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Moving Beyond Tyrosine Hydroxylase to Define Dopaminergic Neurons for Use in Cell Replacement Therapies for Parkinson's Disease

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SHORT RUNNING TITLE: REGULATION OF TH TRANSCRIPTION IN NEUROGENESIS

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Abstract:

Cell replacement therapies are an attractive mode of treatment for neurodegenerative disorders as they have the potential to alleviate or modify disease symptoms and restore function. In Parkinson's disease, the cell type requiring replacement is dopamine-producing neurons of the midbrain. The source of replacement cells is contentious, with opinion still evolving. Clinical trials have previously used fetal brain tissue, however this will likely be superseded by the use of embryonic or induced pluripotent stem cells, due to their greater availability and homogeneity. One significant caveat in the use of any cell sources for therapy is that cells must first be adequately characterised and purified. The gold standard marker in the identification of dopaminergic neurons is tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis, catalyzing the conversion of L-tyrosine to L-DOPA. However there are multiple ways of measuring TH readout, and potential flaws in the fidelity of TH expression. This review will look at the complex regulatory mechanisms that govern different facets of TH expression, including reported differences in TH expression *in vitro* and *in vivo*. We will also examine the regulation of the *TH* gene; assessing the which, the where and the when of TH expression. We will look at how knowledge of regulation of the *TH* gene can be utilised to enhance research efforts. And, finally we will delve into the transcription factors that govern elements of TH expression, and which may prove more effective for defining appropriate dopaminergic neuron precursor cells.

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INTRODUCTION

Cell replacement therapy (CRT) for neurodegenerative disorders is an experimental treatment approach that aims to modify disease progression by replacing disease-vulnerable, dead or damaged cells. A prominent example has been Parkinson's disease (PD), where the aim has been to replace a specific population of dopamine producing (dopaminergic) neurons. Now with over 30 years of experimental data and human clinical trials using fetal tissue, it is apparent that the subtle differences in apparently similar cells translate into functional differences upon transplantation [1]. With the emergence of embryonic stem (ES) and induced pluripotent stem (iPS) cells, the ability to differentiate, sort and select specific cell types has taken on a renewed level of importance.

In the case of CRTs for PD, identification of the requisite characteristics of specific populations of dopaminergic neurons is essential. The gold standard marker used to identify a dopaminergic neuron is the presence of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of dopamine, catalyzing the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). However there are multiple ways of measuring TH readout and some studies are beginning to uncover potential flaws in the fidelity of associating TH expression with post-mitotic dopaminergic neurons. As TH is also necessary for the catalysis of precursors to other catecholamines, its presence is clearly not specific to dopaminergic neurons alone. Further, transplantation studies have demonstrated that fetal tissue taken from the dopaminergic precursor rich regions of the midbrain, the substantia nigra (SN) or the ventral tegmental area (VTA), have different abilities to integrate into the host environment. This prompts the question of when, where, and indeed whether TH is an appropriate marker for transplantable dopamine cells?

GENERATION OF THE NEUROTRANSMITTER DOPAMINE

Three distinct catecholamine molecules are utilised as neurotransmitters in the nervous system: dopamine, noradrenaline (norepinephrine), and adrenaline (epinephrine). These three neurotransmitters are synthesized in catecholamine-producing (catecholaminergic) neurons from the common metabolite tyrosine, via DOPA, dopamine and noradrenaline, to adrenaline. Four enzymes are sequentially involved in the biosynthesis of adrenaline: (1) tyrosine hydroxylase (TH, aka tyrosine 3-mono-oxygenase); (2) aromatic L-amino acid decarboxylase (AADC, or DOPA decarboxylase, DDC); (3) dopamine beta-mono-oxygenase (dopamine beta-hydroxylase, DBH); and (4) noradrenaline N-methyltransferase (phenylethanolamine N-methyltransferase, PNMT) (Figure 1) [2]. As TH catalyzes the first and rate-limiting step of catecholamine synthesis, its expression is necessary for neurotransmitter specification of all catecholaminergic neurons. The expression and activity of the enzymes DBH and PNMT is only essential for the adrenergic phenotype.

PD symptoms, pathology and traditional dopamine replacement strategies

PD is a chronic and progressive condition characterised by the cardinal motor symptoms of resting tremor, rigidity, bradykinesia and postural instability [3, 4]. However, a substantial proportion of people with PD also experience a range of non-motor symptoms such as impairments of executive functions (defined as the ability to plan, organise, and regulate goal directed behaviour) and visuo-spatial processing, as well as a depression and anxiety.

Pathologically, time course studies [reviewed in 4] have demonstrated that neurodegeneration in PD first touches select populations of dopaminergic neurons in the hindbrain (dorsal motor nuclei of the hypoglossus and vagus nerves), the midbrain (giganto-cellular and dorsal part of the raphe nuclei) and the forebrain (anterior olfactory nucleus and olfactory bulb) regions, before extending into the midbrain areas of the SN and VTA [4]. However it is the degeneration of SN dopaminergic neurons - which results in the depletion of striatal dopamine levels - that is recognised as the core pathological event in PD.

While dopamine itself is unable to cross the blood-brain-barrier, treatments have focused on using dopamine precursors or agonists to replace dopamine levels in the striatum. The mainstay of PD pharmaceutical therapy is L-DOPA, first prescribed by George Cotzias in the late 1960s. L-DOPA is very effective in the early stages of PD as it can cross the blood brain barrier (even though only a small percentage of administered L-DOPA actually does). Then, all that is required *in situ* is its conversion to dopamine by the enzyme DOPA decarboxylase (DDC), a reaction catalysed by vitamin B6 (Pyridoxal phosphate). Unfortunately, with time, the efficacy of L-DOPA treatment wanes and patients develop on-off fluctuations with drug induced dyskinesias [5, 6]. Thus CRTs for PD have long held promise as a disease modifying treatment option.

Dopamine replacement via cell therapies

Similar to pharmaceutical approaches, CRTs developed for PD have focused on delivering dopamine producing cells to the striatum. The typical approach using grafts of fetal tissue has been to isolate recently differentiated dopaminergic neurons derived from the ventral mesencephalon (VM) of 6- to 8-week-old human embryos and engraft the tissue into the striatum of PD patients. The VM is not a homogenous population, and contains various cell types which are known to produce both morphologically and functionally distinct dopamine neurons [7, 8] and other neurotransmitter cell types. This is important, as it has clear implications for the progression of PD CRT: upon grafting into the adult striatum, dopaminergic-precursor neuroblasts derived from the ventral midbrain display different abilities to survive and innervate the striatum and surrounding tissues [9, 10]. For example, in a manner reflective of their adult projection pathways, SN precursor cells are able to form connections with the host striatum, whilst VTA grafts tend to form synapses within the graft but do not extend into the surrounding host striatum [9]. Transplantation of an undefined and heterogeneous population of cells can, in the best case simply lead to low survival of grafted cells, and in the worst case lead to unregulated dopamine release or even teratoma formation.

Thus, the identification, isolation and separation of progenitor or newly post-mitotic dopaminergic neurons that will differentiate into fully mature SN dopaminergic neurons is necessary for the clinical advancement of CRTs for PD. Which brings us back to TH, the presence of which is still the gold standard marker of a dopamine neuron.

TH EXPRESSION AS A MARKER OF DOPAMINE NEURONS

Mutation studies and expression patterns

There is no doubt that TH is critical for dopamine production and consequently plays an important role in PD. Severe mutations in the *TH* gene lead to infantile Parkinsonism, with patients being profoundly disabled from infancy (<6 months) [11]. *TH* gene mutations can also cause progressive infantile encephalopathy, in which children have persistent encephalopathy coupled with motor disability. In some cases, no benefit is observed even following directed treatment with L-DOPA [12, 13].

TH expression and its co-localisation with dopaminergic neuronal differentiation has been covered in-depth by many other excellent review articles [14-16], and it is not our intention to revisit this in any detail here. Rather we note that: during development, TH expression is widely accepted to first occur around 10.5 days of embryonic (mouse) development in cells of the intermediate zone of the developing neural tube [17]; BrdU-pulsing demonstrated that TH is expressed before or during withdrawal of a cell from the cell cycle in SN dopamine neurons [18]; and in the ventral midbrain its expression occurs at 11-12 days of embryonic development, which coincides with the appearance of the first postmitotic dopaminergic neurons which migrate to form the SN and VTA over coming developmental days [19, 20]. Thus during development TH expression coincides with cells that are still mitotic or very early postmitotic.

TH based cell sorting strategies

Given the limitations of current cell culture differentiation protocols, which at best generate in the order of 20% dopaminergic neurons [21, 22], fluorescence-activated cell sorting (FACS) could be used to enrich or isolate the cells of interest. Given its expression patterns, TH has been seen by the scientific community as an ideal candidate sorting marker. One such strategy would be to insert a fluorescent gene downstream from the *TH* promoter and then use FACS to isolate the cells of interest. Kessler et al. [23] generated transgenic mice with green fluorescent protein (GFP) expression under control of the TH promoter, randomly inserting a *TH* promoter-GFP plasmid into the genome. Unfortunately, although GFP was expressed in midbrain dopamine neurons, it was also expressed in noncatecholaminergic cells. It may be more appropriate to use a gene that is more specific for dopamine neurons, such as the dopamine transporter (DAT), and the scientific community has starting to explore this alternative; *DAT*-GFP knock-in ES cells have been used to create *DAT*-GFP knock-in mice [24]. Dopamine neurons recovered from these *DAT*-GFP embryonic mouse brains can be purified by FACS and survive replating in tissue culture [24]. Of course, cell source is of critical importance here. What may be an appropriate marker for sorting pre-differentiated ES cells may be totally inappropriate for sorting cells from VM tissue, due to the structural and supportive limitations imposed by a mature neuron *in situ*.

Is TH an appropriate marker of potential dopamine production capability in PD CRTs?

Given that TH expression *in vivo* occurs in both committed precursors and mature dopamine cells, it has proven useful in identifying early stage cells appropriate for grafting, as well as for characterising the dopamine production capabilities of mature cells. As already discussed, there is a pressing need to differentiate and select specific progenitor cells predestined to differentiate into specific subtypes of dopamine neurons (i.e. those characteristic of the SN) for the development of effective CRTs for PD. While selecting a marker based on its *in vivo* role, it needs to be taken into consideration that cells removed from the constrictions imposed by being part of the developmental milieu within the embryo will be subject to different constraints upon expression of genes, and this of course includes *TH*. Unfortunately, extrapolating the usefulness of TH as a marker of specific cell types in highly manipulated circumstances is unlikely to be straightforward.

The *TH* gene has to be responsive to cues to guarantee adequate and appropriate homeostatic expression and function of its enzyme and through this, the production of catecholamine neurotransmitters. This responsiveness occurs developmentally, defining when and where TH expression occurs during brain and peripheral nervous system formation; it occurs on a cell-specific basis, defining exactly which neurons express TH and thus synthesise catecholamines; and it occurs on an even finer scale, with TH expression levels adapting to changing conditions within the environment of the adult nervous system. This distinction is particularly important with the advent of iPS cells and other distinct types of progenitor cells, themselves with distinct origins and range of differentiation capabilities. Within this context, is TH the best marker? To understand the role of TH, and evaluate its potential for defining dopaminergic cells suitable for transplantation in PD, it is essential to understand the regulation of TH expression.

CONTROLLING TH EXPRESSION

Growth and other factors

Expression levels of TH are highly responsive to signalling molecules, both *in vivo* and *in vitro*. Glial cell line-derived neurotrophic factor (GDNF), on top of its well-described role in dopaminergic neuron survival, will also induce expression of a TH transcriptional reporter, as well as endogenous mRNA and protein expression in a panel of human neuroblastoma cell lines [25]. Adding strength to this being a direct mechanism, this induction only occurred in the cellular fraction expressing the Ret receptor tyrosine kinase (a GDNF receptor). The histone deacetylase (HDAC) inhibitor, sodium butyrate, a short chain fatty acid, likewise also regulates TH gene expression [26]. Fibroblast growth factor (FGF) signalling also plays a role; FGF1,2,4, and 9 all induce TH expression, mediated at least in part via MAPK phosphorylation. Certainly, MEK/ERK inhibitors are sufficient to block TH expression [27].

Perhaps not surprisingly, TH gene-expression is homeostatically responsive to dopamine levels, and its transcription is inhibited by dopamine. The dopamine antagonist haloperidol elevates both number and density of TH-positive cells within the pars reticulata of the SN within 8 hours of administration, although the dopamine receptor agonist quinripole showed no alteration in TH staining [28]. The antipsychotic/antihypertensive reserpine (a vesicular monoamine transporter antagonist that inhibits, among other things, dopamine transport) actively decreases transcription from *TH*, as shown by nuclear run-on experiments in rat phaechromatoma PC12 cells, and after injection of reserpine into the rat locus ceruleus and adrenal medulla [29].

Homeostatic response mechanisms and hormone levels regulate TH expression. Progesterone, acting most efficiently through the PR-B receptor, stimulates *TH* transcription [30]. Hypoxia too activates *TH* transcription [31, 32]; nicotine administration causes a constant increase in *TH* gene transcription [33, 34] and a transitional increase in noradrenergic cells of the locus ceruleus; immobilisation stress even activates *TH* gene transcription in the locus ceruleus and adrenal glands. Furthermore, this relationship is dependent upon the duration of the stress [35-37].

These regulators of TH expression have direct implications for the use of TH as a marker *in vitro*. In any cell culture environment, cells are exposed to a suite of different growth factors, many of which will influence the expression of TH, either aberrantly or otherwise. This phenomenon may be transient, with TH expression disappearing following growth factor withdrawal, or more permanent, signifying a conversion to a TH-expressing cellular phenotype and the cell's commitment to differentiate along a particular lineage. Either way, for CRTs to be successful, this issue requires greater attention than it has been given to date.

Epigenetic control mechanisms

As with many non-constitutively expressed mammalian genes, chromatin remodelling regulates the activity of the *TH* promoter. Both nucleosome repositioning and alterations in histone acetylation patterns are associated with changes in promoter activity in distinct cell lines [38]. The HDAC inhibitors, trichostatin A (TSA) and sodium butyrate, induce human *TH* promoter activity both in neuronal and non-neuronal cell lines [39]. Methylation, too, plays an important role in regulating TH expression. CpG island cytosine residues are specifically methylated in neural stem cells (with low TH expression) but not in SH-SY5Y neuroblastoma cells [40]. Treatment of TH-negative cells with the demethylating agent 5-Azacytidine increases the fraction of TH-expressing cells in human neuroblastoma CHP212 cells [41] and human neural stem cells [40]. Thus it is clear that epigenetic programming plays an important role in regulating expression from the *TH* gene.

Cis-elements within the *TH* promoter & enhancers

The *TH* gene is subject to highly complex mechanisms of transcriptional regulation. It is a single copy gene, encoded in human DNA on chromosome 11p15. The human *TH* gene encodes 14 exons, and is expressed as at least 9 alternate transcripts, the consequence of using alternate exons and additional donor sites, and resulting in tissue-specific splicing [15]. Enzymatic properties of different alternate isoforms can vary by as much as 40% [42]. Multiple studies have provided a finely detailed understanding of the *cis*-regulatory elements that are required for the spatio-temporal and cell specific instigation, maintenance, and perturbation of level of TH expression.

Mutations, bioinformatics, reporters and deletion constructs have identified multiple elements that drive the expression of TH. Promoter elements in the region inclusive of 9 kilobases (kb) upstream of the *TH* transcriptional start site (TSS) are sufficient to drive a tissue- and developmental stage-specific expression of the gene in transgenic mice [43]. These same elements drive *TH*-GFP expression in most midbrain dopaminergic neurons of transgenic mice, at least during early development [19]. Interestingly, even the mammoth 9kb promoter region does not drive *exactly* the same pattern of endogenous TH expression, as GFP expression is rapidly lost during foetal development, to be re-established to adult levels later [44]. The cellular distribution of *TH*-eGFP, when compared to TH antibody staining, is far from perfectly faithful: with an 80 to 94% overlap in the SN pars compacta, and a 60 to 85% overlap in VTA [44]. Ectopic *TH*-reporter-driven GFP expression is also

observed in 8 to 13% of cells that do not label with TH antibody [19]. This indicates the existence of multiple control elements that are required for accurate and faithful TH expression; some of which are present within the 9kb upstream of the TSS, and others that clearly are not (at least in a rat *TH* promoter being driven in mouse).

Smaller promoter/enhancer regions also recapitulate certain aspects of TH expression. Two 100 base pairs (bp) elements within 3kb of the *TH* TSS function as bidirectional enhancers, supporting long term, neuron-specific expression [45]. TH expression in most catecholaminergic neurons *in vivo* is conveyed by a 4.5kb region 5′ of the TSS of the rat *TH* promoter [44]. Activity of shorter deletion constructs of the *TH* promoter has been also characterized using transgenic mice [46, 47], while these constructs do recapitulate some aspects of TH expression, the aberrations are rife.

Promoter mutation and analysis has been instrumental in identifying several distinct regions within the relatively proximal promoter region. These *response elements* control different aspects of TH expression (Figure 2). Interestingly, transgenic reporter mice have shown that enhancer elements directing region-specific expression exist as spatially separated functional elements within the *TH* promoter. A hypothalamus regulatory element was localised between -2.5 and -3.4kb of the rat *TH* promoter and a midbrain-specific regulatory element has been localised to between -0.8 and -2.5kb of the TSS [46]. Olfactory bulb-specific elements have not yet been identified and are potentially located outside the 6.0kb assayed in this study [46]. Promoter segment analysis has also defined and localised response elements in a precise manner. Initially, this showed that differing expression of distinct promoter elements was observed in distinct cell lines. Cell lines with differing permissiveness to TH expression either did, or did not, express elements from different locations relative to the *TH* TSS. Human neuroblastoma BE(2)-C-16 cells (which are 50% immuno-positive for TH expression) and human renal carcinoma 293FT cell (which are not permissive for TH expression) will both express some promoter elements together, whereas other promoter elements will only express in the cells permissive to endogenous TH expression [38]. Thus there is a spatial separation of *TH* promoter elements necessary for different aspects of basal expression, cell-type specificity, maintenance and responsiveness.

Kim *et al*. [48] bioinformatically scoured the 5.5kb fragment of human genomic DNA immediately upstream of the *TH* coding region, and located sequences corresponding to a TATA box as well as multiple consensus sites for transcription factors. Consensus binding sequences for both basal (TATA and CRE), and dopaminergic neuron-specific (NBRE and BBE) transcription factors were all readily identified. Two regions within the 5′ flanking region displayed exceptionally high homology. Domains encompassing -2384 to -2323bp and -23 to -65bp relative to the TSS contain NBRE, BBE, CRE elements, which are known to play important roles in dopaminergic neurogenesis [48]. There are several BBE cassettes in promoters of the human, mouse, and rat *TH* genes [49]. The proximal promoter contains multiple transcriptional elements that regulate expression, including response elements to AP-1, AP-2, cyclic AMP, and NGFI-B, as well as an E-box, responsive to rlTF2 and CDP2 [50-55] (Figure 2).

Bioinformatic analyses have been validated in cultured cells and reporter assays. By fusing a luciferase reporter gene to a 3301bp human *TH* promoter fragment (-3174 to +127bp), Kim *et al*. [48] demonstrated activity in human SH-SY5Y neuroblastoma cells, but minimal luciferase activity in human F3 neural stem cells. Deletion analysis identified a repressor element, not active in SH-SY5Y cells, 1.2kb upstream of the TSS, which was responsible for repressing promoter activity by 85% in neural stem cells [48]. The same group [56] later showed, functionally, that a specific NRSE/RE1 site within the human *TH* promoter, located between nucleotides −204 and −184bp relative to the TSS, is critical for repression of *TH* gene expression (deletion studies showed only one of three NRSE/RE sequences was functional). NRSE/RE1 sites are occupied by NRSF/REST protein, which inhibits the transcription of neuron-specific genes in both undifferentiated neuron precursors and cells of non-neuronal origin [56]. NRSF/REST represses expression from the *TH* gene through this *cis*-element, demonstrated using a NRSF/REST mutated reporter [56]. TH expression is also responsive to retinoic acid induction, and this occurs via AP2 binding to multiple functional sites in the *TH* promoter [55]. The HDAC complex participates in *TH* gene regulation through a *cis*-element within the human *TH* promoter, located between nucleotides −204 and −184bp, revealed by experiments using HDAC inhibitors and normal and mutated reporter constructs [56].

Outside the proximal promoter

The first intron of the mouse *TH* gene also contains control elements that result in it being an important mediator for tissue-specific gene expression, as shown by a recent report based on enhanced yellow fluorescent protein (eYFP) knock-in mice reporter system [57]. In this study, eYFP was placed, via homologous recombination, under the control of the endogenous mouse *TH* promoter and used to generate a mouse stem cell line and, from this, knock-in mice. Importantly, in this reporter model, eYFP replaced the first exon and first intron of the mouse *TH* gene, while the other allele continued to express endogenous TH. Analysis of this reporter showed that eYFP and endogenous TH gene expression were not completely overlapping, both in embryonic stem cell-derived neurons and in brain tissues isolated from knock-in mice, in spite of having the entire endogenous mouse *TH* promoter driving the expression of the reporter gene [57]. *Per se*, such a finding indicates that the deleted region of the mouse *TH* gene encodes for *cis*-acting regulatory sequences, which are necessary to confer an accurate tissue-specific *TH* gene expression.

TRANSCRIPTION FACTORS CONTROLLING *TH* EXPRESSION

Transcription factors are a hot topic in the conversion of precursor cells to dopaminergic neurons for use in CRTs: they have the power to reprogram the cellular transcriptional platform from the gene level, driving cells to adopt specific and characteristic phenotypes. Transcription factors are nuclear proteins that regulate the transcription of downstream target genes, dictating when and where many genes are switched on or off, and adjusting levels of transcription. Loss-of-function studies have clearly defined an essential role for the transcription factors nuclear receptor-related subfamily 1 (Nurr1), Foxa2, pituitary homeobox 3 (Pitx3), engrailed (En1) and orthodenticle homologue 2 (Otx2), and others, in dopaminergic neuron differentiation [58-62]. Interestingly, many of the sequence-specific transcription factors that specify and drive differentiation of midbrain dopaminergic neurons also function as direct upstream activators of the *TH* gene.

Nurr1: The *TH* promoter contains three Nurr1-binding motif sequences (NBREs) and TH promoter deletional analysis indicates that less than 1.0kb relative to the TSS of upstream sequence, encompassing the NBRE-like motifs, is responsible for the effects of Nurr1 transactivation of *TH* [63] (Figure 1). Nurr1 activation of *TH* occurs in a cell type-dependent manner and is specific to neural cells, and in a grand act of synchrony in dopaminergic neurogenesis, Nurr1 also regulates the dopamine transporter (*DAT*), and the vesicular monoamine transporter 2 (*VMAT2*) genes necessary for dopaminergic neuron functionality [64]. In hippocampal cells overexpressing Nurr1, TH is ubiquitously expressed. In proliferating cells TH expression occurs at low levels and is upregulated in differentiating conditions. This suggests that Nurr1 overexpression activates *TH* in undifferentiated cells and, additively, in a fully activated neuronal differentiation program. Alternatively, in non-neuronal cells, such as rat FF12 primary skin fibroblast and human 293 kidney cell lines, over expression of Nurr1 fails to activate *TH* expression [53]. These data indicate that Nurr1 alone is not sufficient to activate *TH* in every cellular background (such as non-neural cell lines).

Foxa2: Foxa2 directly binds to the *TH* promoter and dose-dependently activates *TH* gene transcription, which is induced by Nurr1 [65]. Foxa2 promotes *TH* expression only in a subset of neural progenitor cells that express endogenous Nurr1. Furthermore, Foxa2 and Nurr1, simultaneously overexpressed in neural progenitor cells promote *TH* expression in a synergistic fashion.

Pitx3: Pitx3 is a homeodomain-containing protein that recognizes the bicoid binding element in the promoter sequence of *TH*. Co-transfection of Pitx3 with a *TH* reporter construct identified Pitx3 as a trans-acting factor that activates or inhibits reporter gene transcription depending on the permissivity of the cell line to TH expression (TH-positive or TH-negative type). However it is thought that Pitx3 functions to activate *TH* expression *in vivo*, as initiation of Pitx3 transcription coincides with the initiation of TH expression [49]. Regulation of the *TH* gene by Pitx3, at least in mice, differs between two distinct dopaminergic midbrain regions, the SN and the VTA [66]. Like Foxa2, Pitx3 also acts in concert with Nurr1; the cooperation of Nurr1 and Pitx3 is necessary, but not entirely sufficient, to induce a dopaminergic phenotype in midbrain neurons [53, 67]. Messmer et al. [68] showed that simultaneous overexpression of Nurr1 and Pitx3 proteins does not have an additive effect on *TH* transcription, as the level promoted independently by each of the factors in the non-

neuronal human HEK293 cells or mouse D3 ES cells was equivalent to that promoted by simultaneous overexpression of both. Pitx3 also recognizes specific binding elements in the promoter sequences of *Map2*, *DAT* and *VMAT2* genes.

Co-regulatory proteins that activate/repress TH: DJ-1 (also known as PARK7) [69] regulates TH action at two levels, initially transcriptionally and then later to increase the enzyme's activity [70]. PTB-associated splicing factor (PSF) is a transcription repressor that upon binding to the promoter region of the *TH* gene, represses its expression. However, DJ-1 can bind to PSF and in doing so sequesters the PSF/co-repressor complex, resulting in the induction of *TH* gene expression in cultured human cells [69].

Discrepancies between species

One monumental task that must be accomplished is the assimilation of *TH* regulation data from distinct species. These studies have predominantly been conducted on the *TH* genes from human, mouse and rat, so it is apt to first ask, how similar are human, mouse and rat *TH cis*-regulatory sequences? Perhaps not unsurprisingly, sequence analysis shows low overall homology between human, mouse and rat *TH* promoter regions [48]. Comparative analysis of the sequences of the human, mouse and rat *TH* promoters revealed only five small evolutionary conserved regions of high homology [23, 48]. The degree of homology between the human and mouse *TH* promoters is 46.6% [38], whereas the human and rat *TH* promoters share only a 30% degree of homology [48, 71]. The five conserved regions are upstream of the first –194bp from the TSS of the human *TH* promoter and the first 35bp of the untranslated messenger RNA leader of the human *TH* gene [38]. This human *TH* minimal promoter was linked to GFP and transduced into human neuronal progenitor cells (hNPCs) and mouse primary striatal and SN cells. Transduced cells were then treated *in vitro* with a mixture of differentiating agents to enhance *TH* expression. Interestingly, the human *TH* minimal promoter carrying the five conserved regions exhibited a significant degree of specific gene expression only in induced TH-positive hNPCs [38], while it failed to do so in TH-positive differentiated mouse primary striatal cells and in differentiated mouse SN cells. This finding is consistent with differences in the mechanism of *TH* gene regulation between the human and mouse systems. Another striking difference between the human and murine models was observed in a more recent study on Nurr1, which did not affect human *TH* gene expression in hNPCs, in contrast to the mouse and rat systems [72]. Nurr1 is required to transactivate mouse *TH* minimal promoters [54, 63]. However, *TH* gene expression did not depend on a direct Nurr1-mediated transactivation in the human model [72].

Endogenous *TH* gene expression and TH enzymatic activity are reduced only in human cells following *DJ-1*-knockdown; this manipulation has no effect in mouse cells [73]. The transcriptional co-repressor PSF is sequestered away from the promoter region by DJ-1 in human cells. Similarly, mouse DJ-1 associates with mouse PSF; however, as this complex does not target the mouse *TH* promoter, the mouse *TH* gene is not repressed by PSF in mouse cells. Consequently, neither DJ-1 nor PSF have any effect on the regulation of mouse *TH*, but both are critical for the correct regulation of human *TH*.

IS TH ENOUGH?

There are important reasons to continue to explore the complex mechanisms of *TH* gene regulation. Aberrant TH expression could lead to the incorrect assumption that a cell is a fully functional post-mitotic dopamine producing neuron. However, as previously expounded, this may not be the case: induced expression of controlling transcription factors also activates *TH* expression without necessarily inducing dopaminergic neuronal differentiation. For instance over-expression of Pitx3, Nurr1, or both together, induces endogenous *TH* expression as well as a *TH* promoter-reporter construct in a human non-neuronal and mouse embryonic stem cell lines [68]. In hippocampal cells, Nurr1 overexpression elevates *TH* expression 60-fold in proliferating cells and 7-fold above controls following induced differentiation. Surprisingly, Nurr1 had little effect on the proliferation of cells or on the expression levels of markers of dopaminergic differentiation (*Pitx3*, *AADC, c-Ret*, *GFR-α1*, *D2R* and *VMAT2*). Indeed TH expression can be aberrantly induced in developing striatal neurons via the synergistic interaction of acidic fibroblast growth factor (aFGF) and a coactivator (dopamine, protein kinase

A, or protein kinase C activator) [27]. Furthermore, isolated bone marrow mesenchymal stem cells express detectable levels of TH after several passages with no specific neural induction [74].

There are many cases of precursor cells expressing TH and other supposed markers of terminal dopaminergic differentiation and never obtaining electrophysiological maturity. For example, when human embryonic stem cells are induced to differentiate into dopaminergic neurons using a step-wise procedure, TH mRNA and protein expression is strongly induced at the neural precursor expansion stage prior to the withdrawal of mitogenic growth factors. Cells at this stage still express high levels of the proliferative marker ki67 [75].

Alterations in TH expression can also occur in a finely temporally regulated manner. Just because a cell is not currently expressing TH does not mean that it does not have the ability to. Likewise, it does not mean that the given cell will not express TH at another point in time. Indeed, *TH* gene-expression can be activated through a dopamine receptor-mediated mechanism [28]. So in a population of TH-negative neurons in the SN, dopamine agonists can increase the number of TH expressing cells. Thus TH expression waxes and wanes even in mature committed dopaminergic cells, and because of this, cells may be mislabelled as a consequence.

Finally dopaminergic neurons from different brain regions are anatomically, electrophysiologically and synaptically distinct. The ability to sort regionally distinct dopaminergic cells (beyond TH expression) will take on a further level of importance in terms of CRTs, in particular in context of ES and iPS cells.

CONCLUSIONS AND PERSPECTIVES

Appropriate release and uptake of dopamine is essential for normal motor function. For the restoration of synaptic connections and a correctly-regulated dopamine neurotransmitter supply in PD, CRT donor cells need to be equipped with all of the required machinery to appropriately produce, store and release dopamine (including TH, but also AADC, V-MAT2, DAT, and a precise suite of other proteins).

Whilst TH expression is clearly useful for dopaminergic neuron identification at a first glance, to obtain the required cells for CRT purposes requires a far more in-depth analysis. The success of future cell replacement therapies is likely to depend on cell sorting technology (FACS) with multiple markers capable of selecting the optimal cells to maximise clinical benefit [76].

ABBREVIATIONS

bp: Base pairs

CRT: Cell replacement therapy

DAT: Dopamine transporter

DDC: DOPA decarboxylase

DBH: Dopamine beta-hydroxylase

ES: Embryonic stem (cell)

eYFP: Enhanced yellow fluorescent protein

FACS: Fluorescence-activated cell sorting

GFP: Green fluorescent protein

HDAC: Histone deacetylase

hNPC: Human neuronal progenitor cell

iPS: Induced pluripotent stem (cell)

kb: kilobase pairs

L-DOPA: L-3,4-dihydroxyphenylalanine

PD: Parkinson’s disease

PNMT: Phenylethanolamine N-methyltransferase

RE: Responsive element

SN: Substantia nigra

TH: Tyrosine hydroxylase

TSS: Transcriptional start site

VTA: Ventral tegmental area

VM: Ventral mesencephalon

CONFLICTS OF INTEREST

The authors declare that no conflicts of interest exist.

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FIGURES

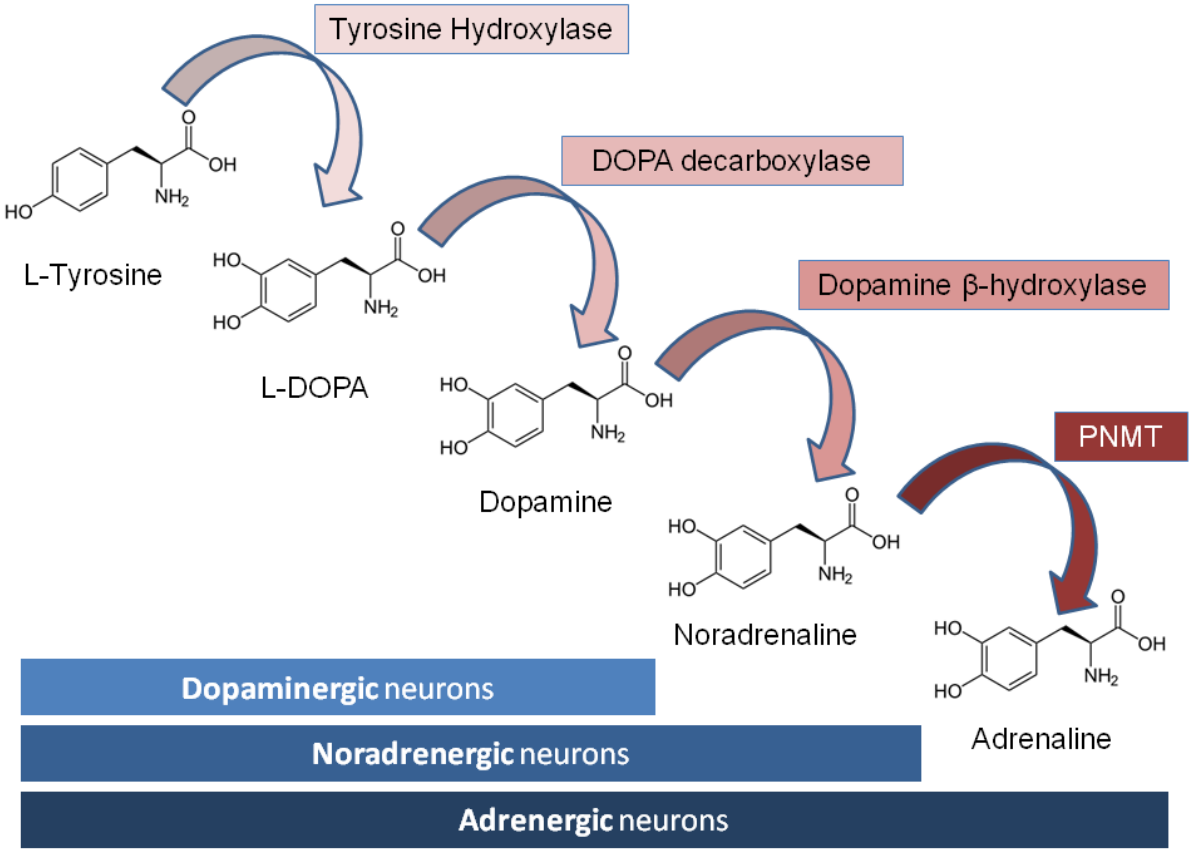

Fig. 1. Generation of catecholamine neurotransmitters. L-Tyrosine is converted to L-DOPA (catalysed by tyrosine hydroxylase), dopamine (catalysed by DOPA decarboxylase), noradrenalin (catalysed by dopamine beta-hydroxylase, DBH), and finally adrenaline (catalysed by phenylethanolamine N-methyltransferase, PNMT) (Figure 1). Shown below are the expression patterns of enzymes and their resultant reactions according to neuronal phenotypes (neurotransmitter production) (References in text).

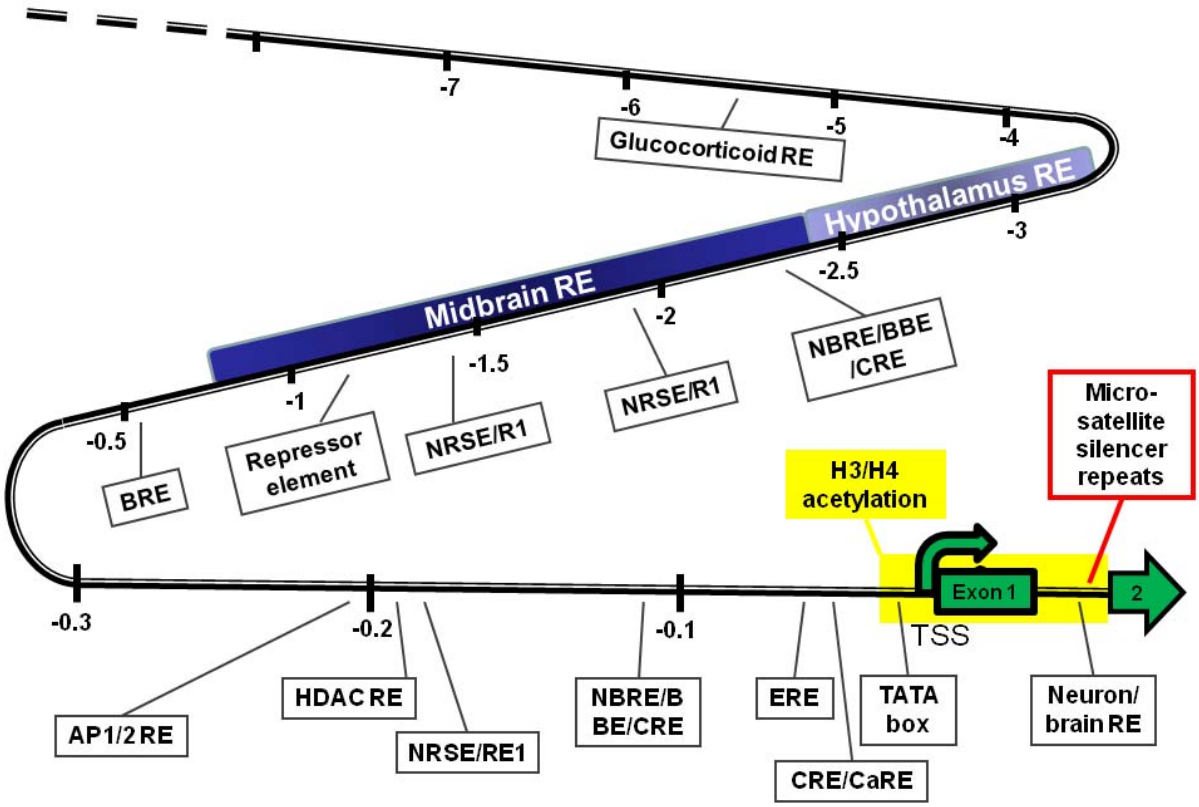

Fig. 2. Response elements (REs) involved in the regulation of *TH* gene expression. TSS: transcriptional start site; RE: response element; CRE: cyclic AMP-response element; CaRE: Ca^{2+} response element; ERE: Estrogen response element; NBRE: Nerve growth factor responsive element; BBE: bicoid-type binding element; NRSE: neuron restrictive silencer element; HDAC: histone deacetylase; AP1/2RE: activating transcription factor 1/2 response element (References in text). Measurement is in DNA kilobases (kb), relative to the *TH* transcriptional start site (TSS).