Published in final edited form as:
J Neurochem. 2004 December ; 91(6): 1332–1343.

Expression profiling upon Nex1/MATH-2-mediated neuritogenesis in PC12 cells and its implication in regeneration

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Abstract

The expression of Nex1 peaks during brain development when neurite outgrowth and synaptogenesis are highly active. We previously showed that Nex1 is a critical effector of the nerve growth factor (NGF) pathway and its overexpression results in spontaneous neuritogenesis. Furthermore, the PC12-Nex1 cells exhibit accelerated neurite extension upon NGF exposure, and have the capacity to regenerate neurites in the absence of NGF. In this study, we identify the repertoire of genes targeted by Nex1 to unravel the molecular mecha nisms by which Nex1 promotes differentiation and regeneration. Our transcriptional analysis reveals that Nex1 modulates a wide spectrum of genes with diverse functions, many of them being key downstream regulators of the NGF pathway, and critical to neuritogenesis, such as microtubules, microtubule-associated proteins (MAPs) and intermediate filaments. We also provide the first evidence that a basic helix-loop-helix (bHLH) protein stimulates the expression of the cyclin-dependent kinase (CDK) inhibitors belonging to the INK4 family, which plays a role in promoting cell-cycle arrest. Finally, we show a dramatic synergistic effect between Nex1 and cAMP, resulting in an impressive regeneration of an elaborate and dense neurite network. Thus, Nex1 has endowed the PC12-Nex1 cells with a distinct combination of gene products that takes part in the complex regulation of neuritogenesis and regeneration.

Keywords

basic helix-loop-helix transcription factor; cyclin-dependent kinase inhibitors; microtubules; neurofilaments; neuronal differentiation; regeneration

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During brain development, newly generated neuroblasts undergo extensive neuritogenesis, which begins immediately after neuronal commitment. During this critical process, specific combinations of extrinsic and intrinsic factors lead to distinct and complex neuronal networks accompanied by appropriate neuronal connectivity. Neurite outgrowth is triggered upon binding of neurotrophic factors to their tyrosine kinase receptors, leading to activation of distinct intracellular signaling pathways such as phosphoinositide 3′-kinase (PI3K), phospholipase C3-γ (PLC-γ) and Ras (reviewed by Kaplan and Miller 2000). The rat pheochromocytoma PC12 cell line is a well established system for the study of neurite outgrowth and neuronal differentiation induced by nerve growth factor (NGF) treatment

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Supplementary Material: The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/JNC/JNC2814/JNC2814sm.htm

Table S1. Complete List of genes regulated by Nex1.

(Greene and Tischler 1976). The intracellular signaling pathways leading to differentiation into sympathetic neuron-like cells have been extensively studied (reviewed by Segal and Greenberg 1996). However, the transcriptional circuitry elicited by these intracellular signaling cascades remains largely unknown. Most of the transcriptional studies focused on the immediate early genes, such as members of the c-*fos* family, c-*jun*, and the two members of the Egr family, *Egr*1 and *Erg*4 (Greenberg et al. 1985; Kruijer et al. 1985; Wu et al. 1989; Crosby et al. 1991; Boss et al. 2001; Levkovitz and Baraban 2002).

In an effort to acquire a more comprehensive knowledge of the intrinsic transcriptional mechanism by which PC12 cells acquire a neuronal phenotype characterized by neurite outgrowth and transition to a post-mitotic state, we focused on the basic helix-loop-helix (bHLH) differentiation factor Nex1/MATH-2, a member of the NeuroD subfamily. Our previous studies have shown that Nex1 is only expressed in PC12 cells upon NGF exposure and behaves as a critical effector of the NGF pathway (Uittenbogaard and Chiaramello 2002). Gain-and-loss of function studies on the members of the NeuroD subfamily (NeuroD, NeuroD2 and Nex1/MATH-2) have shown that they play essential roles in the developing nervous system (reviewed by Bertrand et al. 2002). Their main function is to execute specific neuronal differentiation programs in neuronal-restricted progenitor cells, resulting in cell-cycle arrest and acquisition of specific neural identities. During brain development, Nex1 expression is initiated after determination of neuronal fate, parallels overt neuronal differentiation, and peaks when neurite outgrowth and synaptogenesis are highly active (Bartholoma and Nave 1994; Shimizu et al. 1995; Schwab et al. 1998). Its persistent expression in areas of the adult brain associated with higher brain functions, such as learning and memory formation, suggests a potential role in synaptic plasticity and maintenance of the differentiated state (Schwab et al. 2000).

To dissect the potential functions of Nex1 during the early steps of neuronal differentiation, we generated a stable Nex1-overexpressing PC12 cell line in which Nex1 expression is constitutively regulated by the cytomegalovirus (CMV) promoter (Uittenbogaard and Chiaramello 2002). We found that Nex1 triggers spontaneous neurite outgrowth in the absence of NGF, and that there is a direct correlation between Nex1 expression and the increased expression of the *GAP-43* gene (Uittenbogaard et al. 2003). A plethora of evidence indicates that the GAP-43 protein is critical to the establishment of axonal outgrowth during the initiation and remodeling of neural connections (Benowitz and Perrone-Bizzozero 1991; Aigner et al. 1995; Strittmatter et al. 1995; Mani et al. 2000). Furthermore, our previous study revealed that constitutive expression of Nex1 accelerates the NGF responsiveness of the PC12-Nex1 cells, resulting in a substantial increase of neurite outgrowth (Uittenbogaard and Chiaramello 2002). Finally, we found that Nex1 is a critical effector of the NGF pathway, as constitutive expression of a truncated Nex1 mutant blocks NGF-induced neuronal differentiation.

To further decipher the transcriptional pathway mediated by Nex1 during the early steps of neuronal differentiation and neuritogenesis, we sought to carry out a comprehensive analysis of Nex1-regulated target genes by employing Atlas cDNA expression array in conjunction with immunoblot analysis. We found that overexpression of Nex1 directly or indirectly induces the expression of a wide spectrum of genes, such as cytoskeletal genes, vesicular trafficking/synapse-related genes, transcription factors, cell adhesion and metabolic-related genes. We focused on a repertoire of cytoskeletal proteins known to be involved in neurite outgrowth as well as on the cyclin-dependent kinase (CDK) inhibitors known to favor G1 arrest. This study reports the first evidence that a neuronal-specific bHLH transcription factor modulates the expression of the INK4 family members. Finally, we expanded our analysis to the regeneration-inducing properties of Nex1 in the absence or presence of cAMP. We observed a dramatic synergistic effect between Nex1 and cAMP that resulted in full neurite network regeneration,

suggesting that cAMP brings a signaling component to the PC12-Nex1 cells necessary to achieve a more advanced regeneration program.

Materials and methods

Cell culture and neurite analysis

The PC12-Nex1 cells and the control PC12 cells (ATCC) were grown on collagen I-coated plates (Becton Dickinson Labware, San Jose, CA, USA) under the conditions described in Uittenbogaard and Chiaramello (2002). Differentiation was carried out in the presence of NGF (2.5s murine, Roche Molecular Biochemicals, Nutley, NJ, USA) or dibutyryl cAMP (dbcAMP; Roche Molecular Biochemicals) as indicated in the figure legends. For neurite regeneration studies, PC12 and PC12-Nex1 cells were differentiated with 50 ng/mL NGF for 7 days, and the cells were carefully washed at least five times with NGF-free medium. The neurites were then mechanically sheared by triturating cells in a Pasteur pipette and the cells were re-plated on collagen I-coated plates in the absence or presence of dbcAMP (1 m_M). Regenerated neurites were defined as a phase dark process that was at least two cell diameters in length. The regeneration process was examined at different time points after re-plating cells from three independent experiments; the percentage of neurite-bearing cells was scored on at least 300 cells per experiment and repeated three times.

RNA isolation and cDNA microarray analysis

DNA-free total RNA was isolated from control PC12 and PC12-Nex1 cells using the Atlas Pure Total RNA Labeling System (BD Biosciences Clontech, Palo Alto, CA, USA). RNA concentration was determined spectrophotometrically and RNA integrity was confirmed by electrophoresis in a 1% (w/v) denaturing agarose/formaldehyde gel. The poly(A) RNA fraction was isolated using biotinylated oligo(dT) and streptavidin magnetic beads according to the manufacturer's recommendations. cDNA probes were synthesized using gene-specific primers and labeled with [α^{32}P] dATP (3000 Ci/mM) (Amersham Biosciences, Piscataway, NJ, USA). The ^{32}P-labeled complex cDNA probes were hybridized overnight to Atlas Rat 1.2 II array (Clontech) using Express Hyb hybridization solution at 68°C. After two high stringency washes, the hybridized membranes were exposed on phosphorimager screen and scanned using a Molecular Dynamics Storm Imager (Amersham Biosciences). All cDNA microarrays were performed three times with new filters, and RNA isolated at different times. The images were analyzed and quantified using the Atlas Image 2.01 software (Clontech). The background was determined from the intensity reading around the spotted areas. The results from each filter were normalized using the mean intensity as the reference, i.e. global normalization.

Western blot analysis

PC12 cells and stably transfected PC12-Nex1 cells were lysed in M-Per mammalian protein extraction buffer (Pierce, Rockford, IL, USA) in the presence of a cocktail of protease inhibitors (Roche Molecular Biochemicals) as described in Uittenbogaard and Chiaramello (2002). Cell extracts were spun at 10 000 *g* for 10 min, and protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA, USA). Proteins (40 μg) were resolved on either a 10% NuPAGE Bis-Tris gel or a 3-8% Trisacetate gel (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane. Nitrocellulose membranes were stained with Ponceau S (Sigma, St. Louis, MO, USA), to confirm uniform loading and transfer of proteins, and subsequently blocked with Superblock blocking buffer (Pierce) in phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween-20. The membranes were probed with various primary antibodies listed in Table 1. Equal loading of proteins was verified by probing with anti-peripherin antibody, anti-NF-L antibody or anti-p27^{Kip1} antibody, as shown in the Results section. A horseradish peroxidase-conjugated secondary antibody (Pierce) was used at a 1 :

100 000 dilution, and the antigen-antibody complex was detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Results

Gene expression profiling upon constitutive expression of Nex1 in PC12-Nex 1 cells

We previously showed that overexpression of Nex1 induced spontaneous neurite outgrowth of PC12-Nex1 cells accompanied by an increased expression of the GAP-43 and βIII-tubulin proteins. To gain insight into the molecular mechanism of Nex1-mediated neuronal differentiation in the absence of NGF, we analyzed RNA isolated from naïve control PC12 and PC12-Nex1 cells. Three independent experiments were carried out with total RNA isolated from three distinct samples of control PC12 and PC12-Nex1 cells. RNA was labeled and hybridized to the Atlas Rat 1.2 II array membrane. This array did not include the three following relevant genes: GAP-43, β-III tubulin and α-tubulin. Of the 1176 genes represented in the membrane, we identified 129 (11%) whose expression changed at least twofold upon constitutive expression of Nex1 in the absence of NGF. These included 95 genes whose expression increased and 34 whose expression decreased (see Table S1). Representative genes from the most prevalent categories are shown in Table 2.

Consistent with the Nex1-mediated neuronal differentiation of the untreated PC12-Nex1 cells, many of the differentially-expressed genes encoded proteins involved in neurotransmitter release and synaptic activity, such as synaptotagmin VII and the neuronal isoform synaptogyrin I. Overexpression of Nex1 led to an 18-fold increase in the expression of synapse-associated protein 90/post-synaptic density-95-associated protein 2 (SAPAP2), which is involved in signaling at the post-synaptic density (PSD) via the recruitment of PSD95/SAP90 protein to the plasma membrane (Takeuchi et al. 1997).

Constitutive expression of Nex1 also resulted in increased expression of genes involved in neuritogenesis. These include several microtubule-associated proteins (tau, MAP-1A, MAP-2A/B) and intermediate filaments (NF-M and NF-H) that are key cytoskeletal components for neurite formation and maintenance (Tanaka and Sabry 1995). Interestingly, the expression levels of peripherin and neurofilament NF-L were not affected by the constitutive expression of Nex1.

Nex1 also induced the expression of transcription factors, such as mash-1, Phox-2a and HNF-3γ, known to play important roles during neuronal differentiation, as well as the expression of genes involved in cell adhesion, such as brevican, ryudocan, S-laminin and laminin receptor, which is in agreement with the increased cell adherence observed with the PC12-Nex1 cells (Uittenbogaard and Chiaramello 2002).

Finally, constitutive expression of Nex1 directly or indirectly up-regulated several genes involved in amino acid metabolism and, more specifically, the ornithine metabolic pathway, such as arginosuccinate synthase (ASS), ornithine transcarbamylase (OTC), ornithine aminotransferase (OAT) and ornithine decarboxylase (ODC). ODC, which is a key enzyme in the biosynthesis of polyamines, is implicated in the NGF-induced neuritogenesis of PC12 cells (Feinstein et al. 1985; Volonté and Greene 1990). More recently, it was shown that inhibiting ODC activity blocks the cAMP effect in overcoming inhibition of regeneration by myelin-associated glycoprotein (MAG) and myelin (Cai et al. 2002). Therefore, these observations suggest that Nex1-mediated differentiation is associated with specific metabolic changes. Collectively, this cDNA expression array suggests that Nex1 by itself most likely modulates, directly or indirectly, a wide spectrum of genes with diverse functions in order to mediate differentiation.

Nex1 selectively promotes the expression of intermediate filaments in the absence of NGF

To investigate the mechanism by which Nex1 promotes spontaneous neurite outgrowth in PC12-Nex1 cells, and to validate the cDNA array results, we examined the expression of the intermediate filaments by immunoblot analysis using whole cell extracts from control PC12 and PC12-Nex1 cells. We first initiated our protein analysis on the type III intermediate filament peripherin, as it is critical to the initiation, extension and maintenance of neurites in PC12 cells (Helfand et al. 2003). Figure 1 shows that Nex1 has no effect on peripherin expression at the protein level and thus, corroborates the lack of variation in RNA levels shown by the cDNA array results (Table 2). We then extended our analysis to the type IV intermediate filament α-internexin, since its expression coincides with the early steps of differentiation in the developing brain (Fliegner et al. 1994). The membrane used to detect peripherin was stripped before probing it with a monoclonal antibody against α-internexin. Figure 1 shows a significant increased expression of the α-internexin protein in the presence of constitutive expression of Nex1. On the basis of our cDNA array analysis, we evaluated the protein expression levels of the neurofilament (NF) light (NF-L), medium (NF-M) and heavy (NF-H) subunits in PC12-Nex1 cells. Since our cDNA microarray analysis showed that the RNA levels of NF-L remained unchanged in the presence of Nex1 overexpression, we initially verified by immunoblot analysis that the expression of the NF-L protein remained constant (Fig. 1). The same membrane was used to examine the expression of NF-M and NF-H subunits. The expression levels of both the NF-M and NF-H proteins were increased upon overexpression of Nex1 (Fig. 1) and thus confirmed the cDNA array results (Table 2). Taken together, these results indicate that Nex1 modulates the expression of a selective set of intermediate filaments, which may influence the neurite properties of PC12-Nex1 cells.

Nex1 induces the expression of microtubules and microtubule-associated proteins independently of NGF

In view of our previous findings that Nex1 up-regulates the expression of the βIII-tubulin protein in untreated PC12-Nex1 cells, we extended the scope of our analysis to the expression levels of α-tubulin and several microtubuleassociated proteins (MAPs), as suggested by our cDNA array analysis (Table 2). We included the βIII-tubulin immunoblot analysis to present a complete analysis of the microtubule elements, and to directly compare it with the α-tubulin expression levels. Thus, the membrane was stripped and re-probed with the monoclonal anti-α-tubulin antibody. We observed a similar increased expression level of α-tubulin protein in the presence of constitutive expression of Nex1 (Fig. 2). We then addressed the question of whether tubulin up-regulation in response to overexpression of Nex1 may also be mediated by an up-regulation of MAPs, as suggested by the cDNA array analysis (Table 2). In addition, it is well established that an increase in MAP expression induces microtubule polymerization and therefore, tubulin synthesis through an autoregulatory mechanism (Cleveland et al. 1981;Cleveland 1989). Thus, we evaluated the expression levels of the three MAPs, tau, MAP-1A and MAP-2A/B, in the absence (control PC12 cells) and presence (PC12-Nex1 cells) of Nex1. We found that constitutive expression of Nex1 resulted in increased expression levels of the three MAPs, with MAP-1A showing the strongest magnitude of induction (Fig. 2). Equal loading was verified by visualization of total proteins by Ponceau S staining after transfer to nitrocellulose and blotting with anti-peripherin antibody (data not shown). It is worth noting that control PC12 cells do not express much MAP-1A and MAP-2A/B (Fig. 2). Thus, our findings on the expression levels of the three MAPs are in agreement with the increased RNA levels detected by cDNA array analysis (Table 2). Collectively, this expression profile of the major microtubule cytoskeletal proteins supports our original observation that the PC12-Nex1 cells exhibit spontaneous neurite extension and differentiation.

Nex1 induces the expression of specific G1-phase CDK inhibitors in the absence of NGF

Our previous biochemical analysis revealed that the PC12-Nex1 cells display a generation time (7 days) twice as long as that of the control PC12 cells, and that constitutive expression of Nex1 leads to a dramatic increase in the expression of the $p21^{Cip1}$ cyclin-dependent kinase (CDK) inhibitors (Uittenbogaard and Chiaramello 2002). Since neuronal differentiation is closely linked to cessation of cell proliferation, we performed a detailed analysis of the expression of the members of the CIP/KIP and the INK4 families upon constitutive expression of Nex1, as these CDK inhibitors are believed to favor G1 phase and facilitate terminal differentiation (Elledge et al. 1996). We examined the expression of the three members of the CIP/KIP family, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, by immunoblot analysis. In Fig. 3(a), we included the $p21^{Cip1}$ immunoblot analysis to provide a complete analysis of the CIP/KIP family of CDK inhibitors, as well as to directly compare their expression levels between each other. As expected, we observed a dramatic increase in $p21^{Cip1}$ expression upon constitutive expression of Nex1 (Fig. 3a). The membrane was then stripped and re-probed with either the polyclonal anti-$p27^{Kip1}$antibody or the polyclonal anti-$p57^{Kip2}$ antibody. We found that Nex1 did not alter the expression of the $p27^{Kip1}$ and $p57^{Kip2}$ CDK inhibitors, which were already expressed at significant levels in control PC12 cells (Fig. 3a). This stands in contrast with the negligible levels of $p21^{Cip1}$ expression detected in control PC12 cells (Fig. 3a).

We extended our protein analysis to the four members of the INK4 family, $p15I^{NK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$, as they bind directly to the cyclin D-dependent kinases CDK4 and CDK6 and inhibit their activities (reviewed by Ruas and Peters 1998). As shown in Fig. 3(b), constitutive expression of Nex1 substantially increased the expression of the two CDK inhibitors, $p15^{INK4}$ and $p16^{INK4a}$, whereas Nex1 moderately modulated the expression of the INK4 member $p18^{INK4c}$ (Fig. 3b). In contrast, Nex1 did not appear to affect the expression of the $p19^{INK4d}$ protein (Fig. 3b). Thus, collectively, our results suggest that Nex1 affects the expression of specific CDK inhibitors belonging to either the CIP/KIP or the INK4 family, resulting in increased doubling time of the PC12-Nex1 cells. These results suggest that Nex1 regulation of neuronal-specific genes and cell-cycle regulators links neuronal differentiation and cell-cycle arrest during neurogenesis.

Constitutive expression of Nex1 stimulates cAMP-mediated neuritogenesis

Our previous analysis showed that constitutive expression of Nex1 resulted in accelerated NGF-induced neuritogenesis (Uittenbogaard and Chiaramello 2002). Since NGF induces a transient increase of intracellular levels of cAMP within PC12 cells (Schubert and Whitlock 1977; Schubert et al. 1978), we addressed the question of whether constitutive expression of Nex1 would stimulate cAMP-mediated neuritogenesis. It is well established that dbcAMP-treated PC12 cells only display a modest neurite outgrowth (Gunning et al. 1981). Control PC12 and PC12-Nex1 cells were exposed to 1 mM dbcAMP for different periods of time and neurite processes were determined as described in Materials and methods. After 4 h of dbcAMP treatment, 70% of PC12-Nex1 cells bore neurites (Figs 4a and b), which is a significant increase from the original 25% of neurite-bearing PC12-Nex1 cells observed in the absence of neurotrophic factors (Uittenbogaard and Chiaramello 2002). In contrast, control PC12 cells did not yet display any significant neurite extension (Figs 4a and b). After 24 h of treatment, 90% of PC12-Nex1 cells had started establishing a short but interactive neurite network, whereas only 25% of control PC12 cells underwent neurite initiation as visualized by very short neurite extension (Figs 4a and b). By 48 h of dbcAMP exposure, nearly all of the PC12-Nex1 cells (98%) bore numerous and elongated neurites forming a network, which remained stable after 3 days of treatment (Figs 4a and b). In contrast, control PC12 cells not only did not display additional neurite outgrowth, but also failed to establish a significant neurite network after 3 days of dbcAMP treatment (Figs 4a and b). Therefore, these results indicate that PC12-Nex1 cells show a rapid response to cAMP signaling, resulting in accelerated and increased

neuritogenesis. This observation suggests that constitutive expression of Nex1 triggers a transcriptional program that converges with the cAMP signaling cascade, which most likely also results in enhanced transcription.

Constitutive expression of Nex1 in the presence of cAMP promotes dramatic neurite regeneration

Neurite initiation and elongation occur not only during NGF-induced differentiation, but also during regeneration, after neurites have been severed. In view of our published observations that Nex1 is capable of triggering the neurite regeneration process in the absence of NGF, we sought to determine whether constitutive expression of Nex1 in conjunction with dbcAMP treatment would enhance neurite regeneration and ultimately re-establish a neurite network. For this study, control PC12 and PC12-Nex1 cells were first differentiated with NGF (50 ng/mL) for 7 days. The neurites were then mechanically triturated after extensive washes to remove NGF, as described in Materials and methods. A time-course analysis of neurite regeneration was performed in the absence or presence of dbcAMP (1 mM). Figure 5 shows that dbcAMP treatment of NGF-treated control PC12 cells results in a modest rescue of neurite regeneration when compared with the treated PC12-Nex1 cells. After 1 day of treatment, only 32% of dbcAMP-treated control PC12 cells bore neurites and formed a loose network, which started regressing after 2 days of dbcAMP treatment (Figs 5a and b). In contrast, constitutive expression of Nex1 in the presence of dbcAMP resulted in accelerated and increased neurite regeneration of NGF-treated PC12-Nex1 cells (Figs 5a and b). After 6 h of dbcAMP exposure, 82% of the PC12-Nex1 cells already displayed significant neurite extension whereas in the absence of dbcAMP, only 52% of PC12-Nex1 cells showed regeneration of small neurites (Figs 5a and b). A 1-day exposure to dbcAMP resulted in most of the treated PC12-Nex1 cells (98%) bearing long neurites with extensive branching (Figs 5a and b) and, most strikingly, treated PC12-Nex1 cells regenerating an impressive and elaborate dense neurite network (Fig. 5a). In the absence of dbcAMP, 80% of untreated PC12-Nex1 cells displayed significant long neurite regeneration and a moderate neurite network (Figs 5a and b). The regeneration process observed in untreated and dbcAMP-treated PC12-Nex1 cells remained stable beyond 48 h after neurites were severed (Fig. 5b). Thus, our results indicate that Nex1-mediated regeneration is synergistically enhanced upon cAMP signaling, resulting in full regeneration of an elaborate neurite network. Furthermore, Nex1 provides an important transcriptional program to the regeneration-inducing effect of cAMP.

Discussion

Gain-and-loss of function studies on the members of the NeuroD subfamily have clearly demonstrated their critical roles in the developing nervous system. However, little is known about their transcriptional cascades leading to neuronal differentiation and regeneration. Thus, the main goal of this study was to identify the repertoire of genes targeted by Nex1, using our PC12-Nex1 stable cell line, in order to gain insight into the molecular mechanisms by which Nex1 promotes neuronal differentiation and neurite regeneration. The transcriptional analysis of PC12-Nex1 cells revealed a differential regulation of a wide diversity of genes involved in neuronal cytoskeleton, synaptic activity, cell adhesion, transcriptional machinery, cell-cycle regulators and metabolic activities. In fact, many of the genes directly or indirectly regulated by Nex1 are known to be key downstream regulators of the NGF pathway. This is in accordance with our previous findings that Nex1 expression is triggered upon NGF exposure in PC12 cells and that Nex1 is a critical effector of the NGF pathway (Uittenbogaard and Chiaramello 2002).

Nex1 promotes the expression of several inhibitors of G1 cyclin-dependent kinases

Our results indicate that constitutive expression of Nex1 induces the expression of selective G1 phase-specific CDK inhibitors known to be critical for cell-cycle withdrawal, which precedes neuronal differentiation. It is well established that NGF induces differentiation of PC12 cells by first stimulating the expression of cytoskeletal proteins necessary for neurite outgrowth within the first 3 days of exposure, followed by the up-regulation of specific CDK inhibitors after 3 days of treatment. Among the members of the CDK inhibitors CIP/KIP family, Nex1 only stimulates the expression of $p21^{Cip1}$, which is known to inhibit the kinase activity of CDK4 and CDK6 complexed to cyclin D, and of the CDK2 complexed to either cyclin E or cyclin A (Harper et al. 1995). Consequently, $p21^{Cip1}$ blocks cell-cycle progression in late G1 phase by preventing hyperphosphorylation of the retinoblastoma gene product (pRB), which is an important substrate of G1 cyclin:CDK complexes (reviewed by Weinberg 1995). The expression of $p21^{Cip1}$ is induced upon NGF treatment by a transcription-dependent mechanism involving a combination of transcription factors, such as Sp1 (Yan and Ziff 1997) and Nex1 (Uittenbogaard and Chiaramello 2002). The delay in NGF-induced cell-cycle arrest may reflect a requirement for accumulation of $p21^{Cip1}$ at high enough inhibitory levels. Consistent with this notion is the observation that maximal $p21^{Cip1}$ overexpression achieved by an inducible system results in permanent cell-cycle arrest (Erhardt and Pittman 1998). However, such high levels fail to induce neurite outgrowth and differentiation of untreated PC12 cells, but promote accelerated NGF-induced differentiation (Erhardt and Pittman 1998).

As for the members of the INK4 family, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, which are known to serve as specific G1 phase inhibitors of CDK4 and CDK6 complexed to cyclin D by preventing the phosphorylation of pRB, little is known of their transcriptional regulation or their roles during neuronal differentiation. Constitutive expression of Nex1 leads to a dramatic increase in $p16^{INK4a}$ expression, whereas it only moderately induces the expression of $p15^{INK4b}$ and $p18^{INK4c}$. Therefore, this study provides the first evidence that a bHLH transcription factor regulates the expression of members of the INK4 family. A recent study shows that the HLH Id1, known as a dominant-negative inhibitor of lineage-specific bHLH proteins, negatively regulates the expression of $p16^{INK4a}$ in senescent fibroblasts by interacting with the transcription activators Ets1 and Ets2 (Ohtani et al. 2001). However, it is also plausible that Nex1 may directly regulate the $p16^{INK4a}$ promoter activity by modulating the activity of E-boxes present in the 5′UTR region.

Our results collectively suggest that Nex1 may in part promote neuronal differentiation by inducing the expression of a set of specific CDK inhibitors, although not at levels high enough to induce permanent cell-cycle arrest in the presence of serum. However, this CDK inhibitor expression profiling mediated by Nex1 is consistent with our previous observation that the PC12-Nex1 cells display a generation time twice as long as that of the control PC12 cells (Uittenbogaard and Chiaramello 2002). Furthermore, the induced expression of these CDK inhibitors may be partly responsible for the accelerated NGF responsiveness of the PC12-Nex1 cells and the elimination of the 3-day lag period for NGF-induced neuritogenesis (Uittenbogaard and Chiaramello 2002).

Nex1-mediated neuritogenesis is associated with the accumulation of key cytoskeletal proteins

Previous studies have shown that the expression of the α- and βIII-tubulin genes is modulated by the activity of the conserved bHLH binding sites located in the 5′UTR region (Gloster et al. 1994; Dennis et al. 2002). Therefore, one of the questions addressed in this study was whether Nex1-induced neurite outgrowth of PC12-Nex1 cells is accompanied by the induction of microtubule expression in the absence of NGF. Our results show that Nex1 by itself promotes

the expression of several key microtubule proteins that are known to play an essential role in both the extension and maintenance of neurite outgrowth upon NGF treatment of PC12 cells. Constitutive expression of Nex1 results in an increased expression of α- and βIII-tubulins that is similar to the twofold increase reported upon NGF treatment of PC12 cells (Dubrin et al. 1985). More importantly, Nex1 also induces the expression of microtubule-associated proteins, such as tau, MAP-1 and MAP-2, that are known to be the limiting factors for microtubule assembly necessary for neurite outgrowth (Olmsted 1986; Matus 1991; Maccioni and Cambiazo 1995; Mandelkow and Mandelkow 1995). It was found that tubulin accumulation is not enough to promote neurite outgrowth since it does not lead to microtubule assembly during the first 3 days of NGF treatment of PC12 cells (Dubrin et al. 1985). This is further supported by the observation that the timing of tau and MAP-1 expression is tightly correlated with neurite extension, which occurs after a 3-day lag period of NGF treatment in PC12 cells (Dubrin et al. 1985).

In addition, Nex1 promotes the expression of MAP-2, a marker of dendritic differentiation, which is barely expressed in undifferentiated PC12 cells. Therefore, our results suggest that Nex1-induced differentiation may also include dendritic differentiation via MAP-2-mediated stability of microtubules located in dendrites (Johnson and Jope 1992). Interestingly, a recent study suggests that MAP-2 expression may be up-regulated by NeuroD2 during oleic acid-induced neuronal differentiation (Rodriguez-Rodriguez et al. 2004). Thus, MAP-2 expression may be modulated by distinct members of the NeuroD subfamily depending on the cellular context and extrinsic signaling pathways. This is in accordance with the well established notion that bHLH proteins participate in a complex cross-regulatory network with each other, resulting in partial functional redundancy (reviewed by Bertrand et al. 2002). Close parallels therefore exist between the ability of Nex1 to activate the expression of key microtubules and microtubule-associated proteins and its ability to induce neurite outgrowth in the absence of extracellular signaling.

In agreement with this notion, we show here that Nex1 promotes the co-ordinate induction of the expression of the type IV intermediate filament, α-internexin, NF-M and NF-H. The expression levels of NF-L remain constant and high in both control PC12 and PC12-Nex1 cells. NGF is known to induce the levels of the three neurofilament proteins in PC12 cells in a asynchronous fashion, with NF-H expression only detected at later stages of differentiation (Lindenbaum et al. 1988). During rat CNS development, the three subunits are also sequentially expressed in developing and maturing neurons, with NF-L and NF-M proteins being co-expressed in embryonic immature neurons, and NF-H appearing at later post-natal stages in maturing neurons (Carden et al. 1987). The NF-M and NF-H genes may be direct downstream target genes of Nex1, as their 5′UTR sequences reveal the presence of multiple E-boxes (Shneidman et al. 1992). In contrast, no E-boxes were found in the 5′UTR region of the NF-L gene. The co-ordinate expression of the three subunits suggests that NF triplet polymers are most likely formed in PC12-Nex1 cells. This co-expression is critical, as neurofilaments are obligate heteropolymers *in vivo*, to increase radial growth of axons (reviewed by Lee and Cleveland 1996). Various NF knockout mice have revealed that individual NF subunits assume distinct functions in the formation of the type IV network, such as the NF-L subunit, which is absolutely required for NF-M or NFH assembly into IFs (Zhu et al. 1997). The NF-M subunit plays a significant role in NF-L assembly and translocation from the perikaryon into the axon, as well as in the optimum formation of NF structures and the radial growth of large myelinated axons during development (Jacomy et al. 1999). Finally, the NF-H subunit is a determinant of the microtubule content in axons, suggesting a potential role in modulating microtubule dynamics and functions (Jacomy et al. 1999).

In summary, given the ability of Nex1 to induce the expression of several components of the neuronal cytoskeleton and the synaptic vesicle cycle, it is likely that the ability of Nex1 to

directly up-regulate GAP-43 promoter activity (Uittenbogaard et al. 2003) and, potentially, other components controlling growth cone activity, underlies its role in promoting neurite outgrowth. Indeed, we previously showed that overexpression of the truncated Nex1-mut1 protein lacking the N-terminal transactivation domain in PC12 cells prevented NGF-induced neurite outgrowth (Uittenbogaard and Chiaramello 2002). Our findings are therefore consistent with the embryonic and post-natal Nex1 expression, which parallels overt neuronal differentiation and peaks when neurite outgrowth and synaptogenesis are highly active (Bartholoma and Nave 1994; Schwab et al. 1998).

Signaling effects on Nex1-mediated neuritogenesis and regeneration

Together, the results from the gene expression profiling elucidate part of the underlying mechanism of the PC12-Nex1 cells' phenotypic characteristics. Constitutive expression of Nex1 has endowed these cells with a distinct combination of gene products that take part in the complex regulation of neuritogenesis and regeneration. Although many of these components are also regulated by the NGF pathway, our results show that the PC12-Nex1 cells cannot carry out differentiation to form a mature network solely based on a Nex1 transcription-dependent pathway, suggesting a need for a signaling component. This is consistent with our previous findings that the PC12-Nex1 cells display a robust and accelerated differentiation upon NGF exposure, characterized by a wider cell body and thicker neurites. Similarly, dbcAMP-treated PC12-Nex1 cells display substantial increased neurite outgrowth when compared with dbcAMP-treated control PC12 cells. Several previous reports have actually shown that cAMP levels are rapidly increased in NGF-treated PC12 cells and that the cAMP immediate response involves cytoskeletal reorganization, resulting in neurite initiation (Schubert and Whitlock 1977; Schubert et al. 1978; Gunning et al. 1981). Therefore, our results collectively suggest that constitutive expression of Nex1 generates a pool of key differentiation-related proteins, and that the addition of NGF or dbcAMP brings a complex signaling cascade that acts at several levels to converge with the Nex1-mediated transcription pathway.

The same rationale extents to the early phase of regeneration of NGF-primed PC12-Nex1 cells. Our results suggest that constitutive expression of Nex1 may 'prime' the PC12-Nex1 cells to regenerate neurites by generating a pre-existing pool of regeneration-associated genes (RAGs), such as GAP-43, α-internexin, α1-tubulin and βIII-tubulin (Miller et al. 1989; Tezlaff et al. 1991; Moskowitz and Oblinger 1995; Bomze et al. 2001; McGraw et al. 2002). Furthermore, the activity of this pre-existing pool of RAGs is potentiated by dbcAMP treatment, leading to dramatic neurite regeneration of differentiated PC12-Nex1 cells. Several observations support the notion that part of the underlying mechanisms regulating regeneration recapitulates the early steps of development. First, many RAGs are developmentally-prevalent genes, such as *GAP-43*. In fact, overexpression of a combination of RAGs in transgenic mice enhances axonal regeneration (Bomze et al. 2001). Second, the endogenous levels of cAMP necessary to overcome inhibition of regeneration by myelin and myelin-associated glycoproteins (MAG) decrease with development (Cai et al. 2001) and third, specific regeneration-competent transcriptional cascades may not be sufficiently responsive or maintained in adult neurons following CNS injury.

In conclusion, our results indicate that by manipulating the endogenous transcriptional network, we have altered the intrinsic growth capacity of the PC12-Nex1 cells, which has rendered them more responsive to distinct differentiation signaling pathways, as well as competent to regenerationinducing signals. Thus, manipulation of Nex1 expression by pharmacological or gene therapy procedure may be of benefit for CNS repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by NIH grants R01-NS41391 to AC and by P30HD40677.

Abbreviations used :

bHLH, basic helix-loop-helix; cAMP, adenosine cyclic-3′:5′-monophosphate; CDK, cyclin-dependent kinase; dbcAMP, dibutyryl adenosine cyclic-3′:5′-monophosphate; MAP, microtubuleassociated protein; NF-H, neurofilament heavy subunit; NF-L, neurofilament light subunit; NF-M, neurofilament medium subunit; NGF, nerve growth factor; PBS, phosphate-buffered saline.

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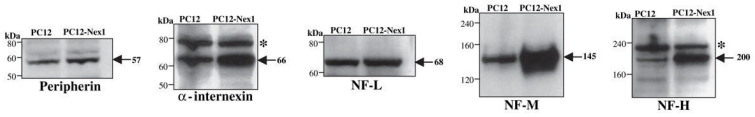

Fig. 1.

Overexpression of Nex1 results in increased expression of specific intermediate filaments in the absence of NGF. The control PC12 and PC12-Nex1 cells were grown on collagen I-coated plates, and cell extracts (40 μg) were subjected to immunoblot analysis with appropriate antibodies as indicated at the bottom of each panel. The antigen-antibody complexes were detected by chemiluminescence. Equal loading was verified by visualization of total proteins by Ponceau S staining and by blotting with the anti-peripherin antibody. The immune-specific polypeptides are indicated by an arrow with the corresponding molecular weight (in kDa) next to it, whereas the non-specific immune reactive polypeptides are indicated by an asterisk. Relevant molecular weight markers are indicated on the left side of each figure. Data shown are representative of at least three independent experiments.

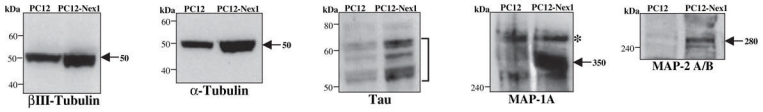

Fig. 2.

Overexpression of Nex1 induces the expression of microtubules and microtubule-associated proteins independently of NGF. The control PC12 and PC12-Nex1 cells were grown on collagen I-coated plates, and cell extracts (40 μg) were subjected to immunoblot analysis with appropriate antibodies as indicated at the bottom of each panel. The antigen-antibody complexes were detected by chemiluminescence. The immune-specific polypeptides are indicated by an arrow with the corresponding molecular weight (in kDa) next to it, or a bracket for the five bands of Tau spanning 52-60 kDa, whereas the non-specific immune reactive polypeptides are indicated by an asterisk. Relevant molecular weight markers are indicated on the left side of each figure. Data shown are representative of at least three independent experiments.

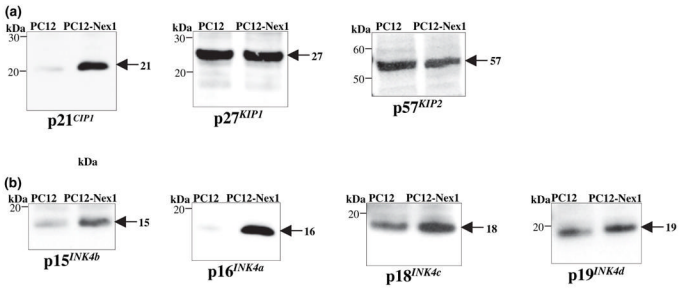

Fig. 3.

Overexpression of Nex1 induces the expression of several inhibitors of G1 cyclin-dependent kinases. (a) Nex1 only induces the expression of the member of the CIP/KIP family, p^{21CIP1}. The control PC12 and PC12-Nex1 cells were grown on collagen I-coated plates, and cell extracts (40 μg) were subjected to immunoblot analysis with appropriate antibodies as indicated at the bottom of each panel. The antigen-antibody complexes were detected by chemiluminescence. Equal loading was verified by visualization of total proteins by Ponceau S staining and by blotting with the anti-$p27^{Kip1}$ antibody. The immune-specific polypeptides are indicated by an arrow with the corresponding molecular weight (in kDa) next to it, whereas the non-specific immune reactive polypeptides are indicated by an asterisk. Relevant molecular weight markers are indicated on the left side of each figure. Data shown are representative of at least three independent experiments. (b) Nex1 stimulates the expression of the members of the INK4 family. The control PC12 and PC12-Nex1 cells were treated and analyzed as described in (a). Equal loading was verified by visualization of total proteins by Ponceau S staining and by blotting with the anti-$p27^{Kip1}$ antibody. The immune-specific polypeptides are indicated by an arrow with the corresponding molecular weight (in kDa) next to it, whereas the non-specific immune reactive polypeptides are indicated by an asterisk. Relevant molecular weight markers are indicated on the left side of each figure. Data shown are representative of at least three independent experiments.

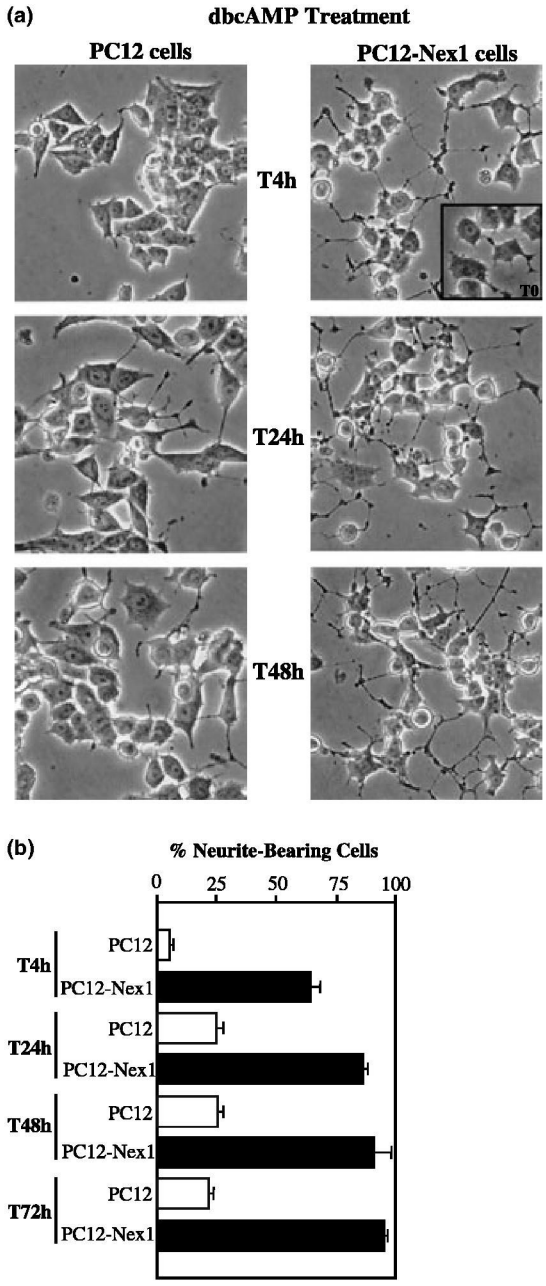

Fig. 4.

Constitutive expression of Nex1 accelerates cAMP-mediated neuritogenesis. (a) Phase-contrast micrographs of control PC12 and PC12-Nex1 cells treated with dbcAMP (1 mM). The cells were grown on collagen I-coated plates and exposed to dbcAMP for different periods of time, as indicated at the side of each panel. The phase-contrast micrograph of PC12-Nex1 cells at To is shown as an inset figure in the panel. The phase-contrast micrographs are representative of at least three independent experiments. (b) Quantification of neurite-bearing cells in the presence of dbcAMP (1 mM). The percentage of neurite-bearing cells was scored on at least 300 cells for each experiment, and three independent experiments were carried out. The open bars represent the control PC12 cells treated with dbcAMP (1 mM), whereas the hatched bars

represent the PC12-Nex1 cells treated with dbcAMP (1 mM). This is a representative graph from a triplicate experiment that was repeated three times. The error bar is the standard deviation of the mean.

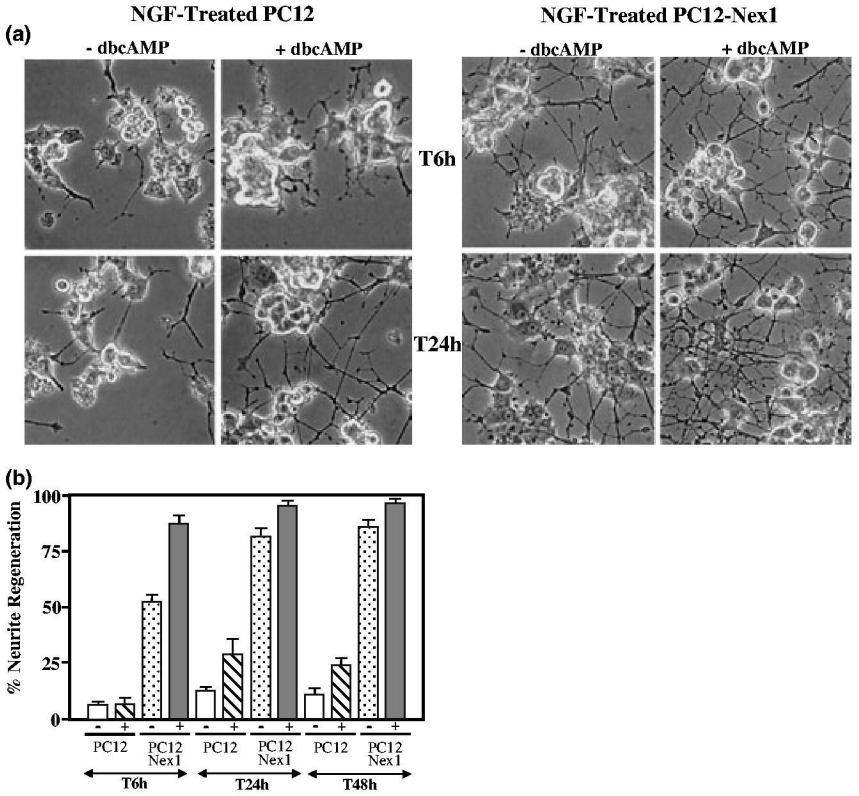

Fig. 5.

Constitutive expression of Nex1 in conjunction with dbcAMP treatment results in dramatic neurite regeneration. (a) Phase-contrast micrographs of NGF-treated control PC12 and PC12-Nex1 cells in the presence or absence of dbcAMP. Control PC12 and PC12-Nex1 cells were grown on collagen I-coated plates and treated with NGF (50 ng/mL) for 7 days, after which the cells were thoroughly washed with NGF-free medium. Neurites were then sheared as described in Materials and methods. The cells were re-plated on collagen I-coated plates in the absence or presence of dbcAMP (1 mM). The regeneration process was analyzed for 48 h. The phase-contrast micrographs are representative of at least three independent experiments. (b) Quantification of neurite regeneration in the absence or presence of dbcAMP. Cells were treated and processed as described above. The percentage of neurite-bearing cells was scored on at least 300 cells from three distinct experiments. This is a representative graph from a triplicate experiment that was repeated three times. The error bar is the standard deviation of the mean. The open bars and the hatched bars represent neurite regeneration from NGF-differentiated control PC12 cells in the absence or presence of dbcAMP (1 mM), respectively. The dotted bars and the shaded bars represent neurite regeneration from NGF-treated PC12-Nex1 cells in the absence or presence of dbcAMP (1 mM), respectively.

Table 1

Primary antibodies used in this study

Antibody	Type[a]	Source (Cat. no.)	Dilution
α-Tubulin	Mouse mAb	Santa-Cruz (sc-5286)	1 : 2000
βIII-Tubulin	Mouse mAb	Covance (mms-435P)	1 : 1500
Tau	Mouse mAb	Chemicon (MAB 3420)	1 : 1250
MAP-1A	Mouse mAb	Chemicon (MAB 362)	1 : 1000
MAP-2A/B	Mouse mAb	Chemicon (MAB364)	1 : 1000
Peripherin	Rb Ab	Chemicon (AB1530)	1 : 800
α-Internexin	Mouse mAb	Chemicon (MAB 1525)	1 : 1000
NF-L	Rb Ab	Chemicon (AB1983)	1 : 2500
NF-M	Rb Ab	Chemicon (AB1987)	1 : 3000
NF-H	Rb Ab	Chemicon (AB1989)	1 : 1000
$p21^{waf/cip}$	Mouse Ab	Santa-Cruz (sc-6246)	1 : 500
$p27^{KIP1.}$	Rb Ab	StressGen (KAP-CC02)	1 : 5000
$P57^{KIP2}$	Rb Ab	Santa-Cruz (sc-8298)	1 : 1500
$P15^{INK4}$	Rb Ab	Santa-Cruz (sc-613)	1 : 10 000
$p16^{INK4}$	Rb pAb	SantaCruz (sc-1207)	1 : 3500
$P18^{INK4c}$	Rb Ab	Santa-Cruz (sc-865)	1 : 1000
$P19^{INK4d}$	Rb Ab	Santa-Cruz (sc-1063)	1 : 1000

[a] Rb (rabbit), Ab (antibody), m (monoclonal).

Table 2

Categories of genes regulated by Nex1. Genes significantly modulated by Nex1 were grouped into functional categories based on information from GenBank. Genes shown were selected from the 129 found to be significantly regulated by Nex1 (p-values ≤ 0.05). The full list is provided as Table S1

Description	Accession no.	Fold regulation
Cytoskeletal genes		
MAP-1A	M83196	2
MAP-2	X54100	2
Tau	X79321	2
NF-M	Z12152	3.3
NF-H	M21964	2
Kinesin-related prorein (KIF-1D)	AJ000696	2
Cytoplasmic dynein intermediate chain 2C	U39046	2.2
Cell adhesion encoding genes		
Brevican	X79881	4.8
Ryudocan	M81786	2
S-laminin	X16563	2
Laminin receptor	M27798	2.1
Thy-1	X03150	- 4.9
TAG-1	M31725	- 6.3
Neuroligin-1	U22952	- 4
Neural cell adhesion molecule L1	X59149	- 3
Vesicular trafficking/synapse-related genes		
SynGAP-b1	AF058789	2
Synaptotagmin VII	U20106	2.1
Synaptogyrin 1	U39549	2
Complexin I	U35098	2
Sortilin	AF019109	2.5
SAPAP2	U67138	18
Synaptogyrin 2	AF039085	- 2
Rabphilin 3	AU12571	- 10
Synaptotagmin VI	U20105	- 3
Complexin II	U35099	- 2
Metabolic-related genes		
Beta-glucoronidase	M13962	3.5
Fumerase	J04473	3.3
Creatine kinase	M14864	2
GM3 synthase	AB018049	2.5
Carnitine octanoyltransferase	U26033	2
Arginosuccinate synthase	M36708	7
Ornithine transcarbamylase	M11266	2.4
Ornithine aminotransferase	M11842	5.2
Ornithine decarboxylase	X07944	2
Guanyl cyclase	M57507	2.3
Glutamate dehydrogenase	X14223	2.2
Acyl-CoA synthase 5	AB012933	- 2
Cytochrome c oxidase subunit VIa	X12553	- 2
Apolipoprotein B	M21842	- 5
Stress response-related genes		
Stress-inducible chaperone Grpe	U62940	2
Mitochondrial genes		
Delta subunit of F1F0 ATPase	U00926	2.2
Mitochondrial processing protease p52	D13907	2.0
Transcription factors and chromatin-related genes		
Mash-1	X53527	2.3
HNF-3gamma	L09648	3.3
Arix	U25967	2
Histone 1T	M28409	- 6