Epigenetic mechanisms in the development and maintenance of dopaminergic neurons

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Summary

Mesodiencephalic dopaminergic (mdDA) neurons are located in the ventral mesodiencephalon and are involved in psychiatric disorders and severely affected in neurodegenerative diseases such as Parkinson's disease. mdDA neuronal development has received much attention in the last 15 years and many transcription factors involved in mdDA specification have been discovered. More recently however, the impact of epigenetic regulation has come into focus, and it's emerging that the processes of histone modification and DNA methylation form the basis of genetic switches that operate during mdDA development. Here, we review the epigenetic control of mdDA development, maturation and maintenance. As we highlight, epigenetic mechanisms play a pivotal role in all of these processes and the knowledge gathered from studying epigenetics in these contexts may aid our understanding of mdDA-related pathologies.

Key words: Brain, Development, Dopamine, Epigenetics, Gene regulation, Midbrain

Introduction

If you turn this page, both the decision you make and the action that you carry out are significantly influenced by the amount of dopamine (DA; see Glossary, Box 1) released from dopaminergic neurons within your brain. Mesodiencephalic dopaminergic (mdDA) neurons located in the substantia nigra pars compacta (SNc; see Glossary, Box 1), in particular, are essential for motor functions whereas mdDA neurons of the ventral tegmental area (VTA; see Glossary, Box 1) and the retrorubal field (RRF; see Glossary, Box 1) are involved in the regulation of emotions and reward (Smidt and Burbach, 2007). Notably, severe loss of SNc mdDA neurons is a pathological hallmark of Parkinson's disease (PD) (Sulzer, 2007). By contrast, mdDA neurons in the VTA and RRF, which remain intact in PD, are part of the mesocorticolimbic system (see Glossary, Box 1), in which defective DA neurotransmission has been implicated in the development of drug addiction, depression and schizophrenia (Nestler, 2000). Given their involvement in neurodegenerative diseases and psychiatric disorders, mdDA neurons have been studied intensively in recent years.

In the last two decades, a variety of molecular approaches have revealed factors that are specific for mdDA neuronal subsets or that guide their spatial organization (Smidt and Burbach, 2007); recently, a pathway that molecularly distinguishes SNc DA neurons from those located in the VTA was identified (Jacobs et al., 2011). In addition to molecular profiling of DA neurons, however, a

second level of complexity has been added by the rapidly emerging field of epigenetics, which has provided new insights into the origin and maintenance of distinctive gene expression patterns in mdDA neurons. Current definitions of epigenetics often emphasize the heritability of changes in gene expression throughout cell divisions that are not due to alterations in DNA sequence. However, perhaps a more appropriate definition of epigenetics, especially when applying this term to mature neurons that cannot divide, is postulated by Adrian Bird: 'The structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' (Bird, 2007). This definition emphasizes the dynamic manner in which epigenetic mechanisms are coordinated and regulate gene expression. In addition, elegant studies have suggested that early life experiences might result in long-lasting epigenetic changes that potentially affect expression throughout life, whereas aging might influence epigenetic regulation in mature neurons (Lu et al., 2004; Liu et al., 2009; Weaver et al., 2004). In this Review, we discuss the epigenetic mechanisms (see Box 2),

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Box 1. Glossary
CpG sites. Areas in the genome that consist of a C-phosphate-G DNA sequence (i.e. cytosine next to a guanine) and can be sites of specific methylation.
Dopamine (DA). A neurotransmitter of the monoaminergic group.
DNA methyl transferases (DNMTs). Enzymes that transfer a methyl group to CpG sites in DNA molecules, thereby influencing transcriptional activity.
Histone acetyltransferases (HATs). Enzymes that transfer acetyl groups to histone complexes.
Histone deacetylases (HDACs). Enzymes that remove acetyl groups from histone complexes.
HDAC inhibitors (HDACis). Chemical compounds that have a repressive effect on the enzymatic activity of HDACs. Specific compounds can selectively inhibit specific HDAC family members.
Mesocorticolimbic system. Dopaminergic network encompassing the VTA/RRF mdDA neurons and the projections towards the prefrontal cortex and ventral striatum (nucleus accumbens).
Polycomb group (PcG) proteins. Transcriptional silencers that can form multiple large protein complexes, regulating lineage choices during development and differentiation via chromatin remodeling and histone modification.
Retro rubral field (RRF). Neuroanatomical region containing the most caudal group of dopaminergic neurons in the brain.
Substantia nigra pars compacta (SNc). Neuroanatomical region with dense groups of DA neurons located in the mesodiencephalon. Named after the presence of a black precipitate in human DA neurons that forms as a consequence of melanin deposits.
Sumoylation. Process of post-translational modification of proteins, whereby small ubiquitin-like modifier proteins (SUMO proteins) are attached to the targeted protein lysine residue.
Ventral tegmental area (VTA). Neuroanatomical region containing DA neurons that mostly project to the prefrontal cortex and are involved in emotion, attention and reward.

Box 2. Epigenetic mechanisms
Multiple layers of nuclear organization are crucial for determining the unique expression patterns of individual cell types. Changes in nuclear organization rely on three distinct processes: (1) chromatin remodeling; (2) DNA modification (no sequence change); and (3) changes mediated by non-coding RNAs (ncRNAs). Ongoing investigations have already related many of these processes to nervous system function in health and disease (for reviews, see Mehler, 2008; Im and Kenny, 2012). Central to understanding epigenetic regulation are the smallest functional units of chromatin, the nucleosomes. Nucleosomes consist of ~147 bp of DNA wrapped around an octamer of four paired histone proteins (H2A2, H2B2, H32, H42) (Luger et al., 1997). Each of the histone proteins has an N-terminal tail that protrudes from the DNA, making these linear amino-acid chains accessible for post-translational modifications, such as acetylation, methylation, phosphorylation and ubiquitylation. The dynamic pattern of these chemical modifications on histones is essential for chromatin remodeling and, ultimately, for the regulation of gene expression. Another epigenetic mechanism involves DNA modification, such as that seen in genomic imprinting, a robust epigenetic process leading to the specific repression of one of the parental alleles. Differential DNA methylation controls regions during spermatogenesis and oogenesis and reflects silencing of one of the alleles, which can be maintained throughout multiple cell cycles by the combined effort of various epigenetic mechanisms, including those mediated by long ncRNAs and histone modifications (Kacem and Feil, 2009). In embryonic stem cells, the majority of genes that are subject to silencing by imprinting are paternally derived. However, during development such discrimination is reversed and in the adult brain maternally derived alleles, in particular, are silenced (Gregg et al., 2010). Although its underlying mechanisms are largely unknown, this shift might reflect a process of spatiotemporal organization during brain development (Keverne et al., 1996).

with emphasis on chromatin remodeling and DNA modifications, that contribute to the development of mdDA neurons. Furthermore, we highlight how these epigenetic control mechanisms might impact human health and disease.

Epigenetics in mdDA neuronal development
The genesis of mdDA neurons involves a number of developmental steps. First, during early development, patterning of the brain induces local regional signaling that specifies the permissive region to induce early mdDA precursors. Thereafter, these progenitors differentiate to assume a neuronal fate, acquire a DA phenotype and then mature. It must be noted that neuronal fate and the DA phenotype are acquired sequentially, and this is accompanied by migration of the early mdDA precursors to their specific ventral regions. Although these processes are likely to be coordinated, how exactly this is orchestrated is not well defined. As such, we discuss each of these events individually. Furthermore, neuronal fate determination in mdDA precursors is not really different to that occurring in other brain regions and neuronal subtypes, and thus follows the same general mechanisms of neuronal differentiation.

Recent studies have shown that, as development progresses, shifts in epigenetic marks contribute to the ongoing changes in expression profiles that reflect these developmental processes. Below, we discuss the various epigenetic mechanisms that are known to operate during each of these stages, beginning with the formation of an mdDA permissive region in the developing brain and leading to the genesis of a mature functional mdDA neuronal population.

Epigenetic regulation of early midbrain development and patterning
The first stage in the formation of the mdDA system is the regionalization of the midbrain to form a permissive area in which mdDA neurons can be produced. This regional permissiveness has been described to depend on the expression of fibroblast growth factor 8 (Fgf8) emerging from the isthmic organizer (the mid-hindbrain border) and on the presence of Sonic hedgehog (Shh) originating from the floorplate ventricular zone (Smidt and Burbach, 2007). The correct gene regulatory events leading to the expression of these genes may rely on regulation by the Polycomb group (PcG; see Glossary, Box 1) proteins, which have been shown to influence gene expression of Shh, Fgf8 and other factors [such as wingless-type MMTV integration site family proteins (Wnts), orthodenticle homeobox-2 (Otx2) and engrailed 1/2 (En1/2)], which are crucial during early mdDA development and patterning (Fig. 1) (Bracken et al., 2006; Smidt and Burbach, 2007). PcG proteins form multi-protein complexes that generally associate with typically pre-modified chromatin (Sauvageau and Sauvageau, 2010) and they are able to stably repress or maintain gene expression by modifying the surrounding chromatin by targeting early development factors, such as homeobox genes (Schuettengruber et al., 2007). Furthermore, PcG proteins are generally associated with embryonic regulation or stem cell renewal through the epigenetic regulation of transcription factors (Sauvageau and Sauvageau, 2010), although a direct role for PcG proteins in midbrain patterning and gene programming is yet to be established. Interestingly, the PcG protein Yin Yang 1 has been shown to be involved in axial patterning of anterior head structures and was described to be a regulator of the engrailed 2 (En2) protein (Kwon and Chung, 2003), which is involved in formation of the mid-hindbrain border and is directly involved in the induction of the DA lineage in vertebrates.

Specifying mdDA neuronal precursors
Once the permissive region is generated, stem cells in the ventricular zone start to undergo initial differentiation programs and a specific set of precursors are identified by the expression of early markers such as LIM homeobox transcription factor 1 alpha/beta (Lmx1a/b), Otx2 and forkhead box A2 (Foxa2; also known as Hnf3b). One epigenetic mechanism involved in the expression of precursor type-specific genes is the recruitment of histone acetyltransferases (HATs; see Glossary, Box 1), particularly CBP/P300 (CREB binding protein, CREBBP), to regulatory sites within these genes. It is noteworthy that translocation of P300 to each of these regions is orchestrated by precursor type-specific signaling, which thus determines distinctiveness (Fig. 1). For example, general neurogenesis is initiated by the upregulation of the neurogenic basic helix-loop-helix (bHLH) protein neurogenin 1 [Ngn1 (also known as Neurog1)], which forms an activator complex with Smad and P300, thereby stimulating histone acetylation and increasing the expression of neuronal specific genes such as NeuroD (Sun et al., 2001) (Fig. 1). At the same time, hypermethylation of a signal transducer and activator of transcription 3 (Stat3)-binding site in the promoter region of the glial fibrillary acidic protein gene (*Gfap*) facilitates methyl CpG binding protein 2 (MeCP2) binding and the subsequent recruitment of a repressive histone deacetylase complex containing Sin3a and histone deacetylase (HDAC; see Glossary, Box 1), which represses the glial phenotype (Cheng et al., 2011). At the switch from neurogenesis to astrogenesis, leukemia inhibitory factor (Lif) and bone morphogenetic protein 2 (Bmp2) signaling upregulate Stat3
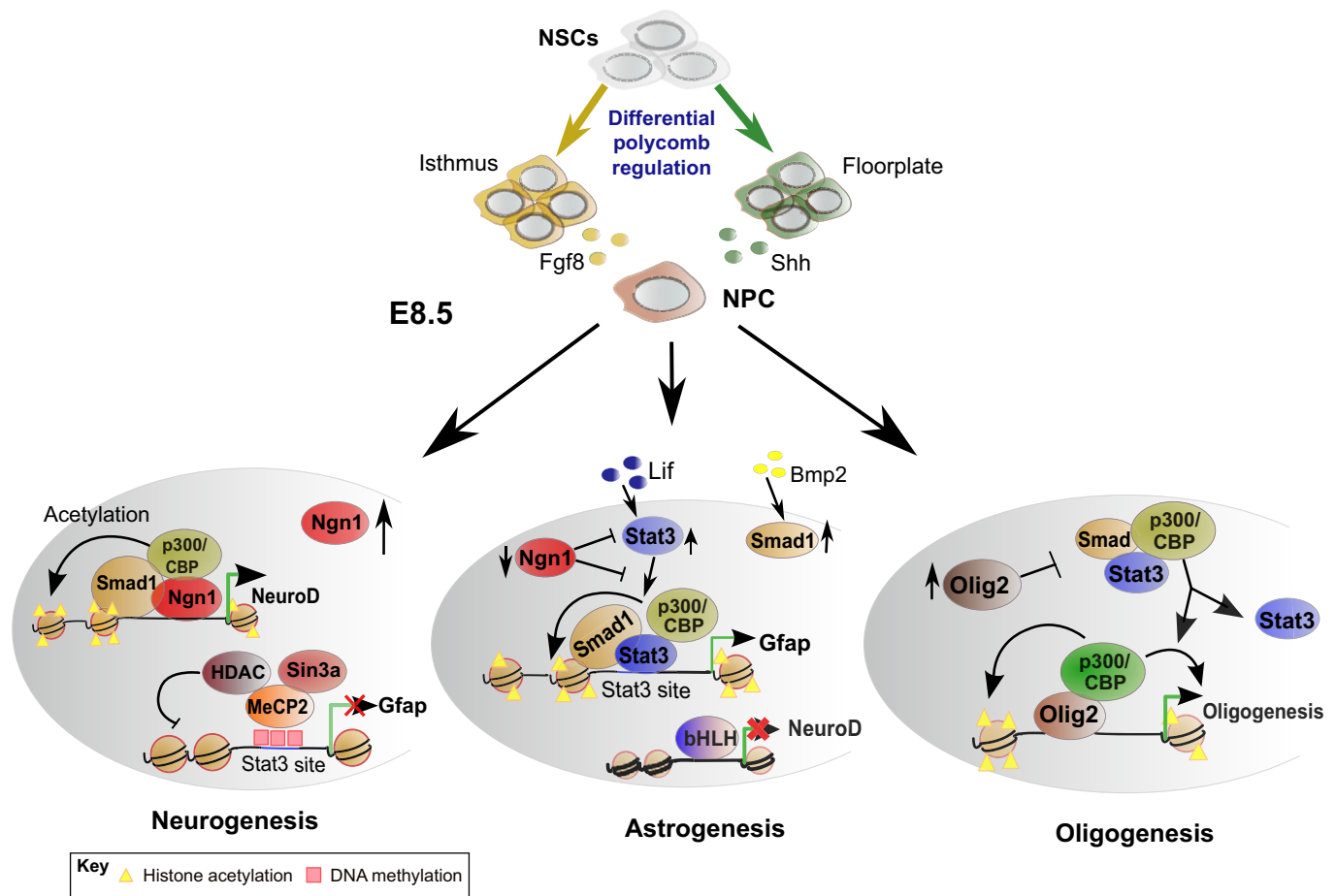
Fig. 1. Model of epigenetic events that can influence the midbrain mdDA permissive region and the developmental programming of specific neuronal cell types. In the early phase of mdDA neuronal development, Polycomb group proteins, via their effects on Fgf8 and Shh expression levels, might play a role in specifying the permissive region within the midbrain. In addition, they might also influence the activation of genes involved in mdDA neuronal specification (not shown). Later in development, additional epigenetic processes, including histone acetylation and DNA methylation, can play key roles in directing cell fate towards the neuronal, astroglial and oligodendrocytic lineages. NPC, neural precursor cell; NSCs; neural stem cells.

and Mad homolog 1 (Smad1), thereby inducing astrogenesis (Nakashima et al., 1999) by downregulation of Ngn1 expression and by enhancing the expression of anti-neurogenic bHLH factors that enable activation of astrocyte-specific genes, such as *Gfap* (Fukuda and Taga, 2005; Hsieh and Gage, 2004) (Fig. 1). Eventually, upregulation of the oligodendrocytic oligodendrocyte transcription factor 2 (Olig2) inhibits the formation of Stat3-P300 activation complexes, via binding to p300, thereby initiating oligodendrogenesis (Fukuda and Taga, 2005) (Fig. 1).

An additional epigenetic check-point in neuronal differentiation may be provided by activity of the nuclear receptor co-repressor 2 (NcoR2; also known as SMRT), which is essential for repression of the H3 trimethyl K27 demethylase, Jumonji domain-containing 3 (Jmjd3; also known as Kdm6b) (Jepsen et al., 2007). In the forebrain, this mechanism is used to suppress retinoic acid (RA) signaling (which directs cells towards a neuronal fate), thereby keeping the cells in a stem cell fate until the appropriate ligand is present, resulting in release of SMRT. It has been shown that RA is present in the developing midbrain field during the first phase of differentiation (Smidt and Burbach, 2007); therefore, this mechanism might play a role in suppressing RA signaling and maintaining stem cell fate until appropriate RA induction occurs in later differentiation stages.

Epigenetics during differentiation to mdDA neurons
Upon initial neurogenesis induced by Ngn2, the mdDA precursors start to express Nur-related protein 1 [Nurr1; also known as nuclear receptor subfamily 4, group A, member 2 (Nr4a2)], an orphan nuclear hormone receptor that is essential for the proper genesis of mdDA neurons and that marks the post-mitotic phase of mdDA development. Reciprocally, the mdDA precursors repress Ngn2 (Smidt and Burbach, 2007). Shortly thereafter, marking the maturation of mdDA neurons, the transcription factor paired-like homeodomain transcription factor 3 (Pitx3) is activated. Nurr1 regulates proteins involved in DA synthesis and transport, whereas dependence on Pitx3 is displayed specifically by the subpopulation of mdDA neurons that ultimately forms the SNc (Smidt et al., 2004; Jacobs et al., 2011). *In vivo*, Nurr1 and Pitx3 have been shown to act in concert at the genomic loci of several genes, thereby affecting transcription of genes involved in DA metabolism (Jacobs et al., 2009a; Jacobs et al., 2011) (Fig. 2A,B). Moreover, these studies have shown that Pitx3-mediated changes in gene expression occur via epigenetic mechanisms.

Analysis of *Pitx3*-null mice revealed that in the absence of *Pitx3*, SMRT-HDAC repressive complexes are present at the Nurr1 transcriptional complex, thereby repressing Nurr1 target genes (Fig. 2C). Upon induction of *Pitx3*, however, the SMRT-HDAC
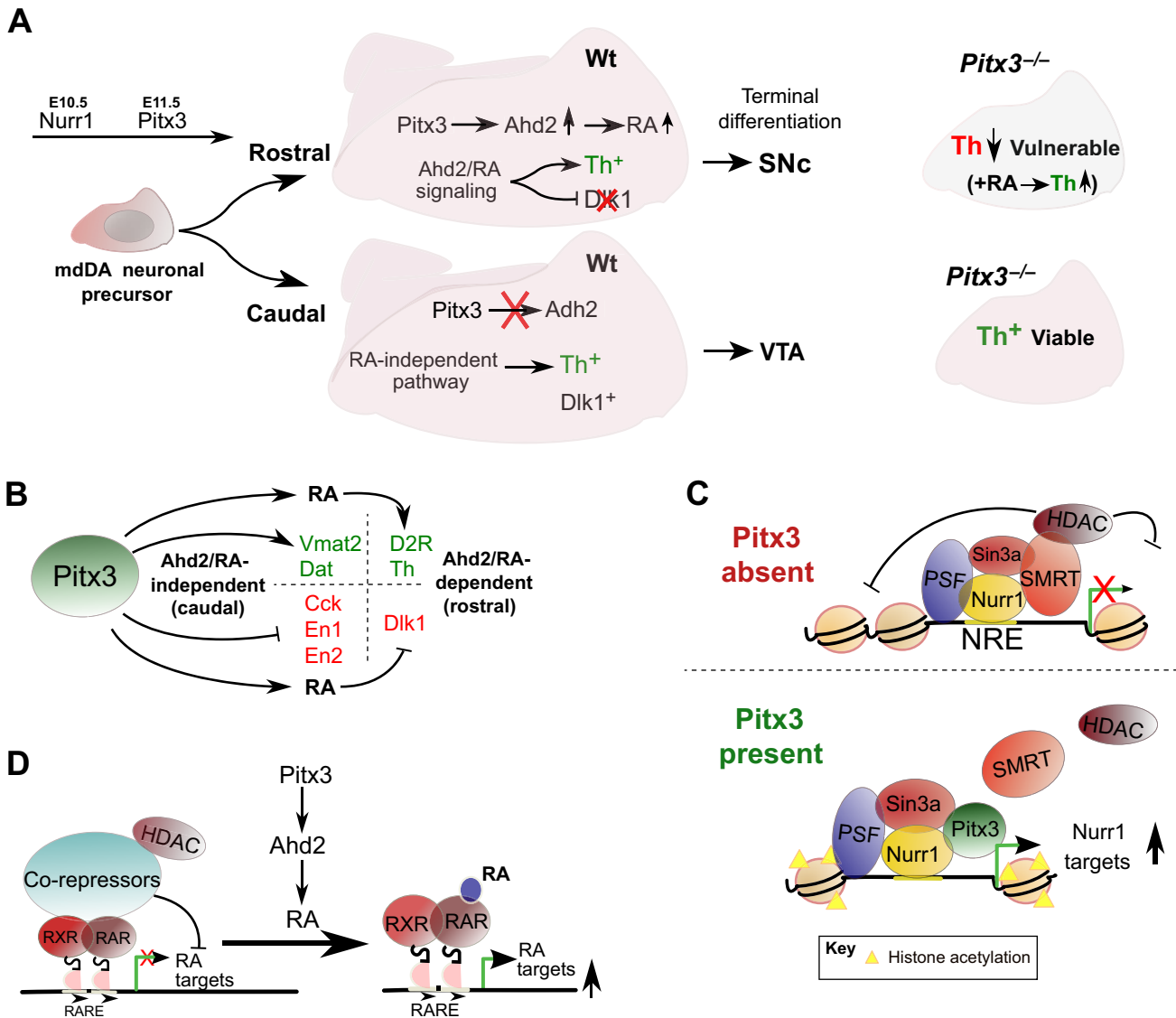
Fig. 2. Molecular processes underlying the development and phenotypic specification of mdDA neurons. (A) The combinatorial action of Pitx3 and Nurr1 determine mdDA subset-specific gene expression. There is specific dependence of rostral mdDA neurons on RA signaling; in rostral mdDA neurons, Pitx3 activates Ahd2, which leads to increased RA production. This, in turn, is important for the induction of Th and the repression of Dlk1. This dependence can be mimicked by application of RA to *Pitx3*-deficient cells (right-hand panel). In caudal mdDA neurons, Pitx3 does not activate Ahd2 but these neurons are able to activate *Th* gene expression without the requirement of signaling through RA. In the absence of Pitx3 mdDA neurons lose *Th* expression and are vulnerable to cell death during development leading to a complete loss of the SNc in the adult stage. Supplementing these cells with RA is able to rescue this phenotype. This dependence on RA signaling is not present in the VTA. **(B)** Pitx3 thus participates in RA-dependent and RA-independent pathways through the subset-specific induction of Ahd2. **(C)** Nurr1 target genes are regulated via HDAC-mediated repression, which can be released by Pitx3 through its interaction with this complex. NRE, Nurr1-responsive element. **(D)** Pitx3-induced RA acts as a ligand for RAR-RXR complexes, which are otherwise in a suppressed state (achieved through protein interactions with co-repressors and HDACs). RARE, RA-responsive element.

complex is removed and Nurr1 target genes are activated. In line with these observations, the application of sodium butyrate (NaB), an inhibitor of class I and II HDACs, to *Pitx3*-deficient explant cultures results in de-repression of Nurr1 target genes (Jacobs et al., 2009a).

A second molecular mechanism by which Pitx3 regulates mdDA neuronal development is Pitx3-induced RA signaling, which is spatially confined to the developing SNc (Jacobs et al., 2007; Jacobs et al., 2011) (Fig. 2A). Pitx3 induces RA synthesis via the upregulation of aldehyde dehydrogenase family 1, subfamily A1 (Aldh1A1; also known as Ahd2), an efficient generator of RA from

vitamin A in mdDA neurons (McCaffery and Dräger, 1994). RA acts as a ligand for RA receptors RAR, RAR-RXR and RXR, which bind to genomic RA-responsive elements (RAREs), leading to release of class II HDACs (Nebbioso et al., 2010), and trigger the expression of RA gene targets. RA-induced activation of RA-responsive elements might also affect the expression of mdDA-specific genes, such as tyrosine hydroxylase (Th; discussed below). Such a direct role for RARs in transcriptional regulation of the *Th* gene was suggested in SK-N-BE(2)C cells, in which RAR bound the promoter of the *Th* gene and induced *Th* expression upon activation of RAR by RA (Jeong et al., 2006) (Fig. 2D). In support of this,

administration of a pan-RAR agonist upregulated *Th* transcript levels in *Pitx3*-deficient embryos (Jacobs et al., 2011). Because RA-dependence within the total mdDA precursor population is restricted to the rostral subset, which is destined to form the SNc, several genes that fulfil a role in mdDA development are differentially regulated by RA signaling (Jacobs et al., 2011). Therefore, RA-induced changes in the chromatin landscape might represent a mechanism that contributes to the formation of distinct epigenetic profiles reflecting the SNc subclass of mdDA neurons. One of the RA targets that is suppressed in the developing SNc (Jacobs et al., 2011) is the imprinted gene delta-like homolog 1 (*Dlk1*) (Gregg et al., 2010; Rogers et al., 2012). Dlk1 is present in both a soluble and a membrane-associated form in the brain, and contains six EGF-like repeats, classifying it into the epidermal growth factor (EGF) superfamily (Jensen et al., 2001). Its expression arises from the paternal allele of the *Dlk1-Gtl2* cluster, which is exclusively expressed in brain tissue and also contains maternally expressed non-coding RNAs (ncRNAs) such as maternally expressed 3 (*Meg3*; also known as *Gtl2*), and paternally expressed deiodinase type III (*Dio3*) and retro transposon-like 1 (*Rtl1*) (Lin et al., 2003; da Rocha et al., 2008; Wilkinson et al., 2007). An intergenic germ-line derived differentially methylated region (IG-DMR) is present on both parental alleles of the cluster, unmethylated on the maternal and methylated on the paternal allele (Lin et al., 2003). Recent studies of the expression of specific parent-of-origin alleles in the mouse brain, described the SNc and VTA as imprinting hot-spots. Among others, *Dlk1* was found to be imprinted in the mdDA system and was found to be expressed from the paternal allele in both the SNc and VTA (Gregg et al., 2010). Moreover, it was recently shown that postnatal loss of *Dlk1* imprinting in stem cells and niche astrocytes regulates neurogenesis (Ferrón et al., 2011). Finally, *Dlk1* was identified as a target of Nurr1 in mdDA neurons and is involved in the regulation of DA transporter (Dat; Slc6a3) expression (Jacobs et al., 2009b; Jacobs et al., 2011). Thus, although *Dlk1* is an imprinted gene, additional regulation of its expression in mdDA neurons might be influenced by Pitx3 and RA-mediated epigenetic regulation, which in turn influences the expression of Dlk1 target genes.

Epigenetic regulation of the *Pitx3* gene itself has been ascribed to two neurotrophic factors: glial cell-derived neurotrophic factor (Gdnf) and brain-derived neurotrophic factor (Bdnf), both of which support the development and survival of mdDA neurons. In the midbrain neuronal field, Gdnf, which is temporally secreted from the basal plate at around embryonic day (E) 10.5-11.5, may stimulate *Pitx3* expression via activation of the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling pathway (Peng et al., 2011). Moreover, Gdnf enhances the expression of Bdnf exclusively in the presence of Pitx3 (Peng et al., 2011) (Fig. 3A). The *Bdnf* gene itself is modulated by extensive methylation, and this facilitates the binding of MeCP2 and subsequent interactions of Hdac1 and Sin3a with the *Bdnf* promoter (Chen et al., 2003; Martinowich et al., 2003). In addition, upon neuronal activity, *Bdnf* transcription is induced through phosphorylation of MeCP2, resulting in the release of MeCP2 and associated repressing proteins (Chen et al., 2003). Another factor acting at the *Bdnf* promoter is cAMP-responsive element binding protein (Creb). Phosphorylation of Creb upon neuronal activity increases the robustness of its association with the *Bdnf* promoter, thereby strengthening transcriptional activation (Martinowich et al., 2003) (Fig. 3B). In addition, Bdnf signaling itself can influence epigenetic regulation. *In vitro* studies have shown that Bdnf signaling induces the cytosolic upregulation of nitric oxide (NO). The chemical addition of NO to glyceraldehyde-3-phosphate
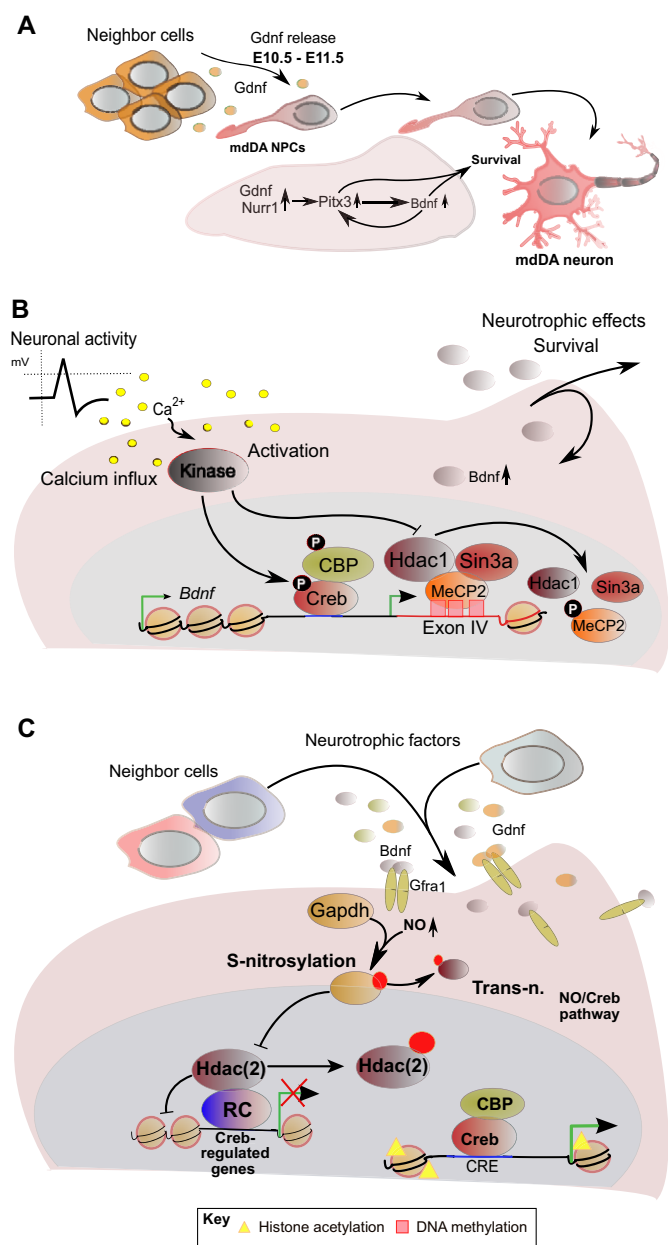
Fig. 3. The effects of extracellular signals on epigenetic programming in dopaminergic and other neuronal cell models. (**A**) The combined action of Nurr1 and Gdnf is able to induce Pitx3, which in turn is able to induce Bdnf expression in a positive-feedback loop towards Pitx3, in *in vitro* dopaminergic cell models. (**B**) In other *in vitro* cell models, neuronal activity, via its effects on Creb phosphorylation (P) and HDAC inhibition, is able to change the epigenetic programming of the *Bdnf* promoter leading to enhanced Bdnf expression. (**C**) In the cortex, neurotrophic factors, such as Bdnf and Gdnf, are also able to influence Creb-regulated genes through epigenetic signaling. In this model, the initial s-nitrosylation (indicated by red ovals) of Gapdh leads to inhibition of HDACs via trans-nitrosylation (Trans-n). CRE, cAMP response element; Gfra1, glial cell line derived neurotrophic factor family receptor alpha 1; NPCs, neural precursor cells.

dehydrogenase (Gapdh), in a process called s-nitrosylation, then facilitates trans-nitrosylation of nuclear proteins such as Hdac2 (Kornberg et al., 2010). This Bdnf-induced s-nitrosylation of Hdac2 results in the release of Hdac2 from chromatin, increases

acetylation of histones in gene promoters, promotes transcription and regulates dendritic growth and branching in rat primary cortical neurons, possibly by activation of Creb. Moreover, NO induces Creb phosphorylation and its recruitment to DNA via s-nitrosylation of nuclear proteins that associate with Creb target genes (Nott et al., 2008; Riccio et al., 2006) (Fig. 3C). A comparable cytosolic effect, whereby nitrosylation inhibits the formation of HDAC-repressive complexes has been suggested (Watson and Riccio, 2009).

These data suggest that the neurodevelopmental effects of Bdnf, in part, might act through release of HDACs from promoters that are involved in neuronal differentiation. In line with this, de-repression of Nurr1 target genes (Jacobs et al., 2009a) upon release of SMRT-HDAC repressive complexes, as described above, might indicate why Bdnf exposure is beneficial for mdDA neuron development and function (Sortwell, 2003). Moreover, it has been shown that Bdnf signaling, RA signaling and NO signaling intersect in the protection of mdDA neurons (Katsuki et al., 2009;

Kurauchi et al., 2011). It is therefore possible that these converging signaling events act via common mechanisms that involve changing the epigenetic state of Nurr1 target genes through release of HDAC-mediated repression.

The generation of mature mdDA neurons: epigenenetic control of *Th* gene expression

In terminally differentiated mdDA neurons, the neurotransmitter dopamine is generated from tyrosine, which is first converted to dihydroxyphenylalanine (DOPA) by the rate-limiting enzyme tyrosine hydroxylase (Th), DOPA in turn, is converted to DA through DOPA decarboxylase (Ddc; also known as Aadc) activity. Therefore, Th is generally used to mark dopaminergic neurons and is present in mature dopaminergic cells, although the colocalization of Th with the terminal differentiation marker Pitx3 is restricted (Maxwell and Li, 2005; Jacobs et al., 2007). Owing to its importance in defining the transmitter phenotype, Th has been subject to numerous investigations, resulting in a detailed insight
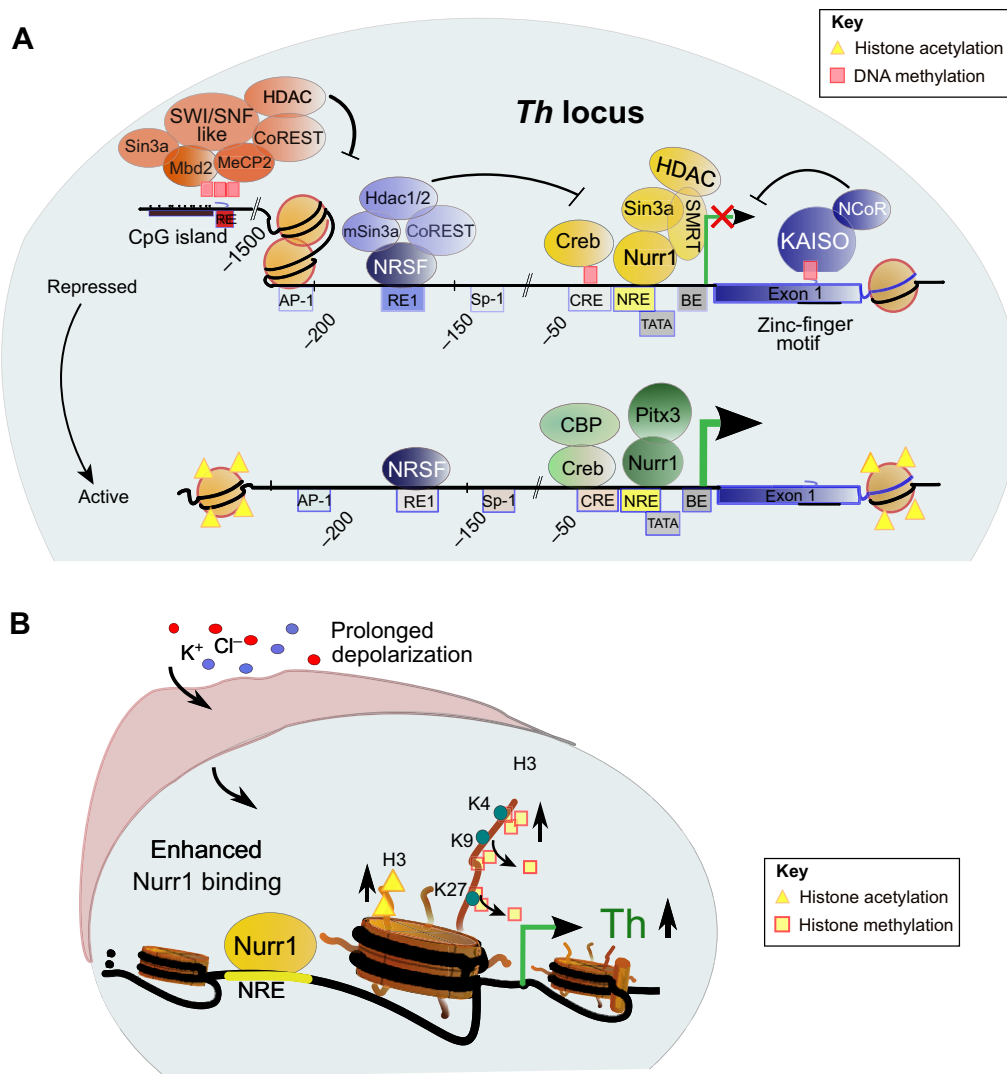
Fig. 4. Epigenetic events at the *Th* promoter. (**A**) An extensive combination of classic and epigenetic regulation is involved in induction of the *Th* gene. Epigenetic complexes at upstream CpG islands and local co-repressor complexes at NRSF- and KAISO-binding positions suppress the classic transcription factor-activating complexes consisting of Creb and Nurr1. (**B**) Prolonged depolarization events in dopaminergic neurons can also influence the epigenetic state of the *Th* promoter resulting in increased Nurr1 binding and modified histone methylation and acetylation patterns and, hence, enhanced *Th* gene expression. AP-1, Fos/jun binding element; BE, basal element; CRE, cAMP response element; RE1, repressive element 1; SP-1, trans-acting transcription factor 1 binding element.

into the complex regional chromatin organization of the *Th* promoter. In this section, we describe recent investigations of *Th* regulation during development (Fig. 4).

Over 15 years ago, the DNA methylation state of three CpG sites (see Glossary, Box 1) in the *Th* promoter region was found to be tissue specific in rodents (Okuse et al., 1993); however, it was not until many years later that an additional regulatory element was revealed in the first exon of the *Th* gene (Arányi et al., 2005). This study revealed that the methylation of one specific CpG in exon 1 of *Th* corresponds to an inactive state. Moreover, based on expectations that this CpG was embedded in a transcription factor (TF)-binding element, it was demonstrated that the repressive zinc finger transcription factor KAISO (also known as Zbtb33) (Daniel and Reynolds, 1999) binds exclusively when this CpG is methylated (Arányi et al., 2005). KAISO is ubiquitously expressed in the brain, with highest levels found in the neocortex, amygdala, hippocampus and cerebellar cortex (Della Ragione et al., 2006). *In vitro*, KAISO can interact with Nco-R complexes and, therefore, through HDAC interaction, might act as an additional modulator of *Th* expression (Fig. 4A). In line with the fact that a single CpG region can keep the *Th* gene in a repressive state, is the observation that *Th* expression could be stimulated in neuroblastoma cells by treatment with the demethylating agent 5-azacytidine (Arányi et al., 2005).

At the level of histone modification, it has been described that the HDAC inhibitors (HDACis; see Glossary, Box 1) trichostatin A (TSA) and sodium butyrate (NaB) can induce *Th* promoter activity in non-neuronal cell lines (Kim et al., 2003). In line with these findings, NaB can restore Th expression in *Pitx3*-deficient mdDA neurons (Jacobs et al., 2009a), as outlined above, and treatment with the non-specific HDAC inhibitor valproic acid [VPA, a potent zinc-dependent class I and II histone deacetylase inhibitor already used commonly as mood stabilizer and anti-epileptic drug (Phiel et al., 2001)], has been shown to upregulate *Th* expression in primary cultures of rat SNc neurons (Monti et al., 2010). VPA inhibits, among others, Hdac1, which may be recruited by pyrimidine tract-binding protein-associated splicing factor (PSF; also known as Sfpq) to the *Th* locus. Interestingly, HDAC recruitment relies on the correct sumoylation (see Glossary, Box 1) of PSF. The PD-associated protein DJ-1 (Park7) inhibits this post-translational modification and thereby increases *Th* expression (Zhong et al., 2006). Therefore, mutations in *DJ-1* (*PARK7*), as found associated with PD (Hague et al., 2003), could result in a lack of epigenetic regulation that may sensitize the *Th* locus towards silencing.

Several pathways that induce or silence *Th* expression during mdDA neuronal development might also be subject to epigenetic regulation. In neural stem cells (NSCs), the neurorestrictive silencer factor repressor element 1 silencing transcription factor (NRSF; also known as Rest), a well-established repressor of >2000 neuronal genes, has been reported to repress the expression of two microRNAs, miR-9 and, in particular, miR-124, in NSCs (Conaco et al., 2006), thereby preventing NSCs from maturing or exiting a self-renewal state (Yoo et al., 2011). *In vitro* studies suggested that the *Th* locus is regulated by a proximal cis-regulatory mechanism that involves Rest, the co-repressor coREST (also known as Rcor2) and HDAC recruitment (Kim et al., 2006; Yang et al., 2011) (Fig. 4A). Studies have shown that a repressive regulatory region upstream of the putative *Th* promoter is fully methylated in NSCs, whereas several CpGs may be demethylated in Th-positive cells. In line with this, an unmethylated CpG (−1868 of the *Th* transcription start site) has been located within a binding site of the methylated CpG-binding proteins Mbd2 and MeCP2, which recruit repressors and may act independently of Rest, serving as an additional repressive element (Kim et al., 2006; Yang et al., 2011).

Interestingly, a role for PcG group (and PcG group-like) proteins in *Th* gene regulation has also been described. In *Caenorhabditis elegans*, the PcG-like proteins SOP-2 and SOR-3 specify and maintain neuronal fate by regulating the expression of DA metabolic enzymes, including the *C. elegans* homolog of tyrosine hydroxylase (CAT-2) (Yang et al., 2007). Although it remains to be determined if PcG proteins act directly on the *Th* locus in vertebrates, an important role for PcG-mediated regulation in mdDA programming seems likely and perhaps needs to be included in the design of somatic cell reprogramming protocols.

Finally, another epigenetic level of *Th* gene regulation has been suggested to act through cellular activity. In rat midbrain primary cultures, prolonged membrane depolarization with potassium chloride enhances Nurr1 recruitment to the *Th* promoter and synchronously increases histone H3 acetylation and H3K4 trimethylation, and decreases histone 3;lysine (H3K9) and histone 3;lysine 27 (H3K27) trimethylation, resulting in improved differentiation of DA neurons (He et al., 2011) (Fig. 4B).

Producing DA neurons via stem cells and reprogramming: can epigenetics play a role?

In order to provide a scalable pool of DA neurons for transplantation purposes in PD patients, continued efforts have been made to direct stem cell reprogramming and differentiation towards the dopaminergic phenotype. Although initial results of cell graft transplantation into the SNc of PD patients were promising, clinical application is severely hampered because of long-term side effects (Björklund et al., 2003), and it has been suggested that the underlying problem is a failure to produce molecularly appropriate mdDA neurons (Smidt and Burbach, 2007). In order to improve mdDA neuron induction protocols, it is essential to unravel both the molecular and epigenetic mechanisms underlying the specification of healthy and functional mdDA neuronal subsets. In recent attempts to generate isogenic pluripotent stem cells by reprogramming somatic cells, the combination of current de-differentiation factors (Caiazzo et al., 2011), the broad-spectrum inhibition of HDACs and DNA methyl transferases (DNMTs; see Glossary, Box 1), and the activation of histone acetyltransferases (HATs) proved to be beneficial (Han et al., 2010), suggesting that re-differentiation of cells that have a more open, stem cell-like chromatin state might be more effective. However, intensive treatment with HDACis, DNMT inhibitors and histone methyltransferase inhibitors might be insufficient to generate full pluripotency of NSCs derived from the adult subventricular zone (Deleidi et al., 2011). However, a recent study focusing on differentiation towards the mdDA neuronal phenotype using non-mesencephalic NSCs, suggested that the administration of TSA, a non-specific inhibitor of class I and II histone deacetylases, combined with the mdDA-inducing factors Shh, Fgf8 and Wnt1, to mimic paracrine signaling in local midbrain development, more efficiently induced differentiation towards mdDA-like neurons (Rössler et al., 2010).

Dopaminergic pathologies: understanding epigenetics might have potential for treatment paradigms

As it is clear that epigenetics are involved in the generation, specification and function of mdDA neurons, understanding the role of epigenetics in maintaining these neurons may well be of

importance with respect to stimulating mdDA neuronal survival in various neurodegenerative diseases and neurological disorders. Below, we describe the epigenetic regulation of mdDA neuronal maintenance and discuss how this might be related to mdDA pathology.

Epigenetic control of and by α-synuclein

In PD, α-synuclein (α-syn; SNCA) is a well-known protein because its aggregates are elementary components of the hallmark lesions known as Lewy bodies (LBs), which colocalize with regions of neuronal cell death (Forno et al., 1996). A number of recent studies have shown that the expression level of the gene encoding α-syn is regulated through epigenetic mechanisms, through a methylated CpG in intron 1 and through HDAC activity (Du et al., 2010; Jowaed et al., 2010; Matsumoto et al., 2010) (Fig. 5A). Therefore, part of the beneficial results observed following HDACis exposure in PD models might be due to a change of the α-syn level in those cells. Furthermore, an indirect effect of α-syn aggregation is the possible inclusion of protein components involved in epigenetic regulation, which could thereby change the epigenetic landscape in affected neurons. In human postmortem brain samples derived from PD

patients and in α-syn-expressing transgenic mice, for example, DNA methyltransferase 1 was reported to accumulate within α-syn aggregates (Desplats et al., 2011). Moreover, it has been reported that α-syn aggregation is involved in repressing the nuclear positioning of HATs (Fig. 5B) (Kontopoulos et al., 2006). This suggests that the inhibition of α-syn aggregation might alleviate the indirect effects on epigenetic regulation in dopaminergic cells.

Influence of pesticides on the epigenetic landscape

Pesticides are well known for their detrimental effects on dopaminergic maintenance (Moretto and Colosio, 2011). Interestingly, these pesticides can influence the epigenetic regulatory mechanisms present in dopaminergic cells. For example, the herbicide paraquat (Song et al., 2011) and the insecticide dieldrin (Song et al., 2010) have been shown to induce histone H3 hyperacetylation in a dopaminergic cell line (N27). For dieldrin, this included rapid histone H4 hyperacetylation, suggesting a direct effect on the HAT/HDAC activity balance. Paraquat, by contrast, induces only H3 hyperacetylation, which was associated with decreased levels of Hdac4 and Hdac7 protein. In both cases, cytotoxicity could be attenuated by the application of the HAT inhibitor anacardic acid (which inhibits CBP/p300/P/CAF). These data suggest that at least part of the cytotoxicity of these pesticides might be caused by epigenetic changes in dopaminergic neurons. The fact that epigenetic changes may be the result of pesticide exposure should be recognized and implemented in understanding the risks of developing Parkinson's disease.

Using epigenetic regulation to promote mdDA neuron survival

Substances that enhance histone acetylation (e.g. HDACis) have been studied for their potent neuroprotective effects in several Parkinsonian models. A promising example of such an HDAC inhibitor, VPA, has been studied most intensively (Kidd and Schneider, 2011) and the first cases of its utilization in relation to PD treatment have been reported recently (Hicks et al., 2011). Notably, not all neuroprotective effects of HDAC inhibition are due to deacetylation of chromatin-bound histones. For example, several HDAC-inhibiting factors, including VPA, are thought to inhibit the activity of glycogen synthase kinase 3β (Gsk3β), which is negatively related to neuronal viability (Huang et al., 2011).

Currently, the majority of studies that relate mdDA neuroprotective effects to HDAC inhibition focus on the involvement of glial cells and release by these cells of neurotrophic or inflammatory factors. VPA pre-treatment of rat primary midbrain cultures, for example, can protect them against lipopolysaccharide (LPS)-induced neurotoxicity by decreasing the release of inflammatory factors from microglial cells (Peng et al., 2005). In a follow-up study, HDAC inhibition was proposed as an underlying mechanism of neuroprotection, strengthened by observations that enhanced histone H3 acetylation correlates with a decrease in inflammatory factors and increased apoptosis in microglial populations (Chen et al., 2007). Moreover, 3-hydrocymorphinan, a dextromethorphan analog, was shown to protect against LPS toxicity by increasing histone H3 acetylation, which resulted in increased neurotrophic factor expression (Zhang et al., 2006). In line with this is the observed upregulation of Bdnf and Gdnf expression upon VPA treatment of *in vitro* cultured astrocytes (Chen et al., 2006). Furthermore, HDAC inhibition enhanced histone H3 acetylation within the *Gdnf* promoter resulting in increased *Gdnf* mRNA levels in primary astrocyte cultures (Wu et al., 2008). In addition, similar capacities were proposed for other HDAC
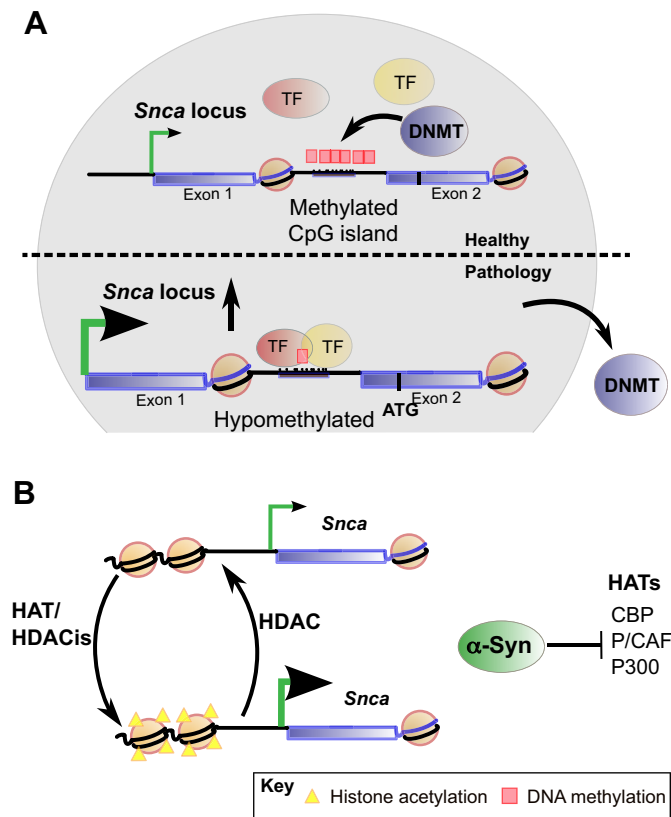
Fig. 5. Epigenetic processes involved in dopaminergic neuronal pathology. (**A**) The gene encoding α-syn (SNCA), which is involved in the pathogenesis of PD, is regulated by Dnmt1-mediated methylation (top panel). α-Syn is upregulated as a consequence of loss of methylation on the *Snca* promoter due to loss of nuclear-localized Dnmt1 (which occurs as a consequence of it being sequestered in α-syn aggregates). (**B**) HDAC inhibitors (green) increase acetylation at the *Snca* promoter and are thus able to increase the level of α-syn, which in turn is able to lower the level of HATs, again through aggregation within α-syn-containing complexes, thereby influencing the epigenetic landscape of the α-syn-containing dopaminergic neurons.

inhibitors, including TSA and NaB and suberoylanilide hydroxamic acid (SAHA) (Kidd and Schneider, 2010; Chen et al., 2012).

Several reports have described the beneficial effects of HDAC inhibitors on DA neuron survival. The HDAC inhibitor phenylbutyrate was shown to protect mdDA neurons, possibly through increased DJ-1 expression, which is involved in *Th* promoter activation as described above (Gardian et al., 2004; Zhou et al., 2011). Moreover, beta-hydroxybutyate, an endogenous and specific inhibitor of class I HDACs (Shimazu et al., 2012), has been reported to protect primary rat mesencephalic neurons from 1-methyl-4-phenylpyridinium [MPP(+)]-induced toxicity (Kashiwaya et al., 2000). Another study reported regulation of heat shock protein 70 (Hsp70) expression through changes to the di- and trimethylation of histone H3 lysine 4 (H3K4me2 and H3K4me3) and p300 (HAT) recruitment (Marinova et al., 2009; Marinova et al., 2011; Leng et al., 2010). As a member of the heat shock protein family, Hsp70 is involved in protein folding, is able to upregulate the apoptotic regulator Bcl2, and is involved in several additional anti-apoptotic mechanisms (Yenari et al., 2005). In addition, urocortin (Ucn), a neuropeptide with HDAC-inhibiting properties, was studied for is protective effects in primary rat mesencephalic cultures, following preliminary neuroprotective reports in other models (Bayatti et al., 2003). The protective effects of Ucn against spontaneous DA neuronal cell death correlated with increased histone H3 acetylation. Besides its HDAC-inhibiting capacity, Ucn inhibits glycogen synthase kinase 3β, and this has been suggested to contribute to the Ucn-driven neuroprotective effects (Huang et al., 2011). Finally, pioglitazone, a peroxisome proliferator-activated receptor γ (Pparγ) agonist, was demonstrated to inhibit indirectly nuclear translocation of Hdac3 in a 3-nitropropionic acid-induced Huntington animal model, thereby partly alleviating neurodegeneration (Napolitano et al., 2011). This suggests that the neuroprotective effect of pioglitazone as described in PD models (Laloux et al., 2012) might rely on inhibition of Hdac3 activity in the nucleus.

However, despite the promising results described here, it has become clear that HDAC inhibitors such as VPA can display detrimental effects in humans. For example, it has been reported that VPA, used as an anti-psychotic drug, can induce parkinsonism (Mahmoud and Tampi, 2011). Therefore, the clinical application of epigenetic-modulating drugs for promoting dopaminergic neuroprotection is at a very early stage of development and a more in-depth validation and understanding through experiments in animal models is essential.

Conclusions

As we have highlighted above, the development of mdDA neuronal precursors and their specification to mature mdDA neurons might rely on many levels of epigenetic regulation. In brief, Polycomb group proteins may be involved in initially defining regional specificity within the midbrain in order to generate an mdDA permissive region and they might additionally have direct influence on crucial mediators of mdDA neuronal specification. Within the mdDA permissive area, precursors then start to differentiate and imprinted factors such as Dlk1 arise, specifying part of the mdDA neuronal phenotype. During the terminal differentiation of mdDA neurons, the regulation of large parts of the transcriptional profile is controlled through inhibition of HDAC occupancy of the involved promoter regions, either through interplay with Nurr1 and/or through RAR-RXR complexes, the latter providing a dual role for RA. In the ventricular zone, RA is involved in maintaining stem cell fate though epigenetic mechanisms, whereas in the

differentiation phase, RA plays a role in fine-tuning mdDA neuronal subset specificity. Furthermore, RA provides positional specification in the induction of mdDA neurons that will become SNc neurons in the adult. These inductive signals could rely on RXR-RAR-directed epigenetic mechanisms, mainly involving HDAC regulation. The complexity of the regulatory events occurring at specific promoter sites in mdDA neurons is best illustrated by the regulation of the rate-limiting enzyme in DA synthesis, tyrosine hydroxylase (Th), and it is clear that a multitude of both epigenetic and classic gene regulatory events are essential for proper *Th* gene regulation.

In the case of cell programming and reprogramming approaches, the ultimate goal is to generate a sizable pool of replacement mdDA neurons. Based on the crucial role of epigenetics in modulating mdDA neuronal fate, epigenetic programming might thus form a new corner stone. From current data, it is clear that some aspects of the mdDA neuronal phenotype can be programmed *in vitro*; however, the stability and efficiency of this process may be increased if the correct epigenetic marks in such cells are properly generated. This might also circumvent the problem of teratoma formation after transplantation procedures using these neurons. Recent advances in genome methylation and acetylation profiling will provide the means to achieve this goal. Concerning PD pathology, the initial data suggest that epigenetic events, including histone acetylation, can also contribute to mdDA neuronal cell loss and the disruption of mdDA neuron maintenance. However, although *in vitro* data were promising and suggested that treatment with HDAC inhibitors might overcome some of this pathology, initial data on VPA treatment in humans suggest that we should be careful in translating these *in vitro* results into a clinical application. Toxic substances and drugs of abuse might also cause a decrease in epigenetic robustness within the short term, whereas aging may be responsible for a slow, ongoing decline in the effectiveness of epigenetic mechanisms to adapt chromatin in order to secure the activity states of vital genes in neuronal viability.

Finally, it seems inevitable that research associated with the mdDA neuronal system needs to embrace epigenome-wide association studies (Rakyan et al., 2011), for which technical limitations are rapidly being surmounted. It seems increasingly likely that promising findings from low-throughput studies, which are reviewed here, will prompt high-throughput studies in a manner comparable to that used in the field of genetics. Such progression will be fruitful for the fundamental understanding of the development, maintenance and specific vulnerability of mdDA neurons and might also facilitate stem cell reprogramming strategies in an ultimate attempt to generate healthy mdDA neurons as a treatment paradigm for PD.

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