The transcription factor orthodenticle homeobox 2 influences axonal projections and vulnerability of midbrain dopaminergic neurons

Chee Yeun Chung,1,2,3 Pawel Licznerski,1,2 Kambiz N. Alavian,1,2 Antonio Simeone,4,5 Zhicheng Lin,6 Eden Martin,7,8 Jeffery Vance7,8 and Ole Isacson1,2,3

1 Neuroregeneration Laboratories, Harvard Medical School, McLean Hospital, Belmont, MA 02478, USA
2 NINDS Udall Center of Excellence in Parkinson’s Disease Research, McLean Hospital/Harvard University, Belmont, MA 02478, USA
3 Harvard NeuroDiscovery Center, Boston, MA 02114, USA
4 CEINGE Biotecnologie Avanzate, Italy and SEMM European School of Molecular Medicine – Naples site, 80145 Naples, Italy
5 Institute of Genetics and Biophysics ‘A. Buzzati-Traverso’, CNR, 80131 Naples, Italy
6 Department of Psychiatry, Harvard Medical School and Mailman Neuroscience Research Centre, McLean Hospital, Belmont, MA 02478, USA
7 Miami Institute for Human Genomics, Miller School of Medicine, University of Miami, Miami, FL 33136, USA
8 NINDS Udall Center of Excellence in Parkinson’s Disease Research, University of Miami, Miami, FL 33136, USA

Correspondence to: Dr Ole Isacson,
Neuroregeneration Laboratories,
Harvard Medical School, McLean Hospital,
115 Mill Street, Belmont, MA 02478, USA
E-mail: isacson@hms.harvard.edu

Correspondence can also be sent to: Dr Chee Yeun Chung,
Whitehead Institute/ MIT,
9 Cambridge Center,
Cambridge, MA 02142,
E-mail: cychung@wi.mit.edu

Two adjacent groups of midbrain dopaminergic neurons, A9 (substantia nigra pars compacta) and A10 (ventral tegmental area), have distinct projections and exhibit differential vulnerability in Parkinson’s disease. Little is known about transcription factors that influence midbrain dopaminergic subgroup phenotypes or their potential role in disease. Here, we demonstrate elevated expression of the transcription factor orthodenticle homeobox 2 in A10 dopaminergic neurons of embryonic and adult mouse, primate and human midbrain. Overexpression of orthodenticle homeobox 2 using lentivirus increased levels of known A10 elevated genes, including neuropilin 1, neuropilin 2, slit2 and adenylyl cyclase-activating peptide in both MN9D cells and ventral mesencephalic cultures, whereas knockdown of endogenous orthodenticle homeobox 2 levels via short hairpin RNA reduced expression of these genes in ventral mesencephalic cultures. Lack of orthodenticle homeobox 2 in the ventral mesencephalon of orthodenticle homeobox 2 conditional knockout mice caused a reduction of midbrain dopaminergic neurons and selective loss of A10 dopaminergic projections. Orthodenticle homeobox 2 overexpression protected dopaminergic neurons in ventral mesencephalic cultures from Parkinson’s disease-relevant toxin, 1-methyl-4-phenylpyridinium, whereas downregulation of orthodenticle homeobox 2 using short hairpin RNA increased their susceptibility. These results show that orthodenticle homeobox 2 is important for establishing subgroup phenotypes of post-mitotic midbrain dopaminergic neurons and may alter neuronal vulnerability.
Keywords: axon; protection; Parkinson’s disease; neuropeptides; transcription factor
Abbreviations: Adcyap = adenylyl cyclase activating peptide; En1 = homeobox protein engrailed-1; LCM = laser capture microdissection; MPP+ = 1-methyl-4-phenylpyridinium; Otx2 = orthodenticle homeobox 2; PCR = polymerase chain reaction; shRNA = short hairpin RNA; SN = substantia nigra; VTA = ventral tegmental area

Introduction

Clinical interest has focused on the midbrain dopaminergic neurons due to their involvement in many neurological and psychiatric diseases. Considerable progress has been made on the biology and development of these neurons in an effort to understand how to treat such devastating illnesses. For example, many transcription factors that determine midbrain dopaminergic neuron phenotype have been identified, including nuclear receptor related 1 protein, pituitary homeobox 3, LIM homeobox transcription factors 1a and 1b, homeobox protein engrailed-1 (En1) and orthodenticle homeobox 2 (Otx2) (Ang, 2006; Prakash and Wurst, 2006). Even within midbrain dopaminergic neurons, there are two major subgroups; A9 dopaminergic neurons in the substantia nigra (SN) and A10 dopaminergic neurons in the ventral tegmental area (VTA). In addition to their anatomical location in the midbrain, their axons project to distinct targets; A9 dopaminergic neurons to the dorsolateral striatum and A10 dopaminergic neurons to the nucleus accumbens, cortex, septum, amygdala and olfactory tubercle. Moreover, these neurons show different susceptibility in Parkinson’s disease. Independent of specific aetiology, A9 dopaminergic neurons in the SN preferentially degenerate, whereas A10 dopaminergic neurons in the adjacent VTA are relatively spared. Our previous study demonstrated that molecular differences between A9 and A10 dopaminergic neurons may explain some aspects of their differential vulnerability (Chung et al., 2005, 2007). Understanding the transcriptional control determining these distinct dopaminergic neuronal populations will be a critical advance in developing therapies for the diseases caused by the dysfunction of these neurons.

Little is known about transcription factors that control the distinct phenotypes of midbrain dopaminergic neurons. Our previous laser capture microdissection (LCM) and microarray study in mice brought to light a well-known transcription factor, Otx2, as a potential contributor to subgroup specification of midbrain dopaminergic neurons since Otx2 messenger RNA levels were highly elevated in adult A10 dopaminergic neurons by approximately 6-fold compared with adult A9 dopaminergic neurons (Chung et al., 2005). Otx2 is a homeodomain transcription factor, whose expression is restricted to the forebrain and midbrain in the nervous system during development (Ang, 2006; Prakash and Wurst, 2006). Otx2 plays an important role in the proliferation and differentiation of midbrain dopaminergic neuronal progenitors during mitosis, demonstrated by studies using various Otx2 mutant mice (Puelles et al., 2004; Vernay et al., 2005; Omodei et al., 2008). In adult mice, elimination of Otx2 expression in the basal midbrain region overlapping the En1 expression domain resulted in a selective loss of axonal projection from dopaminergic neurons in the VTA, despite of an equal reduction in the number of midbrain dopaminergic neurons from both SN and VTA (Borgkvist et al., 2006). In light of these findings, we demonstrate that Otx2 is elevated in post-mitotic A10 dopaminergic neurons in mice and humans, and the levels of Otx2 affect the pattern of A10 axonal projection and vulnerability of midbrain dopaminergic neurons to a Parkinson’s disease-relevant toxin. These results suggest that differential expression of Otx2 between A9 and A10 dopaminergic neurons may be one of many ways to establish subgroup phenotypes of post-mitotic midbrain dopaminergic neurons.

Materials and methods

Quick tyrosine hydroxylase immunostaining and laser capture microdissection

Quick tyrosine hydroxylase immunostaining and LCM were performed according to the previous protocol (Chung et al., 2005, 2007). Briefly, adult C57/B6 mice (Jackson Laboratory, West Grove, PA) were anaesthetized with intraperitoneal sodium pentobarbital (300 mg/kg) and decapitated. The brain was removed and snap-frozen in dry ice-cooled 2-methylbutane (~60°C). Fresh frozen human midbrain blocks were obtained from Harvard Brain Tissue Resource Centre. Brains were cut using a cryostat with 10 µm (for mouse) or 18 µm (for human) thickness, mounted on LCM slides (Arcturus) and immediately stored at ~70°C. The tissue sections were fixed in cold acetone for 5 min, washed with phosphate buffered saline, incubated with rabbit anti-tyrosine hydroxylase (Pel-Freez Biologicals, Rogers, AR; 1:25) for 4 min, washed in phosphate buffered saline and exposed to biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA; 1:25) for 4 min. The slides were washed in phosphate buffered saline, incubated in avidin/biotinylated enzyme complex-horseradish peroxidase (Vectorstain, Vector Laboratories) for 4 min and the staining was detected with the substrate, diaminobenzidine. Sections were subsequently dehydrated in graded ethanol solution (30% each in water, 70% ethanol, 95% ethanol, 100% ethanol and twice for 5 min in xylene). For LCM, the PixCell II System (Arcturus, Mountain View, CA) was used to capture ~100–200 neurons. RNA was isolated using PicoPure RNA isolation kit (Arcturus, Mountain View, CA).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (PCR) was performed according to the previous protocol (Chung et al., 2005). Briefly, RNA samples from A9 and A10 dopaminergic neurons were reverse-transcribed into complementary DNA using Sensiscript reverse transcriptase (Qiagen, Valencia, CA) and oligodeoxythymidylic acid (oligo dt) as the primer. PCR reactions were set up in 25 µl reaction volume using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with 250 nM final concentrations of primers. For each primer pair, triplicates of three to five independently collected A9 and A10 samples were compared to quantify relative gene expression differences between these cells using...
the $^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Beta-actin was used as an internal control gene.

**Animals**

$\text{En}^{\text{Cre/+/Otx2}^{\text{fluox/fluox}}}$ male mice were mated to $\text{Otx2}^{\text{fluox/fluox}}$ female mice in order to generate $\text{En}^{\text{Cre/+/Otx2}^{\text{fluox/fluox}}}$ littermates used as controls. Three to 4-month-old male mice were analysed. All mouse studies were approved by the McLean Hospital Institutional Animal Care and Use Committee.

**Perfusion and tissue handling**

Animals were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbital and were sacrificed by exsanguination with the aid of ice-cold saline perfusion. For immunohistochemistry, the brains were then fixed with a 4% paraformaldehyde solution. The brains were then removed from the skull and placed in fresh 4% paraformaldehyde solution for 1 h and equilibrated through 20 and 30% sucrose solutions and refrigerated until cutting for immunohistochemistry.

**Immunohistochemistry**

Brains were cut while frozen in the coronal plane at a thickness of 40 μm on a sliding microtome and six series of sections were stored in cryoprotectant. One series of sections were processed for visualization of tyrosine hydroxylase via the biotin-labelled antibody procedure. Briefly, following several washes in a phosphate buffered saline solution containing 0.01% Triton X-100 (PBS-T), endogenous peroxidase was quenched in a 3% hydrogen peroxide solution and background staining was then inhibited in a 5% normal goat serum solution. Tissue was then incubated with rabbit anti-tyrosine hydroxylase antibody overnight (1:5000, Pel-Freeze, Rogers, AR). After three washes in PBS-T, sections were sequentially incubated in biotinylated goat anti-rabbit immunoglobulin G (1:500, Vector, Burlingame, CA) for 1 h and the Elite avidin–biotin complex (ABC Kits; Vector, Burlingame, CA) for 1 h separated by three washes in phosphate buffered saline. Tyrosine hydroxylase immunostaining was visualized following a reaction with 3,3-diaminobenzidine (Vector). Sections were then mounted on glass slides, allowed to dry, dipped into distilled H2O, dehydrated through graded alcohol (70, 95, 100%), cleared in xylene and coverslipped with di-n-butylphthalate in xylene mounting medium. For immunofluorescence staining, coverslips were washed with PBS-T and blocked with donkey serum. Coverslips were then incubated with anti-tyrosine hydroxylase (Pel-Freeze), anti-Otx2 (Neuromics, 1:500), or green fluorescent protein (Invitrogen, 1:1000) antibodies overnight and subsequently incubated in the following Alexa secondary antibodies (Invitrogen, Carlsbad, CA). Coverslips were mounted onto glass slides and visualized using confocal microscopy.

**Lentivirus encoding short hairpin RNA production**

Lentiviruses were produced as described (Miyoshi et al., 1998; Dittgen et al., 2004). Briefly, 293T cells (human embryonic kidney) were transfected by using the Perfectin transfection reagent (Genlantis, San Diego, CA) with three plasmids: expression vector and two helper, delta 8.9 and vesicular stomatitis virus G protein plasmids at 4, 30 and 22 μg of DNA per 175 cm cell culture flask. After 48 h, the supernatants were spun at 780g for 5 min, filtered at a 0.45 μm pore size, spun at 83 000g for 1.5 h and the pellet was resuspended in 100 μl of phosphate buffered saline. Titres were adjusted to $1–2 \times 10^6$ μl.

The DNA sequences encoding short hairpin (sh)RNAs: control (non-targeting) and Otx2-targeting were subcloned into a modified version of the lentiviral expression vector pFUGW (Lois et al., 2002) containing the U6 promoter to drive their expression (Dittgen et al., 2004) in front of the ubiquitin promoter—green fluorescent protein expression cassette. The structure of the short hairpin (sh) RNA was sense-loop-antisense. The oligonucleotides used for the construction of shRNA-targeting Otx2 were:

- up: TTTGGGTATGGACTTGCTGCATCCCATCTGTGGCTTC
- down: CGAAAAGGGTATGGACTTGCTGCATCCCATCTGTGGCTTC
- ACGGATGCACGGCAAGTCCCATATCCCTTTTT;
- CGGATGCACGGCAAGTCCCATATCCCTTTTT

Both control (non-coding) and Otx2-targeting hairpins contained the same loop sequence.

**Primary ventral mesencephalic cultures**

Primary cell cultures were performed using embryonic day 12.5 mouse embryos, as described elsewhere (Alberi et al., 2004). Briefly, ventral midbrains were isolated and tissue was dissociated using trypsin–EDTA (Invitrogen). The cells were seeded on poly-L-ornithine-laminin-coated coverslips at ~150 000 per coverslip, in medium, containing Dulbecco’s modified Eagle’s medium-F12, supplemented with 5% foetal calf serum, 0.25% bovine serum albumin (Sigma, Germany), 33 mM glucose, 50 U/ml penicillin, 50 U/ml streptomycin and 1% Fungizone (Invitrogen). The coverslips were incubated at 37°C for up to 3 weeks. The typical numbers of tyrosine hydroxylase-positive cells per coverslip were between 1500 and 1800 cells. All cell counts were normalized against the controls in each experiment. Each of the experimental conditions is represented by at least three coverslips in each experiment.

**Results**

**Orthodenticle homebox 2 is elevated in A10 (ventral tegmental area) dopaminergic neurons**

Our previous microarray analysis demonstrated that Otx2 mRNA was ~6-fold higher in A10 dopaminergic neurons compared to A9 dopaminergic neurons (Chung et al., 2005). In order to validate the mRNA levels of Otx2 in human A9 and A10 dopaminergic neurons without interference from other cell types, we used quick tyrosine hydroxylase immunostaining and the LCM technique to collect dopaminergic neurons selectively from the SN (A9) and the VTA (A10) in fresh frozen mouse (Fig. 1A–C) and human midbrain sections. Quantitative PCR using unamplified RNA isolated from LCM samples revealed that Otx2 mRNA levels were higher in A10 dopaminergic neurons in the VTA compared to A9 dopaminergic neurons in the SN (Fig. 1D). In addition to the elevated mRNA levels, Otx2 immunoreactivity was higher in A10 dopaminergic neurons in the medial VTA showed relatively stronger
Otx2 immunoreactivity compared to neurons in the lateral VTA (Fig. 1H–M). We next investigated the developmental expression of Otx2 in post-mitotic dopaminergic neurons in mouse ventral mesencephalon. Compared to cells in the intermediate zone, post-mitotic tyrosine hydroxylase-positive neurons in the marginal zone showed relatively weak Otx2 immunoreactivity at embryonic day 12 (Supplementary Fig. 1). Otx2 immunoreactivity became stronger in some midbrain dopaminergic neurons at embryonic...
day 15 (Supplementary Fig. 1), exactly when the separation of midbrain dopaminergic axonal projections starts (Hu et al., 2004). At embryonic day 17 when A9 and A10 dopaminergic neurons are clearly segregated, A10 dopaminergic neurons in the VTA showed strong expression of Otx2 compared with A9 dopaminergic neurons in the lateral SN (Fig. 2A–K). Otx2 expression was not exclusive to tyrosine hydroxylase-positive neurons (Fig. 2A–C).

Orthodenticle homeobox 2 regulates known A10-elevated gene expression

Since Otx2 is a transcription factor, we investigated Otx2-mediated gene expression profiles. Our previous microarray analysis of A9 and A10 dopaminergic neurons in adult mice identified two major categories of genes that were elevated in A10 dopaminergic neurons: axon guidance cues and neuropeptides (Chung et al., 2005). We first used the mouse dopaminergic cell line MN9D to overexpress Otx2 via lentivirus. MN9D cells normally exhibit a very low baseline level of Otx2 expression. Compared to control cells overexpressing yellow fluorescent protein with nucleus localization signal, Otx2 overexpression by lentivirus increased known A10-elevated axon guidance cue genes including neuropilin 1, neuropilin 2, and slit2 (Fig. 3A). A10-elevated neuropeptides such as vasoactive peptides and adenylyl cyclase activating peptide (Adcyap) were also significantly increased by Otx2 overexpression in MN9D cells (Fig. 3A). We next utilized primary ventral mesencephalic cultures to validate the results from MN9D cells. Primary ventral mesencephalic cultures were transduced with a lentivirus encoding yellow fluorescent protein–nucleus localization signal or Otx2 to examine the effects of Otx2 overexpression. Lentivirus encoding control shRNA or shRNA against Otx2 were also used to examine the effect of endogenous Otx2 downregulation. In A10 dopaminergic neurons, Otx2 overexpression resulted in an increase in levels of genes such as neuropilin 1, neuropilin 2, slit2, and Adcyap (Fig. 3B), whereas reduction of endogenous Otx2 by shRNA caused a decrease in levels of these genes (Fig. 3B). The degree of Otx2-mediated changes in gene expression was smaller in primary ventral mesencephalic cultures than in MN9D cells, probably because, unlike MN9D cells, dopaminergic neurons comprise only a small portion of heterogeneous primary ventral mesencephalic cultures. Overall, these results from primary ventral mesencephalic culture validated findings from MN9D cells, with the exception of vasoactive peptides.

Orthodenticle homeobox 2 conditional knockout mice lose A10 dopaminergic neuronal projection

Since Otx2 regulated the levels of axon guidance genes, we utilized the En1cre/+;Otx2flox/flox mice to investigate the role of Otx2
in governing the axonal projection of midbrain dopaminergic neurons. In these mice, Otx2 expression is selectively eliminated in the basal midbrain region overlapping the En1 expression domain. A previous report showed that the numbers of dopaminergic neurons were reduced in both SN and VTA to a similar degree, with a corresponding loss of tyrosine hydroxylase-positive fibres in striatum (mainly ventral) and olfactory tubercle in these mice (Borgkvist et al., 2006). We analysed these mice with the hypothesis that Otx2 may affect subgroup identity of midbrain dopaminergic neurons. We analysed known A10 neuronal projection targets including prefrontal cortex, nucleus accumbens, septum, amygdala and olfactory tubercle. Tyrosine hydroxylase immunohistochemistry results revealed dramatic reduction of tyrosine hydroxylase-positive projections in all of these targets in En1cre/+;Otx2flox/flox mice, whereas the projection to dorsolateral striatum, a target of the A9 dopaminergic neuronal projection, was

**Figure 3** Otx2 regulates expression of known A10-elevated genes in MN9D cells and primary ventral mesencephalic (VM) cultures. (A) MN9D cells were transduced with lentivirus encoding yellow fluorescent protein (YFP)-nucleus localization signal or Otx2 and harvested 4 days after transduction for mRNA isolation and quantitative PCR analysis. Overexpression of Otx2 using lentivirus in MN9D cells induced A10 elevated guidance cue molecules including neuropilin 1 (Npn1), neuropilin 2 (Npn2) and slit2; and A10 elevated neuropeptides, vasoactive peptide (VIP) and adenylyl cyclase activating peptide (Adcyap). (B) Primary ventral mesencephalic cultures were transduced with lentivirus encoding yellow fluorescent protein–nucleus localization signal, Otx2, control shRNA or shRNA against Otx2 on in vitro Day 1 and harvested at in vitro Day 8 for mRNA isolation and quantitative PCR analysis. Gene expression profiles in primary ventral mesencephalic cultures confirmed the findings in MN9D cells with the exception of VIP. Data are shown as mean ratios of lenti-Otx2 over lenti-yellow fluorescent protein–nucleus localization signal or lenti-Otx2 shRNA over lenti-control shRNA ± SEM (n = 5 for each condition; *P < 0.05 two-tailed t-test) and are representatives of two experiments with the similar trends. Plexin C1; EphnB3 = ephrin type-B receptor 3; CART = cocaine and amphetamine regulated transcript; GRP = Gastrin releasing peptide; CCK = cholecystokinin; NT-3 = neurotrophin 3.
largely spared (Fig. 4A–F, Supplementary Fig. 2). These results suggest that Otx2 may play a critical role in establishing the projection of A10 dopaminergic neurons.

Orthodenticle homeobox 2 makes dopaminergic neurons less vulnerable to 1-methyl-4-phenylpyridinium toxicity

One of the key characteristics of A10 dopaminergic neurons is reduced vulnerability to toxins. Since Otx2 regulates genes that are known to be protective, such as Adcyap or pituitary adenylate cyclase-activating polypeptide (Takei et al., 1998; Reglodi et al., 2004; Chung et al., 2005), it is plausible that Otx2 may contribute to the reduced vulnerability of A10 dopaminergic neurons. Therefore, we challenged ventral mesencephalic cultures with 1-methyl-4-phenylpyridinium (MPP+) after overexpressing or reducing Otx2 using the lentivirus encoding Otx2 or a shRNA directed against Otx2, respectively. Overexpression of Otx2 in ventral mesencephalic cultures using lentivirus resulted in ~25% increase in Otx2-positive tyrosine hydroxylase-positive neurons (Fig. 5A–C). After 10 μM MPP+ treatment, more tyrosine hydroxylase-positive neurons were observed in Otx2 overexpressing conditions than control yellow fluorescent protein–nucleus localization signal overexpressing conditions (Fig. 5D). On the other hand, reduction of endogenous Otx2 in ventral mesencephalic cultures caused ~25% decrease in Otx2-positive tyrosine hydroxylase-positive neurons in ventral mesencephalic cultures (Fig. 5E–G) and made tyrosine hydroxylase-positive neurons more susceptible to MPP+ toxicity compared to control conditions (Fig. 5H).

Discussion

Orthodenticle homeobox 2 regulates dopaminergic axonal projection

During mouse development, Otx2 is a critical determinant in proliferation and differentiation of mesencephalic dopaminergic progenitors (Vernay et al., 2005; Omodei et al., 2008). In the absence of Otx2 expression in En1<sup>cre/+;Otx2<sup>flox/flox</sup> mice, expression of key transcription factors in midbrain dopaminergic progenitors, LIM homeobox transcription factor 1a and Msh homeobox 1, and the proneural genes neurogenin 2 and achaete-scute homologue ash1 (Mash1) are missing at embryonic day 11.5, and tyrosine hydroxylase-positive neurons are dramatically reduced by embryonic day 12.5 (Omodei et al., 2008). Midbrain dopaminergic neurons become post-mitotic and differentiate at ~embryonic day 12–14 (Prakash and Wurst, 2006). Our data show that, despite the strong regional expression at embryonic day 12.5, Otx2 expression levels within midbrain dopaminergic neurons themselves at embryonic day 12.5 are relatively low, suggesting Otx2 may not have a cell-autonomous role at this time of development. This is different at embryonic day 15, when our data indicate strong Otx2 expression in midbrain dopaminergic
neurons as they start to migrate to take position in either the A9 (SN) or the A10 (VTA) area and send axonal projections to their proper targets (Hu et al., 2004). Our data thus raise the interesting possibility that, once a pool of post-mitotic mature dopaminergic neurons has been determined by regional expression of Otx2 and other transcription factors in the ventricular and intermediate zone, differential expression levels of certain transcription factors such as Otx2 in post-mitotic midbrain dopaminergic neurons may shape their subtype characteristics.

One category of genes elevated in adult A10 (VTA) dopaminergic neurons relate to axon guidance cues, including neuropilin 1, neuropilin 2, ephrin B2, ephrin B3, plexin C1 and slit 2. Even in developing midbrain dopaminergic neurons within ventral mesencephalon culture or cell lines that mimic immature midbrain dopaminergic neurons (MN9D) (Heller et al., 1996), we show that Otx2 overexpression leads to an increase in some of these genes, including neuropilin 1, neuropilin 2 and slit 2. These results suggest that Otx2 may regulate the expression of these genes and that an axonal guidance cue map may be maintained in the adult stage. It is plausible that the differential expression of Otx2 in midbrain dopaminergic neurons observed during embryonic day 15 and embryonic day 17 regulates levels of axonal guidance cues during this critical time for axonal projection, thus contributing to the distinct projections pattern of A9 (SN) and A10 (VTA) dopaminergic neurons. Indeed, reduction of Otx2 in the knockout mice showed the dramatic reduction of A10 (VTA) projections and sparing of A9 (SN) projections despite the fact that dopaminergic neurons in both SN and VTA were reduced in these mice. We suggest that in these mice, the axonal projection of A10 dopaminergic neurons was inhibited due to the reduced levels of A10-elevated guidance cues such as neuropilin 1, neuropilin 2 and slit 2. It remains unclear how the A9 projection to the dorsolateral striatum is preserved despite significant loss of dopaminergic neurons in the SN. It may be that the axonal projections

Figure 5 Otx2 overexpression protects dopaminergic neurons from MPP+ toxicity in primary ventral mesencephalic cultures. Primary ventral mesencephalic cultures were transduced with lentivirus encoding yellow fluorescent protein (YFP)–nucleus localization signal, Otx2, control shRNA or shRNA against Otx2 on in vitro Day 1 and 10 μM MPP+ was applied to the culture at in vitro Day 8. Cultures were fixed at in vitro Day 10 and stained with tyrosine hydroxylase for tyrosine hydroxylase-positive cell counting. When Otx2 was overexpressed by lentiviral delivery of Otx2 with multiplicities of infection (MOI) of 5 and 10 (A–C), dopaminergic neurons were more resistant to 10 μM MPP+ compared to yellow fluorescent protein–nucleus localization signal overexpressing ventral mesencephalic cultures (D). When Otx2 levels were reduced by lentivirus encoding shRNA against Otx2 with an MOI of 10 (E–G), dopaminergic neurons become more vulnerable to 5 μM MPP+ toxicity compared to control shRNA transduced ventral mesencephalic cultures (H). Data are shown as mean ± SEM (n = 5 for each condition; *P < 0.05 two-tailed t-test) and are representatives of two experiments with the similar trends. GFP = green fluorescent protein.
A9 dopaminergic neurons collateralize excessively to compensate for the cell loss. Our preliminary data utilizing Fluorogold retrograde tracing indicate that the projection to the dorsolateral striatum of these mice did not originate from A10 dopaminergic neurons (data not shown). Taken together, our data suggest that Otx2 may be a key transcription factor influencing dopaminergic projection, potentially by elevating levels of certain guidance cues including neuregulin 1, neuregulin 2 and slit2. Although the majority of A10 dopaminergic neurons are Otx2-positive, a small number of dopaminergic neurons in the VTA are Otx2-negative. This suggests that, even within the VTA, subgroups may exist. The presence of Otx2 dopaminergic neurons in the VTA raises two possible interpretations: (i) these neurons still project to the same or similar targets and their axonal projection is controlled by factors other than Otx2 expression; or (ii) these neurons do not project to VTA targets but rather project to the medial or dorsolateral striatum.

Orthodenticle homeobox 2 regulates the vulnerability of midbrain dopaminergic neurons

Another prominent category of genes elevated in A10 (VTA) dopaminergic neurons is neuropeptides and their receptors, including vasoactive peptides, Adcyap, cocaine and amphetamine regulated transcript (CART), gastrin releasing peptide, calcitonin gene related transcript (CART), gastrin releasing peptide, calcitonin gene-nicotinic acetylcholine receptor subunit alpha (nAChRα) and somatostatin. Among these, Otx2 overexpression increased mRNA levels of Adcyap in both MN9D cells and ventral mesencephalic cultures, whereas reduction of endogenous Otx2 levels diminished them in ventral mesencephalic cultures. Adcyap belongs to the family of peptides containing secretin, glucagons and vasoactive peptides (Ogi et al., 1990; Arimura, 1992). It is thought to act as a neurotrophic factor during development as well as a neuroprotective factor against various insults (Arimura, 1998; Vaudry et al., 2000). Adcyap is also neurotrophic for tyrosine hydroxylase-positive neurons in primary ventral mesencephalic culture (Takei et al., 1998; Reglodi et al., 2004). We previously demonstrated that Adcyap protected both PC12 cells and the dopaminergic neurons of primary ventral mesencephalic cultures from MPP+ induced toxicity (Chung et al., 2005). In addition, Adcyap infusion into the SN protects dopaminergic neurons and improves behavioural deficits in a rat model of Parkinson’s disease (Reglodi et al., 2004). These findings suggest an increase in Adcyap levels is neuroprotective in dopaminergic neurons. Adcyap induction by Otx2 overexpression may thus contribute to the resistant nature of A10 (VTA) neurons against toxins.

Considering the complexity of A9 and A10 dopaminergic neuronal differences, it is likely that transcription factors other than Otx2 contribute to altered vulnerability of these cells. For example, in our experimental paradigm, Otx2 only elevated gene expression levels of Adcyap, but failed to control expression of other A10-elevated neuropeptides that are shown to be neuroprotective (Chung et al., 2005).

In conclusion, our data demonstrate a critical role for Otx2 in shaping neurobiological phenotypes of post-mitotic midbrain dopaminergic neurons, including axonal projection and vulnerability to degeneration.

Acknowledgements

The authors thank Dr Vikram Khurana for discussion and reading of the manuscript; Emily Stackpole, Kari Ording, Casper Reske-Nielsen, Kristen Lee and Alyssa Yow for their excellent technical assistance.

Funding

This study was conducted at McLean Hospital. NIH/NINDS P50 (NS39793) to O.I.; Parkinson’s Disease Udall Research Centres of Excellence to McLean/Harvard Medical School, the Michael Stern Foundation for Parkinson’s Disease Research, the Orchard Foundation, the Consolidated Anti-Aging Foundation, Harold and Ronna Cooper Family; Stem Cell Project of Fondazione Roma, Rome Italy to A.S.; National Institutes of Health Grant NS39764.

Supplementary material

Supplementary material is available at Brain online.

References


Otx2 determines dopamine neuron subgroup identity

Brain 2010: 133; 2022–2031 | 2031
