Expression and function of the dense-core vesicle membranes are governed by the transcription repressor REST

Rosalba D’Alessandro, Jacopo Meldolesi

1. Introduction

In eukaryotic cells many cytoplasmic membranes, such as those of the ER, Golgi complex and mitochondria, are ubiquitous. Others, however, are present only in specialized cells. Among these are the membranes of the organelles competent for regulated secretion, the granules/vesicles present only in professional secretory cells. Here we will focus on the membrane of one such organelle, the dense-core vesicle (DCV), expressed by neurons, by neural cells that share with neurons their origin, such as the chromaffin cells, by their differentiated tumors, and by at least some astrocytes [1–5]. When considered together these cells will be referred to as neural cells; the membrane that surrounds the core of their DCVs will be referred to as the DCV membrane.

DCVs are distinct from the synaptic/synaptic-like/clear vesicles, the other main secretory vesicles of neural cells, and from the granules of endocrine cells with which, however, they share some components; from the dense-core vesicles of non-neural cell types, such as platelets; from secretion granules of non-neural glands; and also from lysosomes, part of which appear competent for regulated exocytosis, however with properties different from those of DCVs [2–4].

The presence of DCV membranes in a cell depends on at least two processes: the expression of their components, in particular their proteins; and the sorting of these proteins to the DCVs [2–4,6]. The function of the membranes includes the accumulation of neurotransmitters and ions by specific transporters and pumps, and their fusion with the plasma membrane by a process of regulated exocytosis [2–4]. During the last few decades these processes have been intensely investigated by many laboratories in various cell types. The preferred experimental model employed in our laboratory is composed not by a single type of cell but by clones of the rat pheochromocytoma cell line PC12 exhibiting either the wild-type phenotype, including bona fide DCVs (WT-PC12), or a phenotype lacking DCVs and other properties (Fig. 1(a) and (b)). Studies by us and others have shown the defect of the latter clones to depend on their high levels of the transcription repressor REST (RE-1 specific Silencing Transcription factor), otherwise referred to as NRSF.
rapidly, reliving the expression of many neural proteins and playing a key role in the establishment of the specific neural phenotype (Fig. 2). Because of these properties REST is widely considered as a master factor of neural cell differentiation [7–9].

In the initial studies on REST, the genes and proteins to be investigated were often chosen as markers, paying little attention to their coordinate function. As a consequence, the possibility that REST plays an important role also in mature cells, governing the expression and function of structures such as specific membranes, had not been taken into consideration. Work of the last several years has demonstrated however that this is the case with the DCV membranes. Here we will summarize the evidence obtained by the comparative study of PC12 clones competent and not for neurosecretion. The PC12 clones lacking the latter process will be referred to as the defective or the high-REST PC12 clones.

2. PC12 clones with and without neurosecretion

WT-PC12 cells exhibit a differentiated phenotype characterized by typical DCVs similar, although smaller, to the DCVs of chromaffin cells [Fig. 1(a)]. During long-term treatment with NGF the phenotype of PC12 is progressively converted from cromaffin-like to neuron-like, with outgrowth of neurites, appearance of specific markers and block of proliferation [10]. Because of these properties, PC12 cells have enjoyed great popularity as a model of neurosecretory cells, being employed to investigate a variety of specific processes, from gene expression to neurotransmitter release, neurite outgrowth and neurogenesis.

The typical neurosecretory phenotype, however, is not general among PC12 cells. Studies carried out in several laboratories have identified, in fact, a few spontaneously defective clones characterized by the lack of DCVs (Fig. 1(a) and (b)) and of catecholamine secretion [11–13]. In other neurosecretory cell lines, clones lacking DCVs had also been reported. In those cases, however, the defective phenotype had been shown to disappear with changes of culture conditions or upon prolonged stimulatory treatments. This was shown not the case of the defective PC12 clones. Rather, their cells were found to keep their phenotype even after fusion with WT-PC12 cells, suggesting their properties to depend on the expression of one (or more) repressive factor(s) [12–14]. Initially, the defect of
the PC12 clones was believed to depend on the blocked transcription of only a few neurosecretory genes [12–14]. Soon thereafter, however, it became clear that the defect included additional processes, for example the NGF-induce neurite outgrowth [15]. In contrast other processes, such as the expression of a regulated exocytic vesicle, the enlargeosome, distinct from both the DCVs and the synaptic-like vesicles, were found to lack in WT-PC12 cells, being present in the defective clones [16]. In the first overall study, carried out by microarray analysis of 4200 gene expression, 190 were found to be largely up-regulated and 226 down-regulated in the defective clones. The down-regulated genes connected to neurosecretion were 22, encoding secretory proteins of the chromogranin and secretogranin families, that in WT-PC12 are segregated within the DCV lumen; proteins of the DCV and synaptic-like vesicle membranes (synaptogamins, synaptophysin, SVOP, SV2, ICA512, the monoamine transporter vMAT1, dopamine-β-hydroxylase, the vesicle SNAREs VAMP1 and 2) and the plasma membrane SNAREs, SNAP25 and syntaxin1A. The only genes of the DCV membrane expressed at the same level in WT and defective PC12 clones were those encoding proteins shared by other cell membranes, such as the gene of the H+ pump vATPase, present also in endosomes and lysosomes [17]. Recently, we have carried out an extensive RNA-seq reinvestigation of the WT and defective clones. The results have confirmed and extended the microarray results identifying, in the defective clones, almost 800 down-regulated genes out of a total of over 13,000, including several genes encoding neurosecretory membrane proteins that had been missed in the previous analyses (unpublished).

3. The DCV membrane defect is a consequence of high REST

In the initial studies in the field, the WT and defective PC12 clones were characterized based on their structural and functional differences. However, the cause(s) of these differences remained undefined. To clarify this issue, extensive studies were carried out in our laboratory taking into account various transcription systems including REST, already identified as a key repressor of neural cell-specific genes, abundant especially in undifferentiated or non-neurally differentiated cells [7,8]. At the time, however, knowledge about the repressor was still limited and the specific research tools were not largely available. For quite some time, therefore, our efforts remained unsuccessful.

The first evidence for a role of REST emerged from the studies of Bruce et al. [18] who showed the expression of a number of specific genes and the process of catecholamine release to be markedly reduced in WT-PC12 cells upon transfection of specific REST constructs. Soon thereafter Pance et al. demonstrated the defective PC12 clone isolated in the laboratory [13] to express high levels of REST [19]. In the Pance's clone the transcription of a few DCV genes was increased upon transfection of a dominant-negative construct of REST. However, this increase was not followed by the rescue of the encoded proteins, suggesting low REST to be necessary, but not sufficient for the expression of the PC12 phenotype [19]. Our subsequent studies [20] confirmed part of the data of [18] and [19] and expanded our knowledge about the role of REST. In our defective clones the mRNA levels of the repressor were found to exceed those of WT-PC12 by 50 to 60-fold while the levels of 12 neurosecretory REST targets were decreased, ranging however from 3 to 50-fold (Fig. 1(c) and (d)). At variance with the data of [19] we found that REST does not govern only the expression of the neurosecretion genes. Rather, it does affect in parallel also the expression of the encoded proteins as well as the appearance of DCVs within the cell. Transfection of dominant positive constructs in the WT clones, and of the DNA binding domain of REST in the defective clones resulted in the attenuation and the partial rescue of neurosecretion, respectively (Figs. 3 and 4) [20]. Taken together, our expression results complemented the previous macroarray studies [17] demonstrating that, out of the 12 genes investigated, only one was repressed indirectly while 11 were direct targets of REST repression. In view of the variability of their
repression, however, also the changes of the levels, properties and functions of their encoded proteins were variable [20].

In conclusion, the differential properties characterized in the PC12 clones with and without neurosecretion depend largely on their different levels of REST. In WT-PC12 cells REST is very low, similar to the level reached during differentiation by neurons and chromaffin cells; in the defective clones it is much higher, similar to the level of stem cells and of most non-neural cells [7,8]. These differences should not be considered as static epigenetic properties of the cells. REST, in fact, is a fast turnover protein, dynamically controlled in its expression both at the transcriptional and, especially, the post-translational level [21]. As a consequence, fluctuations of the repressor levels can occur. For example, the levels of REST increase several fold upon prolonged stimulation of neurons, with ensuing attenuation of at least some of their specific properties [22 and unpublished]. In the brain tissue, some of the astrocytes exhibit the expected high levels of REST while others exhibit levels as low as those of neurons. Interestingly, only the low-level astrocytes exhibit DCVs and discharge them upon stimulation [5]. Finally, high REST has been shown to play the role of oncogene in neural cells, of tumor suppressor in non-neural cells [23]. The high level of the repressor present in the defective PC12 clones, therefore, does not appear as a unique curiosity but as a permanent disregulation of a setting system that, in the other cells, appears accurately regulated.

4. Is REST involved in the control of DCV assembly?

The results reported in the previous section demonstrate that indeed REST controls the expression of the DCV genes and of their encoded proteins. Is the lack of these proteins in the high-REST PC12 cells the only cause of their lack of DCVs? In other words, is the competence for DCV expression maintained or not in the high-REST PC12 that lack the proteins of the organelle? The present evidence, although only partial, suggests the latter to be not the case. In fact, when DCV proteins were transfected in the defective PC12 clones they were mistargeted to various organelles as observed also in non-neurosecretory cells: the secretory chromogranins A and B ended up in the constitutive secretion vesicles; dopamine-β-hydroxylase and synaptotagmin 1 in lysosomes and the plasma membrane, respectively [2,4,12,13,20]. These results suggest that the correct DCV assembly occurs in a specific sub-compartment of the TGN that exists only in neurosecretory cells. In this sub-compartment the proteins destined to DCVs are sorted from the other components in transit, such as the proteins destined to constitutive secretion vesicles and lysosomes. If the targeting to this sub-compartment does not occur, the proteins are missorted to other structures.

During the last 25 years the biogenesis of DCVs has attracted great attention, however most of the studies were focused on the generation of the dense core of the vesicle and not on the generation of the membrane. Within the DCV lumen the secretory proteins are aggregated to form the core. As reported in the various reviews of Tooze [see Section 3], the most widely accepted hypothesis, the sorting for entry hypothesis, is that the core proteins are first sorted to the sub-compartment where their aggregation begins, driven by the acidic environment, the high Ca²⁺ and possibly the interaction/metabolism with and by local proteins such as the prohormone convertases. Among the various secretory proteins, chromogranin A has been reported to play the key role for aggregation, inducing the assembly of DCVs even in incompetent cells [24]. Subsequent studies have shown, however, that in the latter cells the dense organelles containing chromogranin A were not DCVs but lysosomes [4]. Other studies have come to the conclusion that the proteins important for aggregation are chromogranin B [25] and/or pro-secretogranin II [26]. The other secretogranins (III, V and VI) and the long list of peptides and enzymes residing at low levels within the granules may not play as important structural roles. Recent data have shown that chromaffin granules, although atypical and of large size, assemble even in mice KO for both chromogranin A and B [27]. In conclusion, the competence for the assembly of the dense core of DCVs in the TGN subcompartment...
of neurosecretory cells appears to depend not on a single but on various lumenal proteins, molecularly quite different from each other, and on their mixtures.

For DCV membrane assembly the most important process appears to be the surface remodeling taking place at the level of immature DCVs. The process is governed by coated vesicles of the Golgi type, composed by clathrin associated with the adaptor complex protein AP-1 and with the GGA proteins. Specifically, the coated vesicles appear to segregate from other domains of the immature DCV membrane, fishing out the non-DCV components of TGN origin still associated with the membrane [3,28,29]. Among the non-DCV components are the M6P receptor and the furin enzyme which are destined to traffic to constitutive vesicles; the SNAREs VAMP4 and syntaxin6 together with synaptotagmin IV, that are responsible for the membrane fusions taking place during DCV maturation, governing the homotypic fusion of immature DCVs and also the traffic of coated vesicle. In fact, the SNAREs and VAMP4 are removed not at the early phases but at the end of the maturation process. Remodeling results also in the DCV membrane enrichment of cholesterol and gangliosides, a property of importance for its rigidity and curvature. Among the factors that regulate the remodeling process are various G proteins such as the monomeric Rab3s, and at least two GEFs, trio and kalirin [30–32].

The maturation of the DCV membrane might not be independent from that of the core. The removal by coated vesicles of membrane domains destined to the DCVs appears to be precluded also by the interaction of the luminal domain of their transmembrane proteins with the surface of the dense-core. Finally, the decrease of the membrane area, taking place during maturation as a consequence of the pinching off of coated vesicles, would not only increase the specificity, but also decrease the volume of maturing DCVs, thus favoring the aggregation of the segregated core proteins [6].

So far we have discussed the mechanisms of DCV assembly, however not whether this process is regulation by REST. The absence of DCVs in the high-REST cells (defective PC12, Fig. 1(b), and non-neurosecretory cells) and the mistargeting of neurosecretion...
proteins transfected into these cells [2,4,12,13,20] suggest this to be most likely the case. In fact the assembly does not appear to depend on the genes encoding proteins that have roles in the immature DCV membrane remodeling because, with the exception of Rab 3A [7], all of them are expressed at WT levels in the high-REST PC12 cells (unpublished). Some information was obtained by taking advantage from the different sensitivity to repression of the various REST target genes. In WT-PC12 in which the REST level had been increased of 4-fold by transfection of a full length construct, the chromogranins were repressed almost completely whereas some membrane proteins were prominent (Fig. 3). In these cells the DCVs, although of smaller volume (~75%), were still present. Their exocytic response induced by a Ca\(^{2+}\) increase appeared largely normal [20]. In defective PC12 in which REST repression had been decreased to a moderate extent by the stable transfection of a DNA binding domain construct, bona fide DCVs remained lacking, yet clear vesicles positive for synaptotagmin1, scattered in the cytoplasm, were discharged by exocytosis upon stimulation [20]. Although the latter results are still preliminary, they seem to suggest the synaptotagmin 1-positive vesicles to be DCVs defective of their dense-core. According to this interpretation, this “empty form” of DCVs would be due to the assembly of the proteins with lower sensitivity to REST repression compared to chromogranins. In order to confirm this possibility various aspects of the process remain to be investigated: whether the assembly of the “empty DCVs” requires the existence, in the cell, of the TGN sub-compartment discussed above, where the assembly of intact DCVs appears to take place; whether a single, or a few, other membrane proteins need to be co-expressed with synaptotagmin1; whether specific regulatory factor(s), not yet identified, are necessary; and so on.

5. DCV membrane function: transmembrane transport processes

Various types of transport, of ions, nucleotides and neurotransmitters, take place across the DCV membrane. The most important ion is calcium. Its high level in the DCV lumen (50–100 mM) is of great importance for protein aggregation. Studies with aequorin have shown the free Ca\(^{2+}\) concentration to be ~50–100 \(\mu\)M, the rest being bound primarily to the low affinity binding proteins, chromogranins and secretogranins. The DCV Ca\(^{2+}\) pool appears to be dynamic, therefore its import needs to occur continuously, apparently via ATPases of the SERCA and SPCA type [33]. These ATPases are not specific of DCVs but occur primarily in the ER and Golgi complex, two structures fully active also in the high-REST cells. Therefore their encoding by genes insensitive to REST, recently observed (unpublished), is not surprising.

Other molecules of the DCV lumen are transported across the membrane not by ATPases but by electrogenic exchangers energized by the steep electrochemical gradient established by the H\(^+\) pump vATPase. Expression of the nucleotide transporter of ATP [34] is independent, whereas the expression of the monoamine transporter vMAT1, predominant in the rat chromaffin cells and PC12, is highly dependent on REST. Vesicular transporters of other amines, such as histamine and serotonin, do not appear to be present in the DCV membranes of PC12 cells. Dopamine-\(\beta\)-hydroxylase [17,20 and unpublished], the enzyme necessary for the conversion of dopamine to noradrenaline, which is associated with the luminal surface of the DCV membrane, is in contrast expressed and down-regulated by REST. In conclusion, the REST dependence of molecular transport across the DCV membrane is not general but appears restricted to catecholamines, the physiologically key neurotransmitter molecules accumulated within the vesicle. When the level of REST increases, the catecholamine content of DCVs is progressively decreased up to its complete disappearance.

6. DCV membrane function: the exocytic discharge

The final step of the DCV membrane function discussed here is its fusion with the plasma membrane by a regulated exocytosis process. Before undergoing fusion the vesicles need to be appropriately processed by cytoskeleton remodeling followed by a series of interactive events between their membrane and the cytosolic face of the plasma membrane. These events are referred to as tethering, direct docking and priming for fusion. The proteins participating in these processes (including Munc13, Munc 18a, complexin and synaptojanpin), which may be common to other membrane fusions taking place within and at the surface of the cell [35–38], appear to be all encoded by genes little sensitive to REST [17].

The situation changes with the proteins of the exocytic fusion machinery, the SNAREs, that assemble the channel through which the continuity of the fusing DCVs and plasma membranes is established: VAMP2, the SNARE of DCVs; SNAP25 and syntaxin1A, the two SNAREs of the plasma membrane [39,40]. The genes encoding these proteins are all repressed by REST, however to different degrees [17,20]. Transcription of the SNAP25 gene is extensively repressed even by few fold increases of REST over the WT-PC12 level; syntaxin1A requires higher levels of REST for repression; VAMP2 is still ~22% active in the high-REST PC12 clones (Figs. 3 and 4) [17,20 and unpublished]. Proteins of the DCV membrane, such as SVO, SV2 and ICA, are also repressed, and the same is true for the soluble monomeric G protein, Rab 3A [17,20 and unpublished], whose association to the DCV membrane is necessary for the exocytosis to take place. The other DCV membrane proteins playing critical roles in regulated exocytosis, the Ca\(^{2+}\) sensors of the synaptotagmin family, are also repressed by REST, although to variable extents, and the same occurs with the voltage-gated Ca\(^{2+}\) channels of the plasma membrane distributed in the proximity of the exocytic sites [17,20 and unpublished]. When the REST repression is decreased to a large extent, bona fide DCVs fully competent for exocytosis are rescued (Fig. 4(c–h)). Because of all these properties there is no doubt that, in high-REST PC12 cells, neurotranscretion is repressed by REST not only at the site of DCV assembly, as discussed previously, but also at the site of their exocytic fusion. In order the block of exocytosis to be complete, however, the REST levels should be very high. At lower levels, in fact, some exocytosis may still occur. An example are the “empty DCVs” negative for chromogranin A and positive for synaptotagmin that assemble when the activity of REST is intermediate between those of WT and high-REST PC12, as we already mentioned. The exocytosis of these vesicles, taking place upon cell stimulation [20], could occur because the repressed SNARE, SNAP25, can be replaced by its homologue, SNAP23 [40], which is not a target of the repressor [17].

7. Conclusion

The results of the last few years, including those obtained by the parallel study of pheochromoctyoma PC12 cell clones expressing much different levels of REST, demonstrate the low REST typical of WT neural cells to be the cause for the expression of the proteins specific of the DCV membrane, for their assembly and for their functions. Although not presented in this review, REST appears to operate similarly also for the membrane of the other neurotransmitter release organelle of neural cells, the synaptic/synaptic-like or clear vesicles [18,20].

A concern that was often raised to question this conclusion was that the supportive results, by us and others, had been obtained working not with tissue neural cells, such as neurons and chromaffin cells, but with clones of a cell line, possibly characterized by peculiar properties. We believe in contrast that the data we have
summarized about the role of REST in the expression and function of the DCV membrane do not apply to the PC12 line and its clones only but are of general validity. Even the cells of the defective PC12 clones are not curiosities but solid research models. These cells, in fact, grow and function as normal, healthy cells [41]. Their synthesis and intracellular traffic, and even the fusions of membranes other than those of DCVs and synaptic-like vesicles appear normal. At the level of the plasma membrane, the defective PC12 cells are fully competent for exocytic processes, not only constitutive, but also regulated, via the participation of SNAREs other than those of DCVs and synaptic-like vesicles. Moreover, the defective PC12 cells appear competent endocytosis. Finally, their defects can be rescued by the decrease of their REST repression (Fig. 4(c–h)) or by the expression of single REST targets [14,16,20]. For example, the neurite outgrowth induced by NGF, which is lacking in these cells (15), is rescued by the expression of p75NTR, a neurotrophin receptor repressed by REST [17 and submitted]. On the other hand, the data obtained with the PC12 clone model agree with data obtained with a growing number of other cells, of cultured lines and of tissues, published [5,23,41] or not yet.

As already mentioned, REST is a protein of fast turnover [21]. Its levels can change or fluctuate even without major changes of its regulatory settings. Significant increases, therefore, can occur in neural cells not only in pre-necrotic states [42], but also in physiological conditions such as during may hours of stimulation. The consequences of these increases, for example the reduction of excitability and of neurosecretion, or the changes in cell metabolism, appear to be relevant resulting in a protection of neural cells. Taken together they could result in fact in an attenuation of signaling across the plasma membrane and across the intracellular membranes, with decreased chances of both apoptosis and necrosis [22,43,44]. Expression and functioning of the DCV membranes participate therefore in a dynamic framework of events taking place in the cell under the govern of REST. Depending on its level the role of REST can vary from the repression of the DCV membrane as a whole, to the repression of some, but not all membrane components, to a subtle regulation of the molecular properties of membranes and other structures participating in the overall functioning of neural cells.

Acknowledgments

We are grateful to the colleagues who participated in the studies of the laboratory reported here, in particular Maria Luisa Malosio, Barbara Borgenovo, Emanuele Cocucci, Andijana Klajn, Ilaria Prada and Sara Negrini. We also thank Gabriella Racchetti for the continuous assistance and support.

References


