HHS Public Access

Author manuscript

J Neurochem. Author manuscript; available in PMC 2016 June 07.

Published in final edited form as:

J Neurochem. 2009 December ; 111(5): 1202–1212. doi:10.1111/j.1471-4159.2009.06404.x.

Vesicular monoamine transporter 2 and dopamine transporter are molecular targets of Pitx3 in the ventral midbrain dopamine neurons

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Abstract

Midbrain dopamine (mDA) neurons play critical roles in the regulation of voluntary movement and their dysfunction is associated with Parkinson's disease. Pitx3 has been implicated in the proper development of mDA neurons in the substantia nigra pars compacta, which are selectively lost in Parkinson's disease. However, the basic mechanisms underlying its role in mDA neuron development and/or survival are poorly understood. Toward this goal, we sought to identify downstream target genes of Pitx3 by comparing gene expression profiles in mDA neurons of wild-type and Pitx3-deficient aphakia mice. This global gene expression analysis revealed many potential target genes of Pitx3; in particular, the expression of vesicular monoamine transporter 2 and dopamine transporter, responsible for dopamine storage and reuptake, respectively, is greatly reduced in mDA neurons by Pitx3 ablation. In addition, gain-of-function analyses and chromatin immunoprecipitation strongly indicate that Pitx3 may directly activate transcription of vesicular monoamine transporter 2 and dopamine transporter genes, critically contributing to neurotransmission and/or survival of mDA neurons. As the two genes have been known to be regulated by Nurr1, another key dopaminergic transcription factor, we propose that Pitx3 and Nurr1 may coordinately regulate mDA specification and survival, at least in part, through a merging and overlapping downstream pathway.

Keywords

aphakia mice; dopamine neuron; Parkinson's disease; Pitx3; substantia nigra pars compacta

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The authors declare no conflicts of interest.

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Among potential transcription factors involved in development and physiological function of midbrain dopamine (mDA) neurons, Nurr1 and Pitx3 may play critical roles in the development of mDA neurons when DA neuronal precursors exit mitotic stage (Riddle and Pollock 2003; Smits and Smidt 2006). Studies with Nurr1 knockout mouse have shown that while it is not essential for the initial formation of DA neuronal precursors, Nurr1 is important for determining the DA neurotransmitter identity and neurotransmission, as well as for the survival and maintenance of DA neurons later on. This conclusion has been derived from, and further supported, by the identification of target genes of Nurr1. So far, the list of known Nurr1 downstream target genes includes tyrosine hydroxylase (TH) (Castillo *et al.* 1998; Saucedo-Cardenas *et al.* 1998; Sakurada *et al.* 1999; Iwawaki *et al.* 2000; Kim *et al.* 2003), aromatic amino acid decarboxylase (Hermanson *et al.* 2003), vesicular monoamine transporter 2 (VMAT2) (Hermanson *et al.* 2003; Smits *et al.* 2003), DA transporter (DAT) (Smits *et al.* 2003), c-Ret (Wallen *et al.* 2001), p57Kip2 (Joseph *et al.* 2003), neuropilin (Hermanson *et al.* 2006) brain-derived neurotrophic factor (Volpicelli *et al.* 2007) and vasoactive intestinal peptide (Luo *et al.* 2007).

Pitx3 is another crucial transcription factor involved in the early development of mDA neurons, especially the substantia nigra pars compacta (SNpc) DA neurons (Hwang *et al.* 2003; van den Munckhof *et al.* 2003; Nunes *et al.* 2003; Smidt *et al.* 2004). Notably, both Nurr1 and Pitx3 are expressed in mDA neurons throughout adulthood, suggesting the interesting possibility that Pitx3 and Nurr1 are important for the maintenance and normal physiology of mature mDA neurons. In line with this, several reports recently showed that mutations of these genes are closely associated with Parkinson's disease (PD) (Le *et al.* 2003, 2008; Grimes *et al.* 2006; Fuchs *et al.* 2009; Bergman *et al.* 2008). Therefore, unveiling regulatory cascade of Pitx3 will help us to understand not only basic mechanisms of early development and physiology of mDA neurons but also pathophysiology of PD.

Despite the functional importance of Pitx3, molecular pathways controlled by Pitx3 are only partially understood (Jacobs *et al.* 2007; Peng *et al.* 2007). In this report, we attempted to identify downstream target genes of Pitx3 on a genomic scale by comparing gene expression profiles of laser-captured mDA neurons from E12.5 wild-type (*wt*) and aphakia (*ak*) mice. Intriguingly, among the list of mostly affected genes by Pitx3 ablation, we identified VMAT2 and DAT genes and found that their *in vivo* expression is significantly compromised during both early embryonic and adult stages of mDA neurons in *ak* mice. In addition, our gain-of-function studies using *in vitro* differentiation of Pitx3-over-expressing mouse embryonic stem cells (mESCs) and chromatin immunoprecipitation (ChIP) analysis further suggested that VMAT2 and DAT are direct target genes of Pitx3. Taken together, we propose that Pitx3, in concert with Nurr1, controls neurotransmission and homeostasis of DA neurons by regulating these two target genes.

Experimental procedures

Animal care

Pitx3-deficient *ak* mice were maintained as previously described (Hwang *et al.* 2005). Animal use was in accordance with Institutional Animal Care and Use Committee of McLean Hospital and followed National Institutes of Health guidelines.

In situ hybridization

For *in situ* hybridization, both *wt* and *ak* mouse embryos of the age of E12.5 and E14.5 were fixed in 4% *p*-formaldehyde/phosphate-buffered saline (PBS) overnight, cryoprotected in 30% sucrose/PBS at 4°C and then frozen in optimal cutting temperature (OCT) compound. Adult brains from 9-week-old mice were snap-frozen on dry ice without OCT. Serial coronal and sagital sections were cut at 14 μm on a cryostat and went through *in situ* hybridization process as described previously (Hwang *et al.* 2003). In this study, probes for *in situ* hybridization were labeled with digoxigenin (DIG) and visualized with an alkaline phosphatase-conjugated anti-DIG antibody using Nitro-Blue Tetrazolium Chloride (NBT)/5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt (BCIP) as a substrate. The DNA fragments used for the riboprobe generation were amplified by PCR using the following primer pairs: TH, forward 5′-GTATACGCCACGCTGAGGG-3′, reverse 5′-ATCCTGGACCCCCTCTAAGG-3′; mouse VMAT2, forward 5′-GCAACTTTTCTAGGGGTTTG-3′, reverse 5′-GTTCCAGAACATGAACTGG-3′; mouse DAT, forward 5′-GGCAGATCTTCCAGACACC-3′, reverse 5′-CAGAGAGGTGGAGCTCATC-3′.

Laser capture microdissection

Laser capture microdissection (LCM) of TH-positive neurons was performed using a PixCell II Laser-capture Microscope (Arcturus, Mountain View, CA, USA) and macro LCM caps (CapSure LCM Caps, Arcturus). Briefly, 10-μm cryosections of the brain were fixed in ice-cold acetone for 5 min, followed by air-drying. After rehydration in PBS for 5 s, the sections were stained for 5 min in PBS containing an anti-TH antibody (diluted 1 : 100; Pel-freez, Rogers, AR, USA) and then were rinsed in PBS for 5 s. Subsequently, the slides were stained with an Alexa Fluor 594 anti-rabbit IgG (1 : 100) (Molecular Probes, Eugene, OR, USA) for 5 min, rinsed with PBS for 5 s, dehydrated by sequential exposure to increasing concentrations of ethanol (30 s in 75%, 30 s in 95% and 1 min in 100%) and then treated with xylene for 2 min. Finally, the slides were air-dried for 5 min before LCM. For our experiments, about 1500 TH-positive neurons were isolated from the adult ventral tegmental area (VTA) and the ventral mesencephalon of E12.5 embryos.

RNA preparation and RT-PCR analyses

Total RNA from the laser-captured neurons was isolated using the PicoPure kit (Arcturus) according to the manufacturer's protocol. Then we used the RiboAmp kit (Arcturus) to perform T7-based linear amplification. The total RNA from the ventral mesencephalic tissue of embryos was prepared using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) followed by treatment with DNase I (Qiagen) and then used for either DNA microarray or RT-PCR

experiment. For RT-PCR analysis, 5 μg of RNA was transcribed into cDNA with the SuperScriptTH First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Samples were amplified in Continuous Fluorescence Detector (MJ RESEARCH, Waltham, MA, USA) using DNA Engine Opticon software under the following conditions: denaturing step at 94°C, 30 s; annealing step at 55°C, 30 s; extension step at 72°C, 30 s for 50 cycles and a final extension step at 72°C, 10 min. The cDNA was analyzed in a PCR assay using the following primers; En1, forward 5′-GTGAGTGTGAGTGTGTTTCCTTGTG-3′, reverse 5′-CTCGTAGTCTGTGGGGTTGTATTTC-3′; guanosine triphosphate cyclohydrolase (GTP) cyclohydrolase I (GTPCH), forward 5′-ATCCCTTCTGAACGACCCTG-3′, reverse 5′-AATTCCCCAAATGTGCTCGG-3′; DAT, forward 5′-CATTGCCACATCCTCCATGG-3′, reverse 5′-TAGGCCAGTTTCTCTCGGAA-3′; VMAT2, forward 5′-GTCCACCTGCTAAGGAAGAA-3′, reverse 5′-CAGGAGACACATGTACACAG-3′; TH, forward 5′-TCCAACCTTTCCTGGCCCAG-3′, reverse 5′-GCATGAAGGGCAGGAGGAAT-3′; glyceraldehyde-3-phosphate dehydrogenase, forward 5′-AGGGCATCTTGGGCTACACTG-3′, reverse 5′-TGGGTGGTCCAGGGTTTCTTAC-3′; cRet, forward 5′-TCCCAGAGTGAGTTACGAGACCTG-3′, reverse 5′-GACAGCCCAAAGTCGGAAAT-3′.

DNA microarray analyses

Two micrograms of each linearly amplified aRNA sample derived from LCM-captured TH-positive neurons was subjected to DNA microarray analyses using the Affymetrix GeneChip Mouse Expression Set 430 2.0 (Affymetrix, Santa Clara, CA, USA). These DNA microarray analyses were performed three times per sample ($n = 3$) in Transcription Analysis Laboratory at California Institute of Technology.

Cultivation and dopaminergic differentiation of mouse embryonic stem cells

The mESC line D3 was obtained from ATCC (Rockland, MD, USA) and cultured on gelatin-coated dishes in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1× non-essential amino acids (Invitogen), 2 mM glutamine (Invitrogen), 0.001% b-mercaptoethanol (Invitrogen), 10% donor horse serum (Sigma, St Louis, MO) and 2000 U/mL human recombinant leukemia inhibitory factor (R&D, Minneapolis, MN, USA). Dopaminergic differentiation of mESCs was carried out using the 5-stage protocol described previously (Lee *et al.* 2000).

Retroviral vector, viral production and titration

The Pitx3-expressing recombinant retroviral vector used in this study was generated by inserting Pitx3 cDNA into the pMS retroviral plasmid. As a control, enhanced green fluorescence protein (EGFP)-expressing retroviral vector was also constructed by inserting EGFP into the same retroviral plasmid. To generate retroviral particles, the recombinant retroviral plasmids were transfected into retroviral packaging cells, 293GPG cells, using Lipofectamine 2000 (Invitrogen). Medium containing secreted retroviruses was collected everyday from day 2 to 5, followed by concentration of viral particles by ultracentrifugation (SW28, 50,000 × g, 90 min). Retroviral titration was carried by Retro-X qRT-PCR Titration Kit (Clontech, Mountain View, CA, USA) following manufacturer's instructions.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed according to manufacturer's protocol (Upstate, Lake Placid, NY, USA). Briefly, 1×10^6 cells of neural precursor cells derived from mESCs were plated in 60 mm plates and cultured for 2 days and then transduced by hemagglutinin (HA)-tagged Pitx3 in retroviral vector. Cells were cultured 2 days more, differentiated into DA neurons for 7 or 14 days, cross-linked with 1% formaldehyde for 10 min and then harvested in the presence of protease inhibitor (EDTA-free complete; Roche). These cells were then lysed and sonicated to generate 200–500 bp DNA fragments. One-tenth of the lysates was used for input control. The remaining lysates were divided by half and treated with 1 μg of polyclonal anti-HA antibody (Upstate) or normal rabbit IgG as a negative control overnight at 4°C. After treating Salmon sperm DNA/Protein A agarose slurry to immunoprecipitated complexes, the precipitates were extensively washed and then eluted in elution buffer (1% sodium dodecyl sulfate, 0.1 M $NaHCO_3$). The cross-linked protein–DNA complexes were reversed by NaCl treatment. The DNA was recovered by phenol extraction and resuspended in 50 μL of H_2O. PCR was performed to detect specifically bound DNA using MasterAmp 2× PCR Premix IN buffer (Epicentre, Madison, WI, USA) using 1 μL of the resuspended sample as a template at 94°C 30 s, 55°C 30 s, 72°C 30 s for 30 cycles with primer sets in 25 μL reaction volume. Primers for VMAT2 are 5′-CATCACGCAGACTTGAAAGAC-3′ and 5′-CGCCTCGCCTTGCTTATCC-3′. Primers for DAT are 5′-GTTAACATTTACCCAACTGG-3′ and 5′-GGACCTCTAAATCAACATG-3′.

Results

Identification of potential downstream target genes of Pitx3 on a genomic scale

In an effort to unveil potential role of Pitx3 in the development and function of mDA neurons, we attempted to identify its downstream target genes on a genomic scale. Toward this goal, we compared gene expression profile of mDA neurons between *ak* (Pitx3-deficient) and *wt* (Pitx3-present) mice. As the outcome could be easily biased by the secondary effects resulting from the absence or death of a specific population of mDA neurons, we performed the laser capture experiments at the development stage soon after Pitx3 started to be expressed (E11.5) and before no obvious differences in the mDA neuronal pattern were detected between *ak* and *wt* mice. In regards to this issue, no apparent differences in the number and distribution of mDA neurons have been detected at E12.5 between *wt* and *ak* mice (Fig. S1), which is in consistent with the previous report from other group (16). On the contrary, a clear defect became evident in SNpc DA neurons from E14.5 (Fig. S2).

About 1500 TH-positive neurons were captured throughout the whole ventral midbrain of both *wt* and *ak* E12.5 embryos by LCM. Total RNAs were isolated, linearly amplified and subjected to DNA microarray analyses using the Affymetrix GeneChip Mouse Expression Set 430 2.0 which includes 45 000 probe sets to analyze the expression level of over 39 000 transcripts and variants from well characterized mouse genes. Our microarray analysis revealed that 243 independent genes were down-regulated twofold or more in *ak* mDA neurons compared with *wt* mice (Table S1). This gene list included many genes that are known to regulate cell cycle/growth, cell migration and transcriptional regulation. Thus, it is

possible that Pitx3 may regulate neurogenesis (DA neuron/precursor generation), neuronal migration and other important functions of DA neurons. Interestingly, among highly affected genes (Fig. 1a, left), included were the DAT and the VMAT2 genes which are primarily responsible for DA reuptake into the nerve terminals and DA storage into synaptic vesicles, respectively. When RT-PCR analyses were performed using total RNAs isolated from independently laser captured tissues, we confirmed that mRNA expression levels of DAT, VMAT2, as well as five randomly chosen additional genes were dramatically reduced by the absence of Pitx3 (Fig. 1a, right).

As both DAT and VMAT genes are known to play critical roles in DA neuron physiology, function and related behavioral effects (Fon *et al.* 1997; Rocha *et al.* 1998; Jones *et al.* 1999), we decided to further investigate the regulation of DAT and VMAT2 gene expression in the absence of Pitx3. First, we performed additional RT-PCR analysis for several mDA neuronal genes using total RNAs independently isolated from captured mDA neurons of E12.5 embryos. This analysis confirmed that the levels of VMAT2 and DAT mRNAs were dramatically reduced in mDA neurons of *ak* embryos compared with those of *wt* embryos (Fig. 1b). On the contrary, there was no significant difference in the level of TH, GTPCH and *Engrailed* 1 (*En*1) mRNAs between *wt* and *ak* embryos at E12.5 (Fig. 1b). Thus, the down-regulation of VMAT2 and DAT gene expressions in the absence of Pitx3 is a specific phenomenon. To address the possibility that these gene expression changes are biased during the LCM procedure, we next dissected the whole ventral midbrain area from *wt* and *ak* E12.5 embryos and prepared total RNAs from these tissues. Quantitative RT-PCR experiments on these RNAs resulted in a very similar result to that of laser captured tissues, confirming that VMAT2 and DAT genes are down-regulated in the absence of Pitx3 (Fig. 1c).

VMAT2 and DAT gene expression, but not TH gene expression, is greatly diminished in mDA neurons of *ak* mice during early embryonic development

To address whether VMAT2 and DAT gene expression is affected *in vivo* at the cellular level, *in situ* hybridization experiment was performed. The level of VMAT2 mRNA was mostly diminished in the ventral mDA neurons of *ak* mice at E12.5, compared with that of the age-matched *wt* mice (Fig. 2a). Interestingly, absence of Pitx3 in *ak* mice did not affect VMAT2 expression in serotonergic neurons of the dorsal raphe nuclei (Fig. 2a). This observation is congruent with the fact that Pitx3 is not expressed in the raphe nuclei. Thus, our results strongly suggest that the mechanism(s) responsible for VMAT2 gene expression is different between mDA and serotonergic neurons and that its expression in mDA neurons is specifically affected by Pitx3. In contrast, there was no noticeable difference in TH gene expression in the ventral midbrain area between *wt* and *ak* E12.5 embryos (Fig. 2a, panels iii and vi; also see Fig. S1). We found the same pattern of differential VMAT2 gene expression in E14.5 embryos of *ak* mice; its expression was largely reduced in ventral mDA neurons, but not in the serotonergic neurons (Fig. 2b).

Dopamine transporter is considered to be a late marker of developing mDA neurons (Perrone-Capano *et al.* 1994). Although DAT mRNA was detected at E12.5 by RT-PCR (Fig. 1), it was not readily detected by the conventional *in situ* hybridization method (data not

shown). As such, we used E14.5 embryos to examine the expression level of DAT gene by *in situ* hybridization. The mRNA levels of DAT as examined by both coronal and sagittal sections were largely diminished in mDA neurons (Fig. 2c). However, it is noteworthy that some noticeable level of DAT mRNA was still remaining in the ventral midbrain of *ak* mice (Fig. 2c, d and h), suggesting that other factor(s) might be also involved in the basal expression of DAT gene. In summary, our *in vivo* gene expression analysis demonstrates that VMAT2 and DAT gene expression is impaired during early development of mDA neurons of *ak* embryos.

Down-regulation of VMAT2 and DAT gene expression is sustained in mDA neurons of adult *ak* mice

We next sought to determine whether expression of VMAT2 and DAT genes is later restored or continues to be down-regulated in mDA neurons of adult *ak* mice. Toward this end, we performed *in situ* hybridization of brain sections of 9-week-old mice. At this stage, we could observe comparable signals for TH mRNA in both *wt* and *ak* mice (Fig. 3a). Given that DA neurons in the SNpc (A9 neurons) are largely diminished in *ak* mice, these TH positive neurons are most likely VTA DA neurons (A10 neurons). Interestingly, similar to embryonic stages, VMAT2 and DAT gene expressions were greatly reduced in *ak* mice as compared with age-matched *wt* mice (Fig. 3a). On the other hand, the level of VMAT2 mRNA was not affected by Pitx3 ablation in noradrenergic neurons of the locus coeruleus (Fig. 3b-ii and iv). This *in situ* hybridization result was further corroborated by RT-PCR experiments using total RNAs isolated from laser captured adult (9-week-old) VTA neurons. As shown in Fig. 3c, VMAT2 and DAT mRNA levels were greatly diminished in the adult *ak* VTA neurons, compared with their *wt* counterpart. In contrast, the expression levels of TH, GTPCH, *En*1 and c-ret genes were shown to be comparable in the *ak* VTA neurons to those of *wt* mice, demonstrating that VMAT2 and DAT gene expression is specifically reduced in the adult VTA neurons of *ak* mouse. Taken together, our results suggest that the effects of Pitx3 on the expression of VMAT2 and DAT genes are not transient, but are still evident in 9-week-old adult mice.

Forced expression of Pitx3 up-regulates VMAT2 and DAT gene transcription in mouse embryonic stem cells

To further substantiate the idea that Pitx3 regulates VMAT2 and DAT gene expression, we next attempted a gain-of-function approach. Toward this goal, we speculated that mESCs are a useful tool because of their pluripotent differentiation capability. DA neuronal derivation *in vitro* from the mESCs were performed using the well-established 5-stage differentiation procedure (Lee *et al.* 2000; Chung *et al.* 2005) (Fig. 4a and b). To deliver an exogenous gene into mESC-derived neural precursors, we used retroviral vectors which normally transduce the majority of the cells (> 90%; Fig. 4c). Using the retroviral system, we delivered Pitx3 gene into cells of neural precursor stage which corresponds to the 16th day of the whole differentiation procedure (Fig. 4a). Forty-eight hours post-transduction, the cells continued to be differentiated for seven more days and were examined for the change of VMAT2 and DAT mRNA levels by both quantitative (Fig. 4d) and real time PCR analyses (Fig. 4e and f). This experiment showed that Pitx3 overexpression robustly increased mRNA levels of both VMAT2 and DAT genes, compared with GFP-retroviral transduction.

Pitx3 directly binds to putative binding motifs in the 5′ upstream regions of the VMAT2 and DAT gene promoters

Our gain-of-function results prompted us to hypothesize that Pitx3 directly activates transcription of VMAT2 and DAT genes. To determine if Pitx3 directly binds to the promoter region(s) of these target genes and activate their transcription, we first determined the consensus Pitx3 binding motif by the random oligonucleotide selection procedure *in vitro* (Blackwell *et al.* 1990). Although the Pitx3-related Drosophila *bicoid* protein is known to bind to the '5-TAATCC-3' motif (Hanes *et al.* 1994), the consensus binding site for Pitx3 has yet to be determined. For the random oligonucleotide selection procedure, we performed gel mobility shift experiments using a mixture of *in vitro* translated Pitx3 protein and the 63bp-long oligonucleotides with 14 degenerative nucleotides inside (5′-GACTACGTCGACAGCGAATTCAGA$(N)_{14}$ CTCGGGATCCATGCTCAGTAGACAG-3′). The Pitx3-oligonucleotide complexes on the gel were isolated and oligonucleotides within the complexes were amplified by PCR with primers flanking the internal degenerative nucleotide sequences. The PCR-amplified oligonucleotides obtained were used for another round of the selection procedure and this procedure was repeated four times. The sequencing analyses of the oligonucleotides obtained after five rounds of the selection procedure showed that Pitx3 recognizes 5′-(C/G)NTAATCC(A/C)-3′ as the consensus binding motif (Fig. S3). Using this sequence motif, we next performed *in silico* analyses to identify potential Pitx3 binding motifs in the VMAT2 gene regulatory regions using the available genomic data base ECR Browser and rVista (http://www.dcode.org). Analysis of the 26 kb upstream promoter region as well as the first exon and intron areas of the VMAT2 gene, revealed seven potential binding sites with less than one nucleotide deviation from the consensus motif (Fig. 5a and b). Among these sites, we analyzed three potential sites (sites 2, 3 and 5) by ChIP assay. We designed oligonucleotide primers to amplify each putative Pitx3 binding site. mESCs were transduced with retroviral vectors expressing HA-tagged Pitx3, subsequently differentiated for 1 week, and Chip assay was carried out with anti-HA antibody or rabbit IgG (control). This ChIP analysis revealed that site 2 is bound *in vivo* by Pitx3 (Fig. 5c). Notably, this site 2 is highly conserved among different species, in particular between mouse and rat (Fig. 5d). Similarly, our *in silico* analysis identified five potential Pitx3-binding sites in the 5′ upstream sequence of DAT gene (Fig. 5e and f), although these sequences did not showed significant sequence homologies among species. When we tested the two most proximal sequences (sites 1 and 2) using the ChIP analysis, site 2 was found to interact with Pitx3 *in vivo* (Fig. 5g).

Discussion

Since its discovery in 1997 (Semina *et al.* 1997; Smidt *et al.* 1997), Pitx3 has been suspected as a transcription factor implicated in the development and/or survival of mDA neurons. It was not until 2003 that the clear involvement of Pitx3 in mDA neuronal development has been demonstrated *in vivo* by several groups including ours (Hwang *et al.* 2003; van den Munckhof *et al.* 2003; Nunes *et al.* 2003; Smidt *et al.* 2004). Our previous report has also shown that heterologous expresion of Pitx3 in mESCs increases A9-like DA neuronal population (Chung *et al.* 2005), further supporting its role in early development of mDA neurons. In addition to its role during early development, Pitx3 may also control important

characteristics of mature mDA neurons as is supported by its continued expression throughout adulthood. However, at this moment, we do not know much about the functional role of Pitx3 and its underlying molecular mechanisms. This poor understanding is, at least in part, because of the limited number of known downstream target genes of Pitx3.

In this study, we sought to identify genes whose expression is down-regulated by Pitx3 ablation by comparing gene expression profile of mDA neurons from *ak* mice to that from *wt* mice. We report the identification of more than 240 genes that are down-regulated at least twofold in the mDA neurons of *ak* mice, many of which are implicated in diverse functions such as neurogenesis, transcriptional regulation and neuronal migration. In particular, we found that the VMAT2 and DAT genes, which critically regulate DA neurotransmission, are in the list of highly regulated genes. Furthermore, we present *in vivo* evidence that the expression levels of both VMAT2 and DAT genes are also diminished not only in developing but also in mature (adult) mDA neurons when Pitx3 is deficient. This result clearly showed that VMAT2 and DAT genes are the downstream genes of Pitx3 *in vivo*.

The reduction of VMAT2 and DAT gene expression in Pitx3-deficient *ak* mice is evident from E12.5 and lasts at least 9 weeks after birth. This reduction is a specific and regulated process, but not because of non-specific down-regulation of genes occurring in degenerating cells. Our conclusion is supported by the following observations: (i) several mDA neuronal markers tested are not affected by Pitx3 ablation (Figs 1 and 2); (ii) although only a portion of mDA neurons are degenerating during the early development of *ak* mice, the reduction of VMAT2 and DAT gene expression occurs all the mDA neurons in the ventral midbrain area; (iii) this reduction is also detected in the surviving mDA neurons in adult *ak* mice (Fig. 3).

In line with the involvement of Pitx3 in the regulation of VMAT2 gene expression, VMAT2 gene expression is detectable from E12 (Schutz *et al.* 1998) in the rat brain, the time point when Pitx3 is also expressed. At this time point, Nurr1, another factor implicated in the regulation of VMAT2 gene expression, is also present in the mDA neurons. Nurr1 was shown to be absolutely required for the induction of both VMAT2 and DAT genes, because Nurr1−/−) mice show no expression of the two genes (Hermanson *et al.* 2003; Smits *et al.* 2003). Our data suggest that Nurr1 by itself is not sufficient for the full expression of both VMAT2 and DAT genes in the ventral midbrain, because *ak* mice display significant reduction of VMAT2 and DAT gene expression even in the presence of normal levels of Nurr1. This result indicates that robust expression of VMAT2 and DAT genes can be detected when both Nurr1 and Pitx3 coexist and lack of either component diminishes the induction of VMAT2 and DAT gene expression. This observation can be explained by the synergistic interaction between Nurr1 and Pitx3, as suggested in a recent study of over-expression of Pitx3 and Nurr1 in embryonic stem cells (Martinat *et al.* 2006). In contrast to Nurr1, a recognizable minimal expression can be detected in the absence of Pitx3 (Figs 1–3), suggesting that, Nurr1 and/or other factor(s) could be responsible for this residual expression.

It is noted that DAT and VMAT2 genes are expressed in other brain regions beyond mDA neurons where Pitx3 is not normally expressed. For example, DA neurons in hypothalamus and frontal cortex that normally do not express Pitx3 exhibit prominent DAT gene

expression (Shimada *et al.* 1992; Roth and Elsworth 1995). This suggests that different activating mechanisms of the DAT gene exist in these neurons. We also detected robust expression of VMAT2 gene in both serotonergic (Fig. 2) and noradrenergic (Fig. 3) neurons and, as expected, the expression levels in these neuronal types were not affected by Pitx3 ablation, further suggesting that independent mechanisms of gene activation exist for the VMAT2 and DAT genes in different subtypes of neurons.

In addition to the critical role in DA neurotransmission, VMAT2 and DAT also play an important role in the cell survival and protection of DA neurons against neurotoxins. In line with this notion, it has been suggested that the expression level of DAT and VMAT2 genes may determine the susceptibility of individual DA neurons to a variety of toxic substances (Miller *et al.* 1999). Furthermore, expression levels of both VMAT2 and DAT genes are significantly reduced in post-mortem SNpc of PD patients compared with controls (Uhl *et al.* 1994; Harrington *et al.* 1996). These observations also corroborate well with recent findings that mutations or altered expression of Nurr1 or Pitx3 are associated with PD (Le *et al.* 2003, 2008; Grimes *et al.* 2006; Fuchs *et al.* 2009).

It is highly probable that both Nurr1 and Pitx3 are critically involved in the development, physiological function and/or survival of mDA neurons (Fig. 6). Recently, many downstream target genes of Nurr1 have been identified and shown to be involved in many important aspects of DA neuronal development and function, such as determination of neurotransmitter identity, DA neurotransmission, cell survival and maintenance, as well as DA neuronal differentiation (Saucedo-Cardenas *et al.* 1998; Sakurada *et al.* 1999; Iwawaki *et al.* 2000; Wallen *et al.* 2001; Hermanson *et al.* 2003, 2006; Joseph *et al.* 2003; Kim *et al.* 2003; Smits *et al.* 2003; Luo *et al.* 2007; Volpicelli *et al.* 2007). In contrast, only a few downstream targets of Pitx3 have been reported yet. TH has been suggested as a gene regulated by Pitx3, but further clarification is needed because of inconsistent reports among various research groups (Cazorla *et al.* 2000; Smidt *et al.* 2000; Maxwell *et al.* 2005; Martinat *et al.* 2006; Messmer *et al.* 2007). Recently, Smidt and his colleagues elegantly showed that aldehyde dehydrogenase 2 (Ahd2 or Aldh1a1) involved in the synthesis of retinoic acid is directly regulated by Pitx3 (Jacobs *et al.* 2007). The present study will further contribute to our understanding about the potential role of Pitx3 in the regulatory cascade of mDA neuronal function, physiology and perhaps survival/maintenance. Notably, our results indicate that Pitx3 and Nurr1 may affect DA neuronal function and survival through the common downstream pathway by merging with the same target genes, including VMAT2 and DAT (Fig. 6). On the other hand, we could not detect any significant difference in the expression of TH and c-Ret genes, the genes known to be regulated by Nurr1, by the ablation of Pitx3 (Figs 1 and 3). Taken together, it appears that Pitx3 and Nurr1 play their own roles in the normal function, physiology and, maintenance of mDA neurons through common as well as distinct target genes. In support of our conclusions, a recent report demonstrated that both Pitx3 and Nurr1 recognize the same areas of many promoters in the genome (Jacobs *et al.* 2009). Further unveiling of the downstream target genes of Pitx3 and Nurr1 will provide us with a more comprehensive and detailed mechanistic understanding about the regulatory cascade of these critical transcription factors and their cross-talks for their control of the development and physiological function of mDA neurons and may lead to novel therapeutic approaches of associated brain disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants MH48866, DC006501, Michael J Fox Foundation for Parkinson's Research, and International Grants from Brain and Stem Cell Research Centers (SC5130 & SC5170) funded by the Ministry of Education, Science and Technology, the Republic of Korea.

Abbreviations used

ak	aphakia
ChIP	chromatin immunoprecipitation
DA	dopamine
DAT	dopamine transporter
GTPCH	GTP cyclohydrolase I
LCM	laser capture microdissection
mDA	midbrain DA
mESCs	mouse embryonic stem cells
PBS	phosphate-buffered saline
PD	Parkinson's disease
SNpc	substantia nigra pars compacta
TH	tyrosine hydroxylase
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area
wt	wild-type

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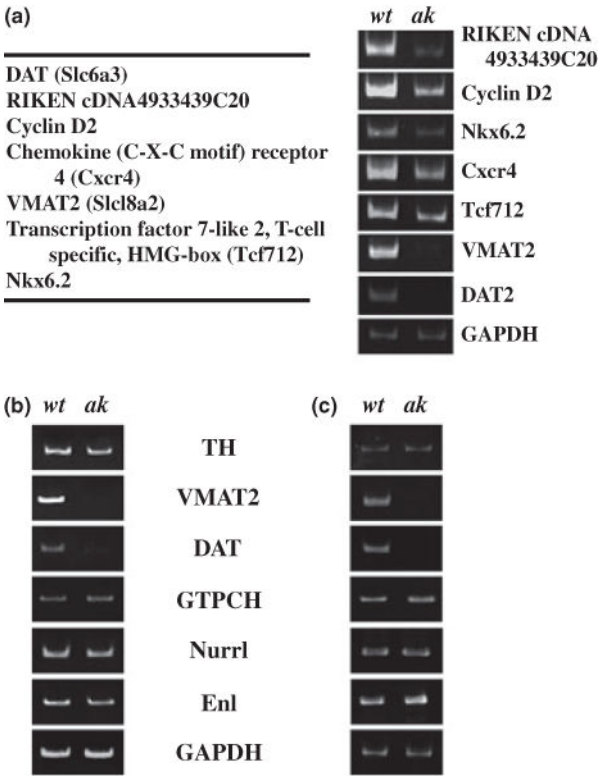

Fig. 1.

Examination of the expression change of dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2) and several other genes by the absence of Pitx3. (a, left) Among genes significantly down-regulated in laser capture microdissection-captured midbrain dopamine (mDA) neurons in aphakia (*ak*) mice as examined by DNA microarray experiment, five genes were randomly chosen in addition to DAT and VMAT2 for RT-PCR analysis. (a, right) RT-PCR analysis confirms that all these seven genes are affected in mDA neurons of *ak* mice compared with those of wild-type (*wt*) mice and thus validates our microarray experiment. In particular, expression of VMAT2 and DAT was most strikingly affected in *ak* mice. (b) The level of several mDA neuronal markers was examined in total RNAs from laser-captured mDA neurons of *wt* and *ak* embryos at E12.5. (c) The level of several mDA neuronal markers was examined in total RNAs from the isolated ventral midbrain tissue of *wt* and *ak* embryos at E12.5.

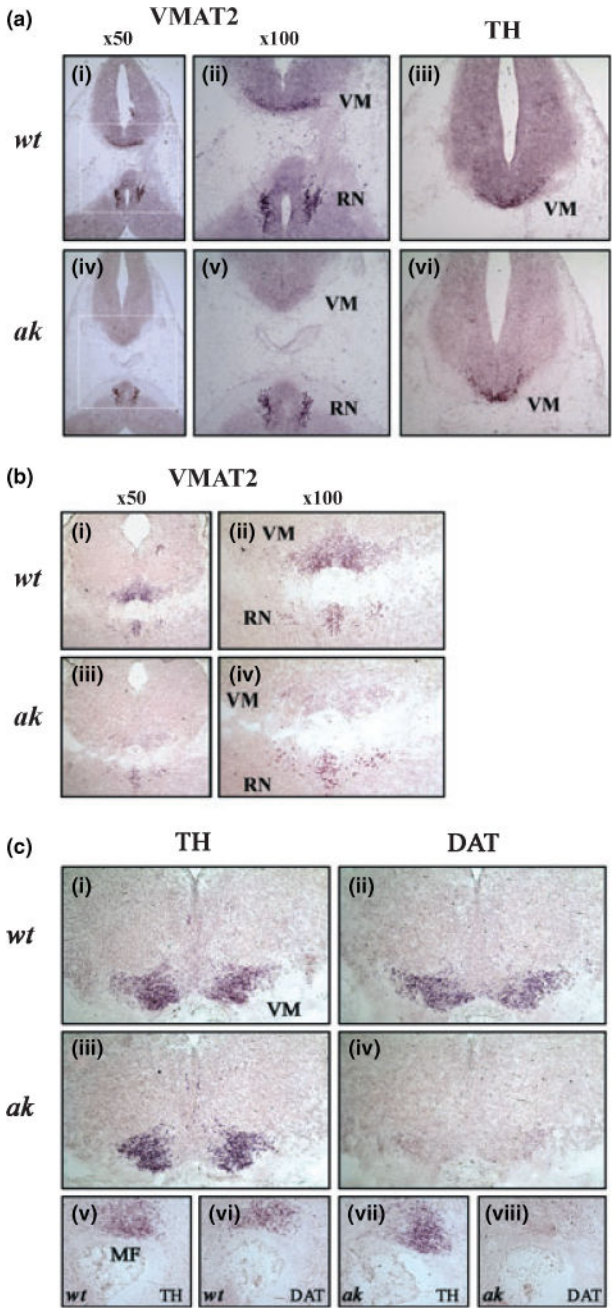

Fig. 2.

Expression of vesicular monoamine transporter 2 (VMAT2) (a, b) and dopamine transporter (DAT) (c) mRNAs in E12.5 (a) and E14.5 (b, c) embryos of wild-type (*wt*) and aphakia (*ak*) mice was examined by *in situ* hybridization. (a) Coronal sections of *wt* E12.5 (i–iii) and *ak* E12.5 (iv–vi) embryos were hybridized with antisense riboprobes for VMAT2 (i, ii, iv, v) and tyrosine hydroxylase (TH) (iii, vi). Panels ii and v are higher magnification of boxed areas in panels i and iv, respectively. (b) Coronal sections of *wt* E14.5 (i, ii) and *ak* E14.5 (iii, iv) embryos were hybridized with antisense riboprobes for VMAT2. Panels ii and v are higher magnification of panels i and iii, respectively. (c) Expressions of TH (i, iii, v, vii) and

DAT (ii, iv, vi, viii) genes in the ventral midbrain of *wt* (i, ii, v, vi) and *ak* (iii, iv, vii, viii) mice at E14.5 were examined by *in situ* hybridization. Panels i–iv are coronal sections and panels v–viii are sagital sections. VM, ventral midbrain; MF, mesencephalic flexure; RN, raphe nuclei.

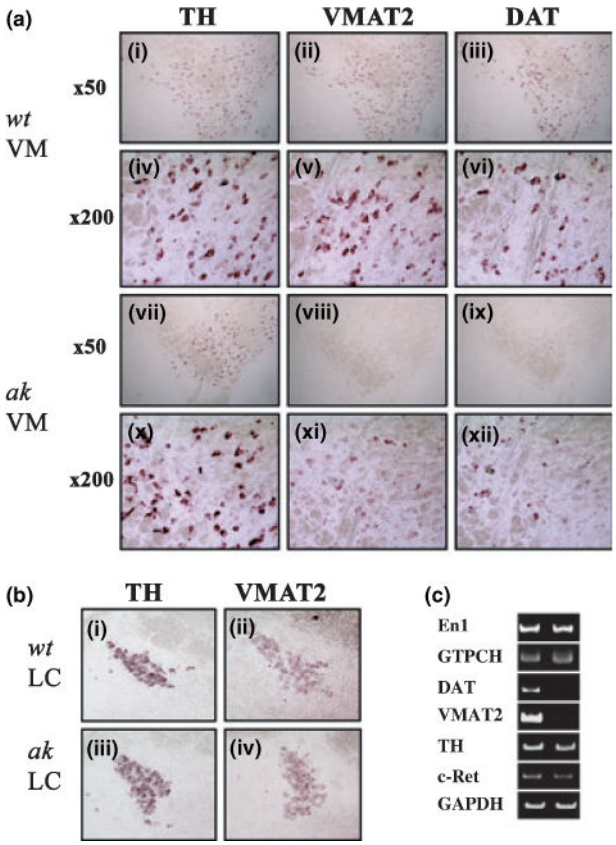

Fig. 3.

Expression of vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) genes is diminished in midbrain dopamine neurons of adult aphakia (*ak*) mice. (a) Expression of TH (i, iv, vii, x), VMAT2 (ii, v, viii, ix) and DAT (iii, vi, ix, xii) genes was analyzed in the ventral midbrain of adult wild-type (*wt*) (i–vi) and *ak* (vii–xii) mice. Panels iv, v, vi, x, xi and xii are higher magnifications of panels i, ii, iii, vii, viii and ix, respectively. (b) The level of TH (i, iii) and AT2 (ii, iv) mRNAs in the locus coeruleus of adult mice were examined by *in situ* hybridization. (c) RT-PCR analyses of several midbrain dopamine neuronal markers in the total RNAs isolated from laser captured adult VTA neurons. VM, ventral midbrain; LC, locus coeruleus.

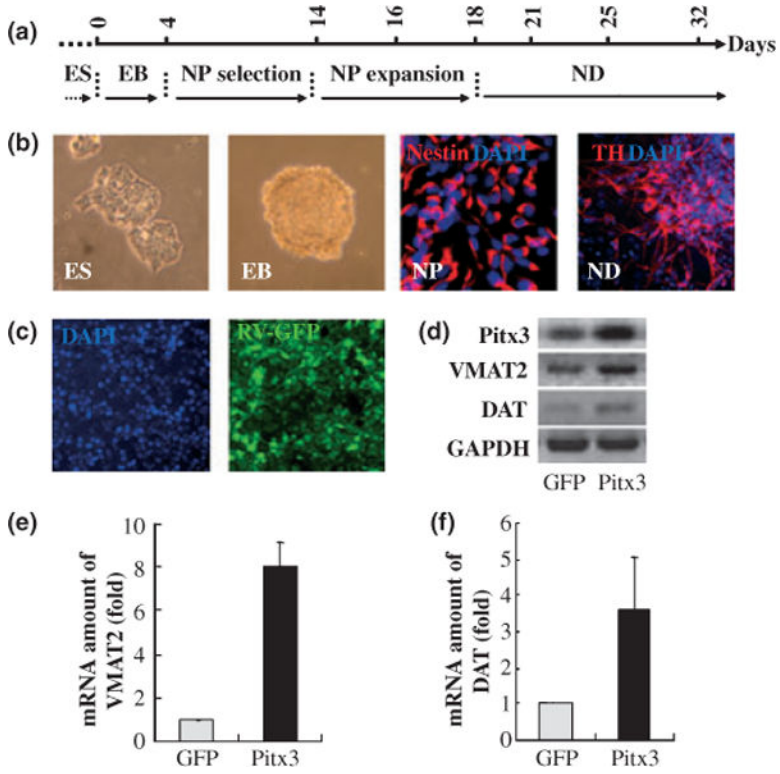

Fig. 4.

Over-expression of Pitx3 induces transcription of endogenous vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) genes during *in vitro* differentiation of mouse embryonic stem cells (mESCs). Total RNAs were isolated from neuronal cells after *in vitro* differentiation of the Pitx3-over-expressing mES cells and then RT-PCR was performed. (a) Diagram of *in vitro* differentiation of mESCs using the 5-stage method. ES, embryonic stem cells; EB, embryoid bodies; NP, neural precursors; ND, neuronal differentiation. (b) Representative images of each stage during mESC differentiation. (c) mESC-derived NPs are efficiently transduced with GFP-expressing retroviruses (about 90%). (e, f) VMAT2 and DAT mRNA levels are analyzed by semi- and real-time PCR analyses. Pitx3 over-expression leads to a robust increase of VMAT2 and DAT transcript levels, while the level of GAPDH transcript was not affected. Quantification based on real-time RT-PCR shows the fold change of endogenous transcripts between cells transfected with GFP control (white bars, control) and Pitx3 transfected cells (black bars, Pitx3). Relative expression of each mRNA was normalized according to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

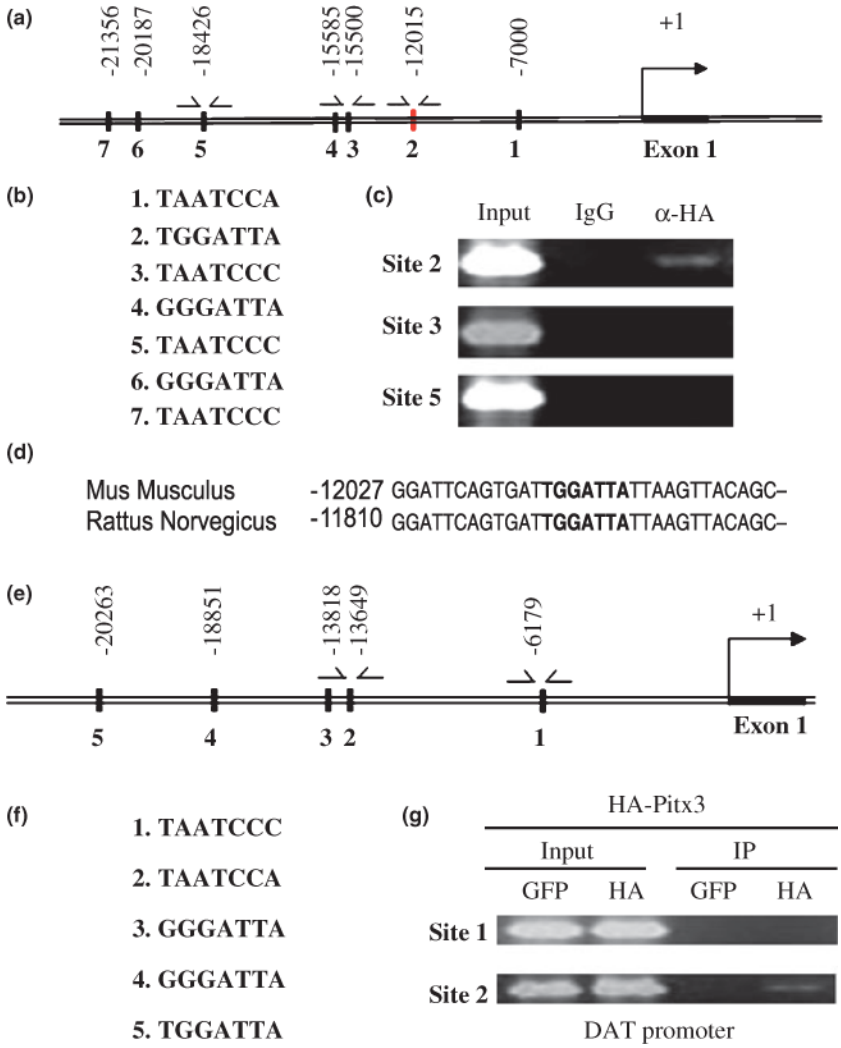

Fig. 5.

Pitx3 specifically binds to putative binding motifs in the 5′ upstream promoter of the vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) gene. (a, e) Seven and five putative Pitx3-binding sites are identified in the 5′ promoter region of the VMAT2 and DAT genes, respectively. (b, f) Their nucleotide sequences are shown in 5′ to 3′ directions. (c, g) Chromatin immunoprecipitation analysis indicates that Pitx3 interacts with both VMAT2 and DAT gene promoters *in vivo*. The protein–DNA complexes were immunoprecipitated using antibodies against anti-HA. As a negative control, rabbit IgG was used. (d) Comparison of nucleotide sequences flanking the site 2 of the VMAT2 gene shows that they are highly conserved between mouse and rat.

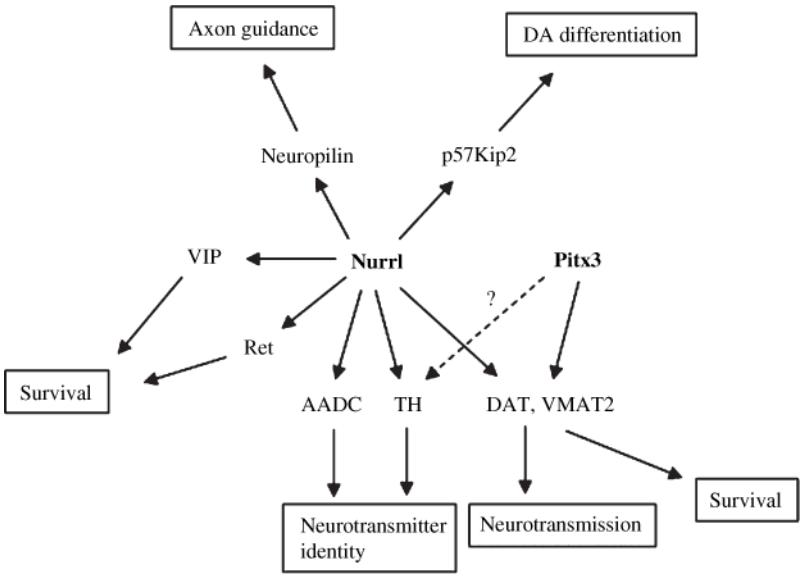

Fig. 6.

Pitx3, in addition to Nurr1, is regulating the expression of vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) genes critically involved in DA neurotransmission of midbrain dopamine neurons. DAT and VMAT2 are also implicated in the survival/vulnerability of midbrain dopamine neurons against neurotoxins.