Sortilin 2010***. A meeting on structural, molecular and cell biology of the *Sortilin* (Vps10p-D) receptor family and related diseases. June 14-17, *2010*. *Aarhus*, Denmark.

3. XIX World Congress on Parkinson's Disease and Related Disorders. December 11th-14th, 2011. Shanghai, China.

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Dorsal root ganglion progenitors differentiate to gamma-aminobutyric acid- and choline acetyltransferase-positive neurons*☆○

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Abstract

This study examined the isolation and differentiation of dorsal root ganglion progenitor cells for therapeutic use in neurodegenerative diseases. Rat embryonic dorsal root ganglia progenitors were isolated and purified using the differential adhesion method combined with cytosine arabinoside treatment. After culture in serum-free medium supplemented with B27, basic fibroblast growth factor and epidermal growth factor, these cells remained viable and survived for more than 18 months *in vitro*. Most cells differentiated to neurons that were immunoreactive for gamma-aminobutyric acid and choline acetyltransferase as detected by immunohistochemical staining. In addition, nerve growth factor and neurotrophic tyrosine kinase receptor expression were also observed in dorsal root ganglion progenitors and differentiated cells. K252a, an inhibitor that blocks nerve growth factor-induced signaling, inhibited cell survival, suggesting the possible existence of a nerve growth factor autocrine loop in these proliferating cells.

Key Words: dorsal root ganglion; neural progenitor; differentiation characterization; nerve growth factor; tyrosine kinase receptor type 1

Abbreviations: DRG, dorsal root ganglion; MAP2, microtubule-associated protein 2; CNP, cyclic nucleotide phosphodiesterase; ChAT, choline acetyltransferase; GABA, gamma-aminobutyric acid; NGF, nerve growth factor

INTRODUCTION

Neural stem cells or progenitors exist in the nervous system[1] and various non-neural peripheral tissues such as gut[2], skin[3], connective tissue[4] and even heart[5] of embryonic or adult mammals. Studies to isolate and characterize such progenitors have attempted to purify particular phenotypes with which to generate pluripotent cell lines, with the objective of increasing the efficacy of therapy with grafts of neuronal precursors or mature neurons into injured neurological systems to replace degenerated neurons such as acetylcholine neurons in Alzheimer's disease and dopaminergic neurons in Parkinson's disease[6]. Many sensory organ diseases resulting from aging or wounding are usually accompanied by sensory nerve fiber loss or sensory neuron degeneration. Thus, it is important to isolate sensory neural progenitors or stem cells for cell replacement therapy of sensory organ diseases[7]. An early study demonstrated that allotransplant and xenotransplant of fetal dorsal root ganglion (DRG) neurons could survive and reinnervate the denervated host peripheral targets in a variety of locations in a ganglioectomized adult rat[8]. In addition, xenografts of human fetal DRGs could extend axons into the central nervous system to form functional connections in the deafferented rat spinal cord[8-9]. Although neural stem/progenitor cells have been isolated from postnatal and adult DRG[10-12], adult otic placode-derived spiral ganglion[13] and inner ear[14], the differentiation status and neurotransmitter phenotype have not been characterized. In addition, embryonic stem cell-derived neuronal precursors can survive and differentiate to glia and neurons after central nervous system transplantation[15-16]. In the present study, DRG progenitors were isolated and purified from embryonic day 17 rats to investigate their proliferation and differentiation capacities. We hope that our study will be helpful for understanding neurogenesis in the peripheral nervous system and for harnessing the potential application of peripheral neural stem cells for the treatment of sensory organ diseases.

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RESULTS

Purification of neuronal progenitors from embryonic day 17 DRG

To obtain a high yield of neurons from DRG neuronal progenitors, cells from embryonic day 17 rat DRGs were purified using the differential adhesion method combined with 0.5 μM cytosine arabinoside treatment. Cultures comprising approximately 90% neurons were obtained after purification (Figure 1).

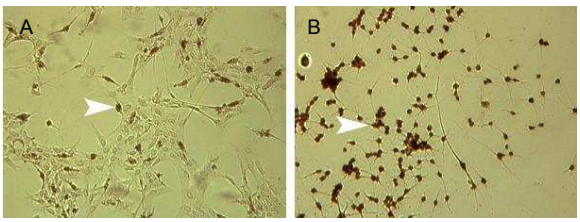

Figure 1 Dorsal root ganglion (DRG) neuronal progenitors before (A) and after (B) purification (magnification × 200).

Cells were plated on coverslips and purified by the differential adhesion method combined with 0.5 μM cytosine arabinoside treatment, after which the cells were fixed and immuno-labeled with a monoclonal anti-microtubule-associated protein 2 antibody.

Positive staining was revealed by the avidin-biotin-peroxidase complex method. Arrows show microtubule-associated protein 2 positive cells.

Progenitors derived from embryonic day 17 DRG can be expanded and passaged long term (Figures 2 and 3)

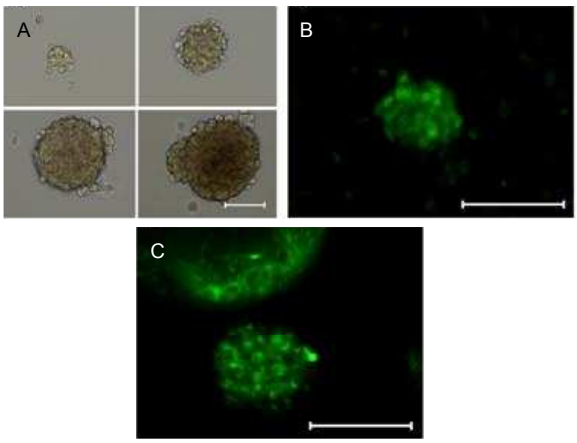

Figure 2 Dorsal root ganglion (DRG) contained self-renewable neurospheres.

(A) Formation of spheres from purified DRG neurons; (B) spheres stained for incorporated 5-bromodeoxyuridine (green); (C) spheres stained for stem/progenitor cell marker nestin and revealed by fluorescein isothiocyanate-conjugated secondary antibody (green).

Samples were visualized *via* fluorescent microscopy. Scale bars represent 25 μm.

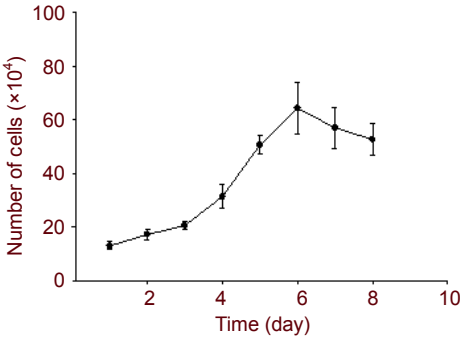

Figure 3 Growth curve of dorsal root ganglion neuronal progenitors at passage ten. 10 000 cells were seeded per well in triplicate in 24-well plates. The number of cells was measured every 24 hours for 8 days. Results represent mean ± SEM of three independent experiments.

To assess the ability of progenitor cells to proliferate, primary spheres were dissociated to a single-cell suspension, and new or secondary spheres were obtained between 1 to 2 weeks (Figure 2). Bromodeoxyuridine (BrdU) incorporation and subsequent immunostaining for BrdU and nestin revealed that neuronal progenitors existed in the embryonic DRG neurons (Figure 2). Cells maintained in culture for periods of more than 1 year retained their dependency on epidermal growth factor and basic fibroblast growth factor. In the early stage of culture (1–5 passages), there was a moderate increase in cell numbers. After passage 5, the growth rate increased, followed by stabilization at passage 15. The growth curve was measured at passage 10, and demonstrated that a logarithmic growth phase occurred 4 to 6 days after sub-culturing, and that the saturated phase occurred on day 6, after which cells aged and died (Figure 3).

Identification of DRG progenitors after differentiation

Co-localization of microtubule-associated protein 2 (MAP2) and cyclic nucleotide phosphodiesterase (CNP)

To investigate the subtype of cells derived from the DRG progenitors, cells were stained for MAP2, glial fibrillary acidic protein and CNP. We observed that most cells were immunoreactive for MAP2, with a high proportion being CNP positive cells, whereas no glial fibrillary acidic protein positive cells were observed. CNP is highly expressed in myelin-forming glial cells and is widely used as a immunohistological marker for oligodendrocytes[17]. However, in the present study, CNP was present in patches throughout the soma and dendrites of cells, and significantly co-localized with MAP2. This expression pattern suggested an important role for CNP in the neuronal progenitors (Figure 4). Western blot analysis confirmed the immunocytochemical findings (Figure 5).

DRG progenitors differentiate to cells co-expressing choline acetyltransferase (ChAT) and gamma-aminobutyric acid (GABA)

To assess the progenitor cell neuronal subtypes, we screened for the presence of neurotransmitters using

antibodies specific for tyrosine hydroxylase (TH), GABA, ChAT and 5-hydroxytryptamine/serotonin (5-HT). We observed both ChAT and GABA positive reactivity in the majority of the cells examined. However, 5-HT and TH were undetectable (Figure 4). Immuno-labeling analysis implied the co-localization of GABA and ChAT[4].

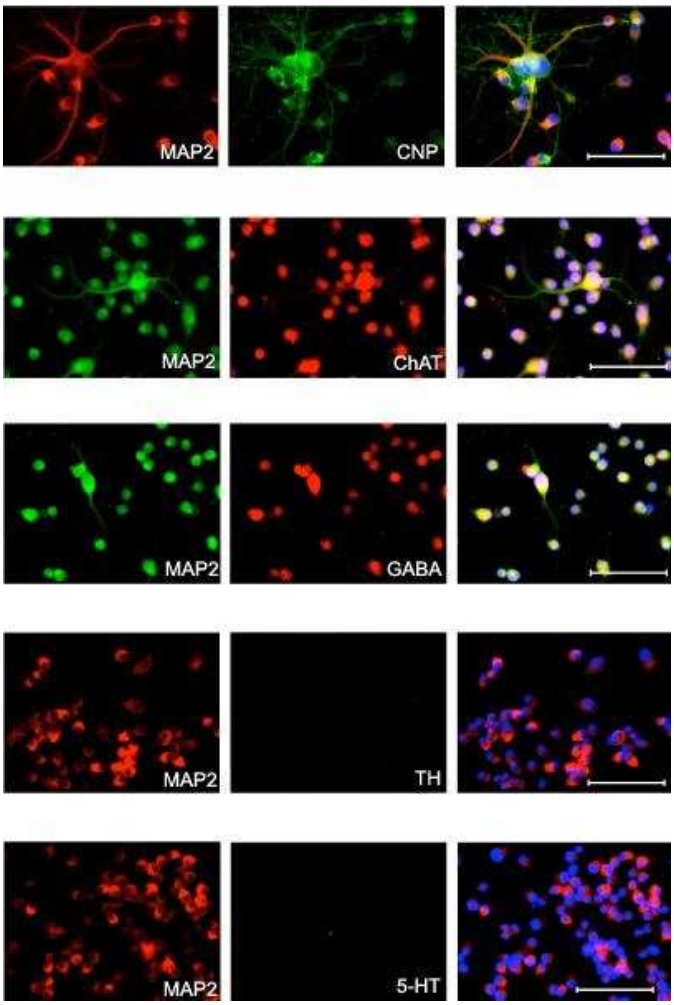

Figure 4 Immunofluorescence labeling of microtubule-associated protein 2 (MAP2), cyclic nucleotide phosphodiesterase (CNP), choline acetyltransferase (ChAT), gamma-aminobutyric acid (GABA), tyrosine hydroxylase (TH) and 5-hydroxytryptamine (5-HT) of differentiated dorsal root ganglion cells.

The left columns represent MAP2-positive cells; the center columns represent CNP, ChAT, GABA, TH and 5-HT positive cells.

Fluorescence was revealed by fluorescein isothiocyanate (green) or tetraethyl rhodamine isothiocyanate (red)-conjugated secondary antibodies. Merged images are shown in the right columns.

Nucleii were counterstained using 4',6-diamidino-2-phenylindole (blue). All images were taken at the same magnification.

Samples were visualized *via* fluorescent microscopy. Scale bars represent 25 μm.

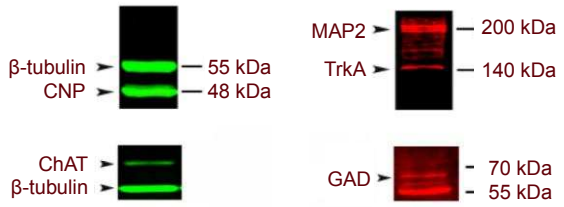

Figure 5 Western blot analysis of cyclic nucleotide phosphodiesterase (CNP), microtubule-associated protein 2 (MAP2), tyrosine kinase (TrkA), choline acetyltransferase (ChAT) and glutamic acid decarboxylase (GAD) proteins in dorsal root ganglion progenitors at passage 25, with β-tubulin as the endogenous control.

The primary antibodies were probed with IR-Dye-880 or IR-Dye -680-conjugated secondary antibodies. Protein bands were detected using the Odyssey Infrared Imaging System.

Tyrosine kinase (TrkA) and nerve growth factor (NGF) expression in DRG progenitors and differentiated neural cells

NGF is essential for the survival, differentiation, and maintenance of many sensory neurons. NGF induces biological effects through binding with receptor tyrosine kinase, TrkA, or the death-like domain containing receptor, $p75^{NTR}$, or both depending on their presence on a given cell and their surface localization. NGF activates TrkA and/or $p75^{NTR}$, triggering signal transduction cascades. Most DRG neurons require NGF for survival in early development[18]. We maintained and analyzed DRG progenitors *in vitro* for more than 1 year without adding exogenous NGF to the culture medium. As DRG cells can express NGF following injury *in vivo* [14], NGF and TrkA may also be involved in the proliferation and differentiation of the progenitor cells. The expression of TrkA and NGF was evaluated in progenitors, and differentiated cells by immunofluorescence. We observed that the majority of the cells were stained positive for TrkA and NGF (Figure 5). Western blot analysis confirmed the expression of TrkA protein, but not NGF protein in those cells (Figure 6), possibly indicating that NGF was either expressed at a very low level or was being rapidly degraded.

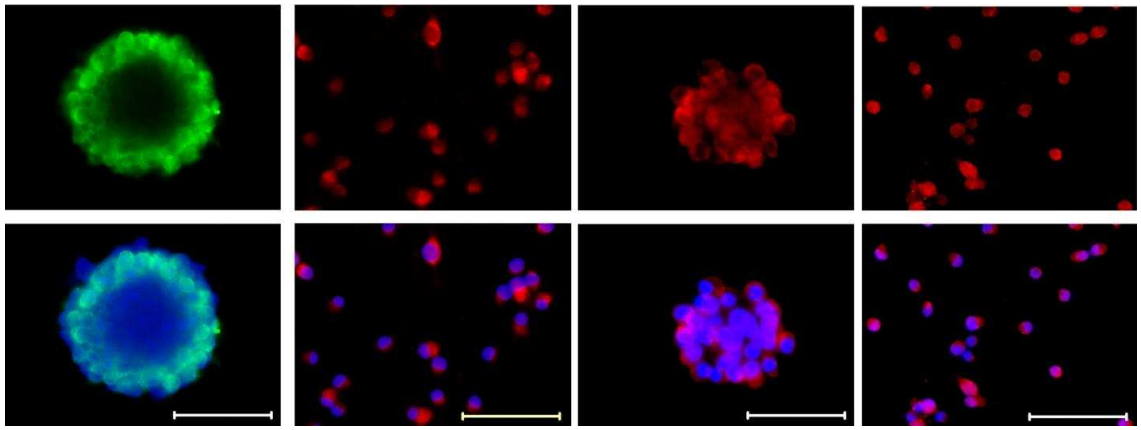

Figure 6 Immunofluorescence labeling for tyrosine kinase (TrkA) and nerve growth factor (NGF) in proliferating and differentiated dorsal region ganglion (DRG) cells.

The left columns represent TrkA-positive cells; the second columns represent TrkA-positive cells in differentiated DRG cells; the third columns represent NGF-positive cells in proliferating DRG cells; and the fourth columns represent NGF-positive cells in differentiated DRG cells.

The fluorescence was revealed by fluorescein isothiocyanate (green) or tetraethyl rhodamine isothiocyanate (red)-conjugated secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). All images were taken at the same magnification. Samples were visualized *via* fluorescent microscopy. Scale bars represent 50 μm.

As abundant TrkA and NGF were detected in differentiated DRG neurons by immunofluorescence, we investigated whether endogenous NGF produced by the cells was required for their maintenance. K252a, an inhibitor which blocks NGF-induced signaling in PC12 cells[19-21] was added to the culture medium. Survival rates of the cells were diminished with increasing concentrations of K252a (Figure 7). This result implied that NGF might have a key role in supporting the survival and function of DRG progenitors.

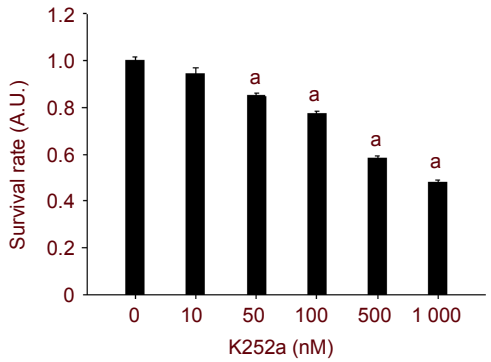

Figure 7 K252a, an inhibitor that blocks nerve growth factor-induced signaling, attenuates the survival of dorsal region ganglion (DRG) progenitors. DRG progenitors were maintained in differentiation medium at 24 hours before incubation with K252a at increasing concentrations.

A cell counting kit-8 assay was used to evaluate cell viability. Data are presented as the percent viability of vehicle-treated control cells. Values are expressed as the mean ± SEM of three independent experiments.

Fisher's least significant difference test was used to analyze the statistical significance of the differences. $^{a}P < 0.01$, *vs.* untreated cells (0 nM) K252a.

DISCUSSION

Previously, several *in vitro* systems have been described for the derivation of neural stem/progenitor cells from the central nervous system or peripheral nervous system[22]. The entire peripheral nervous system is derived from a migratory cell population termed neural crest cells. These cells generate a wide variety of cell and tissue types during embryonic and adult development including cartilage and bone, connective tissue, pigment and endocrine cells as well as neurons and glia amongst many others. Due to these specific properties they have been studied for their potential application in cell-based tissue and disease-specific repair[23]. DRGs are derived from precursors in the neural crest, suggesting that early postnatal DRGs may contain a population of neuronal precursors that retain their capacity for neurogenesis. In the present study, we report the purification of a DRG neuronal stem/progenitor cell, and the further characterization of proliferation and differentiation of these cells.

Progenitors derived from embryonic DRGs can be expanded long term

DRG cells from embryonic day 17 rats were purified using the differential adhesion method followed by treatment with cytosine arabinoside that causes the selective removal of glial cells. After purification, cells were cultured in serum-free medium DMEM/F12 (1:1) supplemented with B27, basic fibroblast growth factor and epidermal growth factor. Cells proliferated slowly in the first 2 to 3 weeks. After this time point, neurospheres were observed and new spheres were generated after each passage. These cells were routinely passaged once every 1 to 2 weeks depending on the density

seeded. The growth curve of the 15th passage cells demonstrated that progenitors from embryonic DRGs could proliferate efficiently. Cells were maintained in culture for more than 1 year and retained their potential for proliferation and differentiation as specialized subtypes. Such long-term proliferation was unexpected and to our knowledge has not been reported previously. The incorporation of BrdU, together with positive nestin immunofluorescence, suggested that the purified cells from embryonic DRG were proliferating[24]. Thus, we termed these cells DRG progenitors.

DRG progenitors exhibit characteristics similar to neural precursors

To investigate the differentiation characteristics of embryonic DRG progenitors, cells were incubated in culture medium with serum and without exogenous basic fibroblast growth factor and epidermal growth factor. Most DRG progenitors expressed MAP2, 40% of which were positive for both MAP2 and CNP. CNP is also present in various cell types in addition to myelinating cells, such as lymphocytes, retinal, liver, muscle, and Purkinje cells and hippocampal neurons[25-27], indicating that CNP is also expressed in some subpopulations of neuronal cells. CNP is a regulator of tubulin polymerization, where it associates with the cytoskeleton and has microtubule-associated protein-like characteristics[28]. Taken together and combined with our findings, these results suggest that CNP may be important in the modulation of the cytoskeleton in the differentiating DRG progenitors.

In addition to glial cells, mature DRGs are composed of many neurons with different morphologies and distinct biochemical properties. How distinct cell fates are generated from an initially homogeneous cell population in the embryonic DRG is a compelling question in developmental biology. Moreover, once DRG precursors aggregate to their final positions, there are still a number of “fate choices” that can occur[29]. The sensory neurons present in mature DRG receive sensory information including pain, temperature, touch and proprioception. Cells in DRG produce multiple neurotransmitters, such as GABA, acetylcholine, and glutamate catecholamine. Tyrosine hydroxylase is expressed in a subpopulation of small DRG neurons in the adult mouse[30].

Since therapeutic applications may require considerable *in vitro* expansion of neural precursors, we next investigated whether the expanded, multi-passage DRG progenitors retained key neural precursor properties. The purified DRG cells formed a subpopulation of neuronal progenitors rather than glial precursors. These cells could proliferate, but also expressed GABA and ChAT simultaneously while undergoing differentiation. Cholinergic neurons have been reported to be immunoreactive for either GABA or its synthesizing enzyme[31]. Furthermore, in the rat cerebral cortex, 88% co-localization of ChAT with GABA was observed in interneurons[32]. Thus, we assumed that the subpopulations of DRG progenitors mainly differentiated to neurons coexpressing acetylcholine and GABA. However, no TH and 5-HT containing cells were detected.

DRG cells express NGF and its receptor TrkA

In vivo, NGF signaling is required for both survival and the differentiation of DRG neural phenotypes[33]. Embryonic DRG neurons initially require NGF for survival *in vitro*. Withdrawal of NGF from DRG neurons isolated at embryonic day 15 within the first 10 days of culture, resulted in apoptotic death. However, by 21 days of culture, the majority of these neurons survived for long periods without exogenous NGF support[34-35]. Recent studies reported that NGF mRNA is expressed in a variety of cell types in the injured spinal cord. NGF mRNA is also up-regulated in DRG neurons after spinal cord injury and the percentage of sensory neurons expressing NGF mRNA correlates with proximity to the lesion epicenter. This suggests that NGF expression in DRG may be up-regulated by damage to the central processes of sensory neurons[36]. In addition, it is likely that DRG neurons will express NGF when stimulated or under special conditions. In this study, the progenitors from embryonic day 17 DRG that were either proliferating or had differentiated were independent of exogenous NGF *in vitro*. We observed NGF and TrkA expression in both proliferating and differentiated cells. However, $p75^{NTR}$ positive cells were not detected (data not shown). In addition, incubation with K252a attenuated cell survival and implied that DRG progenitors expressed NGF for their survival and function.

To conclude, we isolated a subpopulation of DRG neural progenitors, which could be induced to differentiate to neurons co-expressing ChAT and GABA. These cells also expressed TrkA and NGF. These novel DRG progenitor cells represent a useful tool for studying the mechanisms of cell proliferation and differentiation, and have potential application as peripheral neural stem cells for the treatment of sensory organ diseases.

MATERIALS AND METHODS

Design

A parallel controlled *in vitro* experiment.

Time and setting

The experiments were performed at the Laboratory of Molecular and Cellular Neurophysiology, East China Normal University from September 2007 to April 2011.

Materials

Healthy adult Sprague-Dawley rats, weighing 150–200 g, were provided by the Experimental Animal Research Center of Shanghai, China. All rats were maintained in air-conditioned quarters under regulated light and dark periods. For mating, two to three females and two vigorous males were caged together. The vaginal canal was examined in the morning for the presence of sperm or vaginal plug on subsequent days. The day sperm or vaginal plug was found in the vagina was considered as

embryonic day 0.5. At embryonic day 17.5, the mother was sacrificed, and the embryos were removed for dissection. All experimental procedures were carried out in accordance with the guidelines of the National Institutes of Health on animal care.

Methods

Cell culture and growth curve analysis

DRG neurons were isolated and purified using the differential adhesion method combined with treatment with 0.5 μM cytosine arabinoside. Briefly, DRG from all spinal levels were removed, and incubated in pre-warmed Hank's buffered salt solution containing 0.25% trypsin (Invitrogen, Grand Island, NY, USA) for 20 minutes at 37°C. After enzymatic treatment, a single cell suspension was prepared by passing cells through a fire polished Pasteur pipette approximately 15 times, and then cells were plated in non-coated 6-well plates for 50 minutes to remove non-neuronal cells. The unattached cells were collected and cultured in serum-free Dulbecco's modified eagle's medium/F12, supplemented with 2% B27 (Invitrogen), and 0.5 μM cytosine arabinoside (Sigma, St. Louis, MO, USA) was added after 12 hours. Cells were then cultured in proliferation medium Dulbecco's modified Eagle's medium/F12 supplemented with 2% B27, 20 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor-2 (all from Gibco, Grand Island, NY, USA). Half of the medium volume was replaced every 3 days. The primary spheres were dissociated into a single-cell suspension, and new or secondary spheres could be obtained between 1 to 2 weeks. Expanded cultures were passaged routinely when new spheres formed and neared confluence (70–80% confluence). To evaluate the proliferation capacity, 10 000 cells were seeded per well in triplicate in 24-well plates. The number of the cells was counted every 24 hours for 8 days. To induce cell differentiation, spheres were mechanically dissociated into single cell suspensions before being placed on poly-L-lysine and laminin coated coverslips in 24-well plates, and differentiation medium supplemented with 2% fetal calf serum was added and refreshed every 3 days.

Immunohistochemistry for MAP2, ChAT, 5-HT, GABA, glial fibrillary acidic protein, CNP, TH, NGF, TrkA, and BrdU

Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, and then incubated with antibodies specific for MAP2 (Shanghai Branch of Sigma, Shanghai, China), ChAT (Shanghai Branch of Chemicon, Shanghai, China), 5-HT (Shanghai Branch of Chemicon), GABA (Shanghai Branch of Chemicon), glial fibrillary acidic protein (Shanghai Branch of Sigma), CNP (Shanghai Branch of Sigma), TH (Shanghai Branch of Sigma), NGF (Shanghai Branch of Santa Cruz Biotechnology, Shanghai, China), TrkA (Shanghai Branch of Chemicon) and BrdU (Shanghai Branch of Sigma) overnight at 4°C, followed by incubation with an appropriate fluorescein isothiocyanate or tetraethyl rhodamine isothiocyanate-conjugated secondary antibody (Shanghai Branch of Chemicon) for 1 hour at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (Shanghai Branch of Chemicon). The staining for nestin (Shanghai Brance of Millipore, Shanghai, China) was performed using the streptavidin-biotin-alkaline phosphatase (ABC) method using the ABC kit (Shanghai Branch of Vectastain, Shanghai, China) according to the manufacturer's protocol. Stained samples were visualized *via* fluorescent microscopy (Leica DMI4000B). Images were analyzed using Leica Imager, and brightness and contrast were adjusted using ImageJ software (version 1.38x, China).

Western blot method for MAP2, ChAT, GABA, CNP, and TrkA expression

Briefly, protein samples extracted from DRG cells were separated by 7.5–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred onto nitrocellulose membranes (Millipore, China), membranes incubated with 5% bovine serum albumin in Tris-buffer saline at room temperature for 1 hour to inhibit non-specific binding, and then incubated with antibodies specific for MAP2 (Sigma, China), ChAT (Chemicon, China), GABA (Chemicon, China), CNP (Sigma, China), TrkA (Chemicon, China), β-tubulin (Santa Cruz Biotechnology) overnight at 4°C. The primary antibodies were probed with IR-Dye-880 or IR-Dye -680-conjugated secondary antibodies (LI-COR Biosciences, USA). Specific protein bands were detected and measured by Odyssey Infrared Imaging System (LI-COR Biosciences).

Cell counting kit-8 assay

DRG cell viability was detected using a cell counting kit-8 assay (Dojido Laboratories, Japan) according to the manufacturer's protocol. Cells were seeded at 5 000 cells/well in 96-well plates. K252a at various concentrations (0, 10, 50, 100, 500, 1 000 nM; Shanghai Branch of Sigma) was added to the culture for 24 hours before cell counting kit-8 detection.

Statistical analysis

Results were expressed as mean ± SEM. Analyses were performed using SigmaPlot version 10.0 (Systat Software, Chicago, IL, USA). Fisher's least significant difference was used to analyze the statistical significance of the differences. Results were considered statistically significant when $P < 0.05$.

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Author contributions: The work presented here was carried out in collaboration between all authors. Lingli Yu and Yindi Ding defined the research theme, designed methods and experiments, performed the laboratory experiments, analyzed the data, interpreted the results and wrote the paper. All authors have contributed to and approved the manuscript.

Conflicts of interest: None declared.

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Caveolin-1对乳腺癌细胞系MCF-7增殖和存活的影响

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摘要 该文研究窖蛋白(Caveolin-1)对乳腺癌细胞系MCF-7细胞增殖与存活的影响。运用蛋白质印迹方法(Western blot)检测发现, Caveolin-1在5株不同细胞系均只有低表达。运用电穿孔转染方法在乳腺癌细胞系中高表达Caveolin-1, 运用Western blot检测转染后Caveolin-1表达情况发现, 转染后细胞内Caveolin-1表达上升, 并具有生物活性。运用单核细胞直接细胞毒性测定法(MTT)检测发现, 转染后乳腺癌细胞系MCF-7增殖速度降低。运用Western blot方法和免疫荧光(immunofluorescence)方法检测转染后细胞凋亡途径的变化, 磷酸化的P38蛋白含量上升, Bax表达量明显上升。据此推测Caveolin-1抑制MCF-7细胞的增殖和存活, 并诱导基于Bax途径的细胞凋亡。

关键词 Caveolin-1; 乳腺癌; 增殖和存活

细胞膜窖(Caveolae)于上世纪50年代被发现, 是一种囊泡样的细胞膜亚结构, 由位于胞质侧的Caveolin蛋白与膜上的脂筏组成。Caveolin基因家族有三个成员, 即*Caveolin-1*, *Caveolin-2*和*Caveolin-3*。这些都是膜整合蛋白, 在维持Caveolae的功能中起到重要作用[1-2]。*Caveolin-1*是这个基因家族中最早发现, 也是研究最深入的成员。它通过N段序列(61~101)的相互作用形成14~16个单体组成的同聚物, 并通过N端的脚手架域(Caveolin scaffolding domain, CSD)与众多信号转导蛋白内保守的Caveolin结合域(Caveolin binding domain, CBD)产生相互作用[3-4], 使结合后的信号子处于失活状态, 从而实现其对信号通路的负性调控。

Bcl-2基因家族的成员是高等动物细胞生存和死亡信号的重要整合因子[5-6]。目前, 已经发现的Bcl-2基因家族按功能可大致分为两类, 一类具有抑制凋亡作用, 如哺乳动物的Bcl-X1、Bcl-W、Mcl-1、A1、线虫Ced-9等, 而另一类具有促进凋亡作用, 如Bax、Bcl-Xs、Bad、Bak、Bik/Nbk、Bid等。Bax的结构与细菌毒素高度类似; 而体外实验证实, Bax能在脂质体上形成离子通道(巨孔), 并将脂质体中的细胞色素C释放出来。Akt是Bax/Bcl-2的上游调节因子, Akt的激活能抑制Bax的凋亡信号。Akt的活化是通过PI3K信号通路实现的[7], 而Caveolin-1则被证明与PI3K具有相互作用, Caveolin-1的过表达也能通过PI3K机制使成纤维细胞对神经酰胺诱导的死亡敏感化[8]。

近期的研究发现, 多种癌细胞尤其是乳腺癌细胞都出现Caveolin-1的低表达或突变, 并且使用RNAi的方法下调Caveolin-1的表达能引起雌激素水平的下降[9]。因此, Caveolin-1对癌细胞的增殖和存活的抑制作用及其相关细胞信号途径的研究引起了广泛关注。本实验中, 我们运用电转染方法在乳腺癌细胞系MCF-7中表达Caveolin-1, 检测转染前后MCF-7细胞的增殖、存活和相关的Akt-Bax凋亡途径, 了解Caveolin-1可能的作用途径和相关信号通路, 由此了解和研究Caveolin-1影响乳腺癌细胞系MCF-7增殖和存活的机制。

1 材料与方法

1.1 材料

转染试剂盒为Lonza产品, MTT试剂为Invitrogen

收稿日期: 2011-03-01 接受日期: 2011-06-10
上海市国际合作(No.075407059)资助项目
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公司产品, Caveolin-1抗体、Bax抗体和Bcl-2抗体为Santa cruz产品, β-tubulin抗体为Millipore产品, p-P38抗体、Alexa488、Alexa594荧光二抗为Invitrogen产品, p-Caveolin-1抗体为Bioworld产品, 蛋白浓度测定试剂盒为Bio-Rad产品, 红外荧光二抗为Odyssey产品, DAPI核染料为Sigma-Aldrich产品, 人乳腺癌细胞MDA-MB-231、MDA-MB-435、MDA-MB-453、ZR-75-30、B-CAP-37、MCF-7购于中国科学院细胞库, L-15培养基、DMEM培养基、新生牛血清(new born cattle serum, NBCS)均为Gibco产品。

1.2 方法

1.2.1 乳腺癌细胞系的培养 MCF-7、MB-MDA-435、MB-MDA-453、B-CAP-37细胞培养在含有10% NBCS的DMEM-HG培养液中, MB-MDA-231细胞培养在含有10% NBCS的L-15培养液中, ZR-75-30细胞培养在含有10% NBCS的1640培养液中。MB-MDA-231细胞置37 ℃培养箱密闭培养, 其他细胞置37 ℃、5% CO_2、饱和湿度的培养箱中培养。

1.2.2 Western Blot蛋白检测 细胞裂解抽提蛋白, 通过SDS/PAGE电泳(80 μg protein/well), 100 V电压电转1 h, Odyssey封闭液封闭1 h, 加入一抗后于4 ℃振荡孵育过夜。0.5‰ TBST洗膜3×5 min。加入Odyssey二抗, 抗兔(1:5 000), 抗鼠(1:15 000), 室温孵育1 h, 0.5‰ TBST洗膜3×5 min。Odyssey仪器检测条带。用Odyssey公司相应软件对印迹结果进行半定量测定。

1.2.3 MCF-7细胞的转染 在37 ℃、5% CO_2条件下培养MCF-7细胞, 待细胞长至70%以上融合度后, 用胰酶消化, 离心, 制成细胞悬液后计数, 用培养基调整细胞密度为1×10^6/管。根据Amaxa的说明书转染, 选择P-020程序转染MCF-7细胞, 以只加电转液的MCF-7细胞作为空白对照组。在转染好的电转杯中加事先温好的培养基, 转移至6孔板中, 每孔2 mL完全培养基。通过共转报告基因GFP, 转染24 h后, 用荧光显微镜观察细胞内绿色荧光的多寡评估转染效率。

1.2.4 细胞增殖分析 将MCF-7细胞以1.5×10^3/孔的密度接种于96孔板中正常培养, 检测时每孔加入20 μL的MTT溶液(5 mg/mL), 在培养箱中孵育6 h后, 吸去培养液, 每孔加入150 μL DMSO, 用多功能酶标仪检测590 nm下的吸光度值。

1.2.5 免疫荧光检测 将贴有细胞的盖玻片, 以4%多聚甲醛固定30 min, PBS漂洗3×5 min。加封闭剂于37 ℃湿盒中孵育1 h, 吸去封闭剂, 加入一抗后于4 ℃湿盒孵育过夜。PBS漂洗3×5 min, 加入荧光二抗, 孵育1 h, PBS漂洗3×5 min, 再加入核染料DAPI孵育15 min, PBS漂洗3×5 min。加入抗荧光猝灭剂后, 于荧光显微镜下进行观察。

1.2.6 统计学处理 采用Mirosoft excel软件分析。计量资料数据以均数±标准差($\bar{x}\pm s$)表示。单变量配对资料之间的比较采用配对样本t检验。

2 结果

2.1 Caveolin-1在乳腺癌细胞系中的表达

Western blot结果显示, Caveolin-1在MB-MDA-231细胞系中高表达, 而在参与检测的其它5种乳腺癌细胞系, 即ZR-75-30、MB-MDA-435、MB-MDA-453、MCF-7以及B-CAP-37中均只有极少量表达(图1)。

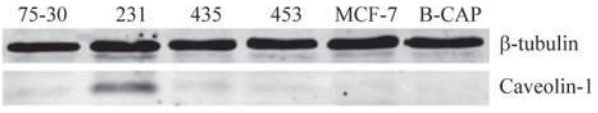


图1 Caveolin-1在6种乳腺癌细胞系中的表达

Fig.1 Expression of Caveolin-1 in 6 breast cancer cell lines

2.2 瞬时转染Caveolin-1的MCF-7细胞的构建

转染24 h后在显微镜下随机选取多个视野, 通过比较明场下细胞数与发绿色荧光的细胞数, 得出转染效率约为40%左右(数据未列入)。在参与检测的6种乳腺癌细胞系中, MCF-7细胞的Caveolin-1表达量最低, 而细胞生长迅速, 因此, 我们选择该细胞系作为后续研究的对象。Western blot结果显示, 转染后MCF-7细胞中有大量Caveolin-1蛋白表达(图2)。

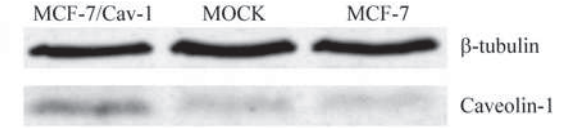
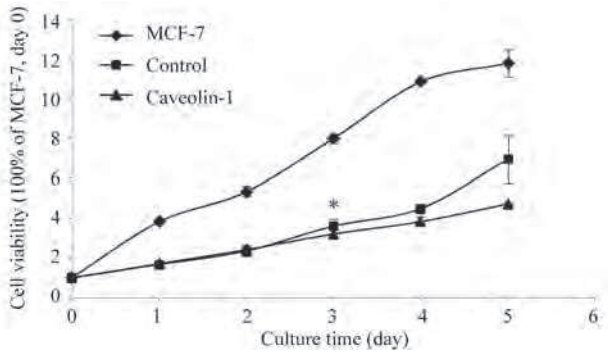


图2 瞬时转染Caveolin-1的MCF-7细胞的Western blot结果

Fig.2 Caveolin-1 expression in transient transfected MCF-7 cells

2.3 转染Caveolin-1后MCF-7细胞增殖率的变化

MTT结果显示, 与未转染Caveolin-1的MCF-7细胞和进行了空转的MCF-7细胞相比, 转染Caveolin-1的MCF-7细胞增殖缓慢(图3)。



转染的MCF-7细胞与空转相比, *P<0.05。

Caveolin-1/MCF-7 cells compared with control, *P<0.05.

图3 MTT法检测转染Caveolin-1后MCF-7细胞增殖率的变化

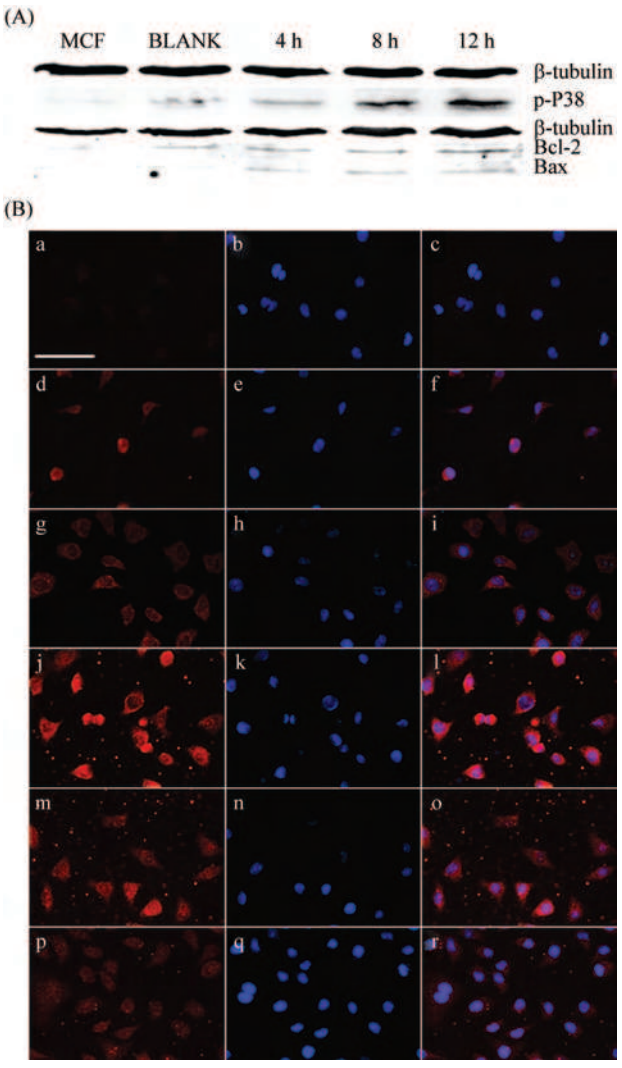
Fig.3 Proliferation rates of MCF-7 cells transfected with Caveolin-1

2.4 转染Caveolin-1对MCF-7细胞凋亡通路的影响

Western blot半定量结果显示, 转染后随着时间推移, 细胞内磷酸化的P38蛋白含量不断上升, Bax表达也不断上升, 而Bcl-2的表达没有明显变化(图4A)。免疫荧光结果显示, 转染的Caveolin-1能作为底物被激酶磷酸化, 转染Caveolin-1后的MCF-7细胞中Bax表达明显上升, 而Bcl-2表达略有降低(图4B)。

3 讨论

细胞膜窖(Caveolae)是一类富含胆固醇糖基化磷脂酰肌醇锚定蛋白以及鞘糖脂等物质的细胞膜亚结构[10], 最近的研究发现, Caveolae的功能不仅仅是参与跨膜物质的转运, 也是细胞信号转导中蛋白分子富集的枢纽[10-11]。C端的44个氨基酸残基使这些同聚体能相互连接[2-3,10,12-13]。Caveoliln-1的N端序列中有一段被称为脚手架区(CSD)的序列, 能与大多数信号蛋白分子中存在的CBD结合, 并对信号转导产生影响。Hulit等[14]的研究发现, Caveolin-1正常表达可以抑制*cyclinD1*基因启动子的活性, 从而抑制cyclinD1的表达, 产生细胞周期阻断。而Glait等[15]则发现Caveolin-1能控制*BRCA1*基因的表达以及产物的细胞定位, 而后者是恶性乳腺癌发生过程中的重



A: 转染Caveolin-1后细胞内P38磷酸化程度变化及Bax/Bcl-2表达变化; B: 转染后24小时细胞内p-Caveolin-1, Bax及Bcl-2的免疫荧光染色结果。a~c: MCF-7细胞的p-Caveolin-1染色, 红色为蛋白染色, 蓝色为染了DAPI的细胞核, 右侧图为叠加后效果(以下同); d~f: MCF-7/Caveolin-1细胞的p-Caveolin-1染色; g~i: MCF-7细胞的Bax染色; j~l: MCF-7/Caveolin-1细胞的Bax染色; m~o: MCF-7细胞的Bcl-2染色; p~r: MCF-7/Caveolin-1细胞的Bcl-2染色。标尺=20 μm。

A: P38 phosphorylation and Bax/Bcl-2 expression after transfection; B: MCF-7 cells were stained at 24 h after transfection. Red: protein staining. Blue: nuclei stained with DAPI. Right is a merged image. In image, a~c: MCF-7 cells stained with p-Caveolin-1 antibody; d~f: MCF-7/Caveolin-1 stained with p-Caveolin-1 antibody; g~i: MCF-7 cells stained with Bax antibody; j~l: MCF-7/Caveolin-1 stained with Bax antibody; m~o: MCF-7 cells stained with Bcl-2 antibody; p~r: MCF-7/Caveolin-1 stained with Bcl-2 antibody. The scale bar is 20 μm.

图4 转染Caveolin-1对MCF-7细胞凋亡通路的影响

Fig.4 The effect of Caveolin-1 in the apoptosis of MCF-7 cells

要角色。另外, 也有研究发现, Caveolin-1的表达能够抑制乳腺癌细胞的非贴壁依赖性生长[16]。这些研究都暗示了Caveolin-1在乳腺癌细胞增殖存活中可能有非常重要的作用。

本文就Caveolin-1对乳腺癌细胞系增殖和生长的作用进行了研究。运用Western blot对6种不同的乳腺癌细胞系中Caveolin-1的表达进行检测发现, 除了MB-MDA-231外, 其余的5种细胞中(ZR-75-30、MDA-MB-435、MDA-MB-453、MCF-7和B-CAP-37)都只有微量的Caveolin-1表达。选择MCF-7细胞系转染Caveolin-1的基因, 获得的瞬时转染细胞能大量表达Caveolin-1蛋白, 并能作为底物被激酶磷酸化, 表明存在一定的生物活性。对转染后的细胞进行了增殖速率的检测, 结果发现, 与空转和未转染的MCF-7细胞相比, 转染Caveolin-1的MCF-7细胞增殖率明显降低。转染后12 h内, MCF-7细胞内Bax的表达水平和P38的磷酸化逐步上升, 在12 h时迅速提高, 而Bcl-2的水平在12 h后反而出现下降。运用Immunofluoresence方法对转染后的细胞信号通路进行了研究, 结果表明, 转染24 h后MCF-7细胞内的Bax表达水平明显上升, 而Bcl-2表达水平略有下降。由于Bax介导的凋亡途径受到PI3K/Akt的调控, 而PI3K能与Caveolin-1产生相互作用, 因此我们猜测, 转染Caveolin-1后, Caveolin-1可能作为抑制乳腺癌细胞系MCF-7生长的因子, 启动MCF-7细胞内Bax介导的凋亡途径, 最终导致细胞凋亡, 从而降低了增殖率。

Caveolin-1在许多类型的组织中都有广泛表达。在不同组织类型的癌细胞中, Caveolin-1既存在高表达, 也存在低表达。而不同的组织类型中, Caveolin-1表达量升高或降低所引起的细胞效应也不尽相同。例如: Caveolin-1在食道癌、膀胱癌、前列腺癌和甲状腺癌(乳头型)中表达均升高[17-19], 而在典型的间质肉瘤、卵巢癌、肺癌及乳腺癌内Caveolin-1的表达则是下调的。在肝癌中, Caveolin-1能够抑制TRAIL诱导的细胞凋亡[20]。在用3T3细胞进行的研究中发现, 在原癌基因*v-Abl*、*Bcr-Abl*、*H-Ras*被激活的3T3细胞中, 失去Caveolin-1后出现非贴壁依赖性生长, 而且这种变化可以被重新表达Caveolin-1逆转, 表明Caveolin-1具有抑癌因子的作用[21]。而另有研究发现, Caveolin-1在原发和转移性前列腺癌中表达增高, 暗示Caveolin-1增高可能与癌发生发展有关[22]。Caveolin-1在癌症发生发展中的这种两面性提示, 在Caveolin-1相关信号通路和Caveolae结构功能上可能存在特异性。因此, 深入研究Caveolin-1在癌症发生发展中的双重作用, 对于深刻理解癌症机理、探寻癌症的新型治疗靶点有着重要意义。

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Effect of Caveolin-1 on Proliferation and Survival of MCF-7 Breast Cancer Cells

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Abstract We Study the effect of Caveolin-1 on the proliferation and survival in MCF-7 breast cancer cells. A very low expression level was detected by Western blot in 6 breast cancer cell lines. Caveolin-1 expression increased dramatically after transient transfection and has its biological activity. The MTT assay showed that the proliferation rate of MCF-7 cells was decreased after the transfection of Caveolin-1. The cell apoptosis relative protein was observed by Western blot and immunofluorescence. The quantity of phosphorylated P38 and the expression of Bax increased. We concluded that Caveolin-1 expression inhibited cells proliferation and survival and induces the Bax mediated cell apoptosis in MCF-7 breast cancer cells.

Key words Caveolin-1; breast cancer; proliferation and survival

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