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Studies on the Nuclear Receptor Nurr1

-Identification of Nurr1-regulated genes

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Stockholm 2004

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-833-5

To my Family

ABSTRACT

The nuclear receptor family comprises more than sixty members, including receptors for steroids, thyroid hormone and retinoids. Many nuclear receptors function as ligand-activated transcription factors that regulate the expression of specific target genes. The family also includes nuclear receptors that lack identified ligands, and these receptors are therefore referred to as orphan receptors. It has recently been shown that some of these orphan receptors are ligand-independent. Nurr1 (NR4A2) is a constitutively active nuclear receptor that belongs to this category of nuclear receptors. Nurr1 is expressed in the central nervous system (CNS) from early embryogenesis into adulthood. Interestingly, Nurr1 is an early expression marker of midbrain dopamine (DA) cells, and gene targeting of Nurr1 in mice leads to agenesis of midbrain DA neurons. The fact that Nurr1 is important for the development of DA neurons has important implications, as these cells regulate motor control and their degeneration is the cause of Parkinson's disease.

The main focus of this thesis was to identify genes that are regulated by Nurr1 and in this way increase our understanding of the role of this transcription factor in DA cells and in other cells that express Nurr1. We used the DA cell line MN9D, which store and produce DA. Expression of Nurr1 in this cell line resulted in an increased DA content of the cells. Furthermore, Nurr1 increased the expression of the DA producing enzyme aromatic L-amino acid decarboxylase (AADC) and the DA transporter vesicular monoamine transporter 2 (VMAT2). Expression analyses showed that the levels of expression of AADC and VMAT2 were deregulated in developing midbrain DA cells of mice in which the Nurr1 gene had been selectively disabled, which suggests that Nurr1 plays a role in DA production and storage.

Using cDNA microarrays, we investigated changes in gene expression induced by Nurr1 in the MN9D cells. This analysis revealed that neuropilin-1 (Nrp1), a receptor for secreted neuronal guidance polypeptides, is regulated by Nurr1 in the cells. Furthermore, Nurr1 regulated the Nrp1 promoter in reporter gene assays. *In situ* hybridization experiments revealed that Nrp1 expression was diminished in Nurr1 knockout mice in the dorsal motor nucleus of the vagus nerve, which suggests that Nurr1 plays a role in the regulation of Nrp1 *in vivo*.

We used MN9D cells to study the effects of Nurr1 on DA cell differentiation. Expression of Nurr1 in the MN9D cells induced cell cycle arrest and morphological differentiation, characterized by neurite extension. We determined the functional requirements for Nurr1-induced differentiation using different Nurr1 derivatives, and the results provide valuable information about the functional role of Nurr1 *in vivo*.

We elucidated the function of Nurr1 in developing DA cells by experiments on embryonic ventral midbrains cultured *in vitro*. Expression of the DA cell marker tyrosine hydroxylase, which is never expressed in the ventral midbrains of Nurr1 knockouts *in vivo*, could be induced in the knockout cultures. Moreover, well-defined nerve fiber bundles were formed in wild-type cultures, but did not form in Nurr1-deficient cultures, giving further evidence that Nurr1 is important for target innervation.

To understand the role of Nurr1 in adult physiology we investigated the impact of a heterozygous deletion of Nurr1 on rewarding behavior in mice. Mice heterozygous for the Nurr1 gene had a lower tendency than wild-type mice to become dependent on ethanol drinking and wheel running, which suggests that Nurr1 is important for reward mechanisms.

Elisabet Hermanson, 2004

ABBREVIATIONS

6-OHDA	6-Hydroxydopamine
AF-1, AF-2	Activation function 1, 2
AADC	Aromatic L-amino acid decarboxylase
BrdU	Bromodeoxyuridine
CARβ	Constitutive active androstane receptor β
CNS	Central nervous system
COUP-TF	Chicken ovalbumine upstream promoter transcription factor
D2R	Dopamine D2 receptor
DA	Dopamine
DAT	Dopamine transporter
DBD	DNA-binding domain
DMN X	Dorsal motor nucleus of the vagus nerve
dox	Doxocycline
E	Embryonic day
EGF	Epidermal growth factor
En	Engrailed
ER	Estrogen receptor
ES	Embryonic stem
FGF	Fibroblast growth factor
FXR	Farnesoid X receptor
GDNF	Glial cell line-derived neurotrophic factor
HNF4	Hepatocyte nuclear factor 4
HRE	Hormone response element
LBD	Ligand-binding domain
L-dopa	Levodopa
LGE	Lateral ganglionic eminence
LRH-1	Liver receptor homolog 1
LXR	Liver X receptor
MPTP	1-Methyl-4-phenylpyridinium
NAc	Nucleus accumbens
N-CoR	Nuclear receptor co-repressor
NBRE	NGFI-B response element
NR	Nuclear receptor
NGFI-B	Nerve growth factor induced gene B
Nrp1	Neuropilin-1
Nor1	Neuron-derived orphan receptor 1
Nurr1	Nur-related factor 1
PCR	Polymerase chain reaction
PD	Parkinson's disease
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
POMC	Pro-opiomelanocortin
QTL	quantitative trait locus
Raldh1	Retinal dehydrogenase 1
RAR	Retinoic acid receptor
RRF	Retrorubral field (also A8)
RT-PCR	Reverse transcription PCR
RORβ	Retinoic acid related receptor β
RXR	Retinoid X receptor
Sema-3A	Semaphorin-3A
SF-1	Steroidogenic factor-1
Shh	Sonic hedgehog
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
SNc	Substantia nigra pars compacta (also A9)
SZ	Schizophrenia
TH	Tyrosine hydroxylase
TR	Thyroid hormone receptor
USP	Ultraspiracle
VDR	Vitamin D receptor
VMAT2	Vesicular monoamine transporter-2
VTA	Ventral tegmental area
VM	Ventral mesencephalon

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II. **Elisabet Hermanson**, Maria Bergsland, Gerard Benoit, Eliza Joodmardi, Marcin Kowanetz, Aris Moustakas and Thomas Perlmann. The nuclear receptor Nurr1 regulates the expression of Neuropilin-1. (2004) *Manuscript.*

III. Diogo Castro, **Elisabet Hermanson***, Bertrand Joseph*, Åsa Wallén, Piia Aarnisalo, Alfred Heller, Thomas Perlmann. Induction of cell cycle arrest and morphological differentiation by Nurr1 and retinoids in dopamine MN9D cells. (2001) *The Journal of Biological Chemistry* 276, 43277-43284.

IV. Nina Törnqvist, **Elisabet Hermanson**, Thomas Perlmann, Ingrid Strömberg. Generation of tyrosine hydroxylase-immunoreactive neurons in ventral mesencephalic tissue of Nurr1 deficient mice. (2002) *Developmental Brain Research* 133, 37-47.

V. Martin Werme, **Elisabet Hermanson**, Andrea Carmine, Silvia Buervenich, Rolf Zetterström, Peter Thorén, Sven-Ove Ögren, Lars Olson, Thomas Perlmann, Stefan Brené. Decreased ethanol preference and wheel running in Nurr1-deficient mice. (2003) *European Journal of Neuroscience* 17, 2418-2424.

INTRODUCTION

Multicellular organisms consist of many different types of cells that play important roles during embryogenesis and in the adult organism. The individual cell gets its identity by the integration of a variety of signals received from surrounding cells or from the cell itself. These signals are converted by a multitude of receptor systems and result in the expression of a certain gene repertoire that, in turn, gives the cell its own specific properties. Some of the receptors are placed on the cell surface, and are activated by hydrophilic ligands, which start a cascade of intracellular events that eventually leads to gene regulation. Other receptors, called nuclear receptors (NRs), are intracellular and can be activated by small lipophilic molecules that easily pass through the cell membrane and bind to the receptors. The activated NRs act as transcription factors that bind to DNA and turn on the transcription of target genes (Fig. 1). It has long been known that NRs are important in processes such as differentiation, metabolism, reproduction, homeostasis and morphogenesis of higher organisms. Significant progress in efforts to understand the underlying mechanisms of nuclear hormone action and signaling came almost two decades ago with the cloning of the first NR, the glucocorticoid receptor. This event was followed by the cloning of a whole family of nuclear receptors, the nuclear hormone receptor family. Another major breakthrough in the study of NRs has been the target disruption of receptor genes in mice, which allows an analysis of the relevance of particular receptors for mammalian physiology and development.

A major challenge in future research is to identify the genes that are regulated by NRs to further understand the role of these receptors in their respective physiological processes.

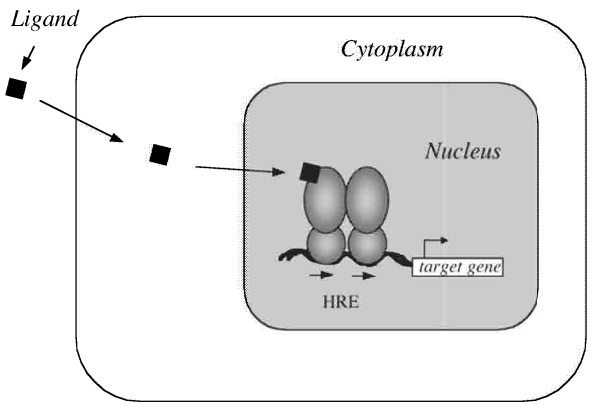

Figure 1. Mechanism of action of nuclear receptors. NRs are activated by small lipophilic ligands that can easily pass the cell- and nuclear membrane. Once in the nucleus, the ligand binds to NRs and regulate transcription by binding to hormone response elements (HREs), normally located in regulatory regions of target genes.

The nuclear receptor family

The NR family contains ligand-activated transcription factors that exert widely different biological responses by regulating target gene expression positively and/or negatively (Laudet and Gronemeyer, 2002). Included in this family of related proteins are receptors for hydrophobic molecules such as steroid hormones (i.e. estrogens, glucocorticoids, progesterone, and vitamin D), retinoic acids (vitamin A derivatives), thyroid hormones and fatty acids. This family also contains a number of receptors for which ligands do not exist, or still have to be identified. These receptors have been termed "orphan receptors" (Enmark and Gustafsson, 1996). The role of NRs in a wide variety of signaling pathways qualifies them as pharmacological targets. Intense research in recent years has identified ligands for several of the orphan receptors, and their roles in physiological functions have been identified (Giguere, 1999). This process founded a novel concept, "reverse endocrinology", where the characterization of the receptor precedes the identification of its ligand and its physiological function (Kliewer et al., 1999). Some of the orphan nuclear receptors may have ligands yet to be discovered, while others may act in a constitutive manner or may be regulated by other means.

Studies on ligand binding and nuclear receptor evolution have suggested that orphan nuclear receptors are the most ancient receptors, and that ligand binding was acquired during NR evolution (Escriva et al., 2000; Escriva et al., 1997).

Scientists have identified more than 300 NRs, using an increasingly complex nomenclature. Therefore, the nomenclature of these receptors has been unified and sorted into a system based on their evolutionary conservation (Committee, 1999).

Nuclear receptor structure

Nuclear receptors are composed of 5-6 regions (A-F), each region having a different character (Fig. 2A) rewieved in (Aranda and Pascual, 2001b; Laudet and Gronemeyer, 2002). The A/B region in the N-terminal part contains a domain involved in transcriptional activation, called the AF-1 (activation function 1) domain. The sequence of the A/B domain is very weakly conserved between species, and the domain varies significantly in length, ranging from 23 (vitamin D receptor) to 550 (glucocorticoid receptor). The A/B domain shows promoter-specific and cell-specific activity, suggesting that it is likely to contribute to the specificity of action among receptor isoforms and that it could interact with cell-type specific factors.

The DNA binding domain (DBD) in domain C is the most conserved domain of nuclear receptors and is involved in sequence-specific DNA recognition. This domain contains two zinc-finger motifs, each of which consists of four cysteines that chelate one Zn+ ion. A region in the first zink finger (designated the P-box) is important for mediating interaction with specific DNA sequences, contributing in this way to the specificity of DNA binding (Umesono and Evans, 1989). In the second zink finger, a motif called the D-box, was shown to be important for dimerization of receptors (Perlmann et al., 1993). In addition, certain receptors, for example the Nurr1/Nor1/NGFI-

B subfamily (see below), contain an A-box, involved in recognition of additional residues upstream of a half site (Fig. 2B) (Wilson et al., 1993a).

The D-domain, termed the hinge region, is not well conserved among the different receptors and functions as a hinge between the DNA-binding domain and the ligand binding domain (LBD), allowing rotation of the DBD. In many cases the hinge region harbors the nuclear localization signal.

The LBD in region E harbors the AF-2 (activation function 2) motif responsible for ligand-dependent transcriptional activation. It also contains regions important for receptor dimerization. The crystal structures of multiple nuclear receptor LBDs have been solved, and these structures are similar in different receptors. The LBDs are formed by 12 conserved α-helical regions, numbered from H1 to H12, and a β-turn. The LBDs are folded into a three-layered antiparallel helical sandwich. A central core layer of three helices is packed between two additional layers to create the ligand-binding pocket. The pocket is lined with hydrophobic amino acids that interact with the ligand. The size of the ligand-binding pockets varies among receptors, where some pockets are small and the ligands occupy most of the space. Other ligand binding pockets are larger, which allow binding of several structurally diverse ligands (Benoit et al., 2004).

The F-domain displays little evolutionary conservation, is only present in some NRs and has an unknown function.

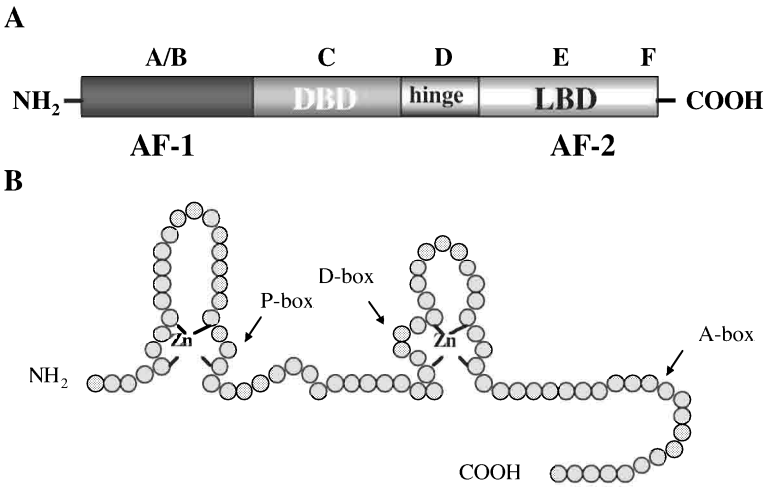

Figure 2 A. Schematic representation of a nuclear receptor. A typical NR is composed of several functional domains. The N-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The conserved DNA-binding domain (DBD), or region C, is responsible for the recognition of specific DNA sequences. The variable hinge region, region D, connects the DBD to the conserved E/F region that contains the ligand-binding region (LBD) as well as the dimerization surface. The LBD also contains the ligand-dependent AF-2 transactivation domain. **B.** The DNA binding domain contains two zink finger motifs. Each motif contains a zink atom that is coordinated by four cysteines, forming a tetrahedral structure. In the first zink finger, amino acid residues in the so called P-box mediate DNA-binding specificity, whereas the D-box in the second finger is important for dimerization interactions between receptors bound to DNA. In some NRs, amino acid residues in a C-terminal extension form the A-box, which is important for recognition of 5' flanking residues of the half-site. (Figure 2B modified from Aranda and Pascual 2001).

DNA binding as monomers, homodimers or heterodimers

Nuclear receptors regulate the transcription of target genes by binding to specific DNA sequences known as "hormone response elements" or HREs (Aranda and Pascual, 2001a). These elements are normally located in the promoter region of the target gene, but they may lie several kilobases upstream or downstream of the promoter (Sap et al., 1990). The HREs are derivatives of the same hexameric DNA half-site motif, PuGGTCA (Pu = A or G). Importantly, this motif represents a consensus sequence that can show significant variations in naturally occurring HREs. The receptors interact with the response element as monomers, homodimers or heterodimers (Fig. 3). Monomeric receptors, such as for example the orphan receptor NGFIB, bind to a single half-site (Wilson et al., 1993a), whereas receptor dimers bind to two recognition motifs. For dimeric HREs, the half-sites can be arranged as palindromes, inverted palindromes, or direct repeats, spaced by zero to five basepairs (Fig. 3). Steroid hormone receptors, such as glucocorticoid receptors, progesterone receptors and androgen receptors, bind as homodimers to palindromes of the AGAACA motif spaced by three nucleotides (Fig. 3). In contrast to the steroid receptors, non-steroidal receptors can bind to HREs arranged as palindromes, inverted palindromes, or direct repeats. Careful analysis of both natural and synthetic HREs has shown that the non-steroidal receptors are most potently bound to DRs and that the spacing of the two HREs is an important determinant for the specificity of the hormonal response. Although several non-steroidal receptors bind DNA as homodimers, they prefer to bind their HREs as heterodimers (Bugge et al., 1992; Kliewer et al., 1992), with the promiscuous heterodimerization partner retinoid X receptor (RXR).

Many of these heterodimers, such as RAR (retinoic acid receptor)/RXR, TR (thyroid hormone receptor)/RXR and VDR (vitamin D receptor)/RXR, are known as "non-permissive" heterodimers (Kliewer et al., 1992). Such heterodimers respond only to ligand-binding to the RXR partner, and not to ligand-binding to RXR. However, in RAR/RXR heterodimers, ligand binding to RAR induces conformational changes in RXR that allow binding of RXR ligands (Botling et al., 1997). RXR can also form "permissive" heterodimers with other NRs, such as LXR (liver X receptor), FXR (farnesoid receptor), PPAR (peroxisome proliferator activated receptor) and Nurr1 (nur-related factor1), where RXR ligands can activate the heterodimer independently of the ligand-binding status of the heterodimer partner (Blumberg and Evans, 1998; Mangelsdorf and Evans, 1995).

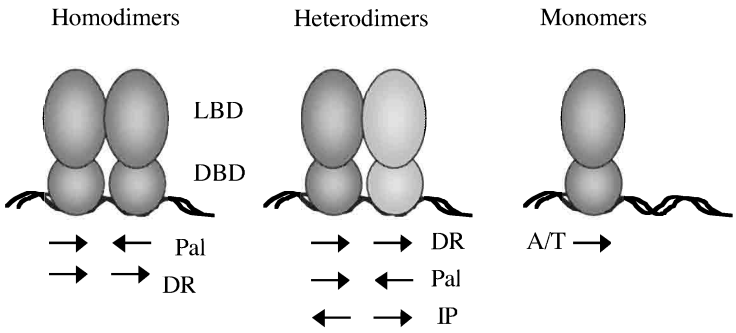

Figure 3. Binding of NRs to hormone response elements (HREs). NRs can bind DNA as homodimers, heterodimers or monomers. Steroid receptors bind as homodimers to palindromic (Pal) HREs. Some orphan receptors (e.g COUP-TF and HNF-4) bind as homodimers to direct repeats (DRs). Heterodimers with RXR (e.g RAR, TR, VDR, PPAR, LXR, FXR, NGFI-B and Nurr1) can recognize diverse HREs in which half-site motifs are arranged as palindromes, direct repeats (DRs), or inverted palindromes (IPs). Monomeric binding requires the half-site motif preceded by a 5'-flanking A/T rich sequence. LBD= ligand-binding domain, DBD= DNA-binding domain.

Transcriptional activation by NRs

NRs are transcriptional modulators that regulate the expression of target genes; often, but not always, as a response to ligand binding. X-ray crystallography studies have contributed significantly to the understanding of the conformational changes that occur in the LBD upon ligand binding. Comparisons of unliganded (apo-) and liganded (holo-) receptors have suggested a "mouse trap" model for ligand-dependent transcriptional activation (Moras and Gronemeyer, 1998). In this model, ligand binding induces a series of conformational changes in the 12 α-helices, the most important being the swinging of helix 12, which in the unligated form is positioned away from the ligand-binding pocket. In its final position, helix 12 forms a "lid" for the pocket that further stabilizes ligand binding by contributing to the hydrophobic environment of the ligand-binding pocket. The second step in transcriptional activation of nuclear receptors is the recruitment of co-activators. The conformational change in helix 12, which corresponds to the AF-2, leads to the exposure of amino acids involved in the recruitment of coactivators. Coactivators bind to NRs via specific motifs composed of one or several LXXLL (L=leucine and X= any amino acid) motifs (Bevan and Parker, 1999; Heery et al., 1997). Coactivators possess or recruit enzymatic activities, and form large coactivator complexes that are reported to have histone acetyltransferase (HAT) activity, capable of acetylating histones (Glass and Rosenfeld, 2000). Histone acetylation opens up the highly structured and condensed chromatin in order to allow the transcriptional machinery to access the DNA. In the third step, the coactivator complex dissociates from the receptor, possibly due to acetylation of the coactivator that decreases the ability to interact with the receptor. Another complex, termed SMCC/DRIP/TRAP is assembled to the receptor (Fondell et

al., 1996; Ito et al., 1999; Rachez et al., 1999). This complex is able to establish contacts with the basal transcription machinery, that in turn initiates transcription of the target gene.

Receptors such as TR, retinoic acid receptor RAR and VDR bind to DNA in the absence of ligand and repress transcription of target genes (Perlmann and Vennstrom, 1995). A search for proteins involved in mediating this silencing effect identified two co-repressor proteins called N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) that attach to unliganded receptors and dissociate upon ligand binding (Chen and Evans, 1995; Horlein et al., 1995; Kurokawa et al., 1995). It was later shown that N-CoR and SMRT are part of a large complex that possesses histone deacetylase activity (Pazin and Kadonaga, 1997). Thus, the active repression in the absence of ligand is due to histone acetylation, which results in highly condensed inactive chromatin.

Ligand-independent activation of NRs

In addition to the well-established ligand-dependent activation of nuclear receptors, several members of the NR superfamily can be regulated by ligand-independent mechanisms. The mechanisms whereby ligand-independent activation is achieved are poorly understood. Several of the NRs, such as progesterone receptor (PR), estrogen receptor α and β (Erα, Erβ) and the orphan receptors chicken ovalbumine upstream promoter transcription factor (COUP-TF) and steroidogenic factor-1 (SF-1), are stimulated by extracellular signals, for example growth factors and DA (Hammer et al., 1999; Power et al., 1991a; Power et al., 1991b; Weigel and Zhang, 1998). These signals in most cases alter the activity of phosphatases or kinases, suggesting that phosphorylation of NRs or their cofactors is involved. Indeed, ER activity is modulated by phosphorylation of specific residues in the AF1 domain, and phosphorylation of the receptor has been shown to induce coactivator recruitment (Tremblay et al., 1999). Several other NRs in addition to ER, for example SF-1 and NGFI-B, are phosphorylated (Rochette-Egly, 2003).

Do all NRs bind ligands in a classical way?

The classical way of looking at nuclear receptors is that binding of ligand to the LBD induces activation of the receptor. However research during the past years has revealed that the LBD can be utilized in several different ways (Benoit et al., 2004). The nuclear receptors that use their LBD in a non-classical way can be divided into three main categories depending on how they utilize their LBD. The first group includes the constitutively active receptors constitutive androstane receptor β (CARβ) and retinoic acid related orphan receptor β (RORβ), which can bind ligands and be deactivated by androstane metabolites and retinoic acid respectively (Forman et al., 1998; Stehlin-Gaon

et al., 2003). The second group includes hepatocyte nuclear factor 4 (HNF4) and the *Drosophila* RXR homolog USP ultra-spiracle (USP), which bind fatty acids and phospholipids respectively in a constitutive manner. Thus, these binding molecules are thought to be constitutive structural cofactors rather than regulatory ligands (Billas et al., 2001; Wisely et al., 2002). Finally, the third category is represented by the constitutively active receptors Nurr1 and DHR38, which completely lack a ligand-binding cavity. Instead, the ligand binding pocket is filled with bulky hydrophobic side-chains (Baker et al., 2003; Wang et al., 2003). More recently the crystal structure for liver receptor homolog 1 (LRH-1) was solved and shown to be empty, but with the potential to bind ligand as the ligand binding pocket was intact (Sablin et al., 2003).

The NGFI-B subfamily

The NGFI-B subfamily of nuclear receptors contains three vertebrate receptors (Nurr1, Nor1 and NGFI-B) and their homologues in *Drosophila* (DHR38) (Fisk and Thummel, 1995) and *C.Elegans* (CNR8) (Kostrouch et al., 1995). The nomenclature for this group of NRs is extremely confusing and many different names have been given to these receptors (Table 1) (Laudet and Gronemeyer, 2002). All members of this family are transcriptional activators that do not require a ligand to be active (Davis et al., 1991; Paulsen et al., 1992). The genomic structures of the vertebrate receptors are remarkably similar in both the DBD (> 90%) and LBD (> 60%), suggesting that the NGFI-B family has evolved from a common ancestral gene.

Table 1. Alternative names for NGFI-B, Nurr1 and Nor1

Receptor	Official name	Species	Other names
NGFI-B	NR4A1	Human	TR3, NAK1, ST-59, NGFI-Bα
		Mouse	Nurr/77, nurr77, N10, NGFI-Bα
		Rat	NGFI-B, NGFI-Bα, TIS1
Nurr1	NR4A2	Human	NOT, NGFI-Bβ, TINUR, Nurr1
	NR4A3	Mouse	Nurr1, NGFI-Bβ
		Rat	RNR-1, NGFIB-β, Nurr1, HZF-3
Nor1		Human	TEC, MINOR, CHN, NOR-1, NGFIB-γ
		Rat	NOR-1, NGFIB-γ

NGFI-B (nerve growth factor induced gene B) was originally cloned as a gene induced by nerve growth factor in the rat pheochromocytoma cell line PC12 (Milbrandt, 1988). Nurr1 (Nur-related factor 1) was isolated from a neonatal mouse brain cDNA library under low-stringency hybridization conditions, using the DBD of the nuclear receptor COUP-TF as a probe (Law et al., 1992). Nor1 (neuron-derived orphan receptor 1) was identified from cultured rat fetal forebrain cells (Ohkura et al., 1994).

Nurr1, Nor1 and NGFI-B bind as monomers to DNA at an octameric sequence (AAAGGTCA) called the "NGFI-B response element" (NBRE), which was initially identified by genetic selection in yeast (Wilson et al., 1991; Wilson et al., 1993a). The NBRE includes two additional alanines preceding the consensus AGGTCA half-site, and it has been shown that recognition of these two residues depends on the A-box in the DBD (Wilson et al., 1992). In addition, Nurr1 and NGFI-B (but not Nor-1) can

heterodimerize with RXR and activate transcription through a DR5 element (Forman et al., 1995; Perlmann and Jansson, 1995; Zetterstrom et al., 1996a). Finally, a third type of DNA binding activity for this group of nuclear receptors has been identified in the regulatory region of the pro-opiomelanocortin (POMC) gene (Philips et al., 1997a). This promoter contains an inverted repeat, called NuRE, containing two NBREs spaced by six nucleotides. Nurr1, Nor1 and NGFI-B can bind the NuRE as homodimers or as heterodimers between NGFI-B and Nurr1 (Maira et al., 1999).

These genes are classified as immediate-early genes, whose expression is induced rapidly but transiently by various stimuli in the absence of *de novo* protein synthesis (Morgan and Curran, 1995). The stimuli that are able to induce expression of the receptors include cAMP, growth factors, peptide hormones and neurotransmitters (Maruyama et al., 1995; Milbrandt, 1988). Furthermore, their expression can also be induced by membrane depolarization, mechanical agitation and magnetic fields (Bandoh et al., 1997; Miyakoshi et al., 1998; Pena de Ortiz and Jamieson, 1996).

The three members of this subfamily are abundantly expressed in the CNS. Nurr1 and Nor1 are expressed during embryogenesis and their expression continues into adulthood, while NGFI-B expression is mainly found in the adult nervous system (Zetterstrom et al., 1996a). The expression patterns of these receptors overlap in some areas, suggesting functional redundancy among the NGFI-B subfamily genes (Saucedo-Cardenas and Conneely, 1996; Xiao et al., 1996; Zetterstrom et al., 1996a; Zetterstrom et al., 1996b). Nor1 and NGFI-B are also found in areas outside the CNS, in, for example, adrenal glands, thymus, lung and testis (Maruyama et al., 1995; Milbrandt, 1988). The expression in thymus is consistent with a suggested role for Nor1 and NGFI-B in the negative selection of immature T-cells (Calnan et al., 1995; Liu et al., 1994; Woronicz et al., 1994; Woronicz et al., 1995). Furthermore, Nurr1, Nor1 and NGFI-B are highly expressed in the hypothalamus, pituitary and adrenal glands, and are thought to play a coordinate role in neuroendocrine regulation of the hypothalamic-pituitary-adrenal axis at multiple levels (Murphy and Conneely, 1997; Murphy et al., 2001; Philips et al., 1997a; Philips et al., 1997b; Wilson et al., 1993b). More recently, it has been shown that in response to apoptotic stimuli, NGFI-B translocates from the nucleus to mitochondria to induce the release of cytochrome c and apoptosis in a manner that does not depend on DNA-binding (Li et al., 2000). Nor-1 has been implicated in the pathogenesis of cancer, as Nor1 is fused to the EWS protein (an RNA-binding protein of unknown function) in patients with extraskeletal myxoid chondrosarcoma (Gill et al., 1995; Labelle et al., 1999).

Surprisingly, no major phenotypes have been described in NGFI-B null mutant mice (Lee et al., 1995). Two papers on Nor1 knockout mice have recently been published. In the first, a subtle phenotype in the inner ear was detected (Ponnio et al., 2002). In contrast, the second report describes a severe phenotype where the embryos die during early embryogenesis due to incomplete gastrulation (DeYoung et al., 2003). Recently, Nor1 and NGFI-B double knockout animals were generated. These mice develop a myeloproliferative disease that leads to death of the mice within three weeks after birth, suggesting that Nor1 and NGFI-B function as myeloid tumor suppressor genes (Conneely, 2004).

Nurr1, which is the main focus of this thesis, is discussed in more detail below.

Nurr1

The mouse Nurr1 (NR4A2) gene is approximately 7 kilobases long and contains 8 exons. It has been mapped to mouse chromosome 2 (Castillo et al., 1997). The human Nurr1 gene (NOT1) has the same structure as the mouse Nurr1 gene and spans a region of 8.3 kilobases (Ichinose et al., 1999; Torii et al., 1999). Several different Nurr1 isoforms have been reported with truncations in both the N- and C-terminal part of the protein, but the physiological roles of these splice variants are still unknown (Castillo et al., 1998b; Castillo et al., 1997; Ohkura et al., 1999).

Nurr1 can form heterodimers with RXR *in vitro,* and the amino acids important for heterodimerization have been identified (Aarnisalo et al., 2002; Perlmann and Jansson, 1995; Sacchetti et al., 2002). It was recently shown that Nurr1-RXR signaling is important for the survival of Nurr1-positive neurons, suggesting a role for Nurr/RXR heterodimers *in vivo* (Wallen-Mackenzie et al., 2003).

Nurr1 contains an AF-2 core that can activate transcription in a cell-type dependent manner (Castro et al., 1999). In contrast to the AF-2 of other nuclear receptors, the Nurr1 AF-2 domain is not involved in binding of classical co-activators (Castro et al., 1999). The N-terminal domain is unusually large and contains a core domain important for transcriptional activation (Nordzell et al., 2004). It was recently shown that Nurr1 lacks a cavity for ligand binding as determined by the crystal structure of the LBD (Wang et al., 2003).

Nurr1 expression is seen mainly in the CNS except for a few Nurr1-positive cells in the developing limb, adult testis, adrenal gland and thymus (Law et al., 1992; Saucedo-Cardenas and Conneely, 1996; Zetterstrom et al., 1996a; Zetterstrom et al., 1996b). The CNS expression of Nurr1 is found in a number of areas both during embryogenesis and postnatally. For example, Nurr1 mRNA is detected in the cortex, hippocampus, thalamus, dorsal motor nucleus of the vagus nerve, cerebellum, olfactory bulb, and spinal cord (Law et al., 1992; Saucedo-Cardenas and Conneely, 1996; Zetterstrom et al., 1996a; Zetterstrom et al., 1996b). Importantly, Nurr1, but not Nor1 or NGFI-B, is expressed in developing DA cells starting from embryonic day (E) 10.5 in the mouse (Zetterstrom et al., 1996b), with a peak of expression shown between E13-15 (Perrone-Capano and Di Porzio, 2000). In accordance with this observation, studies on Nurr1 null mice showed that these mice lack midbrain dopamine (DA) neurons at birth, demonstrating that Nurr1 is essential for the formation of these cells (Zetterstrom et al., 1997). Further studies on the Nurr1 knockouts have shown that DA progenitors are initially formed but as development progresses these midbrain DA progenitors degenerate in the absence of Nurr1 (Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Witta et al., 2000)}. In accordance with the results *in vivo*, Nurr1 expression in cerebellar neural stem cells, together with an unknown astrocyte factor, results in an increased number of cells with a DA neuronal phenotype (Wagner et al., 1999).

The role of Nurr1 in DA cell differentiation is well established, however the immediate target genes and downstream molecular mechanisms for Nurr1 are poorly understood. A few reports on genes regulated by Nurr1 have been published during the last years. For example, Nurr1 binding sites are present in the promoter of the tyrosine hydroxylase (TH) gene (the rate-limiting enzyme involved in DA synthesis) and Nurr1 can transactivate the activity of this promoter (Iwawaki et al., 2000; Kim et al., 2003b;

Sakurada et al., 1999; Schimmel et al., 1999). In addition, Nurr1 has been shown to regulate the transcriptional activity of the 5'flanking region of the dopamine transporter (DAT) gene via an NBRE-independent mechanism (Sacchetti et al., 1999; Sacchetti et al., 2001). Furthermore, the gene for CYP11B2, which is involved in the production of aldosterone in the adrenal gland, was identified as regulated by Nurr1 (Bassett et al., 2003). More recently, Nurr1 was shown to regulate the expression of the bone matrix protein osteopontin (Lammi et al., 2004). Additional, studies have revealed the dependence of Nurr1 for expression of AADC, VMAT2, Ret, and $p57^{Kip2}$ ((Joseph et al., 2003; Smits et al., 2003; Wallen et al., 2001) and results discussed below).

As Nurr1 is encoded by an immediate-early gene, its expression can be induced by a variety of stimuli. Stressful insults, such as ischemia of the rat brain, induce a rapid increase of Nurr1 expression (Honkaniemi and Sharp, 1996; Honkaniemi et al., 1997). Furthermore, Nurr1 expression is regulated in models of neuronal activation. For example, Nurr1 expression is induced by membrane depolarization in PC12 cells, a rat pheocromocytoma cell line often used as a neuronal model system (Greene and Tischler, 1976). In addition, kainic acid, which increases synaptic activity and causes seizures, leads to upregulation of Nurr1-expression in the hippocampus of rats (Crispino et al., 1998; Honkaniemi and Sharp, 1999). It has also been suggested that Nurr1 regulates the activity of neurons in the olfactory bulb, since naris closure leads to decreased expression of Nurr1 in the olfactory bulb (Liu and Baker, 1999). Induction of Nurr1 expression is also seen in cells outside of the CNS, exemplified by the cloning of Nurr1 in rat regenerating liver and in human activated T-lymphocytes (Mages et al., 1994; Scearce et al., 1993). Furthermore, Nurr1 expression is induced in bone cells by the metabolic regulator parathyroid hormone (Tetradis et al., 2001).

The Nurr1 promoter has been characterized and contains a glucocorticoid binding site, a cAMP response element and two c-Jun binding sites (Castillo et al., 1997). Interestingly, inflammatory mediators can activate the Nurr1 promoter *via* cAMP and NFκB response elements, suggesting a role for Nurr1 in inflammatory responses (McEvoy et al., 2002a; McEvoy et al., 2002b).

The DA system

Brain DA cells were originally identified by the Falck-Hillarp histofluorescence method, which identifies fluorescent monoamines upon formaldehyde treatment (Falck et al., 1962). It was found that DA cells were present in the diencephalons (forebrain), the mesencephalon (midbrain), the retina and the olfactory bulb (Dahlström and Fuxe, 1964). The DA neurons of the mesencephalon contain approximately 75% of the total number of brain DA cells (about 40,000 neurons in the rat) and most attention has been given to this group of DA neurons. The reason for this is the fact that midbrain DA neurons have been implicated in mental and neurological disorders (see below). Midbrain DA neurons can be divided into three groups, the lateral groups of the retrorubral field (RRF), the substantia nigra pars compacta (SNc), and the ventral tegmental area (VTA). These three groups, originally defined as A8-A10 respectively, project their axons to different forebrain areas (Dahlström and Fuxe, 1964). The SNc neurons project to the dorsolateral

striatum, called the caudate putamen, and are involved in the control of voluntary movements. This projection is called the nigrostriatal pathway, and is degenerated in patients with Parkinson's disease. VTA neurons project to the subcortical and cortical areas and form the mesolimbocortical system involved in emotional behavior and in mechanisms of natural motivation and reward. The neurons of the RRF are probably involved in interconnecting the VTA and SNc as RRF neurons project to both of these groups (Parent et al., 2000).

There are several milestones in research showing the importance of DA as a neurotransmitter. First, it was concluded that DA was a neurotransmitter (Von Euler and Lishajko, 1957). Second, depletion of DA was shown to cause the symptoms of Parkinson's disease (Carlsson, 1959) and third, striatal and limbic DA originated from mesencephalic DA neurons (Dahlström and Fuxe, 1964).

There are a number of genes whose expression is important for the function of the DA nerve terminal (Fig. 4). The first enzyme involved in the formation of DA is the rate-limiting enzyme tyrosine hydroxylase (TH), which converts the amino acid tyrosine into levodopa (L-dopa). L-dopa is then converted to DA by aromatic L-amino acid decarboxylase (AADC). Dopamine is stored in synaptic vesicles in the nerve terminal and is packed into the synaptic vesicles by vesicular monoamine transporter 2 (VMAT2). Activation of the nerve terminal by an action potential releases DA into the synaptic cleft. Released DA exerts its effect by binding to DA receptors located on the presynaptic and postsynaptic neurons, which in turn give rise to intracellular signals in the neurons. DA in the synaptic cleft is transported back into the nerve terminal by the DA transporter (DAT).

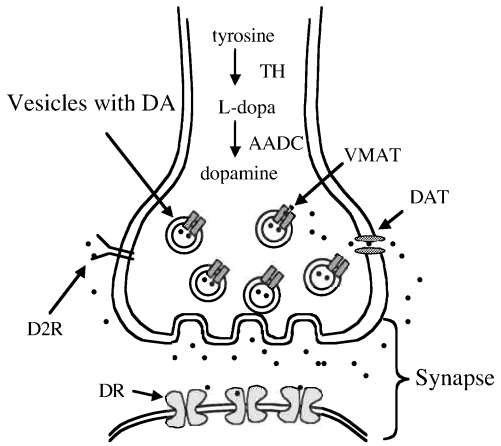

Figure 4. Signal transduction by the transmitter DA at a synapse between two nerve cells. DA is synthesized from tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) and the enzyme amino acid decarboxylase (AADC). Upon release from the presynaptic terminals into the synaptic cleft, DA interacts with postsynaptic dopamine receptors (DR). Neurotransmission is terminated by uptake into presynaptic DA fibers by the dopamine transporter (DAT). DA also activates presynaptic autoreceptors of D2-type (D2R), which are involved in the regulation of dopamine synthesis, metabolism and release. Finally, vesicular monoamine transporter 2 (VMAT2) transports dopamine into synaptic vesicles.

Disorders of the midbrain DA system

Parkinson's disease

Parkinson's disease (PD) is caused by the degeneration of neurons in the SNc and results in loss of the nigrostratal pathway. The cardinal symptoms of PD occur when approximately 70-80% of the ventral mesencephalic dopaminergic cells have undergone degeneration (Bernheimer et al., 1973). Individuals suffering from this disease show muscular rigidity, reduced movement ability, difficulties of inducing movement, slowness of movement and rhythmic resting tremors. Another characteristic feature of PD is the formation of so called Lewy bodies, primarily in the SNc. Lewy bodies are intracytoplasmic spherical inclusions that are immunoreactive for a protein called α-synuclein (Spillantini and Goedert, 2000). Interestingly, in some genetic forms of PD, α-synuclein is mutated, but the function of the gene is unknown (Kruger et al., 1998; Polymeropoulos et al., 1997). Moreover, overexpression of α-synuclein in *Drosophila* results in protein aggregates and PD-like symptoms (Feany and Bender, 2000). Genetic studies of families with inheritable forms of PD have identified two additional proteins that can be linked to PD. Both of these genes, parkin and UCHL1, are involved in the degradation of proteins by ubiquitination (Chung et al., 2003).

Mutations in the Nurr1 gene have been identified in patients with PD. Two mutations in exon 1 of Nurr1 were found to be associated with familial forms of PD. Transfection of this mutated form of Nurr1 into cell lines resulted in lower mRNA expression levels than those in cells transfected with wild-type Nurr1. Furthermore, reporter gene assays revealed that these mutants had lower transcriptional activity (Le et al., 2003). In another study, a variant of Nurr1 in which a single base pair was inserted into exon 6 of the Nurr1 gene was associated with PD. It was speculated that the Nurr1 splicing process might be disturbed in these patients as this base pair insertion is near the junction of exon/intron 6 (Xu et al., 2002; Zheng et al., 2003).

Treatment of PD

There is no treatment for PD that can slow down the degeneration of SNc neurons. The most common therapy is to give L-dopa, which increase the striatal DA levels. Treatment with L-dopa is effective in the earlier and middle stages of the disease but the effect of this drug decreases with the progression of DA cell degeneration (Dunnett and Bjorklund, 1999). Another type of treatment is the transplantation of human fetal mesencephalic tissue into the caudate putamen. The grafted cells survive and innervate the tissue but a major problem is that a large number of embryos are required and a lot of the cells die during the process (Brundin et al., 2000).

Intense research is being conducted to find alternative therapies for PD. An important tool when it comes to testing new therapies and understanding the pathology of PD is the use of animal models. Two commonly used DA cell toxins are 1-Methyl-4-phenylpyridinium (MPTP) and 6-hydroxyDA (6-OHDA), which both destroy DA signaling in the striatum. The identification of factors that can increase the survival of DA cells in toxin-induced animal models is a potential therapy for PD. For example, the dopaminotrophic factor glial cell line-derived neurotrophic factor (GDNF) can rescue the nigrostriatal pathway from toxin-induced degeneration *in vivo* (Beck et al., 1995; Choi-

Lundberg et al., 1997). As an alternative to increasing the survival of DA cells which aims at rescuing endogenous DA cells, transplantation of engineered DA cells serves to replace these cells. An attractive alternative to the treatments discussed above would be to develop technologies that could generate unlimited numbers of DA neurons from either stem cells, or DA-precursor cells derived from small amounts of embryonic tissue (Lindvall, 1999). This issue will be discussed further in "Engineering of DA cells with the help of Nurr1".

Schizophrenia

Schizophrenia (SZ) is a mental illness in which both genetic and environmental factors probably play an important role in the manifestation of symptoms. The disease is complex in origin and cannot easily be explained by a single genetic or environmental component. Efforts in identifying the underlying disturbances in SZ are currently focused on examination of the mechanism of action of drugs that alleviate the symptoms of SZ, examination of neuroanatomical abnormalities in the brains of SZ patients, and examination of candidate genes that confer susceptibility to SZ (Sawa and Snyder, 2002). In the early 1950s the beneficial effects of the drug chlorpromazine on SZ symptoms were discovered. It was later found that this drug blocked DA receptors in the nucleus accumbens (NAc) and prefrontal cortex, areas that regulate emotional behavior. Furthermore, the administration of amphetamine, which acts by releasing DA, was found to increase SZ symptoms. These drug effects led to the "DA hypothesis" for the modulation of SZ symptoms, with excess DA increasing, and decreased DA alleviating, the symptoms (Carlsson, 1988). It has later been shown that other neurotransmitters such as glutamate and seretonin are also involved in the disease (Sawa and Snyder, 2002).

Curiously, mutations in the Nurr1 gene has been linked to schizophrenia and manic depressive disorders. Direct sequencing of the Nurr1 gene revealed two different missense mutaions in the third exon of Nurr1 in two schizophrenic patients, and another missense mutation in the same exon in an individual with manic-depressive disorder. All three mutations, which were situated in the Nurr1 DBD, resulted in a 30-40% decrease in transcriptional activity of Nurr1 dimers (Buervenich et al., 2000).

The DA system in addiction and reward

The ability of drugs of abuse to cause addiction can be viewed as a form of neural plasticity. Indeed, chronic drug exposure has been shown to produce profound biochemical and morphological changes in specific brain regions thought to mediate the reinforcing and addicting actions of the drugs (Nestler and Aghajanian, 1997). Prominent among these brain regions is the mesolimbic DA system, which encompasses dopaminergic neurons in the VTA and their projections to the forebrain, including the nucleus accumbens (NAc). Indeed, chronic exposure to several drugs of abuse, including cocaine, amphetamine, opiates, and alcohol causes a series of common biochemical adaptions in the DA system (Nestler et al., 1993). For example, the levels of tyrosine hydroxylase, specific glutamate receptors and glial fibrillary acid protein (an intermediate filament protein specific to glia) are increased in the NAc, while there is a decreased level of neurofilalments (intermediate filament protein specific to neurons). In the NAc, chronic drug exposure upregulates activity of the cAMP pathway and induces delta FosB, a Fos family transcription factor. The importance of the mesolimbic DA system in

addiction is further supported by the fact that a single injection of most drugs of abuse, including psychostimulants, opioids, ethanol and nicotine, causes the release of DA in the striatum, preferentially in the nucleus accumbens (Di Chiara and Imperato, 1988). Furthermore, withdrawal from addictive drugs decreases extracellular DA release (Rossetti et al., 1992). Both social and psychological factors contribute to addiction, but it is clear from epidemiological studies that genetic factors also weigh in (Nestler, 2000). An important tool for analyzing the genetic influence on reward and addictive behaviors is the comparison of inbred strains of rat and mouse that show robust differences in behavioral and biochemical responses to drugs of abuse. For example, the drug-preferring Lewis and drug-avoiding Fischer rats have been extensively studied (Beitner-Johnson et al., 1991; Guitart et al., 1992). Furthermore, it has been shown that DBA and C57 strains of mice differ in self-administration of addictive drugs (Meliska et al., 1995; Risinger et al., 1998). Accordingly, several groups are attempting to identify the genetic basis of these behavioral differences. Another genetic approach to study addiction is the analysis of transgenic mice that lack or overexpress genes of potential interest in adaptions to drugs of abuse. For example, studies of transgenic mice overexpressing delta FosB provide evidence that delta FosB causes increased sensitivity to behavioral effects of drugs (Nestler et al., 2001).

The dopamine neurons that originates in the VTA and terminates in the NAc not only plays a critical role in the rewarding effects of drugs of abuse, but also regulates natural rewards, such as food, drink, sex and physical exercise (Koob et al., 1998; Wise, 1998). As a result, there is considerable interest in a possible role of this brain region in behavioral addictions such as gambling, eating disorders and exercise addiction,

Mice deficient in TH, VMAT2, D2R or DAT

TH deficient mice die at mid-gestation due to cardiovascular failure. Administration of L-dopa to pregnant females results in complete rescue of the mutant phenotype *in utero*. However, without further treatment these mice die before weaning (Kobayashi et al., 1995; Zhou et al., 1995). However, disruption of the TH gene results in deficiency in both DA and noradrenergic cells. To restore TH expression in noradrenergic cells, the TH coding sequence was targeted to the noradrenergic-specific dopamine-β-hydroxylase promoter by homologous recombination. These dopamine deficient mice were born at expected frequency but became hypoactive and died a few weeks after birth due to lack of feeding (Zhou and Palmiter, 1995). Moreover, they had a marked reduction of dopamine in the brain which led to multiple behavioral abnormalities characterized by reduction in spontaneous locomotion, blockade of metamphetamine induced hyperactivity and defects in active avoidance learning (Nishii et al., 1998).

Newborn VMAT2-/- mice move little, feed poorly and die within a few days after birth (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). VMAT+/- mice are viable and have half as much VMAT2 compared to VMAT2+/+ mice (Takahashi et al., 1997). The lower level of VMAT2 in heterozygous mice results in decreased levels of DA despite a near doubling of DA synthesis rates in the brain (Wang et al., 1997). In addition, MPTP administration resulted in twice as much cell death in DA neurons of heterozygous mice compared to wild-type mice (Gainetdinov et al., 1998). Finally, these

mice showed an increased locomotor response to several drugs of abuse such as cocaine, amphetamine, and ethanol (Wang et al., 1997).

Disruption of DAT results in spontaneous hyperlocomotion despite lower level of TH and dopamine in the striatum. In DAT-/- mice dopamine persist at least 100 times longer in the extracellular space, explaining the biochemical basis for the hyperdopaminergic phenotype (Giros et al., 1996). In addition, these mice do not show any degradation of DA cells after MPTP treatment, in accordance with the fact that MPP^+ (the oxidation product of MPTP) is transported into DA nerve terminals via DAT transport (Gainetdinov et al., 1997).

Dopamine D2 receptors (D2R) are important in striatal processing of motor information received from the cortex. In addition, pre-synaptic D2 autoreceptors are well known to modulate dopamine release. Absence of this gene leads to animals that are bradykinetic and akinetic in behavioral tests and show a significant reduction in spontaneous locomotion (Baik et al., 1995). Thus, this phenotype presents analogies with symptoms characteristic of PD. D2R knockouts are hypoactive and show insensitivity to the hypolocomotor effects of D2 receptor agonists (Boulay et al., 1999). Moreover, these mice exhibit decreased DAT function but do not have any changes in DA release in the dorsal striatum (Dickinson et al., 1999).

Importantly, all of the knockouts described above have fully differentiatiated DA neurons. Thus, expression of these genes is not necessary for the development of DA neurons.

Midbrain DA neuron development

Alterations in DA neurotransmission have been implicated in a number of human disorders including Parkinson's disease, schizophrenia and drug abuse. Understanding how DA cells are formed during embryogenesis may provide important insight for the treatment of these disorders. Transgenic technology, especially the use of homologous recombination to disrupt specific genes to produce knockout mice, has added considerably to the understanding of DA neuron development.

The development of midbrain DA neurons starts with the formation of mitotic DA progenitor cells from neuroepithelial stem cells in the midbrain. An important structure for the development of these cells is the mid-hindbrain junction, also called the isthmus. Around embryonic day E10-E10.5 in the mouse, the first mitotic DA progenitors are converted to post-mitotic differentiating DA neurons. These cells will eventually differentiate into DA neurons with the complete molecular make-up, and they will establish the correct connections with their target areas (Burbach et al., 2003; Simon et al., 2003; Wallen and Perlmann, 2003).

The mid/hindbrain region-the isthmus

The DA neurons are born rostrally to the isthmus, which is a very important barrier that anatomically and molecularly separates the forebrain from the hindbrain. Deregulation of this structure has severe effects on the development of the whole midbrain. The isthmus can be specified by its expression of the signaling molecule FGF-8, which is important for the induction of many different types of neurons, including the midbrain DA neurons.

Interestingly, ectopically applied FGF-8 is capable of inducing an ectopic isthmus structure (Martinez et al., 1999). Several genes such as, Otx1, Otx2, Pax2, Pax5 and Gbx2, En1 and En2 are expressed during the formation of the isthmus, suggesting a role for these genes in the establishment of the isthmus (Hynes and Rosenthal, 1999).

Specification of proliferating DA progenitor cells

The proliferating midbrain DA neurons are specified in the immediate vicinity of the isthmus and the floor plate, a specialized cell type that lies along the CNS ventral midline (Fig. 5). The combined signaling by FGF-8, which is secreted from the isthmus, and Shh, from the floor plate, leads to the induction of mitotic DA progenitor cells (Hynes et al., 1995a; Hynes et al., 1995b; Hynes and Rosenthal, 1999; Ye et al., 1998).

Only one marker specific for the proliferating DA progenitor cells has been reported. Raldh1 (or AHD2), an aldehyde dehydrogenase capable of metabolizing retinaldehyde into retinoic acid (Lindahl and Evces, 1984), is expressed in the ventral midbrain neuroepitelium (Wallen et al., 1999). Its expression continues after cells have stopped proliferating and is later colocalized with postmitotic markers such as TH (Wallen et al., 1999). These findings raise the possibility that retinoic acid is involved in controlling DA cell proliferation.

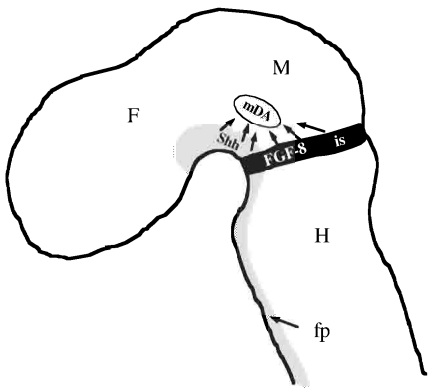

Figure 5. Schematic representation of the location of midbrain DA neurons. The figure shows a sagittal section of an E9 mouse neural tube. The DA neurons are born in the midbrain at the intersection of Shh and FGF-8 signaling, rostrally to the isthmus. FGF-8= fibroblast growth factor 8, fp= floor plate, is= isthmus, shh= sonic hedgehog, mDA= midbrain DA cells, F= forebrain, M= midbrain, H= hindbrain. (Drawing adapted from Simon et al. 2003).

Differentiation of DA neurons

The differentiation of postmitotic DA neurons starts with the expression of Nurr1 at E10.5 and is followed by the expression of the classical DA marker TH at E11.5 in the mouse (Zetterstrom et al., 1997). DA can be detected at E12, and the cells also express AADC and VMAT2 by this time, which allows them to synthesize and store DA (Olson and Seiger, 1972). The neurons also start to migrate into medial and lateral positions, to

form the A8 to A10 areas, and they also start to innervate their target areas. Gene targeting experiments have identified several transcription factors that are important for the development of DA neurons. These include Nurr1 and the homeodomain transcription factors Lmx1b, Ptx3 (also called Pitx3) and En1/En2.

In Nurr1knockout mice the expression of TH is completely lost, but initially the cells express other DA markers, i.e Ptx3, En1, En2 and Lmx1b (Castillo et al., 1998a; Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Zetterstrom et al., 1997). At around E15 to E16 these markers are lost, and an increase in apoptosis is seen at late gestation. Moreover, fluorogold retrograde tracing experiments have shown that Nurr1- deficient neurons are not able to innervate the striatum (Wallen et al., 1999). Opposing results have been reported from another group that showed preserved innervation and cellularity (Witta et al., 2000). In summary, Nurr1 is essential for the generation of midbrain DA cells and most probably for correct target innervation.

Ptx3 is expressed from E11 in TH-positive cells of the VTA and SNc, and this gene is not expressed in any other neurons, making it specific for the mesencephalic DA cells (Smidt et al., 1997). The Ptx3 gene is deleted in the naturally occurring mouse mutant, the *aphakia* mouse, and these mice fail to develop their SNc neurons. Furthermore, the striatal projections of the SNc neurons are lost. The dopaminergic phenotype of the remaining DA neurons is not affected, as they express other DA markers (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003).

Lmx1b is found in the ventral mesencephalon as early as E7.5, and its expression continues in developing and adult DA neurons. Developing DA cells of Lmx1b knock-out mice express TH and Nurr1 but lack Ptx3-positive cells, indicating that Ptx3, but not TH and Nurr1, depends on Lmx1b for its expression. At later stages (E16) expression of TH is lost, showing the importance for Lmx1b in differentiation and maintenance of mesencephalic DA neurons (Smidt et al., 2000).

The engrailed genes, engrailed-1 (En1) and engrailed-2 (En2), are expressed during early midbrain formation (E9), and show a second phase of expression in the developing DA cells from approximately E11 (Danielian and McMahon, 1996; Wurst and Bally-Cuif, 2001). Single knockouts of En1 and En2 have normal DA cells in both the VTA and SNc. Interestingly, a small number of TH positive cells are detected in En1 and En2 double mutants, but they are lost at later stages (Simon et al., 2001). Importantly, the gene targeted Lmx1b and En-mice have structural malformations of their midbrains, which may itself result in defective DA cell differentiation. A summary of the midbrain DA cell development is presented in figure 6.

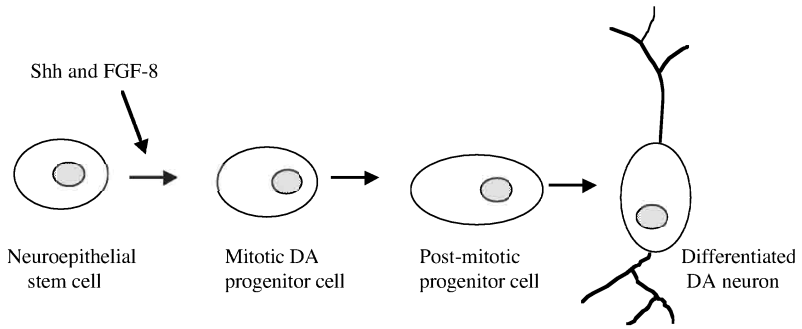

Figure 6. Midbrain DA cell development. Mitotic DA progenitors are formed from neuroepithelial stem cells by the combinatorial action of sonic hedgehog and fibroblast growth factor 8 (FGF-8). Raldh1/AHD2 is expressed in proliferating DA progenitors from approximately E9.5. As the cells become post-mitotic they express Nurr1 from E10.5, followed by En1, En2 and Ptx3 at E11. At E11.5 the cells start to express markers characteristic for differentiated DA neurons, such as TH. Lmx1b is another early marker for mitotic DA progenitors, expressed from E7.5, however its expression is not specific to the DA progenitors (Smidt, 2000).

Engineering of DA cells with the help of Nurr1

Nurr1 knockout studies have shown that Nurr1 is important for the development of DA neurons, suggesting that Nurr1 expression is important for the generation of DA cells *in vitro*. Indeed, several studies have shown that Nurr1 expression can increase the number of DA cells in culture. For example, overexpression of Nurr1 in an immortalized neural stem cell line, together with factors derived from type 1 astrocytes, can induce a ventral mesencephalic DA phenotype in these cells (Wagner et al., 1999). Over 80% of the cells obtained by this method demonstrate a phenotype indistinguishable from endogenous DA neurons. It was shown in another study that a highly enriched population of DA precursors can be generated from embryonic stem (ES) cells stably expressing Nurr1 under very specific culture conditions (Kim et al., 2002). These cells had an increased DA release and a higher expression of dopaminergic markers, such as TH and AADC, compared to ES cells not expressing Nurr1. Furthermore, the Nurr1-expressing ES cells formed functional synapses when grafted into 6-OHDA-lesioned animals, indicating that functional DA neurons were formed. In a third study Nurr1-overexpression in embryonic rat CNS precursors gave rise to DA neurons, characterized by the expression of several DA markers. However, transplantation of these cells into the striatum of 6-OHDA-lesioned rats resulted in poor survival and *in vivo* differentiation (Kim et al., 2003a).

METHODS

The methods used in this thesis work are described in Papers I-V. A summary of the techniques used, with reference to the respective paper, follows below.

Technique	*Paper*
Differential gene expression assays	I, II
Differential display	I
CDNA microarrays	II
Subtractive hybridization	n.p (I)
RT-PCR analysis	I, II, V
Semi-quantitative RT-PCR	I, V
Real-time RT-PCR	II
In situ hybridization	I, II, III, V
DIG-labeled	I, III
Radioactive	II, V
Cryo- and microtome sectioning	I, II, III, V
Genotyping by PCR	I-V
Cell culture work	I-V
BrdU labeling of cells	III
HPLC-analysis of DA content	I
FACS analysis	III
Immunohistochemistry	III, IV
Transfection of DNA	I-III
Reporter gene assay	II, III
Cloning of DNA into expression vectors	I-III
Dissection of mouse embryos	I-V
Microscopy	I-V
DNA-binding assay	III
In vitro tissue cultures	IV
Behavioral studies of mice	V
Western blot	n.p (V)

n.p not published

AIMS OF THE STUDY

The role of Nurr1 in DA cell differentiation is well established, however the immediate target genes and downstream molecular mechanisms for Nurr1 are poorly understood. In addition, very little is known about the role of Nurr1 in adult DA cells, and cells distinct from the DA cells.

The specific aims of the study has been:

- To identify genes that are regulated by Nurr1 (Papers I and II).
- To establish an *in vitro* model system where we can study the effects of Nurr1 in developing DA neurons (Papers I and III).
- To further understand the role of Nurr1 in differentiating midbrain DA cells (Papers I, III and IV).
- To investigate the effect of deleting one Nurr1 allele on drug-induced and natural reward mechanisms (Paper V).

RESULTS AND DISCUSSION

Model systems for the identification of Nurr1-regulated genes

The main focus of this thesis has been to identify genes regulated by Nurr1 to further understand the role of Nurr1 *in vivo*. As Nurr1 is important for the development of DA cells, we chose to use two different dopaminergic model systems to identify these genes. One approach was to compare gene expression in E13.5 ventral midbrains dissected from Nurr1 +/+ and Nurr1 -/- embryos. The idea was to identify genes expressed in the wild-type midbrain DA cells that were not found in knockout cells. In the second strategy we used a dopaminergic cell line, MN9D, where we overexpressed Nurr1 and studied the effect of Nurr1 on gene expression. An important advantage of using ventral midbrain tissue to find differentially expressed genes is that the DA cells are derived from a natural source. A disadvantage with the dissected tissue is that it is difficult to dissect the correct area of the brain, and the amount of tissue is highly limited. Furthermore, the ventral midbrain contains many cell types that do not express Nurr1. The MN9D cells, on the other hand, are easy to culture and high amounts of RNA can be obtained. In addition, they represent a uniform source of DA cells. The drawback with these cells is that they are a fusion between primary embryonic DA cells and a neuroblastoma cell line. Thus, they do not fully represent a natural DA cell source. In addition, the MN9D cells are not perfect DA cells, as they do not express all dopaminergic markers (see below).

MN9D cells

The DA cell line MN9D was generated by somatic cell fusion of primary neurons from mouse embryonic day 14 rostral mesencephalic tegmentum and the neuroblastoma cell line N18TG2 (Choi et al., 1991). This cell line was chosen as a model system for midbrain DA cells as the cells express neuronal-specific markers and show characteristic dopaminergic phenotypes. Importantly, they store and produce high levels of DA, have embryonic properties, and are sensitive to the DA cell toxin MPTP (Choi et al., 1991). In addition, these cells can be induced to extend neurites in response to various stimuli, such as the dopaminotrophic factor GDNF (Choi et al., 2001; Heller et al., 1996; Oh et al., 1996; Swarzenski et al., 1996). Furthermore, the DA content of the MN9D cells can be increased following exposure to an unknown factor derived from immortalized striatal neurons (Heller et al., 2000). In order to study the effect of physiological expression levels of Nurr1, we produced an MN9D cell line in which Nurr1-expression is controlled by the doxocycline-dependent promoter (paper I and (Joseph et al., 2003)).

Mesencephalic DA cells in Nurr1 knockout mice

It is necessary to explain more about the fate of the developing DA neurons in the Nurr1 gene targeted mice, as we chose to use ventral midbrains from E13.5 in our differential screening assays. Initial work describing these mice showed that the dopaminergic markers (TH, Ret, Raldh1/AHD2 and DA receptor D2) are absent at birth (Zetterstrom et al., 1997). Furthermore, TH expression was absent specifically in the mesencephalic DA cells throughout all developmental stages. These results concluded that Nurr1 is necessary for the developmental process generating these neurons (Zetterstrom et al., 1997), which was later confirmed by two other groups (Castillo et al., 1998a; Saucedo-Cardenas et al., 1998). More detailed work on the DA cells of the Nurr1-mutant mice showed that several DA markers, such as Raldh1/AHD2, Ptx3, En1 and En2, are lost between E12.5 and E15.5 (Saucedo-Cardenas et al., 1998; Wallen et al., 1999). Thus, DA-specific genes are induced even in the absence of Nurr1, but further maturation is disrupted. As development progresses, these midbrain DA precursors degenerate in the absence of Nurr1, as seen by an increase in cell death at E18.5 (Saucedo-Cardenas et al., 1998; Wallen et al., 1999). One or several genes, which are induced by Nurr1, should be important for the continued differentiation of the post-mitotic DA progenitors. If these genes are not expressed, the DA cells will be reprogrammed and further maturation is disrupted. In our attempts to find such gene/-s we used E13.5 embryos, as this is a stage where the developing DA cells still express most DA markers and have not reached the stage where DA markers are lost and cell death occurs.

Methods for the identification of differentially expressed genes

In recent years a variety of techniques have been developed to analyze differential gene expression, including comparative expressed sequence tag sequencing (Adams et al., 1995), differential display (Liang and Pardee, 1992; Welsh et al., 1992), PCR-based subtractive hybridization cloning (Diatchenko et al., 1996; Hubank and Schatz, 1994), mRNA hybridization to cDNA microarrays (Duggan et al., 1999), and serial analysis of gene expression (SAGE) (Velculescu et al., 1995). In this thesis work we used subtractive hybridization, differential display and cDNA microarrays with the aim of identifying Nurr1-regulated genes. Subtractive hybridization is a technique that enables comparison of two populations of mRNA and retreivment of clones of genes that are expressed in one population but not in the other. In this method both mRNA populations are first converted to cDNA. The cDNA population that contains the differentially expressed transcript is referred to as tester and the reference cDNA as driver. Tester and driver cDNAs are hybridized and the hybrid sequences are removed. Consequently, the remaining unhybridized cDNAs represents genes that are expressed in the tester, but are absent from the driver cDNA. This cDNA population is then subjected to PCR amplification to enrich for the differentially expressed genes, and the cDNAs of this library is then further characterized (Diatchenko et al., 1996; Hubank and Schatz, 1994). Subtractive hybridization is a powerful technique that requires very little starting material

and it is also known to detect low abundant transcripts. In contrast, it is a complex procedure with a lot of pitfalls.

Differential display is another PCR-based method where the mRNA populations to be compared are first converted to cDNA. Subsequently, arbitrary primers are used to amplify cDNA fragments of varying length from the different cDNA populations. The amplified cDNA fragments are separated on a polyacrylamide gel and side-by-side comparisons of such cDNA patterns reveals differences in gene expression. Differentially expressed cDNA bands can be retrieved, cloned and sequenced for further characterization (Liang and Pardee, 1992; Welsh et al., 1992). Differential display is relatively simple to execute, and is efficient for analyzing small amounts of RNA. In addition, many different samples can be analyzed on a single gel. A limitation of the differential display method is that false positives can be generated during PCR amplification, or in the process of cloning the differentially expressed PCR products. Furthermore, this technique is not very good at detecting low abundant transcripts. In contrast to subtractive hybridization, this method identifies both increases and decreases in gene expression.

CDNA microarrays are coated glass microscope slides where cDNAs from the genes of interest are spotted. Total RNA from both test and reference sample is fluorescently labeled with either Cy5 or Cy3-dUTP using a single round of reverse transcription. The fluorescent targets are pooled and allowed to hybridize under stringent conditions to the clones spotted on the arrays. Measurement of Cy5 and Cy3 emission spectras after excitation with a laser allows determination of the relative amount of transcript present in the cDNA pool by the type of fluorescent signal generated (Duggan et al., 1999). This technique does not require PCR amplification steps that can potentially introduce errors. However, microarray analyses are limited by the fact that rare transcripts are hard to detect. In addition, all genes are not represented on the arrays, a limitation that will probably be less important in the future. More importantly, this technique requires large amount of RNA. Protocols for RNA-amplification have been described, but it is difficult to amplify all mRNAs in a linear fashion (Smith and Greenfield, 2003).

Paper I-Nurr1 regulates DA synthesis and storage in MN9D DA cells

Nurr1 increases DA content and the expression of AADC and VMAT2

We aimed, in the work described in paper I, at finding genes whose expression is deregulated early in the developing DA neurons of Nurr1 knockout mice. Furthermore, we wished to establish an *in vitro* model system in which we could study Nurr1 function. We used MN9D cells for this, where Nurr1 was stably transfected into the cell line under control of a doxocyclin-dependent promoter. Thus, we were able to increase expression of Nurr1 by addition of doxocycline (dox). Furthermore, removal of dox resulted in downregulation of Nurr1 expression.

Our first observation was that Nurr1 had the capacity to increase the DA content of the MN9D cells, as increased expression of Nurr1 doubled the amount of DA in the cells. In addition, Nor1 and NGFI-B, but not RAR or the thyroid hormone receptor TR, were able to increase the dopamine content of the cells, suggesting that the capacity of increasing the DA content is unique for the NGFI-B subfamily. Furthermore, the DA content of the cells was increased in cells transiently transfected with a Nurr1 mutant that is not able to dimerize with RXR, suggesting that Nurr1/RXR heterodimers are not involved in increasing the DA content of the cells (discussed further in Paper III). Binding of Nurr1 to DNA is required for increasing the dopamine content, as a DNA-binding deficient Nurr1 mutant (Paper III and (Aarnisalo et al., 2002)) was not able to increase the DA content of the MN9D cells.

We wished to understand the mechanism behind the Nurr1-dependent increase in dopamine content of the cells, and used semi-quantitative RT-PCR to analyze the expression of genes that are involved in the production, storage and elimination of DA. These analyses showed that the DA-synthesizing enzyme AADC was upregulated in cells that overexpress Nurr1. Furthermore, the expression of VMAT2, which is responsible for the transportation of DA into synaptic vesicles of DA nerve terminals, was increased in cells induced to express Nurr1. Interestingly, VMAT2 was also picked up in a differential display experiment, where we compared gene expression in dissected ventral midbrains from Nurr1+/+ and Nurr1 -/- mice.

We were interested to investigate if the Nurr1-induced expression of AADC and VMAT2 was dependent on continuous expression of Nurr1. Therefore, Nurr1 expression was first induced by addition of dox and subsequently reduced by removal of dox. Our results showed that VMAT2 mRNA expression was reduced upon removal of dox, suggesting that this gene is dependent on continuous expression of Nurr1. In contrast, the expression level of AADC mRNA was not changed when dox was removed.

Next, we were interested in the importance for Nurr1 in regulating VMAT2 and AADC expression *in vivo*. *In situ* hybridization analyses of Nurr1 knockout E13.5 ventral midbrains showed that VMAT2 and AADC expression was downregulated in the DA progenitors at this early developmental stage.

We were able to induce morphological differentiation by transient transfection with Nurr1 or by addition of retinoids to the culture media (Paper III, discussed below). Thus, we were interested to determine if retinoids could increase the DA content of the MN9D cells. In contrast, retinoids decreased the dopamine content and the expression of AADC, suggesting that Nurr1 plays an instructive role in regulation of DA production and storage.

In summary, the results of Paper I suggest that Nurr1 is important for the DA neurotransmitter phenotype, as this NR is capable of inducing the expression of genes important for DA synthesis and storage.

Nurr1-dependent regulation of VMAT2 and AADC

The early downregulation of AADC and VMAT2 expression in the knockout animals supports a relatively direct effect of their regulation by Nurr1. In addition, we observed that AADC and VMAT2 mRNA levels were reduced in E13.5 midbrains of Nurr1

heterozygous mice compared to wild-type mice, suggesting that the lower expression of Nurr1 results in reduced expression levels of AADC and VMAT2 *in vivo*. Furthermore, in the case of VMAT2, the reduction in expression of this gene upon removal of dox in the MN9D cells indicates dependence on sustained Nurr1 expression. In contrast, the fact that we did not find any NBREs or NBRE-like sequences, which have previously been shown to bind Nurr1 (Murphy et al., 1996; Wilson et al., 1991; Wilson et al., 1993a), in the promoter regions of AADC and VMAT2, suggests that Nurr1 regulates these promoters indirectly. In theory, we cannot exclude that Nurr1 can bind to elements distinct from the known target sequences. Therefore, promoter analyses would be necessary to gain further insights into the Nurr1-dependent regulation of VMAT2 and AADC. In addition, the NBREs important for the Nurr1-dependent regulation of these genes may be situated far away from the proximal promoter.

Interestingly, two other studies have reported that Nurr1 induces the expression of VMAT2 and AADC mRNA, further supporting the importance of Nurr1 for expression of these genes (Kim et al., 2002; Kim et al., 2003a).

Nurr1 and the DA transmitter phenotype

Several studies have showed the importance of Nurr1 in the regulation of the DA transmitter phenotype. The first observation was that Nurr1 knockout mice lack expression of TH and that striatal DA levels were reduced in Nurr1 -/- and +/- mice at birth (Castillo et al., 1998a; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). This was followed by the *in vitro* observation that Nurr1 can regulate the tyrosine hydroxylase promoter (Iwawaki et al., 2000; Kim et al., 2003b; Sakurada et al., 1999). Another gene important for the regulation of DA homeostasis, DAT, was shown to be regulated by Nurr1 *in vitro* (Sacchetti et al., 1999; Sacchetti et al., 2001). Recently, a study by Smits *et al.* (Smits et al., 2003) showed the importance of Nurr1 for the expression of DAT *in vivo*. This was supported by the fact that DAT was not expressed in E14.5 mescencephalic DA neurons of Nurr1 gene targeted mice. In this study, the authors also noted the early downregulation of VMAT2 mRNA, but they could still see expression of AADC mRNA, even though this expression was reduced in the Nurr1 -/- embryos. The discrepancy between the two studies could be due to differences in design of the gene-targeting construct, or to differences in detection levels of the AADC mRNA. The fact that AADC was not downregulated in the MN9D cells upon withdrawal of dox indicates that this gene is less dependent on Nurr1 for its expression. In any event, Nurr1 is probably involved in the induction of AADC mRNA, although it might not be necessary for its sustained expression.

Nurr1, VMAT2 and AADC in the adult brain

Nurr1 expression continues in adult DA cells, indicating a function for this protein in the adult brain (Saucedo-Cardenas and Conneely, 1996; Xiao et al., 1996; Zetterstrom et al., 1996a; Zetterstrom et al., 1996b). In addition, mutations in the Nurr1 gene have been identified in patients with PD and manic–depressive disorders, indicating that Nurr1

plays an important role in maintenance of the normal DA cell functions in the adult (Buervenich et al., 2000; Le et al., 2003). We noted that the expression of VMAT2 is dependent on continuous expression of Nurr1. Thus, this result may indicate that Nurr1-dependent expression of VMAT2 is not only important in the developing, but also in adult DA neurons. Controlled regulation of VMAT2 is certainly important, as mice heterozygous for VMAT2 display reduced response to the rewarding effect of amphetamine and enhanced amphetamine locomotion (Takahashi et al., 1997).

The regulation of VMAT2 and AADC by Nurr1 can also have implications for PD. L-dopa treatment is limited by the short duration of behavioral improvement and fluctuating responses to the drug. In theory, increasing the Nurr1 activity would result in increased production (*via* AADC) and storage (*via* VMAT2) of DA. It is interesting in this context to mention that both Nurr1 and VMAT2 have a reduced expression in PD brains (Chu et al., 2001; Miller et al., 1999). Furthermore, endogenous AADC levels are considered to be low in human brains, and it is thought that this enzyme becomes rate-limiting as degeneration of DA neurons progresses in PD (Li et al., 1998; Lloyd and Hornykiewicz, 1972). Interestingly, transplantation of genetically modified fibroblasts expressing AADC and VMAT2 into Parkinsonian rat striata resulted in higher DA levels and prolonged duration of DA release after exogenous L-DOPA administration (Lee et al., 1999). In another experiment 6-OHDA-lesioned rats were transduced with adeno-associated vectors expressing TH or AADC into the striatum. Cotransduction with the two viruses resulted in more remarkable behavioral recovery than that which occurred in rats receiving TH alone, indicating the importance of AADC enzymatic activity (Fan et al., 1998).

VMAT2 has been suggested to protect DA cells from various toxic insults. DA itself can be toxic to neurons and potentially be involved in degeneration of dopaminergic neurons in PD (Willis and Armstrong, 1998). VMAT2 has been shown to protect DA cells from DA-induced toxicity by sequestering DA into synaptic vesicles (Weingarten and Zhou, 2001). In addition mice heterozygous for VMAT2 are more susceptible to the neurotoxic effects of MPTP, suggesting that VMAT2- mediated sequestration of the neurotoxin into vesicles may play an important role in attenuating MPTP toxicity *in vivo* (Gainetdinov et al., 1998; Le et al., 1999; Takahashi et al., 1997).

Nurr1 function in the adult midbrain should ultimately be studied with the help of conditional gene target strategies. In this type of experiment, the Nurr1 activity would be maintained during development and disrupted at later stages.

Evaluation of MN9D cells as a tool for studying Nurr1 function

The MN9D cells proved to be a useful model system for developing DA neurons, as we were able to find Nurr1-dependent mechanisms in the cells that were also relevant *in vivo*. First, we found that Nurr1 increased the DA content, and we identified two genes, AADC and VMAT2, which probably contributed to the DA increase. Next, we could verify that the expression of these genes was indeed down-regulated in Nurr1 knockout mice. The cells are not a perfect model for developing DA neurons, as we could not see the established Nurr1-regulation of DAT. Furthermore, the basal expression of TH was

very high even in the absence of Nurr1 overexpression, and a weak or no increase in this gene was observed when Nurr1 was overexpressed.

Another recent publication showed that the MN9D cells are a useful model for developing DA cells (Joseph et al., 2003). In this report, the cyclin-dependent kinase inhibitor $p57^{Kip2}$ was regulated by Nurr1 in the MN9D cells, and was detected in a pattern similar to that of Nurr1 in the ventral midbrain. Furthermore, $p57^{Kip2}$ was downregulated in ventral midbrains of Nurr1 knockout mice. Analysis of $p57^{Kip2}$ null mutant mice showed a resembling midbrain DA cell phenotype to that of the Nurr1 mutant mice, supporting that $p57^{Kip2}$ is an effector target gene of Nurr1 in DA neuron development.

Future directions

The participation of Nurr1 in regulating the expression of AADC and VMAT2 cannot explain the severe developmental phenotype characterized by degeneration of mesencephalic DA neurons in Nurr1 -/- mice, as mice lacking DA and VMAT2 still generate midbrain DA neurons (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997; Zhou and Palmiter, 1995).

In this context it is also of interest to mention another publication, where the expression of ret, the tyrosine kinase signal transducing receptor for GDNF, was investigated in Nurr1 null mice (Wallen et al., 2001). In this study it was shown that ret failed to be expressed in the developing DA cells, suggesting that ret is dependent on Nurr1 for its transcription. No abnormalities of the midbrain DA cells have been found in mice with targeted deletion of ret, suggesting that this is not a master regulator of DA cell differentiation (Marcos and Pachnis, 1996).

We are still trying to identify other genes regulated by Nurr1 that are important players in DA cell differentiation. We are currently analyzing data from a subtractive hybridization experiment in which the mRNA from E13.5 wild-type midbrains was subtracted from that of Nurr1 knockout midbrains.

Paper II-The nuclear receptor Nurr1 regulates the expression of neuropilin-1

Nurr1 regulates neuropilin-1 expression

We decided to direct the next phase of the project towards identifying other genes that are regulated by Nurr1 to shed further light on additional roles of Nurr1 *in vivo*. Therefore we performed gene array experiments in which we compared non-treated MN9D cells with cells that had been induced to express Nurr1 by the addition of dox. This analysis resulted in the identification of a novel Nurr1-regulated gene, neuropilin-1 (Nrp1). MRNA expression levels of Nurr1 and Nrp1 were monitored in MN9D cells treated with dox. Real-time PCR analyses revealed that Nurr1 mRNA was induced already one hour after

addition of doxocycline, followed by an increased expression of Nrp1 mRNA three hours after addition of dox to the MN9D cells. Nrp1 expression levels were also increased in cells that overexpressed Nor1 or NGFI-B, suggesting that these receptors are also capable of inducing Nrp1 expression. Interestingly, the induced expression of Nrp1 required continuous Nurr1 expression, as removal of dox reduced the amount of Nrp1 mRNA of the cells. Cells transiently transfected with the DNA-binding deficient mutant showed very little or no increase in Nrp1 expression, suggesting that binding of Nurr1 to DNA is important for induction of Nrp1 expression. In addition, cells that expressed the Nurr1 mutant which is not capable of dimerizing with RXR was able to increase Nrp1 expression, suggesting that Nurr1/RXR heterodimers are not required for the induction of Nrp1 expression. However, the levels of Nrp1 expression is slightly reduced in cells transfected with the dimerization mutant, compared to cells transfected with wild-type Nurr1. This result suggests that Nurr1/RXR heterodimers might be involved but not absolutely required for activation of Nrp1 expression in the MN9D cells. Furthermore, the RXR ligand LG268 was able to slightly increase the expression of Nrp1. However, LG268 was not able to increase the Nurr1-dependent expression of Nrp1. Thus, as this ligand is also activating other RXR heterodimers, the effect on Nrp1 expression by LG268 might be due to activation of heterodimers distinct from Nurr1/RXR. Ultimately, treatment of the cells with a ligand specific for Nurr1/RXR heterodimers would clarify the role of this heterodimer in regulation of Nrp1 expression.

Two NBREs were observed in the promoter sequence of the Nrp1 gene, which led us to clone the promoter and couple it to a luciferase reporter. Interestingly, the more upstream NBRE is evolutionary conserved between mouse, rat and human while the second NBRE is conserved between rat and mouse, suggesting that the NBREs are important regulatory sequences. In reporter gene assays we observed Nurr1-dependent activation of the Nrp1 promoter (discussed in detail below).

In situ hybridization analysis revealed that Nurr1 and Nrp1 are co-expressed in a cranial motor nucleus, the dorsal motor nucleus of the vagal nerve (DMN X). Furthermore, expression analyses of E13.5 hindbrains revealed that Nrp1 mRNA levels are downregulated in the DMN X of Nurr1 knockout mice.

Neuropilin-1

Neuropilin-1 (Nrp1) is a single spanning transmembrane protein with a large extracellular domain and a small cytoplasmic domain of about 40 amino acids (Neufeld et al., 2002). Nrp1 functions as a receptor for semaphorin 3A (sema-3A), which is one of six axon-repellent factors belonging to the class-III semaphorin subfamily (Goodman 1999). The biological activity of Nrp1 was originally characterized *in vitro* where it was shown that sema-3A could repel the growing tips of Nrp1-expressing dorsal root ganglion cells (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Several studies have subsequently confirmed the role of Nrp1 axonal repulsion, and in some cases axonal attraction reviewed in (Bagri and Tessier-Lavigne, 2002). The short cytoplasmic domain of Nrp1 is not responsible for the transduction of the repulsing effect. Instead, the signal is conveyed by transmembrane receptors, called plexins, that form complexes with Nrp1 (Takahashi et al., 1999; Tamagnone et al., 1999). In addition, Nrp1 is able to bind certain splice

forms of vascular endothelial growth factor (VEGF), indicating that Nrp1 has a role in the cardiovascular system as well (Soker, 2001; Soker et al., 1998). Interestingly, during embryonic development Nrp1 participate in the regulation of both nervous and cardiovascular development (Kawasaki et al., 1999; Kitsukawa et al., 1997; Kitsukawa et al., 1995).

Why are so few genes regulated?

In microarray experiments in general it is common to find several hundred regulated genes. In contrast, we saw very few changes in gene expression in the MN9D cells. Our aim was to identify genes directly regulated by Nurr1, and we therefore chose to prepare RNA from cells treated with doxocycline for zero, two and eight hours respectively. However, treating the cells with doxocycline for longer periods might increase the number of differentially expressed genes. Another reason for the low number of genes might be that very few genes are actually regulated by Nurr1 in our experimental setup. Curiously, differential expression of a small number of genes was observed in transiently Nurr1-transfected immortalized synoviocyte cells using microarrays (Davies M.R, 2004). Another possible explanation is that the MN9D cells have a basal expression of Nurr1. Indeed, we see expression of Nurr1 mRNA in RT-PCR analyses. However, in other experiments Nurr1 expression induces multiple changes of the MN9D cells, including upregulation of AADC and VMAT2 (Paper II) and morphological differentiation (Paper III), suggesting that the basal expression of Nurr1 is very low.

Regulation of the Nrp1 promoter by Nurr1

Several lines of evidence suggested that Nurr1 regulates expression of Nrp1. First, real-time PCR analyses showed that Nurr1-dependent upregulation of Nrp1 was rapid and could be detected as early as three hours after addition of doxocycline. Second, removal of doxocycline resulted in downregulation of Nrp1. Third, Nrp1 expression was diminished in the DMN X of Nurr1 null mice. The above results together with the evolutionary conservation of the NBREs encouraged us to clone the Nrp1 promoter. Earlier studies have shown that the transcription factor Sp1 and, to a lesser extent, the transcription factor AP-1 and a CCAAT box are involved in activation of the Nrp1 promoter in response to the tumor promoter agent TPA (Rossignol et al., 2003).

We showed that Nurr1 was able to activate the Nrp1 promoter in reporter gene assays, if parts of the Nrp1 promoter were removed. The basal activity of the full-length promoter construct was lower than the activity seen if the more distal part of the promoter was removed, indicating that a repressive region is present in the more proximal part of the promoter. In any event, the presence of the NBREs increases the Nurr1-dependent activation of the promoter. The question is whether Nurr1 activates the promoter directly. Interestingly, we have preliminary data that supports a direct regulation of the Nrp1 promoter by Nurr1. In reporter gene assays, the Nurr1 mutant that is deficient in DNA-binding was not able to activate the 177+448 reporter. In addition, a Nurr1 mutant lacking both AF1 and AF2 could not activate the 177+448 reporter. We are planning to

carry out mutational analysis of the NBREs to confirm the importance of these sites. However, the Nurr1 dependent activation of the promoter is relatively modest, suggesting that more upstream sequences of the Nrp1 promoter or even downstream introns might be important. Reporter gene assays are artificial in the sense that the DNA is not folded into its natural chromatin structure. Therefore, we are also planning to perform chromatin immunoprecipitation experiments to verify that Nurr1 is present on DNA in its natural context. We would also like to study the effects of $p57^{Kip2}$ on Nurr1 activated Nrp1 promoter, as $p57^{Kip2}$ is a known co-factor that negatively influences Nurr1-dependent transcriptional activity (Joseph et al., 2003).

Nurr1 and the DMN X

As Nrp1 is not expressed in the DA cells of the midbrain, we used *in situ* hybridization analysis to identify potential neurons in which Nurr1 and Nrp1 are co-expressed. This would enable us to identify a common role for the two proteins *in vivo*. We found overlapping expression of Nurr1 and Nrp1 in the dorsal motor nucleus of the vagus nerve (DMN X). Furthermore, Nrp1 expression was strongly downregulated in the DMN X of Nurr1 knockout mice, suggesting that Nurr1 regulates Nrp1 expression *in vivo*. The DMN X sends efferent projections in the vagus nerve that innervates the esophagus, lungs, heart and gastrointestinal tract. The expression of Nurr1 is seen in visceral motor neurons of the DMN X and is induced as early as E10.5 (Wallen et al., 2001). Prominent Nurr1 expression has also been detected in this nucleus in the adult rat (Zetterstrom et al., 1996b). Interestingly, the GDNF receptor ret is expressed in Nurr1-positive cells of the DMN X, and the expression of Ret is lost in the Nurr1 mutant mice (Wallen et al., 2001), indicating that this nucleus is affected in Nurr1 null mice.

The lethality of the Nurr1 knockouts is probably not due to the lack of DA neurons, since it has been shown that mice with abolished DA production survive a few weeks after birth (Zhou and Palmiter, 1995). It is thus more likely that Nurr1-expressing cells in another area of the CNS are responsible for this early death. Indeed, the DMN X is known to be involved in the control of breathing. It has recently been demonstrated that newborn Nurr1 knockout mice have a severe respiratory phenotype characterized by hypoventilation, an increased number of apneas and the absence of hypoxic response (Nsegbe et al., 2004). These results suggest that the early death of the Nurr1 mutant pups is due to a lack of respiratory control (Nsegbe et al., 2004). The regulation of Nrp1 by Nurr1 may be important in this context, as the DMN X nerve fibers might not be appropriately routed in homozygous gene-targeted mice due to lack of Nrp1 expression. Curiously, initial studies have indicated that the nerve fibers derived from the DMN X show a subtle disorganization in the Nurr1 -/- mice (Wallen et al., 2001). Furthermore, the vagal nerve is highly defasciculated in Nrp -/- mice (Kitsukawa et al., 1997). Therefore, it would be interesting to compare the nerve fibers of Nrp1 and Nurr1 knockout embryos by tracing studies in which a tracer dye is injected into the DMN X. Another experiment would be to culture DMN X neurons from Nurr1 +/+ and Nurr1 -/- mice and compare their response to sema-3A. In addition, it would be interesting to analyze if inhibition of Nrp1 signaling blocks the Nurr1-induced morphological differentiation (discussed in paper III) in the MN9D cells.

Other interesting aspects

Nurr1 and Nrp1 may have common roles in other cells. For example, expression of both Nurr1 and Nrp1 are induced during ischemia (Beck et al., 2002; Honkaniemi and Sharp, 1996; Honkaniemi et al., 1997). In addition, Nurr1 is upregulated in inflammatory responses while Nrp1 is involved in initiation of the primary immune response (McEvoy et al., 2002a; McEvoy et al., 2002b; Tordjman et al., 2002). Finally, Nurr1 and Nrp1 are expressed in endothelial cells and can potentially share common roles in vascularization (Liu et al., 2003; McEvoy et al., 2002a; Soker et al., 1998).

Paper III-Nurr1 induces cell cycle arrest and morphological differentiation

As it is complicated to study the differentiation of DA cells *in vivo,* we wished to establish an *in vitro* culture system to study Nurr1 function in this process. We therefore used MN9D cells transiently transfected with Nurr1 as our model system.

MN9D cells and differentiation

MN9D cells can be stimulated to extend neurites by various treatments. Paper III describes experiments in which we showed that transient transfection of Nurr1 in the MN9D cells resulted in a drastic decrease in cell proliferation, with arrest in the G1 phase of the cell cycle. This arrest was followed by morphological differentiation, as assessed by the extension of long bipolar neurites. In general, there seems to be no obligatory coupling between cellular decisions to proliferate and differentiate, even though the two behaviors are often controlled simultaneously (reviewed in (Brown et al., 2003)). Interestingly, Nurr1 coupled to the *Drosophila* engrailed repressor domain, resulted in growth arrest without subsequent differentiation of the MN9D cells, suggesting that Nurr1 might promote growth arrest by gene repression. In addition, rapamycin, which stops cells in G1, did not lead to morphological differentiation. These results suggest that cell cycle arrest and differentiation are two separate events, and that Nurr1 has a dual role in mediating both cell cycle arrest and differentiation in the MN9D cells. The mechanism by which Nurr1 induces cell cycle arrest is not clear, but it has been reported that the cell cycle inhibitors $p21^{Cip1}$ and $p57^{Kip2}$ are both upregulated by Nurr1 in the MN9D cells (Joseph et al., 2003).

As the enzyme Raldh1, which is involved in production of the retinoid all-*trans* retinoic acid, is expressed in the area where midbrain DA cells develop (Wallen et al., 1999) we wished to analyze the role of retinoids in MN9D cell differentiation. Our results showed that all-*trans* retinoic acid was able to induce morphological differentiation of the

cells, indistinguishable from the Nurr1-induced differentiation. A number of experiments (discussed below) showed that the effect of retinoids was not *via* Nurr1/RXR heterodimers. The addition of both RAR and RXR ligands resulted in a synergistic effect on differentiation, suggesting that RAR/RXR heterodimers are involved. Furthermore, transient transfections with combinations of dominant negative and wild-type forms of Nurr1 and RAR suggested that retinoids and Nurr1 participate in a common pathway to induce MN9D cell differentiation.

Interestingly, Ptx3 or Lmx1b could not support differentiation of the MN9D cells, further confirming that Nurr1 and Ptx3/Lmx1b induce DA cell differentiation *via* distinct pathways (Smidt et al., 2000).

Proliferation, differentiation and Nurr1 in DA cells in vivo

Ultimately, the results obtained in the MN9D cells should give us clues about how Nurr1 acts *in vivo*. An obvious question, therefore, is if Nurr1 is involved in regulating cell cycle arrest and morphological differentiation *in vivo*. Retrograde tracing experiments in newborn pups demonstrated that the developing DA cells failed to innervate their target areas in the absence of Nurr1 (Wallen et al., 1999). This result, together with the MN9D data, supports the idea that Nurr1 is involved in neurite outgrowth *in vivo*. This issue is further discussed in Paper IV.

During development of the DA cells, Nurr1 is expressed in post-mitotic neurons. This is based on the fact that Nurr1 is excluded from the ventricular layer where proliferating cells are present. In addition, bromodeoxyuridine (BrdU) injections into pregnant mice and subsequent analysis of the embryos showed lack of co-detection of BrdU-labelled cells with Nurr1-expressing cells in the ventral midbrain (Wallen et al., 1999). Thus, we believe that during development of the DA cells, Nurr1 does not play a direct role in mitotic arrest. Instead, the MN9D data suggests that Nurr1 plays a role in stabilizing the non-proliferative state, thereby protecting the cells from re-entry into the cell cycle. Interestingly, studies on knockouts for cyclin-dependent kinase inhibitors, which stop cell cycle progression, have shown ectopic activation of cell proliferation in mature neuronal cells (Zindy et al., 1999). No abnormal cell proliferation has been detected in the ventral midbrain of Nurr1 mutant pups (Åsa Wallén, personal communication), which may indicate that Nurr1 is not involved in the control of proliferation. Alternatively, the normal control of cell proliferation in the absence of Nurr1 *in vivo* could be explained by redundant mechanisms that ensure normal regulation of such important processes, even in the absence of one of the control genes. This idea is supported by the observation that an abnormal increase in cell proliferation in mice deficient in cell cycle regulators often requires the generation of compound knockout animals (Zhang et al., 1999).

Nurr1 or Nurr1/RXR heterodimers?

As mentioned in the introduction, Nurr1 can act as a monomer, a homodimer or a permissive RXR heterodimer. In theory, regulation of Nurr1 activity can therefore occur

by modulating Nurr1 activity (discussed below), or by activation of the heterodimerization partner RXR. Nurr1/RXR heterodimers can be activated by natural RXR ligands, including the retinoid metabolite 9-*cis* retinoic acid (Wallen-Mackenzie et al., 2003) and fatty acids such as docosahexanoic acid (de Urquiza et al., 2000). A relevant question, therefore, is if Nurr1 heterodimerization is important *in vivo*. Two recent reports have suggested that Nurr1 acts as a silent non-ligand binding partner in RXR-mediated signaling. In the first study, it was shown that Nurr1/RXR heterodimers promote the survival of neurons in the mouse and that these heterodimers are active in the developing DA neurons (Wallen-Mackenzie et al., 2003). The second report identified a new signaling pathway mediated by the *Drosophila* Nurr1 homologue DHR38 and the RXR homologue USP, where ecdysteroids activated these receptors (Baker et al., 2003). In addition, two studies have provided an interesting link between the DA system and retinoid signaling in the brain as compound RXR receptor mutant mice show impaired locomotion and downregulation of dopamine D2 receptors in the striatum (Krezel et al., 1998). Furthermore, the dopamine D2 receptor promoter was shown to contain RAR/RXR responsive sites that mediated retinoid-induced transactivation (Samad et al., 1997).

In contrast to the Nurr1/RXR requirement discussed above, several experiments in Paper III presents the results of experiments that suggest that the Nurr1/RXR heterodimer is not involved in induction of morphological differentiation and mitotic arrest in the MN9D cells. First, the RXR agonist SR11237 could not on its own give rise to morphological differentiation. Second, the dimerization- deficient Nurr1 mutant ($Nurr1^{dim}$) was capable of inducing differentiation and cell cycle arrest. Third, transient transfection with Nor1, which is not capable of forming heterodimers with RXR, resulted in differentiated and arrested MN9D cells. In addition, the data presented in Paper I suggest that Nurr1 regulate DA synthesis and storage in a Nurr1/RXR- independent manner. Thus, it is reasonable to conclude that Nurr1 can act as a monomer in some circumstances, and as a heterodimer with RXR in others. However, it is possible that Nurr1 expression in MN9D cells does not reproduce all features of Nurr1 during embryonic development, and additional requirements for the function of Nurr1 in the developing CNS might therefore exist.

Analyses of mice in which the wild-type allele of Nurr1 is replaced by a dimerization-deficient Nurr1 mutant will allow definitive elucidation of the *in vivo* significance of Nurr1 monomers and RXR-Nurr1 heterodimers.

Nurr1, Nor1 and NGFI-B and regulation of proliferation

The results presented in paper III show that Nor1, Nurr1 and NGFI-B are all able to induce cell cycle arrest. As Nor1 and NGFI-B are not expressed in midbrain DA cells, we believe that these three NRs might have a general growth inhibitory role also in non-mesencephalic cells. Our result showing that Nurr1 can induce cell cycle arrest in the neuroblastoma cell line N18TG2 supports this hypothesis. Nurr1 is known to act as an immediate early gene that is rapidly induced after stressful insults to the CNS, i.e. after kainic acid-induced seizures or ischemia (Crispino et al., 1998; Honkaniemi and Sharp, 1996; Honkaniemi and Sharp, 1999; Honkaniemi et al., 1997; Xing et al., 1997). Thus,

the growth inhibitory role of these receptors may be important to prevent uncontrolled proliferation during these conditions.

Data supporting a role for the NGFI-B/Nurr1/Nor1 group of receptors in regulating the cell cycle in cells outside the CNS is gathering. Recently, Conneely presented results from Nor1, NGFI-B double knockouts showing that these two receptors are involved in arresting the cell cycle in myeloid cells (Conneely, 2004). A somewhat contradictive result was obtained in Nor1 knockout mice, where there was a decreased proliferation of nonsensory epithelial cells of the inner ear (Ponnio et al., 2002). Furthermore, NGFI-B has been shown to induce cell cycle arrest in vascular endothelial cells by upregulation of the cycline dependent kinase inhibitor $p27^{Kip1}$ and downregulation of cyclin A (Arkenbout et al., 2003). In addition, it has been suggested that NGFI-B, Nor1 and Nurr1 block the proliferation of smooth muscle cells (Arkenbout et al., 2002).

Regulation of Nurr1 activity

As Nurr1 is involved in the regulation of DA transmitter phenotype (Paper I) and in the differentiation of midbrain DA cells (Paper III and (Castillo et al., 1998a; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997)) it would be of great clinical interest to be able to regulate its activity. As discussed above, one way would be to regulate the activity of the heterodimerization partner RXR. However, the results presented in Papers I and III suggest that Nurr1 function in DA cells may be independent of RXR. Functional studies on the Nurr1 LBD showed that the AF-2 domain is active in some cell types and inactive in others (Castro et al., 1999). This cell-type dependent activity of Nurr1 indicated a regulatory potential of the Nurr1 carboxy-terminal domain, suggesting that ligands may modulate Nurr1 activity *in vivo*. However, it has turned out to be hard to find ligands for Nurr1. The crystal structure of the Nurr1 LBD has recently been solved, and the structure may explain why it has been so difficult to find Nurr1-ligands. It turned out that the ligand-binding pocket of Nurr1 is very small, and that bulky hydrophobic amino acid side chains occupy most of the space, thus preventing ligand binding. The constitutive Nurr1 activity was explained by folding of the AF-2-helix into an active conformation (Wang et al., 2003). Interestingly, the *Drosophila* Nurr1 homologue (DHR38) shares similar structural properties (Baker et al., 2003).

How regulation of the Nurr1 LBD is achieved is not clear, but it probably involves post-translational modifications and/or recruitment of cofactors. According to the structural analysis, Nurr1 lacks a classical coactivator- or corepressor-binding surface. Instead, we believe that cofactors may bind to the most C-terminal amino acids of Nurr1, which are hydrophobic and protrude out of the structure (personal communication, Gérard Benoit). Recently, the ubiquitine ligase PIASγ was shown to interact with the C-terminal domain of Nurr1 and repress Nurr1-dependent transcriptional activation (Galleguillos et al., 2004).

Several lines of evidence show that Nurr1 activity can be regulated *via* domains distinct from the LBD. For example, $p57^{Kip2}$ binds to the N-terminal region and represses Nurr1 activity (Joseph et al., 2003). Furthermore, the atypical orphan NR SHP, which lacks a conventional DBD, can inhibit Nurr1 activity by binding to the Nurr1 DBD (Song

et al., 2004). In addition, we have suggested that phosphorylation of the N-terminal positively influences transcriptional activation of Nurr1 (Nordzell et al., 2004). Moreover, the anti-inflammatory drug 6-mercaptopurine acts as an N-terminal activator of Nurr1 (Ordentlich et al., 2003). Finally, the coactivator SRC-1 can activate Nurr1 dimeric activity via the N-terminal part of Nurr1 (Maira et al., 2003).

An alternative way to regulate the activity of a constitutively active transcription factor like Nurr1 is to regulate the expression of Nurr1 itself. Thus, immediate early genes such as Nurr1, Nor1 and NGFI-B are rapidly induced by various stimuli, as discussed in the introduction.

Paper IV-*In vitro* cultures of ventral mesencephalon from Nurr1- deficient mice

Paper IV describes experiments in which the ventral mesencephalon (VM) was dissected and cultured *in vitro*, and the expression of TH was investigated in the VM from Nurr1 wild-type, heterozygous and knockout embryos. We could induce expression of TH in Nurr1 knockout VM, demonstrating that the DA progenitors in the Nurr1 -/- pups retain their capacity for inducing TH expression. In addition, nerve fiber bundles formed in the VM from Nurr1 +/+ and Nurr1 +/- mice, but not in that from Nurr1 -/- mice.

TH expression can be induced in Nurr1 deficient DA progenitors in vitro

The expression of TH is first detected in the developing DA cells of the mouse at embryonic day E11.5 and occurs one day after the initial expression of Nurr1, which is seen at E10.5. TH is never expressed in Nurr1 knockout mice. Furthermore, studies on the TH promoter have shown that Nurr1 can activate and bind the promoter (Iwawaki et al., 2000; Kim et al., 2003b; Sakurada et al., 1999), suggesting that Nurr1 regulates this gene *in vivo*.

Since the Nurr1-/- DA cells loose their capacity to fully differentiate *in vivo* we wished to study this loss closer by culturing the DA progenitors *in vitro* and monitoring TH expression. Our first goal was to investigate if support from the developing striatum, the target innervation area, could influence the TH expression in VM tissue from Nurr1-/- embryos. Therefore we cultured E10.5 VM, as this is a stage prior to normal onset of TH-expression, together with lateral embryonic eminence (LGE) from wild-type embryos. We could induce TH expression in Nurr1-/- VM under these conditions, suggesting that the LGE supported the expression of TH. Surprisingly, when VM from null embryos was cultured without LGE it was still possible to induce TH expression. To avoid possible interference with the plasma/thrombin clot we chose to change to free-floating cultures at this point. In the free-floating cultures, we could still see TH expression in the Nurr1-/- cultures.

Furthermore, co-expression of TH with Raldh1 showed that it was the DA progenitors that were induced to express TH. The results presented above suggest that TH can be induced in Nurr1-deficient DA progenitors when the tissue is removed from its natural environment. A possible explanation for this finding is that TH expression is induced by pathways that are unrelated to Nurr1. Indeed, previous reports have shown that TH expression can be induced *in vitro* in embryonic neurons that would never have expressed TH *in vivo* (Du and Iacovitti, 1997; Pliego Rivero et al., 1999; Stull and Iacovitti, 1996; Zhou et al., 1996; Zhou et al., 1998). This theory is also supported by a similar study by Eells *et al.* (Eells et al., 2001) in which dissociated midbrain DA cell precursors from Nurr1-/- newborn pups were cultured *in vitro*. These authors could induce expression of TH in Nurr1-/- cells by the addition of forskolin. It is known that forskolin increases cAMP activity which in turn can increase CREB phosphorylation. A subsequent increase in TH may be achieved by activation of CREB sites that are known to be present in the TH promoter. Another possibility for TH induction in Nurr1 null VM could be that Nor1 or NGFIB are induced under our culture conditions and, as these receptors have redundant functions, are able to induce TH expression.

When the free-floating VM tissue of Nurr1 -/- mice was cultured in serum-free conditions, there were very few or no cells expressing TH, while the addition of serum induced TH expression. This suggested that there is a factor in the serum that is able to promote TH -induction. To further investigate which serum-derived factor might be involved, FGF-8, which is involved in the specification of DA progenitors (Crossley et al., 1996; Hynes et al., 1995a; Hynes et al., 1995b; Ye et al., 1998), and epidermal growth factor (EGF), known to create an environment permissive for proliferating progenitor cells (Vescovi et al., 1993), were added to the serum-free medium. These factors did not affect the expression of TH. To elucidate which factor(s) might be involved several additional factors, for example shh, would have to be tested in the same manner.

In trying to translate this data to the *in vivo* situation it is possible to speculate that Nurr1 induces the expression of a factor that affects a nearby cells. This cell could then as a response to this factor send a signal back to the DA progenitor, which results in TH expression. In our culture conditions a factor in the serum may mimic the signal, and thus bypass the need for Nurr1. In this context it is interesting to mention that an unknown factor produced by VM astrocytes was able to induce TH expression in a Nurr1-expressing neuronal stem cell line (Wagner et al., 1999). It would be interesting to see if the astrocyte medium used in this study could induce expression of TH in the Nurr1-/- VM cultures discussed above.

Nurr1 and target innervation

An interesting finding was the formation of a distinct nerve fiber bundle in wild-type VM-LGE co-cultures. Bundles were found in 80% of the wild-type cultures, while only a diffuse nerve fiber network was found in the knockout cultures. These results imply that the LGE stimulates the formation of a nerve fiber bundle in the presence of Nurr1, and that this ability is lost in the absence of Nurr1, suggesting that Nurr1 is important for target innervation *in vivo*.

The question of weather the nigrostriatal pathway is intact in Nurr1 null mice has been a matter of debate. Injections of the retrograde tracing dye Flouro-Gold into the striatum of newborn mice showed that the nigrostriatal innervation was abnormal in the Nurr1 -/-pups (Wallen et al., 1999). In contrast, another study performed by striatal injections of the lipophilic tracer DiI indicated that the innervation was preserved in the Nurr1 null pups (Witta et al., 2000). The data presented above, together with the finding that Nurr1 can induce neurite outgrowth in MN9D cells, suggests a role for Nurr1 in DA axon pathfinding. An experiment in which one could study the formation of DA nerve fibers would be to transplant Nurr1 -/- VM into the striatum of 6-OHDA-lesioned rats.

The issue of wether projections of Nurr1-containing neurons are disturbed in the Nurr1 null mutant mice will be further investigated in the lab. An ongoing project is to generate mice with the gene encoding green fluorescent protein inserted into the Nurr1 locus (Aarnisalo and Perlmann, personal communication). Using these mice it will be possible to study the nigrostriatal projections, and projections of the DMN X axons in Nurr1- deficient embryos. It is interesting to speculate that Nurr1 has a more general role in axon pathfinding in several different CNS neurons.

Paper V-Nurr1 +/- mice have reduced preference for ethanol intake and wheel running

Nurr1 expression continues during adulthood, and several lines of evidence suggest that this NR modulate the function of adult midbrain DA neurons. First, adult Nurr1 heterozygous mice have reduced levels of DA in the midbrain and the NAc, the target area for VTA neurons, suggesting that Nurr1 gene dosage regulate DA levels in the adult brain (Witta et al., 2000). Second, Nurr1 expression is downregulated in the midbrain of cocaine abusers (Bannon et al., 2002). Third, mutations in the Nurr1 gene have been identified in patients with PD and schizophrenia (Buervenich et al., 2000; Chen et al., 2001; Le et al., 2003; Xu et al., 2002; Zheng et al., 2003). Fourth, Nurr +/- mice have been shown to be more susceptible to the DA cell specific toxin MPTP (Le et al., 1999).

Since long, it has been believed that DA signaling in the NAc is important in the mediation of motivation, locomotion and reward (Berridge and Robinson, 1998). Virtually all drugs of abuse, including ethanol, cocaine and nicotine, have been shown to increase DA levels in the NAc (Di Chiara and Imperato, 1988). Moreover, it has been reported that lesions of the VTA and/or NAc attenuate the self-administration of drugs of abuse (Bozarth and Wise, 1986; Pettit et al., 1984; Spyraki et al., 1983; Zito et al., 1985). In addition, it is believed that the NAc is involved in mediating natural reinforces such as food, drink, sex and social interactions (Koob et al., 1998; Wise, 1998).

Given the DA phenotype of the Nurr1 gene targeted mice and the involvement of DA pathways in rewarding mechanisms, we decided to study Nurr1 heterozygous mice in models of natural and drug-induced reward. Ethanol consumption was used as a model for drug- induced reward and wheel running as a model for natural reward. Our results showed that Nurr1 +/- mice were less prone to develop preference for ethanol when they

were given a choice of water or ethanol. The difference in ethanol consumption was not due to differences in taste sensations or ethanol clearance. Curiously, the Nurr1 gene is located within an alcohol preference quantitative trait locus (QTL) on mouse chromosome 2 (Belknap and Atkins, 2001; Phillips et al., 1998a), further linking Nurr1 to ethanol abuse. A QTL is a region of a chromosome that has been shown through genetic mapping to contain one or more genes that contribute to phenotypic differences (Crabbe et al., 1999). Next, natural reward mechanisms were explored by giving the Nurr1 +/+ and +/- mice the opportunity to exercise in running wheels over a three-week period. The wild-type mice steadily increased their running levels during the period tested, while heterozygous mice maintained their running levels at essentially the initial level. This difference in running levels was not due to lower spontaneous motor activity of heterozygous mice, as accessed by monitoring locomotion in activity boxes. In contrast, Nurr1 +/- mice exhibited higher motor activity on the first day of testing, a result that has also been reported by two other groups (Backman et al., 2003; Eells et al., 2002). In summary, the heterozygous mice were less susceptible to natural and drug-induced reward. Interestingly, mice deficient in the DA D2 receptor also show a markedly reduced alcohol preference (Phillips et al., 1998b).

Differences in the Nurr1 promoter of C57/6J and DBA/2J mice

The C57/6J mice strain quickly develops a high preference for ethanol, while the DBA/2J strain is much more resistant to ethanol addiction (Meliska et al., 1995; Risinger et al., 1998). As Nurr1 heterozygous mice were less attracted by ethanol, we wished to analyze if the nucleotide sequence of the Nurr1 gene differed between the two mice strains. Sequencing of the Nurr1 exons and promoter region identified two dinucleotide repeats in the promoter region that differed between the two strains. In the alcohol-preferring C57/6J mice these repeats were longer, suggesting a correlation between ethanol preference and dinucleotid-repeat length in the Nurr1 promoter. Using RT-PCR, we did not find any difference in expression levels of Nurr1 mRNA in VTAs dissected from the respective mouse strains, suggesting that these repeats do not affect Nurr1 expression. However, this method is crude, and differences in gene expression might not be detected. Promoter analyses would be necessary to further evaluate the role of the dinucleotide repeats. Alternatively, these repeats may not be important for the expression of the Nurr1 gene, suggesting that genes distinct from Nurr1 are responsible for differences in ethanol preference between the strains.

Biochemical changes in the NAc and VTA of Nurr1+/- mice

As discussed in the introduction, chronic exposure to drugs of abuse induces biochemical adaptions in the VTA and NAc, characterized by changes in levels of certain proteins. We were therefore interested to analyze the levels of some of these proteins in Nurr1 +/+ and Nurr1 +/- mice. Tissue from the VTA and NAc were dissected from Nurr1 heterozygous and wild-type mice. In western blots the protein levels of TH, glutamate receptor 1, neurofilament and delta FosB were determined. We did not find any

significant differences in protein levels of any of these genes in the respective phenotypes (unpublished data).

Final remarks

The decreased reward behavior in the Nurr1+/- mice might be due to reduced activity of the VTA-NAc pathway. It should also be considered that the presence of Nurr1 in other neuronal pathways might be responsible for the effects seen in Paper V. Interestingly, Nurr1 has been shown to be upregulated in the hippocampus of rats during spatial learning, suggesting that Nurr1 is involved in learning and memory processes (Pena de Ortiz et al., 2000).
It is not clear wether the behavioral defects discussed in Paper V are due to deficiencies occurring during development of the DA system, or if these effects are a result of reduced gene dosage in the adult animals. For this, it is necessary to study inducible Nurr1 knockouts in which Nurr1 is deleted in adult brains.

CONCLUSIONS

This study has provided new insights into the function of the nuclear receptor Nurr1 and the target genes that it regulates. Dopamine MN9D cells were utilized as a model system for midbrain dopamine cells. These cells respond to Nurr1 overexpression by induction of cell cycle arrest and morphological differentiation. In addition, Nurr1 expression leads to an increase in the dopamine content of the cells. Nurr1 was capable of positively regulating the expression of the dopamine-producing enzyme AADC and the vesicular monoamine transporter VMAT2. Interestingly, both AADC and VMAT2 mRNA expressions were deregulated in the developing midbrain DA cells of Nurr1 deficient mice, suggesting that Nurr1 is important for dopamine synthesis and storage.

To identify additional genes regulated by Nurr1, a cDNA microarray experiment was performed, comparing MN9D cells with an induced expression of Nurr1 with non-induced cells. In this screen the axon guidance cell surface receptor Neuropilin-1 was identified as a new Nurr1-regulated gene. In reporter gene assays Nurr1 was able to activate the Nrp1 promoter, which contains two Nurr1 binding sites. Nurr1 and Nrp1 were coexpressed in the DMN X and Nrp1 mRNA was downregulated in this nucleus in Nurr1 knockout mice. These results indicate that Nurr1 dependent differentiation of this nucleus requires regulation of Nrp1.

Ventral midbrains of various Nurr1 genotypes were cultured *in vitro* to further analyze Nurr1-dependent differentiation of DA cells. Expression of TH could be induced in Nurr1 deficient cultures. Furthermore, axon bundles were not identified in cultures lacking Nurr1, supporting a role for Nurr1 in target innervation of DA neurons.

Finally, adult Nurr1 heterozygous and wild-type mice were less attracted by ethanol drinking and wheel running, suggesting that Nurr1 is important for natural and drug induced reward mechanisms.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people that have supported me throughout these years.

Prof. **Ralf Pettersson** for creating an excellent scientific and social environment at the Ludwig Institute.

Prof. **Thomas Perlmann**, my supervisor, who has provided scientific expertise, ideas, suggestions, patience, criticism and numerous other qualities that nourished my work during my years at the Ludwig Institute. Thanks also for nice skiing company in the bumpy slopes of Steamboat Springs.

Past and present members of the Perlmann Lab (in chronological order): **Ludmila Solomin**, **Rolf Zetterström**, **Diogo Castro**, **Mariette Arvidsson**, **Alex Mata**, **Åsa Wallén**, **Helena Pettersson**, **Piia Aarnisalo**, **Joanne Savory**, **Bertrand Joseph**, **Gérard Benoit**, **Eliza Joodmardi**, **Johan Lengqvist**, **Martin Werme**, **Lotta Borgius**, **Stina Friling**, **Susanna Petersson**, **Michal Malewich, Nikolas Volakakis**, **Banafsheh Kadkhodaei**.
Special thanks to: My bench partner **Åsa** for all help and support and for frienship. **Mariette** for frienship and support during the writing of this book, I really appreciated your company. **Ludmila** for introducing me to the Perlmann lab and for friendship. **Diogo** for nice collaboration on the MN9D cells and for all your crazy jokes. **Susanna** for help with this book, and for skiing company. **Eliza** for help with oligo in situ. **Gérard**, for always being very helpful and willing to share your great knowledge in science. **Michal** for your great help with lab and writing matters. **Alex** and **Stina** for good travel company. **Bertrand** for MN9D-tet cells and collaboration. **Helena** for nice dinners.

The Muhr group: **Jonas Muhr**, **Magdalena Bylund**, **Magnus Sandberg** and **Maria Bergsland**. A special thanks to **Maria**, I really enjoyed to work with you, good luck in the Muhr group!

All members of the "Ulf-group" for great lunch-room discussions.

All present and past member of the Ludwig Institute.

Charlotta Linderholm and **Birgitta Freidenfelt** for always being very helpful with all administrative matters.
Mats Anderling and **Ulf Florman** for help with computer-matters
Inger Tollman for help with a lot of different things from ordering of lab-stuff to equipment maintenance. **Erika Enrione Barrera** for help with dishes and solutions.

Collaborators from other labs: **Nina Törnqvist** and **Ingrid Strömberg** for collaboration on the VM culture paper. **Martin Werme** and **Stefan Brené** for collaboration on the

behavioral studies of the Nurr1 heterozygotes. **Eva Lindqvist**, **Karin Lundströmer** and **Lars Olsson** for help with the MN9D paper. **Marcin Kowanetz** and **Aris Moustakas** for help with microarrays.

Eva Severinsson for being and excellent coordinator of the Stockholm Biomedical Research School and for informing me that the TP-lab is an outstanding lab.

This work was supported by the National Network in Neuroscience (NNN). A special thanks to **Britt Arvidsson**, **Lennart Brodin** and **Gilberto Fisone** for taking such good care of all the members of the network.

My friends from the NNN network, **Karin Agerman** and **Karin Hygge-Blakeman**.

Anna Romert, Karin Aase, Hanna Forsberg, Mariette and **Åsa** for good discussions about everything from science to life in general, for nice dinners and friendship.

My friends from the outside-world, what would I do without you? **Lovisa** and **Sten**, **Linda** and **Andreas**, **Hazze**, **Rodrigo**, **Marie and Stefan**, **Carina and Peter**, **Gabriella** and **Jonas**, **Helen** and **Ronnie**, **Marie** and **Bosse**, **Monica** and **Keivan**, **Lars** and **Charlotte**, **Micke** and **Annelie, Björn** and **Veronica, Tomas** and **Mia, Jessica** and **Anders**.
"Syjuntan": **Linda, Lollo, Emma, Chris, Jessica, May-Britt, Lina.**

The whole **Nielsen**-family: **Flemming** and **Sig-Britt**, **Kent** and **Carro**, **Maria** and **Hans**, **Eva** and **Micke**, and all their kids.

Jennifer for updating me on the latest clothes fashion.

My French relatives, the **Lebier** and **Franc** families.

My dear parents, **Gunnar** and **Maja-Stina**, for always believing in me and for your endless support at all times. My brothers **Jonas** and **Christian** and their families, you really mean a lot to me.

Kajsa and **Stina**, our wonderful twin daughters who have turned our world upside-down.

Thomas, the best "sambo" one could ever have! I love you with all my heart.

REFERENCES

Aarnisalo, P., Kim, C. H., Lee, J. W. and Perlmann, T. (2002). Defining requirements for heterodimerization between the retinoid X receptor and the orphan nuclear receptor Nurr1. *J Biol Chem* **277**, 35118-23.

Adams, M. D., Kerlavage, A. R., Fleischmann, R. D., Fuldner, R. A., Bult, C. J., Lee, N. H., Kirkness, E. F., Weinstock, K. G., Gocayne, J. D., White, O. et al. (1995). Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. *Nature* **377**, 3-174.

Aranda, A. and Pascual, A. (2001a). Nuclear hormone receptors and gene expression. *Physiol Rev* **81**, 1269-304.

Aranda, A. and Pascual, A. (2001b). Nuclear hormone receptors and gene expression. *Physiol Rev* **81**, 1269-304.

Arkenbout, E. K., de Waard, V., van Bragt, M., van Achterberg, T. A., Grimbergen, J. M., Pichon, B., Pannekoek, H. and de Vries, C. J. (2002). Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation* **106**, 1530-5.

Arkenbout, E. K., van Bragt, M., Eldering, E., van Bree, C., Grimbergen, J. M., Quax, P. H., Pannekoek, H. and de Vries, C. J. (2003). TR3 orphan receptor is expressed in vascular endothelial cells and mediates cell cycle arrest. *Arterioscler Thromb Vasc Biol* **23**, 1535-40.

Backman, C., You, Z. B., Perlmann, T. and Hoffer, B. J. (2003). Elevated locomotor activity without altered striatal dopamine contents in Nurr1 heterozygous mice after acute exposure to methamphetamine. *Behav Brain Res* **143**, 95-100.

Bagri, A. and Tessier-Lavigne, M. (2002). Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. *Adv Exp Med Biol* **515**, 13-31.

Baik, J. H., Picetti, R., Saiardi, A., Thiriet, G., Dierich, A., Depaulis, A., Le Meur, M. and Borrelli, E. (1995). Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. *Nature* **377**, 424-8.

Baker, K. D., Shewchuk, L. M., Kozlova, T., Makishima, M., Hassell, A., Wisely, B., Caravella, J. A., Lambert, M. H., Reinking, J. L., Krause, H. et al. (2003). The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell* **113**, 731-42.

Bandoh, S., Tsukada, T., Maruyama, K., Ohkura, N. and Yamaguchi, K. (1997). Mechanical agitation induces gene expression of NOR-1 and its closely related orphan nuclear receptors in leukemic cell lines. *Leukemia* **11**, 1453-8.

Bannon, M. J., Pruetz, B., Manning-Bog, A. B., Whitty, C. J., Michelhaugh, S. K., Sacchetti, P., Granneman, J. G., Mash, D. C. and Schmidt, C. J. (2002). Decreased expression of the transcription factor NURR1 in dopamine neurons of cocaine abusers. *Proc Natl Acad Sci U S A* **16**, 16.

Bassett, M. H., Suzuki, T., Sasano, H., White, P. C. and Rainey, W. E. (2003). The NGFIB family of nuclear receptors regulates adrenal aldosterone production. *Mol Endocrinol.*

Beck, H., Acker, T., Puschel, A. W., Fujisawa, H., Carmeliet, P. and Plate, K. H. (2002). Cell type-specific expression of neuropilins in an MCA-occlusion model in mice suggests a potential role in post-ischemic brain remodeling. *J Neuropathol Exp Neurol* **61**, 339-50.

Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A. and Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* **373**, 339-41.

Beitner-Johnson, D., Guitart, X. and Nestler, E. J. (1991). Dopaminergic brain reward regions of Lewis and Fischer rats display different levels of tyrosine hydroxylase and other morphine- and cocaine-regulated phosphoproteins. *Brain Res* **561**, 147-50.

Belknap, J. K. and Atkins, A. L. (2001). The replicability of QTLs for murine alcohol preference drinking behavior across eight independent studies. *Mamm Genome* **12**, 893-9.

Benoit, G., Malewicz, M. and Perlmann, T. (2004). Digigng deep into the pockets of nuclear receptors. *Trends in Cell Biology* **submitted**.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K. and Seitelberger, F. (1973). Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci* **20**, 415-55.

Berridge, K. C. and Robinson, T. E. (1998). What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Res Brain Res Rev* **28**, 309-69.
Bevan, C. and Parker, M. (1999). The role of coactivators in steroid hormone action. *Exp Cell Res* **253**, 349-56.
Billas, I. M., Moulinier, L., Rochel, N. and Moras, D. (2001). Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. *J Biol Chem* **276**, 7465-74.
Blumberg, B. and Evans, R. M. (1998). Orphan nuclear receptors--new ligands and new possibilities. *Genes Dev* **12**, 3149-55.
Botling, J., Castro, D. S., Oberg, F., Nilsson, K. and Perlmann, T. (1997). Retinoic acid receptor/retinoid X receptor heterodimers can be activated through both subunits providing a basis for synergistic transactivation and cellular differentiation. *J Biol Chem* **272**, 9443-9.
Boulay, D., Depoortere, R., Perrault, G., Borrelli, E. and Sanger, D. J. (1999). Dopamine D2 receptor knock-out mice are insensitive to the hypolocomotor and hypothermic effects of dopamine D2/D3 receptor agonists. *Neuropharmacology* **38**, 1389-96.
Bozarth, M. A. and Wise, R. A. (1986). Involvement of the ventral tegmental dopamine system in opioid and psychomotor stimulant reinforcement. *NIDA Res Monogr* **67**, 190-6.
Brown, G., Hughes, P. J. and Michell, R. H. (2003). Cell differentiation and proliferation--simultaneous but independent? *Exp Cell Res* **291**, 282-8.
Brundin, P., Karlsson, J., Emgard, M., Schierle, G. S., Hansson, O., Petersen, A. and Castilho, R. F. (2000). Improving the survival of grafted dopaminergic neurons: a review over current approaches. *Cell Transplant* **9**, 179-95.
Buervenich, S., Carmine, A., Arvidsson, M., Xiang, F., Zhang, Z., Sydow, O., Jonsson, E. G., Sedvall, G. C., Leonard, S., Ross, R. G. et al. (2000). NURR1 mutations in cases of schizophrenia and manic-depressive disorder. *Am J Med Genet* **96**, 808-13.
Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992). RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. *Embo J* **11**, 1409-18.
Burbach, J. P., Smits, S. and Smidt, M. P. (2003). Transcription factors in the development of midbrain dopamine neurons. *Ann N Y Acad Sci* **991**, 61-8.
Calnan, B. J., Szychowski, S., Chan, F. K., Cado, D. and Winoto, A. (1995). A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* **3**, 273-82.
Carlsson, A. (1959). The occurrence, distribution and physiological role of catecholamines in the nervous system. *Pharmacol Rev* **11**, 490-3.
Carlsson, A. (1988). The current status of the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* **1**, 179-86.
Castillo, S. O., Baffi, J. S., Palkovits, M., Goldstein, D. S., Kopin, I. J., Witta, J., Magnuson, M. A. and Nikodem, V. M. (1998a). Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. *Mol Cell Neurosci* **11**, 36-46.
Castillo, S. O., Xiao, Q., Kostrouch, Z., Dozin, B. and Nikodem, V. M. (1998b). A divergent role of COOH-terminal domains in Nurr1 and Nur77 transactivation. *Gene Expr* **7**, 1-12.
Castillo, S. O., Xiao, Q., Lyu, M. S., Kozak, C. A. and Nikodem, V. M. (1997). Organization, sequence, chromosomal localization, and promoter identification of the mouse orphan nuclear receptor Nurr1 gene. *Genomics* **41**, 250-7.
Castro, D. S., Arvidsson, M., Bondesson Bolin, M. and Perlmann, T. (1999). Activity of the Nurr1 carboxyl-terminal domain depends on cell type and integrity of the activation function 2. *J Biol Chem* **274**, 37483-90.
Chen, J. D. and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377**, 454-7.
Chen, Y. H., Tsai, M. T., Shaw, C. K. and Chen, C. H. (2001). Mutation analysis of the human NR4A2 gene, an essential gene for midbrain dopaminergic neurogenesis, in schizophrenic patients. *Am J Med Genet* **105**, 753-7.
Choi, H. K., Won, L. A., Kontur, P. J., Hammond, D. N., Fox, A. P., Wainer, B. H., Hoffmann, P. C. and Heller, A. (1991). Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Res* **552**, 67-76.
Choi, W. S., Chun, S. Y., Markelonis, G. J., Oh, T. H. and Oh, Y. J. (2001). Overexpression of calbindin-D28K induces neurite outgrowth in dopaminergic neuronal cells via activation of p38 MAPK. *Biochem Biophys Res Commun* **287**, 656-61.
Choi-Lundberg, D. L., Lin, Q., Chang, Y. N., Chiang, Y. L., Hay, C. M., Mohajeri, H.,

Davidson, B. L. and Bohn, M. C. (1997). Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* **275**, 838-41.
Chu, Y., Cochran, E. J., Mufson, E. J. and Kordower, J. H. (2001). Reduction of Nurr1 expression in substantia nigra of patients with Parkinson's disease. In *Society for Neuroscience abstract, No 967.1*, (ed.
Chung, K. K., Dawson, V. L. and Dawson, T. M. (2003). New insights into Parkinson's disease. *J Neurol* **250 Suppl 3**, III15-24.
Committee, N. R. N. (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**, 161-3.
Conneely, O. M., Mullican, S., Milbrandt, J. (2004). Essential and redundant roles of Nor1 and Nurr77 in regulation of myelopoiesis. In *Nuclear Receptors: Orphan brothers*, (ed.
Crabbe, J. C., Phillips, T. J., Buck, K. J., Cunningham, C. L. and Belknap, J. K. (1999). Identifying genes for alcohol and drug sensitivity: recent progress and future directions. *Trends Neurosci* **22**, 173-9.
Crispino, M., Tocco, G., Feldman, J. D., Herschman, H. R. and Baudry, M. (1998). Nurr1 mRNA expression in neonatal and adult rat brain following kainic acid-induced seizure activity. *Brain Res Mol Brain Res* **59**, 178-88.
Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-8.
Dahlström, A. and Fuxe, K. (1964). Evidence for the Existence of Monoamine-Containing Neurons in the Central Nervous System. I. Demonstration of Monoamines in the Cell Bodies of Brain Stem Neurons. *Acta Physiol Scand* **62**, SUPPL 232:1-55.
Danielian, P. S. and McMahon, A. P. (1996). Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature* **383**, 332-4.
Davies M.R, R. S., Harding C.J, Downey-Jones M and Needham M. (2004). Nurr1 can induce pro-inflammatory gene expression in synoviocytes through a mechanism distinct from the corticotrophin releasing hormone pathway. In *Nuclear Receptors: Orphan Brothers*, (ed.
Davis, I. J., Hazel, T. G. and Lau, L. F. (1991). Transcriptional activation by Nur77, a growth factor-inducible member of the steroid hormone receptor superfamily. *Mol Endocrinol* **5**, 854-9.
de Urquiza, A. M., Liu, S., Sjoberg, M., Zetterstrom, R. H., Griffiths, W., Sjovall, J. and Perlmann, T. (2000). Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* **290**, 2140-4.
DeYoung, R. A., Baker, J. C., Cado, D. and Winoto, A. (2003). The orphan steroid receptor Nur77 family member Nor-1 is essential for early mouse embryogenesis. *J Biol Chem* **278**, 47104-9.
Di Chiara, G. and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U S A* **85**, 5274-8.
Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D. et al. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci U S A* **93**, 6025-30.
Dickinson, S. D., Sabeti, J., Larson, G. A., Giardina, K., Rubinstein, M., Kelly, M. A., Grandy, D. K., Low, M. J., Gerhardt, G. A. and Zahniser, N. R. (1999). Dopamine D2 receptor-deficient mice exhibit decreased dopamine transporter function but no changes in dopamine release in dorsal striatum. *J Neurochem* **72**, 148-56.
Du, X. and Iacovitti, L. (1997). Protein kinase C activators work in synergy with specific growth factors to initiate tyrosine hydroxylase expression in striatal neurons in culture. *J Neurochem* **68**, 564-9.
Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P. and Trent, J. M. (1999). Expression profiling using cDNA microarrays. *Nat Genet* **21**, 10-4.
Dunnett, S. B. and Bjorklund, A. (1999). Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature* **399**, A32-9.
Eells, J. B., Lipska, B. K., Yeung, S. K., Misler, J. A. and Nikodem, V. M. (2002). Nurr1-null heterozygous mice have reduced mesolimbic and mesocortical dopamine levels and increased stress-induced locomotor activity. *Behav Brain Res* **136**, 267-75.
Eells, J. B., Rives, J. E., Yeung, S. K. and Nikodem, V. M. (2001). In vitro regulated expression of tyrosine hydroxylase in ventral midbrain neurons from Nurr1-null mouse pups. *J Neurosci Res* **64**, 322-30.
Enmark, E. and Gustafsson, J. A. (1996). Orphan nuclear receptors--the first eight years. *Mol Endocrinol* **10**, 1293-307.

Escriva, H., Delaunay, F. and Laudet, V. (2000). Ligand binding and nuclear receptor evolution. *Bioessays* **22**, 717-27.

Escriva, H., Safi, R., Hanni, C., Langlois, M. C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R. and Laudet, V. (1997). Ligand binding was acquired during evolution of nuclear receptors. *Proc Natl Acad Sci U S A* **94**, 6803-8.

Falck, B., Hillarp, N.-Å., Thieme, G. and Torp, A. (1962). Fluorescence of catechol amines and related compounds condensed with formaldehyde. *J Histochem Cytochem* **10**, 348-354.

Fan, D. S., Ogawa, M., Fujimoto, K. I., Ikeguchi, K., Ogasawara, Y., Urabe, M., Nishizawa, M., Nakano, I., Yoshida, M., Nagatsu, I. et al. (1998). Behavioral recovery in 6-hydroxydopamine-lesioned rats by cotransduction of striatum with tyrosine hydroxylase and aromatic L-amino acid decarboxylase genes using two separate adeno-associated virus vectors. *Hum Gene Ther* **9**, 2527-35.

Feany, M. B. and Bender, W. W. (2000). A Drosophila model of Parkinson's disease. *Nature* **404**, 394-8.

Fisk, G. J. and Thummel, C. S. (1995). Isolation, regulation, and DNA-binding properties of three Drosophila nuclear hormone receptor superfamily members. *Proc Natl Acad Sci U S A* **92**, 10604-8.

Fon, E. A., Pothos, E. N., Sun, B. C., Killeen, N., Sulzer, D. and Edwards, R. H. (1997). Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. *Neuron* **19**, 1271-83.

Fondell, J. D., Ge, H. and Roeder, R. G. (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc Natl Acad Sci U S A* **93**, 8329-33.

Forman, B. M., Tzameli, I., Choi, H. S., Chen, J., Simha, D., Seol, W., Evans, R. M. and Moore, D. D. (1998). Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* **395**, 612-5.

Forman, B. M., Umesono, K., Chen, J. and Evans, R. M. (1995). Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* **81**, 541-50.

Gainetdinov, R. R., Fumagalli, F., Jones, S. R. and Caron, M. G. (1997). Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J Neurochem* **69**, 1322-5.

Gainetdinov, R. R., Fumagalli, F., Wang, Y. M., Jones, S. R., Levey, A. I., Miller, G. W. and Caron, M. G. (1998). Increased MPTP neurotoxicity in vesicular monoamine transporter 2 heterozygote knockout mice. *J Neurochem* **70**, 1973-8.

Galleguillos, D., Vecchiola, A., Fuentealba, J. A., Ojeda, V., Alvarez, K., Gomez, A. and Andres, M. E. (2004). PIASgamma represses the transcriptional activation induced by the nuclear receptor Nurr1. *J Biol Chem* **279**, 2005-11.

Giguere, V. (1999). Orphan nuclear receptors: from gene to function. *Endocr Rev* **20**, 689-725.

Gill, S., McManus, A. P., Crew, A. J., Benjamin, H., Sheer, D., Gusterson, B. A., Pinkerton, C. R., Patel, K., Cooper, C. S. and Shipley, J. M. (1995). Fusion of the EWS gene to a DNA segment from 9q22-31 in a human myxoid chondrosarcoma. *Genes Chromosomes Cancer* **12**, 307-10.

Giros, B., Jaber, M., Jones, S. R., Wightman, R. M. and Caron, M. G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606-12.

Glass, C. K. and Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* **14**, 121-41.

Goodman , e. a. (1999). Unified nomenclature for the semaphorins/collapsins. Semaphorin Nomenclature Committee. *Cell* **97**, 551-2.

Greene, L. A. and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A* **73**, 2424-8.

Guitart, X., Beitner-Johnson, D., Marby, D. W., Kosten, T. A. and Nestler, E. J. (1992). Fischer and Lewis rat strains differ in basal levels of neurofilament proteins and their regulation by chronic morphine in the mesolimbic dopamine system. *Synapse* **12**, 242-53.

Hammer, G. D., Krylova, I., Zhang, Y., Darimont, B. D., Simpson, K., Weigel, N. L. and Ingraham, H. A. (1999). Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. *Mol Cell* **3**, 521-6.

He, Z. and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* **90**, 739-51.

Heery, D. M., Kalkhoven, E., Hoare, S. and Parker, M. G. (1997). A signature motif in

transcriptional co-activators mediates binding to nuclear receptors. *Nature* **387**, 733-6.
Heller, A., Freeney, A., Hessefort, S., Villereal, M. and Won, L. (2000). Cellular dopamine is increased following exposure to a factor derived from immortalized striatal neurons. *Neurosci Lett* **295**, 1-4.
Heller, A., Price, S. and Won, L. (1996). Glial-derived neurotrophic factor (GDNF) induced morphological differentiation of an immortalized monoclonal hybrid dopaminergic cell line of mesencephalic neuronal origin. *Brain Res* **725**, 132-6.
Honkaniemi, J. and Sharp, F. R. (1996). Global ischemia induces immediate-early genes encoding zinc finger transcription factors. *J Cereb Blood Flow Metab* **16**, 557-65.
Honkaniemi, J. and Sharp, F. R. (1999). Prolonged expression of zinc finger immediate-early gene mRNAs and decreased protein synthesis following kainic acid induced seizures. *Eur J Neurosci* **11**, 10-7.
Honkaniemi, J., States, B. A., Weinstein, P. R., Espinoza, J. and Sharp, F. R. (1997). Expression of zinc finger immediate early genes in rat brain after permanent middle cerebral artery occlusion. *J Cereb Blood Flow Metab* **17**, 636-46.
Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K. et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377**, 397-404.
Hubank, M. and Schatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* **22**, 5640-8.
Hwang, D. Y., Ardayfio, P., Kang, U. J., Semina, E. V. and Kim, K. S. (2003). Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. *Brain Res Mol Brain Res* **114**, 123-31.
Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995a). Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.
Hynes, M., Poulsen, K., Tessier-Lavigne, M. and Rosenthal, A. (1995b). Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell* **80**, 95-101.
Hynes, M. and Rosenthal, A. (1999). Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. *Curr Opin Neurobiol* **9**, 26-36.
Ichinose, H., Ohye, T., Suzuki, T., Sumi-Ichinose, C., Nomura, T., Hagino, Y. and Nagatsu, T. (1999). Molecular cloning of the human Nurr1 gene: characterization of the human gene and cDNAs. *Gene* **230**, 233-9.
Ito, M., Yuan, C. X., Malik, S., Gu, W., Fondell, J. D., Yamamura, S., Fu, Z. Y., Zhang, X., Qin, J. and Roeder, R. G. (1999). Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol Cell* **3**, 361-70.
Iwawaki, T., Kohno, K. and Kobayashi, K. (2000). Identification of a potential nurr1 response element that activates the tyrosine hydroxylase gene promoter in cultured cells. *Biochem Biophys Res Commun* **274**, 590-5.
Joseph, B., Wallen-Mackenzie, A., Benoit, G., Murata, T., Joodmardi, E., Okret, S. and Perlmann, T. (2003). p57(Kip2) cooperates with Nurr1 in developing dopamine cells. *Proc Natl Acad Sci U S A* **100**, 15619-24.
Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T. and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126**, 4895-902.
Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K. et al. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* **418**, 50-6.
Kim, J. Y., Koh, H. C., Lee, J. Y., Chang, M. Y., Kim, Y. C., Chung, H. Y., Son, H., Lee, Y. S., Studer, L., McKay, R. et al. (2003a). Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. *J Neurochem* **85**, 1443-54.
Kim, K. S., Kim, C. H., Hwang, D. Y., Seo, H., Chung, S., Hong, S. J., Lim, J. K., Anderson, T. and Isacson, O. (2003b). Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. *J Neurochem* **85**, 622-34.
Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T. and Fujisawa, H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* **19**, 995-1005.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H. and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* **121**, 4309-18.
Kliewer, S. A., Lehmann, J. M. and Willson, T. M. (1999). Orphan nuclear receptors: shifting endocrinology into reverse. *Science* **284**, 757-60.
Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. and Evans, R. M. (1992). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* **355**, 446-9.
Kobayashi, K., Morita, S., Sawada, H., Mizuguchi, T., Yamada, K., Nagatsu, I., Hata, T., Watanabe, Y., Fujita, K. and Nagatsu, T. (1995). Targeted disruption of the tyrosine hydroxylase locus results in severe catecholamine depletion and perinatal lethality in mice. *J Biol Chem* **270**, 27235-43.
Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J. and Ginty, D. D. (1997). Neuropilin is a semaphorin III receptor. *Cell* **90**, 753-62.
Koob, G. F., Sanna, P. P. and Bloom, F. E. (1998). Neuroscience of addiction. *Neuron* **21**, 467-76.
Kostrouch, Z., Kostrouchova, M. and Rall, J. E. (1995). Steroid/thyroid hormone receptor genes in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **92**, 156-9.
Krezel, W., Ghyselinck, N., Samad, T. A., Dupe, V., Kastner, P., Borrelli, E. and Chambon, P. (1998). Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science* **279**, 863-7.
Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J. T., Schols, L. and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet* **18**, 106-8.
Kurokawa, R., Soderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M. G. and Glass, C. K. (1995). Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature* **377**, 451-4.
Labelle, Y., Bussieres, J., Courjal, F. and Goldring, M. B. (1999). The EWS/TEC fusion protein encoded by the t(9;22) chromosomal translocation in human chondrosarcomas is a highly potent transcriptional activator. *Oncogene* **18**, 3303-8.
Lammi, J., Huppunen, J. and Aarnisalo, P. (2004). Regulation of the Osteopontin Gene by the Orphan Nuclear Receptor Nurr1 in Osteoblasts. *Mol Endocrinol*.
Laudet, V. and Gronemeyer, H. (2002). The nuclear receptors factsbook: London: Academic Press.
Law, S. W., Conneely, O. M., DeMayo, F. J. and O'Malley, B. W. (1992). Identification of a new brain-specific transcription factor, NURR1. *Mol Endocrinol* **6**, 2129-35.
Le, W., Conneely, O. M., He, Y., Jankovic, J. and Appel, S. H. (1999). Reduced Nurr1 expression increases the vulnerability of mesencephalic dopamine neurons to MPTP-induced injury. *J Neurochem* **73**, 2218-21.
Le, W. D., Xu, P., Jankovic, J., Jiang, H., Appel, S. H., Smith, R. G. and Vassilatis, D. K. (2003). Mutations in NR4A2 associated with familial Parkinson disease. *Nat Genet* **33**, 85-9.
Lee, S. L., Wesselschmidt, R. L., Linette, G. P., Kanagawa, O., Russell, J. H. and Milbrandt, J. (1995). Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). *Science* **269**, 532-5.
Lee, W. Y., Chang, J. W., Nemeth, N. L. and Kang, U. J. (1999). Vesicular monoamine transporter-2 and aromatic L-amino acid decarboxylase enhance dopamine delivery after L-3, 4-dihydroxyphenylalanine administration in Parkinsonian rats. *J Neurosci* **19**, 3266-74.
Li, H., Kolluri, S. K., Gu, J., Dawson, M. I., Cao, X., Hobbs, P. D., Lin, B., Chen, G., Lu, J., Lin, F. et al. (2000). Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* **289**, 1159-64.
Li, X. M., Juorio, A. V., Qi, J. and Boulton, A. A. (1998). L-deprenyl induces aromatic L-amino acid decarboxylase (AADC) mRNA in the rat substantia nigra and ventral tegmentum. An in situ hybridization study. *Mol Chem Neuropathol* **35**, 149-55.
Liang, P. and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-71.
Lindahl, R. and Evces, S. (1984). Rat liver aldehyde dehydrogenase. II. Isolation and characterization of four inducible isozymes. *J Biol Chem* **259**, 11991-6.
Lindvall, O. (1999). Engineering neurons for Parkinson's disease. *Nat Biotechnol* **17**, 635-6.
Liu, D., Jia, H., Holmes, D. I., Stannard, A. and Zachary, I. (2003). Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of

Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. *Arterioscler Thromb Vasc Biol* **23**, 2002-7.
Liu, N. and Baker, H. (1999). Activity-dependent Nurr1 and NGFI-B gene expression in adult mouse olfactory bulb. *Neuroreport* **10**, 747-51.
Liu, Z. G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M. and Osborne, B. A. (1994). Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* **367**, 281-4.
Lloyd, K. G. and Hornykiewicz, O. (1972). Occurrence and distribution of aromatic L-amino acid (L-DOPA) decarboxylase in the human brain. *J Neurochem* **19**, 1549-59.
Mages, H. W., Rilke, O., Bravo, R., Senger, G. and Kroczek, R. A. (1994). NOT, a human immediate-early response gene closely related to the steroid/thyroid hormone receptor NAK1/TR3. *Mol Endocrinol* **8**, 1583-91.
Maira, M., Martens, C., Batsche, E., Gauthier, Y. and Drouin, J. (2003). Dimer-specific potentiation of NGFI-B (Nur77) transcriptional activity by the protein kinase A pathway and AF-1-dependent coactivator recruitment. *Mol Cell Biol* **23**, 763-76.
Maira, M., Martens, C., Philips, A. and Drouin, J. (1999). Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. *Mol Cell Biol* **19**, 7549-57.
Mangelsdorf, D. J. and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**, 841-50.
Marcos, C. and Pachnis, V. (1996). The effect of the ret- mutation on the normal development of the central and parasympathetic nervous systems. *Int J Dev Biol* **Suppl 1**, 137S-138S.
Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. *Development* **126**, 1189-200.
Maruyama, K., Tsukada, T., Bandoh, S., Sasaki, K., Ohkura, N. and Yamaguchi, K. (1995). Expression of NOR-1 and its closely related members of the steroid/thyroid hormone receptor superfamily in human neuroblastoma cell lines. *Cancer Lett* **96**, 117-22.
McEvoy, A. N., Bresnihan, B., Fitzgerald, O. and Murphy, E. P. (2002a). Corticotropin-releasing hormone signaling in synovial tissue vascular endothelium is mediated through the cAMP/CREB pathway. *Ann N Y Acad Sci* **966**, 119-30.
McEvoy, A. N., Murphy, E. A., Ponnio, T., Conneely, O. M., Bresnihan, B., FitzGerald, O. and Murphy, E. P. (2002b). Activation of nuclear orphan receptor NURR1 transcription by NF-kappa B and cyclic adenosine 5'-monophosphate response element-binding protein in rheumatoid arthritis synovial tissue. *J Immunol* **168**, 2979-87.
Meliska, C. J., Bartke, A., McGlacken, G. and Jensen, R. A. (1995). Ethanol, nicotine, amphetamine, and aspartame consumption and preferences in C57BL/6 and DBA/2 mice. *Pharmacol Biochem Behav* **50**, 619-26.
Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* **1**, 183-8.
Miller, G. W., Erickson, J. D., Perez, J. T., Penland, S. N., Mash, D. C., Rye, D. B. and Levey, A. I. (1999). Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease. *Exp Neurol* **156**, 138-48.
Miyakoshi, J., Tsukada, T., Tachiiri, S., Bandoh, S., Yamaguchi, K. and Takebe, H. (1998). Enhanced NOR-1 gene expression by exposure of Chinese hamster cells to high-density 50 Hz magnetic fields. *Mol Cell Biochem* **181**, 191-5.
Moras, D. and Gronemeyer, H. (1998). The nuclear receptor ligand-binding domain: structure and function. *Curr Opin Cell Biol* **10**, 384-91.
Morgan, J. I. and Curran, T. (1995). Immediate-early genes: ten years on. *Trends Neurosci* **18**, 66-7.
Murphy, E. P. and Conneely, O. M. (1997). Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. *Mol Endocrinol* **11**, 39-47.
Murphy, E. P., Dobson, A. D., Keller, C. and Conneely, O. M. (1996). Differential regulation of transcription by the NURR1/NUR77 subfamily of nuclear transcription factors. *Gene Expr* **5**, 169-79.
Murphy, E. P., McEvoy, A., Conneely, O. M., Bresnihan, B. and FitzGerald, O. (2001). Involvement of the nuclear orphan receptor NURR1 in the regulation of corticotropin-releasing hormone expression and actions in human inflammatory arthritis. *Arthritis Rheum* **44**, 782-93.
Nestler, E. J. (2000). Genes and addiction. *Nat Genet* **26**, 277-81.

Nestler, E. J. and Aghajanian, G. K. (1997). Molecular and cellular basis of addiction. *Science* **278**, 58-63.
Nestler, E. J., Barrot, M. and Self, D. W. (2001). DeltaFosB: a sustained molecular switch for addiction. *Proc Natl Acad Sci U S A* **98**, 11042-6.
Nestler, E. J., Hope, B. T. and Widnell, K. L. (1993). Drug addiction: a model for the molecular basis of neural plasticity. *Neuron* **11**, 995-1006.
Neufeld, G., Cohen, T., Shraga, N., Lange, T., Kessler, O. and Herzog, Y. (2002). The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends Cardiovasc Med* **12**, 13-9.
Nishii, K., Matsushita, N., Sawada, H., Sano, H., Noda, Y., Mamiya, T., Nabeshima, T., Nagatsu, I., Hata, T., Kiuchi, K. et al. (1998). Motor and learning dysfunction during postnatal development in mice defective in dopamine neuronal transmission. *J Neurosci Res* **54**, 450-64.
Nordzell, M., Aarnisalo, P., Benoit, G., Castro, D. S. and Perlmann, T. (2004). Defining an N-terminal activation domain of the orphan nuclear receptor Nurr1. *Biochem Biophys Res Commun* **313**, 205-11.
Nsegbe, E., Wallen-Mackenzie, A., Dauger, S., Roux, J. C., Shvarev, Y., Lagercrantz, H., Perlmann, T., Herlenius, E., Mata de Urquiza, A., Petersson, S. et al. (2004). Congenital hypoventilation and impaired hypoxic response in Nurr1 mutant mice
Nurr1-RXR heterodimers mediate RXR ligand-induced signaling in neuronal cells
Defining an N-terminal activation domain of the orphan nuclear receptor Nurr1. *J Physiol* **17**, 3036-47.
Nunes, I., Tovmasian, L. T., Silva, R. M., Burke, R. E. and Goff, S. P. (2003). Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc Natl Acad Sci U S A* **100**, 4245-50.
Oh, Y. J., Swarzenski, B. C. and O'Malley, K. L. (1996). Overexpression of Bcl-2 in a murine dopaminergic neuronal cell line leads to neurite outgrowth. *Neurosci Lett* **202**, 161-4.
Ohkura, N., Hijikuro, M., Yamamoto, A. and Miki, K. (1994). Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells. *Biochem Biophys Res Commun* **205**, 1959-65.
Ohkura, N., Hosono, T., Maruyama, K., Tsukada, T. and Yamaguchi, K. (1999). An isoform of Nurr1 functions as a negative inhibitor of the NGFI-B family signaling. *Biochim Biophys Acta* **1444**, 69-79.
Olson, L. and Seiger, A. (1972). Early prenatal ontogeny of central monoamine neurons in the rat: fluorescence histochemical observations. *Z Anat Entwicklungsgesch* **137**, 301-16.
Ordentlich, P., Yan, Y., Zhou, S. and Heyman, R. A. (2003). Identification of the antineoplastic agent 6-mercaptopurine as an activator of the orphan nuclear hormone receptor Nurr1. *J Biol Chem* **278**, 24791-9.
Parent, A., Sato, F., Wu, Y., Gauthier, J., Levesque, M. and Parent, M. (2000). Organization of the basal ganglia: the importance of axonal collateralization. *Trends Neurosci* **23**, S20-7.
Paulsen, R. E., Weaver, C. A., Fahrner, T. J. and Milbrandt, J. (1992). Domains regulating transcriptional activity of the inducible orphan receptor NGFI-B. *J Biol Chem* **267**, 16491-6.
Pazin, M. J. and Kadonaga, J. T. (1997). What's up and down with histone deacetylation and transcription? *Cell* **89**, 325-8.
Pena de Ortiz, S. and Jamieson, G. A., Jr. (1996). HZF-3, an immediate-early orphan receptor homologous to NURR1/NOT: induction upon membrane depolarization and seizures. *Brain Res Mol Brain Res* **38**, 1-13.
Pena de Ortiz, S., Maldonado-Vlaar, C. S. and Carrasquillo, Y. (2000). Hippocampal expression of the orphan nuclear receptor gene hzf-3/nurr1 during spatial discrimination learning. *Neurobiol Learn Mem* **74**, 161-78.
Perlmann, T. and Jansson, L. (1995). A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes Dev* **9**, 769-82.
Perlmann, T., Rangarajan, P. N., Umesono, K. and Evans, R. M. (1993). Determinants for selective RAR and TR recognition of direct repeat HREs. *Genes Dev* **7**, 1411-22.
Perlmann, T. and Vennstrom, B. (1995). The sound of silence. *Nature* **377**, 387-8.
Perrone-Capano, C. and Di Porzio, U. (2000). Genetic and epigenetic control of midbrain dopaminergic neuron development. *Int J Dev Biol* **44**, 679-87.
Pettit, H. O., Ettenberg, A., Bloom, F. E. and Koob, G. F. (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology (Berl)* **84**, 167-73.
Philips, A., Lesage, S., Gingras, R., Maira, M. H., Gauthier, Y., Hugo, P. and Drouin, J. (1997a). Novel dimeric Nur77 signaling

mechanism in endocrine and lymphoid cells. *Mol Cell Biol* **17**, 5946-51.
Philips, A., Maira, M., Mullick, A., Chamberland, M., Lesage, S., Hugo, P. and Drouin, J. (1997b). Antagonism between Nur77 and glucocorticoid receptor for control of transcription. *Mol Cell Biol* **17**, 5952-9.
Phillips, T. J., Belknap, J. K., Buck, K. J. and Cunningham, C. L. (1998a). Genes on mouse chromosomes 2 and 9 determine variation in ethanol consumption. *Mamm Genome* **9**, 936-41.
Phillips, T. J., Brown, K. J., Burkhart-Kasch, S., Wenger, C. D., Kelly, M. A., Rubinstein, M., Grandy, D. K. and Low, M. J. (1998b). Alcohol preference and sensitivity are markedly reduced in mice lacking dopamine D2 receptors. *Nat Neurosci* **1**, 610-5.
Pliego Rivero, F. B., McCormack, W. J., Jauniaux, E., Stern, G. M. and Bradford, H. F. (1999). Forskolin-induced expression of tyrosine hydroxylase in human foetal brain cortex. *Brain Res Dev Brain Res* **114**, 201-6.
Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. et al. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276**, 2045-7.
Ponnio, T., Burton, Q., Pereira, F. A., Wu, D. K. and Conneely, O. M. (2002). The nuclear receptor Nor-1 is essential for proliferation of the semicircular canals of the mouse inner ear. *Mol Cell Biol* **22**, 935-45.
Power, R. F., Lydon, J. P., Conneely, O. M. and O'Malley, B. W. (1991a). Dopamine activation of an orphan of the steroid receptor superfamily. *Science* **252**, 1546-8.
Power, R. F., Mani, S. K., Codina, J., Conneely, O. M. and O'Malley, B. W. (1991b). Dopaminergic and ligand-independent activation of steroid hormone receptors. *Science* **254**, 1636-9.
Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P. and Freedman, L. P. (1999). Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* **398**, 824-8.
Risinger, F. O., Brown, M. M., Doan, A. M. and Oakes, R. A. (1998). Mouse strain differences in oral operant ethanol reinforcement under continuous access conditions. *Alcohol Clin Exp Res* **22**, 677-84.
Rochette-Egly, C. (2003). Nuclear receptors: integration of multiple signalling pathways through phosphorylation. *Cell Signal* **15**, 355-66.
Rossetti, Z. L., Hmaidan, Y. and Gessa, G. L. (1992). Marked inhibition of mesolimbic dopamine release: a common feature of ethanol, morphine, cocaine and amphetamine abstinence in rats. *Eur J Pharmacol* **221**, 227-34.
Rossignol, M., Pouyssegur, J. and Klagsbrun, M. (2003). Characterization of the neuropilin-1 promoter; gene expression is mediated by the transcription factor Sp1. *J Cell Biochem* **88**, 744-57.
Sablin, E. P., Krylova, I. N., Fletterick, R. J. and Ingraham, H. A. (2003). Structural basis for ligand-independent activation of the orphan nuclear receptor LRH-1. *Mol Cell* **11**, 1575-85.
Sacchetti, P., Brownschidle, L. A., Granneman, J. G. and Bannon, M. J. (1999). Characterization of the 5'-flanking region of the human dopamine transporter gene. *Brain Res Mol Brain Res* **74**, 167-74.
Sacchetti, P., Dwornik, H., Formstecher, P., Rachez, C. and Lefebvre, P. (2002). Requirements for heterodimerization between the orphan nuclear receptor Nurr1 and retinoid X receptors. *J Biol Chem* **277**, 35088-96.
Sacchetti, P., Mitchell, T. R., Granneman, J. G. and Bannon, M. J. (2001). Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism. *J Neurochem* **76**, 1565-72.
Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D. and Gage, F. H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* **126**, 4017-26.
Samad, T. A., Krezel, W., Chambon, P. and Borrelli, E. (1997). Regulation of dopaminergic pathways by retinoids: activation of the D2 receptor promoter by members of the retinoic acid receptor-retinoid X receptor family. *Proc Natl Acad Sci U S A* **94**, 14349-54.
Sap, J., de Magistris, L., Stunnenberg, H. and Vennstrom, B. (1990). A major thyroid hormone response element in the third intron of the rat growth hormone gene. *Embo J* **9**, 887-96.
Saucedo-Cardenas, O. and Conneely, O. M. (1996). Comparative distribution of NURR1 and NUR77 nuclear receptors in the mouse central nervous system. *J Mol Neurosci* **7**, 51-63.
Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., De Mayo, F., Burbach, J. P. and Conneely, O. M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic

precursor neurons. *Proc Natl Acad Sci U S A* **95**, 4013-8.
Sawa, A. and Snyder, S. H. (2002). Schizophrenia: diverse approaches to a complex disease. *Science* **296**, 692-5.
Scearce, L. M., Laz, T. M., Hazel, T. G., Lau, L. F. and Taub, R. (1993). RNR-1, a nuclear receptor in the NGFI-B/Nur77 family that is rapidly induced in regenerating liver. *J Biol Chem* **268**, 8855-61.
Schimmel, J. J., Crews, L., Roffler-Tarlov, S. and Chikaraishi, D. M. (1999). 4.5 kb of the rat tyrosine hydroxylase 5' flanking sequence directs tissue specific expression during development and contains consensus sites for multiple transcription factors. *Brain Res Mol Brain Res* **74**, 1-14.
Simon, H. H., Bhatt, L., Gherbassi, D., Sgado, P. and Alberi, L. (2003). Midbrain dopaminergic neurons: determination of their developmental fate by transcription factors. *Ann N Y Acad Sci* **991**, 36-47.
Simon, H. H., Saueressig, H., Wurst, W., Goulding, M. D. and O'Leary, D. D. (2001). Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J Neurosci* **21**, 3126-34.
Smidt, M. P., Asbreuk, C. H., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat Neurosci* **3**, 337-41.
Smidt, M. P., Smits, S. M., Bouwmeester, H., Hamers, F. P., van der Linden, A. J., Hellemons, A. J., Graw, J. and Burbach, J. P. (2004). Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. *Development* **131**, 1145-55.
Smidt, M. P., van Schaick, H. S., Lanctot, C., Tremblay, J. J., Cox, J. J., van der Kleij, A. A., Wolterink, G., Drouin, J. and Burbach, J. P. (1997). A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc Natl Acad Sci U S A* **94**, 13305-10.
Smith, L. and Greenfield, A. (2003). DNA microarrays and development. *Hum Mol Genet* **12 Spec No 1**, R1-8.
Smits, S. M., Ponnio, T., Conneely, O. M., Burbach, J. P. and Smidt, M. P. (2003). Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. *Eur J Neurosci* **18**, 1731-8.
Soker, S. (2001). Neuropilin in the midst of cell migration and retraction. *Int J Biochem Cell Biol* **33**, 433-7.
Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735-45.
Song, K.-W., WangL., Kang, H.-Y., Lee, M.-O. and D.D., M. (2004). The atypical orphan nuclear receptor SHP regulates orphan nuclear receptor Nurr77-mediated CYP17 gene expression in mouse testis. In *Nuclear Receptors: Orphan brothers*, (ed.
Spillantini, M. G. and Goedert, M. (2000). The alpha-synucleinopathies: Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. *Ann N Y Acad Sci* **920**, 16-27.
Spyraki, C., Fibiger, H. C. and Phillips, A. G. (1983). Attenuation of heroin reward in rats by disruption of the mesolimbic dopamine system. *Psychopharmacology (Berl)* **79**, 278-83.
Stehlin-Gaon, C., Willmann, D., Zeyer, D., Sanglier, S., Van Dorsselaer, A., Renaud, J. P., Moras, D. and Schule, R. (2003). All-trans retinoic acid is a ligand for the orphan nuclear receptor ROR beta. *Nat Struct Biol* **10**, 820-5.
Stull, N. D. and Iacovitti, L. (1996). Acidic fibroblast growth factor and catecholamines synergistically up-regulate tyrosine hydroxylase activity in developing and damaged dopamine neurons in culture. *J Neurochem* **67**, 1519-24.
Swarzenski, B. C., O'Malley, K. L. and Todd, R. D. (1996). PTX-sensitive regulation of neurite outgrowth by the dopamine D3 receptor. *Neuroreport* **7**, 573-6.
Takahashi, N., Miner, L. L., Sora, I., Ujike, H., Revay, R. S., Kostic, V., Jackson-Lewis, V., Przedborski, S. and Uhl, G. R. (1997). VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc Natl Acad Sci U S A* **94**, 9938-43.
Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H. and Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* **99**, 59-69.
Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M. et al. (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* **99**, 71-80.

Tetradis, S., Bezouglaia, O., Tsingotjidou, A. and Vila, A. (2001). Regulation of the nuclear orphan receptor Nur77 in bone by parathyroid hormone. *Biochem Biophys Res Commun* **281**, 913-6.
Tordjman, R., Lepelletier, Y., Lemarchandel, V., Cambot, M., Gaulard, P., Hermine, O. and Romeo, P. H. (2002). A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat Immunol* **3**, 477-82.
Torii, T., Kawarai, T., Nakamura, S. and Kawakami, H. (1999). Organization of the human orphan nuclear receptor Nurr1 gene. *Gene* **230**, 225-32.
Tremblay, A., Tremblay, G. B., Labrie, F. and Giguere, V. (1999). Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Mol Cell* **3**, 513-9.
Umesono, K. and Evans, R. M. (1989). Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57**, 1139-46.
van den Munckhof, P., Luk, K. C., Ste-Marie, L., Montgomery, J., Blanchet, P. J., Sadikot, A. F. and Drouin, J. (2003). Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. *Development* **130**, 2535-42.
Velculescu, V. E., Zhang, L., Vogelstein, B. and Kinzler, K. W. (1995). Serial analysis of gene expression. *Science* **270**, 484-7.
Vescovi, A. L., Reynolds, B. A., Fraser, D. D. and Weiss, S. (1993). bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11**, 951-66.
Von Euler, U. S. and Lishajko, F. (1957). Dopamine in mammalian lung and spleen. *Acta Physiol Pharmacol Neerl* **6**, 295-303.
Wagner, J., Akerud, P., Castro, D. S., Holm, P. C., Canals, J. M., Snyder, E. Y., Perlmann, T. and Arenas, E. (1999). Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. *Nat Biotechnol* **17**, 653-9.
Wallen, A. and Perlmann, T. (2003). Transcriptional control of dopamine neuron development. *Ann N Y Acad Sci* **991**, 48-60.
Wallen, A., Zetterstrom, R. H., Solomin, L., Arvidsson, M., Olson, L. and Perlmann, T. (1999). Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. *Exp Cell Res* **253**, 737-46.
Wallen, A. A., Castro, D. S., Zetterstrom, R. H., Karlen, M., Olson, L., Ericson, J. and Perlmann, T. (2001). Orphan nuclear receptor nurr1 is essential for ret expression in midbrain dopamine neurons and in the brain stem. *Mol Cell Neurosci* **18**, 649-63.
Wallen-Mackenzie, A., Mata de Urquiza, A., Petersson, S., Rodriguez, F. J., Friling, S., Wagner, J., Ordentlich, P., Lengqvist, J., Heyman, R. A., Arenas, E. et al. (2003). Nurr1-RXR heterodimers mediate RXR ligand-induced signaling in neuronal cells. *Genes Dev* **17**, 3036-47.
Wang, Y. M., Gainetdinov, R. R., Fumagalli, F., Xu, F., Jones, S. R., Bock, C. B., Miller, G. W., Wightman, R. M. and Caron, M. G. (1997). Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. *Neuron* **19**, 1285-96.
Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N. P. and Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* **423**, 555-60.
Weigel, N. L. and Zhang, Y. (1998). Ligand-independent activation of steroid hormone receptors. *J Mol Med* **76**, 469-79.
Weingarten, P. and Zhou, Q. Y. (2001). Protection of intracellular dopamine cytotoxicity by dopamine disposition and metabolism factors. *J Neurochem* **77**, 776-85.
Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D. and McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res* **20**, 4965-70.
Willis, G. L. and Armstrong, S. M. (1998). Orphan neurones and amine excess: the functional neuropathology of Parkinsonism and neuropsychiatric disease. *Brain Res Brain Res Rev* **27**, 177-242.
Wilson, T. E., Fahrner, T. J., Johnston, M. and Milbrandt, J. (1991). Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* **252**, 1296-300.
Wilson, T. E., Fahrner, T. J. and Milbrandt, J. (1993a). The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Mol Cell Biol* **13**, 5794-804.
Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J. and Parker, K. L. (1993b). The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Mol Cell Biol* **13**, 861-8.

Wilson, T. E., Paulsen, R. E., Padgett, K. A. and Milbrandt, J. (1992). Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. *Science* **256**, 107-10.
Wise, R. A. (1998). Drug-activation of brain reward pathways. *Drug Alcohol Depend* **51**, 13-22.
Wisely, G. B., Miller, A. B., Davis, R. G., Thornquest, A. D., Jr., Johnson, R., Spitzer, T., Sefler, A., Shearer, B., Moore, J. T., Willson, T. M. et al. (2002). Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. *Structure (Camb)* **10**, 1225-34.
Witta, J., Baffi, J. S., Palkovits, M., Mezey, E., Castillo, S. O. and Nikodem, V. M. (2000). Nigrostriatal innervation is preserved in Nurr1-null mice, although dopaminergic neuron precursors are arrested from terminal differentiation. *Brain Res Mol Brain Res* **84**, 67-78.
Woronicz, J. D., Calnan, B., Ngo, V. and Winoto, A. (1994). Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* **367**, 277-81.
Woronicz, J. D., Lina, A., Calnan, B. J., Szychowski, S., Cheng, L. and Winoto, A. (1995). Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis. *Mol Cell Biol* **15**, 6364-76.
Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat Rev Neurosci* **2**, 99-108.
Xiao, Q., Castillo, S. O. and Nikodem, V. M. (1996). Distribution of messenger RNAs for the orphan nuclear receptors Nurr1 and Nur77 (NGFI-B) in adult rat brain using in situ hybridization. *Neuroscience* **75**, 221-30.
Xing, G., Zhang, L., Heynen, T., Li, X. L., Smith, M. A., Weiss, S. R., Feldman, A. N., Detera-Wadleigh, S., Chuang, D. M. and Post, R. M. (1997). Rat nurr1 is prominently expressed in perirhinal cortex, and differentially induced in the hippocampal dentate gyrus by electroconvulsive vs. kindled seizures. *Brain Res Mol Brain Res* **47**, 251-61.
Xu, P. Y., Liang, R., Jankovic, J., Hunter, C., Zeng, Y. X., Ashizawa, T., Lai, D. and Le, W. D. (2002). Association of homozygous 7048G7049 variant in the intron six of Nurr1 gene with Parkinson's disease. *Neurology* **58**, 881-4.
Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-66.
Zetterstrom, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-50.
Zetterstrom, R. H., Solomin, L., Mitsiadis, T., Olson, L. and Perlmann, T. (1996a). Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. *Mol Endocrinol* **10**, 1656-66.
Zetterstrom, R. H., Williams, R., Perlmann, T. and Olson, L. (1996b). Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. *Brain Res Mol Brain Res* **41**, 111-20.
Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W. and Elledge, S. J. (1999). p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev* **13**, 213-24.
Zheng, K., Heydari, B. and Simon, D. K. (2003). A common NURR1 polymorphism associated with Parkinson disease and diffuse Lewy body disease. *Arch Neurol* **60**, 722-5.
Zhou, J., Bradford, H. F. and Stern, G. M. (1996). Induction of dopaminergic neurotransmitter phenotype in rat embryonic cerebrocortex by the synergistic action of neurotrophins and dopamine. *Eur J Neurosci* **8**, 2328-39.
Zhou, J., Pliego-Rivero, B., Bradford, H. F., Stern, G. M. and Jauniaux, E. R. (1998). Induction of tyrosine hydroxylase gene expression in human foetal cerebral cortex. *Neurosci Lett* **252**, 215-7.
Zhou, Q. Y. and Palmiter, R. D. (1995). Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. *Cell* **83**, 1197-209.
Zhou, Q. Y., Quaife, C. J. and Palmiter, R. D. (1995). Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. *Nature* **374**, 640-3.
Zindy, F., Cunningham, J. J., Sherr, C. J., Jogal, S., Smeyne, R. J. and Roussel, M. F. (1999). Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. *Proc Natl Acad Sci U S A* **96**, 13462-7.

Zito, K. A., Vickers, G. and Roberts, D. C. (1985). Disruption of cocaine and heroin self-administration following kainic acid lesions of the nucleus accumbens. *Pharmacol Biochem Behav* **23**, 1029-36.