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Expression of the neurosecretory process in pc12 cells is governed by rest

Rosalba D'Alessandro,* Andrijana Klajn,* Laura Stucchi,* Paola Podini,† Maria Luisa Malosio† and Jacopo Meldolesi*,†,‡,§

*Vita-Salute San Raffaele University, Center of Excellence in Cell Development, Milan, Italy
†Scientific Institute San Raffaele, Milan, Italy
‡Istituto Nazionale di Neuroscienze, Milan, Italy
§IIT Network, Research Unit of Molecular Neuroscience, Milan, Italy

Abstract

The neurosecretory process is acquired during differentiation and can be lost en block by differentiated cells. To investigate the role of REST/NRSF, a transcription repressor, in the maintenance of the process we studied two PC12 clones, one wt and one defective, expressing low and high levels of endogenous RE-1 silencing transcription (factor) (REST), respectively. Stable transfection of constructs demonstrated that REST represses 10 genes coding for proteins of neurosecretory vesicles and their exocytosis, eight including and two lacking the REST-binding sequence, RE-1. Of these genes, those of chromogranins were strongly repressed by fewfold increases of REST, those of VAMP2 and syntaxin1a required much higher levels. Moreover, in wt cells transfected with an active construct the dense-core vesicles, still competent for regulated exocytosis, were much smaller, with lighter cores; in defective cells, the dominant-negative construct induced the rescue of many vesicle/exocytosis genes but not of those of chromogranins. Small dense-core vesicles, exocytized upon stimulation, were rescued when the construct-transfected defective cells were transfected also with chromograninA or treated with trichostatinA, a blocker of histone deacetylases. Our results identify REST, working by direct and indirect mechanisms, as the factor governing the maintenance of the neurosecretory process and the properties of dense-core vesicles in PC12 cells.

Keywords: chromogranins, dense-core vesicle, gene expression, regulated exocytosis, SNAREs.
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The neurosecretory process, by which neurons and neurosecretory cells store their neurotransmitters and/or neurohormones within unique organelles and discharge them by regulated exocytosis, is the central event of neurosecretion, acquired by the cells in the course of their progression from pluripotent stem cells to restricted lineages. Extensive studies carried out during the last decade (reviewed by Ballas and Mandel 2005; Wu and Xie 2006) have shown that this progression depends on a complex signaling network orchestrated by the repressor element 1-silencing transcription factor (REST, also referred to as NRSF), operating also through the involvement of additional factors, active at the transcriptional and post-transcriptional level (Conaco *et al.* 2006; Ma 2006; Wu and Xie 2006). Binding of REST to a specific DNA sequence distributed in its numerous target genes, the repressor element 1 (RE-1), entails the assembly, at the N and C terminal domains of the repressor, of two protein complexes, including histone and DNA modifying enzymes. The activity of these complexes consists in the repression of transcription. The differential development of

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Address correspondence and reprint requests to Jacopo Meldolesi, Vita-Salute San Raffaele University, DIBIT, via Olgettina 58, 20132 Milan, Italy. E-mail: meldolesi.jacopo@hsr.it

Abbreviations used: DBD, DNA-binding domain; DBD/REST, dominant negative construct of REST; DCV, dense-core vesicle; FITC, fluorescein isothiocyanate; GC, Golgi complex; hChgA and ChgA, human and rat chromogranin A; hChgB and ChgB, human and rat chromogranin B; HDAC, histone deacetylase; ICA512, Islet cell antigen 512; Q-SNARE and R-SNARE, target and vesicle SNAREs; RE-1, responsive element 1; REST, RE-1 silencing transcription (factor); Scg2, secretogranin 2; SLMV, synaptic-like microvesicles; Stx1a, syntaxin 1a; SynI, synapsin I; Syp1, synaptophysin 1; Syt1, synaptotagmin 1; TH, tyrosine hydroxylase; TRITC, tetramethyl-rhodamine isothiocyanate; TSA, trichostatin A; VAMP, vesicle associated protein; wt, wild-type.

the cells depends, therefore, on whether expression of REST remains stable or undergoes a rapid drop. The first case is typical of non-neurosecretory cells, in most of which REST is expressed at high levels throughout the whole life; the second is typical of neuronal/neurosecretory cells which acquire their specific phenotype following the expression of many RE-1-positive genes essential for their function (Bruce *et al.* 2004; Ballas and Mandel 2005; Conaco *et al.* 2006; Mortazavi *et al.* 2006; Wu and Xie 2006; Buckley 2007; Otto *et al.* 2007).

In addition to its key role during differentiation, REST appears involved also in the maintenance of the differentiated neuron/neurosecretory cell phenotype. Studies in hippocampal neurons have revealed that various conditions, such as hypoxia and epilepsy, induce transient increases of the repressor followed by the decreased expression of at least two RE-1-positive genes, those of the AMPA and μ opioid receptors, and by increased risks of cell death (Calderone *et al.* 2003; Jia *et al.* 2006; Formisano *et al.* 2007). So far, however, a detailed study in the area has been carried out only in PC12, a pheochromocytoma cell line characterized by very low levels of the endogenous repressor (Malosio *et al.* 1999). Genome microarray analysis of wild-type (wt) PC12 cells (Bruce *et al.* 2006) revealed that stable transfection of two positive REST constructs is followed by the down-regulation of 141 RE-1-positive genes, including a few coding for proteins of the secretory process: the dense core vesicle (DCV) cargo protein, chromograninA (ChgA), the vesicle membrane protein SV2; synaptotagmin4 (Syt4), which can act as the Ca^{2+} sensor of vesicle exocytosis in alternative to synaptotagmin1 (Syt1) (Wang *et al.* 2003); and SNAP25, one of the two Q-SNAREs that mediate vesicle exocytosis (Jahn and Scheller 2006). The direct control of these genes by REST was confirmed by ChIP analysis of the DNA and by the de-repression of their mRNA in the cells infected by the dominant negative construct of the repressor. Moreover, conventional electron microscopy revealed, in the REST-transfected PC12 cells, a decrease of the number of DCVs. In addition, the same cells exhibited an altered uptake of noradrenaline and failed to release the catecholamine upon depolarization, but not upon administration of the Ca^{2+} ionophore A23187 (Bruce *et al.* 2006).

Concomitantly to these studies on wt PC12, others were carried out (Pance *et al.* 2006) in one of the few available PC12 clones incompetent for the neurosecretory process. In spite of their expression of many features typical of differentiated PC12, the cells of these clones lack both the neurosecretory vesicles (DCVs and synaptic-like microvesicles, SLMVs) and the proteins that mediate and regulate their exocytosis (Corradi *et al.* 1996; Borgonovo *et al.* 1998; Malosio *et al.* 1999; Pance *et al.* 1999). In the clone studied by Pance *et al.* (2006), characterized by a high level of REST, infection of a dominant negative construct was found to activate the expression of the genes coding for ChgA, synaptophysin1 (Syp1) and Syt4, revealed by the rescue of their mRNAs. The corresponding proteins, however, remained inappreciable, suggesting the existence, in addition to the transcriptional control by REST, of a post-transcriptional control independent of the repressor (Pance *et al.* 2006).

Taken together, the results of Bruce *et al.* (2006) and Pance *et al.* (2006) demonstrate that, in PC12 cells, REST has a role in the expression of the neurosecretory process. These previous studies, however, were carried out primarily by molecular biology, focusing on the expression of the mRNAs, and not according to a cell biological approach, focused on the proteins and the cellular events. Therefore, little was known about the role of the repressor in the expression, the structure and the exocytic discharge of neurosecretory vesicles. These issues have now been taken into consideration. Two PC12 clones, one competent and the other defective of neurosecretion, characterized by very low and high levels of REST expression (Fig. S1a and b), were investigated after stable transfection with a full-length and a dominant-negative REST construct, respectively. Other transfections and treatments were also administered to these cells. The results obtained, on the one hand, revealed several new aspects of the neurosecretory process governed by REST; on the other hand, identified new properties of DCVs and of their exocytic discharge.

Materials and methods

Cloned wt PC12 and PC12-27 cells as well as the hChgB construct and the IgG_{2a} anti-ChgB monoclonal were as in (Borgonovo *et al.* 2002); the IgG1 anti-hChgB monoclonal was the gift of W. Huttner, Dresden Germany; the anti-ChgA and anti-Scg2 polyclonals, of A. Laslop, Innsbruck Austria, and P. Rosa, Milan Italy, respectively; the 12C11-1 anti-REST monoclonal, of D. J. Anderson, Pasadena CA; the anti-ICA512 monoclonal, of M. Solimena, Dresden Germany; the goat and rabbit polyclonals against the Syt1 lumenal domain, of A. Malgaroli, Milan Italy; the MycREST and DBD/REST constructs, of N. Buckley, Leeds UK. Other antibodies were from commercial sources: anti-REST polyclonal, Upstate Cell Signaling, Lake Placid NY, USA; anti-Syt1, anti-Syp, anti-Rab3a, anti-VAMP2, anti-SNAP25 and anti-ß-tubulin monoclonals, Synaptic Systems, Göttingen, Germany; anti-Stx1a monoclonal, Sigma–Aldrich, St. Louis, MO, USA; anti-green fluorescent protein monoclonal, Roche, Indianapolis IN, USA; anti-Myc monoclonal, Abcam, Cambridge, UK; anti-GAPDH monoclonal, Biogenesis, Oxford, UK; FITC-conjugated and tetramethyl-rhodamine isothiocyanate-conjugated goat anti-mouse and anti-rabbit antibodies; goat anti-mouse IgG subclasses, Southern Biotech, Birmingham AL, USA; Alexafluor 647-conjugated anti-mouse antibodies, Molecular Probes, Eugene, OR, USA; horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, Bio-Rad, Hercules, CA, USA. trichostatin A (TSA) and cycloheximide were from Sigma-Aldrich; the BCA Protein Assay Kit from Pierce, Rockford, IL, USA.

Cell cultures

The wt PC12 and PC12-27 clones and subclones were grown at 37°C as in Cocucci *et al.* (2004).

Stable and transient transfections

Lipofectamine 2000^{TM} (Invitrogen, Carlsbad CA, USA), was used for both transient and stable transfections. Wt PC12 cells transfected with the Myc-tagged cDNA of full-length REST, MycREST (Zuccato *et al.* 2003), and PC12-27 cells transfected with the Myc-tagged dominant negative construct DBD/REST (Wood *et al.* 2003) together with the pcDNA3.1/hygro(+) vector carrying hygromycin resistance, were selected with the antibiotic. Stable subclones were grown in complete medium supplemented with 200 or 500 μg/mL of hygromycin B (Invitrogen), respectively. The DBD/REST construct was also transiently transfected in the PC12/MycREST10 clone; the pcDNA3.1/hygro(+) vector fused with hChgB-green fluorescent protein cDNA was transiently transfected into the PC12-27/DBD subclone and used 48 h thereafter. Full-length cDNA of hChgA inserted in pEXP1 vector carring puromycin resistance was stably transfected in the PC12-27/DBD subclone. Upon transfection, the cells were grown in complete medium supplemented with puromycin 10 μg/mL.

siRNA

RNA oligonucleotides (sense sequence AACAGUUCGUACAUCACAUCCtt) and a control 19-bp scrambled sequence with 3′ dT overhangs (Ambion, Austin Texas) were used for REST gene silencing. PC12-27 and PC12-27/DBD cells were transfected with either the siRNA (60 nM) or with the scrambled sequence by the lipofectamine reagent (Invitrogen), once or twice at 24 h distance, and analyzed by western blotting 24 and 48 h after the first transfection.

Q-PCR

Total RNAs, extracted according to the RNeasy Mini Kit (Qiagen, Valencia CA), were used for RT-PCR according to the SuperScript TM III First-Strand Synthesis System (Invitrogen). The synthesized cDNAs were templates for quantitative PCR (Taqman methodology), performed by the RT-PCR and DNA sequencing Service of IFOM-IEO Campus (Milan, Italy). Rqmin/Rqmax indicates the SE (confidence 95%).

Cell extractions; western blotting

Cell extracts in TD buffer containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF and protease inhibitors, were rocked (15 min) and then centrifuged (13 000 *g*, 4°C, 15 min). Fixed amounts of protein, separated by SDS-PAGE and transferred to nitrocellulose filters, were processed as in Cocucci *et al.* (2004). Photographic development was by chemiluminescence (ECL Western Blotting Detection reagent of Amersham Biosci., Little Chalfont, UK or Femto Signal of Pierce). Signals were acquired by the Densitometer SI and Image Quant (Amersham Biosci.). Quantified western blot bands (averages of at least 3 experiments ± SD) were expressed as % of the corresponding values in wt PC12 cells.

Immunofluorescence

Cells on coverslips fixed with 4% formaldehyde (10 min), quenched in 0.1 M glycine, washed, permeabilized in 0.1% Triton X-100 and immunolabeled as in Cocucci *et al.* (2004), were studied in Perkin-Elmer Ultraview ERS confocal microscope (Perkin-Elmer, Waltham, MA, USA). For surface immunolabeling the cells, stimulated or not for 1 min with 2 μM ionomycin, were fixed in 95% ethanol at 20°C, then washed and processed without detergent permeabilization to be finally labeled for Syt1 (lumenal domain), ChgB and ChgA, alone or in combination. Image deconvolution was carried out in a wide field microscope of the Delta Vision system (Cocucci *et al.* 2004). Fluorescence values were calculated on single cells by the ImageJ program (rsb.info.nih.gov/ij).

Electron microscopy

Monolayers of wt and PC12-27 clones and of stably transfected subclones were fixed with 2% glutaraldehyde in phosphate buffer, washed, post-fixed in 2% OsO_4 in phosphate buffer and embedded in Epon. For immunogold labeling monolayers were fixed with a 4% formaldehyde/0.25% glutaraldehyde in phosphate buffer, washed, detached and recovered by centrifugation. Extensively washed pellets were embedded in London Resin white. Ultrathin sections on nickel grids were exposed to the anti-ChgB (90 min, phosphate-glycine buffer), washed and immunolabeled with gold particles (coated with anti-mouse IgGs, 6 or 10 nm), washed, post-fixed with 1% glutaraldehyde and finally stained in sequence with uranyl acetate and lead citrate. Ultrathin sections (60 nm) were studied and photographed in a Leo 912 electron microscope. The distribution, cytoplasmic density and radii of DCVs located away from the Golgi complex were measured manually on images chosen at random and printed at 50 000X. Correction of the radius measurements and calculations for volumes were made as in Parsons *et al.* (1995).

Results

Repression by REST of vesicle/exocytosis gene expression

In the initial experiments (Fig. 1), we investigated the expression of 10 genes coding for proteins of neurosecretory vesicles (specific of DCVs or present also in the second type of vesicle present in PC12, the SLMV) and of their exocytic discharge. These genes, that previous studies (Malosio *et al.* 1999; Grundschober *et al.* 2002) had shown to be down-regulated in the PC12 clones defective of neurosecretion (discussed later), are indicated from here-on as vesicle/exocytosis genes. Five of the 10 genes (encoding the two major DCV cargo proteins, ChgA and ChgB; the vesicle membrane proteins, Syp1 and ICA512; and the exocytic Q-SNARE, SNAP25), are well known REST targets (Bruce *et al.* 2004; Bruce *et al.* 2006). Three genes (encoding the exocytic Ca^{2+} sensor of the vesicle membrane, Syt1, the Q-SNARE, syntaxin1a (Stx1a) and the R-SNARE, VAMP2) were recently shown to harbour a REST-specific RE-1 sequence (Otto *et al.* 2007). So far, however, they have not been recognized as REST targets. The last two genes (encoding the minor cargo protein of DCVs, secretogranin2 (Scg2) and the small G protein, Rab3a) have never been reported to harbour RE-1 sequences. In addition, we also

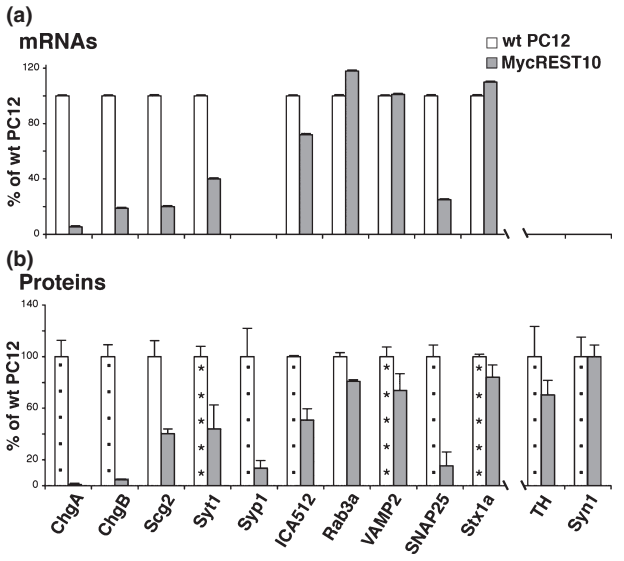


Fig. 1 Expression of ten vesicle/exocytosis genes in the wt PC12 clone and in the stably transfected PC12/MycREST subclones. The quantitative gene expression data (expressed, here and in the following figures, as % of wt PC12) are representative of both the PC12/MycREST10 and PC12/MycREST8 subclones (see Fig. S1). Columns in a refer to the mRNAs (±Rqmin/Rqmax); columns in b refer to proteins (±SD) and include also three genes not investigated at the mRNA level: Syp1, TH and Syn1. In b the dots on the white columns identify the RE-1-positive genes (Bruce *et al.* 2004); the stars on the white columns mark VAMP2, Syt1 and Stx1a, three genes recognized recently as RE-1 positive (Otto *et al.* 2007), which, however, have not been investigated in detail yet. Western blots representative of the data in b are shown in Fig. S2.

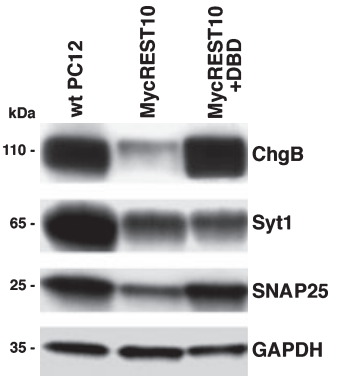


Fig. 2 Effects of the dominant-negative construct DBD/REST on the expression of vesicle/exocytosis proteins in PC12/MycREST10 cells. The levels of the ChgB and SNAP25 proteins, much lower in the PC12/MycREST10 subclone with respect to the wt clone, were re-established to the wt levels when the subclone was transiently transfected with the dominant negative construct, DBD/REST. In contrast, Syt1, which was less intensely decreased in the PC12/MycREST10 subclone, was increased only slightly by the transient transfection of DBD/REST.

studied the proteins encoded by two genes, the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) and synapsin1 (Syn1), that previous studies, carried out in other neurosecretory cells as well as in silico, had shown to be targets repressed by REST (Bruce *et al.* 2004; Sun *et al.* 2005; Otto *et al.* 2007). In PC12 cells, however, these genes were expected to differ from the other genes investigated because in our neurosecretion-defective clones they had been found to be expressed at levels close to wt (Malosio *et al.* 1999; Grundschober *et al.* 2002).

The cells employed in the experiments of Fig. 1 were from a wt PC12 clone extensively used in our previous studies (Malosio *et al.* 1999; Grundschober *et al.* 2002) and from two subclones isolated there-from. These subclones (8 and 10 in Fig. S1c) were those exhibiting the highest levels (4–5-fold the endogenous REST, Fig. S1c and d) of the protein coded by a stably transfected, full-length MycREST construct. Other clones expressing much lower or inappreciable levels of the protein (Fig. S1c) were discarded. In order to assure comparability, the mRNA and protein results, obtained by quantitative PCR and by densitometric scanning of specific western blot bands, respectively, are presented in the text and in all figures as percent of the wt PC12 values.

The changes in expression of the 10 genes observed in the MycREST-transfected cells are illustrated in Fig. 1. Both the mRNAs and the proteins encoded by the genes of the two main cargos, ChgA and ChgB, of Syp1 (only protein) and of SNAP25 were decreased 80% or more. Smaller, but considerable decreases were observed with the products of the Scg2, Syt1 and ICA512 genes (Fig. 1a and b). The products of the three remaining genes, of Rab3a, VAMP2 and Stx1a, were either unchanged (mRNAs, Fig. 1a) or decreased only slightly (proteins, Figs 1b and S2), and the same occurred for the proteins encoded by the TH and Syn1 genes (Fig. 1b).

Further experiments were carried out to establish whether the repression of protein expression induced by the stable MycREST transfection was relieved by the abrogation of the REST function. To this end, MycREST-PC12 cells were further transfected, however, in this case transiently, with DBD/REST, a dominant-negative, truncated and Myc tagged construct (employed also later), lacking both the N and C terminal repressor domains, which competes with endogenous REST for RE-1 binding. As shown in Fig. 2, DBD/REST induced an almost complete rescue of ChgB and SNAP25, two of the proteins most markedly down-regulated in the MycREST-transfected cells. The rescue of Syt1 was in contrast only partial. This result could be due to differences not in gene expression but in the intracellular traffic and turnover of the proteins (Heindel *et al.* 2003; Aikawa *et al.* 2006), too slow in the case of Syt1 to sustain large changes within the time frame of the transient transfection experiments.

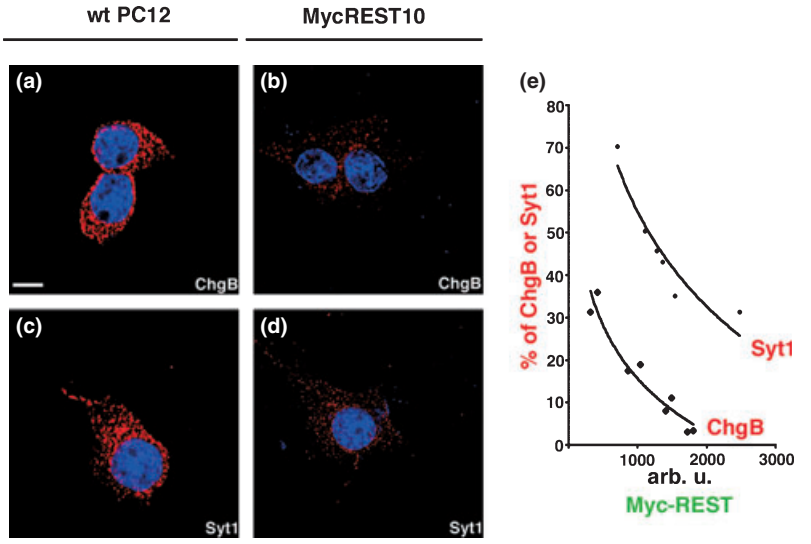


Fig. 3 Dependence of ChgB and Syt1 expression on the levels of the MycREST protein. Panels a and b illustrate examples of immunofluorescence deconvolved images of ChgB; panels c and d, of Syt1, in wt PC12 and PC12/MycREST10 cells. Panel e shows the expression of Syt1 and ChgB in single PC12/MycREST10 cells, plotted as a function of the MycREST protein (averages of at least 10 cells/point) and expressed as % in the wt PC12 clone. Notice that the expression of both proteins decreases with the increase of MycREST (expressed in Arbitrary Units), and that ChgB appears more sensitive to the repressor than Syt1. The blue color of nuclei of a–d is due to DAPI staining. The bar in a, valid also for b–d, corresponds to 5 μm.

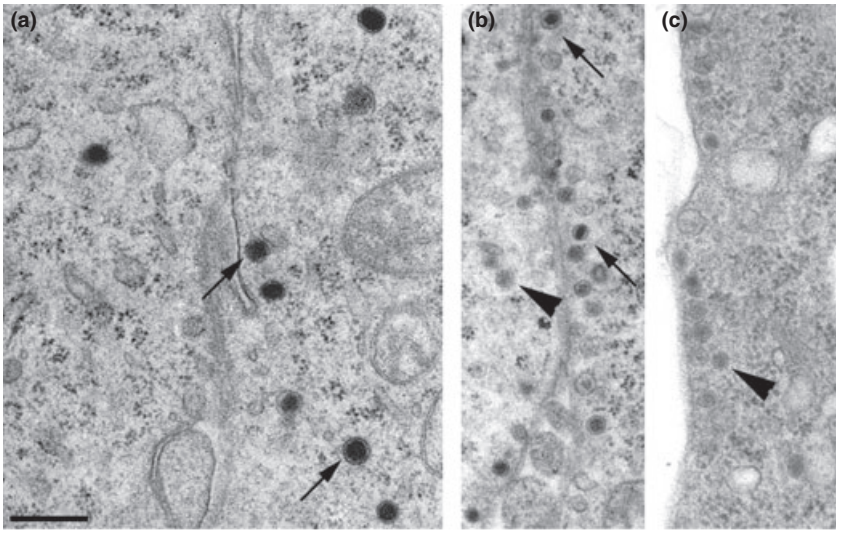
Expression of the proteins as shown in Fig. 1 was investigated also at the single cell level by using immunofluorescence. Fig. 3a–d compares examples of wt and MycREST-PC12 cells expressing ChgB (a and b) and Syt1 (c and d). The decrease of the two proteins in the transfected cells, more evident for ChgB, was variable from cell to cell. This variability is illustrated in Fig. 3e, where the average fluorescence intensities of the two proteins, measured by quantitative imaging in groups of randomly selected MycREST-PC12 cells, are plotted as a function of the MycREST protein expression. As can be seen, the degree of down-regulation of both ChgB and Syt1 varied among the cells in strict correlation with the levels of the construct protein.

Taken together the results of Figs 1–3 demonstrate that MycREST, stably expressed in PC12 cells at an average level a fewfold higher than the low endogenous level, repressed some, but not all the vesicle/exocytosis genes investigated. The repression was variable among the genes and correlated with the levels of REST expression when studied in single cells. It affected not only the well known targets of REST but also the Scg2 gene, where a REST-specific, RE-1 binding sequence has never been reported. As a whole, the genes encoding the cargo proteins were more repressed than those encoding the vesicle membrane proteins. The three vesicle/exocytosis genes and the genes of TH and Syn1, exhibiting similar expression levels in the wt clone and in the transfected subclones, could be insensitive to REST. Alternatively, they could be sensitive, however, only to high concentrations of the repressor.

REST affects the DCV volume and density

The effects of REST on DCVs were investigated by electron microscopy. The well known appearance of the organelles in wt PC12 cells, and their distribution both in the proximity of the plasma membrane and in the rest of the cytoplasm (Watanabe *et al.* 1983), are illustrated in Fig. 4a. In the MycREST/PC12 subclones, dense-core organelles positive for ChgB (Fig. 4e) were present, however, they were small. Fig. 4 illustrates their structure (panels b–d) and provides also a quantitative summary of their properties. On the average, their number was decreased only moderately (-35%) with respect to the DCVs of wt PC12, and their distribution was mostly in the proximity of the plasma membrane. The major differences were in volume, which was much smaller (on the average ~24% of wt) and in the core, which was lighter, of variable density even within the same cell, and often surrounded by a clear halo (Fig. 4b–d).

The data given so far do not establish whether the small dense vesicles of the MycREST cells are discharged by regulated exocytosis, as wt DCVs, or by constitutive exocytosis. Three approaches were used to answer this question. In order to investigate the turnover of their cargo proteins ChgA and ChgB, resting wt and MycREST-transfected PC12 cells were incubated for up to 6 h with cycloheximide, a blocker of protein synthesis, and then analyzed by western blot. In these conditions regulated exocytosis takes place at a rate much slower than constitutive exocytosis. Therefore, the cellular levels of the cargo proteins are expected either to remain almost constant or to drop



Dense-core vesicles	wt (80)	MycREST10 (361)
Cytoplasmic density (number/μm^2)	1.33	0.86
Radius (corrected, nm)	52.60±6.50	33.44±3.03
Calculated volume (corrected, nm^{3}x10^3)	611 (100%)	143 (23.45%)
% adjacent to the PM	40	78

Fig. 4 DCVs in wt and PC12/MycREST10 cells. Panel a shows the typical ultrastructure of DCVs (arrows) in the wt clone; panels b–d that of the dense vesicles in the PC12/MycREST10 subclone. Notice that the vesicles of the subclone are much smaller and that their cores exhibit variable densities, with some approaching the density of wt DCVs (arrows) and others distinctly lighter (arrowheads). Moreover, most of these vesicles were distributed in the proximity of the plasma membrane. Panel e shows that the vesicles of the PC12/MycREST10 cells are immunogold-positive for ChgB (large arrows). The bar in a, valid also for b–d, is of 300 nm; that in e is of 50 nm. The table to the bottom right reports the results (averages ± SD) of a comprehensive morphometric analysis of DCVs and dense vesicles in wt and PC12/MycREST10 cells. The number of analyzed organelles is given below the indication of the cells. The values of radii (±SD) and volumes were corrected according to Parsons *et al.* (1995). The calculated volumes are given both in absolute terms and, in brackets, as % of the values in the wt clone. DCVs adjacent to the plasma membrane (PM) means distributed at <100 nm distance from the cell surface.

within a few hours, depending on the type of exocytosis (Malosio *et al.* 2004). The results showed that, in spite of their large difference in expression in the wt and the MycREST PC12 cells, the Chgs maintained almost constant levels throughout the 6 h experiment in both (Fig. 5a and not shown). These results strongly suggest that discharge of the small dense vesicles takes place by regulated exocytosis. Consistently, when the MycREST-transfected cells were exposed to the Ca^{2+} ionophore, ionomycin (2 μM, 15 min), a drug known to induce the regulated exocytosis of neurosecretory vesicles, they exhibited ChgB decreases proportionally even larger than those of wt PC12 (Fig. 5b). Finally, 1 min stimulation with ionomycin (2 μM) was enough to induce the appearance, at the surface of both wt and MycREST PC12 cells, of the Syt1 domain that in the discrete vesicle is oriented to the lumen (lumenal domain), together with the cargo protein, ChgB (Fig. 5d and f). Both these proteins were almost inappreciable at the surface of resting cells processed without permeabilization (Fig. 5c and e). Taken together the results confirmed the regulated exocytic nature of the small dense organelles of PC12/MycREST cells and excluded them to be discharged constitutively. These vesicles can therefore be considered as peculiar, but authentic DCVs.

Defective PC12 cells: effects of REST function repression

PC12-27 is the PC12 clone defective of neurosecretion and completely lacking DCVs isolated and most extensively investigated in our laboratory (Corradi *et al.* 1996; Kasai *et al.* 1999; Borgonovo *et al.* 2002; Grundschober *et al.* 2002). Its pattern of expression of the 10 vesicle/exocytosis gene resembled that observed in the MycREST-PC12 subclones. The levels of both mRNA and proteins, however, were much lower (compare Fig. 6a–d and Fig. S3 to Fig. 1a and b and Fig. S2). The products of six genes, i.e., those of the three cargo proteins, ChgA, ChgB and Scg2, of the Q-SNARE, SNAP25, and of the vesicle membrane proteins, Syt1 and Syp1, were inappreciable or hardly detectable (Fig. 6a and c). The remaining four genes exhibited different expression. ICA512 and the small G protein Rab3a were very low in terms of protein (Fig. 6d) but exhibited considerable mRNA levels (30 and 38% of wt, Fig. 6b); in contrast the considerable mRNA levels of the Q-SNARE Stx1a and the R-SNARE VAMP2 (10 and 39% of wt, Fig. 6b) were

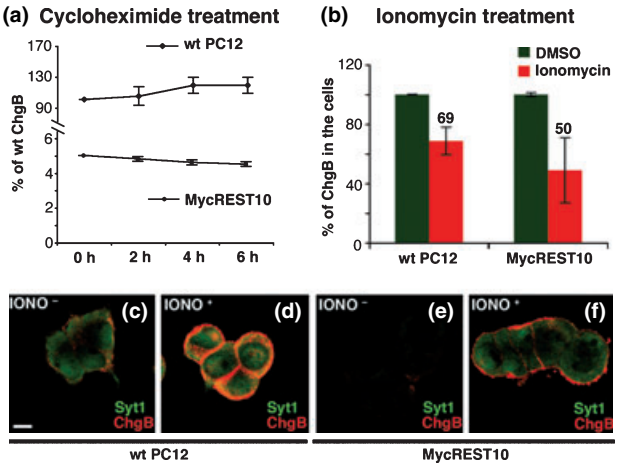


Fig. 5 Turnover of ChgB and exocytic discharge of DCVs upon ionomycin stimulation of wt and PC12/MycREST10 cells. Panel a shows that ChgB (±SD), although expressed at much different levels in resting wt and MycREST10 PC12 cells, remains stable in both cases during a 6 h treatment with the blocker of protein synthesis, cycloheximide. Panel b shows that, when stimulated with ionomycin (2 μM, 15 min), the wt and MycREST10 PC12 cells exhibit large decreases in their ChgB (±SD), corresponding on the average to the 31 (wt) and 50 (MycREST) % of their content at rest, due to discharge to the medium (not shown). Panels c–f show the surface immunolabeling for the lumenal domain of Syt1 and for ChgB in the non-permeabilized wt (c and d) and MycREST10/PC12 (e, f) cells. At rest this immunolabeling was low (panel c, wt cells) or apparently absent (panel e, MycREST10/PC12 cells). Upon stimulation with ionomycin (2 μM, 1 min) there was a considerable increase in both types of cells (panels d and f, respectively). The bar in c, corresponding to 5 μm, is valid also for d–f.

matched by similar levels of the two proteins (Fig. 6d). Interestingly, the two additional proteins related to neurosecretion that in wt PC12 cells had been little affected by the MycREST transfection, i.e., TH and Syn1, (see Fig. 1b), exhibited the highest levels among the proteins investigated in the defective PC12-27 cells, corresponding to ∼ two-thirds of the wt PC12 (Fig. 6d).

The defective PC12-27 cells were stably transfected with the dominant-negative, truncated and Myc tagged construct, DBD/REST, already introduced in Fig. 2, and several subclones were isolated there-from. Four of these subclones, which exhibited high levels of the protein encoded by the construct (from ∼20 to 40-fold the endogenous REST in wt cells; calculations made by comparison to MycREST), were analyzed. All the cells of the four subclones appeared positive in their nucleus for Myc immunofluorescence, with some degree of heterogeneity among them (Fig. 6e). Detailed studies were carried out in the richest of these subclones, subclone 5 (PC12-27/DBD5).

In terms of vesicle/exocytosis gene expression, the DBD/REST transfection induced largely variable effects (Fig. 6a–d). The two main cargos, ChgA and B, remained almost inappreciable (<1% of wt). Syt1 and SNAP25 were still low at both the mRNA (∼3%) and protein (2.8 and 5.9%) level, and the same was true with the Syp1 protein (5.4%) (Fig. 6a and c). Rab3a maintained the considerable mRNA level of untransfected PC12-27 cells (∼30%) and increased its protein from 4% to 19% of wt (Fig. 6b and d). The other three genes exhibited distinctly higher levels, however, with opposite dissociations between mRNA and protein: in the case of ICA512, 214% mRNA with 4.7% protein; in the case of VAMP2 and Stx1a ∼50% mRNA with 98 and 120% protein (Fig. 6b and d; Fig. S3). The additional two proteins encoded by RE-1-positive genes, TH and Syn1, increased their levels from ∼65 to 100 and 148%, respectively (Fig. 6d).

The DBD/REST subclones were investigated also by morphological techniques. A weak, but distinct punctate fluorescence signal, spread through the cytoplasm, could be revealed in the cells immunolabeled with the anti-Syt antibody. In contrast, these cells remained negative for ChgB (Fig. 6f). When investigated by electron microscopy the cells exhibited the expected complement of most cytoplasmic organelles. However, no DCVs of any size and density were seen in the sections of the over 100 cells investigated (Fig. 6g). While at rest, the non-permeabilized cells were surface-negative for both Syt1 and ChgB (Fig. 6h), upon stimulation with ionomycin (1 min, 2 μM) the lumenal domain of Syt1, but not ChgB, appeared at the cell surface (Fig. 6i).

Summing up, the transfection of the dominant negative construct at levels lower than the endogenous REST (∼40 vs. 60-fold the wt level) induced the partial rescue of some, but not all vesicle/exocytosis genes. In particular, the two major cargo proteins remained inappreciable while various membrane proteins increased to only very low levels. The surface appearance of Syt1 upon ionomycin stimulation suggested in the transfected PC12-27/DBD cells some rescue of regulated exocytosis of vesicles lacking the cargo but positive for the membrane protein.

Stable transfection of ChgA rescues DCVs in PC12-27/DBD cells

Although greatly variable in extent, the rescue, in PC12-27/DBD cells, of vesicle membrane and exocytosis proteins and the surface appearance of the Syt1 lumenal domain upon stimulation suggested that the neurosecretory process had been re-established by the dominant negative construct. In the PC12-27/DBD cells, however, the discharged vesicles appeared still missing the main cargo proteins, the Chgs. In order to check for this possibility, we decided to investigate the cells after stable transfection with human ChgA (hChgA). Previous studies had established that, when hChgA is transfected in the defective PC12-27 and other non-secretory cells, it is addressed not to the regulated, but to the constitutive secretion, being completely discharged within 3 h (Malosio *et al.* 2004). If, on the other hand, the

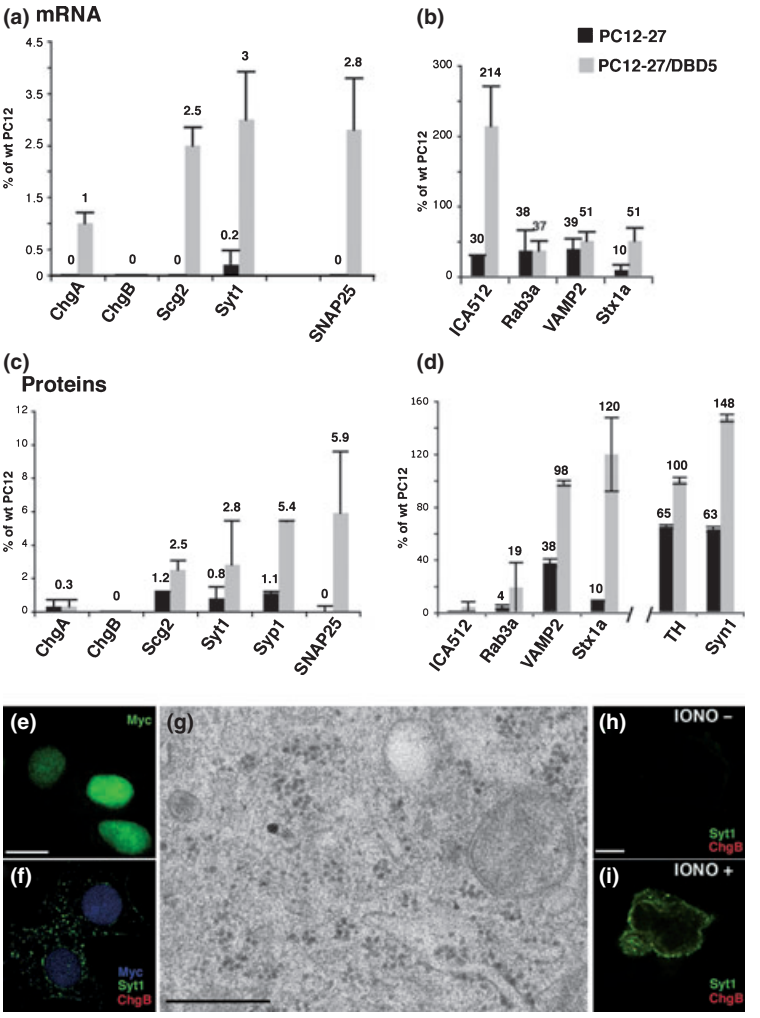


Fig. 6 Gene expression, ultrastructure, immunolabeling and surface labeling for Syt1 and ChgB in the defective PC12-27 and the stably transfected PC12-27/DBD cells. The mRNAs (a and b) and proteins (c and d) encoded by the vesicle/exocytosis genes, expressed by the defective PC12-27 clone and by its PC12-27/DBD subclone 5, are given as % (±SD) of the wt PC12 (discrete values on top of the columns). The left panels (a and c) illustrate the little expressed genes, the right panels (b and d) the more abundantly expressed genes. Typical western blots of the data in panels c and d are shown in Fig. S3. Panel e illustrates the variable expression of the DBD/REST construct in three representative PC12-27/DBD cells, as revealed by Myc immunofluorescence. Panel f shows an example of deconvolved images where the PC12-27/DBD cells are positive for Syt1, distributed in puncta scattered in the cytoplasm, and negative for ChgB. The blue color of nuclei is due to DBD/REST immunolabeled with anti-Myc-Alexafluor 647. The ultrastructure of the PC12-27/DBD cells, exhibiting no DCVs, is shown in panel g. Panels h and i show that the non-permeabilized PC12-27/DBD cells are surface-negative for the lumenal domain of Syt1 and ChgB when fixed at rest (panel h) and become positive for Syt1, but not for ChgB, upon stimulation with ionomycin (2 μM, 1 min, panel i). The bar in panel e, valid also for f, and that in panel h, valid also for i, are of 3 μm; that in panel g is of 300 nm.

neurosecretory process is active in the PC12-27/DBD cells, the stably transfected protein should be stored within DCVs and discharged by regulated exocytosis. As a reference, useful to distinguish the regulated and constitutive discharge, we also investigated PC12-27/DBD cells transfected, not stably but transiently, with the human ChgB (hChgB). Previous studies in wt PC12 cells had shown that, under these conditions, hChgB is expressed in only a fraction of the cells (~50%) at levels that exceed the capacity of the regulated compartment. As a consequence the protein is largely released by constitutive secretion (Malosio *et al.* 2004).

Figure 7 shows the results obtained with the PC12-27/DBD subclone (referred to as PC12-27/DBD/hCghA) stably transfected with a hChgA construct. The level reached by the protein in these cells was considerable, over 15% of wt (Fig. 7a). In contrast, the endogenous ChgA and the other main cargo protein, ChgB, remained inappreciable, as in PC12-27/DBD (see Fig. 6). When investigated by cell incubation with cycloheximide, the hChgA level of the

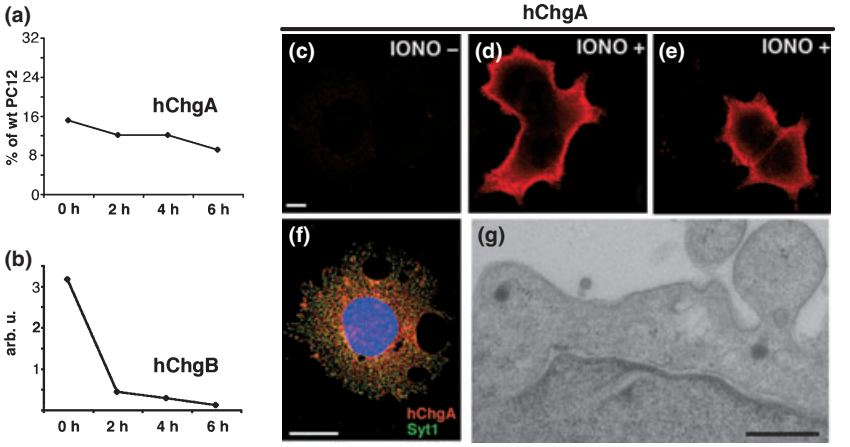


Fig. 7 PC12-27/DBD cells transfected with hChgA (stably) or hChgB (transiently). Turnover of the two proteins; stimulation-induced surface appearance of hChgA; immunofluorescence for hChgA and Syt1; rescue of DCVs. Panels a and b illustrate the evolution of the transfected hChgA (% of ChgA in wt PC12) and hChgB (arbitrary units) during a 6 h incubation of the cells in the presence of cycloheximide. Notice that the level of the transfected hChgA declines only slowly whereas that of the transiently transfected hChgB drops almost completely during the first 2 h. Panel c shows that the non-permeabilized PC12-27/DBD/hChgA cells are surface negative for hChgA while at rest; panels d and e show the surface appearance of the Chg upon cells stimulation with ionomycin (2 μM, 1 min). Panel f is an example of deconvolved images showing the partial coincidence of the immunolabeling for the transfected hChgA and the endogenous Syt1 in puncta distributed in the cytoplasm. The blue color of the nuclei is due to DAPI. Panel g shows the conventional ultrastructure of the PC12-27/DBD/hChgA cells exhibiting small DCVs in the cytoplasm. The bar in panel c, valid also for panels d and e is of 5 μm; that in panel f is of 3 μm; that in panel g is of 300 nm.

stably transfected PC12-27/DBD/hChgA cells declined only slowly, reaching ~65% of the initial value after 6 h (Fig. 7a). In contrast, hChgB transfected transiently in the PC12-27/DBD cells was discharged almost completely during the first 2 h (Fig. 7b). At rest, the transfected, non-permeabilized PC12-27/DBD/hChgA cells exhibited at their surface almost no trace of hChgA (Fig. 7c), which in contrast became quite evident upon cell stimulation with ionomycin (Fig. 7d and e). Taken together these results confirm that, depending on the conditions of transfection, the two cargo proteins were addressed primarily to distinct secretion pathways, regulated for hChgA and constitutive for hChgB.

In the PC12-27/DBD/hChgA cells permeabilized before immunolabeling, the stably transfected hCghA was largely distributed in discrete puncta spread in the cytoplasm. In the dually immunolabeled cells, these puncta partially coincided with those positive for Syt1 (Fig. 7f). At the ultrastructural level the PC12-27/DBD/hChgA cells exhibited few, but typical dense vesicles, similar in their small size (average radius, 35.5 ± 9.0 nm; number of measured vesicles, 50) to those observed in the wt cells transfected with MycREST (see Fig. 4b–d). The core density of these DCVs was, however, higher and the distribution was not only in the proximity of the plasma membrane but also in the rest of the cytoplasm (Fig. 7g). We conclude that, in PC12-27/DBD cells, the stable supply of one of the missing main cargo proteins, hChgA, was enough to rescue small DCVs destined to be discharged by regulated exocytosis.

Attenuation of the REST activity by block of histone deacetylases

Two attempts were made to further attenuate the repression of transcription by the endogenous REST in PC12-27/DBD cells. Upon exposure to a specific REST siRNA the protein level of the repressor decreased, reaching values < 25% after 48 h of a two siRNA transfection protocol. In these cells the Syt1 was increased, however ChgB remained inappreciable (Fig. S4) and the DCVs failed to reappear. Therefore, the approach was not pursued.

Other experiments were made by the use of TSA, a blocker of histone deacetylases (HDACs). HDAC1 and HDAC2 are major enzymes of the REST repressor complexes. Therefore, TSA has been widely used in the literature to attenuate the effects of REST (Belyaev *et al.* 2004; Ballas and Mandel 2005; Ballas *et al.* 2005). However, TSA is not specific for the two HDACs but inhibits with similar potency also the nine other members of the family which can act upon recruitment by other transcription factors (de Ruijter *et al.* 2003). Therefore, in addition to the attenuation of the REST function, this drug can induce REST-independent effects.

TSA was applied for 12–18 h to wt PC12, defective PC12-27 and PC12-27/DBD cells, at concentrations from 0.1 to 1.5 μM. In the wt cells the drug induced significant increases of the main cargo proteins, the Chgs, with no appreciable change of the vesicle membrane and exocytosis proteins (not shown). In the defective PC12-27, the effects were wider and concentration-dependent. Even at the highest concentration,

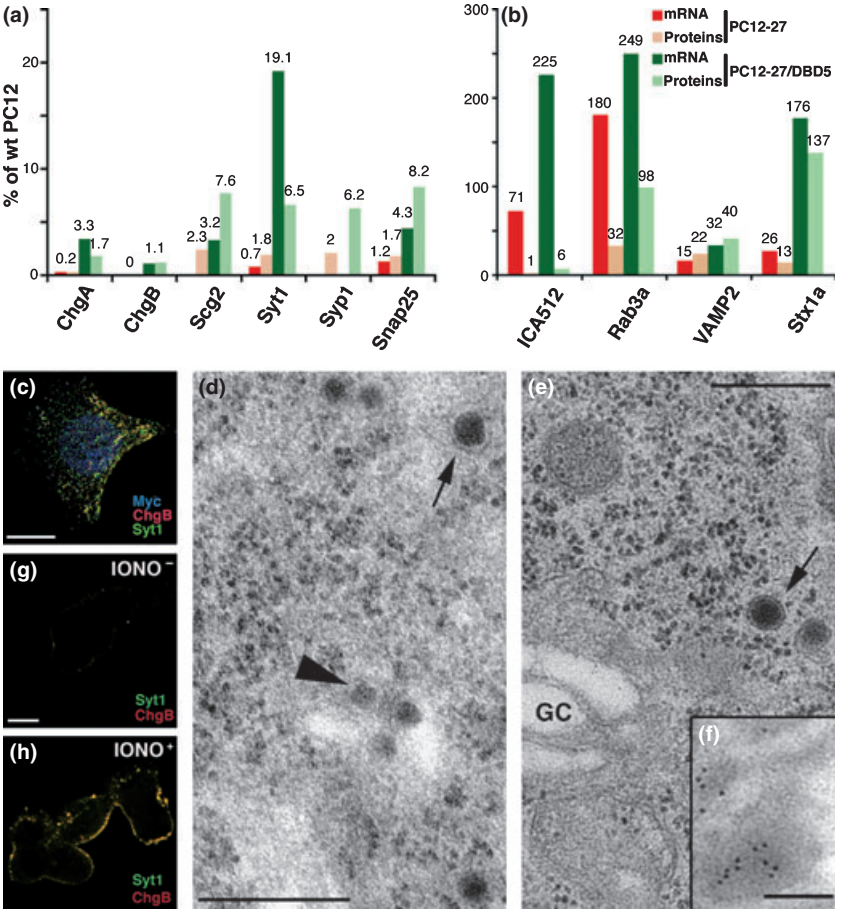


Fig. 8 Effects of TSA on the expression of the vesicle/exocytosis genes, on the immunofluorescence and the electron microscopy of the defective PC12-27 and the PC12-27/DBD cells. Panels a and b show the expression of the vesicle/exocytosis genes in terms of both mRNA and protein in PC12-27 and PC12-27/DBD cells pre-treated with TSA (1.5 μM, 18 h). Panel a includes the genes expressed to low levels, panel b those expressed to higher levels. Typical western blots of these protein data are shown in Fig. S5. Panel c shows a deconvolved image of puncta partially co-labelled for ChgB and Syt1 spread in the cytoplasm of a PC12-27/DBD cell pre-treated with TSA. The blue color of the nucleus is due to REST/DBD labeled by anti-Myc-Alexafluor 647. Panels d and e illustrate the rescue of DCVs in the cytoplasm of the same cells. These DCVs are small (average radius of ~35 nm; measured organelles 130), with cores of variable density, high (arrows) or low (arrowheads). Panel f shows that these DCVs are immunogold positive for ChgB. Panel g shows the lack of Syt1 and ChgB on the surface of resting, non-permeabilized PC12-27/DBD cells pre-treated with TSA; panel h the appearance of Syt1 and ChgB at the surface of the same cells upon stimulation with ionomycin (2 μM, 1 min). The bars in c, valid also for g and h, correspond to 3 μm; the bar in d, valid also for e, to 300 nm; that in f, to 50 nm.

however, TSA induced no rescue of ChgA and ChgB (mRNA and protein). In contrast, the expression of other vesicle/exocytosis genes was increased, going from maxima of 0.5–1% for a few membrane proteins to 150% for the Rab3a mRNA (Fig. 8a, b and S5). In PC12-27/DBD cells (Fig. 8a, b and S5), the increases induced by TSA were larger. With 1.5 μM for 18 h also the two Chgs (1.7 and 1.1% for the ChgA and ChgB proteins) became appreciable. The vesicle membrane, Scg2 and SNAP25 proteins rose to 6–8.2% levels (Fig. 8a and b), Rab3a and Stx1a to and over 100%, while VAMP2 was the only protein to decrease, from 98% without TSA (Fig. 6d) to 40% (Fig. 8b). Also in this case there were mRNA/protein dissociations, the most extensive being that of ICA512 (225 and 6%, respectively, Fig. 8b).

When investigated by immunofluorescence, the PC12-27/DBD cells treated with TSA exhibited numerous discrete puncta spread in the cytoplasm, positive for both the Chgs (ChgB in Fig. 8c) and Syt1. At the electron microscope these puncta appeared as small DCVs, immunogold positive for ChgB (Fig. 8f), similar in many respects to the DCVs already described in the PC12/MycREST and the PC12-27/DBD/hChgA cells (see Figs 4b–d and 7g): average radius, 35 ± 6 nm (number of measured organelles, 130); cores of variable density; thin halos frequently present around the core (Fig. 8d, e). Surface immunofluorescence of non-permeabilized PC12-27/DBD cells pre-treated with TSA revealed a rapid appearance of Syt1 together with ChgB upon stimulation with ionomycin (2 μM, 1 min) (compare in

Fig. 8 panels g and h), suggesting also in this case the discharge of DCVs by regulated exocytosis.

A further series of experiments was carried out in PC12-27/DBD/hChgA cells exposed to 18 h pre-treatment with TSA (1.5 μM). In these cells the puncta immunolabeled for hChgA were positive not only for Syt1 (Fig. 9a) but also for the endogenous ChgB (Fig. 9b). Moreover, the DCVs revealed by electron microscopy shared properties with those found in the PC12-27/DBD cells exposed to either hChgA transfection or TSA treatment (low frequency, variable density of the core, clear halo). Their radius, however, appeared moderately larger (42.1 ± 11.1 nm; number of measured vesicles: 100) (Fig. 9c and d; cfr to Figs 7g and 8d and e). Finally, when these cells were incubated with cycloheximide, the transfected hChgA and the endogenous rChgA exhibited a similar, slow decline (Fig. 9e), compatible with their co-storage within regulated exocytic organelles.

Discussion

The involvement of REST in the maintenance of neurosecretion in differentiated cells, initially suggested by the large difference of the repressor levels in wt and defective PC12 cells (Malosio *et al.* 1999), was demonstrated recently by the study of the effects induced by repressor constructs. In particular, Bruce *et al.* (2006), working in wt PC12, showed that positive constructs repress quite a few genes involved in vesicular traffic, including four coding for vesicle/exocytosis proteins; and Pance *et al.* (2006) found that three mRNAs (but not the proteins) coded by the same genes were de-expressed in a defective PC12 clone upon infection of a dominant negative construct. Whether these effects were accompanied, in the two PC12 models, by cell biological changes remained unknown, except for the decrease of the DCV number, with ensuing alterations of the catecholamine homeostasis, observed in the wt cells transfected with positive constructs (Bruce *et al.* 2006).

Our work, carried out by using tools similar to those employed by Bruce *et al.* (2006) and Pance *et al.* (2006), i.e., REST constructs and PC12 cells, wt and defective, was focused specifically on a cell biological process, the neurosecretory process, investigated by an ample experimental approach. Ten vesicle/exocytosis genes were chosen to obtain information on the expression of major proteins involved in the process: cargo proteins, destined to be discharged upon stimulation; vesicle membrane proteins; the SNAREs that mediate the exocytic fusion of vesicles with the plasma membrane; and a small regulatory G protein. Except for the cargos and ICA512, all these proteins are specific not only of DCVs (which, being more abundant and easy to recognize, have been investigated in detail) but also of the other type of neurosecretory vesicles present in PC12 cells, the SLMVs. Part of our results refer therefore to both types of vesicles taken together, the others are specific of DCVs.

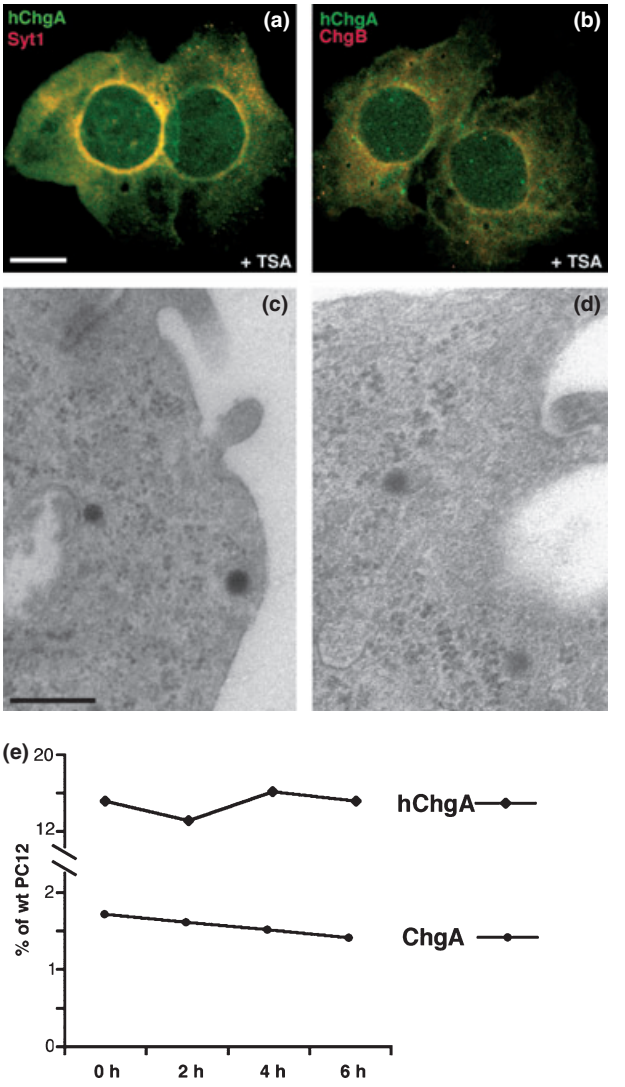


Fig. 9 Effects of TSA on the PC12-27/DBD/hChgA cells: co-distribution of hChgA with the endogenous Syt1 and ChgB; rescue of DCVs; co-stability of hChgA and endogenous ChgA. Panels a and b show the co-distribution of the stably transfected hChgA with two endogenous DCV proteins, Syt1 (a) and ChgB (b), in PC12-27/DBD/hChgA cells pre-treated with TSA (1.5 μM, 18 h). Panels c and d show the DCVs scattered in the cytoplasm of those cells. A morphometric analysis revealed their size to be larger than that of the DCVs in PC12-27/DBD/hChgA cells and PC12-27/DBD cells pre-treated with TSA: average radius of 42.1 nm, corresponding to a volume of 51% of the DCV in wt PC12. Panel e shows that, in resting PC12-27/DBD/hChgA cells pre-treated with TSA, the levels of both transfected hChgA and the endogenous ChgA remained stable during 6 h incubation with cycloheximide. The bar in a, valid also for b, is of 3 μm; that in c, valid also for d, is of 300 nm.

By the parallel use of wt and defective PC12 cells, rich and completely devoid of DCVs, respectively, we were able to investigate the effects of both moderate and large increases of REST. The largest increases of the repressor obtained in wt

cells by the stable transfection of the full length, positive construct MycREST were of only 4–5-fold. This level may be of interest for the understanding of some physiological and pathological events. High levels, on the other hand, reported not only in the defective PC12 cells but also in other neurosecretory cells, are suspected to have a role in the pathogenesis of diseases, such as pheochromocytomas and diabetes (Huynh *et al.* 2006; Martin *et al.* 2007). The partial abrogation, in the defective PC12 cells, of the REST function by the dominant negative construct DBD/REST, alone and combined with other treatments, made possible the investigation of the genes that are repressed only by high levels of REST. In addition, this approach was able to induce, for the first time, the reappearance of DCVs and of their exocytic discharge.

Differential regulation of vesicle/exocytosis gene expression

The differential sensitivity to REST of the various target genes was already known, depending on a variety of factors: from the number (one or more), the binding affinity and the genomic location of their RE-1 sequences to the chromatin environment and the neighboring transcriptional activators and repressors (Majumder 2006; Ooi and Wood 2007). Also known was the fact that a gene, repressed by REST during development, can become little sensitive or insensitive to the repressor within a differentiated cell (Belyaev *et al.* 2004; Bruce *et al.* 2004; Hohl and Thiel 2005; Sun *et al.* 2005). The results we have now obtained in stably transfected wt and defective PC12 cells demonstrate that also a group of genes coding for functionally coordinate proteins, the vesicle/exocytosis genes, are differentially repressed by REST acting in a concentration-dependent fashion. In particular, some of these genes (such as those of the Chgs, Syp1 and SNAP25) were severely repressed by the 4–5-fold increase of REST induced in wt PC12 by the transfection of MycREST, whereas other genes (Rab3a, VAMP2, Sytx1a) were unaffected. Consistently with these results, the resistant genes, although significantly repressed in the defective clone PC12-27, approached or even exceeded the wt levels when this clone was stably transfected with the dominant negative construct, DBD/REST. This transfection, on the other hand, was apparently ineffective on the Chgs but was able to induce some rescue when combined with the HDAC inhibitor, TSA. This result may be due to the inhibition of HDAC1 and HDAC2, two enzymes of the REST complexes. However, the members of the large HDAC family are all inhibited by TSA and can be recruited not only by REST but also by various other transcription factors. Therefore, the possibility that the observed effects of TSA were due, at least in part, to one or more mechanisms independent of REST cannot be excluded.

The mechanisms by which REST controls the vesicle/exocytosis gene expression appear also complex. Out of the 10 genes investigated eight, from the most sensitive Chgs to the most resistant VAMP2 and Stx1a, harbor RE-1 binding sequences (Bruce *et al.* 2004; Otto *et al.* 2007). The repression of these genes can therefore be direct, induced by the DNA binding of REST. However, the two remaining genes (coding for the cargo protein Scg2 and for the small G protein Rab3a) were never shown to harbor RE-1 sequences. Moreover, dissociations between mRNA and protein expression were seen, especially with Rab3a and ICA512. Therefore, the expression of vesicle/exocytosis genes appears to require the involvement, together with REST, also of additional factors active at the transcriptional and post-transcriptional level. Specific studies on the additional factors that control the neurosecretory process have not been carried out yet. Studies on genes other than the vesicle/exocytosis genes suggest these factors to include transcription factors, RE-1-positive or not, and various miRNAs (Wu and Xie 2006; Buckley 2007; Ishizuka *et al.* 2007; Otto *et al.* 2007).

Exocytic vesicles

The differential changes in the vesicle/exocytosis gene expression induced by REST were paralleled by extensive changes in the DCVs. In their previous paper Bruce *et al.* (2006) reported large decreases of the organelle number in the wt PC12 stably transfected with positive REST constructs. In contrast, changes in the organelle structure were not mentioned.

The dense vesicles that we have now observed in our wt PC12/MycREST cells were profoundly different from typical DCVs. Specifically, they were much smaller (∼24% in volume) and their core, often surrounded by a thin clear halo, exhibited a lower and variable density. The identification of these vesicles as authentic DCVs was confirmed not only by their ultrastructure but also by their ChgB immunogold labeling, the slow turnover of their ChgB in resting cells and their rapid exocytic discharge upon stimulation. Interestingly, the small DCVs rescued in the PC12-27/DBD cells stably transfected with hChgA or treated with TSA, were similar to those observed in the MycREST/PC12 cells (calculated volume ∼30% of wt). According to this, it is worth mentioning that all the attempts made to rescue DCVs in PC12 cells: by stable transfection with hChgA or treatment with TSA (this work); by many types of stimulation; by stable transfection of secretory and membrane proteins, of transcription factors, of other agents (Corradi *et al.* 1996; Kasai *et al.* 1999; Malosio *et al.* 1999, 2004; unpublished results), had remained unsuccessful. Therefore, at variance with the results reported in other cells (Kim *et al.* 2001; Huh *et al.* 2003; Beuret *et al.* 2004), the rescue of DCVs in PC12-27 cells appears possible only after the attenuation of the REST function such as that induced by the transfection of the DBD/REST construct.

Changes in the DCV volume, especially decreases, are very uncommon in the literature. The few changes reported so far in specifically treated or diseased cells were, in fact, mostly increases in volume (Kim *et al.* 2005; Gondre-Lewis *et al.* 2006; Wu *et al.* 2006). How do the various neurosecretory cells keep the volume of their vesicle constant is unknown. In our PC12/MycREST cells, the average decrease in the total DCV surface area (~60%) appeared in rough agreement with the average decrease in the membrane proteins (Syt1, Syp1, ICA512, VAMP2), whereas that of the cargo proteins was much greater (>90%). The balance between the two types of proteins might therefore contribute to fix the size of DCVs: excess of membrane could lead to small volumes, excess of cargo to large volumes. This possibility appears consistent also with the results with PC12-27/DBD cells stably transfected with hChgA and also treated with TSA, which exhibited DCVs significantly larger than those of the same PC12-27/DBD cells exposed to only one treatment (TSA or hChgA transfection only).

Exocytic discharge

In wt PC12 the regulated exocytosis of neurosecretory vesicles is mediated by the well known synaptic SNAREs, VAMP2, SNAP25 and Stx1a (Jahn and Scheller 2006). In contrast, defective PC12-27, which lack DCVs completely (Malosio *et al.* 1999), do not exhibit any appreciable exocytosis of this type (Kasai *et al.* 1999). The differential repression of vesicle/exocytosis genes by REST was expected to induce changes of this function, both in wt and defective cells.

In our experiments, stimulation of exocytic discharge was triggered with the Ca^{2+} ionophore, ionomycin, which induces the cytosolic Ca^{2+} concentration to increase independently of the physiological cell signaling. Therefore, differences in the discharge responses among the investigated cells could not be due to differences in processes other than exocytosis. In MycREST PC12 cells, the DCVs were small and less dense than the wt DCVs. At each exocytic event, therefore, the discharged quantum of Chgs was smaller than in wt PC12. The small DCVs, however, were rapidly discharged, as revealed by the surface co-appearance of the lumenal domain of Syt1 and ChgB. This co-appearance was expected because, in spite of their secretory nature, the Chgs are known to be released slowly from the DCV core upon exocytosis. In the PC12-27/DBD cells, on the other hand, the rescue of regulated exocytosis was suggested by the stimulation-induced surface appearance of the Syt1 domain, taking place, however, without Chgs, which are not expressed by these cells. This rescue was confirmed by the results obtained when the same cells were stably transfected with hChgA. The PC12-27/DBD/hChgA cells, in fact, exhibited not only small DCVs but also the stimulation-induced surface appearance of the transfected cargo protein. Neither of these results were obtained when hChgA was transfected in the defective PC12-27. In PC12 cells, therefore, two important processes concerning Chgs, their expression and their discharge by exocytosis, are both repressed by REST, however, the first is repressed already at low levels of the repressor, the second only at high levels. The mechanism of this difference has not been investigated in PC12. Previous studies in chromaffin cells, however, had shown that VAMP2 and Stx1a, the two SNARE proteins present at wt level in the PC12-27/DBD cells, can establish an exocytic complex, and thus mediate exocytosis, by interacting not only with SNAP25, which is low, but also with SNAP23 (Sorensen *et al.* 2003), which is expressed at wt levels in PC12-27 cells (Grundschober *et al.* 2002).

Conclusion

The neurosecretory process is of key importance in animal physiology. By the parallel investigation of ten specific genes we have now shown that its expression is not controlled by a single, "en block" program, as previously envisaged (Malosio *et al.* 1999). The process, in fact, appears orchestrated by REST working in collaboration with other factors acting at various levels of the gene expression pathway (Ballas and Mandel 2005; Conaco *et al.* 2006; Wu and Xie 2006; Buckley 2007). The complexity of this program has apparently profound consequences at the cell biological level, concerning also the structure and the function of DCVs.

The low level of REST is a property common to many, and possibly all differentiated neurosecretory cells, including chromaffin cells and pancreatic beta cells (unpublished). Moreover, small oscillations of these levels are probably frequent. Therefore, part of our results, obtained in the wt cells of the well known PC12 cell line, may ultimately be of importance also in the study of physiological cell systems. Moreover, recent studies are beginning to unravel the role of REST in various diseases (Ooi and Wood 2007), including neurosecretory tumors (Coulson 2005) such as human pheochromocytomas (Huynh *et al.* 2006). Our data about the defective PC12-27 cells, and the attenuation of their phenotype induced by partial abrogation of REST function could therefore be envisaged as a model, useful for the future investigation of various neurosecretory diseases.

Acknowledgments

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 Endogenous REST mRNA and protein in wt and neurosecretion-defective PC12-27 clones; expression of the stably transfected MycREST in wt PC12 clones and its effects on the expression of vesicle/exocytosis proteins.

Fig. S2 Typical western blots of vesicle/exocytosis proteins of the wt PC12 clone and of the PC12/MycREST10 subclone.

Fig. S3 Typical western blots of vesicle/exocytosis proteins in the defective PC12-27 clone and in the PC12-27/DBD5 subclone.

Fig. S4 Effects of the siRNA down-regulation in the PC12-27/DBD5 subclone.

Fig. S5 Typical western blots of vesicle/exocytosis proteins in the defective PC12-27 clone and in the PC12-27/DBD subclone exposed or not to TSA.

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