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European Journal of Pharmacology 405 (2000) 55–62

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Genetic pathways in the developmental specification of hypothalamic neuropeptide and midbrain catecholamine systems

J. Peter H. Burbach *

Section of Molecular Neuroscience, Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, University Medical Center, Universiteitsweg 100, 3584 CG Utrecht, Netherlands

Accepted 28 June 2000

Abstract

The neuropeptide concept concerns the diverse and broad physiological functions of neuropeptides in behavioral adaptation. Neuropeptides like vasopressin and corticotropin-releasing hormone can coordinate multiple brain functions due to the anatomical organization of the neurons producing them. The cell bodies are focally positioned in the hypothalamus and send long-reaching efferents to limbic and brainstem areas. Likewise, midbrain dopamine systems coordinate emotional behaviors and movement control by specific connectivity of neurons in the midbrain to limbic and striatal centers, respectively. The fundament of the functions of these signalling molecules is laid out during development when transmitter identity and connectivity are specified. This is a highly controlled process involving multiple transcription factors and growth factors acting together in genetic pathways. Here, the genetic pathways enrolling in developing vasopressin, corticotropin-releasing hormone, and midbrain dopamine neurons are discussed.

Keywords: Neuropeptide; Vasopressin; Corticotropin-releasing hormone; Dopamine; Homeobox gene; Transcription factors; Development

1. Introduction

From the neuropeptide concept, we learned that neuropeptides are produced by specialized, peptidergic neurons, and after processing and release, act as modulators of integrated brain functions (De Wied, 1987; De Wied and Burbach, 1999). Likewise, they are involved in complex behaviors such as learning and memory processes and adaptation. Neuropeptide systems like the vasopressin, pro-opiomelanocortin (POMC) and corticotropin-releasing hormone systems are anatomically positioned to coordinately control a wide array of brain activity: they are expressed by a limited set of clustered neurons in the hypothalamus from which they send out projections to limbic and brainstem areas. They come into play when the organism is challenged to adapt its physiological and behavioral activities to changes in the environment, and often do so by modulating ongoing neurotransmission (De Wied, 1969; De Wied et al., 1993).

Classically, neurotransmitters are the chemical messengers propagating electrical signals from one neuron to the other. Their activity is essential for basal functioning of the nervous system. While many neurotransmitter systems act locally in brain structures, others act over a long range and connect different brain structures. The dopamine neurons of the midbrain are exemplary for the latter. They consist of two functionally different systems: one connecting midbrain dopamine neurons of the substantia nigra to striatal motor centers, the other consisting of midbrain dopamine neurons in the ventral tegmental area and innervating limbic forebrain structures. Consequently, these systems function in the control of movement and emotional behaviors, respectively. Common to neuropeptide system, such as the vasopressin and corticotropin-releasing hormone systems, and the midbrain dopamine systems is anatomical connectivity from a focal center in the brain to specific target neurons over a long distance. This connectivity is

* Corresponding author. Tel.: +31-30-253-8848; fax: +31-30-253-8859, +31-30-253-9032.

E-mail address: j.p.h.burbach@med.uu.nl (J.P.H. Burbach).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00541-0

essential for the organizing role that their secreted products can exert. Both the identities of the product, a neuropeptide or a neurotransmitter, and the connectivity are established during development. In particular, during terminal differentiation, neurons are set-up for functions during maturity as an endpoint of genetic pathways. These pathways generally involve specific transcription factors, which drive programs of gene expression.

With the identification of transcription factors operating in specific sets of neuropeptide or neurotransmitter neurons, insight is being obtained about the gene expression programs that drive the development of all properties of neurons to function appropriately (reference). Applied to vasopressin, corticotropin-releasing hormone and dopamine neurons, the identification of genetic pathways of development and the associated gene expression programs provide perspectives to the biological functioning, plasticity, regulation and maintenance of these important systems. Here, the current state of research in this direction is presented. This chapter focuses on transcription factors and their developmental functions, highlighting neurons that are fundamental to the neuropeptide concept, and neurons that were among the first classical neurotransmitter system.

2. Development of hypothalamic neuropeptide-expressing neurons

The neurohypophysial hormones vasopressin and oxytocin have contributed importantly to the neuropeptide concept (De Wied, 1969, 1987; De Wied et al., 1993; De Wied and Burbach, 1999). Originally, the hypothalamo–neurohypophysial system was considered as the only site of their synthesis and secretion. The hypothalamo–neurohypophysial system consists of magnocellular neurons grouped in the supraoptic and paraventricular nucleus which mainly supply these hormones to the peripheral blood circulation via release from nerve endings in the posterior pituitary gland. The peptides released from the hypothalamo–neurohypophysial system act on peripheral target cells and should be considered canonically as "hormones". We now know that additional parvocellular vasopressin neurons have different functions. Vasopressin produced by parvocellular vasopressin/corticotropin-releasing hormone neurons in the paraventricular nucleus is the key to the control of release of POMC peptides from the anterior pituitary gland. It is this corticotropin-releasing activity of vasopressin that initiated the discoveries on the behavioral functions of vasopressin (De Wied, 1965, 1969). Neurons in the suprachiasmatic nucleus, amygdala and bed nucleus of the stria terminalis also produce vasopressin, but deliver the peptide exclusively to other brain structures. Thus, there is a clear differentiation of peripheral vs. central functions of vasopressin which originates from the hypothalamo–neurohypophysial system and parvocellular neurons, respectively. This difference is reflected in the requirement of vasopressin gene sequences for cell-specific expression and in regulation in these different systems. Still, there are similarities in genetic pathways that control development of magnocellular neurons and parvocellular vasopressin/corticotropin-releasing hormone-expressing neurons of the paraventricular nucleus, suggesting that they share a common origin, and perhaps, a physiological significance.

The present ideas about the mechanisms underlying developmental origin and differentiation of the hypothalamo–neurohypophysial system follow the general notion that neurons are committed early on by an interaction between endogenous gene expression programs and extracellular signals provided by growth factors and contact with neighboring cells. These forces also mediate migratory direction and axonal pathfinding. While anatomical descriptions of the developing hypothalamo–neurohypophysial system are scarce, details on the developmental events enrolling in magnocellular neurons have recently contributed by analyses of mice in which specific transcription factors were inactivated. These now provide insights in molecular mechanisms of magnocellular neuron development.

Magnocellular neurons have been traced back to embryonic day (E) 10.5 using [^{3}H]thymidine incorporation and histochemical methods. Magnocellular neuron progenitor cells arise in the neuroepithelium of the ventricular region around E 10.5 in the mouse and E 12.5 in the rat (Altman and Bayer, 1978, 1986; Karim and Sloper, 1980; Okamura et al., 1983; Nakai et al., 1995). They migrate laterally, and can be detected at E 11.5 in the anterior hypothalamus of the mouse as a cluster of cells separated from the ventricular surface. Two streams of cells migrate from this cluster towards the hypothalamus, one following a lateral–ventral direction which will form the supraoptic nucleus, and the other following the midline which will form the paraventricular nucleus. Migration takes place between E 10.5 and 14.5, whereafter, axonal extensions are formed. These fibers start to innervate the neurohypophysis from E 14.5 onwards in the mouse (Korteweg, 2000). As early as E 15.5, vasopressin and oxytocin transcripts are present in different groups of neurons. Oxytocin expression is seen first in the paraventricular nucleus at E 15.5 and in the supraoptic nucleus at E 18.5 (Jing et al., 1998).

This description of the birth of vasopressin and oxytocin magnocellular neurons implies an early commitment of neuroblasts to become magnocellular neurons, specific migratory events to hypothalamic end stations, and the induction of one of the two peptide genes, vasopressin or oxytocin, respectively. Recently, insights in the genetic pathways driving these developmental events have been obtained by the identification of transcription factor genes expressed in these neurons, and by the analysis of the phenotypes of mice in which these genes were inactivated.

These transcription factors are the homeobox genes *Brn2* and *Otp*, and the basic-helix-loop-helix-PAS (bHLH-PAS) genes *Sim1* and *Arnt2*.

The POU-homeobox gene *Brn2* was the first gene that caused a phenotype specific for hypothalamic neuropeptide neurons, when inactivated. It now appears that this phenotype is shared with other genes that are upstream of *Brn2*, and converge on this gene.The null mutation of *Brn2* in the mouse results in a defect in migration of early magnocellular neurons around E 12.5 (Schonemann et al., 1995; Nakai et al., 1995). These $Brn2^{-/-}$ neurons are eventually lost during development, and as a consequence, no magnocellular neurons are present at birth and no hypothalamo–neurohypophysial system has been established. Very similar phenotypes have been obtained for mouse null mutants in which *Sim1*, *Arnt2* or *Otp* was inactivated (Michaud et al., 1998, 2000; Acampora et al., 1999), again caused by early defect of magnocellular neurons which fail to migrate normally. Based on analysis of *Brn2* expression in these *Otp* null mutants, it has been proposed that the bHLSH-PAS genes, *Sim1* and *Arnt2* are required for induction or maintenance of expression of *Brn2*. These two genes cooperate through physical interaction of their gene products (Crews and Fan, 1999). The *Sim1* and *Arnt2* proteins need to dimerize in order to act on target genes. The *Brn2* gene is assumed to be one such target gene. The homeobox gene *Otp* acts parallel to *Sim1–Arnt2* on the maintenance of *Brn2*. In addition, *Otp* already functions at the level of neuroblast proliferation (Acampora et al., 1999). The data can be summarised in a genetic pathway operating in developing magnocellular neurons as proposed by Acampora et al. (1999) (Fig. 1).

Surprisingly, not only magnocellular neurons require these transcription factors, but also parvocellular corticotropin-releasing hormone/vasopressin neurons of the paraventricular nucleus. They display the same requirement for these four transcription factor genes. Other peptidergic hypothalamic neurons, i.c. TRH and somatostatin neurons, require one or more of the genes in a different mode (Fig. 1). This suggests that there are common mechanisms in the development of the vasopressin magnocellular neuron, the oxytocin magnocellular neuron, and the corticotropin-releasing hormone/vasopressin parvocellular paraventricular nucleus neuron, and that these neurons share a certain relatedness. It also indicates that the terminal differentiation into the final type of neurons and the induction of the neuropeptide gene require additional mechanisms. The relatedness of the two magnocellular types is also illustrated in adulthood, by the overlap in expression of vasopressin and oxytocin by magnocellular neurons. In the lactation period, a proportion of magnocellular neurons acquires the expression of both the vasopressin and oxytocin genes (Mezey et al., 1987). The data suggest that magnocellular neurons are derived from the same neuroblasts, however, direct evidence for a common progenitor cell is lacking. This is partly due to the fact that the discriminating property, namely, expression of the vasopressin, oxytocin or corticotropin-releasing hormone gene, is only visible relatively late in development, i.e. after E 14.5. It has been demonstrated that at E 16.5, vasopressin and oxytocin transcripts exist in separate populations of neurons (Jing et al., 1998). The observation that a single cluster of calbindin D-28K positive cells is present in the anterior hypothalamus (Nakai et al., 1995) suggests that a common progenitor may exist.

The functional cooperation of *Brn2*, *Sim1*, *Arnt2* and *Otp* during development may be retained in the control of gene expression in the mature neurons. *Otp* and *Brn2* are expressed in adult magnocellular neurons and parvocellular corticotropin-releasing hormone/vasopressin neurons; *Sim1* and *Arnt2* have not been investigated. The putatively reduced level of *Brn2* expression in heterozygous $Brn2^{+/-}$ mice correlates with a reduced basal expression of vasopressin and oxytocin mRNAs. It should also be noted that no change in *Brn2* occurred in a stress model that altered the enhanced expression of corticotropin-releasing hormone (Kovacs and Sawchenko, 1996). Thus, the roles of *Brn2*, *Otp*, *Sim1* and *Arnt2* in the control of adult neuropeptide gene expression in magnocellular neurons and vasopressin/corticotropin-releasing hormone neurons need to be further defined. In particular, the interactions between the transcription factors themselves may contribute unique properties to these cells. For example, we found that *Brn2* and *Otp* can each weakly stimulate promoter activity of the corticotropin-releasing hormone gene in an appropriate neuronal cell context, but that the presence of the two factors together leads to synergistic stimulation (Asbreuk et al., unpublished). Perhaps the dosage of these factors, possibly in combination with additional genes, can also play a role in the selection of the neuropeptide genes to be expressed in the terminal phase of differentiation.

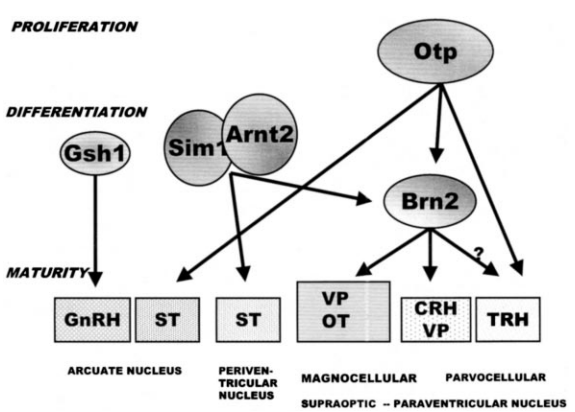

Fig. 1. Genetic pathways in the development of peptidergic neurons of the neuroendocrine hypothalamus. The involvement and hierarchy of transcription factors have been derived from null mutants (Acampora et al., 1999; Michaud et al., 1998, 2000; Nakai et al., 1995; Schonemann et al., 1995).

3. Induction and differentiation of midbrain dopamine neurons

Because of the implications in mental and neurological disorders, dopamine belongs to the most intensively studied neurotransmitters of the brain. dopamine systems have been the main target of neuropharmacology for almost 40 years. The dramatic neurological consequences of degeneration of midbrain dopamine neurons in Parkinson's disease highlights the functions of this dopamine system in the control of motor behavior at the level of the striatum (Barbeau et al., 1962). The antipsychotic effects of dopamine receptor antagonists have pointed to the midbrain dopamine systems as a potential cause of affective and emotional disorders, but proof for a "dopamine hypothesis of schizophrenia" is weak (Janssen and Niemegeers, 1959; Wagner et al., 1966; Meltzer and Stahl, 1976). Still, the midbrain dopamine system is an essential component in emotional and reward behaviors, and developmental disorders in these behaviors may involve the midbrain dopamine neurons. Because of these two types of biological functions, midbrain dopamine systems are still subject of studies that aim to delineate the fundamental neurobiology of dopamine neurons. These are concerned with development, cell-specific gene expression and regulation, molecular pharmacology of dopamine receptors and transporters, and with genetic association of dopamine-related genes and mental disorders, e.g. dopamine receptor variants and linkage to schizophrenia. The genetic programs of specification of midbrain dopamine neurons, i.c. the question of which genes and gene cascades determine the dopaminergic identity of these neurons, and their specific connectivity in mesencephalic-striatal and -limbic pathways, may open new therapeutic strategies in dopamine-associated disorders.

The development of midbrain dopamine neurons is largely dependent on the proper development of the midbrain–hindbrain border that precedes the induction of midbrain dopamine neurons. Genes involved in the boundary formation will have effects on the emergence of midbrain dopamine neurons. The first midbrain dopamine neurons arise at the most ventral rim of the neuroepithelium lining up along the mesencephalic flexure of the ventral midbrain, where the expression of the growth factors Fgf8 and Shh interact (Ye et al., 1998). Before the expression of dopamine-specific markers, ventral midbrain markers are present in these cells. Among the earliest markers of the region are *En1*, *En2*, *Wnt1*, *Pax2* and *Pax5*. The expression of *En1* and *En2* is maintained by the expression of the signalling molecule *Wnt1* (Danielian and McMahon, 1996). Recently, another homeobox family member was implicated in the early specification of the ventral midbrain. This factor, *Lmx1b*, is expressed at E 7.5 in the ventral mes- and diencephalon and remains expressed in the adult in brain structures derived from these areas including midbrain dopamine neurons (Smidt et al., 2000).

The first specific signs of the birth of midbrain dopamine neurons shortly follow the induction of the orphan nuclear hormone receptor *Nurr1* (E 10.5), although the expression pattern is not restricted to these neurons and extends in a large field in the mesencephalon and diencephalon (Zetterstrom et al., 1996a,b, 1997). Shortly after *Nurr1*, expression of the key enzyme in dopamine synthesis tyrosine hydroxylase is initiated and completed (E 12.5) (Smidt et al., 1997; Saucedo-Cardenas et al., 1998). This initiation is parallel to the induction of the midbrain dopamine-specific homeobox gene *Ptx3* (Smidt et al., 1997). In short, one can describe the course of developmental decisions leading to mes dopamine neurons in the following stages (Fig. 2).

(I) dopamine progenitor cells are defined by the intersection actions of Fgf8 and Shh.

(II) The genes *En1*, *En2*, *Wnt1*, *Pax2* and *Pax5* are involved in the induction of Shh and are probably necessary in the differentiation programme of the midbrain dopamine progenitor cells together with the recently identified player *Lmx1b*.

(III) Just before the dopamine phenotype emerges *Nurr1* is activated.

(IV) With the induction of tyrosine hydroxylase, which requires *Nurr1*, and of *Ptx3*, the midbrain dopamine neurons are born. At present, no later emerging developmental proteins have been identified.

(V) Finally, midbrain dopamine neurons acquire the complete molecular make-up required for dopamine neurotransmission, and for establishment of appropriate connectivity with efferent and afferent neurons.

Around stage II, dopamine progenitor cells stop proliferating and enter into a terminal differentiation programme. This transition is a milestone in the birth of midbrain dopamine neurons. Therefore, recent studies on factors potentially involved in terminal differentiation are highlighted below.

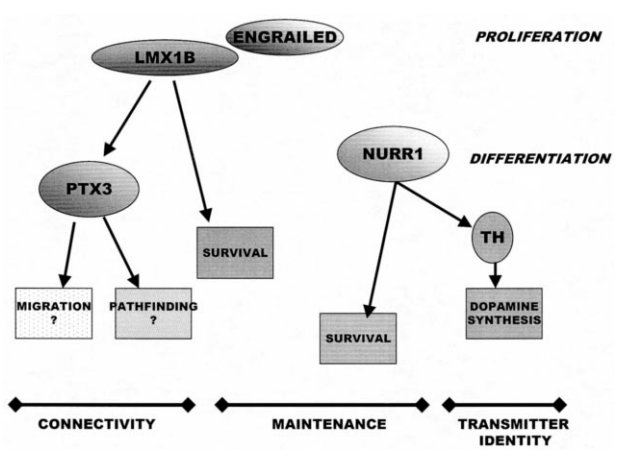

Fig. 2. Genetic pathways in the development of midbrain dopamine systems. The involvement and hierarchy of transcription factors have been derived from null mutants (Saucedo-Cardenas et al., 1998; Smidt et al., 2000; Zetterstrom et al., 1997). Speculatively, the cellular functions in which genetic pathways participate are indicated.

We entered the field of terminal differentiation of midbrain dopamine neurons by searching for homeobox genes expressed in the adult ventral midbrain, reasoning that factors exerting functions during late development are often employed in the mature state as well. This concept is well illustrated by the development of the anterior pituitary gland (Dasen and Rosenfeld, 1999; McEvilly and Rosenfeld, 1999). *Ptx3* was identified and characterized. Midbrain dopamine neurons are the only neurons that express the homeobox gene *Ptx3*. Such exclusivity is unique in the brain and suggests that *Ptx3* serves a function unique to midbrain dopamine neurons, but hitherto unknown.

In situ hybridization on mouse embryos from E 8.5 to 16.5 showed the correlation of *Ptx3* expression with the development of midbrain dopamine neurons. At E 11.5, a small layer at the ventral surface of the mesencephalic flexure expressed *Ptx3*. At E 12.5, a complete field of tyrosine hydroxylase-positive, *Ptx3*-positive neurons has been obtained. These *Ptx3*-positive cells are restricted to the marginal layer of the mesencephalic tegmentum. This group of about 50 cells corresponds to the first tyrosine hydroxylase-expressing cells in the developing rodent brain. At later stages, the expression remains restricted to the midbrain dopamine system and this association is conserved in adult rat brain. This has been demonstrated by 6-hydroxy dopamine lesions and by analysis of human substantia nigra tissue. Extraneural *Ptx3* expression is found in the developing skeletal muscle, in the tongue and in the developing eye lens (Semina et al., 1997; Smidt et al., 1997).

3.1. Nurr1 and the dopaminergic phenotype in Nurr1 null mutants

Nurr1 is an orphan member of the orphan nuclear hormone receptor superfamily of transcription factors (Law et al., 1992). It is expressed in several unrelated regions of the central nervous system (Xiao et al., 1996; Saucedo-Cardenas and Conneely, 1996). It has a relatively wide field of expression in the embryonic midbrain, covering the entire ventral region. Only a small proportion of *Nurr1*-expressing neurons overlap with the midbrain dopamine progenitor neurons. Thus, its brain expression is not uniquely linked to midbrain dopamine neurons as in the case of *Ptx3* (Zetterstrom et al., 1996a,b). The onset of expression in midbrain dopamine neurons is at E 10.5 in the mouse, just before the induction of the dopamine markers tyrosine hydroxylase and *Ptx3* (E 11.5) (Zetterstrom et al., 1996a,b, 1997). The expression of *Nurr1* is maintained in the adult stage, albeit in a more limited pattern, but including the midbrain dopamine system (Saucedo-Cardenas and Conneely, 1996; Xiao et al., 1996; Backman et al., 1999). The Nurr1 protein has therefore functions during development and in the mature brain. This protein being a nuclear orphan receptor, is of special pharmacological interest since interference of its functions might be achieved if a selective ligand can be found. The function of *Nurr1* in the brain has been addressed by the creation of null-mutant mice. Mice with a targeted deletion of the *Nurr1* gene develop until gestation but die soon after birth. Analysis of the brain of these animals showed that in the newborn animals, no midbrain dopamine neurons could be detected by using markers as Adh2, cRet and tyrosine hydroxylase (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Castillo et al., 1998). Although it was initially concluded that the absence of *Nurr1* causes "agenesis" of midbrain dopamine neurons (Zetterstrom et al., 1997), several additional studies demonstrated a more refined role of *Nurr1* in midbrain dopamine neurons. These neurons in E 12.5 *Nurr1* null mutants fail to induce tyrosine hydroxylase, but express all other markers investigated, including HNF 3β, cRet, Adh2, *Ptx3* and *Lmx1b*. Thus, it appears that the midbrain dopamine neurons are initially generated and that the only marker that is initially missing is tyrosine hydroxylase. The developmental program of midbrain dopamine neurons does proceed in the *Nurr1*$^{-/-}$ animals as illustrated by the proper induction of the midbrain dopamine specific homeobox gene *Ptx3* (Saucedo-Cardenas et al., 1998) and maintenance of *Lmx1b* (Smidt et al., 2000). The perinatal survival of these *Nurr1*$^{-/-}$ midbrain dopamine neurons is not entirely clear, since conflicting findings exist (Saucedo-Cardenas et al., 1998; Le et al., 1999; Wallen et al., 1999). Whether these cells are lost gradually or maintained post-natally may depend on secondary influences, such as genetic background of *Nurr1* mutants.

The specificity of the phenotype is remarkable since *Otp* dopaminergic neurons that express *Nurr1* are not affected by the ablation of *Nurr1* (Saucedo-Cardenas et al., 1998; Baffi et al., 1999; Le et al., 1999). It has been tentatively speculated that the specificity of the phenotype to the midbrain dopamine neurons lies in the fact that there might be cross-talk between *Nurr1* and the midbrain dopamine specific homeodomain gene *Ptx3*. Such direct interactions have been described for the nuclear hormone receptor *Ftz*-F1 and the homeodomain gene *Ftz* (Yu et al., 1997). Recent studies in cultured neuronal stem cells showed that *Nurr1* can enhance the number of tyrosine hydroxylase-positive cells, but there was no effect of *Ptx3* in the absence nor presence of *Nurr1* (Sakurada et al., 1999).

We speculate that the degeneration of the midbrain dopamine neurons in *Nurr1*$^{-/-}$ mice is not only due to the fact that there is no dopamine synthesis due to the lack of tyrosine hydroxylase, but that *Otp* functions of *Nurr1* in specific survival of these neurons are involved. Possibly, the *Nurr1*$^{-/-}$ midbrain dopamine neurons cannot form or maintain the connections to their targets and therefore, the neurons degenerate as is found after transection of the projecting axons. The absence of tyrosine hydroxylase alone is probably not sufficient for the severe neuronal loss that is found in *Nurr1*$^{-/-}$ mice, since

tyrosine hydroxylase mice still form projections to the striatum (Zhou and Palmiter, 1995).

Alternatively, *Nurr1* may not directly control the induction of tyrosine hydroxylase, but rather function parallel in a switch from proliferating stem cells to non-proliferating differentiating cells and be more fundamental in the control of the cell cycle. In midbrain dopamine neurons, tyrosine hydroxylase seems to be expressed only in differentiating neurons and mark the transition in the cell cycle. In any case, it is intriguing to know why *Nurr1* displays this marked function in the development of midbrain dopamine neurons, and not in the many other neuronal populations in which it is expressed. Vice versa, olfactory, diencephalic and adrenal dopamine cells do not involve *Nurr1* at all. This unique role of *Nurr1* to midbrain dopamine neurons is likely linked to other specific aspects of the developmental cascades in midbrain dopamine neurons.

3.2. Lmx1b and the dopamine phenotype of Lmx1b null mutants

Lmx1b is a member of the LIM (annotated according to the initial three factors with common protein motif) homeodomain family and is known to be an essential regulator of dorso-ventral patterning of the developing limbs and mutations evoke the nail patella syndrome (Dreyer et al., 1998; Chen et al., 1998).We identified a restricted expression pattern of *Lmx1b* in the brain characterized by high expression in the substantia nigra and ventral tegmental area. Neural *Lmx1b* expression starts already at E 7.5 (Johnson and Chen, personal communication) and is maintained in the adult substantia nigra and ventral tegmental area (Smidt et al., 2000). Early developmental brain expression is extending anterior of the midbrain dopamine region into the ventral hypothalamic area. More posterior, *Lmx1b* expression shifts to dorsal in the hindbrain and extents to the dorsal part of the spinal cord. This expression shift to dorsal seems to coincide with the position of the midbrain–hindbrain border (unpublished; Adams et al., 2000). In the ventral midbrain, *Lmx1b* is expressed in midbrain dopamine neurons. This midbrain dopamine expression of *Lmx1b* also exists in the human melanin-containing substantia nigra dopaminergic neurons (Smidt et al., 2000). The intrinsic potential of the homeobox gene *Lmx1b* as a developmental regulator together with the fact that *Lmx1b* is expressed earlier than *Nurr1*, tyrosine hydroxylase and *Ptx3*, suggests that *Lmx1b* may act genetically as an upstream activator of these genes, and may be involved in preparing the region for genesis and differentiation of the midbrain dopamine system. To validate this hypothesis, we analysed expression of tyrosine hydroxylase, *Ptx3* and *Nurr1* in brain sections of homozygous *Lmx1b* knock out mice (Chen et al., 1998) of stage E 12.5, when the field of expression of tyrosine hydroxylase and *Ptx3* is first complete. *Lmx1b*$^{-/-}$ embryos contained tyrosine hydroxylase-positive cells in the ventral tegmentum, but these cells did not express *Ptx3*, indicating that *Ptx3* is not necessary for tyrosine hydroxylase expression.

Further support for the conclusion that tyrosine hydroxylase-positive cells can develop independent of *Ptx3*, are gain-of-function experiments in vitro and in vivo. It has been shown that *Ptx3* does not alter the number of tyrosine hydroxylase-positive cells in neural stem cell cultures, but *Nurr1* does (Sakurada et al., 1999). Furthermore, ectopic expression of *Ptx3* in the dorsal midbrain driven by the *En2* promoter did not result in the induction of dorsalized tyrosine hydroxylase- or *Nurr1*-positive neurons (Rosenthal and Burbach, unpublished).

As mentioned above, *Nurr1* is expressed widely in the midbrain overlapping with the midbrain dopamine neurons. Notably, in the tyrosine hydroxylase-expressing cells in the ventral tegmentum of the *Lmx1b* knock out animals, the expression of *Nurr1* was not affected. The data obtained from *Nurr1* and *Lmx1b* null mutants lead to the differentiation of two genetic pathways that operate in parallel in developing midbrain dopamine neurons (Fig. 2). One pathway links *Nurr1* to the induction of tyrosine hydroxylase. The function of this pathway is to specify the identity of the neurotransmitter that will be employed by these neurons. The other pathway positions *Lmx1b* upstream of *Ptx3*. Disruption of this pathway by deletion of *Lmx1b* leads to rapid loss of tyrosine hydroxylase-positive neurons after E 12.5, but it is uncertain if the same effect will be obtained when *Ptx3* is inactivated. Humans heterozygous for a mutation in the *Ptx3* suffer from cataract and anterior segment mesenchymal dysgenesis (ASMD), in line with early eye expression of *Ptx3* (Semina et al., 1998), but have no apparent neurological abnormalities. This may indicate that the contribution of *Ptx3* in midbrain dopamine neurons is recessive. Its characteristics of brain expression are remarkable, but its function remains to be elucidated.

4. Concluding remarks

One reason why the neuropeptides vasopressin and corticotropin-releasing hormone can play such important and diverse roles in behavioral and endocrine adaptation, is the way the neurons producing them are organized. Characteristic for both neuropeptides is the focal origin in a limited set of hypothalamic neurons, and the wide, long-range innervation of different limbic and brainstem areas, and connections to the blood circulation. In this aspect, they resemble long-range neurotransmitter systems, like the midbrain dopamine systems that can anatomically be distinguished in functionally different systems. Developmental decision in neurons about the products to employ as chemical signal and about the target structures to innervate, are driven by genetic pathways involving transcription factors. These factors do not operate autonomously,

but are in constant interplay with signal from neighboring cells. In midbrain dopamine systems, different genetic pathways operate in parallel, likely serving the specification of different aspects of the dopamine neurons. Here, we still lack the factors that detail if the dopamine neuron will be a substantia nigra participating in motor control or a ventral tegmental area neuron participating in emotional behaviors. Surprising about hypothalamic vasopressin, oxytocin and corticotropin-releasing hormone neurons is that they share a genetic pathway for migration and developmental maintenance. If this pathway is also involved in the terminal selection process of neuropeptide gene induction and connectivity, or that this terminal process is independent remains to be elucidated. In either case, additional factors must still be involved. Knowing these will help understand the mechanisms by which neuropeptides can exert their modulating functions in complex behaviors, such as learning, memory, and adaptation. The neuropeptide concept has originally posed such questions and will stimulate to do so in the future.

Acknowledgements

I would like to thank my colleagues who have contributed data mentioned in this review. Mrs. Ria van Vlaardingen is gratefully acknowledged for her help in preparing the manuscript.

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