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Identification of a Dopaminergic Enhancer Indicates Complexity in Vertebrate Dopamine Neuron Phenotype Specification

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Abstract

The dopaminergic neurons of the basal ganglia play critical roles in CNS function and human disease, but specification of dopamine neuron phenotype is poorly understood in vertebrates. We performed an *in vivo* screen in zebrafish to identify dopaminergic neuron enhancers, in order to facilitate studies on the specification of neuronal identity, connectivity, and function in the basal ganglia. Based primarily on identification of conserved non-coding elements, we tested 54 DNA elements from four species (zebrafish, pufferfish, mouse, and rat), that included 21 genes with known or putative roles in dopaminergic neuron specification or function. Most elements failed to drive CNS expression or did not express specifically in dopaminergic neurons. However, we did isolate a discrete enhancer from the *otpb* gene that drove specific expression in diencephalic dopaminergic neurons, although it did not share sequence conservation with regulatory regions of *otpa* or other dopamine-specific genes. For the *otpb* enhancer, regulation of expression in dopamine neurons requires multiple elements spread across a large genomic area. In addition, we compared our *in vivo* testing with *in silico* analysis of genomic regions for genes involved in dopamine neuron function, but failed to find conserved regions that functioned as enhancers. We conclude that regulation of dopaminergic neuron phenotype in vertebrates is regulated by dispersed regulatory elements.

Keywords

dopamine; enhancers; zebrafish; CNS; basal ganglia

Introduction

The basal ganglia and their dopaminergic neurons play critical roles in CNS function with vital roles in initiation and regulation of movement, limbic emotional responses, and reward-mediated aspects of behavior and learning. Several human diseases including Parkinson's disease, attention-deficit hyperactivity disorder, Tourette's syndrome, and addiction behaviors have been linked with pathology of basal ganglia dopaminergic neurons

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(Nussbaum and Ellis, 2003; Albin and Mink, 2006; Hyman, Malenka, and Nestler, 2006). In mammals, groups of dopaminergic neurons are located in discrete nuclei in the telencephalon, diencephalon, and mesencephalon. The mesodiencephalic dopaminergic (mesDA) neurons constitute the largest fraction of dopaminergic neurons in the brain (roughly 75%; Wallen and Perlmann, 2003) and include three distinct sets of nuclei (Dahlstroem and Fuxe, 1964): the substantia nigra pars compacta (group A9), the retrorubral field (group A8), and the ventral tegmental area (group A10).

The mesDA system is highly complex both in its organization and function. Its neurons integrate pathways of information from the striatum and cortex in which there is somatotopic representation of distinct body parts, and also sorting of parallel functional pathways for different cortical modalities (such as limbic behaviors or motor function (DeLong and Wichmann, 2009). Accordingly, mesDA neurons are heterogeneous with respect to their development (Smidt et al., 2000), projections, intrinsic electrophysiologic properties (Lammel et al., 2008), and neurotransmitter identities.

Specification of mesDA neurons is controlled by a developmental cascade of transcription factors (Abeliovich and Hammond, 1997; Smidt and Burbach, 2007). However, it is not known whether this hierarchical cascade alone is sufficient to specify mesDA neuron identity (including neurotransmitter status and axon projections), or whether additional information is necessary to provide more precise identities for subtypes of mesDA neurons. For example, the orphan nuclear receptor *nurr1* is necessary for generation and maintenance of mesDA neurons, as assayed by absent tyrosine hydroxylase (TH) expression in knock-out mice (Zetterstroem et al., 1997; Saucedo-Cardenas et al., 1998; Wallen et al., 1999). However, in *nurr1* knock-out mice, some neurons destined to express TH still differentiate partially; nigrostriatal projections still develop normally; and the neurons express other markers (such as cholecystokinin) specific to dopaminergic neurons (Witta et al., 2000). *nurr1* is only partially responsible for specifying dopamine neuron-specific gene expression: while *vmat2* and *dat* require *nurr1, aadc* expression is induced independently (Smits et al., 2003). Therefore, while *nurr1* is necessary for terminal differentiation of mesDA neurons including the expression of several dopamine-neuron specific biosynthetic enzymes and receptors, it does not regulate other key elements of mesDA identity such as axon pathfinding, and does not appear to affect development of other dopaminergic neuron groups in the CNS (Baeckman et al., 1999).

Another key unanswered question is how the genes necessary for dopaminergic neuron function are regulated. Multiple genes necessary for dopamine neuron function, survival, and axon pathfinding must be coordinately expressed in the correct subset of neurons. Elegant work from *C. elegans* has shown that a single cis-regulatory element and associated transcription factor (Ast-1) are necessary and sufficient for establishing dopamine neuron neurotransmitter identity (Flames and Hobert, 2009). Whether such a system is present in vertebrates is unknown. Because of the complexity of dopaminergic neuron development, as well as the involvement of the mesDA neurons in disease processes, identifying a discrete enhancer element specific for mesDA neurons would facilitate studies on the specification of neuronal identity and function in the basal ganglia.

In the present study we have performed an *in vivo* screen in zebrafish to identify dopaminergic neuron-specific enhancers. In zebrafish, dopaminergic neurons are present in the diencephalon but not the mesencephalon (Holzschuh et al., 2001; Kastenhuber et al., 2009), with projections in the adult to the subpallium (striatum) (JLB, unpublished data; Rink and Wulliman, 2001; Rink and Wulliman, 2002; Kastenhuber et al., 2009). Further, chemical ablation of the diencephalic dopaminergic (diDA) neurons phenotypically mimics loss of mesDA neurons in mammals (Lam et al., 2005; McKinley et al., 2005; Wen et al.,

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2008). We have identified a minimal 4.5kb enhancer element associated with the *otpb* gene that is sufficient to drive expression in specific dopaminergic neurons of the diencephalon in zebrafish. However, this enhancer (otpb.A) only drives expression in a subset of CNS dopaminergic neurons, and analysis of other dopaminergic-specific gene regions failed to identify a discrete enhancer with function in CNS neurons. Further, we were unable to detect any conservation between the sequence of the otpb.A enhancer element and the roughly 50 kb genomic neighborhoods (the most likely location of regulatory regions) of other genes specific to dopaminergic neurons. Our analysis of zebrafish dopaminergic gene regulatory regions reveals that conserved DNA elements are widely dispersed over large genomic loci.

Materials and Methods

Fish stocks and embryo raising

Adult fish were bred according to standard methods. Embryos were raised at 28.5°C in E3 embryo medium and staged by time and morphology (Kimmel et al., 1995). For *in situ* staining, embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 3 hours at room temperature (RT) or overnight (O/N) at 4°C, washed briefly in PBS, dehydrated, and stored in 100% MeOH at -20°C until use.

Transgenic fish lines and alleles used in this paper were the following: Tg*(otpb.A:egfp)*^{zc48} (official ZFIN nomenclature Tg(*otpb:EGFP*)^{zc48}), Tg(*fezf2:egfp*)^{zc55}, Tg(*pitx3:egfp*)^{zc50}, Tg(*f.TH.A:egfp*)^{zc56}, Tg(*otpb.A:GAL4*)^{zc57} (official ZFIN nomenclature Tg(*otpb:Gal4-VP16*)^{zc57}), Tg(*otpb.I:GAL4*)^{zc66}, and Tg(*UAS:GFP*) [(Tg(5xUAS:GFP)^{nkuasgfp1a} - kind gift of K. Kawakami]. Lines are available upon request.

Immunohistochemistry and *In situ* hybridization

Following fixation and dehydration in methanol, embryos were rehydrated, permeabilized using proteinase K [10μg/mL in PBST (PBS with 0.1% Tween-20)] at 28°C for 60′ (8′ for 24hpf; 20′ for 36hpf; and 30′ for 48hpf) without rocking, washed twice in PBST for 5′ then re-fixed for 15′. Embryos were then washed in PBST, blocked in PBST/1% DMSO/2% BSA/5% normal goat serum (NGS), and then incubated O/N in a primary antibody solution diluted in PBST/1%DMSO/2%BSA/2%NGS at 4°C. The next day embryos were washed in PBST/1%DMSO/1%NGS for a minimum of 6 hours, followed by incubation O/N with secondary antibodies, and washing the following day. Antibodies and concentrations used were rabbit polyclonal anti-tyrosine hydroxylase 1:400 (Millipore), mouse monoclonal anti-GFP 1:400 (Millipore), Cy-3 anti-rabbit 1:400, and Alexa 488 anti-mouse 1:400.

Double immunohistochemistry/*in situ* labeling was performed by permeabilization using 0.1% collagenase in PBST, re-fixation for 10′ with 4% PFA, and then performing anti-GFP antibody staining and detection in PBST using rabbit polyclonal anti-GFP 1:400 (Millipore #11122) followed by anti-rabbit Alexa 488 1:250 (Invitrogen). Following washing in PBST, embryos were fixed for 1 hour, washed with PBST, then re-permeabilized using 0.1% collagenase at RT. Whole-mount *in situ* labeling for *dat* (Holzschuh et al., 2001) and *isotocin* (Blechman et al., 2007) was then performed, followed by plastic sectioning, as previously described (Bonkowsky and Chien, 2005).

Genomic PCR and Enhancer Cloning

PCR primers used to clone genomic fragments are listed in Table S1. PCR and cloning of genomic fragments into pDONR P4-P1R was performed as described (Bonkowsky et al., 2008). The identity of the genomic fragments was confirmed by restriction enzyme digests and partial sequencing. Unless otherwise specified, the minimal promoter used for expression in Gateway constructs was the adenovirus E1b TATA box with the carp β-actin

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5′-UTR fragment (Kwan et al., 2007;Bonkowsky et al., 2008). Specific plasmids used for cloning were pME-basEGFP (middle entry clone with EGFP preceded by minimal promoter), pDestTol2pA2, pDestTol2CG2, pDestTol2CR3 (pDestTol2pA3 with *cmlc2:*TagRFP transgenesis marker); pME-basGal4-VP16$_{413\text{-}470}$ (Koester and Fraser, 2001;Ogura et al., 2009) was used for generation of GAL4 transgenic lines. pME-gata2EGFP is a middle entry clone with EGFP proceeded by the *gata2a* minimal promoter (Meng et al., 1997;Bessa et al., 2009). pME-cfosEGFP is a middle entry clone with EGFP preceded by the mouse *c-fos* minimal promoter (Dorsky et al., 2002).

Injection of DNA constructs and raising of stable transgenic lines was performed essentially as described (Fisher et al., 2006; Kwan et al., 2007; Bonkowsky et al., 2008). Patterns of enhancer expression were confirmed by transient injections of each construct (>100 embryos per construct), as well as isolation of two or more independent stable transgenic lines (in cases where stable transgenics were isolated). Plasmids and specific PCR conditions are available upon request.

Microscopy and image analysis

Image acquisition and analysis were performed essentially as described previously (Suli et al., 2006). Images of embryos processed for immunohistochemistry were taken using a confocal microscope; embryos were taken step-wise into a solution of 80% glycerol/20% PBST, then mounted on a glass slide with a #0 coverslip fixed into place over a well made using electrical tape. NIH ImageJ software (W. Rasband, NIMH) was used to merge slices to create maximal intensity z-stack projections.

Comparative genomic analysis

Cross-species non-coding conservation was determined by examination of the zebrafish genome assembly Zv5 at the UCSC genome browser (http://genome.ucsc.edu/) (Siepel et al., 2005). The “DA motif” was defined using the position weight matrix scoring from Flames and Hobert (2009). Sequence comparisons for DA motifs and cross-gene comparisons were done using sequence from Zv7. DA motif searches of DNA fragments were performed using ConSite (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) (Sandelin et al., 2004). Genomic DNA comparisons between non-coding regions of genes were performed using Shuffle-LAGAN in rVISTA (Brudno et al., 2004; Frazer et al., 2004). Genomic regions chosen for comparison were centered on each coding regions, encompassing 86.5kb from the *ddc* locus, 51.3kb from the *slc6a3* locus, 76.1 kb from the *th1* locus, and 42.2 kb from the *slc18a2* locus. Shared synteny was determined from genetic maps from the Ensembl and UCSC genome browsers (Hubbard et al., 2009; Rhead et al., 2009).

Results

Screen for dopaminergic neuron enhancers

We undertook a screen to identify genomic DNA fragments which might serve as enhancers to drive expression in dopaminergic neurons of the zebrafish brain, with minimal expression in other neuron types. The identification of potential enhancer fragments was based on the concept of conserved non-coding sequence serving as potential enhancers (Allende et al., 2006; Gomez-Skarmeta et al., 2006; Pennachio et al., 2006). This assumes that regions of nucleotide conservation (usually of greater than 60-70% identity) between species in regions of conserved syntenic blocks of genes may function as enhancers (or silencers) of transcription.

We cloned genomic fragments using PCR into a Tol2 transposon-based vector (Kawakami, 2004; Kwan et al., 2007; Villefranc et al., 2007). Genomic DNA fragments were chosen

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based on their location in relative proximity to a target gene (described below), location upstream of the first coding exon (for some targets), and conservation of non-coding sequence (using the UCSC genome web browser http://genome.ucsc.edu/) (Kent et al., 2002; Bejerano et al., 2005). To visualize expression driven by the potential enhancers, the DNA fragments were cloned immediately upstream of a minimal promoter followed by GFP (Kwan et al., 2007; Villefranc et al. 2007). We have previously demonstrated that this minimal promoter is competent to drive expression in diverse CNS cell types without ectopic expression (Bonkowsky et al., 2008). We also tested the *gata2a* and *c-fos* minimal promoters using previously characterized enhancers (Dorsky et al., 2002; Bessa et al., 2009), but found higher rates of ectopic expression in non-target tissues (JLB, unpublished data). To test for expression, we injected one-cell stage embryos and looked for GFP expression from 12hpf through 96hpf. The first expression of *th* in zebrafish is between 16-20hpf (Holzschuh et al., 2001). Transient expression was analyzed in 100-200 embryos; if we observed consistent CNS expression in a region that potentially had overlap with diDA neurons, we raised stable transgenic lines for characterization. Characterization of stable transgenic lines consisted of double immunohistochemistry for GFP and for tyrosine hydroxylase (TH).

We used a comprehensive strategy to identify potential target genes, choosing them from two classes (Table 1). The first class were genes with known or putative roles in dopaminergic neuron specification [including *FEZ family zinc finger 2* (*fezf2*)*, LIM homeobox transcription factor 1 alpha 2* (*lmx1a.2*)*, muscle segment homeobox E* (*msxE*)*, neurogenin 1* (*ngn1*)*, nuclear receptor subfamily 4 group A member 2a* (*nr4a2a*, a homolog of mammalian *nurr1*)*, orthopedia homolog a* and *b* (*otpa* and *otpb*), and *paired-like homeodomain transcription factor 3* (*pitx3*)] (Abeliovich and Hammond, 2007;Smidt and Burbach, 2007). The second class were genes with roles in dopaminergic neuron function: *aromatic acid decarboxylase* (*ddc*, previously known as *aadc*), *dopamine receptor* (*drd2b*), *tyrosine hydroxylase* (*th*, to be distinguished from its paralog *th2*), *dopamine transporter* (*slc6a3*, previously *dat*), and *vesicular monoamine transporter 2* (*slc18a2*, previously *vmat2*). In some cases we tested for overlap of expression of a particular gene with *th* by double-labeling for its mRNA by *in situ* hybridization, and TH by immunohistochemistry (data not shown). In addition to analyzing zebrafish genomic fragments for potential dopaminergic enhancer activity, we also tested elements from pufferfish, mouse, and rat. Genomic DNA fragments were also cloned from pufferfish (*Fugu rubripes*) because of its compact genome size and the assumption that intergenic regions would be enriched for sequences that could serve as enhancers (Brenner et al., 1994). Enhancers from mouse and rat were chosen because of their known expression in dopaminergic neurons (such as the rat TH promoter) (Schimmel et al., 1999), or expression in the mesodiencephalon (VISTA Enhancer Browser- Visel et al., 2007). Finally, we tested a BAC with GFP inserted at the mouse *slc6a3* region (GENSAT1- BX1837) (Gong et al., 2003).

We tested 54 fragments from 21 different genes. In many cases no CNS expression was seen. We found that DNA fragments isolated from regions near genes encoding transcription factors were more likely to function *in vivo* as enhancers, a phenomenon that has also been noted for other tissue types (Sandelin et al., 2004; Woolfe et al., 2005). For fragments that functioned as CNS enhancers during development, most showed either minimal or no overlap with TH-positive neurons (as for Tg(*fezf2:egfp*)^{zc55} and msxE:EGFP, Figure 1A-C and Figure S2E-G) or had widespread expression in non-TH neurons (Tg(*pitx3:egfp*)^{zc50}, Tg(*f.TH.A:egfp*)^{zc56}, and Tg(*lmx1a.2:egfp*) (Figure 1D-E and Figure S2A-D). *pitx3* has subsequently been shown not to express in dopaminergic neurons in zebrafish (Filippi et al., 2008). Expression patterns from the transgenic lines Tg(*lmx1a.2:egfp*), Tg(*fezf2:egfp*)^{zc55} and Tg(*pitx3:egfp*)^{zc50} appear to match with the known expression domains of *lmx1a.2, fezf2* and *pitx3*, respectively (Figure S2; Blechman et al., 2007; Filippi et al., 2008),

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indicating that our enhancer lines recapitulate endogenous gene expression at least to some extent.

Since it is possible that some enhancers failed to function *in vivo* because they might require a specific promoter (Gehrig et al., 2009), we also tested two of our genomic DNA fragments with two additional alternate promoters (*gata2a* and *c-fos*). We tested a fragment from the *ddc* gene, and the optb. A enhancer (described further below). The *ddc* fragment did not drive expression with any of the three promoters (data not shown). Expression driven by the otpb.A enhancer in transient injections was similar with all three promoters (Supplemental Figure S1), although more ectopic non-CNS expression (compared to the stable transgenic otpb.A line and the endogenous *otpb* expression pattern) was seen using the *c-fos* and *gata2a* minimal promoters. Another possibility is that a gene's endogenous promoter might be necessary for enhancer-driven expression. However, we tested the known endogenous promoter for *slc6a3* in one of our constructs, and for many other tested fragments, their size and location immediately upstream of the translation start site makes it very likely that they include the endogenous promoter (including fragments for the genes *th, drd2b, Fugu dat, otpa, Fugu otpb, and slc18a2).*

We characterized most enhancers that had CNS expression by generation of a stable transgenic line, and have maintained some of these lines (Table 1). The failure of some DNA fragments to function as enhancers despite high cross-species non-coding conservation might be due to function as a repressor or silencer of expression; to the fragment regulating expression at a different (non-embryonic) stage; or to insufficiency of the element in isolation to drive expression.

Analysis of genomic regions of dopaminergic neuron phenotype genes

Because of our difficulty in identifying a discrete enhancer with expression specific to dopaminergic neurons (with one exception, see below), we sought to address how dopaminergic neuron phenotype is regulated in vertebrates. To address whether vertebrate dopaminergic neuron phenotype requires a single, discrete cis-regulatory element, or a dispersed complex code of binding sites, we examined a core group of genes whose expression is relatively specific to dopamine neurons, using both *in silico* and *in vivo* approaches. For our core group of dopaminergic phenotype genes we chose genes expressed by all neurons that use dopamine as a neurotransmitter, including the rate-limiting enzyme for dopamine synthesis *th*, the enzyme for converting L-dopa to dopamine *ddc*, the pre-synaptic uptake receptor *slc6a3*, and the cytosol to synaptic vesicle transporter *slc18a2*.

First, we examined whether the genomic neighbors for the dopaminergic phenotype genes are conserved across evolutionary time. For vertebrate genes, conserved synteny of genes in “genomic regulatory blocks” is associated with dispersal of cis-regulatory elements amongst the coding exons of the different genes (reviewed in Kikuta et al, 2007). We compared conserved synteny between human, mouse, zebrafish, and pufferfish regions for the genes *ddc, slc6a3, th*, and *slc18a2* (Figure 2A). In all cases, there was at least partial conservation of synteny between zebrafish and other vertebrates, suggesting that necessary cis-regulatory elements may be present in these regions.

Next, we looked for conserved cross-species conservation in genomic regions surrounding DA neuron-specific genes, and tested *in vivo* the ability of different genomic fragments to act as enhancers. Surprisingly, none of the genomic fragments we tested *in vivo* drove expression in DA neurons, or even in the CNS (Table 1; Figure 2B). Further, this was despite the presence in many of the fragments of multiple copies of the “DA motif” (Flames and Hobert, 2009), as well as high cross-species conservation in some fragments. We found that the presence of the DA motif was no more frequent (and with no higher matrix scores)

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than in a CNS enhancer not expressed in DA neurons (foxP2-enhancerA, Bonkowsky et al., 2008). We also constructed and tested a DA motif (5′-gcagaggaggaagagtggaga-3″) triplet multimer, fused to a basal promoter and GFP, but did not find specific CNS expression. A separate study has also tried to identify a DA-specific enhancer from the *slc6a3* region, and tested an 11-kb fragment encompassing the transcription start and regions upstream (Bai and Burton, 2009). This 11-kb enhancer drove expression in dopaminergic neurons of the pre-tectal region, but not in other dopaminergic neurons, and also had ectopic expression in many CNS cell groups, implying the absence of both necessary enhancer elements as well as of silencing elements. Together, these results show that in vertebrates a single discrete DA motif is not sufficient for expression in DA neurons.

We also performed a detailed expression analysis of potential enhancers from the *Fugu th* genomic region (Figure S3). The original transgenic line Tg(*f.TH.A:egfp*)^{zc56} showed expression in most diDA neurons (Figure 1), but also had expression in many non-DA neurons. Although we tested a large number of fragments and transgenic lines based on this original enhancer (Figure S3), none gave specific expression in DA neurons alone.

While we did not find that the DA motif was sufficient for expression in dopaminergic neurons, an alternative explanation is that a different conserved element is used in vertebrates. To try to identify whether there were other elements in the loci of the dopamine phenotype genes that might specify for DA neuron expression, we did a comparative analysis of the zebrafish genomic loci for *th, ddc, slc6a3*, and *slc18a2*, to identify highly conserved (>70%) regions of 50bp or more. We found 42, 53, and 65 regions in the *th* locus that were highly conserved with regions in the *slc6a3, slc18a2*, and *ddc* loci, respectively; and only 1 region that was conserved in all 4 genomic loci (Figure 2B). These regions of conservation were dispersed over the entirety of each genomic locus. Further, our previous *in vivo* analysis had tested some of these regions which failed to drive CNS expression. Similarly, genomic fragments for the *th* and *slc6a3* regions partially overlapping the fragments we tested had also been tested *in vivo* by other groups (Meng et al., 2008 and Bai and Burton, 2009, respectively; Figure 2B) and had also failed to drive specific dopaminergic neuron CNS expression.

We used BLAST and CLUSTALW analyses of the most highly conserved regions shared by the different gene loci to look for conserved DNA motifs. No common shared sequence motifs were identified in these highly conserved regions. We conclude that there is not an obvious single candidate cis-regulatory element that controls expression in vertebrate dopamine neurons, and that the core regulatory elements necessary for dopaminergic expression are widely dispersed.

This dispersed pattern of non-coding conservation in the dopamine pathway genes, together with our *in vivo* testing of specific genomic fragments, strongly argues that regulation of the genes necessary to maintain a dopaminergic phenotype is complex in vertebrates. In contrast to *C. elegans*, each group of dopaminergic neurons in vertebrates may require a distinct combinatorial code to establish its mature phenotype.

Characterization of *otpb* enhancer

From the enhancer screen (Table 1) we found a genomic DNA fragment (otpb.A) from the region upstream of the *orthopedia-b* (*otpb*) gene that drove expression in TH-positive neurons of the diencephalon with minimal expression in other neurons (Figure 3). *otpb* encodes a homeobox transcription factor necessary for dopaminergic neuron development in zebrafish and in mouse (Del Giacco et al., 2006;Ryu et al., 2007). Double-labeling for GFP and for TH in the transgenic line Tg*(otpb.A:egfp)*^{zc48} of embryos at 72hpf showed co-expression in the diencephalon, primarily in dopaminergic neuron groups 4 and 6 (based on

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the nomenclature of Rink and Wullimann, 2002) (Figure 3E-E″). Expression of GFP in Tg(*otpb.A:egfp)*^{zc48} was first visible between 18-24hpf, and became more widespread between 36-48hpf (Figure 3F-G″).

TH labels all catecholaminergic neurons, including adrenergic, noradrenergic, and dopaminergic types. To confirm that the TH-positive neurons labeled by the otpb.A enhancer were in fact dopaminergic, we performed double-labeling for the dopamine transporter (*slc6a3*) gene. *slc6a3* encodes a reuptake transporter of dopamine that is specifically expressed in dopaminergic neurons and not other catecholaminergic neuron types (Nirenberg et al., 1996; Nirenberg et al., 1997; Holzschuh et al. 2001). We used Tg(*otpb.A:GAL4*)^{zc57}, in which GAL4-VP16 (Koester and Fraser, 2001) drives expression under the control of otpb.A (Figure 4A-A″), to analyze co-expression. Interestingly, when Tg(*otpb.A:GAL4*)^{zc57} was crossed to Tg(*UAS:GFP*), expression of GFP was found in all TH-positive neurons of the diencephalon, in contrast to the original Tg(*otpb.A:egfp*)^{zc48} line, in which not all diDA neurons were labeled. This may be due to a position effect of the original Tg(*otpb.A:egfp*)^{zc48} line, or to stronger expression due to amplification by the GAL4/UAS system (Koester and Fraser, 2001). We observed that diencephalic neurons expressing GFP in the diencephalon also co-expressed *slc6a3* (Figure 4B-B″), confirming that they were dopaminergic. The otpb.A enhancer also drives expression in the rostral diencephalon, in the neurosecretory preoptic (NPO) neurons that require *otpb* expression for their development (Blechman et al., 2007). Double-labeling in Tg(*otpb.A:GAL4*)^{zc57};Tg(*UAS:GFP*) embryos for GFP and for *isotocin* (the chief neurohypophysial peptide expressed in the NPO- Unger and Glasgow, 2003) revealed that most of this more rostral group co-expressed both markers (Figure 4C-C″). Other neuroendocrine-specific genes are also co-expressed with the otpb.A reporter in the NPO region (J. Schweitzer, H. Loehr, W. Driever, J.L.B., manuscript in preparation). These results show that the otpb.A enhancer specifically recapitulates *otpb* gene expression in non-dopaminergic NPO cells and most if not all diDA neurons.

Regulation of dopaminergic identity and the *otpb* enhancer

otpb is necessary for development of the diDA neurons and is expressed in all diDA neurons as well as in the NPO cells (Ryu et al., 2007; Loehr et al., 2009). In an effort to identify a minimal sufficient region for diDA expression, we tested multiple genomic fragments in the *otpb* genomic locus, including a more distal genomic region (otpb.D) (Figure 5A-C). Some of the fragments failed to drive any CNS expression (fragments otpb.B, otpb.D, otpb.F, and otpb.H), while some overlapping fragments drove essentially identical expression in approximately 22-24 diDA neurons (enhancers otpb.C, otpb.E, and otpb.G) (Figure 5C). otpb.I recapitulated only part of the original otpb.A pattern (Figure 5C, D). To demonstrate that this partial expression was in fact due to an absence of necessary cis-binding elements, and not simply low levels of expression, we tested the otpb.I fragment (a 444-bp fragment derived from optb.A) both when driving GFP directly, as well as driving GAL4-VP16 expression in a Tg(*UAS:GFP*) background. optb.I only drove partial expression in the NPO and diDA neurons, even when using the GAL4/UAS:GFP system (Figure 5D). Thus, otpb.A must contain other important sequences not included in otpb.I, consistent with our hypothesis that expression in diDA neurons is regulated by multiple independent cis-regulatory binding sites spread out over a large genomic region.

Since we had identified a relatively small region in otpb.A (4.5 kb) that was sufficient for expression in diDA neurons, we wondered whether this region contained motifs that would be shared with other genes expressed in diDA neurons. We performed a comparative analysis of the otpb.A genomic region (using the sequence obtained by direct sequencing of the cloned fragment), with the regions surrounding the *ddc, slc6a3, TH*, and *slc18a2* genes using rVISTA. In addition, we examined the region upstream of *otpa* in zebrafish, a paralog

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of *otpb*. We failed to observe any significant conservation (>70%) of sequence from the otpb.A region with *otpa*, or *ddc, slc6a3, th*, or the *slc18a2* gene regions. Therefore, the cis-regulatory sequences, and by extension the transcription factors that bind to these sequences, are probably different for *otpb* compared to *otpa* or to the dopaminergic phenotype genes.

Discussion

Through a detailed screen for enhancers that drive expression in CNS dopaminergic neurons, we have identified a single discrete enhancer that functions in diencephalic dopaminergic (diDA) neurons of the zebrafish. This enhancer fragment, otpb.A, drives expression specifically in diDA neurons and in NPO neurons of the hypothalamus. Despite testing a large number of potential enhancers (54 fragments from 21 genes) with conserved non-coding conservation, most of the genomic regions we tested failed to have CNS expression in embryos, with the exception of regions derived from transcription factor genes. Other groups have also noted previously that genomic regions derived from locations near transcription factors are more likely to act as enhancers (Sandelin et al., 2004; Woolfe et al., 2005).

The reason(s) why certain genomic fragments did not work as enhancers are uncertain. A fragment might work as a silencer of expression; it might regulate expression at a non-embryonic stage (for example, Fujimori, 2009); or it might drive expression at very low levels, although we have tested fragments from *th, slc6a3*, and *ddc* driving GAL4-VP16 and failed to see expression when injected into UAS:GFP transgenic embryos (data not shown). Another possibility is that the endogenous promoter associated with a gene might be necessary for enhancer-driven expression (Gehrig et al., 2009). However, for *slc6a3* we included its known endogenous promoter in one of our constructs, and for *th, drd2b, Fugu dat, otpa, Fugu otpb*, and *slc18a2* we tested large fragments immediately upstream of the translation start site, which were very likely to encompass the endogenous promoter. Furthermore, we have tested two alternative minimal promoters (from *c-fos* and *gata2a*), and did not find substantive differences in expression compared to the E1b-based minimal promoter that we used. We suggest a model in which single DNA elements in isolation are insufficient to specify dopaminergic neuron phenotype in vertebrates. This is based on our work examining the otpb.A enhancer in detail, and our analysis (*in vivo* and *in silico*) of the genomic regions surrounding the dopaminergic phenotype genes. However, our strategy of using non-coding conservation as a marker of potential enhancers is of limited use in cases where the genomic annotation is incomplete. For example, in zebrafish a second *tyrosine hydroxylase* paralog has recently been identified (*th2*) (Candy and Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010), but which does not have significant expression until 3-4 dpf. Alternative strategies for identifying enhancers have also recently been described; for example, using tissue-specific ChIP-seq to identify p300 binding sites (Visel et al., 2009). To test more formally our model that multiple combined DNA elements regulate dopaminergic expression, ideally we would like to have a locus-spanning BAC with a recombineered GFP cassette, and compare it to isolated genomic fragments either alone or in combination. However, for the *slc6a3* locus for example, there is no available BAC (database searches, JLB, and personal communication, Sanger Institute, zebrafish sequencing group) (presumably in part because of its telomeric location).

Our goal of identifying a vertebrate dopaminergic enhancer was only partially successful, in contrast to work in *C. elegans* that identified a simple "DA motif" that is necessary for terminal selection of neuron phenotype (Flames and Hobert, 2009). Loss of the DA motif leads to loss of expression in dopaminergic neurons, and ectopic expression of the transcription factor *ast-1* (which binds the DA motif) is sufficient to induce a dopaminergic phenotype. Flames and Hobert propose a model in which dopaminergic neurotransmitter

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status is regulated by a single terminal selector gene and its corresponding *cis*-motif (the “bar code model”- Spitzer, 2009). The concept of “terminal selector genes” is appealing and several examples have been demonstrated in *C. elegans* (Hobert, 2008). However, the organization of both gene structure and the nervous system are considerably less complex in *C. elegans* compared to vertebrates. Most enhancers in *C. elegans* are located in the 1-2 kb immediately 5′ of the translation start (Okkema and Krause, 2005). The nervous system of *C. elegans* is considerably simpler than that of vertebrates. For example, *C. elegans* hermaphrodites have a total of eight dopaminergic neurons, with projections only to the nerve ring and nerve cord (reviewed in Nass and Blakely, 2003). Thus, the regulation of dopaminergic phenotype in *C. elegans* matches the relative simplicity of its dopaminergic circuits.

Using both bioinformatics and *in vivo* testing, we were unable to isolate a compact enhancer from the genomic loci of dopaminergic phenotype genes (*th, slc6a3, slc18a2*, and *ddc*). Rather, we found multiple conserved motifs dispersed across large genomic regions around these genes. While in some cases we tested these motifs *in vivo* and did not detect enhancer activity, we did not test all of these motifs, nor did we test them in combination. Further, the otpb.A enhancer, which drives expression in dopaminergic neurons, does not share any detectable motifs with neighboring genomic regions of other dopaminergic phenotype genes, and furthermore cannot be reduced to a compact DNA module that is sufficient for expression in dopamine neurons. The otpb.I subfragment (444bp) of otpb.A only expresses in 3-4 dopamine neurons, compared to 20-30 for the original otpb.A fragment, and this does not appear to be due to low levels of expression. Therefore, otpb.I does not have all the necessary cis-information to regulate expression in dopaminergic neurons. It is still formally possible that both our *in vivo* and *in silico* analyses have failed to detect a small, conserved *cis*-motif in the genomic regions of the dopaminergic phenotype genes or in the otpb.A enhancer.

The otpb.A enhancer drives expression in most of the diencephalic dopamine neurons in zebrafish, as well as in neuroendocrine cells, matching *otpb*'s endogenous expression pattern (Del Giacco et al., 2006; Ryu et al., 2007). Despite extensive efforts, we were unable to isolate a minimal region of the otpb.A enhancer that was sufficient for either dopaminergic or neuroendocrine expression alone. Thus, regulation of the otpb.A enhancer appears to be coordinated across the entire 4.5 kb region. *In silico* analysis did not identify sequences shared with the genomic regions of *otpa* or of dopaminergic phenotype genes. The otpb.A enhancer does provide a valuable tool for investigating zebrafish diencephalic dopamine neuron development and function, with the potential for dopamine neuron specific-expression by using combinatorial expression approaches (EF, CBC, and JLB, unpublished data).

We conclude that in vertebrates dopaminergic cell identity regulation is dispersed over large genomic regions, and that a complex regulatory system is necessary for expression of a dopaminergic phenotype. This is consistent with other studies showing that vertebrate gene expression can depend upon widely dispersed cis-elements (Komisarczuk et al., 2009), in “genomic regulatory blocks” (reviewed in Kikuta et al., 2007). These findings suggest that dopaminergic cell identity is regulated by a mosaic of factors that dictate not only the dopaminergic neurotransmitter phenotype, but also other elements of neuronal identity such as synaptic targets and function (Figure 6). Our findings support a model in which distinct groups of dopaminergic neurons use unique solutions to achieve a dopaminergic phenotype.

Supplementary Material

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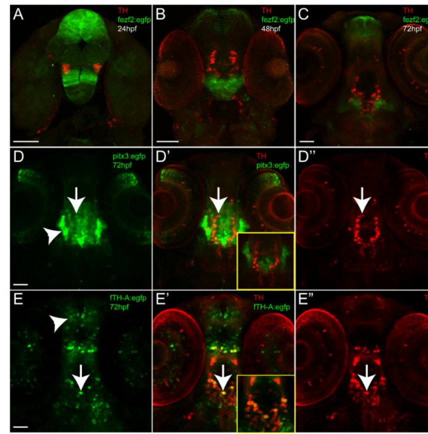

Figure 1.

Examples of stable transgenic enhancer lines generated; ventral views, anterior to the top, double-immunohistochemistry for GFP and TH (green and red), confocal z-stacks. Scale bars are 50 µm. (A-C) Time-series of Tg(*fezf2:egfp*)^{zc55} expression at 24hpf, 48hpf, and 72hpf, shows non-overlap of TH and GFP expression. (D-D″) Tg(*pitx3:egfp*)^{zc50} and (E-E″) Tg(*f.TH.A:egfp*)^{zc56} at 72hpf, shows partial overlap of enhancer expression and TH (arrows), but also wide spread expression of the enhancer in non-TH neurons (arrowheads). Insets in D′ and E′ show single confocal plane of double-labeling, emphasizing the minimal overlap of TH and GFP labeling.

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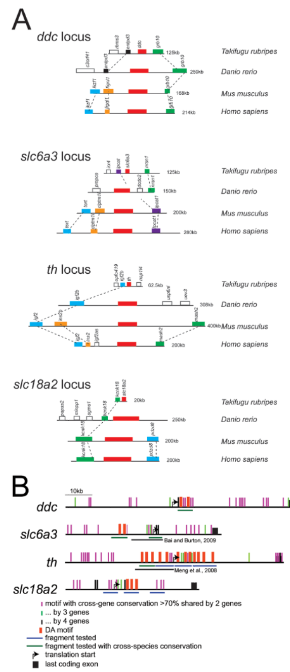

Figure 2.

(A) Gene order and conserved synteny at *ddc, slc6a3, th*, and *slc18a2* loci between pufferfish, zebrafish, mouse, and human; scale is approximate (indicated to right). Zebrafish has partial conservation of synteny with mammalian orthologs. (B) Schematic representation of conserved DA motifs in zebrafish dopamine pathway genes (scale is approximate). DA motifs in tested genomic fragments are represented by red boxes shown above; enhancer fragments tested are shown below. If significant cross-species conservation (from the UCSC genome site) was present the enhancer fragment is labeled in green, otherwise the enhancer fragments are shown in blue. Shared (non-coding) DNA elements between 2 genes are shown as vertical purple lines above the gene locus; elements shared by 3 genes are shown as green vertical lines; and by all 4 genes as black vertical lines. None of these enhancers was sufficient to drive expression in dopaminergic neurons *in vivo*. Also shown are the genomic fragments tested by Meng et al. (2008) and Bai and Burton (2009), which also failed to recapitulate *th* and *dat* expression, respectively.

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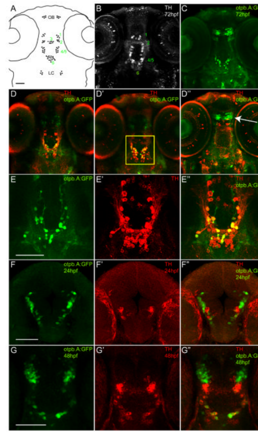

Figure 3.

Characterization of Tg*(otpb.A:egfp)*^{zc48}; confocal images of whole mount embryos double-labeled for GFP and for TH immunohistochemistry, ventral views, anterior to the top. Scale bar is 50 μm. (A) Schematic diagram of TH-positive cell groups in the zebrafish brain at 72hpf, based on Rink and Wullimann, 2002. (B) Confocal z-stack projection of TH immunohistochemistry at 72hpf in the zebrafish brain. (C) Confocal z-stack projection of GFP immunohistochemistry in Tg*(otpb.A:egfp)*^{zc48}. (D-D″) Confocal z-stack projections at different dorsal-ventral levels in the brain of Tg*(otpb.A:egfp)*^{zc48} at 72hpf, showing co-expression of TH and GFP in diDA neuron groups 4 and 6, but not groups 1 and 2 (arrow); arrowhead points to the NPO neurons. (E-E″) Magnified views of the region boxed in B″, showing extensive overlap of GFP-positive neurons in the diencephalon with TH expression. (F-F″) Expression at 24hpf (G-G″) Expression at 48hpf.

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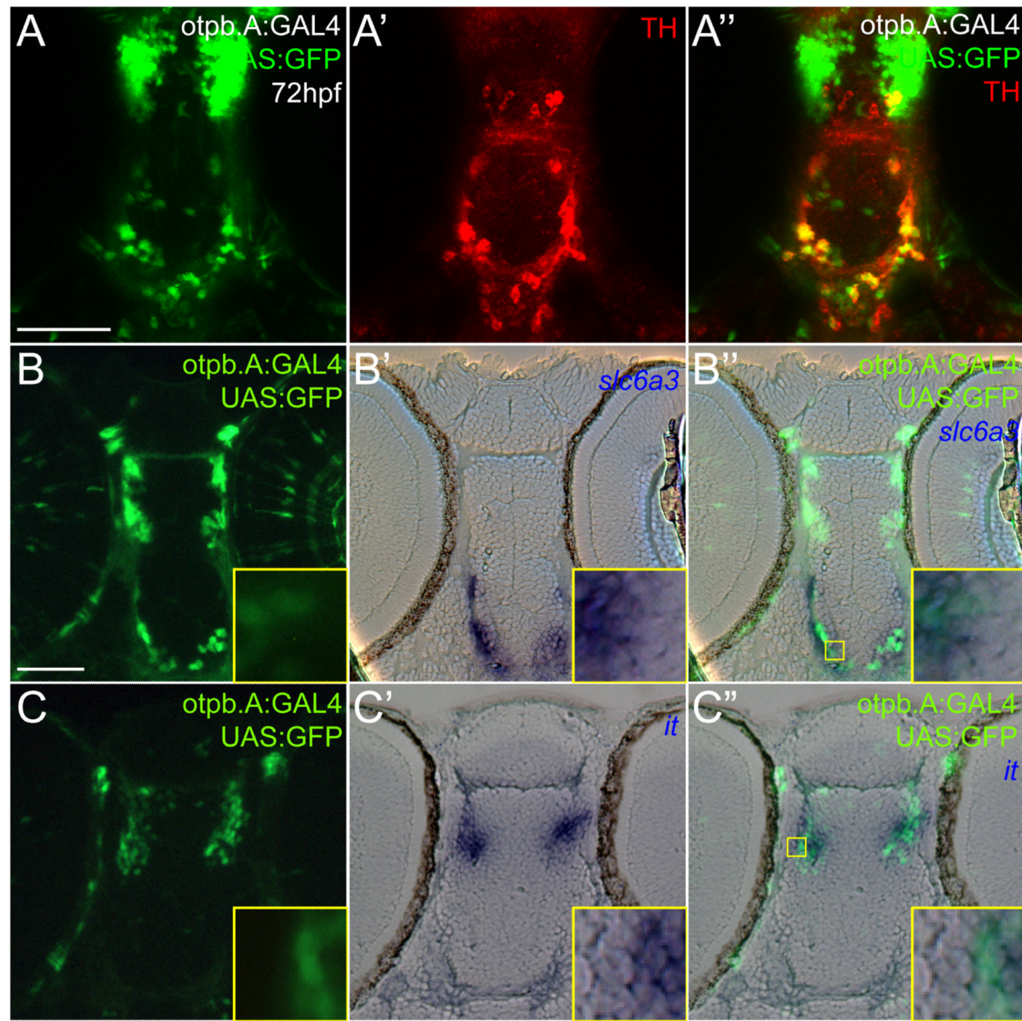

Figure 4.

Characterization of otpb.A expression in dopaminergic and neuroendocrine cells. Embryos at 72hpf, Tg(*otpb.A:GAL4*)^{zc57}, Tg(*UAS:GFP*). Scale bar is 50 μm. (A-A″) Confocal z-stack projection of whole-mount embryos double-labeled for GFP and for TH immunohistochemistry, ventral views, anterior to the top. (B) and (C) Plastic horizontal sections of double-labeled Tg(*otpb.A:GAL4*)^{zc57}; Tg(*UAS:GFP*) embryos at 72hpf, anterior to the top. Higher magnification inset showing co-localization is indicated by yellow boxed area and is shown at bottom right of each panel. (B-B″) Labeled for GFP antibody (B) and *slc6a3* mRNA (B′), GFP-positive neurons in the diencephalon co-express *slc6a3*, confirming their identity as dopaminergic. (C-C″) Labeled for GFP antibody (C) and *isotocin* mRNA (C′), rostral diencephalon GFP-positive NPO neurons co-express GFP and isotocin.

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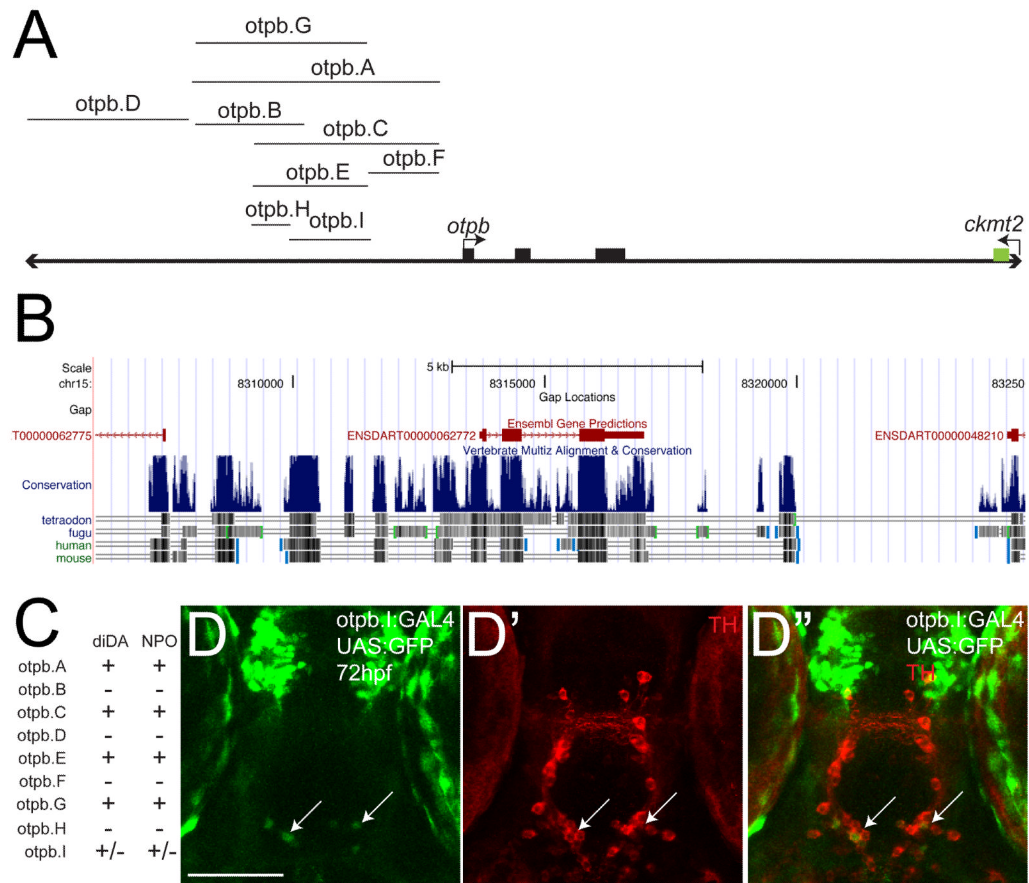

Figure 5.

Genomic structure and characterization of *otpb* genomic locus enhancers. (A) *otpb* genomic region (not to scale). Coding exons are shown as solid black boxes. DNA fragments tested for enhancer activity are shown. Region pictured is approximately 20kb; 3′-most exon of *ckmt2* is shown. (B) Screenshot from the UCSC genome browser (http://genome.ucsc.edu) showing the location of the exons of genes, relative to regions of cross-species conservation (shown as vertical blue lines). Increasing conservation is indicated by increasing height and/or density of the blue lines. Species used to determine the conservation are shown below. (C) Summary table of expression patterns of the different enhancers at 72hpf in the CNS with respect to expression in the diencephalic dopaminergic neurons (diDA) and neurosecretory preoptic nucleus (NPO). (D-D″) Confocal z-stack projection of whole-mount Tg(*otpb.I:GAL4*)^{zc57} embryos at 72hpf, shown crossed to Tg(*UAS:GFP*) and double-labeled for GFP (D) and for TH (D′) immunohistochemistry, ventral views, anterior to the top. Arrows point to the sparse diDA neuron expression.

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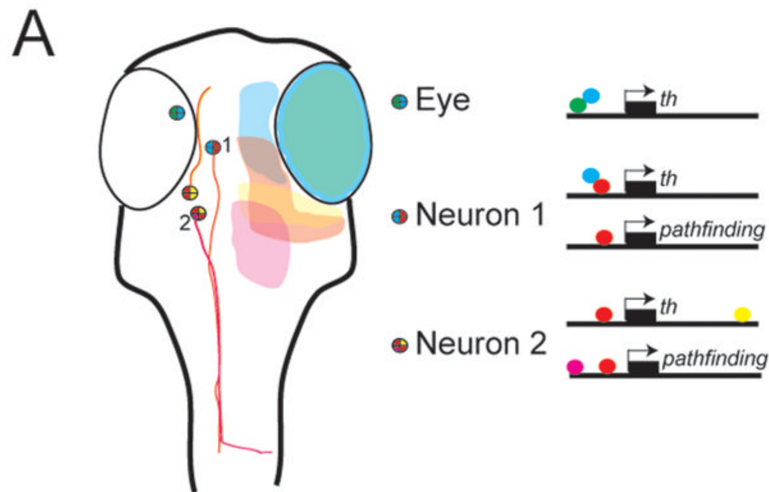

Figure 6.
Model of dopaminergic neuron phenotype specification in vertebrates. Different groups of dopamine neurons are specified by different combinations of transcription factors, specifying both their dopamine phenotype and other aspects of their identity (such as their synaptic targets).

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Table 1

CNS dopaminergic neuron enhancer screen: DNA elements tested.

Table of genomic DNA fragments tested *in vivo*, grouped according to functional category, gene, and species. Position relative to translation start site (chosen instead of transcriptional start site because of variability in zebrafish genome annotation), and enhancer fragment size are also listed. Primer sequences are in Table S1.

Gene	Enhancer	Species	Category+	CNS Expression*	Stable/Allele″	Position^ (kb)	Fragment Size (kb)
ddc		Dr	F	-		6.6	3.4
slc6a3	A	Dr	F	-		-0.7	1.9
	E	Dr	F	-		-5.1	5.1
slc6a3 (BAC)	BX1837**	Mm	F	-			
drd2B	A	Dr	F	-		-0.2	6.7
f.slc6a3	A	Fr	F	-		-0.4	4.5
	B	Fr	F	-		1.7	3.8
fezl		Dr	T	+		-3.4	3.7
LB[1]	219	Mm		-		FOXP2 intragenic	1.3
	298	Mm		+		SHFM1-DLX5	0.7
	304	Mm		+		JMJD2C-PTPRD	0.6
Lmx1a.2	A	Dr	T	+	+	-0.1	5.2
	B	Dr	T	+	+	-2.0	1.3
	C	Dr	T	+	+	-0.1	2.5
msxE		Dr	T	+	+	-0.1	2
ngn1[2]	3.1delLATE	Dr	T	+	+	0	2.4
nurr1		Dr	T	-		4.5	4.5
otpa		Dr	T	-		1.0	2.4
f.otpb	A	Fr	T	-		-0.1	2.0

Gene	Enhancer	Species	Category+	CNS Expression*	Stable/Allele″	Position^ (kb)	Fragment Size (kb)
	B	Fr	T	-		-2.1	1.8
otpb	A	Dr	T	+	+; zc48	-4.8	4.4
	B	Dr	T	-		-4.8	1.8
	C	Dr	T	+	+	-4.1	3.7
	D	Dr	T	-		-7.2	2.5
	E	Dr	T	+	+	-4.1	2.7
	F	Dr	T	-		-1.3	1.3
	G	Dr	T	+	+	-4.8	3.4
	H	Dr	T	-		-4.1	1.1
	I	Dr	T	+	+	-2.8	0.4
pitx3	A	Dr	T	+	+; zc50	-0.3	6.7
f.TH	A	Fr	F	+	+; zc56	-5.4	5.1
	B	Fr	F	+	+	-2.7	2.7
	C	Fr	F	-	-	-5.4	3.1
	D	Fr	F	-	-	-8.0	2.7
	E	Fr	F	+	+	-2.6	0.8
	F	Fr	F	-	-	-1.1	1.3
	G	Fr	F	+	+	-7.7	7.8
	H	Fr	F	-	-	-6.1	6.0
	I	Fr	F	-	-	-6.1	3.5
	J	Fr	F	-	-	-2.6	0.3
	K	Fr	F	+	+	-2.3	0.5
	L	Fr	F	+	+	-2.2	0.4
	M	Fr	F	+	+	-2.3	0.3
	O	Fr	F	-	-	-1.8	0.7
rat TH[3]		Rn	F	-		-0.1	4.5
th	A	Dr	F	-		-12	2.3
	B	Dr	F	-		-4.7	4.5

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Gene	Enhancer	Species	Category+	CNS Expression*	Stable/Allele″	Position^ (kb)	Fragment Size (kb)
	C	Dr	F	-		-2.1	5.8
	D	Dr	F	-		7.3	6
uchL1	A	Dr	F	-		-0.8	1.1
slc18a2	A	Dr	F	-		0.1	0.3
	B	Dr	F	-		-3.1	1
	C	Dr	F	-		-10.6	1
	D	Dr	F	-		0.1	2

*Expression was evaluated from 12hpf through 96hpf.

**GENSAT1 BAC clones (Gong et al., 2003).

+"Category" refers to the gene type-F: functional (function of dopamine neurons), T: transcription factor involved in specifying dopamine neurons.

″Stable indicates that a stable transgenic line was generated and tested. If the line has been maintained this is indicated by an allele number.

^"Position" is relative to translation start site (negative is upstream); except for Lawrence-Berkeley clones, which lists flanking or intragenic region.

Abbreviations: LB: Lawrence-Berkeley clones; Fr: *Fugu rubripes* (pufferfish); Mm: *Mus musculus* (mouse); Rn: *Rattus norvegicus* (rat); Dr: *Danio rerio* (zebrafish).

[1] Visel et al., 2007;

[2] Blader et al., 2004;

[3] Schimmel et al., 1999.