From the DEPARTMENT OF NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

FROM STEM CELLS TO NEURONS: A BMPy RIDE

Therese Andersson

Karolinska Institutet

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ABSTRACT

Pinpointing the mechanisms behind the formation of the vertebrate development has been an intriguing and challenging task for researchers for several decades. In the 1980s two independent groups managed to isolate cells from the inner cell mass of a mouse blastocyst and to culture and expand them *in vitro*, while maintaining their pluripotency. The field of research based on these embryonic stem cells (ESCs) has since exploded and they have turned out to be valuable tools for *in vitro* studies of developmental pathways and cell fate specification. In the future, this research might lead to the use of the human counterparts of these cells as a source for cell replacement therapies. More recently the isolation of neural stem cells (NSCs) from the fetal and adult brain also made it possible to study later events of neural specification *in vitro*. Unlike embryonic stem cells however, these NSCs have potency restriction, being multipotent as a result of cell specialization during development.

In this thesis, I have studied extrinsic and intrinsic factors that can promote neuronal fates from ESCs and fetal NSCs. In Parkinson's disease, midbrain dopamine neurons progressively degenerate. Using a rodent model for this neurodegenerative disease, we show that implantation of ESCs at low doses efficiently generate functional neurons that integrate with the host tissue and relieve the parkinsonian symptoms. We further demonstrate that transgene overexpression of Nurr1, a transcription factor known to be important for maturation and maintenance of dopamine neurons, significantly increase the yield of dopamine neurons after *in vitro* differentiation. Furthermore, these cells produce and secrete dopamine as a response to depolarization. Together, these studies show potential for ESCs as a source for cell replacement therapies.

Bone morphogenetic protein (BMP) 4 is a secreted factor and a member of the TGF-β superfamily. BMP4 has been shown to inhibit neural cell fates at early stages of embryonic development. After neural specification, BMP4 promotes astrocytic while inhibiting oligodendrocytic cell fates, an activity by BMP4 also seen in NSCs. Furthermore, the differentiation downstream of BMP4 in NSCs is dependent on the cell density, inducing mesenchymal differentiation only at low plating densities. We show that in NSCs BMP4 dramatically increases the expression of its own antagonist, noggin. Futhermore we show that the increase in noggin expression as a consequence of BMP stimulation results in the plating density effects on mesenchymal differentiation. We further show that BMP4 is sufficient for neuronal differentiation at high cell densities but that co-stimulation with Wnt3a is required for efficient generation of AMPA responsive neurons.

Finally, we show that BMP4 causes a significant increase in the expression of CxxC5, a putative inhibitor of Wnt-signalling in NSCs. We demonstrate that CxxC5 modulates Wnt-signalling in NSCs and that the expression pattern is overlapping with both BMP4 and Wnt3a in the developing telencephalon. Our results postulate a novel point of interaction between BMP and Wnt signalling.

LIST OF PUBLICATIONS

I. Björklund LM, Sanchez-Pernaute R, Chung S, **Andersson T**, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Kim KS, Isacson O.
Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model.
Proc Natl Acad Sci USA, 2002, 99, 2344-2349.

II. Chung S, Sonntag KC, **Andersson T**, Björklund LM, Park JJ; Kim DW, Kang UJ, Isacson O, Kim KS.
Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons.
Eur J Neuroscience, 2002, 20, 139-145.

III. **Andersson T**, Duckworth JK, Fritz N, Södersten E, Uhlén P, Hermanson O.
Noggin and Wnt3a enable BMP4-dependent differentiation of telencephalic stem cells into GluR-agonist responsive neurons.
Submitted.

IV. **Andersson T**, Södersten E, Duckworth JK, Bryja V, Hermanson O.
CxxC5 is a novel BMP4-regulated modulator of Wnt-signaling in neural stem cells.
Submitted.

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LIST OF ABBREVIATIONS

AADC	Aromatic L-amino acid decarboxylase
BMP	Bone morphogenetic protein
CNS	Central nervous system
DA	Dopamine
DAT	Dopamine transporter
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
GFAP	Gamma-aminobutyric acidGlial fibrillary acidic protein
MAP2	Microtubule associated protein 2
MBP	Myelin basic protein
NSC	Neural stem cell
SMA	α-Smooth muscle actin
SHH	Sonic hedgehog
TGF-β	Transforming growth factor β
TH	Tyrosine hydroxylase

1 INTRODUCTION

1.1 NEURAL INDUCTION AND SPECIFICATION

1.1.1 Identifying the organizer and its 'shortcomings'

Development consists of a series of cell divisions and other events, starting with the unicellular fertilized egg (zygote) and ending with the mature adult organism. The developing organisms have always puzzled researchers: what are the mechanisms steering the cell fate decision of progenitor cells, into the appropriate phenotype at the right time and at the right place? Neurulation begins with the thickening of the ectoderm, forming the neural plate. Starting at the anterior end of the embryo, the lateral edges of the neural plate roll upwards and fuse into the neural tube. Meanwhile, cell proliferation at the posterior end of the embryo elongates the trunk and neural plate. The anterior end partitions into several vesicles representing the future prosencephalon (forebrain), mesencephalon (midbrain) and rhomencephalon (hindbrain), while the posterior end forms the spinal cord.

One groundbreaking experiment in the understanding of neural induction was carried out by Hans Spemann and his student Hilde Mangold in the early 1920s, where they transplanted a piece from one particular part of an amphibian embryo, at the gastrula stage, to another part of a second embryo. They showed that the grafted tissue had the ability to induce neural differentiation of the host tissue, which would otherwise give rise to non-neural tissue (Spemann and Mangold, 1924). They named this part of the embryo "the organizer", defined by its ability to "exert an organizing effect on its environment". These results led to the concept of "neural induction", and the task of identifying the molecules actually exerting this induction started.

Since the identification of the Spemann and Mangold organizer, the concept of organizing regions has been further validated through the identification of regions with similar functions in avian (Hensen's node/avian organizer) and mammals (node) (Beddington, 1994; Waddington, 1932). However, there are several reports deviating from the traditional model where the organizer is needed and sufficient for induction of neural tissue. Beddington showed that although the node is sufficient to induce a secondary axis in mammals, this axis is truncated lacking the most rostral part (Beddington, 1994). One crucial difference between amphibian (and fish) from avian and mammal embryogenesis is the lack of extra embryonic tissue (amnion/yolk sac) in amphibians. Furthermore, while mammals and avians are born/hatched in a relative fully developed physical form, amphibians are hatched as tadpoles and go through metamorphosis to develop into the adult animal. Extra embryonic tissue has been shown to induce forebrain development in chick providing an organizer-independent neural inducing pathway (Eyal-Giladi, 1970). As mentioned above, the mouse node is not sufficient to induce a complete secondary axis. In concordance with this, it was also reported that the anterior visceral endoderm (AVE) is necessary for the expression of the key transcription factor Hesx1 in the prospective forebrain (Thomas and Beddington, 1996).

Together these reports suggest that rather than depending only on the organizer, the development of the central nervous system requires the cooperation of several different regions. Thus, while the traditional model of neurulation primarily is based on amphibian development, one has to take into consideration the species at hand when studying neural induction and development.

1.1.2 Identification of the molecular mediators of neural induction

Amphibian embryos consist of an animal and a vegetal part, where the animal part develops into ectoderm and mesoderm and the vegetal part into endoderm. One pioneering study in 1969 showed that the mesoderm was formed from the animal part only when it was in contact with the vegetal part (Nieuwkoop, 1969). Fibroblast growth factor (FGF) synergized with another extrinsic soluble molecule, transforming growth factor β (TGF-β) to induce mesoderm (Kimelman and Kirschner, 1987; Slack et al., 1987). A few years later TGF-β was also shown to be able to induce mesoderm in mammals (Smith et al., 1990).

During that time, it was also shown that disruption of the normal cell-cell interactions by dissociation of epidermal tissue *in vitro* led to the expression of neural markers (Grunz and Tacke, 1989). Delaying the reaggregation for 5 hours led exclusively to neuronal differentiation (Grunz and Tacke, 1989). Shortly thereafter, the soluble polypeptides noggin and later chordin were identified as a neuralizing factors secreted from the organizer (Smith and Harland, 1992) and the proof that this neuralizing effect could be inhibited by bone morphogenetic protein (BMP) 4 (Piccolo et al., 1996; Zimmerman et al., 1996). Together with the finding that inhibition of the activin II receptor, receptor for the BMPs, led to neural differentiation in the absence of the notochord (Hemmati-Brivanlou and Melton, 1994), these reports transformed the view of the role of the organizer, from being a producer of factors that directly induced neural differentiation, to rather being a source of factors that will inhibit other alternative differentiation pathways. It has later been shown that in Xenopus, the anterior, but not the posterior, neural ectoderm is specified at the blastula stage, i.e. before the formation of the organizer, through a noggin and chordin dependent mechanism, and further that FGF signalling at the blastula stage is required for subsequent neural differentiation (Kuroda et al., 2004; Streit et al., 2000).

Apart from neural induction, a central question when trying to understand the formation of the nervous system is: what instructs a developing neural cell to form in a particular place at a specific time? In 1954 Nieuwkoop published a description of a complementary model of neural induction. He proposed that the induction of the nervous system takes place in two steps: firstly the initial induction of the forebrain and subsequently the caudalization of part of it (Nieuwkoop and Nigtevecht, 1954). Intrinsic factors have an important role in this process. In the late 1970s/early 1980s, a gene cluster of homeotic genes, later called the Hox genes, were identified (Lewis, 1978; McGinnis et al., 1984; Scott and Weiner, 1984). The various combinations of these genes turned out to define the spatial-specific differentiation and the expression pattern along the anterior-posterior axis (Lewis, 1978; McGinnis et al., 1984; Scott and Weiner, 1984).

According to the Nieuwkoop model, both neurulation and subsequent CNS regionalization and cell type specification in the CNS are induced and regulated by the organizer. Therefore, many of the identified factors related to neural induction continue to play important roles in these processes (see Fig. 1 for expression pattern). Morphogens are diffusible proteins that form a gradient and subsequently stimulate the development in an organism in a concentration dependent manner. The identification and proof of principle of the first morphogen, Biocoid (*Bcd*), was done by the Nüsslein-Vollhard lab using the fruit fly as a model organism showing that transplantation of wild-type anterior cytoplasm rescued the *Bcd* knockout phenotype (Frohnhöfer et al., 1986). A few years later the protein identity and gradual expression pattern was also reported (Driever and Nüsslein-Volhard, 1988a; Driever and Nüsslein-Volhard, 1988b). A model for the chemistry behind morphogenesis was postulated already in the early 1950s by Turing, using the first computer simulation in biology (Turing, 1952). This was later modified by Gierer and Meinhardt who showed that the combination of local self-enhancement must be coupled with longer-range inhibition for pattern formation (Gierer and Meinhardt, 1972). In the early 1990s it was shown that the dorso-ventral patterning was controlled by signals from the notochord and the floor plate, later identified as a morphogenic signalling via Sonic hedgehog (Echelard et al., 1993; Krauss et al., 1993; Yamada et al., 1991). As we will see below, extracellular molecules as Wnts and BMPs have also important roles in these processes. The expression patterns of various secreted factors are shown in Fig. 1. It was further shown in Drosophila that gurken-torpedo signalling is involved in the polarization of both major body axes (dorso-ventral and anterior-posterior) (Gonzalez-Reyes et al., 1995) and that the TGF-β molecules, lefty and nodal, regulate the left-right asymmetry (Levin et al., 1995; Meno et al., 1996).

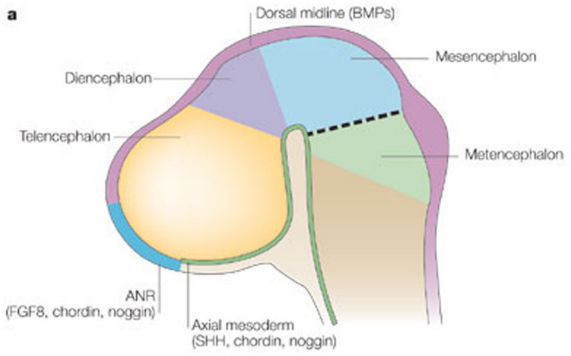

Fig. 1 **A schematic picture displaying regions that express soluble factors.** BMPs are expressed in the dorsal midline and FGF8 in the anterior neural ridge (ANR) and Shh in the axial mesoderm and the BMP antagonists Chordin and Noggin are expressed both in the ANR and axial mesoderm. *Adapted from Liu and Niswander (Liu and Niswander, 2005).*

1.2 WNT SIGNALLING IN THE DEVELOPING NERVOUS SYSTEM

The Wnt signalling pathway is conserved between many species and has been shown to be of importance in many developmental and other cellular processes. Wnt ligands are extracellular proteins, which prior to secretion, are modified by glycosylation and palmityolation, making them hydrophobic and prone to localize close to the membranes (Wainwright et al., 1988; Willert et al., 2003). Wnt signalling is triggered by the binding of a Wnt ligand to a Frizzled receptor located in the cell membrane. To date, nineteen Wnt ligands and ten Frizzled receptors have been identified in mammals (Nusse, 2005). Classically, three different intracellular signalling pathways are described downstream of Wnt: 1) The canonical pathway that signals through β-

catenin, 2) Planar cell polarity and 3) the calcium (Ca^{2+})-pathway. Examples of Wnts that primarily signal through the canonical pathway are Wnt3a and Wnt8c, while Wnt5a and Wnt11 are traditionally classified as a non-canonical Wnts. However, in some cellular systems, the classical classification of downstream Wnt signalling pathways might not be as straightforward.

Wnts are expressed in the developing nervous system in overlapping and complementary patterns (Parr et al., 1993). Canonical Wnt signalling blocks the early neural inducing activity of FGFs, via inhibition of BMP signalling. Non-canonical Wnts do not exert this effect (Wilson et al., 2001). Following the formation of the neural tube, canonical Wnts participate in the anterior-posterior patterning of the developing neural tube. Inhibition of canonical Wnts is important for early patterning of forebrain and diencephalon (Satoh et al., 2004). At later stages however, Wnts exert a neural promoting effect, as shown by the lack of hippocampal formation in the absence of Wnt3a (Lee et al., 2000a). This is further demonstrated through the interplay between FGFs, BMPs and Wnts in cortical patterning (Shimogori et al., 2004). Lately, Wnts have been shown to be important for neuronal differentiation, both in the CNS (Castelo-Branco et al., 2003) and the PNS (Lee et al., 2004).

1.3 BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β superfamily. BMP activity was first identified in 1965 for its role in the autoinduction of bone formation (Urist, 1965). It took two decades however before the proteins responsible for the induction were identified (Luyten et al., 1989; Wozney, 1992; Wozney et al., 1988). To date the BMP family has grown to over twenty members that, based on homology and evolutionary considerations, can be divided into at least five subgroups (Mehler et al., 1997). Apart from bone formation, BMPs display a regulatory role in several other developmental processes, including cell survival, morphogenesis, lineage restrictions and commitment, proliferation and apoptosis (Graham et al., 1996; Massagué, 1996; Tanabe and Jessell, 1996). TGF-β ligands seem to be conserved between invertebrates and vertebrates, consistent with an evolutionary important role during development (Rusten et al., 2002).

BMPs are synthesized as large precursors, which are cleaved by proteolytic enzymes. The active form can consist of a homo- or heterodimer of two related BMPs. Signals from BMPs are mediated by a subgroup of serine/threonine kinase receptors belonging to the TGF-β receptor family. The receptors consist of a complex between one type I and one type II receptor. Binding of the ligand to the receptor complex induces a phosphorylation of the type I receptor mediated by the type II receptor. Subsequently the signal is passed on to receptor activated Smad proteins (Smad1,-5 & -8) which in turn will bind to a common mediator, Smad4 (Chen et al., 1997; Hoodless et al., 1996; Nishimura et al., 1998). The complex is translocated to the nucleus and acts as a transcription regulator.

The activity of BMP is regulated at the extracellular level by antagonists, like chordin and noggin (Piccolo et al., 1996; Zimmerman et al., 1996), and at an intracellular level

by receptor inactivation or by interference with the Smad complex. Smad6 binds to the type I receptor and prevent the binding and phosphorylation of Smad1 and 5 (Hata et al., 1998; Imamura et al., 1997), while Smad ubiquitin regulatory factor 1 (Smurf1) interacts with Smad1 and 5 and mediates the degradation of these proteins (Zhu et al., 1999).

BMPs are highly expressed in the dorsal midline and have been reported to play important roles in the patterning of the nervous system. Active BMP signalling has been demonstrated to play a critical role in patterning the dorsal midline and to be involved in regulation of gene expression, cell proliferation and apoptosis (Furuta et al., 1997; Hérbert et al., 2003; Hérbert et al., 2002). There is also evidence that BMPs exert crosstalks with other major signalling pathways like Notch and Wnt (Dahlqvist et al., 2003; Ille et al., 2006; Machold et al., 2007).

1.3.1 BMP4 and noggin in the developing nervous system

BMP4 null mutant embryos die between E6.5 and E9.5 with severe malformations (Winnier et al., 1995). Most embryos do not develop beyond E6.5 and have very little or no mesoderm (Winnier et al., 1995). It thus appears that in the developing embryo the first distinct role for BMP4 is the induction of mesoderm. At the late gastrula stage BMP4 is expressed in the ectoderm and is responsible for the induction of epidermis (Hemmati-Brivanlou and Melton, 1997; Tanabe and Jessell, 1996; Wilson and Hemmati-Brivanlou, 1995; Winnier et al., 1995). Transgenic BMP4 overexpression enhances astrocytic differentition and inhibits the generation of oligodendrocytes (Gomes et al., 2003). In the early developing brain *Bmp4* is expressed in the most anterior dorsal neuroectoderm and the expression is maintained in the dorsal forebrain (Furuta et al., 1997).

Noggin is a secreted polypeptide that binds BMP2, - 4 and -7 with high affinity and thus prevent the BMP from binding to its receptor (Zimmerman et al., 1996). Noggin was first identified as a dorsalizing RNA expressed in the vegetal regions that will become the organizer in Xenopus (Smith and Harland, 1992). The balance between noggin and BMP4 is important for normal development. Although noggin is considered to be a neuralizing factor in Xenopus and has been suggested to be important for adult neurogenesis in mice (Lim et al., 2000), there are also reports showing that noggin blocks neurogenesis in the developing neocortex (Li and Loturco, 2000). This suggests that the noggin/BMP balance functions in a context dependent manner.

1.4 STEM CELLS

A “stem cell” is defined as an immature cell that has the ability to self-renew as well as to differentiate into several different cell types. Over the last three decades stem cells with diverse properties and from many different tissues have been isolated e.g. neural crest stem cells (Lo and Anderson, 1995; Stemple and Anderson, 1992), embryonic stem cells (blastocyst) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998) and fetal and adult neural stem cells (Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Reynolds and Weiss, 1992; Temple, 1989); Johe, 1996}.

1.4.1 Embryonic stem cells

Embryonic stem cells (ESCs) are isolated from the inner cell mass of the pre-implantation stage embryo, the blastula. The first reports demonstrating successful isolation and *in vitro* maintenance of mouse ESCs came in 1981 from two independent groups (Evans and Kaufman, 1981; Martin, 1981). ESCs are pluripotent cells that can be maintained as such or be differentiated into most cell types in the embryo (Desbaillets et al., 2000; Nagy et al., 1990; Nagy et al., 1993). Furthermore, the isolation of ESCs provided a novel foundation for the generation of genetic models and knockout mice (Nagy et al., 1990; Nagy et al.).

The differentiation of ESCs can be steered either through the exposure to exogenous factors or by genetic manipulation to alter the expression of intrinsic factors, such as transcription factors. The versatile differentiation potential inbuilt in ESCs together with the ability of generating significant amounts of these cells, pinpoints their potential as an alternative cell source for cell replacement therapies. For therapeutic applications focused on neurodegenerative diseases, such as Parkinson's disease (see 1.6), it is important to understand how different factors affect the generation of dopamine neurons from ESCs. To date, there are several protocols differentiating ESCs into neural tissues and specifically into dopamine neurons (Kawasaki et al., 2000; Lee et al., 2000b; Ying et al., 2003).

1.4.2 Neural stem cells

In the developing CNS, a population of immature cells lining the ventriciles of the neural tube give rise to diverse popul(Wilson and Hemmati-Brivanlou, 1995)ations of neurons, astrocytes and oligodendrocytes (see 1.5.2). These cells are called neural "stem cells" (NSCs). Neural stem cells (NSCs) have been isolated both from the fetal and adult central nervous system (Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Reynolds and Weiss, 1992; Temple, 1989); Johe, 1996}. Only a subpopulation of the isolated cells are actual stem cells and a common method to estimate stem cell properties is to culture the cells as suspension cultures and let them form floating cell aggregates, the so called neurospheres assay (Reynolds and Rietze, 2005). NSCs can be grown in both adherent and neurosphere cultures and notably, the estimation of the number of actual stem cells in culture varies a lot between adherent and neurosphere cultures (Reynolds and Rietze, 2005; Temple, 2001).

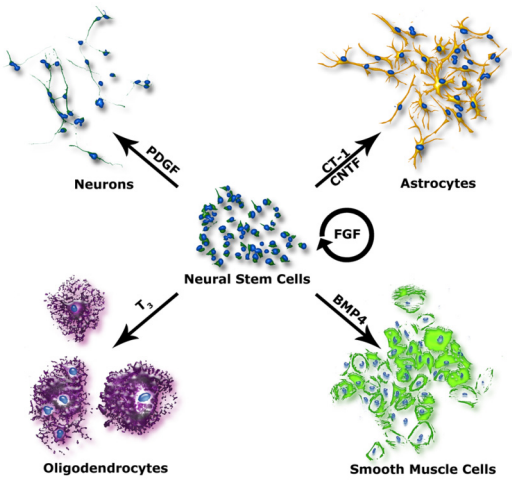

Fig. 2.
NSCs differentiate selectively in response to extrinsic factors. Platelet derived growth factor (PDGF) cause neuronal differentiation, cilial neurotrophic factor (CNTF) and cardiotropin 1 (CT-1) induce astrocytic differentiation, thyroide hormone 3 (T3) gives rise to oligodendrocytes and BMP4 generate mesenchyme while FGF keeps the NSCs at a undifferentiated, proliferative state.
Adapted from Teixeira et al., 2007

Telencephalic fetal NSCs can be isolated from the anterior neural tube and expanded as adherent cultures *in vitro* in the presence of FGF2 (Hermanson et al., 2002; Johe et al., 1996). Exposure to single factors like CNTF/CT-1, PDGF, T_3 and BMP4 can induce differentiation into various cell fates (Fig. 2). Exposure of these neural stem cells (NSCs) to fetal bovine serum (FBS) or BMP4 induces a density dependent differentiation into a variety of fates including mesenchyme, astrocytes, and neurons (Gajavelli et al., 2004; Rajan et al., 2003; Tsai and McKay, 2000) (Duckworth *et al*, manuscript). Mesenchyme is absent at higher densities, while astrocytic differentiation is supported both at high and low plating densities, although the astrocytes have a markedly changed morphology (Rajan et al., 2003; Tsai and McKay, 2000). In addition, BMP4 has been shown to signal through Frap-STAT and SMAD signalling pathways at high and low plating density respectively. Mesenchymal differentiation is dependent on SMAD signalling whilst astrocytic differentiation is dependent on the Frap-STAT pathway (Rajan et al., 2003). It has previously been proposed that BMPs (BMP6 & 7 and GDF5 & 6) induce neural crest stem cell differentiation programmes in NSCs (Gajavelli et al., 2004). In addition to this, recent data suggests that the differentiation programme downstream of BMP4 mimics the formation of choroid plexus, providing an alternative CNS-related differentiation programme for mesenchymal differentiation (Duckworth *et al*, manuscript). Furthermore, our results show that NSCs plated at high density differentiate into AMPA responsive neurons when exposed to BMP4 and Wnt3a, supporting previous findings that show a co-operation between BMP and Wnts during neuronal differentiation in the hippocampus (Shimogori et al., 2004). Mesenchymal differentiation downstream of BMP4 was shown to depend on active Notch signalling (Dahlqvist et al., 2003) (Duckworth *et al*, manuscript), further displaying the importance of cellular context for appropriate BMP signalling response. The density dependent cell fates downstream of BMP4 are shown in Fig. 3.

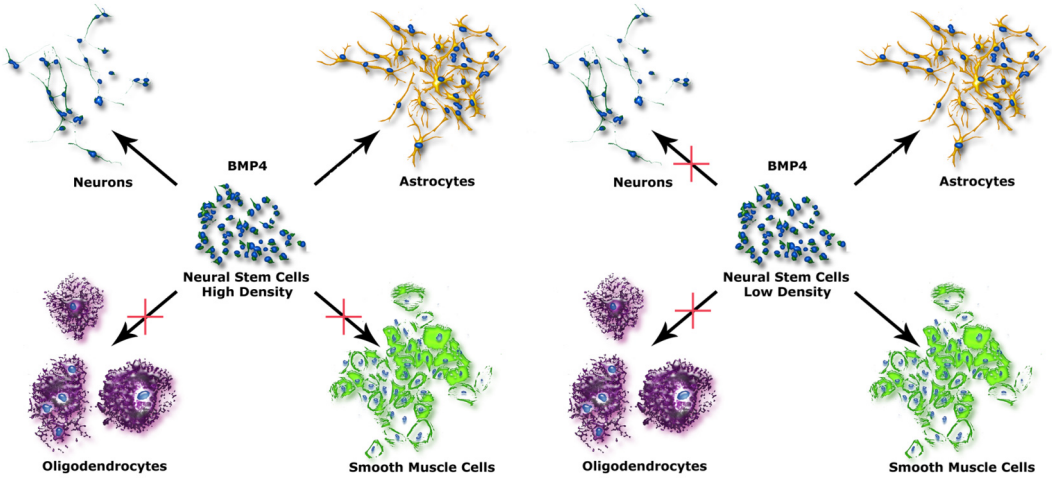

Fig. 3. BMP4 causes a density dependent differentiation, giving rise to neurons and astrocytes at high plating densities and mesenchyme and astrocytes at low. Oligodendrocytic differentation is always blocked by BMP4. *Modified from (Teixeira et al., 2007).*

1.5 PARKINSON'S DISEASE

Parkinson's disease is one of the most common diseases affecting the elderly population. The main motor symptoms consist of tremor, rigidity, bradykinesia and postural instability. In patients with Parkinson's disease, one of the main affected neuronal populations are the dopaminergic neurons located in the ventral mesencephalon projecting to the dorsal striatum. This population progressively degenerates depleting the striatum of dopamine. Traditionally, Parkinson's disease is treated with L-dopa, a dopamine precursor that is metabolised to dopamine in the body. As the disease progresses however, the patients frequently develop dyskinesias associated with the dosages of L-dopa used. There are also surgical alternatives including deep brain stimulation, pallidotomy and cell replacement therapies.

1.5.1 Cell replacement therapies

Cell replacement therapies seek to replace degenerated cells with an external cell source. For Parkinson's disease, transplantations using dopaminergic tissue from human fetal ventral mesencephalon (VM) have demonstrated the capacity for cell mediated recovery in grafted patients (Kordower et al., 1996; Lindvall et al., 1988; Mendez et al., 2000; Piccini et al., 1999; Piccini et al., 2000). Although the development of dyskinesias in some patients has been reported, postulating unregulated production of dopamine from grafted fetal neurons (Freed et al., 2001), these cells display a similar auto-inhibition mechanism of spontaneous DA-release in the response to apomorphine as has been reported in "normal" circuits (Strecker et al., 1987; Zetterström and Ungerstedt, 1984). It should be noted that the procedures for cell preparation and transplantation differ between labs and that these procedures, together with the cell sources, remain the most debated issue to date.

1.6 MIDBRAIN DOPAMINE NEURONS AND NURR1

Characterization of the localization of monoamines (such as dopamine) in the lower brain stem was reported in the mid 1960s (Dahlström and Fuxe, 1964). In the midbrain there are three dopamine neurons nuclei: A8, A9 and A10 (Dahlström and Fuxe, 1964). A9 projects to the dorsal striatum and is the population that is most affected by Parkinson's disease.

The induction of dopaminergic neurons is regulated by a balance of FGF8, Shh and contact mediated signals (Hynes et al., 1995a; Hynes et al., 1995b; Wang et al., 1995). Nurr1 is a transcription factor that belongs to the steroid-thyroid hormone receptor superfamily of transcription factors and is essential for specification and maintenance of dopaminergic neurons in the ventral mesencephalon but not for other dopaminergic neurons (Castillo et al., 1998; Le et al., 1999; Saucedo-Cardenas et al., 1998; Wallén et al., 1999; Zetterström et al., 1997). Nurr1 has been reported to directly activate TH gene expression in a cell dependent manner (Kim et al., 2003; Sakurada et al., 1999). Furthermore, Nurr1 overexpression in mouse postnatal cerebellum NSCs (C17.2) co-cultured with midbrain-derived astrocytes efficiently induces differentiation into dopaminergic neurons (Wagner et al., 1999).

2 AIMS

1) Seek to optimize the use of embryonic stem cells (ESCs) for cell replacement therapies for Parkinson's disease by:
    a. decreasing cell number in grafted material to restrict cell autonomous inhibition of the differentiation into neurons.

    b. increasing the ESCs capability to differentiate into dopaminergic neurons by Nurr1-overexpression.

2) Further the understanding of BMP4-signaling in neural differentiation by:
    a. identifying novel signalling targets and studying their function in neural stem cells (NSCs).

    b. studying how BMP4 mediates differentiation of NSCs to multiple cell fates in a cell density dependent manner.

3 RESULTS AND DISCUSSION

3.1 MOUSE ESCs DIFFERENTIATE INTO FUNCTIONAL DOPAMINE NEURONS AFTER GRAFTING TO RODENTS

It has previously been shown that mouse embryonic stem cells (ESCs) differentiate into neurons when transplanted into the kidney capsule of mice (Deacon et al., 1998). Furthermore previous reports suggest that when extrinsic signals, like BMPs, are blocked, embryonic ectoderm differentiate into neurons (Tanabe and Jessell, 1996; Wilson and Hemmati-Brivanlou, 1995; Winnier et al., 1995). Assuming that ESCs follow an intrinsic "default pathway" to differentiate into neurons, we hypothesised that low numbers of ESCs would differentiate into neurons more efficiently due to the decreased intra-graft cell-cell interactions.

Implantation of low doses of ESCs, cultured to form embryoid bodies for 4 days, to the striatum of Parkinsonian rodents led to differentiation into dopaminergic neurons and integration with the host brain. To characterize the grafts we quantified tyrosine hydroxylase (TH) immunoreactive cells and showed that from 1000-2000 implanted cells, we got in an average ~2000 TH+ cells/graft. Further characterization showed that the TH+ cells colocalized with several other markers and did not express dopamine β–hydroxylase confirming that they were dopaminergic neurons rather than noradrenergic neurons. Some TH+ neurons expressed calbindin, a marker for A10, and some expressed the aldehyde dehydrogenase 2 (AHD2), which is primarily expressed in A9 dopamine neurons. All TH+ cells expressed calretinin, which is co-expressed with TH in the midbrain (both A9 and A10). Several serotoninergic neurons were also found in the grafts (about half as many as the dopaminergic neurons).

Grafted animals were scored according to the rotational response to amphetamine before and after transplantation. After 7 weeks, grafted animals showed a significant decrease in the number of rotations compared to sham controls and they continued improving for the last rotational analysis done at 9 weeks post transplantation. Further, the normality of the grafts was assessed using positron emission tomography (PET) and functional magnetic resonance imaging (MRI) showing that cells in the grafts bound the specific dopamine transporter ligand [^{11}C]CFT and did respond to amphetamine in a similar manner to normal dopamine neurons.

3.2 NURR1 EXPRESSION IN ESCs CAN INCREASE THE YIELD OF DOPAMINERGIC NEURONS AFTER *IN VITRO* DIFFERENTIATION

Dopaminergic neurons can be produced *in vitro* from growth factor expanded embryonic day 12 (E12) ventral mesencephalic (VM) precursor cells (Studer et al., 1998) or embryonic stem cells (Kawasaki et al., 2000; Lee et al., 2000b). Furthermore, Nurr1 transfected progenitor clones cultured with midbrain type 1 astrocytes also differentiate into dopaminergic neurons (Wagner et al., 1999). In addition, Nurr1 has been shown to be critical for the induction and maintenance of midbrain dopaminergic neurons (Zetterström et al., 1997). Therefore, we hypothesized that overexpression of Nurr1 in embryonic stem cells would increase the yield of dopaminergic neurons after

in vitro differentiation. We stably transfected ESCs with a plasmid with the open reading frame of Nurr1 under the control of the elongation factor 1 promoter, a promoter that has been shown to maintain a stable expression throughout the *in vitro* differentiation procedure (Chung et al., 2002). The plasmid also encoded the neomycin resistance gene for selection of transfected cells. After expanding the transfected ESCs plated at low density we picked several colonies for further expansion and subsequent analysis of the Nurr1 mRNA expression. Two clones with significant Nurr1 expression levels were used for subsequent *in vitro* differentiation following a modified version of the protocol proposed by Lee *et al* (Lee et al., 2000b).

In contrast to the study presented by Wagner *et al* (Wagner et al., 1999) we showed that the Nurr1 expression is stably maintained throughout the differentiation procedure. Compared to controls, Nurr1-overexpression increased the mRNA expression of all dopaminergic neurons markers tested. Quantifications of β-tubulin immunoreactive cells showed a maintained amount of neurons, while the TH/β-tubulin ratio showed a significant increase in TH+ neurons. Addition of known enhancer of dopaminergic differentiation, Shh, FGF8 and Ascorbic Acid, to the media during the last stage of differentiation lead to a synergistic increase in the amount of TH+ neurons, showing that Nurr1 cooperate with known enhancers of dopamine neuron differentiation. To further characterize the TH+ neurons we looked for the expression of other dopaminergic markers, showing that most TH+ neurons co-expressed markers for dopaminergic neurons, excluding noradrenergic neurons. Meanwhile, Nurr1 overexpression did not seem to affect other neuronal or astrocytic marker as assessed by immunocytochemistry. We further showed that, in response to potassium chloride induced depolarization, the obtained neurons did release dopamine into the media and that the concentration of dopamine in the Nurr1-overexpressing cultures was significantly higher than that in the control cultures.

Previous reports contrastingly showed that on one hand, Nurr1 acts directly on the TH-promoter inducing TH expression (Sakurada et al., 1999) while, on the other, that Nurr1-overexpression is not enough for the differentiation into TH+-neurons (Wagner et al., 1999). Our results suggest that Nurr1 overexpression in ESCs is enough to generate robust amounts of TH+ neurons. Further studies regarding Nurr1's effect on the TH-expression showed that Nurr1 does indeed binds directly to the TH-promoter, although in a cell dependent manner (Kim et al., 2003). Further analysis of the Nurr1 overexpressing ESCs clones revealed efficient differentiation into functional midbrain dopaminergic neurons also when differentiated on PA6 stromal cells as well as an ability to develop into integrated TH+ neurons when transplanted (Chung et al., 2005).

3.3 BMP4 INDUCES A DIFFERENTIAL GENE EXPRESSION IN NEURAL STEM CELLS ALREADY AFTER 3 HOURS

To identify novel targets of BMP signalling we exposed rat embryonic NSCs to BMP4 for 3 and 10 hours and then used microarray analysis to measure changes in mRNA expression profile from BMP-stimulated cells to that of cells kept in the presence of FGF2. Analysis using the R and bioconductor method LIMMA revealed multiple potential targets. Already after 3 hours, 162 spots showed a putative differential expression differing more than 1.5 fold from the FGF2 control. We selected several

genes to validate the microarray using quantitative RT-PCR and showed that about 90% of the genes were regulated as assessed by the microarray, although to much larger magnitudes.

BMP4 is known to inhibit oligodendrocytic differentiation and in a concordance with this Olig1 as well as Id2 and Id4, known mediators of this effect, were upregulated. We further identified the known Notch target gene Hey1, as well as the known BMP4 inhibitor, noggin, and a novel gene CxxC5.

3.4 BMP4 INDUCES THE EXPRESSION OF CxxC5, A NOVEL WNT-SIGNALING MODULATOR, IN NSCs

CxxC5 is a homologue to the known Wnt-inhibitor Idax (CxxC4). Idax has been shown to regulate Wnt-signalling by binding to and inhibiting the Wnt-signaling mediator Dishevelled (Hino et al., 2001; Michiue et al., 2004). Analysis of the differential expression downstream of BMP4 identified CxxC5 as a potential BMP4 target and further validation showed that CxxC5 was up-regulated downstream of BMP4 independently of protein synthesis. Further, we showed that CxxC5 indeed binds to Dishevelled, a mediator of Wnt-signalling. Idax has been shown to be located in the cytoplasm. Contrasting, CxxC5 was located in the nucleus and as well as the cytoplasm, though mainly in spots close to the nucleus. Co-expression of Dishevelled and CxxC5, led to co-localization in these spots as assessed by confocal and epifluorescent microscopy. These results indicate that CxxC5 might function as a modulator of Wnt signalling in a similar manner to that of Idax. However, the differences in subcellular localization between Idax and CxxC5 suggest that the role of CxxC5 could be different from that of Idax.

In situ hybridization revealed that the expression pattern for CxxC5 is overlapping with both BMPs and Wnts. Overexpressing CxxC5 in NSCs decreased the response to Wnt stimulation as assess by the expression of the Wnt-target gene Axin-2. Furthermore, exposure to BMP4 decrease the Axin2 levels with ~40% within 3hrs. However, the cellular response of NSCs exposed to BMP4 for 90 min before addition of Wnt3a varied between biological replicas. Axin-2 levels were either increased or decreased by pre-treatment of BMP4 (data not shown). Further the variation in response to Wnts depended on the plating density of the NSCs. Together these results showed that the cellular context is of utmost importance for the response and the interplay between BMPs and Wnts.

The co-dependence of BMPs and Wnts for normal development of the telencephalon as has been shown in several independent studies (Shimogori et al., 2004). The mechanism for this dependence however has not yet been identified. Further studies will reveal if CxxC5 (and/or Idax) can function as mediators of the interplay between BMPs and Wnts.

3.5 BMP4 INDUCED NOGGIN MODULATES THE DENSITY DEPENDENT DIFFERENTIATION DOWNSTREAM OF BMP4 IN NSCs

BMP4 induces a density dependent differentiation of NSCs, inducing mesenchymal differentiation at low plating densities (LD) and astrocytic differentiation at both low and high plating densities (Tsai and McKay, 2000). Several studies have focused on the cell fate choice between mesenchyme and astrocytes, implicating cell-cell interactions (Tsai and McKay, 2000), and the activation of different signalling pathways downstream of BMP4 (Rajan et al., 2003). Thus far, there are no reports on the role of secreted factors, nor further characterization of the cell fates obtained in high density (HD) cultures after BMP4 stimulation

Under normal culture conditions, NSCs stimulated with BMP4 do not differentiate to mesenchyme at HD as assessed by the absence of smooth muscle actin (SMA) immunoreactive cells. However, decreasing the concentration of any secreted factors by growing cells in increased media volume, while maintaining the BMP4 concentration, caused the reappearance of some SMA+ cells. This experiment indicates that cell-cell interaction is not enough for the inhibition of mesenchymal differentiation. To further assess if BMP4 stimulated HD cultures secrete factors that will influence the differentiation of NSCs, conditioned media from BMP4 stimulated HD cultures were collected every 24 hours and used to culture low density (LD) cultures. Under these conditions, conditioned media supported astrocytic but not mesenchymal differentiation..

Going back to the gene list identified from the microarray experiment (see above), noggin was identified as one of only two factors annotated to the extracellular space using gene ontology (GOTree) that were up-regulated already after 3 hours of BMP4 exposure. Noggin is a known BMP inhibitor binding with high affinity to BMP4 and prevent the ligand/receptor interaction (Zimmerman et al., 1996). Noggin turned out to be strongly up-regulated independently of protein synthesis, and further to be present in the cell culture media of BMP4 stimulated NSCs in higher concentration at HD then at LD.

Hypothesising that the BMP4 induced noggin-expression participates in the inhibition of mesenchymal differentiation in BMP4 stimulated HD cultures we sought to remove noggin from the media collected at the time for media change (every 48 hours). Depletion of noggin from the conditioned media collected from BMP4 stimulated HD cultures increases BMP4 dependent differentiation when used to culture LD cultures. Most notably there was an increase in the total amount of SMA+ cells/well comparing noggin depleted conditioned media compared to control conditioned media. Therefore unlike the previous experiment where conditioned media from HD cultures only supported astrocytic differentiation in LD cultures, depleting conditioned media of noggin supported astrocytic as well as mesenchymal differentiation. This supports a role for noggin in the inhibition of mesenchymal differentiation of HD NSCs stimulated with BMP4.

Not surprisingly, mesenchymal differentiation was completely blocked when noggin was added alone or together with BMP4, mimicking spontaneous differentiation under control conditions (FGF2 withdrawal/no addition of mitogen).

3.6 BMP4 INCREASES NEURONAL DIFFERENTIATION IN NSCs BUT NEEDS WNT3A TO EFFICIENTLY GENERATE AMPA RESPONSIVE NEURONS

Noggin is traditionally considered to be a neurogenic factor and has been shown to antagonize BMPs in the sub-ventricular zone and thus allowing adult neurogenesis (Lim et al., 2000; Smith and Harland, 1992). Contradictory reports show that BMP4 trigger and noggin inhibits neurogenesis in cells isolated from the neocortical ventricular zone (Li et al., 1998; Piccini et al., 1999). It has further been postulated that cross-regulation of BMPs, Wnts and FGFs pattern the developing telencephalon, and that BMPs are needed for the development of hippocampal neurons (Shimogori et al., 2004). Thus, the precise role of BMPs in neural and neuronal differentiation and development remains to be determined.

Our results suggest, that in addition to the well-established role in promoting astrocytic differentiation, BMP4 induce neuronal differentiation in HD NSCs. As expected, addition of noggin blocks the BMP4 induced neuronal differentiation and strongly inhibits the astrocytic differentiation. Further analysis of the neurons showed that only about 5% did respond according to a neuronal profile as assessed by Ca^{2+} imaging and stimulation with ATP and the glutamate receptor (GluR) agonsit AMPA. Co-stimulation with BMP4 and Wnt3a however gave rise to functional neurons as assessed by Ca^{2+}-imaging.

4 CONCLUSIONS

Paper I and II:

1) Embryonic stem cells can differentiate to dopaminergic neurons and integrate with the host brain providing functional recovery in models of Parkinson's disease.

2) Transgenic overexpression of Nurr1, an intrinsic determinant of dopaminergic differentiation, can influence the differentiation of embryonic stem cells into a desired dopaminergic subtype.

Paper III and IV:

3) BMP4 up regulates a novel Wnt-signalling modulator, CxxC5, in NSCs.

4) A BMP4 mediated increase in noggin expression levels mediates the plating density dependent differentiation of NSCs, contributing to the inhibition of mesenchymal differentiation at high plating density.

5) BMP4 increases neuronal differentiation in NSCs plated at high density, although only a fraction of these neurons display functional characteristics.

6) BMP4 and Wnt3a stimulated NSCs efficiently generate neurons that are responsive to AMPA and ATP and thus display functional characteristics.

5 MATERIALS AND METHODS

This is a brief summary of the experimental procedures used in studies I-IV. For details, please see Appendix.

EMBRYONIC STEM (ES) CELL CULTURE (I, II).
The mouse blastocyst-derived ES cell line D3 was obtained from American Type Culture Collection. Undifferentiated ES cells were maintained on gelatin-coated dishes in DMEM supplemented with 2 mM glutamine, 0.001% ß-mercaptoethanol, nonessential amino acids,10% donor horse serum, and human recombinant leukemia inhibitory factor (LIF; 2,000 units/ml). Early passage cultures were frozen in 90% horse serum & 10% DMSO, and aliquots of cell vials were stored in liquid nitrogen. ES cells were differentiated into embryoid bodies (EBs) on nonadherent bacterial dishes for four days in EB medium, as described above except for removing LIF and exchanging horse serum with 10% fetal bovine serum. The EBs were then plated onto adhesive tissue culture surface. After 24 hours (hrs) in culture, selection of neuronal precursor cells was initiated in serum-free ITSFn medium. After 6±10 days of selection, cells were trypsinized and nestin+ neuronal precursors were plated on polyornithine and fibronectin coated coverslips in N2 medium supplemented with laminin and FGF2. After expansion for four days, FGF2 was removed to induce differentiation to neuronal phenotypes. In some cases, recombinant Sonic Hedgehog-N-terminus and FGF8 was added during neuronal precursor expansion stage and ascorbic acid was added during neuronal differentiation stage for further induction of dopaminergic phenotype (Lee et al., 2000b). Cells were eventually fixed 15 days after starting neuronal differentiation. Alternatively, ES cells were incubated for 4 days before the cells were transferred to a 15-ml sterile culture tube and allowed to settle, spun at 1,000 rpm for 5 min, and then collected and rinsed once in Ca^{2+} - and Mg^{2+} -free Dulbecco's PBS. After rinsing, 1.5 ml of trypsin solution was added, and the cells were incubated for 5 min at 37°C and then triturated with fire-polished Pasteur pipettes with decreasing aperture size to fully dissociate the cells. Finally, ES cells were spun at 1,000 rpm for 5 min, allowing the trypsin solution to be replaced with 200 µl of culture medium, and the viability and concentration of the ES cells were determined by using a hemocytometer after staining with acridine orange and ethidium bromide.

TRANSPLANTATION PROCEDURES (I).
Preanesthesia was given 20 min before animals were anesthetized with ketamine and xylazine. Animals then were placed in a Kopf stereotaxic frame. Each animal received an injection of 1.0 µl (0.25 µl min) of ES cell suspension or vehicle into two sites of the right striatum using a Hamilton syringe. A 2-min waiting period allowed the ES cells to settle before the needle was removed. Animals received 1,000–2,000 ES cells per µl. Twenty-five rats received ES cell injections, and 13 rats received sham surgery by injection of vehicle (medium). Six rats showed no graft survival and five rats died before completed behavioral assessment and were found to have teratoma-like tumors at postmortem analysis. A set of five rats that did not receive full behavioral testing was analyzed histologically.

STABLE TRANSFECTION AND ISOLATION OF NURR1-EXPRESSING ES CELL LINES (II).
D3 cells were transfected using the lipofectamine plus reagent. Stably transfected cells were selected in ES medium containing 500 mg/ml Neomycin. Neomycin-resistant clones were isolated from individual colonies, expanded, and total RNAs were prepared

from each clone. After DNaseI treatment, expression of transfected constructs was tested by RT-PCR.

TELENCEPHALIC NSC CULTURE (III, IV).
NSCs were obtained from the dissociated cerebral cortices of timed pregnant Sprague Dawley E15.5 rat embryos and cultured as described (Brunkhorst et al., 2005; Hermanson et al., 2002; Johe et al., 1996). Briefly NSCs were cultured in serum-free DMEM:F12 media enriched with N2 supplement and grown on poly-l-ornithine/fibronectin coated cell culture dishes. Cells were maintained in a proliferative state using 10ng/ml FGF2. After the second passage, cells were plated at 500cells/cm^2 (low density) or 10,000cells/cm^2 (high density) and allowed to proliferate for 24hrs prior to commencement of the experiments. To induce or block differentiation of NSCs, FGF2 was removed and 10ng/ml BMP4, 10ng/ml Wnt3a or 100ng/ml noggin were used. Addition of mitogen, soluble factors or inhibitors was carried out every 24hrs and media was changed every 48hrs. For inhibition of protein synthesis, 10-20μg/ml cycloheximide was added to the media at least 15mins before exposure to BMP4. For gene expression profiling using microarrays, NSCs were exposed to BMP4 or kept in FGF2 for 3 or 10hrs where after the cells were lysed and RNA extracted. As a control RNA from the time for stimulation start was also extracted. RNA was amplified and labeled with Cy3 or Cy5 and hybridized onto Agilent whole rat genome array (G4131A). Both BMP4 and FGF2 samples where co-hybridized with RNA from the starting point and a dye-swap was performed. The arrays were normalized and the differential gene expression analyzed using the R and bioconductor based method LIMMA.

ETHICAL PROTOCOLS (I-IV).
All animal experiments were approved by the committee for animal research ethics at the Harvard University, Boston, U.S.A., and by the Stockholm regional committee for animal research ethics, Stockholm, Sweden.

CONDITIONED MEDIA EXPERIMENTS (IV).
NSCs plated at 10,000cells/cm^2 (donors) were exposed to BMP4 for 24hrs before the first harvest of media. 1/5 of the total culture volume (200μl) was removed, centrifuged and used for culturing NSCs plated at 500cells/cm^2 (recipients). 200μl of fresh media replaced the removed volume before addition of BMP4 to the donor wells. Every 48hrs all media was replaced in the donor cultures. Media used for culturing the recipient cultures was replenished every day for 7 days before fixation and analysis by immunocytochemistry. For noggin depletion, conditioned media from BMP4-exposed NSCs plated at 10,000cells/cm^2 (donors) was incubated with a goat polyclonal anti-noggin specific antibody or mouse monoclonal IgG and Gamma Bind sepharose beads. The suspension was centrifuged and used to culture NSCs plated at 500cells/cm^2 (recipients). The beads were washed and resuspended in loading buffer. The suspension was incubated at 95°C for 5mins, put on ice for 1min, centrifuged at max speed at room temperature for 1min and stored at -70°C until analyzed using immunoblotting. To increase the concentration of secreted and added factors, we conditioned media for 24-48hrs before harvesting. The media used for culturing the recipient cultures was replenished every day for 7 days, before fixing and analysis of the recipient cultures.

PLASMID CONSTRUCTIONS (I-IV).
Open reading frames of clones were amplified using Taq-polymerase and gene specific primers and cloned using a TOPO ta kit. Isolated clones were sequenced (KI seq –

express) before subsequent use in overexpression experiments. For the creation of GFP fusion proteins in III, PCR was performed as before with other primers, the PCR product was purified and cut with restriction enzymes and ligated into the HindIII and EcoRI sites of pNGFP-EU or pCGFP-EU (III). Clones were then sequenced. To generate the pEF/Nurr1/IRES/hrGFP plasmid in paper II, mouse Nurr1 cDNA was PCR-amplified and inserted into the Sal I and BstE II sites of the pEF/IRES/hrGFP vector (II). The insertion of Nurr1 cDNA into the vector was confirmed by restriction digestion and sequence analysis. The Nurr1 construct was further confirmed by a transient cotransfection assay, where it activated a reporter construct containing Nurr1-binding sites (Murphy et al., 1996). A DNA cassette containing the neomycin resistant gene was inserted into both pEF/IRES/hrGFP and pEF/Nurr1/IRES/hrGFP using cre recombinase. Each construct with the neomycin resistant cassette was confirmed by restriction digestion.

OVEREXPRESSION BY NUCLEOFECTION (III).
NSCs were nucleofected using program A-33 on the Nucleofector (Amaxa) according to manufacturers instructions. Experiments were commenced 24hrs after nucleofection, during which NSCs were maintained in FGF2.

IMMUNOCYTOCHEMISTRY (I-IV).
Cells were fixed in 4% paraformaldehyde (PFA), rinsed with PBS and occasionally incubated with blocking buffer. Cells were then incubated overnight with primary antibodies diluted in PBS. The following primary antibodies were used: Chicken: anti-GFP (Chemicon); Goat: anti-calretinin (Swant); Mouse: TuJ1 (Covance), anti-TH, anti-GalC (Chemicon) anti-SMA, anti-MAP2 (Sigma); anti-c-myc (Santa Cruz Biotechnology); Rabbit: anti-ß-tubulin (Covance), anti-5HT (DiaSorin), anti-ChAT (Chemicon), anti-GABA, anti-glutamate (Sigma), anti-GFAP (DAKO), anti-synaptotagmin (a gift from Dr. P. Löw, KI), anti-TH (Pel-Freez); Rat: anti-DAT, anti-MBP (Chemicon); Sheep: anti-TH (Pel-Freez), anti-AADC (Chemicon). After additional rinsing in PBS, the cells were incubated in fluorescent-labelled secondary antibodies in PBS for 30-60 min at room temperature. After rinsing, sections were mounted and nuclei visualized using Vectashield containing DAPI.

IMMUNOHISTOCHEMISTRY (I).
After implantation of ES cells, animals were terminally anesthetized by pentobarbital and perfused intracardially with heparin saline followed by PFA. Brains were postfixed, equilibrated in sucrose, sectioned at 40 µm on a freezing microtome, and collected in PBS. Sections were rinsed in PBS, preincubated in 4% normal donkey serum (Jackson ImmunoResearch), and incubated with: Mouse: anti-GFAP, anti-NeuN (Chemicon), anti-calbindin (Sigma), anti-PCNA (Santa Cruz Biotechnology), anti-SSEA1 (Hybridoma bank); Goat: anti-Ki-67 (Santa Cruz Biotechnology); Rabbit: anti-5HT (Incstar), anti-DBH, anti-GABA (Chemicon), anti-ALDH (a gift from Dr. R. Lindahl), anti-ChAT (Roche); Rat: anti-DAT (Chemicon); anti-M6 (Hybridoma bank); Sheep: anti-TH (Pel-Freez), and anti-AADC (Chemicon) diluted in PBS. After rinsing, sections were incubated in fluorescent-labeled secondary antibodies in PBS, rinsed, and mounted onto gelatin-coated slides and coverslipped in gel mount.

***IN SITU* HYBRIDIZATION (III).**
Heads from embryos at gestational age E10.5, 12.5 or E14.5 were fixed in PFA and then transferred to sucrose. They were then frozen and kept in -70°C until cryostat-sectioned as 12µm sections. Prior to hybridization, sections were dried, fixed, and

permeabilized with proteinase K. After further processing, the sections were pre-hybridized with hybridization buffer. Hybridization was carried out at 70°C over night with DIG-labelled probes. After post-hybridization washes, sections were incubated with blocking buffer before being stained with an anti-DIG antibody (Roche). Finally, slides were incubated with chromogen components until sufficiently stained. DIG-probes were produced according to the manufacturer's recommendations using DIG RNA labeling mix (Roche). Sections were analyzed using standard light microscopy.

IMMUNOBLOTTING AND IMMUNOPRECIPITATION (III, IV).

For immunoprecipitation (III), COS7 cells were transfected with GFP-Cxxc5 or empty vector control individually or cotransfected with a full-length Xdishevelled using Lipofectamine 2000. Immunoprecipitation, immunoblotting and culturing of Cos-7 and HEK293 cells were performed essentially as previously described (Bryja et al., 2007).
For immunoblot analysis of NSC media (IV), media was collected, concentrated and resolved on SDS-PAGE and subsequently blotted; protein species were detected with an affinity purified polyclonal goat anti-noggin (R&D Systems) antibody.

RNA ISOLATION AND QUANTITATIVE/SEMIQUANTITATIVE RT-PCR (I-IV).

RNA was isolated using standard protocols and contaminating DNA removed using RNase free DNase I. First strand cDNA synthesis was obtained using SuperScript™ Preamplification Kit (Life Technologies) or High Capacity cDNA Reverse Transcription kit (Applied Biosystems). PCR reactions were carried out using an Eppendorf Thermocycler. For the semiquantitative PCR, cDNA templates were normalized by amplifying actin-specific transcripts and levels of gene transcription were detected by adjusting PCR cycling and primer design in such a way that each primer set amplified its corresponding gene product at its detection threshold to avoid saturation effects. For quantitative RT-PCR 0.5x Platinum SYBR green mix (Invitrogen) was used and run on Applied Biosystems 7300 Real Time PCR system using the PCR-program recommended by Invitrogen for Platinum SYBR green. Primers used for the quantitative RT-PCR were designed to span an intron wherever possible. To further exclude DNA contamination, -RT (no reverse transcriptase) samples were run as controls. Data were analysed using the statistical programming language R (http://www.r-project.org/).

ANALYSIS OF CATECHOLAMINES (I).

Differentiated ES cells in were treated with N3 medium supplemented with KCl and Pargyline and the media were collected after 30 min and concentrated solutions of perchloric acid (PCA) were added. For measurement of catecholamine cell contents, cells were harvested in PCA/EDTA. These deproteinated samples were centrifuged and the supernatants were kept at ±80 °C until further analysis. Samples were analysed for their catecholamine content by reverse-phase HPLC as described previously (Wachtel et al., 1997). The flow rate of the mobile phase through the system was 0.8 ml/min, and the potential of the guard cell was set at 330 mV. The potential of the first electrode in the analytical cell was set at 0 mV, the second at 310 mV. L-DOPA, dopamine, dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) were identified by retention time and quantified based on peak height. The limit of detection for all compounds was <1 pg. DA content of each sample was normalized with the amount of total cellular proteins. For protein measurement, after harvesting cells in PCA/EDTA, precipitates were resuspended and sonicated. The protein content was measured by the colorimetric Bradford assay.

CALCIUM RECORDINGS (IV).

Calcium experiments on neurons and astrocytes derived from NSCs were carried out in Krebs-Ringer's solution adjusted to pH 7.4. Cells were incubated in Fluo-3/AM together with Pluronic F-127 using Krebs-Ringer's solution. Coverslips were mounted in a temperature controlled chamber and clamped onto a Zeiss Axiovert 100M microscope, equipped with a C-Apochromat 25X/0.8NA water immersion objective, connected to a Lambda LS xenon-arc lamp, Lambda 10-3 filter-wheel, and a smartShutter (all Sutter). Images were acquired at 0.2Hz with an EMCCD camera controlled by the acquisition software MetaFluor (Molecular Devices). Perfusion with Krebs-Ringer's solution was used for Ca^{2+} recordings, and drugs were applied in the bath.

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