



UNIVERSITÀ DEGLI STUDI DI MILANO

Facoltà di Medicina e Chirurgia

**Scuola di Dottorato in Scienze biochimiche
Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina**

CORSO DI DOTTORATO DI RICERCA IN BIOCHIMICA CICLO XXVI

BIO/10

Tesi di dottorato di ricerca

**THE TRANSCRIPTION FACTOR REST HAS A KEY ROLE IN THE CONTROL OF
PROLIFERATION AND NEURITE OUTGROWTH IN NEURAL PC12 CELLS.**

Tesi di dottorato di:

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Coordinatore: Chiar.mo Prof. Sandro SONNINO

Anno Accademico 2012/2013



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ABBREVIATIONS USED

4E-BP1	Factor 4E binding protein 1
Akt	Protein kinase B (PKB)
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CFSE	5-(6)-carboxyfluorescein succinimidylester
Cm	Clamp capacitance
Dapi	4'6-diamidino-2-phenylindole-dihydrochloride
DBD	Dominant negative construct
DMEM	Dulbecco's modified eagle's medium
ECL	Enhanced Chemiluminescence
Endo-IWR 1	Wnt pathway inhibitor
ERK	Mapk1: mitogen-activated protein kinase 1
FACS	Fluorescence activated cell sorter
FCIII	Fetal clone III
FCS	Fetal clone serum
FCS	Forward scatter
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3 β	Glycogen synthase kinase-3 beta
H2B	Histone IIb
hr	hours
HRP	Horse radish peroxidase
IF	Immunofluorescence
IRS1	Insulin receptor substrate 1
kDa	kilodaltons
LB	Lysis buffer
Map2	Microtubule-associated protein 2
MFI	Means fluorescent intensity
min	Minute
miRNA	microRNA
mM	millimolar
NGF	Nerve growth factor protein
NR	Nerve growth factor and rapamycin together
NRSF	Neuron-restricted silencer factor
NW	Nerve growth factor and wortmannin together
p75 ^{NTR}	Low-affinity nerve growth factor receptor (also called p75 neurotrophin receptor)

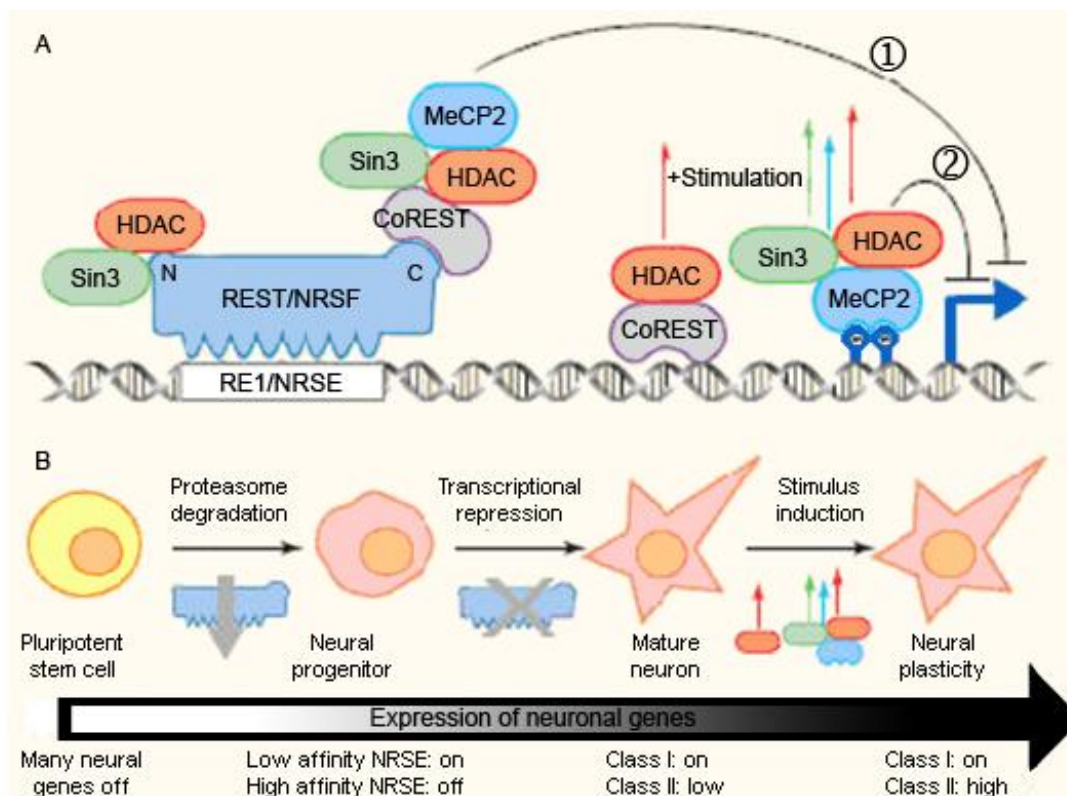
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pF	Picofarad
PKC	Protein kinase C
R	Rapamycin
REST	Repressor Element-1 silencing transcription factor
RT	Room temperature
RT-PCR	Real time-PCR
s.e.m.	
S6	Ribosomal protein
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
TBS	Tris buffered saline
TCF-LEF	T-cell factor and lymphoid enhancing factor
TRITC	Tetramethyl-rhodamine isothiocyanate
TrkA	Neurotrophic tyrosine kinase, receptor, type 1 (also called tropomyosin-related kinase A)
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
WB	Western blotting
WT	Wild type
μm	micromolar

I. INTRODUCTION

I.1. Molecular mechanisms governing neurosecretion competence

I.1.1. *REST/NRSF regulation of neurogenesis*

REST (also known as NRSF), a transcription repressor identified simultaneously by two laboratories in 1995 (Chong et al., 1995; Schoenherr and Anderson, 1995), was first recognized as the controller of a few neural cell-specific genes and then proposed to play the role of the master gene in the neuron differentiation program (Ballas and Mandel, 2005). The level of REST is high in stem cells and in non-neural cells, and this prevents the expression of many genes specific of neural cells. These genes, together with additional genes also repressed by REST, include in their promoter, or in other regulatory areas, one or more sequences known as RE-1, specific for the REST binding.



The REST complexes repress neurogenesis by targeting and blocking the transcription of their substrates (from Coulson et al., 2005)

Bound REST works as a scaffold at two sites located near its N and the C termini, permitting the binding of co-factors and of numerous enzymes. Almost two thousand genes, potential targets of REST, might thus be repressed in their transcription (Bruce et al., 2004; Otto et al., 2007).

During differentiation of neural precursors, the level of the repressor drops due primarily to the increase of its proteasomic turnover, and this changes the state of the REST-dependent genes, making possible the expression of hundreds of them. (See the detailed Figure-Abstract here above).

1.1.2. The role of REST in cell function: from the molecular to the cellular studies.

Extensive studies carried out since the discovery of REST revealed a number of interesting properties of the neuron differentiation and of the mechanisms involved in the latter process (reviews: Ballas and Mandel 2005; Ooi and Wood, 2007; Majumder, 2006). Concomitantly, REST was shown to play a critical role in the growth of tumors, not only neural, but also non-neural (reviews: Majumder, 2006; Tomasoni, Negrini et al., 2013). These studies, however, were mostly focused on molecular processes such as gene expression and its control. No major interest was paid to the role of REST in cell biology, in particular in the integrated processes that sustain the functioning of neural cells. Indeed, only one such process, i.e. neurosecretion, was investigated in detail. In 2006 the group of Neal Buckley (Bruce et al., 2006) reported that the phenotype of the neurosecretory cell PC12, a pheochromocytoma cell similar to chromaffin cells (Greene and Tischler, 1976), was profoundly affected by the transfection of REST constructs, with down regulation of 141 genes including a few coding for proteins of the secretory process, such as the two chromogranin cargo proteins and one SNARE, SNAP25. The direct control of REST on these events was confirmed by the decrease, in the cells stably transfected with a repressor, of the dense-core vesicles, typical of catecholamine-secreting cells, and by the rescue of some repressed genes in these stably transfected cells re-transfected with a dominant negative construct of the repressor.

I.2. The PC12 cell model

1.2.1 Neurosecretion is a REST-dependent process.

PC12 are peculiar in many respects (Greene and Tischler, 1976). Years ago their well known heterogeneity was shown to include clones that miss spontaneously the dense-core vesicles (Corradi et al., 1996; Pance et al., 1999). Pance et al. (2006), by studying one such defective clones, were able to find properties analogous to the PC12 transfected with the REST constructs of Bruce et al. (2006). However they failed to observe any rescue of the neurosecretory phenotype upon expression in the defective cells of dominant-negative REST constructs. The subsequent studies of D'Alessandro et al. (2008), carried out by the comparison of a wild-type clone of PC12 (referred to from here-on as wtPC12) with the defective PC12-27 clone initially characterized by Corradi et al., demonstrated that numerous neurosecretion genes are repressed by REST; that transfection of REST in the wtPC12 induced the repression of these genes accompanied by a decrease of the dense-core vesicles to 20% of their usual size; that the dominant negative construct of REST, when accompanied by the inhibitor of histone deacetylase, trichostatin A, did induce the rescue not only of single neurosecretion proteins but of whole dense-core vesicles (DCVs) that were discharged by exocytosis upon increase of the cytosolic Ca^{2+} concentration. In conclusion, therefore, although some aspects of the process were still unclear, the cellular process neurosecretion was shown conclusively to be governed by REST.

1.2.2 PC12-27 as a high REST neural cell model

In the course of a study on the heterogeneity of the PC12 cell line, a clone was isolated (named PC12-27: Clementi *et al.*, 1992) that, although retaining various aspects of the neuroendocrine phenotype, is incompetent for regulated neurosecretion. Morphological, biochemical and molecular investigations of this clone (Corradi *et al.*, 1996; Borgonovo *et al.*, 1998) provided evidence of its lack not only of both types of secretory vesicles, the clear vesicles and the DCVs (as shown in Figure 1), but also of components of the neurosecretory machinery, e.g. the SNARE proteins (syntaxin 1, SNAP 25 and VAMP2), the granins (chromogranin B-CgB- and secretogranin 2-SgII), and the Ca^{2+} -sensor (synaptotagmin 1), together with the catecholamine uptake

and release processes. In contrast, the components of the other structures in the secretory pathway (*i.e.* ER, Golgi cisternae and TGN) were all apparently unchanged. Moreover, the ER-Golgi-TGN transport of proteins and the constitutive line of secretion were functional (Malosio *et al.*, 1999).

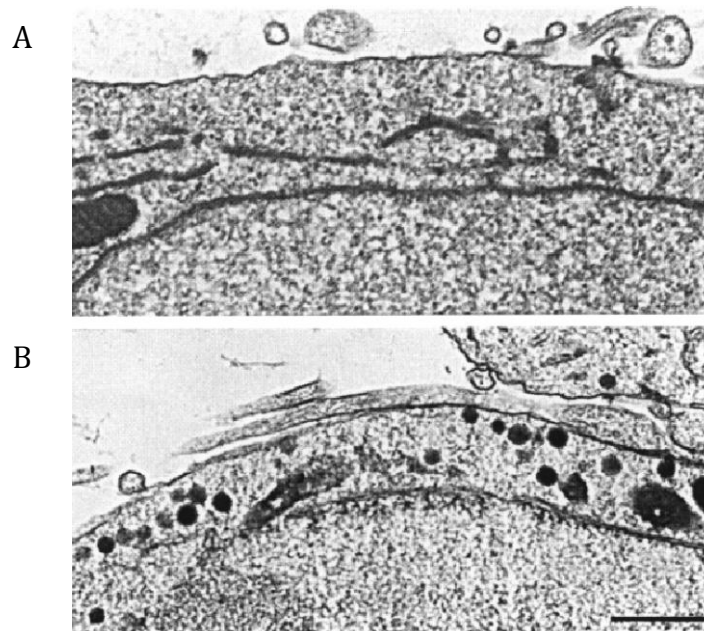


Figure 1. Ultrastructure of PC12-27 and wt PC12 cells processed by the quick freezing-freeze drying procedure (Grohovaz *et al.*, 1996) (from Malosio *et al.*, 1999).

The figure illustrates the electron microscopy of defective PC12-27 (A), where the cytoplasmic layer adjacent to the plasmalemma is devoid of vesicular profiles, and of wtPC12 (B) where the same layer contains many typical DCVs. Clear vesicles are present in wtPC12, however they cannot be identified without immunolabeling. Cells were processed by the quick freezing-freeze drying procedure (Grohovaz *et al.*, 1996).

	Wild-type PC12	PC12-27
ER markers:		
BiP	+	+
calreticulin	+	+
calnexin	+	+
Golgi complex markers:		
mannosidase II	+	+
β COP	+	+
Rab 6	+	+
Neurosecretion markers:		
<i>Cytoskeleton:</i>		

neurofilament H subunit	+	+
N-kinesin	+	+
synapsin I	+	+
<i>Signaling:</i>		
α -latrotoxin receptor	+	+
N-type Ca ²⁺ channel	+	+
XL α _s G protein	+	+
tyrosine hydroxylase	+	+
DCGs and clear vesicle markers:		
D β H	+	-
vAChT	+	-
chromogranin B	+	-
secretogranin II	+	-
synaptophysin	+	-
VAMP2	+	-
synaptotagmin I	+	-
<i>t-SNAREs:</i>		
syntaxin 1A	+	-
SNAP25	+	-
<i>SNARE regulators:</i>		
rbSec1/munc18	+	-
rab3A	+	-
<i>Membrane recycling:</i>		
AP180	+	-
AP2	+	+
dynamin	+	+/-
synaptojanin	+	+
amphyphysin	+	+

Table 1. Expression of markers of the ER, Golgi, DCGs, clear vesicles and of various properties of neurosecretory cells in the wtPC12 and PC12-27 clones (from Malosio *et al.*, 1999)

The REST dependence of neurosecretion, a typical process of neural cells, was not surprising. The project of my work in the laboratory was to investigate whether the REST dependence was limited to that process or concerned also other functions of PC12 cells. For these studies the laboratory did use the same wtPC12/PC12-27 clone model employed by D'Alessandro *et al.* in 2008. Our approach consisted in the comparative analysis of a typical neural cell clone, with the usual very low level of REST, and of another PC12 clone, with a REST level 50-80 fold higher. As already mentioned, these clones had been characterized in detail in previous studies (Clementi

et al., 1992; Corradi et al., 1996; Borgonovo et al., 1998; Malosio et al., 1999; D'Alessandro et al., 2008). Therefore they were a very favorable model for our investigation.

1.2.3. Two new processes governed by REST: proliferation and NGF signaling/neurite outgrowth.

My study was focused in sequence on two functions, proliferation and neurite outgrowth. Mature neurons do not proliferate, however the other neural cells do during their whole life. Thus, low REST, which is shared by all neural cells, does not seem to prevent the process. The question investigated was whether REST has anything to do with proliferation and, in case, what are the mechanisms of its effects. Neurite outgrowth is in contrast a typical property of neural cells, taking place in response to appropriate growth factors. Beginning with the first paper dealing with the isolation of PC12 (Greene and Tischler, 1976), hundreds of papers have investigated the neurite outgrowth induced by long-term exposure to NGF. Among these papers was the paper by Leoni et al. (1999) demonstrating that PC12-27 cells lack this property. For quite some time the defective neurite outgrowth of PC12-27 cells was attributed to the lack of the tyrosine kinase receptor of NGF, TrkA. A detailed investigation of the problem, however, had never been done.

The results reported in this thesis bring new evidence in both the already mentioned areas, proliferation and NGF signaling. PC12-27 cells were found to grow much faster than the wtPC12, and this property was shown to depend on REST, due however to a mechanism different from the usual transcription repression, i.e. a posttranscriptional decrease of the GAP protein TSC2. The latter decrease is shown to operate not via the conventional TSC2 signaling way, i.e. via the decreased inhibition of Rheb and, as a consequence, via the increased activity of mTORC1. Rather, the decreased TSC2 induced by high REST is shown to operate via a decreased turnover of β -catenin, with increased transcriptional activity of the latter. TrkA, on the other hand, was found to be expressed at the same level in both the wtPC12 and the PC12-27 cells. Part of the signaling triggered by the neurotrophin, i.e. that mediated by the ERKs 1 and 2, was the same in the two clones. Missing in the PC12-27 cells was in

contrast the other NGF receptor, p75^{NTR}, whose gene was found to be a typical target of REST endowed with two RE-1 sequences in its promoter. This result was unexpected because the role of p75^{NTR} has been considered for long time to consist primarily in the increased affinity of TrkA for its ligand, NGF. Our results demonstrate that, in contrast, p75^{NTR} is needed for a critical step of TrkA signaling, i.e. the activation of the PI3K-Akt cascade. Moreover, such activation is shown to be necessary for the neurotrophin to induce neurite outgrowth to occur.

In conclusion, the two projects of our research have demonstrated the critical role of REST in two cellular processes, proliferation and neurite outgrowth, in which the dependence on the repressor was unknown. In addition, they have revealed the mechanisms by which these effects of REST take place, bringing new information in two fields of great importance, the multiplicity of the TSC2-dependent cascades and the signaling at NGF receptors, important in general for all neural cells.

II. RESULTS

In this thesis we will report the results of two research lines both concerning the role of REST in important cellular function, proliferation (section III) and neurite outgrowth (section IV) of neural cells. In both cases our studies have identified the molecular mechanisms involved.

III. Differential proliferation of PC12-27 with respect wtPC12.

To investigate the role of REST in proliferation, and subsequently in neurite outgrowth, we took advantage of two PC12 clones extensively characterized in our laboratory: wtPC12 and PC12-27 (Malosio et al., 1999; Grundschober et al., 2002). Whereas the first expresses the very low levels of REST typical of mature neural cells, the second spontaneously expresses levels of REST ~50 fold higher (D'Alessandro et al., 2008). The low and high REST levels account for full or defective competence for neurosecretion, respectively (D'Alessandro et al., 2008).

III.1. Proliferation of PC12-27 cells is faster than that of wtPC12.

In addition to their distinct neurosecretory competence, the two clones revealed clear differences in shape, size and cytoskeletal organization (Fig. 1A). wtPC12 cells exhibited the quasi-spherical phenotype typical of the cell line during growth (Greene and Tischler, 1976), whereas PC12-27 cells appeared larger, spread out and strictly adherent to the culture surface (Fig. 1A). The distribution of actin, which was ordered and concentrated in the cortical cytoplasm of wtPC12 was, by contrast, mostly spread in thick fibers running through the whole cytoplasm in PC12-27 cells. Paxillin, an adaptor protein of the complex linking the actin cytoskeleton to the plasma membrane integrins, was also differently distributed in the clones, being concentrated, in wtPC12 cells, in many discrete, small and closely adjacent puncta over the basal plasmalemma; and, in PC12-27 cells, much larger structures, prominent especially in finger-like protrusions surrounding the cell profile of PC12-27 cells (Fig. 1A). The

differences in surface area of attached wtPC12 and PC12-27 cells were quantified by patch-clamp cell capacitance assay (Racchetti et al., 2010).

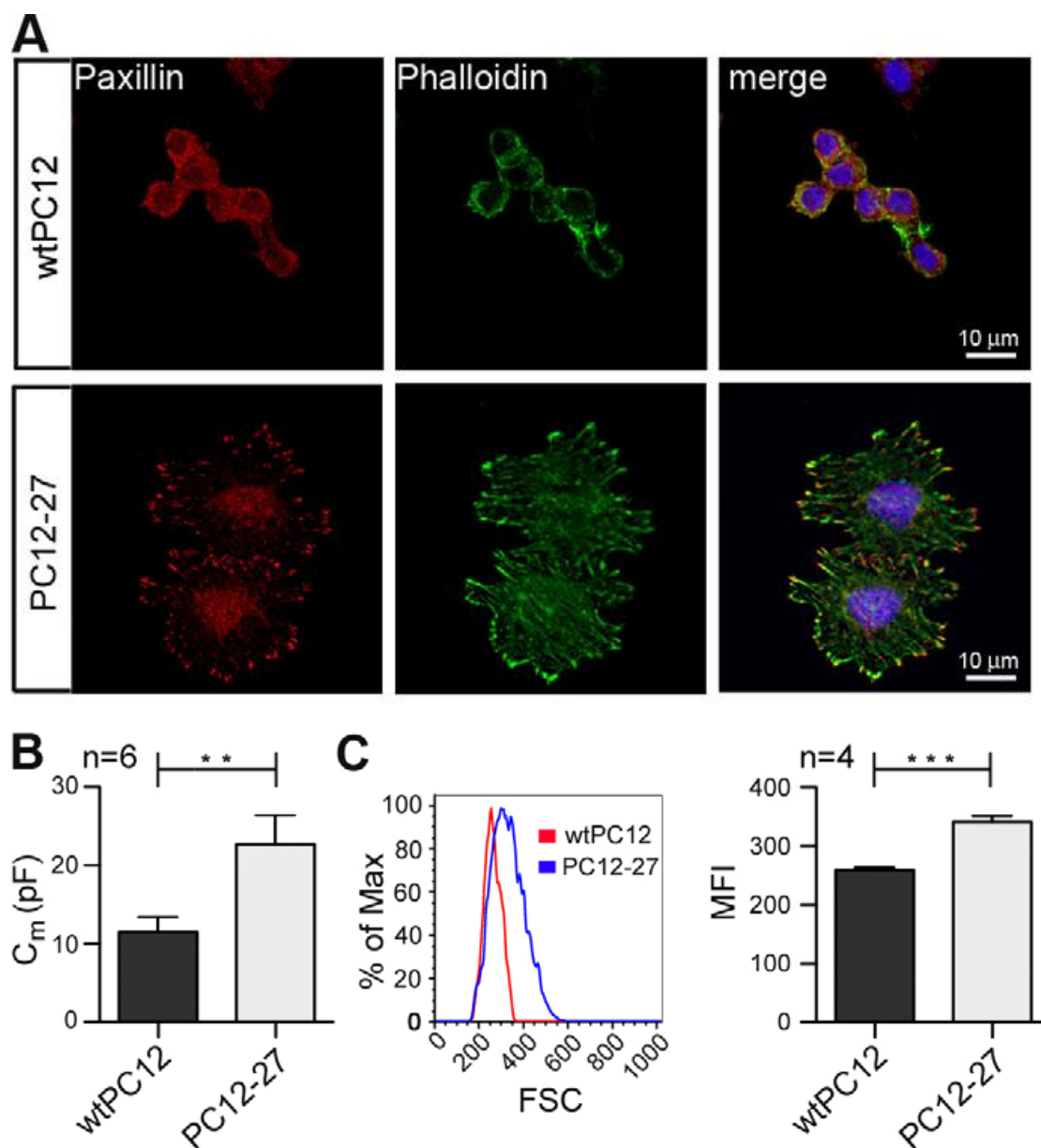


Fig. 1. PC12-27 cells reveal larger size and surface compared with wtPC12 cells. (A) wtPC12 and PC12-27 cells, fixed and dually stained with antibodies against paxillin (left) and phalloidin–FITC conjugate (middle), were analyzed by confocal microscopy and image deconvolution. Nuclei were stained with DAPI. Images on the right were obtained by merging the three individual channels. Scale bar: 10 μ m (B) The surface area of attached wtPC12 and PC12-27 cells was evaluated by whole-cell patch clamp capacitance (C_m) assays. Data shown, expressed in pF, are means \pm s.e.m. of the results in 13 wtPC12 and 6 PC12-27 cells. (C) The volume of wtPC12 and PC12-27 cells was evaluated by cytofluorimetry. Representative histograms of the forward scatter (FSC) (left) and means \pm s.e.m. of quantified results of four independent experiments (right) are shown. MFI, means fluorescence intensity.

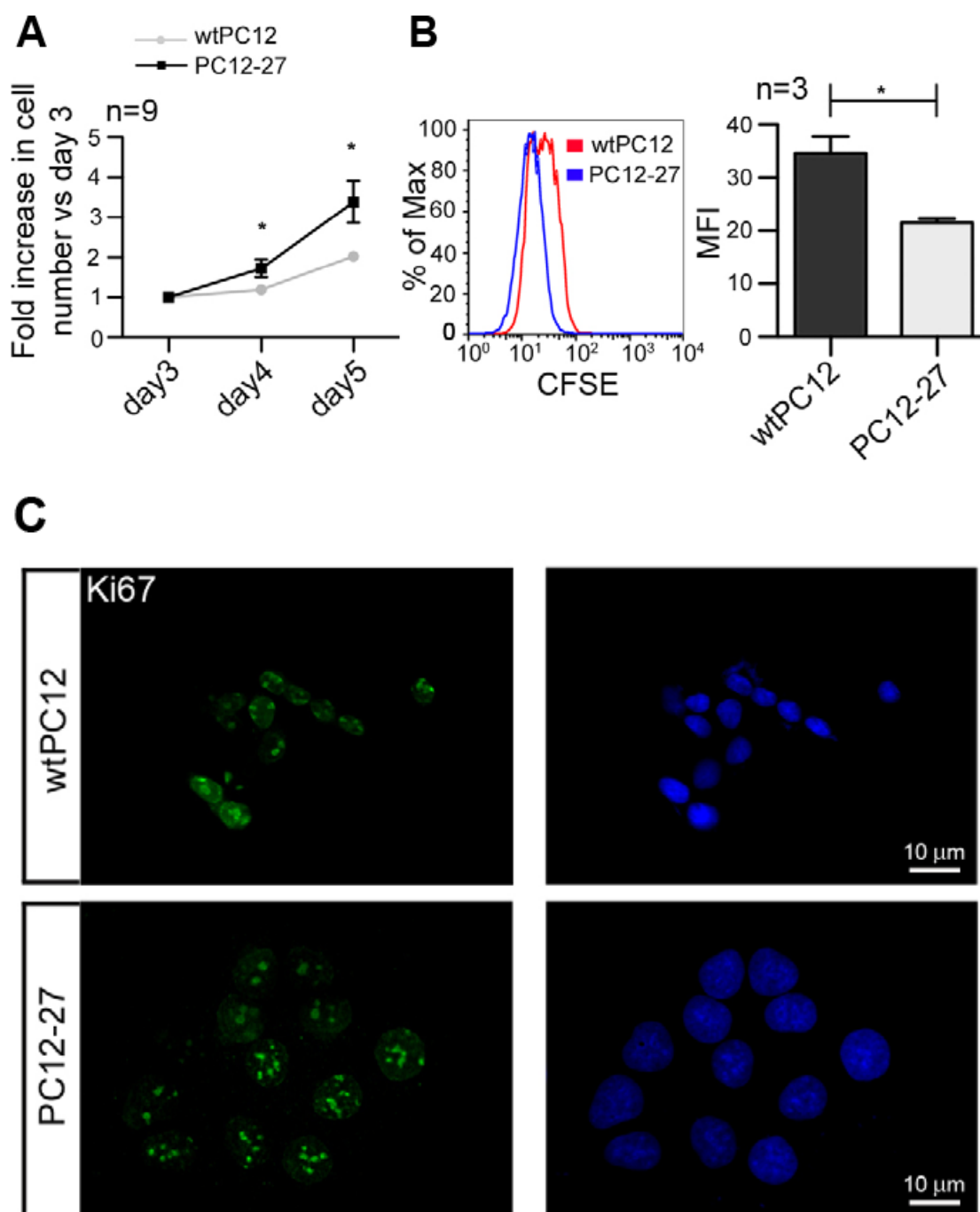


Fig. 2. Differential proliferation of wtPC12 and PC12-27 cells. (A) wtPC12 and PC12-27 cell proliferation was measured daily by viable Trypan-Blue-assisted counting of triplicate wells. The data shown are means \pm s.e.m. of nine independent experiments expressed as fold increases in cell number versus day 3. Proliferation was revealed also by two additional techniques. (B) Flow cytometry of wtPC12 and PC12-27 cells labeled with the CFSE fluorescent dye: a representative day 5 histogram (left) and means \pm s.e.m. of quantified results in three experiments with replicate wells (right) are shown. (C) Immunofluorescence confocal microscopy of the same cells dually stained in the nucleus with anti-Ki67 pAb and DAPI. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Scale bars: 10 μ m

When compared with wtPC12, PC12-27 cells showed almost double capacitance values (22.7 ± 3.7 versus 11.4 ± 2.0 pF, corresponding to 1634 versus 892 mm^2) (Fig. 1B). Likewise, when the cells were analyzed by flow cytometry, PC12-27 cells reproducibly showed a significantly larger forward scatter (FSC), which is proportional to cell size (Fig. 1C).

We also noticed that PC12-27 cells reproducibly reached confluence faster than wtPC12 cells. To investigate the possibility that high levels of REST confer a proliferative advantage, we analyzed the two clones by daily, viable Trypan-Blue-assisted counting and established their single cell division rates by the 5-(6)-carboxyfluorescein succinimidylester (CFSE) dilution assay. Compared with wtPC12 cells we found that, starting by day 4 after seeding, PC12-27 cells accumulated to higher numbers (Fig. 1D), and this was due to a faster rate of their division on a per cell basis (Fig. 1D,E).

To exclude the possibility that PC12 cells entering senescence might contribute to the observed differences, expression of Ki67, a indicator of active proliferation, was investigated. Fig. 1F shows that Ki67 immunolabeling was intense and followed the distribution of chromatin in the nuclei of both wtPC12 and PC12-27 cells, characterized by small and large areas, respectively. This result supports the notion that the growth advantage of PC12-27 over PC12 cells (depicted in Fig. 2A) is caused by increased proliferation of the former, rather than by premature senescence of the latter. Thus, low and high REST-expressing PC12 cells show differences not only in neurosecretion (D'Alessandro et al., 2008), but also in cell size, shape and proliferation rate.

III.2. The faster proliferation rate of high-REST PC12-27 cells reflects downregulation of TSC2 and increased β -catenin co-transcriptional activity

The general phenotype of PC12-27 cells, combined with their faster rate of proliferation, was reminiscent of the phenotype previously reported for HeLa cells defective of rictor (Sarbasov et al., 2004). Rictor is a member of the mammalian target of rapamycin protein kinase complex 2 (mTORC2). Knockdown of rictor results in a defect of mTORC2 accompanied by overstimulation of mTORC1 with

ensuing inhibition and activation, respectively, of the signaling cascades governed by the two mTOR complexes (Sarbasov et al., 2004). To investigate whether mTORCs have a role in wtPC12 and PC12-27 cells, we assayed the phosphorylation of target proteins downstream of the two complexes. S6 and 4E-BP1, commonly used as readouts of mTORC1 activity, were phosphorylated in both wtPC12 and PC12-27 cells, however, to a higher extent in the latter. By contrast, phosphorylation of Akt at S473, a readout of mTORC2 activity, and of the Akt substrate, glycogen synthase kinase 3 β (GSK3 β) at S9, was lower in the high-REST PC12-27 cells when compared with the low-REST wtPC12 cells (data not shown).

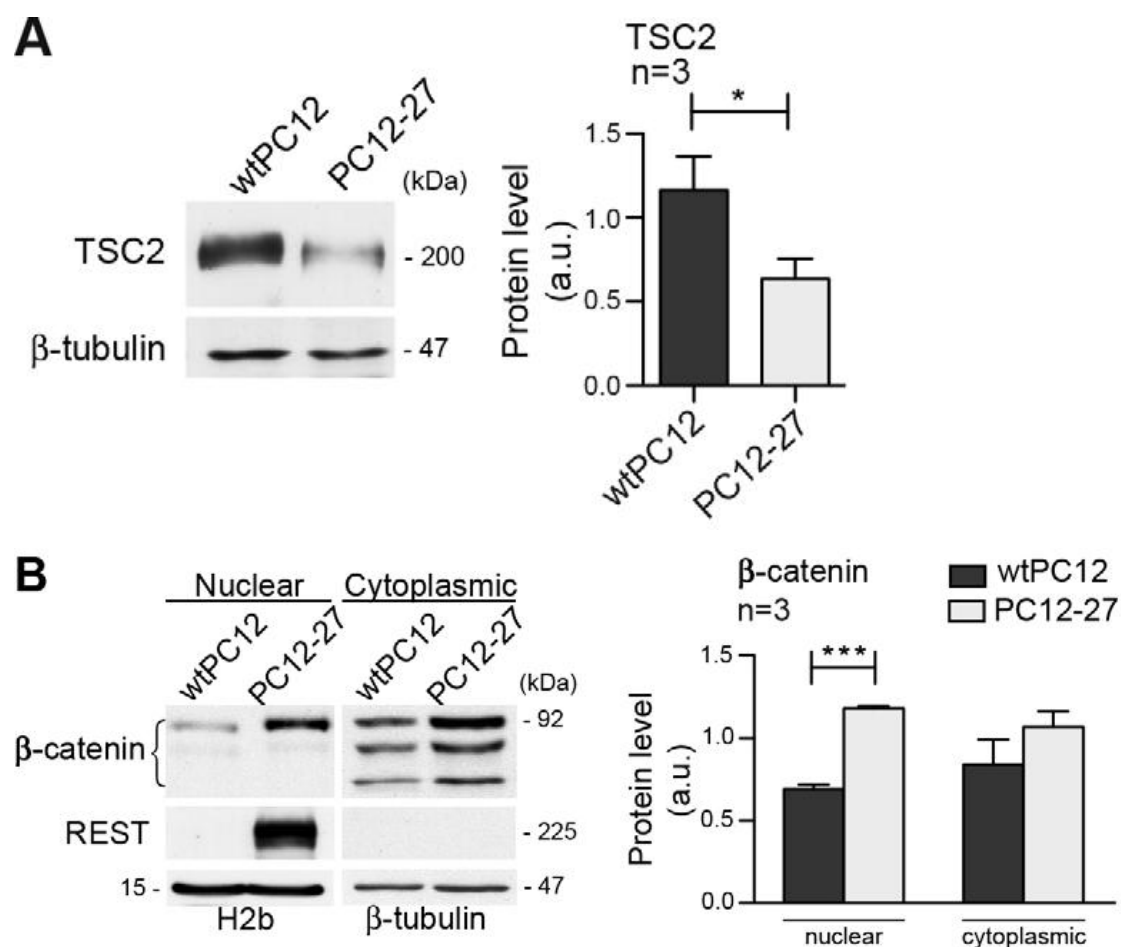


Fig. 3. In high-REST PC12-27 cells, reduced TSC2 levels correlate with β -catenin nuclear accumulation. (A) Lysates and (B) nuclear and cytosolic fractions of wtPC12 and PC12-27 cells were analyzed by SDS-PAGE and western blotting. β -tubulin and the histone H2b were used for normalization. Representative western blots, with molecular size markers (indicated here and in the following figures in kDa), are shown to the left; means \pm s.e.m. of the results of three independent experiments quantified by densitometry, on the right. a.u., arbitrary units.

Thus, in PC12-27 cells, the signaling of both mTORC1 and mTORC2 appears to be deregulated. To establish whether the proliferative advantage of PC12-27 cells was dependent on the increased activity of mTORC1, we investigated the effect of the inhibitory drug rapamycin. In spite of the marked inhibition of the mTORC1 phosphorylation, the proliferation of high-REST PC12-27 cells was largely insensitive to the drug (data not shown). Because of the well-known inhibitory action of rapamycin on mTORC1, and in spite of the caveats associated with the use of pharmacological tools, these results strongly suggest that the faster proliferation of PC12-27 cells depends only to a minor extent on the kinase. Because of this, mTORC1 was not investigated any further. We therefore turned our attention to the regulatory steps upstream of mTORC1. A main controller of the latter kinase is the tuberous sclerosis (TSC) complex, which is composed of two proteins, TSC1 and TSC2. The complex, by its binding to the small GTPase Rheb, inhibits mTORC1. Concomitantly, the TSC complex promotes mTORC2 signaling (Huang et al., 2008; Huang and Manning, 2009; Laplante and Sabatini, 2009). The TSC1–TSC2 complex can have an impact on cell proliferation through its positive control of the turnover of β -catenin (Mak et al., 2003; Jozwiak and Wlodarski, 2006; Barnes et al., 2010). We thus investigated the expression of the TSC complex and β -catenin in wtPC12 and PC12-27 cells. Although TSC1 levels did not differ to a significant extent (data not shown), TSC2 levels were consistently and significantly lower (–45%) in PC12-27 compared to wtPC12 cells (Fig. 3A).

This was due to posttranscriptional event(s), because *Tsc2* mRNA levels were similar in wtPC12 and PC12-27 cells (data not shown). In line with reduced TSC2 levels, expression of β -catenin was higher in PC12-27 cells when compared with wtPC12 cells, with significantly higher distribution to the nucleus (Fig. 3B). This correlated with a higher β -catenin-dependent transcriptional activity (revealed by a luciferase reporter assay, Fig. 4A) and with the higher expression of known β -catenin–TCF–LEF target genes *Myc*, *Rest* (Willert et al., 2002; Nishihara et al., 2003) and (to a lower extent) also *Ccnd1* (Fig. 4B).

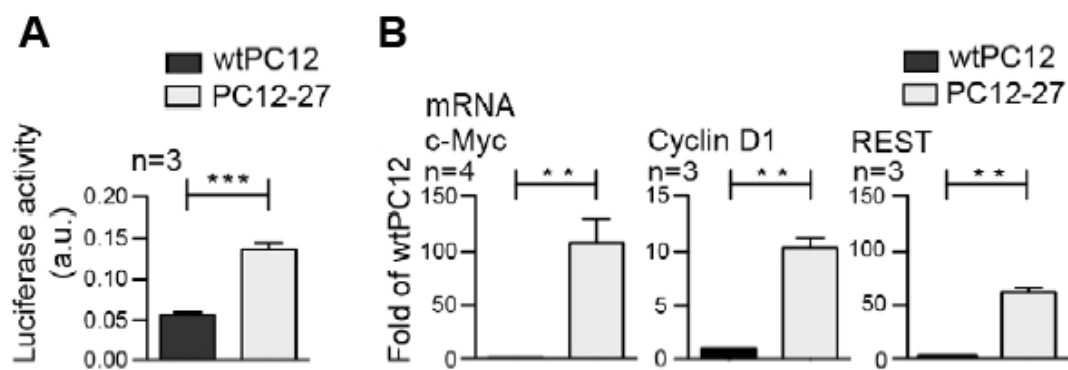


Fig. 4. Increased β -catenin-dependent transcription and proliferation in PC12-27 cells. (A) β -catenin co-transcriptional activity was evaluated by a luciferase reporter assay. wtPC12 and PC12-27 cells were transfected and the luciferase activity was measured 24 hours later. Results shown are means \pm s.e.m. from three independent experiments. (B) Expression of β -catenin target genes in wtPC12 and PC12-27 cells. mRNAs encoding Myc, cyclin D1 and REST were assayed by RT-PCR and values were normalized to the GAPDH housekeeping gene and then expressed relative to control wtPC12 cells. The results shown are means \pm s.e.m. from three independent experiments.

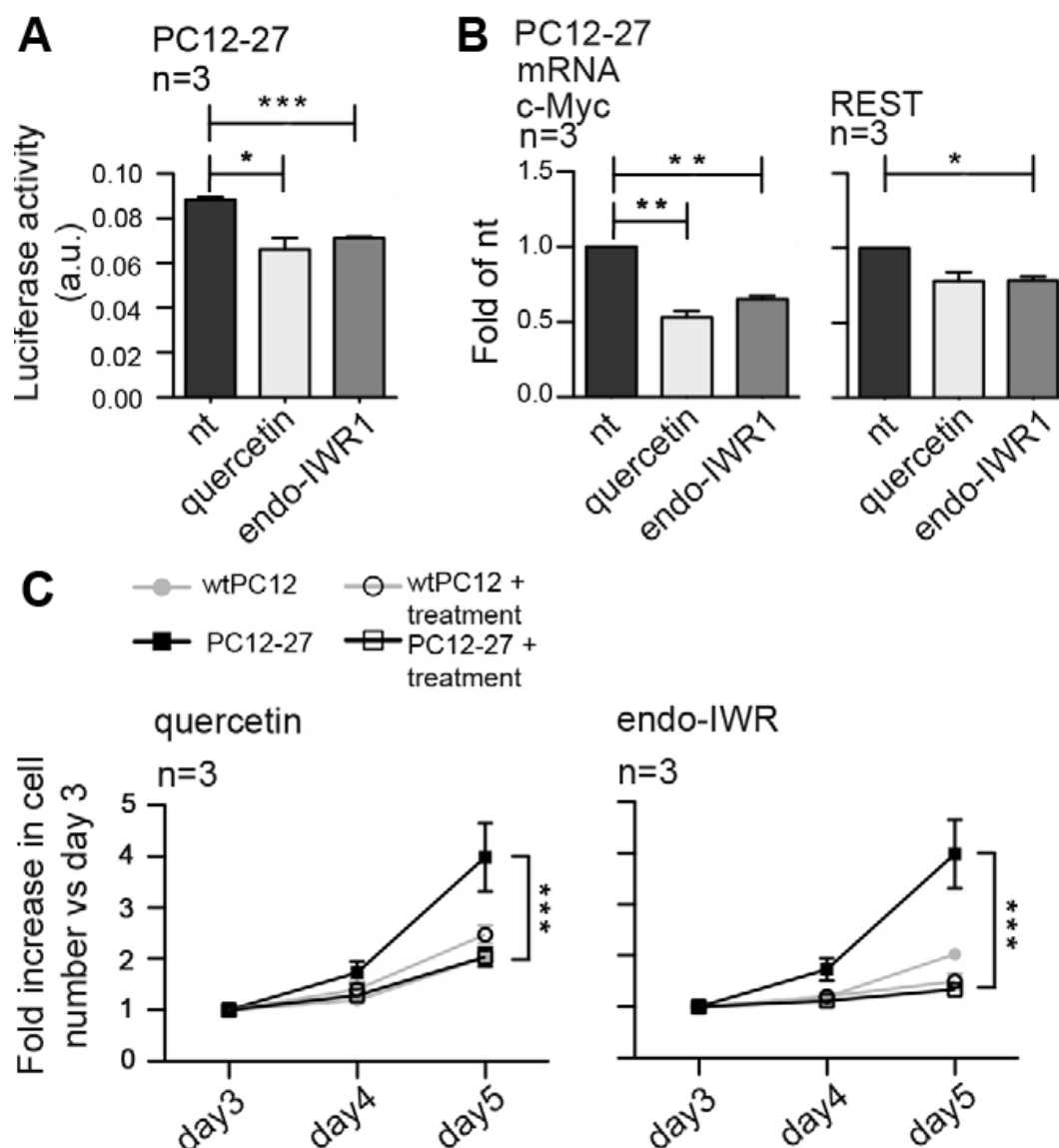


Fig. 5. The increased proliferation of PC12-27 cells depends on β -catenin. (A) β -catenin co-transcriptional activity evaluated by the luciferase reporter assay: effects of quercetin (a blocker of β -catenin-dependent transcription, 100 μ M) and endo-IWR1 (that favors β -catenin degradation, 10 μ M) administered from the fourth and fifth day of culture. Nt, untreated cells. Results shown are means \pm s.e.m. from three independent experiments. (B) Expression of Myc and Rest genes in PC12-27 cells, effects of quercetin and endo-IWR1, conditions and processing and presentation of the results as in 4B. Results shown are means \pm s.e.m. from three independent experiments. (C) wtPC12 and PC12-27 cell proliferation measured daily by viable Trypan-Blue-assisted counting of triplicate wells as in Fig. 2A. Treatment with quercetin and endo-IWR1 as in A. Results shown are means \pm s.e.m. from three independent experiments. Significance shown between PC12-27 cells without and with drug. ***P<0.001; **P<0.01; *P<0.05.

To investigate whether β -catenin transcription activity was indeed responsible for the higher target gene expression and the proliferation advantage of PC12-27 cells, we adopted a pharmacological approach using two drugs known to operate by different mechanisms. We took advantage of quercetin, a blocker of the β -catenin–TCF-LEF transcription (Park et al., 2005) and of endo-IWR1, which favors β -catenin degradation (Chen et al., 2009). In PC12-27 cells, both drugs inhibited to a significant extent the β -catenin-dependent reporter gene expression (Fig. 5A) and the expression of Myc and Rest (Fig. 5B), whereas in wtPC12, these effects were smaller and non-significant (not shown). Moreover, the two drugs abrogated the proliferation advantage of high-REST PC12-27 cells (Fig. 5C). Taken together, results obtained by the comparison of wtPC12 and PC12-27 cells link REST levels to TSC2 levels and to β -catenin nuclear activity, which is critical for cell proliferation.

III.3. REST, TSC2 and β -catenin, inter-connected in a feed-forward loop, control PC12 cell proliferation

The results reported so far (Figs 1-5), which reveal differences in structure, signaling and proliferation between the two PC12 clones, the low-REST wtPC12 and the high-REST PC12-27, suggested that REST, TSC2 and β -catenin might be interconnected in a signaling loop controlling proliferation of PC12 cells. In view of the considerable differences in gene expression existing between the two clones (Grundschober et al., 2002), however, the possibility of the results to be correlative, rather than conclusive, could not be excluded. To directly prove the link of REST, TSC2 and β -catenin we carried out gene complementation and down-regulation experiments in wtPC12 cells.

First, the cells were infected with lentiviral vectors encoding GFP (control cells) or for GFP–Myc-tagged REST, and then sorted by FACS. Forced REST up-regulation (Fig. 6A,B) caused a down-regulation of TSC2 with a gain of β -catenin levels (Fig. 6C) and activity (Fig. 6D,E). These changes were accompanied by changes of protein phosphorylation expected in low-TSC2 cells, i.e. increased phosphorylation of the mTORC1 targets, S6 and 4E-BP1, and decreased phosphorylation of the mTORC2

target Akt and of GSK3 β (data not shown). REST-infected PC12 cells also revealed a proliferation advantage when compared with control-infected cells (Fig. 6F).

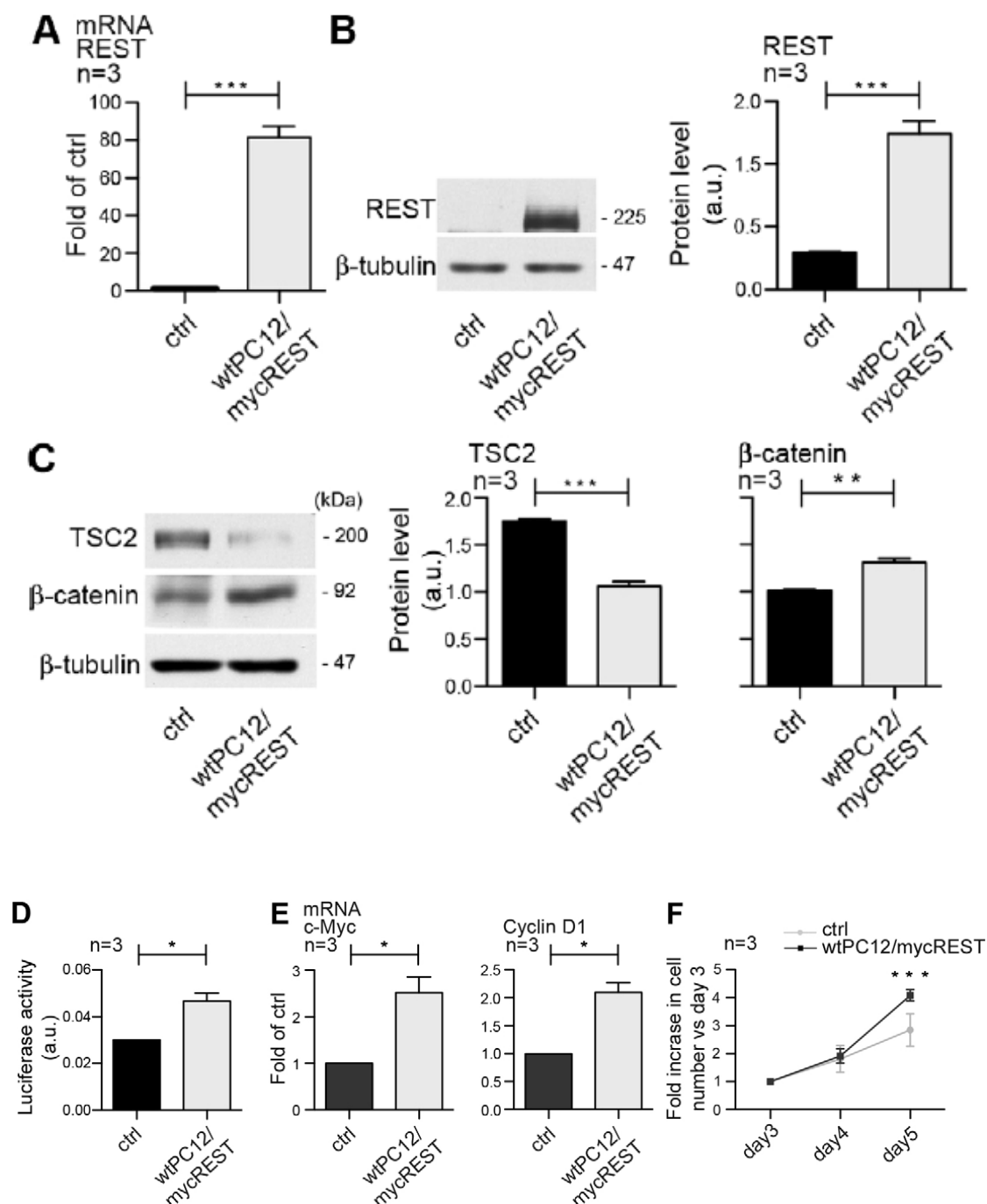


Fig. 6. Expression of REST, TSC2 and β -catenin, and of their targets. (A) Expression of Rest mRNA. (B) Levels of REST in the two infected cell populations, representative western blots also showing β -tubulin used for normalization (left); and means \pm s.e.m. of the results of three independent experiments quantified by densitometry (right). (C) TSC2 and β -catenin in the two infected populations, presentation as in A. (D) β -catenin co-transcriptional activity evaluated by the luciferase reporter assay in the two infected cell populations. Conditions and

processing and presentation of the results (from three independent experiments) as in Fig. 4A. (E) Expression of β -catenin target genes. Conditions, processing and presentation of the data (from three independent experiments) as in Fig. 4B. (F) Proliferation of the two infected cell populations measured daily by viable Trypan-Blue-assisted counting of triplicate wells; means \pm s.e.m. from three independent experiments as in Fig. 1D. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

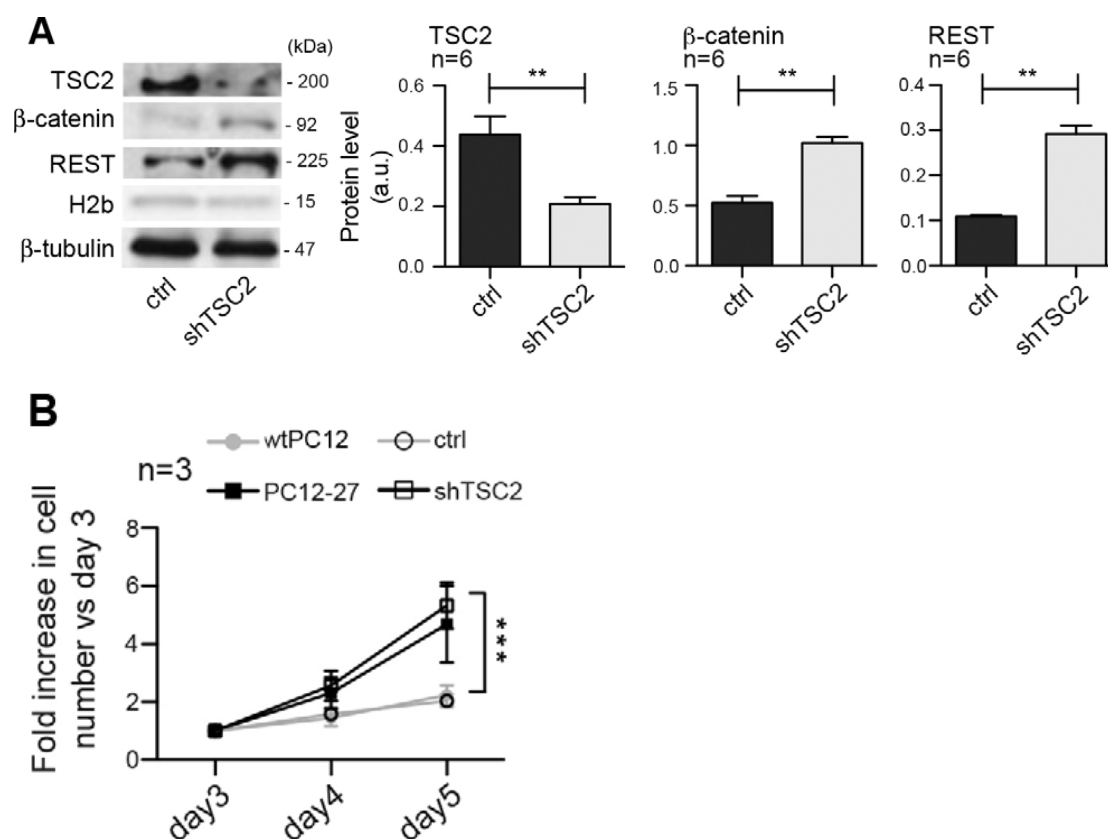


Fig. 7. TSC2 regulates β -catenin levels and cell proliferation in wtPC12. wtPC12 cells were stably transfected with the control construct (ctrl) or the shRNA TSC2 construct (shTSC2). (A) Representative western blots of cells infected with the two constructs showing the levels of TSC2, β -catenin and REST together with H2b and β -tubulin used for normalization (left); and means \pm s.e.m. of the results of six independent experiments quantified by densitometry are shown on the right. (B) wtPC12, PC12-27, control and shTSC2 cells proliferation measured daily by viable, Trypan-Blue-assisted counts of triplicate wells; data are means \pm s.e.m. from three independent experiments as in Fig. 1. Significance in A is shown between control and shTSC2-transfected cell populations.

We next investigated the effects of TSC2 knockdown by shRNA and of over-expression of a constitutively active form of β -catenin. TSC2 down-regulation correlated with increased accumulation of REST and β -catenin that proved to be

transcriptionally active (Fig. 7A). In addition, cells are accumulated to greater numbers compared with control wtPC12 and similarly to PC12-27 cells (Fig. 7B).

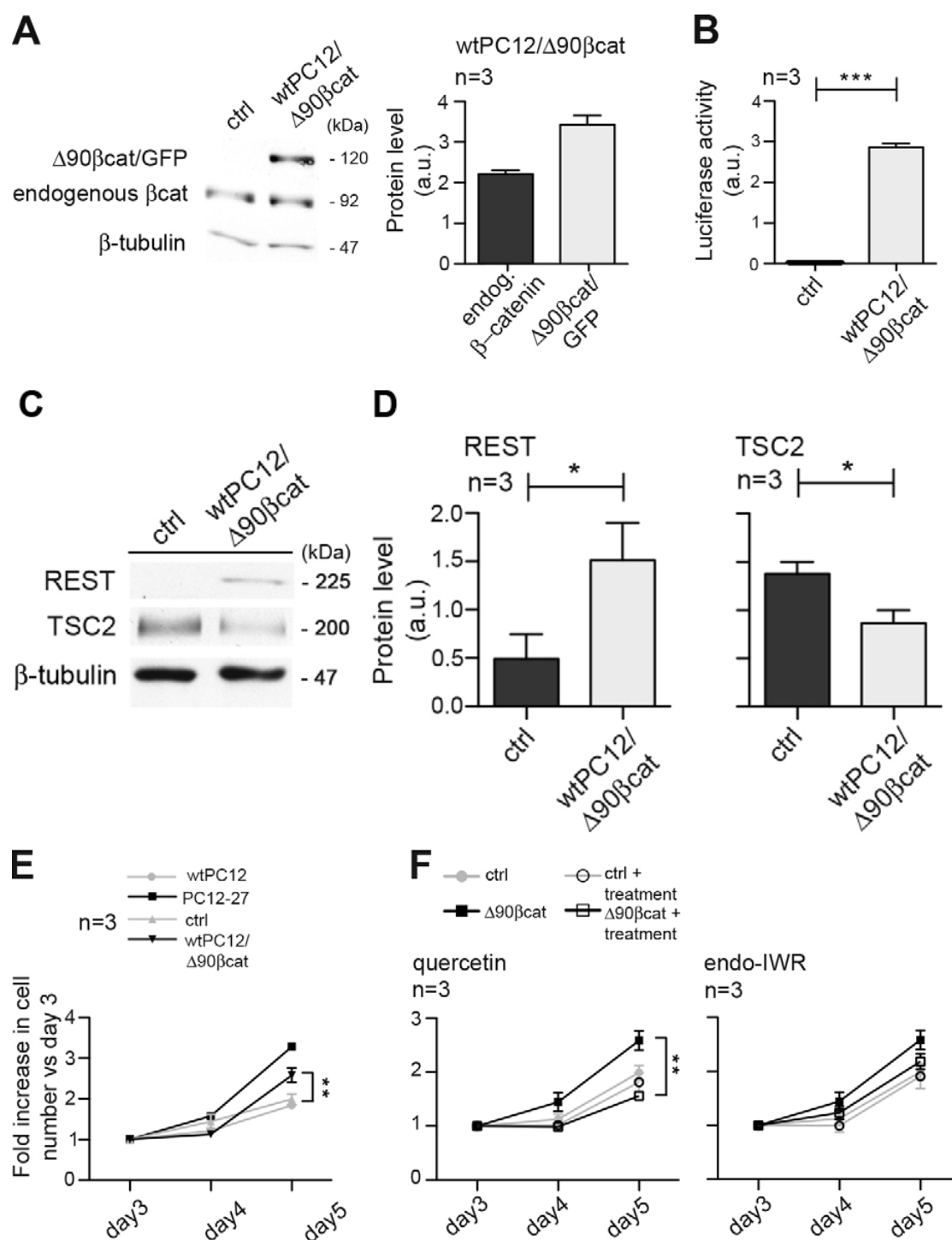


Fig. 8. β -catenin regulates REST and TSC2 levels and cell proliferation in wtPC12. wtPC12 cells were stably transfected with either an empty construct (ctrl) or a construct encoding an N-terminal-truncated form of β -catenin ($\Delta 90\beta\text{cat}$). (A) Representative western blots of cells infected with the control and $\Delta 90\beta\text{cat}$ constructs showing the levels of $\Delta 90\beta\text{cat}$ -GFP and endogenous β -catenin together with β -tubulin

used for normalization (left); and comparison of the levels (means \pm s.e.m.) of the two forms of β -catenin, results of three independent experiments quantified by densitometry (right). (B) β -catenin co-transcriptional activity evaluated by the luciferase reporter assay in the control- and $\Delta 90\beta$ cat-transfected cells. Conditions and processing/presentation of the results (from three independent experiments) as in Fig. 4A. (C) Representative western blots of cells infected with the control and the $\Delta 90\beta$ cat constructs showing the levels of REST and TSC2 together with β -tubulin used for normalization (left); the means \pm s.e.m. of the results of three independent experiments about the REST and TSC2 levels quantified by densitometry are shown on the right. (D) Control and $\Delta 90\beta$ cat-transfected cell proliferation measured daily by viable Trypan-Blue-assisted counts of triplicate wells. Comparison with non-transfected wtPC12 and PC12-27 (left). The results are means \pm s.e.m. from three independent experiments. Significance shown is between control and $\Delta 90\beta$ cat. (E) Effects of quercetin (middle) and endo-IWR (right) employed as in Fig. 5, in the control and $\Delta 90\beta$ cat populations. The results are means \pm s.e.m. from three independents. Significance shown is between $\Delta 90\beta$ cat and $\Delta 90\beta$ cat + drug cell populations. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

In PC12 cells stably transfected with a β -catenin construct, the expression of $\Delta 90\beta$ cat was reflected by gained β -catenin co-transcriptional activity (Fig. 8A, B) and the levels of the REST protein were increased, whereas TSC2 levels were decreased (Fig. 8C). Moreover, the cells also revealed a proliferation advantage that was similar to that of PC12-27 cells when compared with parental and control cells (Fig. 8D). Interestingly, this advantage was largely abrogated by quercetin (Fig. 8E, left panel), but not by endo-IWR (Fig. 8E, right panel). Thus, forcing up-regulation of REST, down-regulation of TSC2 or increased transcriptional activity of β -catenin promote reciprocal changes in their relative expression levels, and have an impact on wtPC12 cell proliferation. These results, which recapitulate in wtPC12 the properties of PC12-27 cells, directly link REST, TSC2 and β -catenin in a feed-forward loop favoring PC12 cell proliferation.

IV. Neurite outgrowth

A key property of PC12 cells, based on which the cells have been often employed as a neuron cell model, is their response to NGF with the outgrowth of neurites (Greene and Tischler, 1976). This property is however absent in the high REST PC12-27 cells. The mechanism by which the high REST of PC12-27 prevents

the outgrowth response had never been thoroughly investigated. In this section, labeled IV, we will report our findings obtained about the mechanism by which high REST represses this process.

IV. 1. NGF receptor expression and signaling

The first task of this study was the analysis of the expression and functioning of the NGF receptors, TrkA and p75^{NTR}, compared in two PC12 clones, the wtPC12 and the high REST PC12-27. In contrast to previous hypotheses (Leoni *et al.*, 1999; Schulte *et al.*, 2010), the two clones, analyzed under resting conditions (10% serum in the medium), were found to be close both in terms of mRNA and protein of the TrkA receptor. In contrast p75^{NTR}, which is prominent in wtPC12, was inappreciable in PC12-27 cells at both the mRNA and the protein level (Fig. 9A, B). These results could be due to a direct repression of p75^{NTR} expression by the high REST of PC12-27 cells. In fact the gene of the receptor includes in its promoter two RE-1 (Tab. 1), the DNA sequence specific of REST binding (Wu and Xie, 2006). In contrast, no RE-1 sequence is present in the promoter of TrkA (Bruce *et al.*, 2004). TrkA and p75^{NTR} were immunolabeled both before and after detergent permeabilization of the cells, to reveal their surface and total complement, respectively.

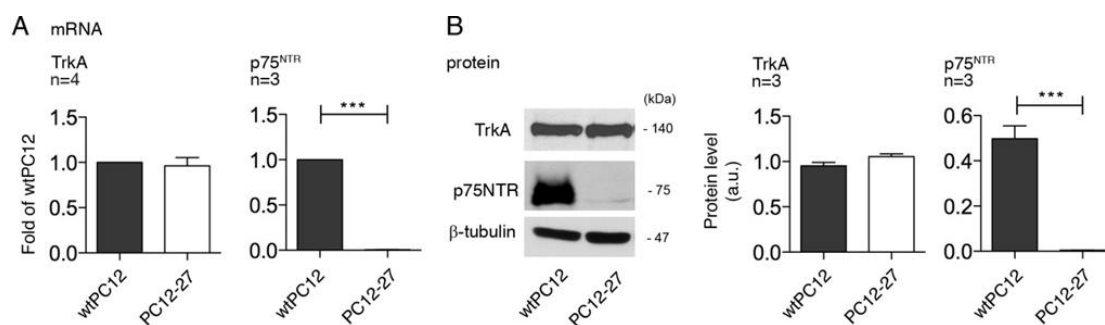


Fig. 9. Expression of TrkA and p75^{NTR}. The mRNA and protein of the two receptors were revealed in the two clones by RT-PCR (A) and western blotting (B). Notice the lack of p75^{NTR} in the PC12-27 clone. Here, and in the following figures, the number of gels analyzed quantitatively is given by the numbers over the panels; the numbers flanking the gels are the MDa of the immunolabeled proteins, given only in the figure showing the protein for the first time. The significance of the results, given as means of 6 experiments \pm s.e.m., is calculated with respect to the sample labeled 0 in each cell population. *P<0.05; **P<0.01; ***P<0.001.

<http://broad.mit.edu/~xhx/projects/NRSE/>

TTCAGCACTGGAGACTGAGGCC

CCCAGACCCTAGGAGAGAGGCT

Tab.1 The two RE-1s (the DNA sequences specific of REST binding) present in the promoter of p75^{NTR} gene

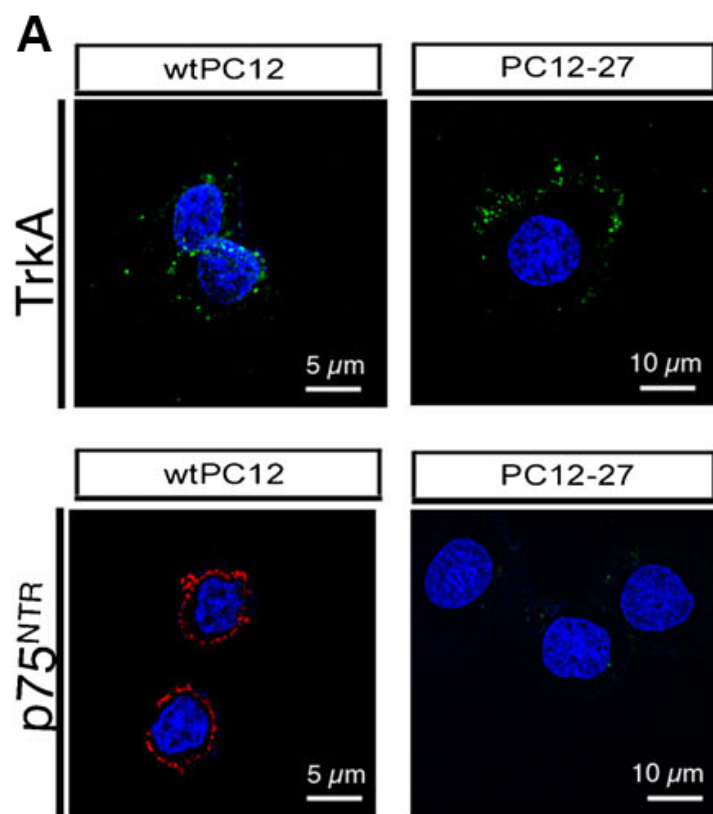


Fig. 10. Surface immunolabeling of the two receptors in the wtPC12 and PC12-27 cells (A). The fractions of the total receptors distributed to the surface, given as percentages, are shown (B). Scale bars: 5 μm (left), and 10 μm (right).

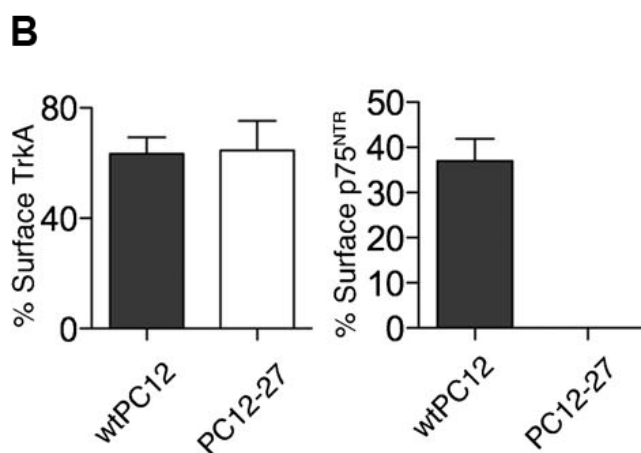


Fig. 10 shows that TrkA, expressed by all cells of the two clones, was mostly exposed at the cell surface while p75^{NTR}, inappreciable in PC12-27, was ~40% surface-exposed and ~60% retained within the wtPC12 cells.

In order to investigate the repression of p75^{NTR} gene by REST, we analyzed the levels of the receptor after transient transfection of either the whole repressor or a mycREST dominant negative construct (DBD) of REST in wtPC12 and PC12-27 cells, respectively. The results show the quantification of western blots that illustrate no appreciable variation of p75^{NTR} protein induced by the REST constructs in the two clones (Fig. 11).

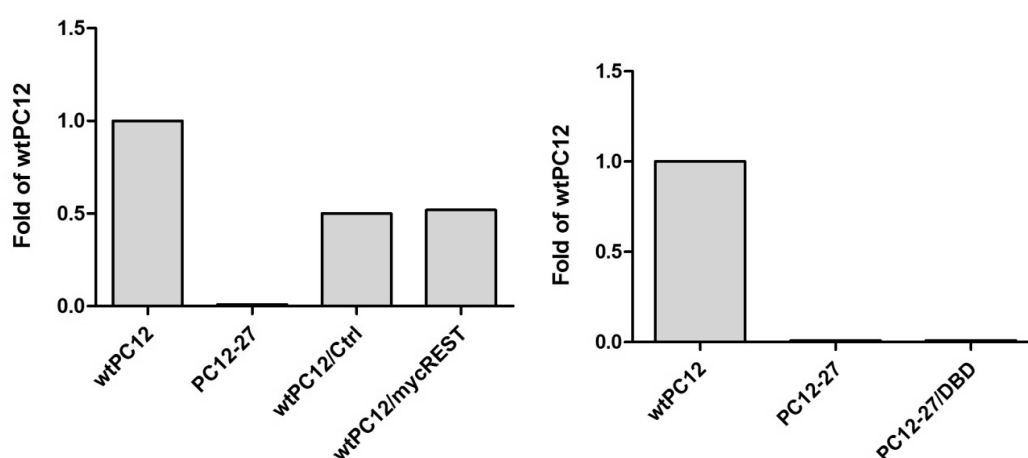


Fig. 11. Relative level of p75^{NTR} protein expression in PC12 and PC12-27 cells after transient transfection of mycREST (A) and DBD (B) constructs. The levels of the receptor, hardly appreciable in wt PC12 cells, decreased after over-expression of REST also in the ctrl cells transfected with the empty vector. In PC12-27 transfected with a dominant negative construct of REST, no appreciable increase of the protein was observed.

IV.2. The NGF signaling cascade:

We next investigated the effects of NGF on its receptor signaling. During preliminary studies the effects on ERK 1 and 2 (ERK 1/2) and Akt phosphorylation induced in the wtPC12 and PC12-27 clones by various concentrations of NGF: 2, 25 and 100 ng/ml were measured. At the low concentrations the effects induced by the neurotrophin were inappreciable or small. Only with the highest concentration many of the differences induced by NGF reached the level of significance. In view of these preliminary results, the subsequent studies were most often carried out using NGF at

100 ng/ml, a concentration widely employed in recent studies of the literature (see, among others, Koch *et al.*, 2008; Miranda *et al.*, 2001; Pincheira *et al.*, 2009; Wang *et al.*, 2013).

IV.2.a. TrkA auto-phosphorylation

In a first series of phosphorylation studies the wtPC12 and PC12-27 cells were incubated in low (1%) serum medium for 24 hr before treatments, and then analyzed in the same medium. The time-course of the TrkA phosphorylation at various tyrosine residues during the first 20 min of NGF treatment is illustrated in Fig. 12.

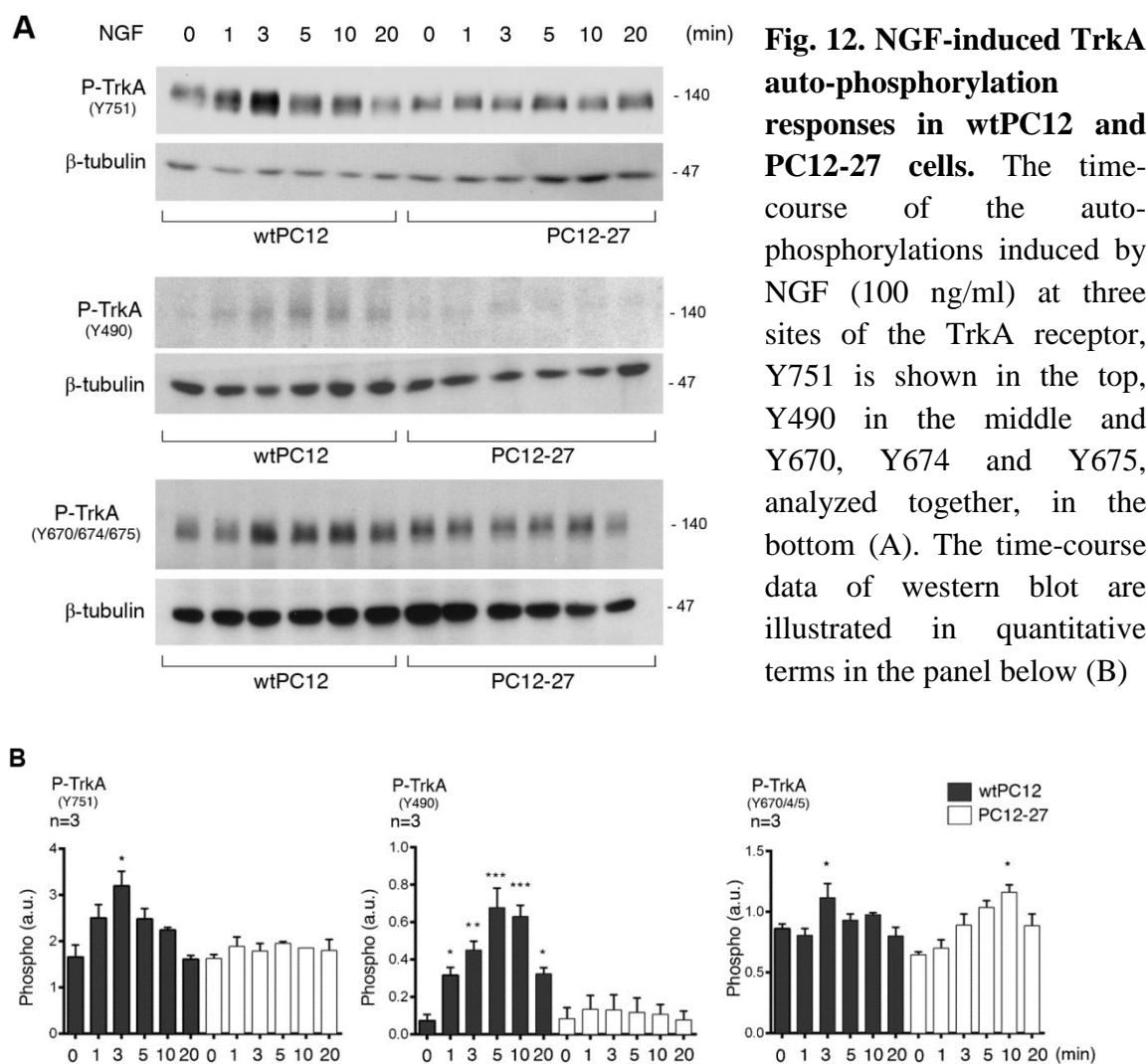


Figure 12 shows that in the wtPC12, the Y751 site was rapidly phosphorylated, reaching the highest level at 3 min and then declining to the resting level. In PC12-27 cells the Y751 phosphorylation, evident at rest, failed to increase significantly during the stimulation. The phosphorylation of the Y490 site was well appreciable only in the wt cells, with the highest values at 5–10 min, whereas the phosphorylation of the Y670, Y674 and Y675, three sites of limited importance for TrkA signaling (Biarc *et al.*, 2013) that were investigated together, was similar in the two clones, with only limited changes induced by NGF stimulation (Fig. 12).

Considering together the data of Figs 9-12 we conclude that the level of TrkA is similar in the two, low and high REST PC12 clones. In contrast, the NGF-induced auto-phosphorylation of the receptor, especially that of the Y490 site, is defective in PC12-27 cells. This might be due to the lack of cooperation of TrkA with the other NGF receptor, p75^{NTR}, which lacks in the PC12-27 cells.

IV.2.b. Phosphorylation of ERK and Akt.

We next investigated the two major signaling cascades triggered by NGF in PC12 cells, the ERK and the PI3K cascades, analyzed by measuring the specific phosphorylation of the ERK 1/2 and Akt kinases, respectively (Fig. 13, 14). The expression levels of ERK 1/2 were close in the wtPC12 and PC12-27 clones (Fig. 14A), however their NGF-induced phosphorylation at the T202/Y204 sites exhibited different time-courses. In the wtPC12 cells the phosphorylation, low at time 0, reached at 3 min high levels that were maintained for the rest of the experiments (20 min).

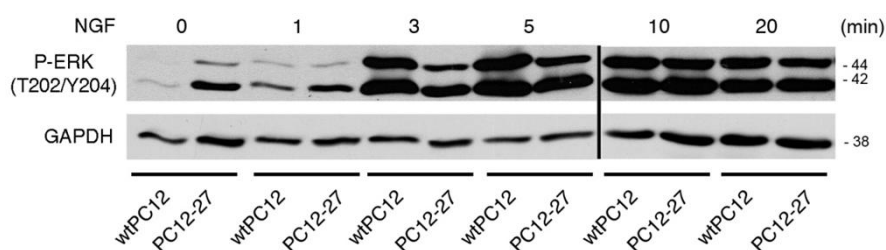


Fig. 13. Time course of the ERK signaling cascades in wtPC12 and PC12-27 cells. The time-course of the ERK 1/2 phosphorylation induced by NGF (100 ng/ml) at the T202/Y204 sites.

In PC12-27 cells, the resting level was higher than that of wtPC12. The NGF-induced increase occurred, however it was delayed, reaching top levels similar to those of stimulated wtPC12 only after 10 min and thereafter (Fig. 13).

The investigation was pursued with longer treatments. In these cases, in addition to NGF, we investigated the effects of the classical blocker of the mTORC1 complex, rapamycin, and of the combination NGF/rapamycin. This approach intended to explore the possible role of a negative, mTORC1-induced feed-back that, in the case of insulin and various other growth factors, has been reported to affect the intracellular signaling cascades (Hsu *et al.*, 2011; Huang and Manning, 2009). In neural cells, a mTORC1-induced feed-back had been demonstrated only in relation to a cAMP-induced differentiation (Chin *et al.*, 2010). If the process exists also in relation to the NGF/TrkA system, a blocker of mTORC1 such as rapamycin was expected to block it and thus to increase the NGF-induced response. In contrast, the lack of effect of rapamycin would suggest the lack of the feed-back inhibitory process.

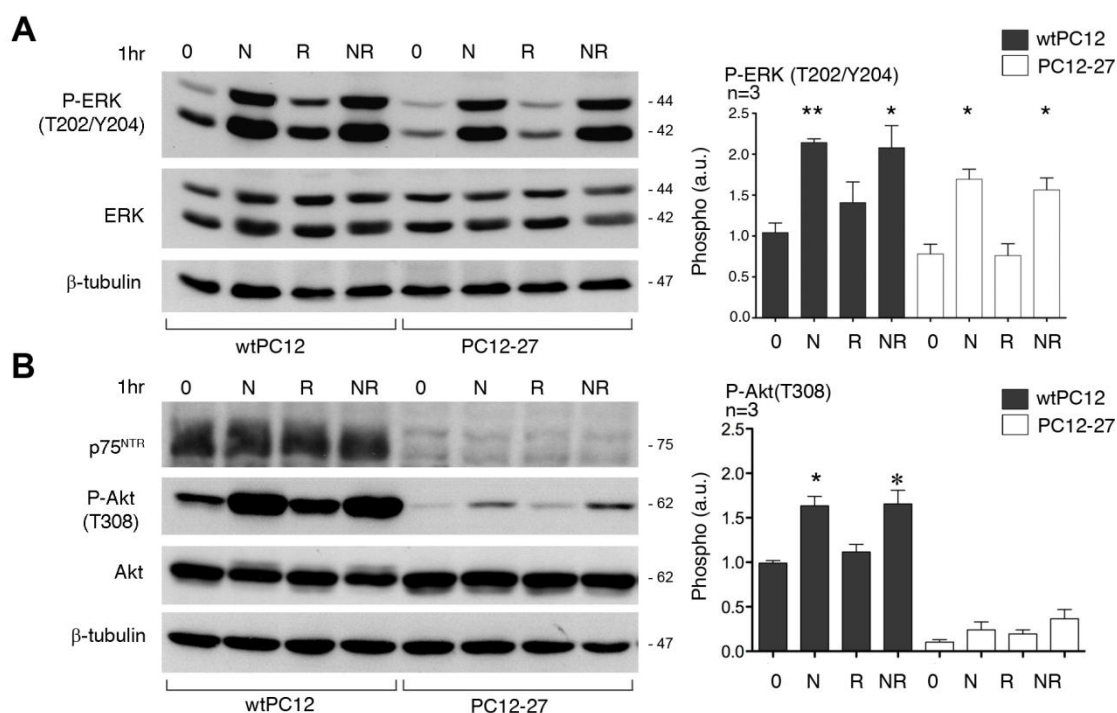


Fig. 14 ERK and Akt(T308) phosphorylation responses: effects of NGF, rapamycin and both. A) The phosphorylation induced at the T202/Y204 sites of ERK 1/2 in wtPC12 and PC12-27 cells maintained for 1hr at rest (0), with NGF (N, 100 ng/ml), rapamycin (R, 1 μ M) and the two together (NR). (B) The responses

induced by the same treatments at the P-Akt(T308). In each cell sample the levels of the ERK (B), Akt and p75^{NTR} (C) proteins did not change during the experiments. The data on P-ERK 1/2(T202/Y204) and P-Akt(T308) are also shown in quantized terms on the right of panels B and C. The numbers flanking the gels are the MDA of the immunolabeled proteins. The statistical analysis and the significance of the differences are shown by the asterisks as specified in the legend for Fig. 10.

The ERK results obtained by 1 hr treatments with NGF, rapamycin and NGF/rapamycin are shown in Fig. 14A. Upon NGF treatment, the levels of ERK 1/2 were unchanged whereas those of their phosphorylation were almost doubled in wtPC12 and significantly increased, although to a moderately lower extent, in PC12-27 cells. In contrast, both the resting and the NGF-induced levels of ERK 1/2, phosphorylation appeared unchanged in both clones upon treatment with rapamycin (Fig. 14A).

Also the levels of Akt were similar in the wtPC12 and PC12-27 cells, with no changes induced by the various treatments with NGF and rapamycin investigated (Fig. 14B). As far as the phosphorylations, that of Akt(T308), indicative of the PI3K cascade, increased slowly in the wtPC12 cells, whereas in the PC12-27 cells it remained apparently unchanged during the first 20 min of NGF treatment (data not shown). One hr (Fig. 14B) or longer (up to 48 hr, data not shown) treatments of wtPC12 cells with NGF induced significant increases of the Akt(T308) phosphorylation. Rapamycin alone, administered for 1 or 24 hr, modified neither the basal nor the NGF-induced Akt(T308) phosphorylation of wtPC12 (Fig. 14B and data not shown).

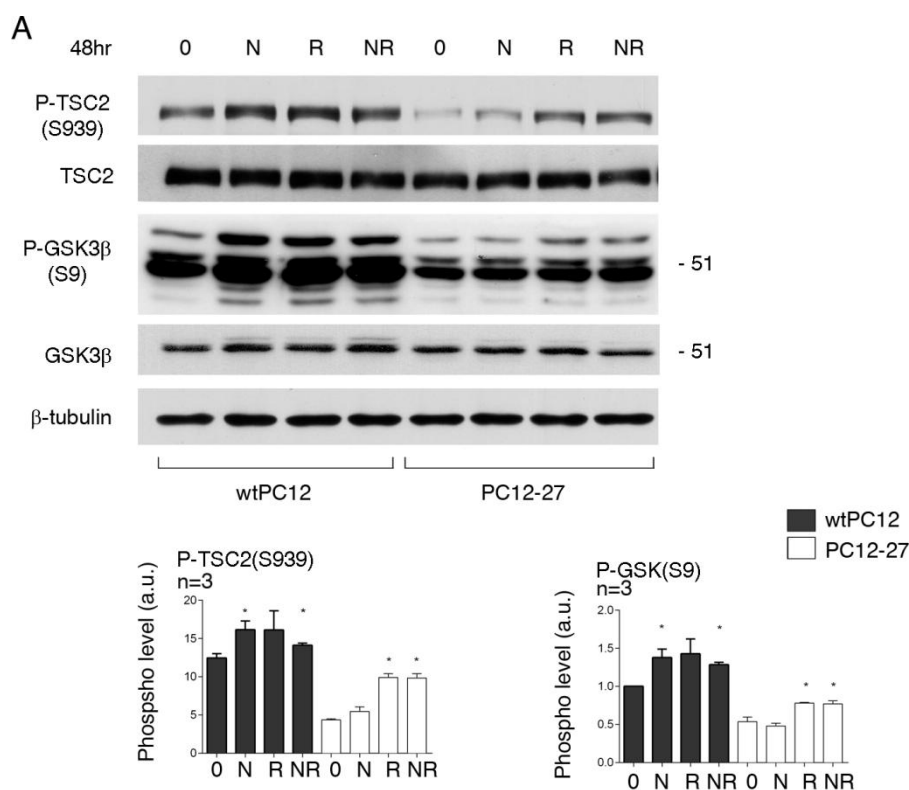


Fig. 15 Phosphorylation of two Akt targets, TSC2(S939) and GSK3 β (S9), induced by NGF and rapamycin in wtPC12 and PC12-27 cells. The changes in phosphorylation of TSC2(S939) and GSK3 β (S9) induced in the two cell clones by 1 hr treatment with NGF (N, 100 ng/ml), rapamycin (R, 1 μ M) or the two together. The numbers flanking the gels are the MDa of the immunolabeled proteins. Compared to the results of P-Akt(T308), shown in Fig. 14B, the changes of the two targets induced by rapamycin are larger, especially in the PC12-27 cells that are unresponsive to NGF.

In the PC12-27 cells the basal phosphorylation of Akt(T308) was much lower than that of wtPC12. NGF and, to a lower extent, also rapamycin (administered alone or together for 1 (Fig. 14B) or 24 hr (data not shown) did apparently induce some increases of P-Akt(T308) which however remained statistically non significant (Fig. 14B).

Two targets of Akt, TSC2(S939) and GSK3 β (S9), exhibited phosphorylation patterns different from those of Akt(T308). Specifically, the increases in the wtPC12 cells were smaller, while those induced by rapamycin were larger than those of Akt(T308) (Fig. 14). In the PC12-27 cells, the resting phosphorylation of TSC2(S939) and GSK3 β (S9) was low. In these cells no significant increase was induced by NGF. In contrast, rapamycin induced significant increases (Fig. 15). Taken together with the data of Fig. 14B, the data of Fig. 15 suggest that some mTORC1-induced feed-back

inhibition of the PI3K cascade may operate in PC12-27. In wtPC12 cells, however, no sign of the feed-back was appreciable.

IV.2.c. mTORC1 and mTORC2

Our previous studies had shown mTORC1 to be moderately more active, and mTORC2 much less active in the high REST PC12-27 cells compared to the wtPC12 cells (Section III.2 and Tomasoni *et al.*, 2011). In order to investigate the possible involvement in these differences of the NGF receptor signaling and of its mTORC1-dependent feed-back inhibition, we analyzed the direct read-outs of mTORC1 and mTORC2, P-S6(S235/236) and P-Akt(S473), respectively. The expression levels of the mTORC1 target, S6, were similar in the two clones. Its basal S235/236 phosphorylation, moderately higher in the PC12-27 cells, was increased upon 1 and 48 hr treatment with NGF, however only in the wtPC12 clone (Fig. 16A and data not shown). The mTORC1 blocker rapamycin, administered alone or together with NGF for 1 to 24 hr, dissipated completely the basal and largely also the NGF-stimulated phosphorylation of the mTORC1 read-out, S6(S235/236), as expected (Fig. 16A and data not shown). We conclude that the moderate differences of mTORC1 activity between wtPC12 and PC12-27 cells, already reported in the two resting clones (Tomasoni *et al.*, 2011), were largely independent of their different NGF signaling.

Fig. 14B had already shown the levels of Akt to be similar in the two clones. These levels were unchanged by 24–72 hr treatment with NGF, 24 hr treatment with rapamycin or by NGF associated to rapamycin during the last 24 hr (Fig. 16B and data not shown).

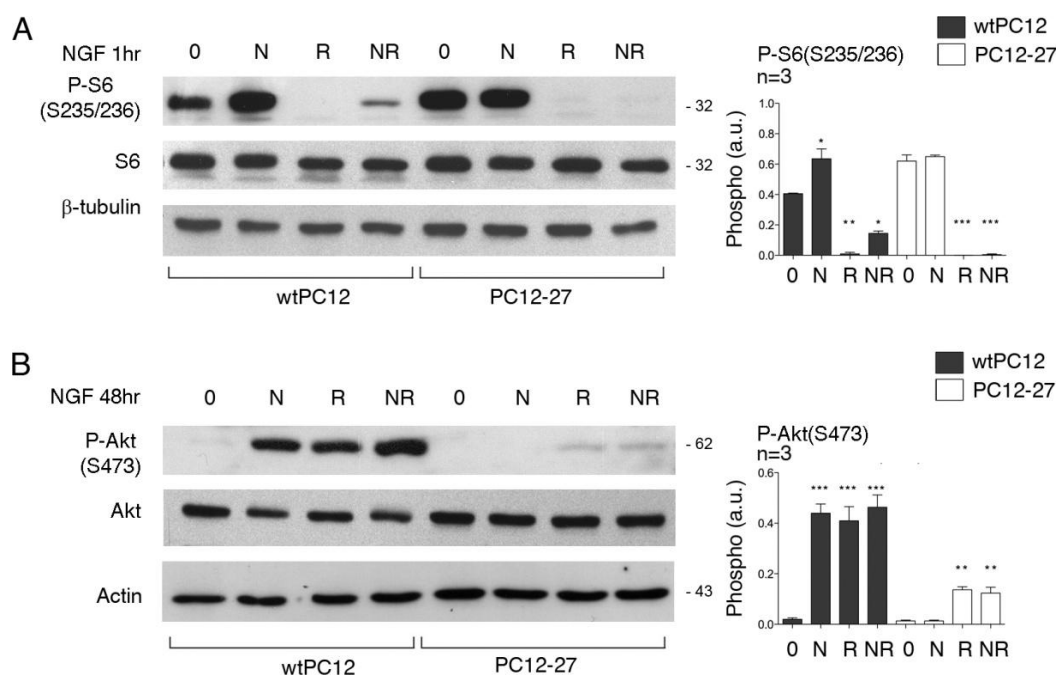


Fig. 16 Read-outs of mTORC1 (P-S6(S235/236)) and mTORC2 (P-Akt(S473)) in wtPC12 and PC12-27 cells. (A,B) wtPC12 and PC12-27 cells were treated for 48 hr with no stimulant (0), with NGF (N, 100 ng/ml), with rapamycin in the last 24 hr (R, 0.1 μ M) and with the two together (NR). The quantization of the data is on the right panels. The levels of the S6 and Akt proteins were not changed by the treatments. The numbers flanking the gels are the MDa of the immunolabeled proteins. Statistical analysis and significance of the differences is given as specified in the legend for Fig. 1.

In terms of phosphorylation, the results with the direct mTORC2 read-out, Akt(S473) were quite different in wtPC12 and PC12-27 cells. In the wtPC12, 1 to 24 hr treatment with NGF induced increases of 15–20-fold (Fig. 16B and data not shown). Treatment of wtPC12 with rapamycin induced increases similar to those induced by NGF. The two increases, however, were not additive when the cells were exposed to the combined NGF/rapamycin treatment (Fig. 16B and data not shown). In the PC12-27 clone, the resting phosphorylation of Akt(S473), distinctly lower than that of wtPC12, was not changed significantly by NGF. In contrast rapamycin did increase the P-Akt(S473) by over 5-fold. The combination with NGF did not change the increased P-Akt(S473) induced by rapamycin (Fig. 16B and data not shown).

IV.2.d. PI3K dependence of the pathways

In parallel experiments, the key role of the PI3K cascade in the control of mTORC2 was confirmed by the use of the PI3K inhibitor wortmannin. In both the wtPC12 and PC12-27 clones treated with NGF for 48 hr, treatment with the drug (0.3 mM) for the last 10 min attenuated considerably the P-TSC2(S939) and P-GSK3 β (S9) and eliminates completely the P-Akt(S473) phosphorylation (Fig. 17).

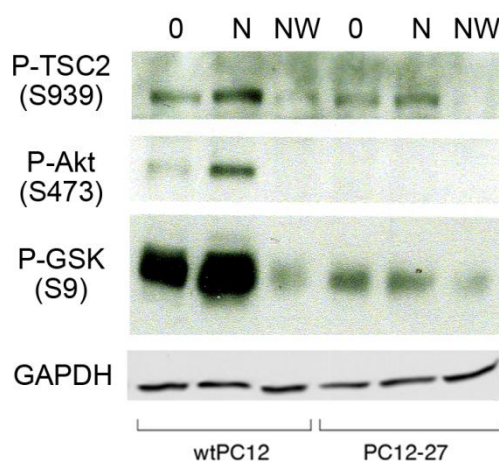


Fig. 17. mTORC2 read-outs were analyzed after wortmannin treatment in wtPC12 and PC12-27 cells. The phosphorylation of Akt(S473) induced by NGF (N, 100 ng/ml, 60 min) in wtPC12 cells was completely dissipated by the addition of wortmannin (NW, 0.3 mM) during the last 10 min.

Summing up, the results show that p75^{NTR} has a role in the control of mTORC2 activity, revealed by the direct read-out Akt(S473). The findings with PC12-27 cells strengthens, at the mTORC2 level, the non-significant rapamycin results of the PI3K cascade dependent phosphorylation of Akt(T308) illustrated in Fig. 14B. In these cells the low mTORC2 activity, unaffected by NGF, appears to be affected by the mTORC1-dependent feed-back inhibition process inhibited by rapamycin.

IV.3. Transient transfection of p75^{NTR} in PC12-27 increases mTORC2 activity

In order to confirm the involvement of p75^{NTR} in mTORC2 activation and in the differentiation process, defective PC12-27 cells were transiently transfected with the full length receptor cDNA. Transient transfection resulted in great increases of the P-

Akt(T308) and P-GSK3 β phosphorylations, the read-outs of the mTORC2 activity, whereas P-ERK was unchanged (Fig. 18).

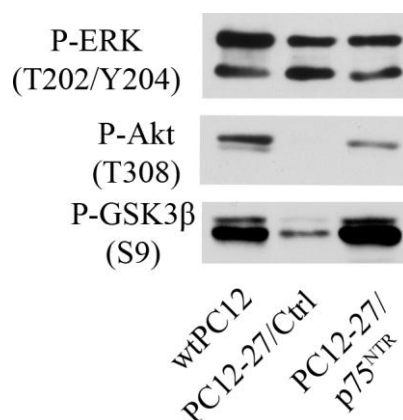


Fig. 18. Transient transfection of p75^{NTR} in defective PC12 cells induces mTORC2 activation. Panel shows that transient transfection of full length receptor leads-out the phosphorylation of Akt(T308) and GSK3 β (S9), whereas there is no difference in ERK activation.

IV.4. Generation and characterization of PC12-27/p75^{NTR} stable clones

PC12-27 cells were stably transfected with a construct of p75^{NTR} human full length cDNA and the screening of the clones for p75^{NTR} expression was performed by western blot analysis. Figure 19 shows the western blot detection of positive clones, however their level of expression is variable. Taking into consideration the level of the receptor in PC12 wt, we did choose one of these sub-clones, sub-clone 8, that from here-on is named PC12-27/p75^{NTR}.

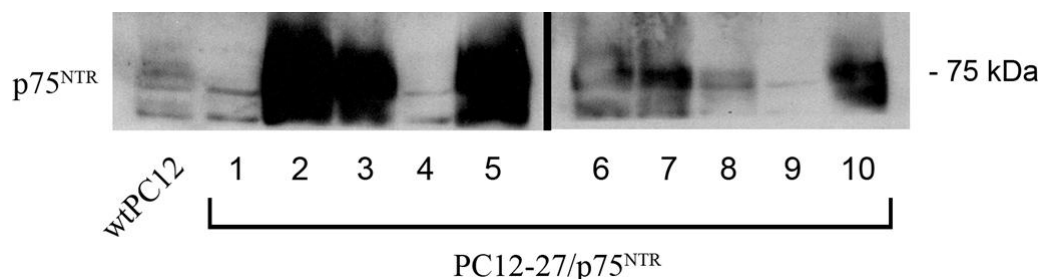


Fig. 19. Expression of p75^{NTR} in the stably transfected PC12-27 sub-clones. As demonstrate, sub-clone 8 expresses level of p75^{NTR} comparable at those of PC12 wt, whereas sub-clones 1,2,3,5 and 10 over-express the receptor, and sub-clones 1,4 and 9 express only low levels.

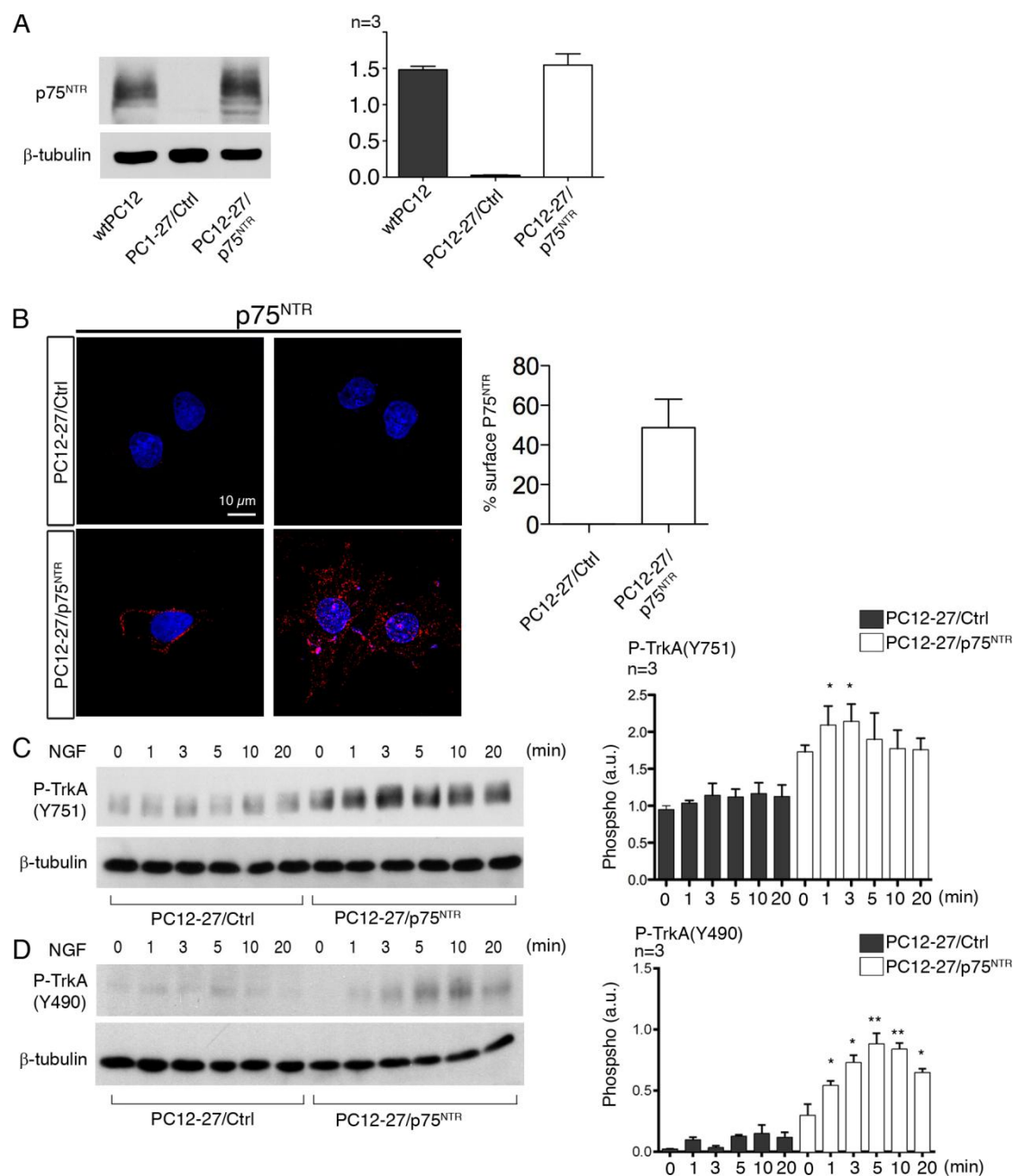


Fig. 20. Expression of p75^{NTR} and time-course of TrkA auto-phosphorylation at the Y751 and Y490 sites in PC12-27 cells transfected with the vector, empty (PC12-27/Ctrl) or including the full length p75^{NTR} (PC12-27/p75^{NTR}). (A) The western blot of p75^{NTR} in wtPC12, PC12-27/Ctrl and PC12-27/p75^{NTR} cells. Quantization of the data, documenting the similar levels of the receptor in the wtPC12 and PC12-27/p75^{NTR} cells, is on the right. (B) Surface immunolocalization of p75^{NTR} in the PC12-27/Ctrl and PC12-27/p75^{NTR} cells. The quantization of the results is on the right. Scale bar: 10 μ m. (C,D) The time-course of the TrkA auto-phosphorylation at the Y751 and Y490 sites induced by NGF (100 ng/ml) in the two transfected sub-clones, PC12-27/Ctrl and PC12-27/p75^{NTR}. The quantization of these data is shown

on the right. Statistical analysis and significance of the differences is given as specified in the legend of Fig. 10.

IV.5. NGF signaling in wtPC12 and PC12-27 cells: role of p75^{NTR}

The sub-clone, PC12-27/p75^{NTR} exhibited a surface distribution of p75^{NTR} similar to those observed in the wtPC12 (compare Fig. 20A,B to Fig. 10A,B). To exclude possible artifacts due to hyper/hypo-expression or altered distribution of the receptor, the sub-clone was selected for subsequent studies, using as control a PC12-27 sub-clone transfected with the empty vector (PC12-27/Ctrl).

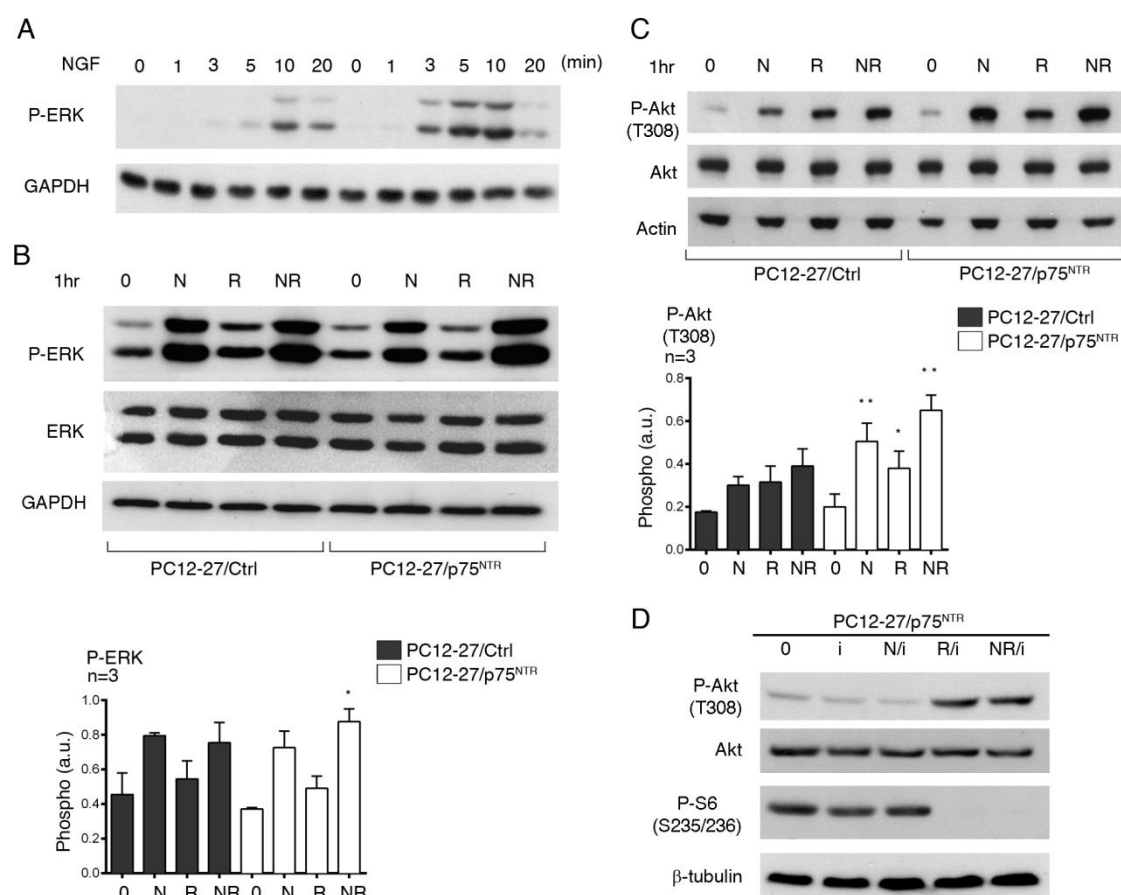


Fig. 21. Stable expression of p75^{NTR} and time-course of TrkA auto-phosphorylation at the Y751 and Y490 sites in PC12-27 cells transfected with the vector, empty (PC12-27/Ctrl) or including the full length p75^{NTR} (PC12-27/p75^{NTR}). A is a time course of the NGF responses; B and C the responses to NGF, rapamycin and the two together, with the quantitation of the results below the Western

blots; D the same in the PC12-27/p75^{NTR}, however in the presence of the TrkA inhibitor, Calbiochem 648450.

During the first 20 min treatment of PC12-27/p75^{NTR} cells with NGF, the Y751 phosphorylation of TrkA increased markedly (Fig. 20C), similar to the wtPC12 cells, whereas that PC12-27/Ctrl cells resembled that of the non-transfected PC12-27 cells, i.e. it did not change significantly. At the Y490 site the differences of phosphorylation observed between the cells transfected with and without p75^{NTR} were even larger. In the PC12-27/Ctrl cells this phosphorylation remained almost inappreciable, as in the non-transfected PC12-27 cells, whereas in the PC12-27/p75^{NTR} cells it increased significantly and rapidly upon NGF addition, reaching a maximum at 5–10 min, as in the wtPC12 (Fig. 20D). The study of the two cascades, of ERK and PI3K, confirmed the marked changes of the NGF signaling induced in PC12-27 cells by the expression of p75^{NTR}. In the case of ERK the phosphorylation of ERK 1/2(T202 and Y204), induced by 1–20 min treatment with NGF, exhibited a faster rate in the PC12-27/p75^{NTR} cells compared to the PC12-27/Ctrl cells (Fig. 21A), similar to the faster rate of the wtPC12 compared to the PC12-27 cells shown in Fig. 13.

Also the responses induced in the PC12-27/Ctrl and PC12-27/p75^{NTR} cells by 1 hr treatment with NGF, alone or with rapamycin, resembled the responses induced by the same treatments in the PC12-27 and wtPC12 cells, respectively. With rapamycin alone the changes were small and non significant in both transfected PC12-27 cell sub-clones (Fig. 21B). In contrast, in the case of P-Akt(T308), the increases in PC12-27/p75^{NTR} cells induced by NGF were larger than in PC12-27/Ctrl (Fig. 21C). Also with the two Akt targets, TSC2(S939) and GSK3 β (S9), the phosphorylations induced by NGF and also by rapamycin in the PC12-27/p75^{NTR} were distinctly larger than those in the PC12/Ctrl cells (Fig. 22).

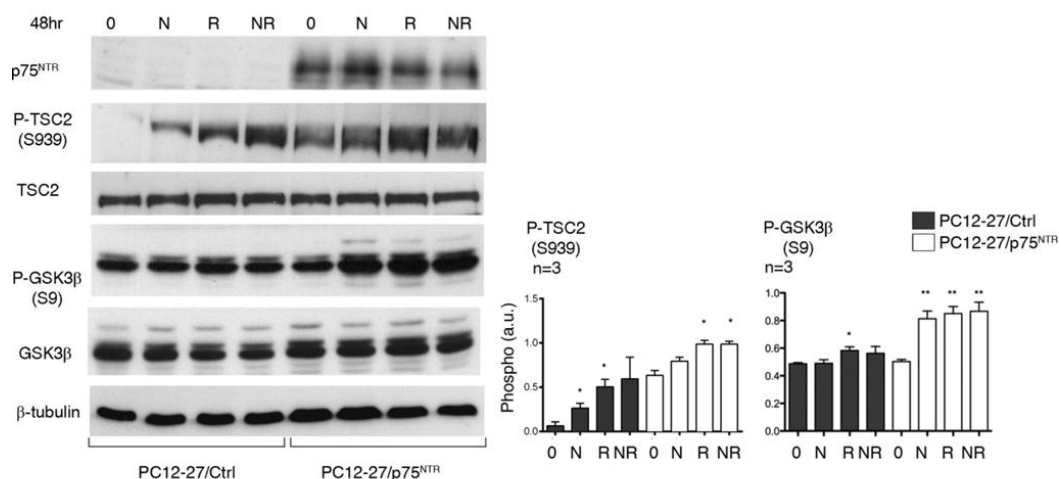


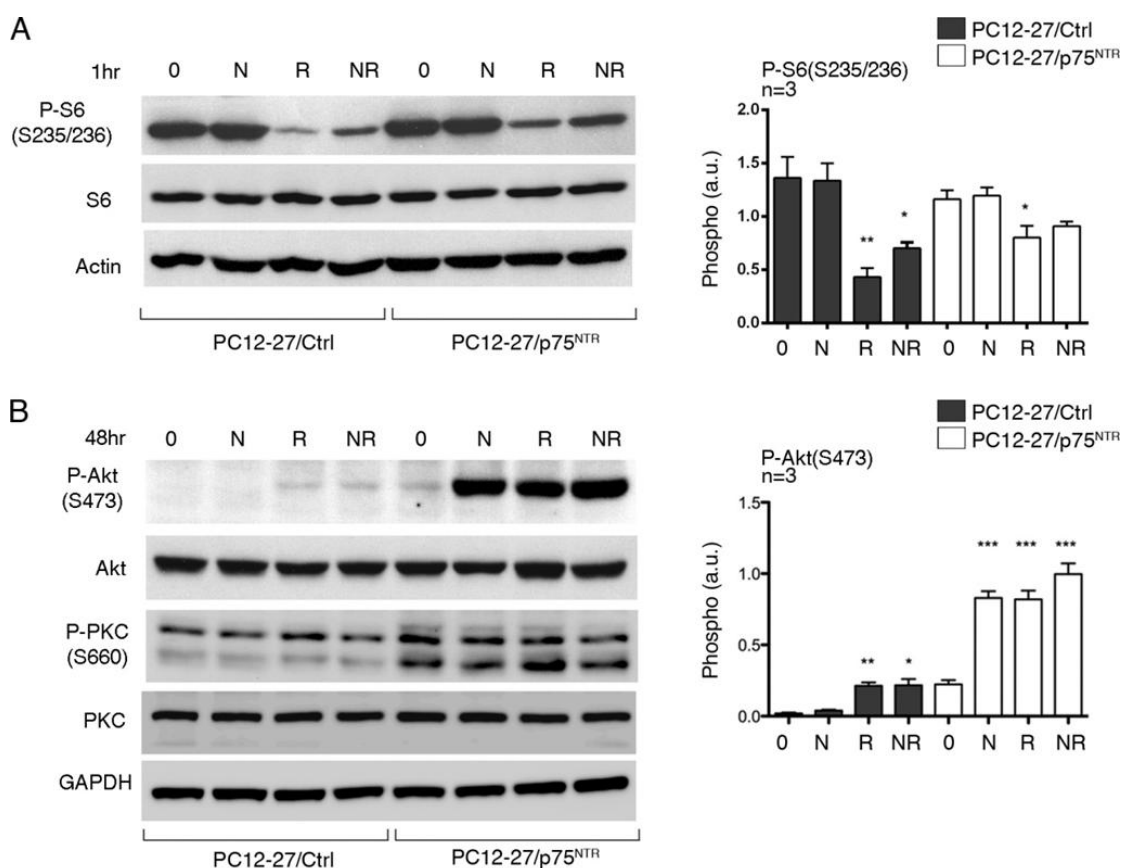
Fig. 22. Phosphorylation of two Akt targets, TSC2(S939) and GSK3β(S9), induced by NGF and rapamycin administered for 48 and 24 hrs, respectively, in PC12-27/Ctrl and PC12-27/p75^{NTR} cells. The results in the PC12-27/Ctrl cells resemble those obtained with the PC12-27 cells. The transfection of p75^{NTR} induces an increased phosphorylation that in the case of GSK3β(S9) occurs not only upon treatment with rapamycin (R, 1 μM) but also with NGF (N, 100 ng/ml).

A question about the signaling of the PC12-27/p75^{NTR} cells was whether the increases of the ERK and PI3K cascades induced by the expression of p75^{NTR} were dependent on the transfected receptor only or on its cooperation with TrkA. To answer this question we repeated the experiments of Fig. 21B,C by employing PC12-27/p75^{NTR} cells pretreated with a specific inhibitor of the TrkA receptor, Calbiochem 648450 (Yamashita and Tohyama, 2003). Fig. 21D shows that the increased phosphorylation of Akt(T308) induced by NGF was prevented by the pretreatment of the cells with the drug. In order to be generated, the NGF-induced signal requires therefore the two receptors, TrkA and p75^{NTR}, to be activated concomitantly. The cooperation with p75^{NTR}, however, does not seem to occur only with TrkA. In fact, the responses triggered by rapamycin, alone or together with NGF, were apparently unchanged by the pretreatment of the PC12-27/p75^{NTR} cells with the TrkA inhibitor (Fig. 21D). In PC12 cells the site of action of the mTORC1-dependent feed-back inhibition, the process blocked by rapamycin, is unknown. In other cell types, however, this site has been proposed to coincide with the insulin receptor substrate 1 (IRS1) (Tremblay *et al.*, 2007). This or another post-receptor site may therefore operate in the cooperation of the feed-back inhibition with p75^{NTR}. In conclusion, the re-establishment in the PC12-27 of a TrkA/p75^{NTR} ratio analogous to the ratio in

wtPC12 was found to rescue the NGF signaling from a partially inactive to a fully active state.

IV.6. mTORC1 and mTORC2 in PC12 cells: role of p75^{NTR}

The lack of p75^{NTR} could be the cause of the different activity of mTORC1 and mTORC2 in PC12-27 cells. Fig. 23A shows that, with mTORC1, the change induced by the stable expression of the receptor was minor. With respect to the PC12-27/Ctrl cells the phosphorylation of the direct (S235/236) S6 read-out was in fact only moderately lower in the resting and NGF-treated PC12-27/p75^{NTR} cell and the inhibitory effect of rapamycin was also lower (Fig. 23A). The situation was profoundly different with mTORC2 (Fig. 23B). In the PC12-27/Ctrl cells the read-out P-Akt(S473) was similar to the non-transfected PC12-27 cells, i.e. it was unresponsive to NGF and increased only little after treatment with rapamycin, (compare Fig. 23B to Fig. 16B).



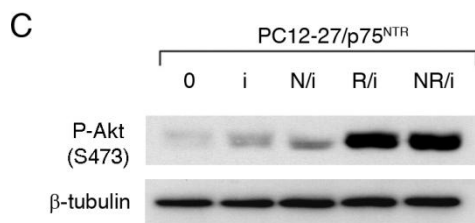


Fig. 23. mTORC1 and mTORC2 in the PC12-27/Ctrl and PC12-27/p75^{NTR} cells; effects of the TrkA inhibitor. (A) The expression of p75^{NTR} does not change the responses of the mTORC1 read-out, P-S6(S325-326) to 1 hr treatment with NGF (N, 100 ng/ml). Rapamycin (R, 1 μ M) induces inhibition of the mTORC1 read-out phosphorylation, which is more extensive in the PC12-27/Ctrl cells. (B) The p75^{NTR} transfection induces the rescue of the mTORC2 read-out P-Akt(S473) phosphorylation which is increased markedly by both NGF (N,100 ng/ml) and rapamycin (R, 1 μ M) The phosphorylation of another mTORC2 read-out, PKCa, was high in the PC12-27/p75^{NTR} cells already at rest, with no appreciable changes induced by the treatments. The quantized data of panels A and B in PC12-27/Ctrl and PC12-27/p75^{NTR} cells are given on the right panels. (C) Two hr treatment with the TrkA receptor inhibitor, Calbiochem 648450 (I, 10 nM), removed the response triggered in the PC12-27/p75^{NTR} cells by 1 hr treatment with NGF (N,100 ng/ml), leaving however unchanged that triggered by rapamycin (R, 1 μ M), administered alone or combined to NGF. These results demonstrate 1) that the NGF response is mediated by the cooperation of the TrkA and p75^{NTR} receptors; and 2) that the mTORC1-induced feed-back block by rapamycin cooperates with p75^{NTR} working however not at the TrkA receptor but at a post-receptor site. Statistical analysis of the differences on the right in panels A and B is given as specified in the legend for Fig. 10.

In contrast, in the PC12-27/p75^{NTR} cells the phosphorylation of the read-out after NGF, rapamycin and the two together was much stronger, approaching values similar to those observed in the wtPC12 (compare Fig. 23B to Fig. 16B). Moreover, similar to the results of P-(T308)Akt (Fig. 21D), also the responses of the mTORC2 read-out P-(S473)Akt induced by treatment with NGF in the PC12-27/p75^{NTR} cells were inhibited by the specific TrkA blocker drug, Calbiochem 648450 (Yamashita and Tohyama, 2003). In contrast, the responses to rapamycin, acting by removal of the mTORC1 feed-back inhibition, were unchanged (Fig. 23C).

Together with the data of Fig. 21D these results confirm the cooperation between the TrkA and the p75^{NTR} signaling to be necessary for the mTORC2 activation unless the signal is triggered not at the level of TrkA but at a post-receptor site. Summing up, the expression of p75^{NTR} in the PC12-27 cells appears to modify

both the signaling of NGF and the activity of mTORC2, bringing them to levels approaching those of wtPC12.

IV.7. About the phenotype of PC12-27/p75^{NTR} cells

We then investigated, in the PC12-27/p75^{NTR} cells, a few properties known to distinguish the PC12-27 from wtPC12 cells, i.e. the very high REST (D'Alessandro *et al.*, 2008), the low TSC2 and the high β -catenin (Section III and Tomasoni *et al.*, 2011). The question was whether these properties also depend on the lack of the p75^{NTR} receptor. Figure 24A shows that this is not the case. In fact the levels of the three factors in the PC12-27/p75^{NTR} cells were as high as in the PC12-27/Ctrl cells. Also a few functional properties dependent on the high β -catenin, i.e. the luciferase assay of its transcription activity, the expression of a transcription target, the cMyc oncogene, and the high cell proliferation (Section III and Tomasoni *et al.*, 2011), were not changed significantly by the p75^{NTR} transfection (Fig. 24B-D).

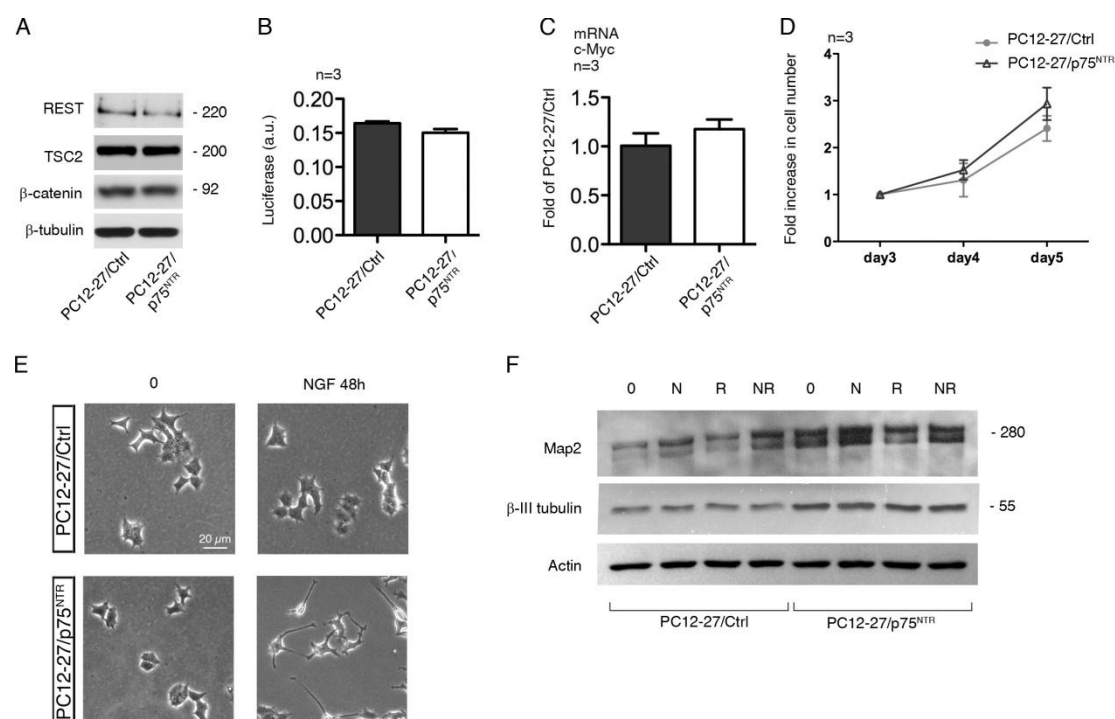


Fig. 24. Phenotype of the PC12-27/Ctrl and PC12-27/p75^{NTR} cells. (A–D) No difference exists between PC12-27/Ctrl and PC12-27/p75^{NTR} in a number of important features: the levels of REST, TSC2 and β -catenin proteins (A); the β -catenin-dependent transcription revealed by a luciferase assay (B); the expression of

the β -catenin-target gene, cMyc (C); the rate of cell proliferation (D). (E) Phase contrast images of the PC12-27/Ctrl and PC12-27/p75^{NTR} before and after a 48 hr treatment with NGF (100ng/ml). Scale bar: 20 μ m. (F) The expression of two neuronal markers, Map2 and β -III tubulin, in PC12-27/Ctrl and PC12-27/p75^{NTR} cells incubated for 48 hr with no treatment, with NGF (N, 100 ng/ml), rapamycin (R, 0.1 μ M during the last 24 hr) and the two together.

Finally, we investigated whether, and to what extent, the expression of p75^{NTR} modifies two aspects of the phenotype sensitive to NGF that are greatly defective in PC12-27 cells, the outgrowth of neurites and the expression of neuron-type markers. Fig. 24E compares the morphology of the PC12-27/Ctrl and PC12-27/p75^{NTR} cells, at rest and upon 48 hr treatment with NGF. The flat structure of PC12-27/Ctrl cells, similar to that of the non-transfected PC12-27 cells (Section III and Tomasoni *et al.*, 2011) was hardly affected by the 48 hr treatment with NGF. In the PC12-27/p75^{NTR} cells, on the other hand, the shape was not changed much, however the NGF-induced neurite outgrowth response was evident in terms of both number of neurites sprouted per cell and average neurite length (Fig.24E). In the resting PC12-27/Ctrl cells the levels of the two neuronal markers investigated, Map2 and β -III tubulin, were low. Treatment for 48 hr with NGF, 24 hr with rapamycin or the two together induced only small or no increases. In PC12-27/p75^{NTR} cells, the resting levels of the two markers were higher, however the increases induced by NGF and rapamycin were small and non significant (Fig.24F).

IV.8. Stable silencing of p75^{NTR} by miRNA in PC12 cells:

In order to investigate the possible involvement of the p75^{NTR} receptor in the regulation of the mTORC2 activity we carried out experiments in sub-clones of wtPC12 cells in which the expression of the receptor had been down-regulated by the stable transfection of a specific miRNA. Fig. 25 shows results with a wtPC12 sub-clone with p75^{NTR} level reduced to approximately 20%, i.e. with a large decrease which however was lower than that of PC12-27 cells, where the receptor is inappreciable.

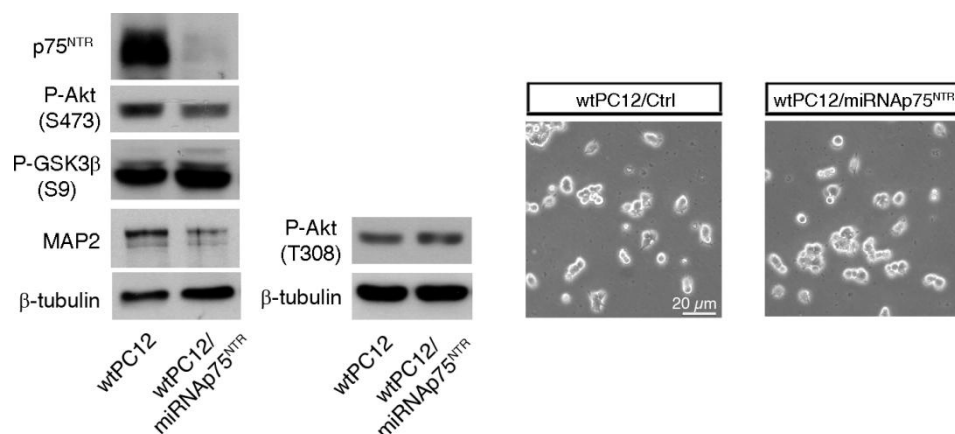


Fig. 25. Phenotype of the PC12-27/Ctrl and PC12-27/p75^{NTR} cells. In the subclone illustrated here, in which the level of the receptor was decreased of about 80%, the decrease of the mTORC2 read-out P-Akt(S473) was decreased of about 35%. These changes were apparently ineffective on the PI3K cascade since the P-Akt(T308) and P-GSK3β(S9) were unchanged, while the level of the neuronal marker MAP2 was significantly decreased. The general phenotype of the wtPC12 cells was apparently unaffected by the miRNA expression. Scale bar: 20 μm.

The miRNA transfection did not modify the general phenotype of the wtPC12 cells, that remained largely spherical, different from the flat shape of the PC12-27 cells (Tomasoni *et al.*, 2011). Likewise, the P-Akt(T308) and the P-GSK3β(S9) were unchanged. In contrast, the Akt(S473) phosphorylation was decreased of about 35%, suggesting the mTORC2 activity to be reduced (Fig. 25).

V. DISCUSSION

The data reported in this thesis about the role of REST in PC12 cells are quite numerous and, in many respects, unexpected. The possibility that high REST induces proliferation of neurosecretory cells had been reported especially in relation to neural fast-growing and aggressive tumors (reviews: Coulson, 2005; Majumder, 2006; Negrini et al, 2013). In these cases, however, a precise mechanism of the REST stimulation had never been identified. The condition of PC12 cells is quite different from that of the above tumors. The cell line originates from a pheochromocytoma, a differentiated, slow-growing, non-aggressive tumor. Therefore the findings we did obtain by comparative investigation of the two PC12 clones, characterized one by the typical low level of REST and the other by a high level of the transcription repressor, had apparently little to do with those on tumors reported previously. Mechanistically, our findings appeared reasonable when we found the increase of REST to be accompanied by a decrease of TSC2, a GAP protein known to govern negatively the activity mTORC1. The mechanism by which the TSC2 decrease is induced by high REST is unclear. Certainly the effect is not due to the typical function of REST, the transcription repression, because the level of the TSC2 mRNA was not decreased in PC12-27 cells. At this stage we can only hypothesize that the decrease of the TSC2 protein is due to its increased turnover induced by REST indirectly, via the activation of an ubiquitinase system that remains to be identified.

V.1. REST, TSC2 and β -catenin govern proliferation working as a signaling/effector loop.

However, the increased activity of mTORC1, was shown to account for only a minor fraction of the fast proliferation of the high REST PC12-27 cells. The major contribution to the proliferation was mediated in fact by the increase of β -catenin, dependent of the decrease of its fast metabolism and as a consequence, of its localization to the nucleus. A negative control of the TSC complex on the β -catenin metabolism had been reported (Jozwiak and Wlodarski 2006; Barnes et al., 2010), however this control pathway, at variance with the TSC complex-mTORC1 pathway,

had failed to become popular. In this case the REST-TSC2 was found to be particularly efficient because β -catenin, together with its classical co-transcription targets, including cMyc and cyclin D1, was found to increase the transcription of REST. Thus REST, TSC2 and β -catenin appear to control the proliferation of PC12 cells working as a loop (Fig. 23). Evidently each of these factors has multiple functions, many of which previously known. Our work has now identified a new, important function, operative in neural cells, in which the three factors operate coordinately. Additional aspects of the data we have presented in the section III of this thesis can be found in the paper we have published about this work (see Tomasoni, Negrini et al., 2011). The model in Figure 26, taken from that paper, illustrates the REST control of proliferation as emerged from our studies.

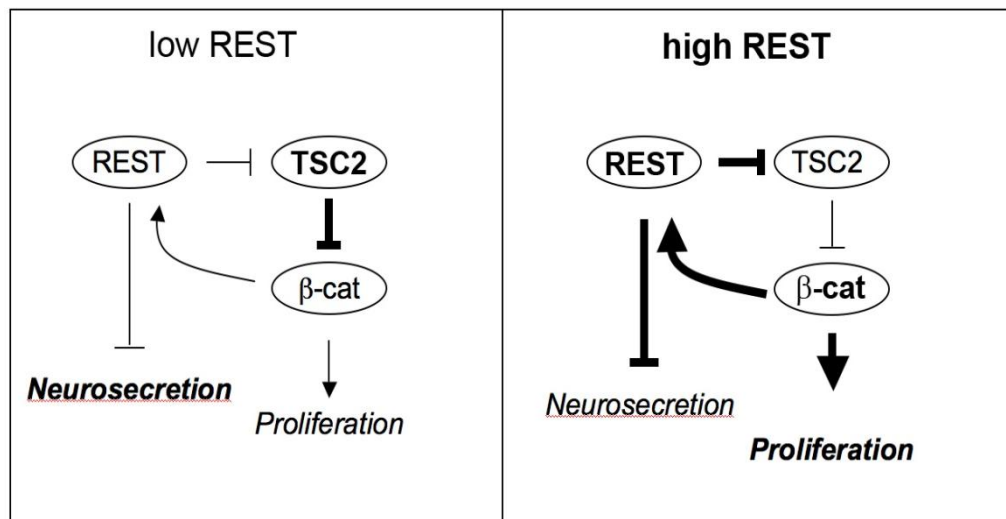


Fig. 26. A model of the feed-forward loop signaling paradigm governing PC12 cell proliferation and neurosecretion. The figure summarizes the results illustrated in Figs 1–7, emphasizing the coordinated regulation of the two cell functions. Changes of one (or more) of the interconnected factors, REST, TSC2 and β -catenin (β -cat), impact the whole signaling loop and affect both proliferation and neurosecretion as shown by the variable thickness of the connections. Other processes, not yet investigated, might be impacted as well. In low-REST cells (left) repression of TSC2 is weak, the TSC1–TSC2 complex reinforces the proteasome degradation of β -catenin, transcription of the β -catenin–TCF–LEF target genes, including the oncogenes and REST, is low. As a consequence proliferation of cells (dependent on β -catenin) is low whereas neurosecretion (repressed only marginally by low levels of REST) is high. In high-REST cells (right), repression of TSC2 is strong, with ensuing increase of β -catenin and of the β -catenin–TCF–LEF oncogene and REST transcription. As a consequence, proliferation is high whereas neurosecretion is repressed to a considerable extent by the high levels of REST (See the detailed Figure-Abstract here above).

V.2. The regulation of neurite outgrowth was still largely unknown.

The section IV of this thesis deals with another key issue in our neural cell investigation, i.e. the mechanism that govern the outgrowth of neurites. This property is general among neural cell lines. In most of them, however, the response visible upon stimulation with various factors, although present is quite small. In contrast, in the case of wtPC12 the outgrowth induced by long-term treatment with NGF is extensive, similar to the one occurring in the initial phase of in vitro differentiation of neurons. The difference between wtPC12 and neurons appears in the subsequent phase, because neurons convert one of the neurites into a typical axon, whereas wtPC12 don't. Thus wtPC12 are a model of the initial phase of neural cell differentiation, that was employed in thousands of studies published in the last 30 years. The high REST PC12-27 cells differ from their wt counterparts because their NGF-induced neurite outgrowth is almost non-existent. The comparative investigation of the two PC12 clones was therefore offering the possibility to clarify whether REST is involved in the control of the outgrowth and what is the mechanism of this control.

V.3. A new role for p75^{NTR}.

The initial hypothesis put forth to explain the lack of outgrowth in the PC12-27 cells (and in another clone that is now known to be of high REST as well, D'Alessandro et al., 2008) was the lack in the defective cells of the tyrosine kinase receptor of NGF, TrkA (Leoni et al., 1999). This hypothesis appeared reasonable because TrkA is believed to govern almost completely the responses induced by the factor, with the second receptor, p75^{NTR}, playing only minor roles: the increase of the TrkA affinity for their ligand or a number of other cooperative activities. When however we did investigate the expression of the NGF receptors in the high REST PC12-27 cells we were surprised to find that TrkA was unchanged, whereas p75^{NTR} was almost non-existent. This finding, on the one hand, was reasonable because, from the data of the literature, the p75^{NTR} gene was shown to include two RE-1 sequences in its promoter; and because, on the other hand, the response to NGF of PC12-27 cells was not absent but specifically missing of one pathway, the PI3K-Akt pathway, and

not that of ERK1/2. The reinforcement by P75^{NTR} of the TrkA-induced activation of the PI3K-Akt cascade had been envisaged (Roux et al., 2001). However its consequences, in particular the activation of mTORC2 and the key role of the two receptor cooperation in neurite outgrowth have never been reported. Our experimental approach permitted us to investigate comparatively the activation of the cascade pathway signaling not only in wtPC12 and PC12-27, but also in the latter stably transfected with either an empty vector or p75^{NTR} at levels analogous to those of the wtPC12. Taken together the results revealed without any doubt that TrkA activation in unable to trigger the PI3K-Akt cascade unless its works cooperatively with p75^{NTR}; that the lack of this activation precludes the activation of mTORC2 (which depends on PI3K), and thus of the cascade of events governed by the latter kinase; and that one of the PI3K-dependent processes was just the neurite outgrowth. Clearly the PC12-27 subclone transfected with p75^{NTR} was not at all identical to wtPC12. The subclone, in fact, maintained its high level of REST and, among other things, showed to be more sensitive to the feed-back inhibition on the receptor signaling induced by the activation of the mTORC1 complex. Nevertheless the data we have obtained provided new information about the coordinate functioning of the two NGF receptors and about the intracellular cascades triggered by their activation. A partial presentation of the data of our Section IV, illustrating the events induced by NGF in cells expressing only TrkA or TrkA together to p75^{NTR} is shown in Fig. 24.

V.4. Conclusion.

In conclusion, our two subsequent investigation of two important chapters of cell biology, proliferation and neurite outgrowth in PC12 cells, have revealed new aspects of the role of REST in those neural cells. REST is not only a controller of a few properties specific of neural cells such as neurosecretion, but is involved in multiple functions and properties, not only proliferation and neurite outgrowth but probably many others, that remain to be investigated. A critical aspect for the future studies will be the choice of the cellular models that can influence significantly the effects of REST, making the various REST-sensitive genes available or not to the repression of the transcription factor.

VI. MATERIALS AND METHODS

VI.1. Cell cultures.

The rat pheochromocytoma wtPC12 and PC12-27 clones, and the various subclones, were grown in Dulbecco's Modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with either 1% (starvation) or 10% horse serum (Euroclone) together with 5% fetal clone serum III (FCIII) (Hyclone Laboratories, Logan, UT). Starvation and complete medium were supplemented also with 2mM ultraglutamine and 100 U/ml penicillin and streptomycin (Lonza). All cells were grown at 37°C, in a 5% CO₂ humidified atmosphere.

VI.2.1. Stable and transient transfections.

For stable transfection of p75^{NTR}, PC12-27 clone cells at about 40% confluence in 10 cm diameter dishes were treated with 10µl of lipofectamine 2000TM reagent (Invitrogen) dissolved in 5ml of medium without antibiotics supplemented with 0.3 µg of plenty-OE-sRFP plasmid including the full length human p75^{NTR} together with 0.3 µg of pcDNA3.1Hygro(+) vector carrying the hygromycin resistance gene (Invitrogen). The controls received the vectors only. After 5 hour treatments the above medium was exchanged with the complete medium, and 48 hours after transfection the cells were split in 10 cm dishes at 1:8 ratio for selection in complete medium supplemented with 500 µg/ml of hygromycin B (Invitrogen). Screening of the subclones for the expression of p75^{NTR} construct was performed by western blotting. The wtPC12/miRNA-p75^{NTR} clones were produced by co-transfection of cells in 10 cm diameter dish with 9 µg of pRRLsinPPT plasmid including the p75^{NTR} miRNA, to downregulate the receptor, and 1 µg of pcDNA3.1Hygro(+), using the same procedure employed for the overexpression. The hygromycin B concentration used for the selection of these subclones was 200 µg/ml.

Transient transfections of the PC12-27 clone with mycREST, DBD-REST and p75^{NTR} constructs were performed using cells at about 40% confluence in 10 cm diameter dishes containing 5ml of medium without antibiotics supplemented with 10 µg of the plasmids and 10µl of lipofectamine 2000TM. The controls received the vectors only. After 5 hours the media were exchanged with the complete medium,

and 48 hours after transfection the cells were used for protein extraction. The transfections were confirmed by RT-PCR and immunoblotting.

VI.2.2. Expression plasmids.

The plenty-OE-sRFP vector including the full length human p75^{NTR} cDNA, and the pRRLsinPPT vector with the p75^{NTR} miRNA were both generous gifts of P.A.Barker (Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada). The dominant negative construct of REST, corresponding to the sequence encoding the DNA-binding domain (amino acid residues 234-437) fused to a Myc epitope and inserted into the pAdTrack-CMV vector, was the generous gift of E. Cattaneo (Department of Pharmacological Sciences and Centre of Excellence on Neurodegenerative Diseases, University of Milan, Italy). The pcDNA3.1/Hygro(+) vector, used in the controls and for the co-transfections, was from Invitrogen.

VI.2.3. mRNA isolation and Real-Time PCR

Total cellular RNA was extracted from cultured cells according to the RNeasy Mini Kit protocol (QIAGEN, Valencia, CA, USA), and its concentration was determined by spectrophotometry. Total RNA (1-2 µg) was used to generate cDNA templates for RT-PCR, using the random hexamers provided with the RevertAid First Strand cDNA Synthesis (from Thermo Scientific) kit. RT-PCR was performed on a LightCycler® 480 System (FastStart DNA Master SYBR Green I of Roche Appl. Sci.) according to a standard protocol, using 50 ng cDNA as template. Values are expressed either as fold of wt or as PC12-27/control (Ctrl) cell ratio.

Primer sequences used for PCR amplification of human p75^{NTR} gene were the following: forward p75^{NTR}: GTGGCCAGAATAACATTCCTATCGC; reverse p75^{NTR}: GAAGGCAAGTTTTGGGGTGATGGTG

Primers were used at the final concentration of 500 nM. Values were normalized to the concentration of calmodulin mRNA.

VI.3.1. Protein Assays; Western Blotting

In order to generate the total cell extracts, the cells of the various clones and subclones were washed in PBS and solubilised on ice in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, together with

phosphatase inhibitor (Roche) and protease inhibitor (Sigma) cocktails. The mixtures were incubated for 15 min at 4°C and then centrifuged at 14,000 rpm for 15 min. The pellets were discarded and the supernatants recovered. To investigate the time-course of TrkA phosphorylation the incubated cells were transferred rapidly to ice and then suspended in ice-cold lysis buffer containing 10 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P40, 1% (w/v) sodium deoxycholate, with protease and phosphatase inhibitors. The lysates were cleared by centrifugation at 16.000 g for 20 min at 4°C, and the supernatants were analyzed (Takahashi et al., 2011).

Protein concentrations were determined by the BCA assay. Appropriate amounts (most often 30 µg) were separated by SDS-PAGE and then transferred to nitrocellulose filters (Whatman) by overnight incubation at 250 mA. Filters were first blocked for 1 h at room temperature (RT) with 5% non-fat dry milk in Tris Buffered Saline (TBST) containing 200 mM NaCl, 50mMTris pH7.4, with 0.5% Tween20; then incubated for 2h at room temperature or, alternatively, overnight at 4°C, with the primary antibody diluted in TBST-1% BSA-0.2% sodium azide; washed 5 times for 10 min in TBST, and then incubated for 1 h at room temperature with the appropriate peroxidase-conjugated secondary antibody (1µg/ml) in 5% non-fat dry milk in TBST. Upon further 5, 10 min washings in TBST, they were finally developed with either the ECL chemiluminescence detection system (Amersham Pharmacia) or the Femto Signal (Pierce). Western blot bands were quantified by the ImageJ program (rsb.info.nih.gov/ij), normalized to markers that do not change their concentration during the experiment (β -tubulin, actin or GAPDH) that had been immunolabeled in parallel. Data are expressed as arbitrary units (a.u.).

VI.3.2. Immunofluorescence and bright field microscopy

Cell monolayers plated on coverslips were fixed for 10 min at room temperature in 4% formaldehyde dissolved in PBS, quenched with 0.1 M glycine, then permeabilized for 20 minutes a room temperature in PBS containing 0.2% Triton X-100 and 1% BSA, and washed. They were then incubated with primary antibodies in dilution buffer (DB, containing PBS and BSA 1%) for 1 h at room

temperature. Then, they were extensively washed in PBS, exposed to fluorescence-conjugated secondary antibodies in DB for 1 h at RT, washed again and finally mounted in gelvatol. In some cases nuclei were stained with DAPI. The cells were analysed in a Perkin-Elmer Ultraview ERS confocal and Delta Vision microscopes. Image deconvolution was performed in a wide-field microscope of the Delta Vision system.

VI.3.3. Antibodies and chemicals:

The anti-p75^{NTR} extracellular domain (REX) rat polyclonal antibody (pAb) was a gift of L. Reichardt (Departments of Physiology, University of California, San Francisco). The other antibodies were from the following commercial sources: anti-P-Akt(S473), anti-P-Akt(T308), anti-GSK3 β , anti-PGSK3 β (S9), anti-TSC2, anti-PKC α (S660), and anti-S6 monoclonal antibodies(mAbs); anti-TrkA, anti-P-TrkA(Y490), anti-ERK 1/2 and anti-P-ERK 1/2(T202/Y204), anti-P-TSC2(S939), anti-Akt and anti-P-S6(S235/236) rabbit pAbs, Cell Signaling; anti-P-TrkA(Y751) and anti-P-TrkA(Y670/Y674/Y675) rabbit pAb: Invitrogen; anti-TrkA C20 extracellular domain rabbit pAb, anti-actin goat pAb: Santa Cruz; anti-p75^{NTR} rabbit pAb, Promega; anti-REST rabbit pAb: Upstate; anti- β tubulin mAb and anti-actin rabbit pAb: Sigma; anti- β -catenin mAb: BD Transduction; anti-Map2 mAb, Millipore; anti- β -III tubulin mAb, Covance; FITC-conjugated and TRITC-conjugated goat anti-rabbit pAbs, and goat anti-mouse IgG subclasses: Southern Biotech.; horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit pAbs: Bio-Rad. NGF was from Alomone; the BCA Protein Assay Kit from Pierce; rapamycin and the TrkA inhibitor 648450 from Calbiochem; the fluorescent DNA binding probe DAPI, wortmannin and other chemicals, from Sigma–Aldrich.

VI.4. Materials

The ECL western blotting detection reagents and chemiluminescence films were from Amersham Pharmacia; the BCA protein protein assay kit and SuperSignal West Femto Maximum Sensitivity Substrate were from Pierce. The SDS-PAGE apparatus was from Hoefer. The Maxi Kit for EndoFree plasmid DNA purification and the RNeasy Mini Kit for total RNA isolation were from QIAGEN. The Mini Kit for plasmid DNA isolation and purification was from Promega.

Lipofectamine 2000™ for plasmid DNA transfection, the ThermoScript™ and the RevertAid First Strand cDNA Synthesis were from Thermo Scientific. Competent cell TOP10 was from Invitrogen; the 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) from Sigma-Aldrich.

VI.5. Statistical analyses

The significance of the data was assessed using the two-tailed unpaired t-test and the Anova test, making reference to unstimulated samples of both controls and variously stimulated cell preparations. Data shown are means \pm s.e. The number of experiments is specified in the Figures or Figure legends. $P < 0.05$ is considered significantly different. In the Figures, *** means $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

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