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Tiia Pönniö

Analyzing the Function of Nuclear Receptor Nor-1 in Mice

UNIVERSITY OF JYVÄSKYLÄ

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ABSTRACT

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Yhteenveto: Hiiren tumareseptori Nor-1:n toiminnan tutkiminen
Diss.

Nuclear receptor Nor-1, together with Nur77 and Nurr1, belongs to the NR4A subfamily of nuclear receptors. Human Nor-1 protein was identified in a chromosomal translocation in extraskeletal myxoid chondrosarcoma. This suggests that Nor-1 may play a role in chondrocyte proliferation. The precise role of Nor-1 in mammalian development has not been previously established. Therefore, to examine the spatiotemporal expression pattern of *nor-1* and the physiological consequences of *nor-1* ablation, nor-1 knock-out mice were generated by insertion of the *lacZ* gene into the *nor-1* genomic locus. Despite *nor-1* expression during chondrocyte development, no defect in the outgrowth of the skeleton was detected in nor-1$^{-/-}$ mice. However, disruption of the *nor-1* gene resulted in a partial bidirectional circling phenotype in the adult mice. The circling behavior can be explained by a defect in the proliferative continual growth of the semicircular canals. The semicircular canals are a part of the vestibular system of the inner ear, a structure responsible for maintaining balance. Nor-1 was also essential for the development of the murine hippocampus; in the absence of Nor-1 the CA3 pyramidal cell layer was dispersed. This was accompanied by a loss of a subset of cells in the CA1 field. In addition, postnatal axonal growth of the dentate gyrus granule cell and mossy cell axons was reduced. These developmental defects in the hippocampus resulted in seizure susceptibility to a convulsant kainic acid in the adult nor-1$^{-/-}$ mice. Behavioral testing revealed that nor-1$^{-/-}$ mice also suffered from heightened fear. Examination of the mediators in the inflammatory pathway suggested Nurr1, and not Nor-1 or Nur77 involvement in the pathogenesis of rheumatoid arthritis. Taken together, the NR4A family members may have both shared and specific functions.

Key words: Arthritis; hippocampus; limb development; Nor-1; NR4A subfamily; nuclear receptor; seizure susceptibility; semicircular canal.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on publications, which are referred to in the text by the following numerals:

I Pönniö, T., Burton, Q., Pereira, F.A., Wu, D.K. and Conneely, O.M. 2002: The nuclear receptor nor-1 is essential for proliferation of the semicircular canals of the mouse inner ear. Mol. Cell. Biol. 22: 935-945

II Pönniö, T. and Conneely, O.M. 2004: Nor-1 regulates hippocampal axon guidance, pyramidal cell survival and seizure susceptibility. Manuscript.

III Pönniö, T., Weeber, E.J., Sweatt, J.D. and Conneely, O.M. 2004: Increased fear behavior in mice lacking nuclear receptor nor-1. Manuscript.

IV McEvoy, A.N., Murphy, E.A., Pönniö, T., Conneely, O.M., Bresnihan, B., FitzGerald, O. and Murphy, E.P. 2002: Activation of nuclear orphan receptor NURR1 transcription by NF-κB and cyclic adenosine 5′-monophosphate response element-binding protein in rheumatoid arthritis synovial tissue. J. Immunol. 168: 2979-2987.

I have also included analysis of *nor-1* expression in the limb (section 5.5.2) and the consequences of *nor-1* ablation during limb development (section 5.5.3). These results are not described in the publications.

RESPONSIBILITY OF TIIA PÖNNIÖ IN THE ARTICLES COMPRISING THIS THESIS

Article I: Quiana Burton did the paint-filling analysis of the inner ears and the *BMP-4/nor-1* in situ hybridization experiments at embryonic day 11.5 with specimens I had dissected and processed. The rest of the experiments were done by me.

Article II: I carried out the experiments described in this study.

Article III: Dr. Edwin Weeber did the electrophysiology measurements. Technician Karen Brown from the behavioral test core facility helped me with the behavioral testing.

Article IV: I cloned the long (-1329/+132) and short (-396/+373) *nurr1* promoter region vectors described in this study.

The analysis of Nor-1 function in the skeleton described in section 5.2.2 and 5.2.3 was carried out by me.

All the work was done under the supervision of Professor Orla M. Conneely who participated in the planning of the experiments and writing of the articles.

ABBREVIATIONS

AF	activation function
BMP	bone morphogenic protein
C/A	commissural/associational
CNS	central nervous system
CREB	cAMP responsive element-binding protein
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
DBD	DNA-binding domain
E	embryonic day
EMC	extraskeletal myxoid chondrosarcoma
ES	embryonic stem (cell)
ESW	Ewing sarcoma virus
IML	inner molecular layer
IL	interleukin
i.p.	intraperitoneally
KA	kainic acid
KO	knock-out
H&E	hematoxylin and eosin
HPA	hypothalamic-pituitary-adrenal gland
LBD	ligand-binding domain
LTP	long-term potentiation
NF-kB	nuclear factor-kappaB
NR	nuclear receptor
nor-1	nor-1 gene
Nor-1	Nor-1 protein
nor-1$^{-/-}$	nor-1 knock-out/lacZ knock-in
O/N	overnight
P	postnatal day
PTHrP	parathyroid hormone-related protein
PBS	phosphate buffered saline
PG	prostaglandin
POMC	proopiomelanocortin
RA	rheumatoid arthritis
RE	response element
RT	room temperature (21-24°C)
RXR	retinoid X receptor
TCR	T-cell receptor
TF	transcription factor
TLE	temporal lobe epilepsy
TNF-α	tumor necrosis factor-α
SCC	semicircular canal
SCD	semicircular duct
VTA	ventral tegmental area

1 INTRODUCTION

The nuclear receptor (NR) superfamily of transcription factors (TFs) comprises a large group of structurally related proteins that program developmental, physiological, and behavioral responses to a variety of chemical signals, most of which are nuclear receptor ligands (Evans 1988). The ligand activated nuclear receptors for steroid and thyroid hormones and certain fat soluble vitamins serve as prototypes for this family of TFs. Upon binding of their cognate ligand they become active dimeric TFs capable of interacting with specific *cis*-acting DNA elements to regulate the expression of target genes (Kumar & Chambon 1988, Tsai et al. 1988, Beato 1989). The high degree of amino acid conservation within the DNA-binding domain (DBD) of NRs has been widely exploited to generate probes that have been used for screening cDNA libraries to isolate novel members of the NR family. Using this approach, NR Nurr1 was identified from a neonatal mouse brain library in the laboratory of Dr. Orla Conneely (Law et al. 1992). The Nurr1 protein is closely related to a previously cloned Nur77 (Hazel et al. 1988).

Nor-1 was originally cloned from primary cultures of rat forebrain neurons undergoing apoptosis (Ohkura et al. 1994) and, together with Nur77 and Nurr1, forms the NR4A subfamily of NRs (Committee 1999). Although the NR superfamily was originally identified as ligand-activated TFs, the NR4A subfamily members do not require an exogenously added ligand for their activity *in vitro* and are therefore termed orphan NRs (Law et al. 1992, Wilson et al. 1993, Wang et al. 2003, Wansa et al. 2003). They are particularly similar in their DBDs, and moderately so in their putative ligand-binding (LBD) and N-terminal transactivation domains (Ohkura et al. 1994). Due to the close similarity between these proteins, they can all bind and activate transcription using the same response element, termed NBRE (Wilson et al. 1991, Scearce et al. 1993, Ohkura et al. 1994).

An interesting and unique feature of the NR4A subfamily is that they are encoded by immediate early genes. They are rapidly induced when cells are activated by extracellular stimuli, including growth factors (Hazel et al. 1988, Milbrandt 1988, Bandoh et al. 1995). In addition, Nurr1 and Nur77 can bind and regulate the expression of hypothalamic *corticotrophin releasing factor* (CRF) and

are induced in anterior pituitary cells in response to CRF (Murphy & Conneely 1997). In the pituitary Nurr1 and Nur77 in turn induce the expression of *proopiomelanocortin* (POMC) and at the level of the adrenal gland (in response to the adrenocorticotrophic hormone) regulate the expression of *steroid-21-hydroxylase,* (Wilson et al. 1993). Thus, both constitutive and inducible expression of the NR4A subfamily members can contribute to their tissue-specific functional roles and serve to expand their functional repertoire.

Nurr1 and Nur77 function *in vivo* has been previously characterized. Loss of the Nurr1 protein by null mutation in mice results in early postnatal death (cause unknown) and agenesis of midbrain dopaminergic neurons (Zetterstrom et al. 1997, Castillo et al. 1998, Saucedo-Cardenas et al. 1998). Interestingly, in humans these midbrain dopamine neurons function in the control of movement and their loss is the cause of Parkinson's disease. In contrast, targeted mutation of *nur77* has failed to show an overt aberrant phenotype, suggesting that the function of this protein is redundant with Nor-1 and Nurr1 (Crawford et al. 1995, Lee et al. 1995).

At the start of this research project little information was available on the spatiotemporal expression, physiological roles or target genes regulated by the third family member, Nor-1. Northern blot hybridization studies in rat and human indicated that *nor-1* mRNA is constitutively expressed in the fetal brain (Ohkura et al. 1994, Ohkura et al. 1996). Therefore, Nor-1 may play a role during the development of brain structures. In addition, *nor-1* expression is detected in the adult brain, thymus and heart. Identification of the human *nor-1* gene through its involvement in a chromosomal rearrangement associated with human extraskeletal myxoid chondrosarcoma (EMC) (Labelle et al. 1995) predicted that this protein may play an important role in chondrocyte proliferation.

Because of the very interesting results obtained from the nurr1 knock-out (KO) mice in our laboratory and the absence of phenotype in the nur77 KO mice, possibly due to redundancy between the NR4A family members, we were interested in the physiological role of Nor-1. Therefore, in an effort to elucidate the role of Nor-1 *in vivo*, the *nor-1* gene was interrupted using homologous recombination by insertion of the *lacZ* gene into the genomic *nor-1* locus (nor-1$^{-/-}$ mice).

2 REVIEW OF THE LITERATURE

2.1 The nuclear receptor superfamily

2.1.1 General concepts

Nuclear receptors provide multicellular organisms with a way to directly control gene expression in response to a wide range of developmental, physiological and environmental cues (Evans 1988). Their activity can be controlled by at least three distinct mechanisms: 1) binding of a small lipophilic ligand, 2) covalent modification, usually in the form of phosphorylation, and 3) protein-protein interactions, generally through contacts with other TFs.

Nuclear receptors are composed of four conserved interacting functional structures. These are the activation domain, the DBD, the hinge region and the LBD (Fig. 1). The activation domain displays the most variability in terms of both length and primary sequence. It contains the transcriptional activation function (AF)-1. The activation domain can interact directly with steroid receptor coactivators to enhance the activity of the receptor complex (Onate et al. 1998). NRs have been historically divided into type I receptors (essentially the classical steroid receptors), which undergo nuclear translocation upon ligand activation and bind as homodimers to *cis*-acting DNA elements composed of inverted repeats of two types of core sequence motifs, AAGGTCA and AGAACA, and type II receptors, which are often retained in the nucleus regardless of the presence of a ligand, and usually bind as heterodimers with retinoid X receptor (RXR) (McKenna 2003).

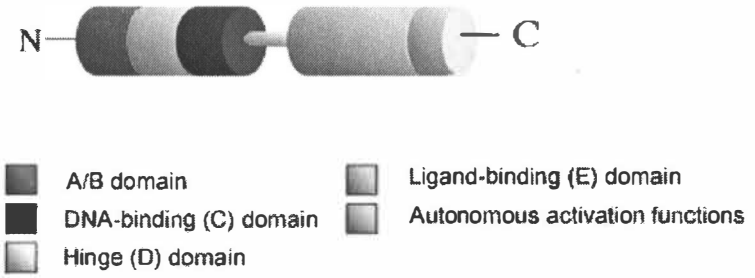

FIGURE 1 Shared functional domains of the nuclear receptor superfamily. General structure of NRs. AF-1 is embedded in the N terminus of type I NRs and AF-2 in the C terminus of all NRs. The A/B domain is prominent in type I NRs, and is considerably smaller in many type II receptors (McKenna 2003).

Nuclear receptors bind DNA as monomers, homodimers, and heterodimers (Glass 1994) and their recognition sites are referred to as hormone response elements. The DBD is the most conserved domain, composed of Cys_4 zinc finger modules encoded by 66-70 amino acid residues that sets the nuclear receptors apart from other DNA-binding proteins (Berg 1989, Klug & Schwabe 1995).

The LBD is a multifunctional domain that mediates ligand binding, dimerization, interaction with heat shock proteins, nuclear localization, and transactivation (Giguere 1999). Although quite variable in primary sequence, ligand-induced transactivation is dependent on the presence of a highly conserved motif, AF-2 localized at the carboxy-terminal end of the LDB (Wurtz et al. 1996).

To modulate transcription NRs interact with coregulatory proteins. They have been shown to associate with various components of the general transcription machinery, co-repressors, and co-activators (Beato & Sanchez-Pacheco 1996, Horwitz et al. 1996, McKenna et al. 1999, McKenna 2003). NRs can also regulate transcription *via* direct interactions with other TFs, a process that does not depend on DNA binding (Pfahl 1993, Karin 1998). In particular, the glucocorticoid receptor has been shown to antagonize AP-1 and nuclear factor-κB (NF-κB) activities *via* transcriptional interference (Jonat et al. 1990).

2.1.2 Orphan nuclear receptors

Classic members of the NR superfamily were cloned on the basis that known hormones were transducing their physiological functions through binding to ligands (Evans 1988). Once it was acknowledged that NRs shared extensive similarity at the nucleotide sequence level, particularly in the DBD, a search for new members was undertaken using low-stringency screening of cDNA libraries with DBD fragments as probes. Surprisingly, no ligands could be linked to some of the new putative receptors on the basis of their structure alone, and they were referred to as orphan NRs.

The vast majority of orphan NRs possess all the functional domains that characterize classic NRs. With the exception of DAX-1 and SHP, they recognize

specific hormone response elements through the well-conserved DBD (Seol et al. 1996, Sem et al. 1997, Zhao et al. 1998). They also have a highly recognizable LBD. However, since the LBD mediates multiple functions besides ligand binding, its presence may signify activities other than ligand binding.

2.2 The NR4A subfamily of orphan nuclear receptors

According to the most recent nomenclature the NR4A subfamily consists of three proteins termed NR4A1, NR4A2 and NR4A3 (Committee 1999). However, due to the familiarity and wide use of the following names, they are referred here to as Nur77, Nurr1 and Nor-1, respectively. These members have multiple other names proposed by different researchers, as summarized in TABLE 1.

TABLE 1 Nomenclature for the NR4A subfamily (Maruyama et al. 1998)

	Human	Rat	Mouse
NR4A1	TR3	NGFI-B	**Nur77**
	NAK1		N10
	ST59		TIS1
NR4A2	NOT	RNR-1	**Nurr1**
	TINUR	HZF-3	
NR4A3	MINOR	NOR-1	**Nor-1**
	TEC		
	NOR-1		
	CHN		

Nor-1 (Neuron-derived orphan receptor-1) was identified from rat forebrain cultures undergoing apoptosis (Ohkura et al. 1994). The *nur77* gene was first cloned as a serum-inducible gene expressed during the G0/G1 transition phase (Hazel et al. 1988) and subsequently identified as a nerve growth factor-inducible gene (NGFI-B) in differentiating rat cells (Milbrandt 1988). *Nurr1* (nur-related factor 1) was cloned as a gene induced by membrane depolarization (Law et al. 1992).

The genomic structures for these receptors are remarkably similar; they share more than 90% identity in the amino acid sequences within the DBD, suggesting that they have evolved from a common ancestral gene (Chang et al. 1989, Watson & Milbrandt 1989, Ohkura et al. 1996, Saucedo-Cardenas et al. 1997). Despite the high similarity in their DBD, the divergence in other domains, particularly in the N-terminal transactivation domain, may result in differences in tissue expression and immediate early responses among these proteins.

2.2.1 Mechanism of action

The NR4A subfamily members can bind DNA as monomers, homodimers and heterodimers. In fact, Nur77 was the first NR identified to bind DNA as a monomer. The homodimer binding site consists of two inverted NBREs (NGFI-B response element, AAAGGTCA) spaced by 6 bp originally identified by genetic selection in yeast (Wilson et al. 1991, Wilson et al. 1993). In contrast to NBRE binding by monomers, the Nur response element (NurRE) binds homodimers of Nur77, Nurr1 and Nor-1 in addition to heterodimers (Nurr1/Nur77, Nor-1/Nur77) (Maira et al. 1999). The NurRE identified at the promoter of the *POMC* gene is an inverted copy of two NBRE sites with two differences each, separated by 6 bp.

The two response elements, NBRE and NurRE can mediate different cellular responses. For example, in T-cell hybridomas, T-cell receptor (TCR) activation induced NurRE but not NBRE reporters (Philips et al. 1997a). In the pituitary, Nurr1 and Nur77 mediate stress response through both NBRE and NurRE on the POMC promoter (Murphy & Conneely 1997, Philips et al. 1997a, Maira et al. 1999). Nor-1, although capable of interacting with NurRE and NBRE, can only bind the POMC NurRE site and together with Nur77, activate transcription as heterodimers (Maira et al. 1999).

Both Nur77 and Nurr1, but not Nor-1, can also bind to a response element composed of direct repeats spaced by 5 nucleotides (DR5 RE) as heterodimers with RXR. On these elements, the heterodimer complex is efficiently induced by rexinoids (Perlmann & Jansson 1995, Zetterstrom et al. 1996, Aarnisalo et al. 2002). Therefore, Nurr1 and Nur77 define a pathway for vitamin A or retinoid and growth factor signaling pathways. Nur77 and Nurr1 heterodimerize with RXR through a C-terminal domain, in which Nor-1 lacks three amino acids in the area believed to be involved in forming the interaction with RXR (Bourguet et al. 1995, Zetterstrom et al. 1996) thereby explaining the lack of heterodimerization between Nor-1 and RXR.

In addition, Nor-1 unlike Nurr1 or Nur77 interacts with a homeobox-containing protein, Six3, through NBRE possibly regulating expression during late stages of forebrain development (Ohkura et al. 2001). In summary, homo- and heterodimerization of the NR4A subfamily members is a way of specifying target sequences and their potency of transactivation.

2.2.2 Activation

Although the steroid/thyroid superfamily was originally identified as a family of ligand-activated TFs, the NR4A subfamily does not appear to require an exogenously added ligand for their activity (Law et al. 1992, Wilson et al. 1993, Wang et al. 2003). In fact, in the Nor-1 LBD the hydrophobic cleft characteristic of NRs is displaced with a very hydrophilic surface which probably accounts for the inability of the LBD to efficiently mediate transcriptional activation (Wansa et al. 2003). The N-terminal AF-1 domain of Nor-1, however, is necessary for recruitment of a cofactor such as the steroid receptor coactivator

(SRC). In addition, the Nurr1 LBD adopts a canonical protein fold resembling that of agonist-bound, transcriptionally active LBDs in NRs, however, the structure has two distinctive features. First, the Nurr1 LBD does not contain a cavity as a result of the tight packing of side chains from several bulky hydrophobic residues in the region normally occupied by ligands. Second, Nurr1 lacks a classical binding site for coactivators (Wang et al. 2003).

Instead of being regulated by ligands, the NR4A proteins are classified as immediate early genes whose expression is induced rapidly by a variety of stimuli including cAMP, growth factors and membrane depolarization (Milbrandt 1988, Law et al. 1992, Bandoh et al. 1995, Ohkubo et al. 2000). In addition, several lines of evidence suggest that their activity is also regulated through phosphorylation, and the extent of phosphorylation is dependent on the stimulus. In the central DBD, Nur77 is phosphorylated at a serine residue in the A-box (Davis et al. 1993, Hirata et al. 1993), repressing the DNA-binding and transactivational activity of Nur77 (Katagiri et al. 1997, Li & Lau 1997). The cellular localization of Nur77 can also be modified by phosphorylation (Katagiri et al. 2000). Thus, both constitutive and inducible expression of these TFs and covalent modification of the expressed proteins can contribute to their tissue specific functional roles.

2.2.3 Expression pattern

The three NR4A receptors display both overlapping and distinct spatiotemporal expression patterns throughout mammalian development. Both *nurr1* and *nor-1* are widely expressed in the developing rodent nervous system, whereas *nur77* expression is strongest in the adult brain (Saucedo-Cardenas & Conneely 1996, Xiao et al. 1996, Zetterstrom et al. 1996, Maruyama et al. 1997a). It is noteworthy, that the family members have overlapping expression patterns in many areas of the adult brain such as the neocortex, hippocampus and cerebellum. While constitutive *nurr1* expression appears predominantly in the brain, both *nur77* and *nor-1* are found in a variety of tissues outside the central nervous system (Saucedo-Cardenas & Conneely 1996, Zetterstrom et al. 1996, Bandoh et al. 1997, Crispino et al. 1998).

A striking difference in the expression pattern is the presence of *nurr1* but absence of both *nur77* and *nor-1* in the substantia nigra and the ventral tegmental (VTA) area of the adult midbrain (Saucedo-Cardenas & Conneely 1996).

2.2.4 Physiological functions

Both Nur77 and Nor-1 have been shown to play a role in the negative selection of developing T-cells. During development in the thymus, immature thymocytes expressing self-reactive TCRs (T-cell receptors) are eliminated from the developing T-cell repertoire by apoptosis (Nossal 1994). This process of negative selection is critical for generating a peripheral T-lymphocyte population with low potential autoreactivity. Nur77 and Nor-1 (but not Nurr1)

are strongly induced following stimulation of TCRs, which leads to apoptosis of the self-reactive immature thymocytes (Cheng et al. 1997). In addition, overexpression of a dominant-negative Nur77 and the use of antisense *nur77* mRNA abrogates TCR-mediated apoptosis (Woronicz et al. 1994, Calnan et al. 1995, Weih et al. 1996). However, both the extent and rate of thymocyte death in nur77$^{-/-}$ mice were unimpaired (Lee et al. 1995), suggesting redundancy between Nur77 and Nor-1 in thymocytes.

The NR4A family members are highly expressed in the hypothalamus, pituitary and adrenal glands (Bandoh et al. 1997, Maruyama et al. 1997a). Therefore, a function for the NR4A subfamily has been implicated in regulating the hypothalamic-pituitary-adrenal (HPA) axis that is activated in response to stress. Following a stressful stimulus, CRF is synthesized in the hypothalamus and released to increase the transcription of POMC in the pituitary. POMC is the precursor of ACTH, which is released from the pituitary to regulate adrenal steroidogenesis. Both Nur77 and Nurr1 can regulate the expression of *CRF* and *POMC* by interacting with a specific *cis*-acting sequence in their proximal promoter regions in the hypothalamus and pituitary, respectively (Murphy & Conneely 1997, Philips et al. 1997a, Philips et al. 1997b). Despite these observations analysis of the activity of the HPA axis in nur77$^{-/-}$ mice did not reveal any disturbances in this pathway either (Crawford et al. 1995). However, *nurr1* expression was induced three-fold in the adrenal gland of the nur77$^{-/-}$ mice, suggesting that in this pathway Nurr1 may compensate for the loss of Nur77 (Crawford et al. 1995).

In mammals the corpus luteum of the ovary plays a central role in the regulation of cyclicity and maintenance of pregnancy. In the absence of fertilization and implantation, the corpus luteum loses the ability to secrete progesterone and undergoes luteolysis. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is involved in the inhibition of progesterone production and luteal regression in many mammalian species (Niswender et al. 2000). All the NR4A family members are expressed in the ovary where they may regulate the transcriptional activity of ovarian steroidogenic genes, and thereby play a role in ovulation (Law et al. 1992, Richards et al. 1995, Maruyama et al. 1997a, Stocco et al. 2000, Park et al. 2001, Park et al. 2003). Moreover, Nur77 mediates the $PGF_{2\alpha}$ induction of *20α-hydroxysteroid*, a steroidogenic enzyme involved in the catabolism of progesterone during luteolysis through a Ca^{2+}-calmodulin-dependent mechanism (Stocco et al. 2002).

Although the NR4A proteins do not have a clearly defined role in vascular function, vascular endothelial growth factor induces their expression in human endothelial cell cultures, indicating they might play a role in the regulation of endothelial function. This is further supported by the finding that Nur77 is involved in the control of endothelial cell proliferation (Arkenbout et al. 2003, Gruber et al. 2003).

2.2.5 The NR4A subfamily and disease

There is increasing evidence for the involvement of the NR4A subfamily in cancer. In many human cancers, tumor-specific chromosomal rearrangements can create TFs with the ability to transform cells in which they are expressed (Rabbitts 1994). The *Ewing sarcoma virus* (EWS) gene is involved in various human malignancies by way of chromosomal translocation. The recurrent t(9,22) chromosomal translocation identified in EMC was found to generate a fusion protein between ESW and TEC (translocated in extraskeletal chondrosarcoma, hNOR-1) (Labelle et al. 1995, Clark et al. 1996). In addition, two other chromosomal translocations involving Nor-1 (TEC/TAF2N and TEC/TCF12) in these tumors have been described (Attwooll et al. 1999, Panagopoulos et al. 1999, Sjogren et al. 1999, Sjogren et al. 2000). A link between the NR4A family and cancer has also been suggested in several studies *in vitro* (Maruyama et al. 1997b, Wu et al. 1997a, Wu et al. 1997b). Significantly, *TR3* (hNur77) is expressed more highly in prostate cancer areas than in adjacent normal tissue, suggesting that TR3 may play a role in development or progression of prostate cancer (Uemura & Chang 1998). TR3 can be induced by apoptosis-inducing agents, and antisense *TR3* reduces cell death caused by these apoptotic reagents, further suggesting that increasing *TR3* expression could be used in combination to improve the efficacy of prostate cancer therapy.

Activated smooth muscle cells are a hallmark of pathological vascular processes, including atherosclerosis (Ross 1999). Atherosclerotic lesions are active sites of inflammation and immune responses, and cytokines activate the chronic development of the disease (Young et al. 2002). The NR4A subfamily is expressed in human atherosclerotic lesions, and inhibition of their activity results in enhanced lesion formation, whereas overexpression decreases lesion formation (Monajemi et al. 2001, Arkenbout et al. 2002).

The VTA and substantia nigra of the midbrain contain dopamine cell bodies the loss of which leads to Parkinson's disease in humans. Despite the wide overlap in expression between the NR4A family members, the developing ventral midbrain is positive only for *nurr1* mRNA. In accordance, using a mouse knock-out model, Nurr1 was shown to be essential for both survival and final differentiation of the ventral midbrain dopaminergic precursor neurons (Zetterstrom et al. 1997, Castillo et al. 1998, Saucedo-Cardenas et al. 1998).

Neurons arising from the VTA project to the limbic system and cortex to regulate emotional and reward behavior (Self & Nestler 1995). Disturbances in this system are implicated in schizophrenia and addictive behavioral disorders (Ritz et al. 1987, Seeman et al. 1997) and dysfunction of retinoid-mediated transcription may be a factor in the etiology of schizophrenia (Goodman 1998). In consequence, as in Parkinson's disease, mutations in the *nurr1* gene has been also implicated in schizophrenia and manic-depressive disorder (Buervenich et al. 2000, Le et al. 2003). In addition, *nor-1* and *nur77* are induced by psychoactive drugs such as cocaine and morphine in the accumbens nucleus and caudate putamen (Werme et al. 2000).

2.3 Inner ear and its role in maintaining balance

The inner is essential for balance and coordinated movement. The mouse inner ear initially forms as a thickening of the ectoderm, termed the otic placode, between rhombomeres 5 and 6 in the hindbrain that invaginates to become the otocyst by embryonic day (E) 9.5. The mature inner ear contains the cochlea and vestibular organs, which are responsible for hearing and balance, respectively (Fig. 2). The vestibular part consists of two principle sets of sensory structures; the otolith organs (in the sacculus and utriculus) sense linear acceleration and gravity and the crista ampullaris (in the semicircular canals) that sense angular acceleration. The membranous labyrinth (non-sensory and sensory epithelium) is contained within an osseous structure (tympanic bone) of the same shape, floating within a cushion of perilymph and filled with endolymph. Stimuli related to vestibular functions are endogenously produced by movement of the endolymph through the semicircular canals (SSCs) and by the position of the otoliths within the utricle and saccule.

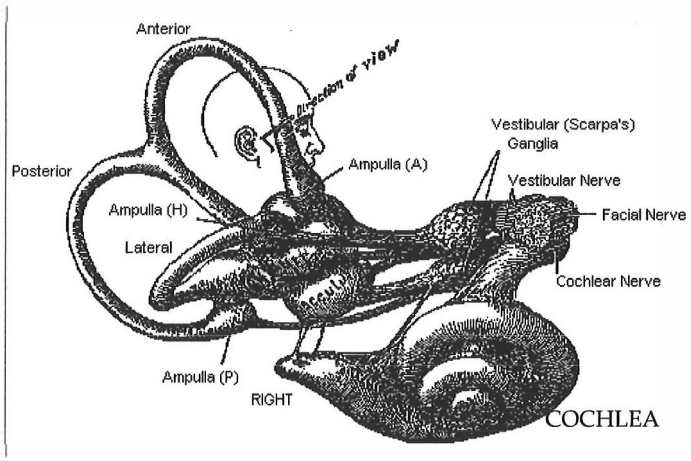

FIGURE 2 Structure of the membranous part of the inner ear. The ampullas contain the sensory receptor hair cells of the semicircular canals.

The SCCs are arranged in three dimensions perpendicularly to each other. They originate from bilayered outpocketings emerging past mid-gestation at E11.5 in the mouse (Martin & Swanson 1993). The lateral wall of each epithelial outpocketing delaminates from the underlying mesenchyme and grows towards the corresponding medial wall forming a fusion plate. Subsequently the fusion plate disappears in order to form the closed tubular form of the canal by E13.5. At this stage all the major structural changes associated with SCC formation are completed and the shape resembles that of an adult inner ear. Inner ear differentiation and formation is followed by a period of continual canal growth. The stages of development of the semicircular canals have been categorized into 4 steps: 1) outgrowth, 2) patterning and specification, 3) fusion

and resorption, and 4) continual growth. The membranous parts of the SSCs within the tympanic bone are referred to as semicircular ducts (SCDs).

2.4 Hippocampal formation

The hippocampus is located on the medial temporal lobe and is part of the so-called limbic system (Paxinos 1994). The limbic system plays a critical role in memory consolidation and expression of emotions. The hippocampal formation refers to the Ammon's horn (hippocampus proper; subiculum and CA1-CA3 fields) and the dentate gyrus (Fig. 3). The hippocampus consists of two major principal cell types, the pyramidal cells of the Ammon's horn and granule cells of the dentate gyrus.

Hilar mossy cells are the source of the commissural/associational (C/A) fibers of the dentate gyrus. They terminate in the inner molecular layer (IML) of the dentate gyrus and make excitatory connections with the granule cell dendrites (Swanson 1976, Hjorth-Simonsen & Laurberg 1977). The mossy fibers represent the axonal projections of the dentate gyrus granule cells and their terminals form excitatory synapses with the apical dendrites of CA3 pyramidal cells and hilar mossy cells (Frotscher et al. 1991).

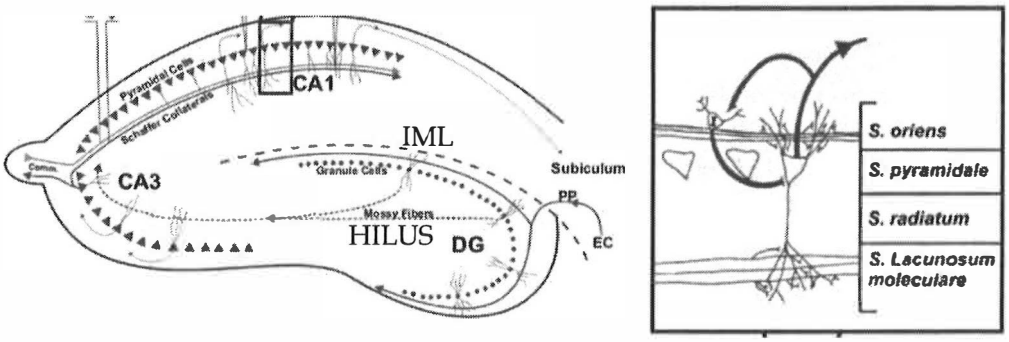

FIGURE 3 Organization of the hippocampus. The hippocampus consists of two main cell layers: the pyramidal cell layer of the Ammon's horn (CA1-CA3) and the granule cells of the dentate gyrus (DG). Hilar mossy cell axons terminate in the inner molecular layer (IML) of the dentate gyrus. The granule cell axons, the mossy fibers ,terminate at CA3.

2.5 Skeletal development

The skeleton is composed of two tissues, cartilage and bone, which together comprise three cell types: chondrocytes located in cartilages and osteoblasts and osteoclasts in bone. The development of bone can proceed by either

endochondral ossification, in which cartilage is first produced and later replaced by bone, or by intramembranous ossification in which ossification occurs without the intervention of a cartilage precursor (Thorogood 1993). The long bones and vertebrae are formed by endochondral ossification, while craniofacial bones are formed by intramembranous ossification.

The differentiation of chondrocytes is preceded by a series of patterning events that determine the position, number and shape of skeletal elements. In case of the limbs, the intermediate and lateral plate mesoderm form a limb bud which is covered by a thick epithelial layer, the apical ectodermal ridge (Fallon & Kelley 1977). The skeletal elements are formed in a proximal to distal sequence beginning with the humerus at E10.5 and branching distally to form condensations for the ulna and radius at E11.5 (Hall & Miyake 1992). As limb digit development continues through E14.5, the shaft of the cartilage model (diaphysis) is surrounded by a perichondrial envelope and contains at each end a region of articular cartilage that is not covered by the perichondrium (Ducy & Karsenty 1998, Karsenty 1998). Differentiation begins at the center of the diaphysis and extends outward towards the ends of the cartilage model.

During endochondral bone development, the cartilage template is formed when mesenchymal cells condense between E10.5 and E12.5 to form the initial shape of the bone (Karsenty 1998). Differentiation into chondrocytes begins by E12.5. The chondrocytes then undergo a program of proliferation and maturation through a prehypertrophic stage to become differentiated hypertrophic chondrocytes (Poole 1991) (Fig. 4). The hypertrophic chondrocytes exhibit a number of changes that lead to differences in the composition and properties of the cartilage matrix allowing invasion of blood vessels. At the time of vascular invasion from the perichondrium, the hypertrophic chondrocytes die through apoptosis, and the osteoblasts brought in by the blood vessels begin depositing bone matrix.

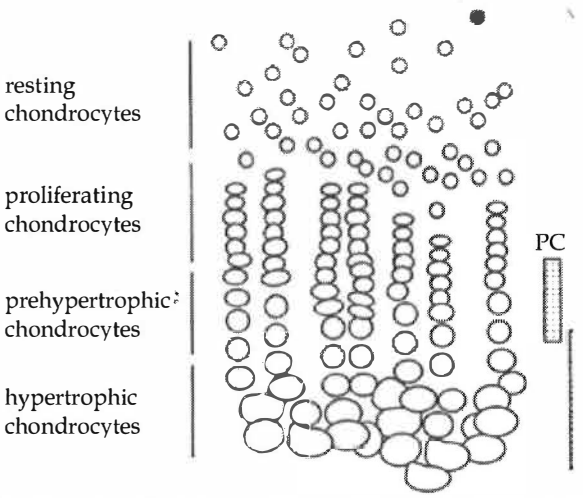

FIGURE 4 Differentiation pathway of cartilage cells at the growth plate. (PC) perichondrium. Modified from (Karsenty 1998).

2.6 Rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common form of inflammatory arthritis and it affects multiple peripheral joints. It is characterized by synovial hyperplasia, immune cell infiltration, cartilage destruction, and bone erosion (Gay et al. 1993, Muller-Ladner et al. 1996, Tak & Bresnihan 2000).

The neuroendocrine and the immune system interact in response to inflammatory stimuli. The HPA axis plays an important role in regulating and controlling immune responses. CRH, one of the main hormones of the axis, is also released outside the brain, at the site of inflammation. Increased levels of CRH are found in RA synovial tissue (tissue lining the joint capsule) and modulation of CRH-mediated signaling is an important mechanism regulating inflammatory events (McEvoy et al. 2002). Inflammation causes activation and proliferation of the synovial lining, expression of inflammatory cytokines and chemokine-mediated recruitment of additional inflammatory cells. A cycle of altered cytokine and signal transduction pathways and inhibition of programmed cell death contribute to synoviocyte- and osteoblast-mediated cartilage and bone destruction. Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are considered to be the key cytokines in the development of RA.

Previously Nurr1 has been shown to regulate *CRH* expression in human inflammatory joint disease (Murphy et al. 2001). Therefore, synovial Nurr1 regulated by locally produced cytokines, may be a general mediator of an autocrine regulatory inflammatory cascade that serves to amplify the inflammatory response by increasing synovial *CRH* expression.

3 RESEARCH AIMS

Since the late 1980s, four general experimental approaches have been adopted in attempts to elucidate the physiological pathways regulated by orphan receptors. The first is to establish the temporal and spatial patterns of expression of transcripts during development and in adult tissues. If restricted, this pattern provides valuable information as to which developmental or physiological processes an orphan receptor is likely to influence and which areas are likely to be adversely affected by orphan receptor ablation. The second approach is to exploit the similarity of the DBD of NRs to predict *cis*-acting core motifs that bind to specific orphan receptors and mediate transactivation of synthetic target genes in cell-based transactivation assays. The third approach is screening strategies to identify ligands that regulate the activity of specific orphan receptors. This approach is not suitable, however, to the analysis of all receptors (including the NR4A subfamily) due to their ability to regulate transcription in a ligand-independent manner. The fourth approach is to examine the physiological consequences of the ablation of specific orphan receptors by gene targeting using homologous recombination. In addition, including a reporter gene in the targeting vector allows detection of the expression of the targeted gene in heterozygous mice. Therefore, to gain more insight into the role of Nor-1 protein, we generated nor-1^{lacZ} mice.

The specific research aims were:

- To establish the spatiotemporal expression pattern of *nor-1* during murine development.
- To establish the consequences of Nor-1 protein ablation *in vivo*.
- To identify new pathways for Nor-1 function.

4 SUMMARY OF MATERIALS AND METHODS

A more detailed description of the materials and methods can be found in the original publications.

4.1 Generation of the nor-1 knock-out mice (I)

The targeting vector was constructed in the plasmid pKos (Stratagene), with a 5.2 kb cassette containing in the 5′→3′ direction an internal ribosomal entry site (IRES), β-galactosidase gene and a neomycin resistance cassette flanked on its 5′-side by the Mc-1 promoter. The targeting vector was inserted into exon 2 of the *nor-1* gene replacing the region encoding amino acids 212-231 located upstream of the DNA-binding domain of the Nor-1 protein. The targeting vector was linearized at its 3′-end and used to electroporate 129SvEv embryonic stem (ES) cells. Two independent targeted ES cell clones were used to generate chimeric mice. Both clones yielded chimeric mice that contributed to the germline.

All protocols involving the use of animals for the following experiments were in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and Baylor College of Medicine Institutional Animal Care and Use Committee.

4.2 Staining for β-galactosidase (I, II)

Whole embryos and P1 heads were fixed for 30 minutes in 2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 in phosphate-buffered saline (PBS) (E10.5-E12.5) or in 2% paraformaldehyde for 2 hours (E13.5-P1) and washed in PBS. The tissues were then incubated overnight (o/n) in an X-gal staining solution at 37°C. After staining, the samples were washed with PBS, re-fixed in 10%

formalin o/n, dehydrated in graded ethanols, cleared in histoclear and embedded in paraffin. Sections were cut at 6 µm and counter-stained with nuclear fast-red. Frozen 20 µm sections were fixed in 2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 in PBS and stained in X-gal staining solution 2-3 hours at room temperature (RT).

4.3 TUNEL assay for apoptosis (I, II)

The heads of embryos and adult brains were fixed in 10% formalin o/n, processed to paraffin and cut at 6 µm. For staining, the slides were rehydrated and digested with Proteinase K (40 ng/ml) for 7 minutes. After washes with PBS, the sections were blocked with TdT buffer and incubated with TdT/dUTP mix at 37°C for 1 hour. The signal was detected using Vectastain Elite and peroxidase substrate kits (Vector). Methyl green was used as a counter-stain.

4.4 Immunohistochemistry (I, II, III)

The tissues were fixed in Bouin's fixative o/n, washed in 70% ethanol, processed for paraffin sectioning and cut to 6 µm. For E-cadherin staining, heads were fixed in 4% paraformaldehyde for 2 hours, cryoprotected in 30% sucrose o/n, embedded in O.C.T embedding medium (VWR), and cut to 7 µm. For phalloidin detection, heads were fixed in 4% paraformaldehyde, and the inner ears dissected and processed as whole-mounts. The specimens were stained according to standard avidin-biotin immunohistochemical procedures (Vector laboratories). Primary antibodies were against PCNA (1:1000, Santa Cruz), E-cadherin (1:1200, Calbiochem), phalloidin-fluorescein isothiocyanate (FITC) (2 µg/ml, Sigma) calbindin (1:500, Calbiochem) and calretinin (1:500, Calbiochem). Secondary antibodies included goat anti-mouse IgG (PCNA), goat anti-rabbit IgG (calbindin and calretinin) and FITC-conjugated goat anti-rat IgG (E-cadherin) from Vector laboratories and Sigma-Aldrich.

4.5 In situ hybridization (I, II)

Sections for radioactive and dioxygenin in situ hybridization were fixed in Bouin's fixative and in paraformaldehyde, respectively. The *nor-1* probe was prepared by reverse transcriptase PCR-amplifying nucleotides 1089-1518 according to rat cDNA (accession #D38530). The primers used were 5' GCCCAGCACCTCCATGTACTTC and 3'CAGCAGGCTGGACGCGGTAGGG. The resulting 430-bp fragment was cloned into vector pCRII (Invitrogen).

4.6 Timm's sulphide silver staining

20 μm frozen sections were thawed for 10 minutes and rinsed in PBS for 5 minutes, then incubated in 0.4% sodium sulfide for 15 minutes at 4°C. After fixation with 4% paraformaldehyde for 15 minutes, the slides were rinsed with PBS and dehydrated in graded ethanols. After drying for 1 hour, the slides were stained for 40 minutes in the dark in the staining solution (240 ml 25% gum Arabic with 10.25 g citric acid, 9.45 g sodium citrate in 30 ml ddH_2O, 3.73 g hydroquinone in 60 ml ddH_2O and 2 ml of 0.51 g silver nitrate in 3 ml ddH_2O). After staining, slides were washed with dH_2O, counter-stained with hematoxylin-eosin, dehydrated and coverslipped.

4.7 Alcian blue/Alizarin red staining

After removing the skin and viscera, the animals were fixed in 95% ethanol before staining in ethanolic Alcian blue (Sigma) acidified with glacial acetic acid. After rinsing, they were counterstained with Alizarin red (Sigma) and cleared for storage in 2% KOH glycerol as described (Hogan 1994).

4.8 Induction of limbic seizures (I)

Kainic acid (KA) (Sigma) was dissolved in saline solution and administered intraperitoneally (i.p.) at 15 mg/kg or 25 mg/kg body weight. After KA injection, seizure activity was observed for 45 minutes and scored by a blinded observer according to a previously described scoring method (Morrison et al. 1996). For induction of nurr1 and nur77 mRNA in the hippocampus, KA was injected at 20 mg/kg bodyweight and mice were sacrificed 30 minutes after the first tonic clonic convulsion.

4.9 Open field test (III)

The open-field domain consisted of a square area (43 cm X 43 cm) surrounded by Plexiglass walls with a lighted field in a room with otherwise standard lighting. By means of eight photoreceptor beams on each side of the test area, which divided the field into 16 quadrants, the activity of an animal was determined and recorded. The animal was released in the center of the field and allowed to roam the open field for 15 minutes. Activity was recorded from the number of photo-beam disruptions in each quadrant to give the total distance traveled and center:total activity ratio.

4.10 Rotorod (III)

The test was performed on an accelerating rotorod apparatus (Ugo Basile) with a 3-cm diameter rod starting at an initial rotation of 4 RPM accelerating to 40 RPM over 5 minutes. Mice were tested for the time spent on the rod during each of four trials per day, for 3 consecutive days.

4.11 Light/dark exploration (III)

The light/dark and mirror boxes consisted of a polypropylene chamber (44x21x21 cm) unequally divided into two chambers by a black partition which had a small opening. The large chamber was open and brightly illuminated. Initially the mouse was placed in the illuminated side and allowed to move freely between the two chambers for 10 minutes. The time taken to enter, number of entries and time spent in the dark/mirror chamber were recorded.

4.12 Resistance-to-capture (III)

The resistance to being picked up was measured on a 7-point resistance-to-capture scale: 0= easy to pick up, 1=vocalizes or shies away from hand, 2= shies away from hand and vocalizes, 3= runs from hand, 4= runs away and vocalizes, 5= bites or attempts to bite, 6= launches jump attacks.

4.13 Fear conditioning (III)

Fear conditioning and testing was performed in a standard conditioning chamber placed inside a noise-reducing solid cabinet with a one-way peephole used for inconspicuous observation. On the training day, the mouse was placed in the conditioning chamber and after a two-minute acclimation period, a conditioning stimulus consisting of a continuous 30-second tone (2.8 kHz, 80 Db) was initiated. The last second of the tone was paired with the unconditioned stimulus which consisted of a 1 mA foot shock. Conditioning was assessed and quantified by observing freezing behavior, defined as a complete lack of movement of the animal, determined by direct observation of the animal every 5 seconds. Contextual fear conditioning was assessed 24 hours following the fear conditioning session by observing the extent of freezing for 5 minutes following the reintroduction of the mouse into the conditioning chamber. Associative fear conditioning was assessed by placing the mouse in an empty plastic cage with the addition of a novel odor (vanilla extract), light and

other visual cues. Baseline freezing was measured for 3 minutes before tone delivery, and learning was determined by the extent of freezing through 3 minutes of continuous tone.

4.14 Passive avoidance (III)

Mice were placed singly in the illuminated side of a two-compartment apparatus. On the mouse entering the dark compartment a door was closed between the two compartments to prevent escape and a mild electric shock was given at an intensity of 0.5 mA. The retention test took place 24 hours after training with a maximum latency of 300 seconds.

4.15 Electrophysiology (III)

Adult mice were sacrificed and the brains rapidly removed and briefly submerged in ice-cold cutting saline. All solutions were saturated with 95% O_2 and 5% CO_2. Hippocampal slices (400 µm) were equilibrated in a 50% cutting saline, 50% ACSF solution at RT for a minimum of 30 minutes. Slices were transferred to an interface chamber supported by a nylon mesh and allowed to recover for a minimum of 1 hour prior to recording. Extracellular field recordings were obtained from the area CA1 and CA3 stratum radiatum. Stimulation was supplied with a bipolar teflon-coated, platinum electrode and recording was obtained by means of a glass microelectrode filled with ACSF. Tetani used to evoke CA1 LTP consisted of one set of stimuli. Each set consisted of two trains of 100 Hz frequency stimulation for 1 second with each train separated by a 20-second interval. Stimulus intensities were adjusted to give pEPSPs with slopes that were ≤50% of the maximum determined from an input /output curve. The 50% maximum stimulus intensity was used in all the LTP experiments. Experimental results were obtained only from those slices which exhibited stable baseline synaptic transmission for a minimum of 30 minutes before the LTP-inducing stimulus was administered.

4.16 Statistical analysis (I, II, III)

Data collected from each experimental group are expressed with standard error of mean (SEM). Student's t test was used in the statistical analysis.

5 SUMMARY OF THE RESULTS

5.1 Generation of the nor-1$^{-/-}$ mice (I, II, III)

The *nor-1* gene was disrupted by the introduction of a *β-galactosidase* reporter gene and a *neomycin* selection cassette downstream of the ATG translation initiation codon and upstream of the DNA-binding domain of Nor-1. Two independent targeted ES cell clones were used to generate chimeric mice. Both clones yielded chimeras that contributed to the germline (I, Fig. 1).

To confirm that the *β-galactosidase* gene introduced into the *nor-1* locus reports the correct spatiotemporal expression of *nor-1* in heterozygote mice, we compared β-galactosidase staining to that of *nor-1* mRNA observed by in situ hybridization (I, Fig. 1). These analyses showed similar patterns of expression of both the *β-galactosidase* reporter gene and endogenous *nor-1* mRNA transcripts.

Heterozygote nor-1 mice (nor-1$^{+/-}$) were indistinguishable from wild-type animals, and mating between nor-1$^{+/-}$ mice resulted in viable nor-1$^{-/-}$ animals. Genotype analysis indicated that nor-1$^{-/-}$ mice were born in expected Mendelian ratios.

While the adult nor-1$^{-/-}$ mice were normal in gross appearance, a partial bidirectional circling (I), seizure susceptibility (II) and increased fear behavior (III) was observed.

5.2 Development and function of the inner ear in nor-1$^{-/-}$ mice (I)

5.2.1 Expression of *nor-1* in the inner ear

The bidirectional circling behavior observed in the nor-1$^{-/-}$ mice is characteristic of mouse mutants with functional defects in the vestibule of the inner ear (Deol 1968, Lyon 1996). Therefore, we examined whether *nor-1* is expressed during

development of the vestibular system by using β-galactosidase as a reporter of *nor-1* gene expression in the heterozygous nor-1 mice (I, Fig. 1).

The earliest expression of *nor-1*^{lacZ} during vestibular development was detected in the otocyst at E11.5 (I, Fig. 2B). This timing coincides with the first appearance of the bilayered outpocketings that give rise to the semicircular canals (SCCs). By this time, specification of the regions that will give rise to the sensory (marked by *BMP-4* expression) and non-sensory epithelium has also occurred. The non-overlapping expression pattern of *nor-1* and *BMP-4* at this stage shows that *nor-1* was specific to the non-sensory epithelium of the developing inner ear (I, Fig. 2D,E). The expression of *nor-1* at E12.5 was localized in the SSC-forming fusion plates (I, Fig. 2F) in a pattern that overlaps that previously observed for *netrin-1*. By E13.5 the shape of the inner ear resembles that of an adult. At this stage and onwards, the expression of *nor-1* in the semicircular ducts (SCD) was limited to the inner edge (I, Fig. 2G). In addition, *nor-1* expression was seen in the non-sensory epithelial wall of the ampulla (SCC sensory organ) (I, Fig. 2K) and utricle at P1 (I, Fig. 2L).

5.2.2 Morphological defects

The restricted spatiotemporal expression pattern of *nor-1* during formation of the inner ear suggested that Nor-1 may play a role in the morphogenesis of the vestibule. To determine whether morphogenesis of the vestibule was affected in the nor-1$^{-/-}$ mice, membranous labyrinths of the inner ear were paint-filled and analyzed as whole-mounts. The overall morphology of the nor-1$^{-/-}$ vestibule seemed unaffected (I, Fig. 3B,D,F). However, the diameter of the SCDs was clearly reduced and their ampullas were severely flattened (I, Fig. 3D,F).

To determine whether the structural abnormalities of the non-sensory epithelium of the vestibule were associated with defects in the sensory areas, the adult hair cell stereocilia were examined by phalloidin staining. No apparent disorganization in the nor-1$^{-/-}$ mice was observed either in the crista ampullaris (I, Fig. 4B) or the macula utriculi (I, Fig. 4D). This is consistent with the fact that these parts of the inner ear do not express *nor-1*.

5.2.3 Decreased continual outgrowth of the semicircular canals

To determine whether the reduction in SCD size was associated with an increase in apoptosis, the inner ears were analyzed by TUNEL staining (I, Fig. 5). No significant difference was detected in the amount of cell death in the SCDs in the nor-1$^{-/-}$ animals compared to their littermates.

In order to examine whether the CSD defect is due to reduced proliferative outgrowth, the proliferative index in these epithelial cells was analyzed by PCNA staining (I, Fig. 6). No difference in the number of proliferating cells between the nor-1$^{-/-}$ and control SCDs was observed at E13.5 (I, Fig. 6A,F). However, PCNA staining at E15.5 demonstrated a significant reduction in the amount of proliferating cells in the nor-1$^{-/-}$ mice relative to their littermates (I, Fig. 6B,G). Quantitation of PCNA-positive cells from the

anterior SCDs demonstrated that the decrease in proliferation is first observed at E15.5 and continues through the canal outgrowth phase until P1 (I, Fig. 6S).

Due to the restricted expression of *nor-1* at the inner edge of the semicircular ducts, Nor-1 must regulate continual outgrowth of the SCDs in a paracrine manner.

5.2.4 Loss of cell differentiation

The restricted expression pattern of *nor-1*^{lacZ} in the non-sensory inner edge of the mature SCD identified a previously uncharacterized specification of the non-sensory epithelium. Surprisingly, the intensity of the β-galactosidase staining was decreased by E17.5 in all of the nor-1$^{-/-}$ SCDs examined (data not shown), being barely detectable at P1 (I, Fig. 7D). Interestingly, until E15.5 the *nor-1*^{lacZ}-expressing cells appeared normal and were columnar in shape (I, Fig. 7F); however, at P1 their shape had flattened in the nor-1$^{-/-}$ animals (I, Fig. 7H). Despite the significant alteration in the cellular morphology of the inner epithelium, the expression of *netrin-1*, a marker for the inner epithelium whose expression overlaps with *nor-1* was retained in the nor-1$^{-/-}$ mice (I, Fig. 7O).

Even though the function of these *nor-1*-expressing cells is not known, their tall columnar shape implies they could be polarized with the apical surface facing the endolymphatic fluid. In line with this, E-cadherin, a cell adhesion protein crucial for maintaining the polarity and integrity of epithelial cells, was lost in the nor-1$^{-/-}$ mice at P1 (I, Fig. 7L). The morphological changes observed in the inner canal epithelium of the nor-1$^{-/-}$ mice thus indicated that Nor-1 is necessary for maintaining the polarity of a specialized cell group in the inner SCD epithelium, in addition to regulating the proliferation of neighboring cells.

5.3 Analysis of the hippocampus of nor-1$^{-/-}$ mice (II)

5.3.1 Seizure susceptibility phenotype

On the basis of an observation that some of the adult nor-1$^{-/-}$ mice exhibited brief freezing spells with tonic posturing, we examined the seizure susceptibility of the nor-1$^{-/-}$ mice to a convulsant L-glutamate analog kainic acid (KA) (Morrison et al. 1996) (II, Fig. 1). Within a few minutes of i.p injection of KA (25 mg/kg), mice became motionless and progressed to head bobbing, rigid posture with forelimb extension and falling. However, a clear difference in the susceptibility to KA-induced seizures was observed between the nor-1$^{-/-}$ and nor-1$^{+/+}$ mice. Nor-1$^{-/-}$ mice had a significantly shorter latency to continued convulsions than nor-1$^{+/+}$ mice (II, Fig. 1A) and developed several severe maximal seizures during the 45-minute observation time while most of the nor-1$^{+/+}$ mice developed only 1 tonic clonic convulsion (Fig. 1B). Nor-1$^{-/-}$ mice also exhibited enhanced sensitivity to KA; 60% nor-1$^{-/-}$ mice died after continuous

tonic clonic convulsions, whereas only 1/8 nor-$1^{+/+}$ mouse died. These results indicated an important role for Nor-1 in modulating convulsant stimulus-induced limbic seizure activity.

5.3.2 Expression of *nor-1* in the hippocampus

Seizures are sometimes associated with increases in hippocampal exitability. To determine whether *nor-1* is expressed during hippocampal development, we examined its expression during the embryonic and postnatal stages using β-galactosidase staining of nor-$1^{+/-}$ animals. Staining for nor-1 was observed in the hippocampal primitive plexiform layer at E14.5 (II, Fig. 1C). By P0, *nor-1* expression was observed in the granule cell layer of the dentate gyrus (II, Fig. 1D). The majority of granule cells develop postnatally during the first 3 weeks of life and *nor-1* expression persisted in these cells in the adult hippocampus (II, Fig. 1E). In addition to the granule cell layer, *nor-1* was strongly expressed in the subiculum, in the pyramidal cell layer (CA1-CA3) (II, Fig. 1E) and in the hilar cells of the dentate gyrus, most of which are mossy cells, at P0 and in adult mice (II, Fig. 1D,F).

5.3.3 Histology

One possible explanation for the differential susceptibility of nor-$1^{-/-}$ mice to KA is that deletion of *nor-1* leads to abnormal development of the hippocampus. To test this hypothesis, we examined the integrity of the nor-$1^{-/-}$ hippocampus by hematoxylin-eosin (H&E) staining of coronal brain sections. The hippocampal formation appears grossly normal in the nor-$1^{-/-}$ mice: both the CA1-CA3 fields of the pyramidal cell layer and the granule cell layer of the dentate gyrus are formed (II, Fig. 2B). Examination of the hippocampal subfields at a higher magnification reveals that the nor-$1^{-/-}$ granule cell layer is comparable to the nor-$1^{+/+}$ dentate gyrus (II, Fig. 2D). However, the Ammon's horn does not show a compact pyramidal cell layer in any of the nor-$1^{-/-}$ mice analyzed (II, Fig. 2F,H).

5.3.4 Loss of CA1 pyramidal cells

Because the pyramidal cell layer was not compact (appeared disorganized) in the adult nor-$1^{-/-}$ mice, we examined the development of this hippocampal layer in early postnatal animals at the level of CA1. H&E-stained coronal sections of P0 animals showed that the nor-$1^{-/-}$ CA1 pyramidal cell layer appeared histologically comparable to the nor-$1^{+/+}$ layer (II, Fig. 3A,E). In contrast, by P7 the disorganization phenotype of the CA1 pyramidal cell layer became evident in all of the nor-$1^{-/-}$ animals (II, Fig. 3F) and was enhanced at P14 (II, Fig. 3G). By P21 the extent of disorganization was comparable to that seen in the adult nor-$1^{-/-}$ mouse (II, Fig. 3H).

In addition to clear disorganization of the pyramidal cell layer in nor-$1^{-/-}$ mice, a decrease in the CA1 neuronal cell numbers was observed. By P7 nor-$1^{-/-}$

mice showed decreased numbers of CA1 pyramidal neurons attaining significance by P14 in all of the nor-1$^{-/-}$ mice examined (II, Fig. 3K).

To confirm that the cell loss in the CA1 pyramidal cell layer was due to increased apoptosis, a TUNEL assay was performed. More TUNEL–positive cells were detected in the CA1 pyramidal cell layer of the nor-1$^{-/-}$ mice than nor-1$^{+/+}$ control mice at birth and clearly at P5 (II, Fig. 3I,J,L). From P7 forward, only occasional TUNEL-positive cells were found in sections from both genotypes (data not shown). This result suggests that the reduction in the CA1 pyramidal neurons in the nor-1$^{-/-}$ mice was caused by increased cell death. Interestingly, this loss of pyramidal CA1 neurons in the nor-1$^{-/-}$ mice was restricted to the early postnatal stages of development and did not progress with age.

Even though the CA3 field of the pyramidal cell layer also did not form a compact layer in any of the nor-1$^{-/-}$ mice, no cell loss was observed (data not shown), indicating a specific role for Nor-1 in the survival of a subset of CA1 pyramidal cells in the hippocampus.

5.3.5 Defect in the granule cell mossy fiber outgrowth

The mossy fiber system is the excitatory projection by which the dentate gyrus projects to the stratum lucidum of the CA3 that develops during the first postnatal weeks in a rodent (Gaarskjaer 1986). Calbindin is normally expressed in the granule cells, including their dendrite and axonal projections (Seress et al. 1993). Therefore, to examine the integrity of these granule cell projections, we examined the staining pattern of calbindin. Despite retained calbindin immunoreactivity in the dentate molecular layer (dendrites) and granule cell bodies, calbindin staining was reduced in the mossy fibers (axons) in the nor-1$^{-/-}$ mice already at P7 (II, Fig. 4B) and P14 (II, Fig. 4D). The reduction in calbindin staining was clearly evident in the adult nor-1$^{-/-}$ mice (II, Fig. 4F). This reduction in mossy fibers in the nor-1$^{-/-}$ mice was confirmed by neuronal process-specific Sevier-Munger silverstaining (II, Fig. 4H). In addition, the mossy fibers were visualized using Timm's sulphide silver staining (Fig. 6). No aberrant fiber growth, either into the granule cell layer or the CA3 pyramidal cell layer was observed in the nor-1$^{-/-}$ mice.

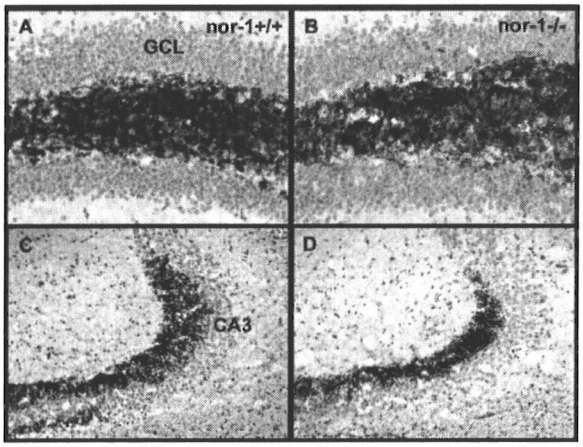

FIGURE 6 Timm's staining at the level of the dentate gyrus (A,B) and CA3 pyramidal cell layer (C,D). GCL: granule cell layer.

5.3.6 Defect in mossy cell associational fiber outgrowth

The mossy cells in the hilus of the dentate gyrus are the main origin of the C/A projections to the IML of the dentate gyrus exciting the granule cells (Frotscher et al. 1991). Since mossy cells and their projections contain calretinin, immunostaining for this calcium-binding protein was used to monitor the main C/A projections. The calretinin–positive axon belt that can easily be identified in the IML of the dentate gyrus in the P14 nor-$1^{+/+}$ mice (II, Fig. 5A) was strongly reduced in the nor-$1^{-/-}$ dentate gyrus (II, Fig. 5B). This reduction in calretinin-positive mossy cell axons was observed also in the adult nor-$1^{-/-}$ mice especially in the suprapyramidal blade (II, Fig. 5D,F).

To determine whether the loss of calretinin staining in the mossy cell axons was accompanied by a loss of mossy cell bodies, the distribution of calretinin-positive cells in sections of ventral hippocampus of adult mice was examined. In contrast to the reduced calretinin immunoreactivity observed in the mossy cell axons innervating the IML of the dentate gyrus, there was no reduction in the number of calretinin-positive cell bodies, most of which are mossy cells, in the nor-$1^{-/-}$ mice (II, Fig. 5H). This suggests a defect specifically in the outgrowth of the mossy cell axons. Commissural fibers from the mossy cells project across the midline and terminate primarily in the infrapyramidal blade of the hippocampus, while majority of the associational fibers innervate the suprapyramidal blade of the ipsilateral side. Because calretinin staining was only slightly reduced in the infrapyramidal blade of the nor-$1^{-/-}$ hippocampus (II, Fig. 5D), indicating retained commissural fiber input, we examined calretinin staining at the midline. Comparison of nor-$1^{+/+}$ (II, Fig. 5I) and nor-$1^{-/-}$ mice (II, Fig. 5J) confirmed that these axons cross the midline, indicating that the defect in mossy cell axon outgrowth mostly affected the associational fibers.

5.3.7 Expression of *nurr1* and *nur77* in the hippocampus

Because expression of *nurr1* and *nur77* in the early postnatal hippocampus has not been described, we examined their expression by *in situ* hybridization. At P5, a time cell loss occurred in the nor-1$^{-/-}$ mice, *nurr1* was expressed in the CA1 pyramidal cells (II, Fig. 6A,B). This suggests that Nurr1 is unable to compensate for Nor-1 function in these cells. Expression of *nur77* was not observed at this time-point (data not shown). However, both genes are expressed in the adult pyramidal cell layer and in a very low amount in the dentate gyrus (II, Fig. 6C,D). Previously, *nurr1* and *nur77* expression has been shown to be induced upon KA injection (Honkaniemi & Sharp 1999). To examine whether this response was normal in the nor-1$^{-/-}$ mice, we injected mice with KA. Both *nurr1* and *nur77* expression in the dentate gyrus was highly induced in the nor-1$^{+/+}$ (II, Fig. 6E,G) and in the nor-1$^{-/-}$ mice (II, Fig. 6F,H).

5.4 Behavioral observations, learning and memory (III)

5.4.1 Heightened fear in nor-1$^{-/-}$ mice

Our observation that deletion of *nor-1* resulted in defects in the postnatal development of the dentate gyrus and CA1 pyramidal cell layer prompted us to examine other hippocampus-mediated functions such as exploratory behavior, learning and memory.

Nocturnal rodents will naturally tend to explore a novel environment. A conflict situation between the tendency to explore and the initial tendency to avoid the unfamiliar occurs when an animal is exposed to a new environment (neophobia). When placed into a novel environment such as an open-field, (III, Fig. 1A), light/dark exploration (III, Fig. 2A-C) or a passive avoidance box (III, Fig. 3B), nor-1$^{-/-}$ mice had increased fear responses. Since no motor impairments in the rotorod test was observed (III, Fig. 1C), it is likely that the nor-1$^{-/-}$ mice showed decreased activity in a novel situation because of heightened fear rather than lack of the coordination required to explore. This was further supported by the increased fear response of the nor-1$^{-/-}$ mice in the resistance-to-capture test that is independent of exploratory behavior (III, Fig. 2D).

5.4.2 Learning and memory

Due to the observed hypoactivity of the nor-1$^{-/-}$ mice in a novel environment, it was difficult for us to evaluate hippocampus-dependent learning and memory processes using standard behavioral testing (III, Fig. 3). At the molecular level, learning and memory formation is thought to occur through changes in synaptic plasticity. Paired pulse facilitation (PPF) and long-term potentiation (LTP) are increases in synaptic efficacy believed to resemble processes underlying short-term and long-term memory formation, respectively. While

examination of the nor-1$^{-/-}$ hippocampus revealed cell death in the CA1 pyramidal cell layer, neither basal synaptic transmission (III, Fig. 4A), PPF (III, Fig. 4B) or LTP (III, Fig. 4C) was affected in these mice. In addition, despite the axonal outgrowth defect observed in the granule cell mossy fibers projecting to the CA3 pyramidal cell layer, basal synaptic transmission (data not shown) and LTP (III, Fig. 4D) was normal at these synapses. In summary, on the basis of the electrophysiological data, nor-1$^{-/-}$ mice exhibited normal hippocampal synaptic function, implying that memory formation may be normal in these mice despite the drastic structural defects.

5.4.3 Loss of calbindin-negative CA1 pyramidal cells

The regulation of intracellular calcium concentration is critical for synaptic transmission and neural plasticity, both of which are generally accepted as underlying memory processes (Baudry 1992, Bliss & Collingridge 1993). It was surprising to us that LTP was normal in the nor-1$^{-/-}$ mice despite the substantial cell loss in the CA1 pyramidal cell layer. Calbindin is a calcium-binding protein strongly expressed in the CA1 field of the hippocampus and is required for maintenance of LTP (Molinari et al. 1996). Therefore, we examined the CA1 pyramidal cell layer by calbindin immunostaining. The number of calbindin-positive cells was unaffected in the nor-1$^{-/-}$ mice (III, Fig. 5) possibly explaining the normal LTP induction in these mice.

5.5 Function of the NR4A subfamily in the skeleton

Extraskeletal myxoid chondrosarcoma is a tumor identified in human patients that appears to originate in the primitive cartilage-forming mesenchyme and is of chondroblast origin (Springfield et al. 1996). Tumorigenesis is associated with cartilaginous differentiation in the form of neoplastic hyaline cartilage. The identification of Nor-1 involvement in a chromosomal rearrangement associated with EMC, together with the fact that *nor-1* (like *nurr-1* and *nur77*) is regulated by the parathyroid hormone in osteoblasts (Ducy & Karsenty 1998, Karsenty 1998, Tetradis et al. 2001a, Tetradis et al. 2001b, Pirih et al. 2003), suggests that Nor-1 may play a role in the skeleton.

5.5.1 Rheumatoid arthritis (IV)

Nurr1 has been previously shown to regulate *corticotropin-releasing hormone* expression in human inflammatory joint disease (Murphy et al. 2001). Therefore, we were interested in the signaling pathways that the NR4A family members may regulate in rheumatoid arthritis.

Nurr1 protein was markedly induced in the human RA synovial tissue (IV, Fig. 1) and its expression in synoviocyte cultures was increased by cytokines IL-1β, TNF-α and PGE_2 (IV, Fig 2-5). Regulation by PGE_2 was CREB-1

dependent (IV Fig. 7) and IL-1β and TNF-α induced NF-κB binding to the *nurr1* promoter (IV, Fig. 6).

To determine whether the inflammatory mediators are capable of regulating the expression of *nurr1* promoter, β-galactosidase reporter plasmids containing proximal promoter regions of the *nurr1* gene were cloned and tested in transfection experiments (VI, Fig. 5). These experiments showed that TNF-α and PGE_2 enhanced the transcriptional activity of the large (-1329/+132) *nurr1* promoter region (VI, Fig. 5A, left panel). Similar to the large *nurr1* promoter, the short (-396/+373) *nurr1* promoter was efficiently induced by PGE_2 but not significantly, by TNF-α treatment (VI, Fig. 5A, right panel). This suggests, that *cis*-acting elements mapping between -1329/-396 are required for the TNF-α-mediated activation and sequences within the short promoter can confer PGE_2 responsiveness to the *nurr1* promoter.

This data provides direct evidence for the involvement of Nurr1 in inflammatory pathways that are central to the pathogenesis of RA. Unlike *nurr1*, inflammatory treatment resulted in rapid but very modest induction of *nor-1* and *nur77* (IV, Fig. 2C), indicating that the major cytokine-regulated member of the NR4A subfamily is Nurr1.

5.5.2 Expression of *nor-1* in the skeleton

The results in Fig. 7 demonstrate that *nor-1* was not expressed in the limb bud mesenchyme during the early patterning stage of limb development (A) but expression initiated at E12.5 (B) in the mesenchyme of the cartilage anlagen of the forelimbs (C). This is coincident with the onset of active chondrocyte differentiation. At E14.5, we observed expression of *nor-1* in the proliferative, prehypertrophic chondrocytes and in the perichondrium (D). The hypertrophic stage of differentiation was associated with a decrease in *nor-1* expression (E). Additional sites of expression were also observed at this stage in the articular region at the end of the cartilage model (F). This region will later give rise to the synovial joints. Expression continued in these regions through E14.5 (G). As development progressed through P1, the differentiated chondrocytes in the diaphysis have undergone apoptosis and are replaced by bone matrix laid down by invading osteoblasts. Our data indicated that *nor-1* expression is lost in bone at P1 but is strongly expressed in the synovial capsule (H).

Taken together, the expression of *nor-1* in the skeleton was localized in the appendicular skeleton (C-G) and ribs (I) but not in cranial bone (B), indicating that Nor-1 is likely to play a role in endochondral rather than intramembranous bone formation.

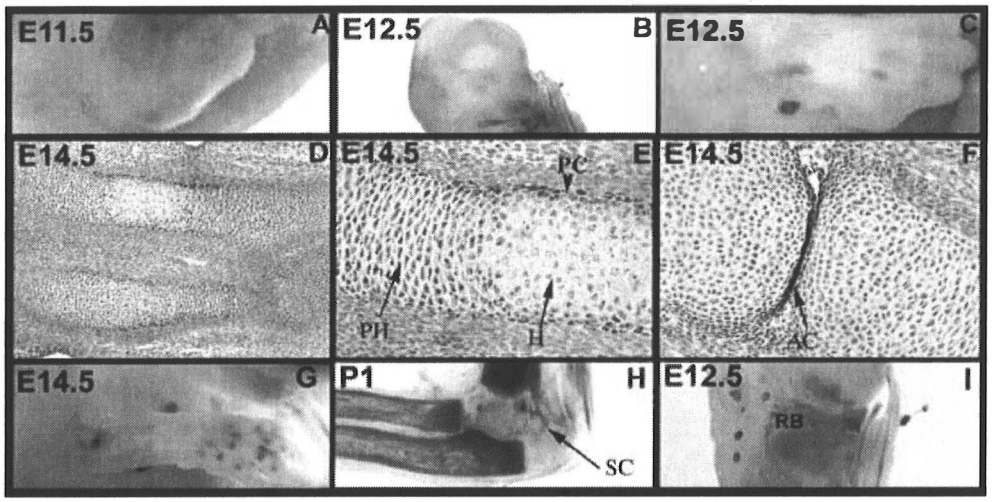

FIGURE 7 Expression of *nor-1* gene during endochondral bone development. Panel A, E11.5 limb bud, B: E12.5 cranial skeleton, C: E12.5 forelimb, D and E: E14.5 forelimb sections showing differentiating chondrocytes in the cartilage model (PH, prehypertrophic chondrocytes, H, hypertrophic chondrocytes, PC, perichondrium), F: E14.5 articular cartilage (AC) at ends of diaphysis, G: E14.5 forelimb, H: synovial capsule (SC) in P1 synovial joint, H: E12.5 ribs (RB).

5.5.3 Examination of the nor-1$^{-/-}$ skeleton

The results of the spatiotemporal analysis summarized above predicted that Nor-1 may play an important role in chondrocyte differentiation during normal endochondral ossification. In order to examine whether ossification was normal in the nor-1$^{-/-}$ mice, P1 skeletons were stained with Alcian blue/Alizarin red staining for cartilage and bone, respectively (Fig. 8). No defect in the level of ossification was observed in the nor-1$^{-/-}$ mice as shown at the level of the forelimb (B) or ribs (D). In addition, there appears to be no defect in the length of the bones, indicating that Nor-1 is not required for endochondral ossification and bone growth.

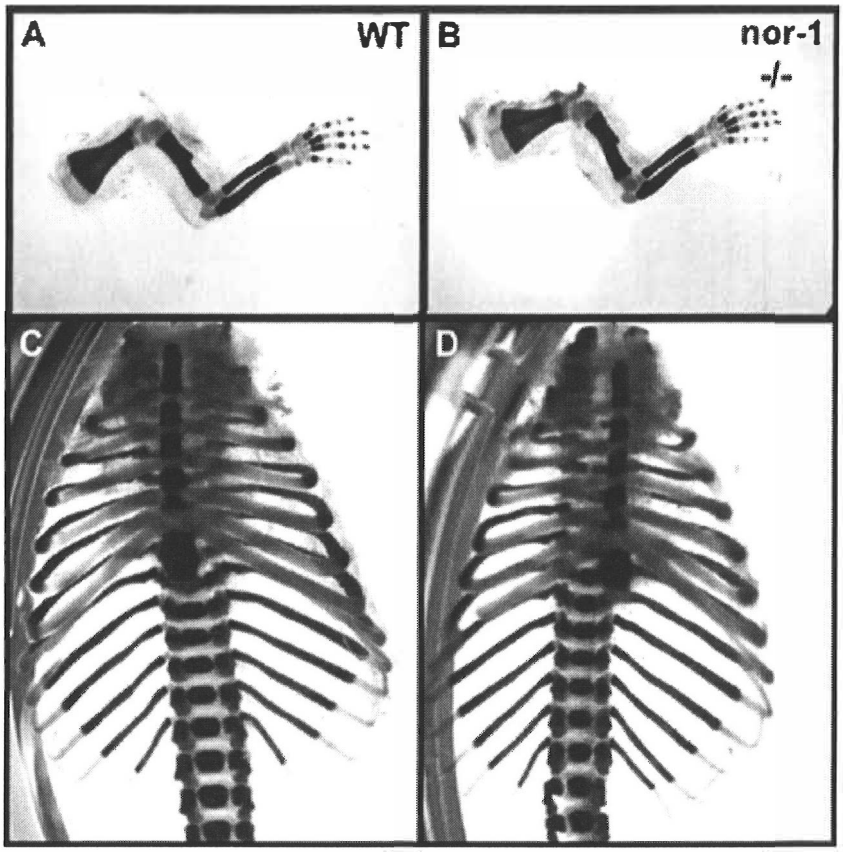

FIGURE 8 Alcian blue/Alizarin red staining of P1 skeletons. Panel A: P1 forelimb WT, B: P1 forelimb nor-1$^{-/-}$, C: P1 ribs WT, D: P1 ribs nor-1$^{-/-}$.

6 DISCUSSION

Cells are constantly exposed to extracellular stimuli that can cause death, support survival, induce or maintain differentiation, and alter cell morphology and synaptic connectivity. Activation of immediate early genes is pivotal to these processes (Herschman 1991). In this project, we have identified immediate early gene *nor-1* essential for cell survival, proliferation, maintenance of cell differentiation and induction of axonal growth.

6.1 Nor-1 as an inducer of proliferation

Human chondrosarcoma tumors represent approximately 10% of all primary malignant bone tumors (Springfield et al. 1996). EMC is one subtype of chondrosarcomas, first described as a rare soft tissue tumor occurring primarily in the musculature that appears to originate in the primitive cartilage-forming mesenchyme of chondroblast origin (Fletcher & McKee 1985, Fletcher et al. 1986). The molecular mechanisms leading to the formation of EMC are unknown, however the ESW/TEC (human Nor-1) fusion protein found in EMC possibly plays a role in tumorigenesis by inappropriately activating the transcription of genes involved in cell proliferation normally regulated by TEC. In fact, the EWS/TEC fusion protein is approximately 270-fold more active as a transcriptional activator on the NBRE element (Labelle et al. 1999) and it interacts with the same transcriptional coactivators as the native TEC protein (Maltais et al. 2002).

The identification of Nor-1 involvement in a chromosomal rearrangement associated with EMC, together with our localization of *nor-1* expression to proliferative chondrocytes (Results, Fig. 7), predicted that Nor-1 may play a role in chondrocyte proliferation. However, analysis of the nor-1$^{-/-}$ skeleton by Alcian blue/Alizarin red staining did not reveal any defects in bone growth (Results, Fig. 8). This indicates that Nor-1 is not required for the proliferation of chondrocytes during development in mice.

A function for Nor-1 in proliferation in other systems has also been previously suggested (Hedvat & Irving 1995), that is supported by our finding that loss of Nor-1 lead to a decrease in proliferation of the SCDs of the inner ear. Therefore, Nor-1 target genes in the inner ear include those responsible for proliferation.

Unfortunately, only a few molecules possibly regulating proliferation of mouse SCDs have previously been described. Studies in chicken have demonstrated that the outgrowth of SCCs is regulated by a BMP-4 signaling pathway that is mediated by Msx-1 (Chang et al. 1999). Surprisingly, we did not detect transcripts for either *BMP-4* or *Msx-1* during murine canal outgrowth. Instead, the SCD epithelium was positive for *Msx-2* (data not shown). The *Msx-2* expressing area was opposite to that of *nor-1*, but appears in the same region previously reported for *Msx-1* in the chicken, suggesting that Msx-2 may carry out a similar function in mouse SCDs. Interestingly, Msx-2 is an inducer of chondrocyte proliferation in mice (Satokata et al. 2000), indicating that an Msx family member could be a target for human Nor-1 in the pathway leading to EMC.

6.2 Function for Nor-1 in seizure susceptibility

Epilepsy is a group of heterogeneous syndromes affecting over 50 million people worldwide. The development of epilepsy (epileptogenesis) and resistance to current medical therapy are recognized as two of the major problems in epilepsy treatment today (Stables et al. 2002). Animal models have played a fundamental role in gaining an understanding of the physiological and behavioral changes associated with human epilepsy.

Genes known to be linked into epilepsy are categorized to molecules that directly alter membrane excitability or synapse release (ion channels, receptors and transporters) and proteins that regulate the developmental assembly of inhibitory and excitatory circuits (TFs, intracellular signaling molecules, regulators of proliferation, migration and cell death) (Noebels 2003b). The majority of genes discovered to date for human epilepsies are inherited disorders of ion channels. The identification of Nor-1 as an essential regulator of developmental pathways that ultimately control epileptogenic activity suggests that identification of Nor-1 target genes whose expression is disrupted in the hippocampus of nor-1$^{-/-}$ mice may provide a novel therapeutic point of intervention to control seizure activity.

Normal function of the hippocampus is dependent on the proper development of its intrinsic circuitry. Dentate gyrus hilar mossy cells send out axons to the IML and make excitatory synapses on proximal dendrites of granule cells. In turn the mossy cells receive excitatory synapses from the granule cell axons, the mossy fibers. The granule cells also exite the CA3 pyramidal cells in the stratum lucidum (Paxinos 1994). This positive feedback system is considered to have special implications regarding hyperexcitability

and seizures in both human and animal models of TLE (Cronin & Dudek 1988, Sutula et al. 1989, Houser et al. 1990, Babb et al. 1991, Mello et al. 1993, Lemos & Cavalheiro 1995, Coulter et al. 1996, Wuarin & Dudek 1996). The loss of proper circuitry in the nor-$1^{-/-}$ mice between the mossy cell→granule cell→CA3 pyramidal cell pathway could produce this kind of abnormal hyperexcitability and the development and spread of limbic seizure activity. To explain the defects in the granule cell and mossy cell axons in the nor-$1^{-/-}$ mice, Nor-1 target genes may involve guidance cues and/or signaling pathways within the growing axon that promote axonal outgrowth.

In fact, a fundamental issue in developmental neurobiology is how neurons establish precise connections to distant target cells. To date several proteins, such as the Semaphorin, Netrin, Ephrin and Slit families have been identified as playing critical roles in axonal guidance (Skutella & Nitsch 2001). However, the mechanisms responsible for guiding granule cell and mossy cell fibers to their appropriate synaptic targets are not well understood.

In the mammalian central nervous system, the hippocampal mossy fibers have received a lot of attention because epileptic conditions cause aberrant mossy fiber sprouting. The ability of granule cell mossy fibers to establish their connections in the stratum lucidum of the hippocampal area CA3 is developmentally regulated. Early in development, mossy fibers may use guidance cues expressed by hippocampal neurons to establish appropriate innervation of CA3. In contrast, mossy fibers formed later in development appear to find their synaptic targets using axon guidance cues whose expression pattern is dependent on the presence of existing mossy fibers (Nguyen et al. 1996, Cremer et al. 1997, Hastings et al. 2002).

Even though the molecular pathways for mossy fiber outgrowth are not characterized to date, both attractant and repellent cues appear to guide their lamina-appropriate outgrowth. One repellent cue to stay away from inappropriate targets is received from the CA1 pyramidal cell region, possibly by a signaling cascade involving cAMP (Mizuhashi et al. 2001). In addition, mouse KO models that have defects in mossy fiber outgrowth include the limbic-system-associated membrane protein (Pimenta et al. 1995) and NCAM-deficient mice (Cremer et al. 1997). In these KO models mossy fibers do not receive repellent cues from the CA3 pyramidal cell layer resulting in aberrant mossy fiber growth into the pyramidal cell layer. In contrast to this, even though mossy fibers are reduced in the nor-$1^{-/-}$ mice they show lamina-appropriate termination in the stratum lucidum of CA3 (II, Fig.4). This indicates that in the CA3 pyramidal cells, Nor-1 does not function in the pathway repelling ingrowing mossy fibers.

The CA3 pyramidal cell layer provides also an attractant cue for ingrowing mossy fibers (Mizuhashi et al. 2001). Therefore, the lack of Nor-1 protein involvement in the attractant pathway in the CA3 pyramidal cell layer may result in the defects observed in mossy fiber outgrowth.

The commissural/associational (C/A) fibers from the mossy cells of the hilus terminate in the IML and entorhinal fibers in the outer molecular layer of the dentate gyrus, respectively. Several studies indicate that entorhinal afferents

are guided to their correct termination zone by Cajal-Retzius cells that are transiently present within the outer molecular layer during development (Del Rio et al. 1997, Super et al. 1998, Ceranik et al. 1999, Ceranik et al. 2000). Unlike the entorhinal fibers, the C/A fibers arrive late in development, when granule cell dendrites are already in place and could present signals for these ingrowing fibers (Cowan et al. 1980, Bayer & Altman 1987, Soriano et al. 1994). In fact, evidence from the reeler mouse mutant suggests that growing C/A fibers receive unidentified postsynaptic cues from the granule cells (Stirling & Bliss 1978, Stanfield et al. 1979, Deller et al. 1999, Drakew et al. 2002, Gebhardt et al. 2002). In the nor-1$^{-/-}$ mice the loss of mostly associational fibers was evidenced by the absence of calretinin staining in the IML of the suprapyramidal blade. This suggests that Nor-1 may provide an attractant guidance signal for these ingrowing axons from the granule cells (II, Fig.5).

In addition to classical guidance cues, the cadherin family mediates axonal navigation, axon target recognition, and synapse formation during brain development (Shapiro & Colman 1999). Both N-cadherin and E-cadherin amounts are differentially regulated in the hippocampus after pilocarpine-induced seizures, indicating that they may play a role in mossy fiber sprouting (Fasen et al. 2002). In addition, E-cadherin immunostaining was upregulated in the pyramidal cell neurons that survived pilocarpine treatment, suggesting that E-cadherin may support survival of these cells. This is an interesting observation because we established that loss of cell polarization in nor-1$^{-/-}$ SCDs was accompanied by loss of E-cadherin staining (I, Fig. 7) and the early postnatal death of CA1 pyramidal neurons in the nor-1$^{-/-}$ mice. Importantly, the E-cadherin promoter has a binding site for Nor-1 (NBRE). Therefore, Nor-1 may regulate cell identity/survival by directly regulating *E-cadherin* expression. In addition, Msx-1 and Msx-2 (a possible Nor-1 target in the SCDs) also have been implicated in the regulation of cadherin-mediated cell adhesion and cell sorting (Lincecum et al. 1998).

6.3 Redundancy between members of the NR4A subfamily

In the normal brain hippocampus is a region known to be essential for learning and memory. Based on our electrophysiological data, nor-1$^{-/-}$ mice appear to have normal paired-pulse facilitation (short-term memory) and long-term potentiation (long-term memory) both at the Schaffer collateral (CA3-CA1) and mossy fiber (granule cell-CA3) synapses (III, Fig.4). However, the precise role of Nor-1 in learning and memory remains elusive due to the novelty-induced hypoactivity observed in the nor-1$^{-/-}$ mice. Hippocampal deficits are often specifically reflected by reduced performance in the spatial learning task of the Morris water maze (Morris et al. 1982, Morris & Frey 1997). Unfortunately, due to deficits in the swimming ability observed in some of the nor-1$^{-/-}$ mice (I) we were unable to reliably perform this test either.

Impairment of open-field exploration behavior has previously been identified in other mouse models with affected hippocampal architecture such as PLC-β1 and CHL1 (Bohm et al. 2002, Montag-Sallaz et al. 2002). However, the nor-1$^{-/-}$ mice exhibited not only reduced exploratory activity, but increased fear (III, Fig.2 and 3B). Fear sensitization is thought to arise from hyperexcitation of fear circuits in the brain (Adamec 1997, Rosen & Schulkin 1998). Both the amygdala and hippocampus are known to participate in the formation of fear memories (Cahill & McGaugh 1998, LeDoux 2000, Pitkanen et al. 2000). Behavioral stress as well as amygdalar stimulation interferes with synaptic plasticity in the hippocampal formation (Ikegaya et al. 1995, Frey et al. 2001). Therefore, we cannot exclude possible involvement of the amygdala in the observed fear behavior in the nor-1$^{-/-}$ mice. However, the substantial developmental defects of the hippocampus are likely to be the underlying cause of the behavior.

The major challenge to understanding the function of Nor-1 *in vivo* is the redundancy that exists between the NR4A subfamily members. This is a common theme among members of gene families (reviewed in Tautz 1992). The lack of a phenotype in learning or memory in the nor-1$^{-/-}$ mice may be due to overlapping expression patterns of the NR4A subfamily members in the hippocampus suggesting that they may function redundantly in regulating gene expression in the hippocampus. In fact, learning of a spatial food search task is accompanied by elevated levels of *nurr1* mRNA in the hippocampus (Pena de Ortiz et al. 2000). Nurr1 was however, unable to compensate for Nor-1 in survival of the CA1 pyramidal cell layer in the early postnatal stages (II, Fig.6B). In addition, transgenic mice carrying dominant negative Ca^{2+}/calmodulin-dependent kinase IV (CaM-KIV) are impaired in hippocampus-dependent memory, and interestingly, both *nor-1* and *nur77* are induced by CaM-KIV *in vitro* (Inuzuka et al. 2002).

Structural components of the extracellular matrix, growth and differentiation factors, signaling molecules and transcription factors are important for proper morphogenesis during endochondral ossification (Erlebacher et al. 1995). Signals that control skeletal development and maintenance include the TGF-β superfamily, especially the BMPs. The rate of chondrocyte differentiation has to be strictly regulated both temporally and spatially so that proper shape and length of the bone is achieved. The first factor shown to control chondrocyte differentiation was the parathyroid hormone-related protein (PTHrP). PTHrP$^{-/-}$ mice show premature differentiation into hypertrophic chondrocytes associated with dwarfism (Karaplis et al. 1994). Therefore, PTHrP keeps chondrocytes in the proliferative pool and delays chondrocyte hypertrophy via the PTHrP receptor, a G-protein-coupled receptor that acts via the cAMP pathway (Karaplis et al. 1994, Lanske et al. 1996). The perichondrium functions during chondrogenesis to regulate the rate of chondrocyte differentiation, and its removal results in increased hypertrophic differentiation. PTHrP function in the perichondrium is regulated by *Indian hedgehog* expressed in the prehypertrophic and hypertrophic chondrocytes (Bitgood & McMahon 1995).

Despite strong *nor-1* expression in the perichondrium and prehypertrophic chondrocytes, no apparent defect was found in the differentiation of the skeleton in nor-1$^{-/-}$ mice (Results, Fig.8). This lack of phenotype in the nor-1$^{-/-}$ skeleton, neither in proliferation nor differentiation, may indicate a redundancy in function between the NR4A family members. As mentioned, *nor-1*, like *nurr-1* and *nur77* are all regulated by the parathyroid hormone in osteoblasts. In addition, even though a detailed expression pattern of *nurr1* is not available, *nurr1* is expressed in the developing limb (Zetterstrom et al. 1996). Redundancy between these family members during skeletal development can be addressed by further analysis of their expression pattern and analysis of the skeletons in the compound KO mice.

NR4A family members are coexpressed in a variety of tissues. It appears that one of the members may be preferred in a particular pathway. For example, on the basis of our observation of the inflammatory pathway, although all three members are present in primary rheumatoid arthritis and normal synoviocyte cells, only Nurr1 is markedly induced by proinflammatory mediators such as TNF-α (IV, Fig.2C). Rheumatoid arthritis has a prevalence of 0.5-1.5% in industrialized nations. The precise cause of this disease remains unknown and there is no cure. Treatment is currently focused on reducing the pain, inflammation and damage caused by it. On the basis of our results, modulation of Nurr1 activity may be an additional target for the development of therapy in human rheumatoid arthritis.

In our laboratory we have also crossed heterozygous nor-1 and nur77 mice (received from Dr. Jeffrey Milbrandt, Washington University) to obtain nor-1/nur77 double KO mice. These mice rapidly developed myeloid leukemia, which resulted in 100% lethality prior to the fourth week of life. This leukemia is characterized by anemia, leukocytosis with blast crisis, and myeloid infiltration into non-hematopoietic tissues such as the liver and lung. It is associated with increased proliferation of a subset of myeloid progenitors (unpublished observations by Shannon E. Mullican). This dramatic phenotype in the nor-1KO/nur77KO mouse model provides strong evidence that Nor-1 and Nur77 play a redundant and very critical tumor suppressor role in murine development.

Finally, the identification of Nor-1 as a cellular survival factor, regulator of axonal growth and as a potential target for anti-cancer drug development underscores the importance of further understanding the molecular mechanisms of Nor-1 function.

6.4 Methodical aspects

The observation that some of the nor-1$^{-/-}$ adult mice exhibited freezing behavior that may be considered as spontaneous seizures lead us to examine the integrity of the hippocampus in these mice. Spontaneous seizures may be brief, infrequent, age-dependent or behaviorally silent and therefore difficult to detect

on the basis of observation alone (Noebels 2003a). In addition, the lowered threshold for KA-triggered seizures does not confirm that the nor-1$^{-/-}$ mice were epileptic. Therefore, to confirm the existence and extent of spontaneous seizure activity in the nor-1$^{-/-}$ mice, electroencephalograph analysis of the hippocampus will be necessary.

Timm's staining recognizes the high concentration of zinc in mossy fiber terminals, and is generally acknowledged to be the most reliable method for identifying mossy fibers. Therefore, in addition to calbindin staining of mossy fibers (II, Fig.4), we used Timm's staining to confirm the reduction of mossy fibers in the nor-1$^{-/-}$ mice (Results, Fig.6). Two main types of morphological change that might contribute to seizure-prone state are neuronal loss and axonal reorganization of neurons (Houser 1999). Both types of alterations may lead to abnormal circuitry within the affected brain region. Timm's staining can reveal aberrant mossy fiber growth into the IML of the dentate gyrus observed frequently in the brains of epileptic patients and in animal models of epilepsy. Interestingly, despite reduced outgrowth of the mossy fibers to the CA3 field of the hippocampus in the nor-1$^{-/-}$ mice, no aberrant outgrowth to the IML was observed. Therefore, the cell loss and decreased fiber outgrowth in the dentate gyrus, possibly resulting in abnormal circuitry within the hippocampus, are likely to result in the seizure susceptibility phenotype even though the exact mechanism cannot be established conclusively.

The majority of ventral hilar mossy cells are calretinin-positive and the vast majority of calretinin-positive cells in the ventral hippocampus are mossy cells (Blasco-Ibanez & Freund 1997, Fujise et al. 1998). Some interneurons in the hilus may also be calretinin-positive; however, these cells are a small minority and their exact identity has not been established. Therefore, it is possible that some of the *nor-1*-expressing cells in the hilus are in fact interneurons and not only mossy cells.

Ideally, the whole CA1 field of the hippocampus should be counted to analyze the extent of cell loss in the nor-1$^{-/-}$ mice. However, the CA1 and CA2 subfields are difficult to distinguish from each other. In addition, the hippocampal pyramidal cell layer increases in size in a dorso-ventral direction. Therefore, it is difficult to obtain sections at identical positions. Because of the lack of expertise in hippocampal histology, we opted for a representative cell count from a specific area of the CA1 field (the arch).

7 CONCLUSIONS

The present study aimed at understanding the physiological function of the orphan nuclear receptor Nor-1. To elucidate the possible systems where Nor-1 may function, *nor-1* expression was analyzed during embryogenesis (data not shown). The expression profile suggested that Nor-1 may play a role in early central nervous system development. However, examination of the nor-1$^{-/-}$ brain did not reveal any embryonic brain defects (data not shown). This could be the result of an absence of Nor-1 developmental function or a functional redundancy with Nurr1. A more detailed analysis of the spinal cord and midline crossing axons, however, is required due to the non-overlapping expression pattern of *nor-1* and *nurr1* in the spinal cord (Zetterstrom et al. 1996). Instead, the nor-1$^{-/-}$ mice, which showed neurodegeneration and neuronal hyperexcitability, offer an alternative approach with which to investigate the molecular pathways that can trigger axonal growth and firing, cell survival and proliferation.

The main conclusions of the study are:

Nor-1 is expressed in the

- developing inner ear; non-sensory epithelium of the semicircular canals and the utricle.
- hippocampus; pyramidal cells and dentate gyrus granule and hilar cells.
- limb; perichondrium, hypertrophic chondrocytes and synovium.

The physiological roles of Nor-1 in mice are

- Induction of proliferation in the developing semicircular ducts of the inner ear by a paracrine manner.
- Maintenance of polarization of non-sensory epithelial cells in the inner edge of the semicircular ducts.
- Regulation of cell positioning in the CA3 pyramidal cell layer of the hippocampus during development.

- Promotion of survival of calbindin-negative CA1 pyramidal cells of the hippocampus during early postnatal development.
- Support outgrowth of dentate gyrus granule cell and mossy cell axons during development.

Possible Nor-1 target genes identified include

- Msx-2.
- E-cadherin.

In addition, the NR4A family members may be expressed in the same tissue, but a certain family member may be preferred in a particular pathway such as Nurr1 in inflammation associated with rheumatoid arthritis.

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YHTEENVETO

Hiiren tumareseptori Nor-1:n toiminnan tutkiminen

Hormonit säätelevät reseptoriensa välityksellä sikiönkehitystä ja monia elimistön keskeisiä fysiologisia perustoimintoja. Reseptorit esiintyvät solun sisällä joko solukalvolla, solulimassa tai tumassa. Steroidihormonit ja D-vitamiini ovat tyypillisiä solunsisäisten reseptoreiden kautta vaikuttavia signaalimolekyylejä, ligandeja. Aktivoitunut reseptori-ligandi -kompleksi sitoutuu dimeerinä kohdegeenin DNA:ssa sijaitsevaan hormonivaste-elementtiin. Tämä saa aikaan muutoksen kohdegeenin ilmenemisessä. Klassisten ligandiasitovien hormonireseptoreiden lisäksi soluissa on useita orporeseptoreita, jotka eivät tarvitse ligandia aktivoituakseen. Tällaisia ovat esimerkiksi tumareseptori 4A (NR4A)-ryhmään luokitellut proteiinit Nur77, Nurr1 ja Nor-1. Näiden kolmen proteiinin aminohapposekvenssit ovat hyvin samankaltaisia, etenkin DNA:ta sitovan alueen kohdalla. Ne ilmentyvät useissa soluissa normaaliolosuhteissa ja aktivoituvat esimerkiksi hypotalamuksessa, aivolisäkkeessä ja munuaisten lisäkuoressa stressin seurauksena. Lisäksi fosforylaation avulla voidaan muuttaa NR4A-proteiinien toimintaa.

Ihmisillä Nor-1-proteiinin aktivoituminen Ewing-sarkoomavirukseen liittyneenä aiheuttaa luustosta lihakseen leviävää syöpää. Kyseisen syövän uskotaan johtuvan Nor-1-proteiinin säätelevien molekyylien yliaktivoitumisesta. Jyrsijöillä *nor-1* ilmentyy sikiönkehityksen aikana, etenkin aivoissa. Täten Nor-1 mahdollisesti säätelee aivojen kehitystä.

Väitöskirjatutkimuksen tarkoituksena oli kartoittaa *nor-1*-geenin ilmentymistä ja toimintaa. Nor-1-proteiinin toimintaa hiiren eri kehitysvaiheissa tutkittiin katkaisemalla *nor-1*-geenin geneettinen tieto homologisen rekombinaation avulla hiiren kantasoluissa (nor-1-poistogeeninen hiiri). Kohdevektorin sisältämän β-galaktosidaasi-reportterigeenin avulla voidaan myös tutkia *nor-1*-geenin ilmentymistä heterozygooteissa hiirissä.

Nor-1-poistogeenisillä hiirillä ilmeni käyttäytymishäiriöitä, jotka ovat yleisiä hiirillä joiden sisäkorvan tasapainoaisti on vioittunut. Ensimmäisen osajulkaisun tutkimuksessa saatiin selville, että *nor-1* ilmentyy sisäkorvan kaarikäytävien sisäosan soluissa sikiönkehityksen aikana. Tasapainohäiriöt aikuisilla hiirillä todennäköisesti johtuvat kaarikäytävien solujen puutteellisesta jakautumisesta ja siitä johtuvasta kaarikäytävien ahtaudesta. Nämä tulokset vahvistavat Nor-1:n merkitystä solujen jakautumisen säätelijänä. Lisäksi kaarikäytävien sisäosien solut, joissa *nor-1* ilmentyy, menettävät epiteelille tyypillisen solumorfologiansa. Todennäköisesti Nor-1 säätelee solujen muotoa ylläpitävää E-cadheriini-adheesioproteiinia.

Tasapainohäiriöiden lisäksi nor-1-poistogeeniset hiiret ovat myös yliherkkiä glutamaattireseptoreita aktivoivan kainaattihapon aiheuttamille kohtauksille; ne saivat useita toonisia kouristuksia ja suurin osa hiiristä menehtyi voi-

makkaisiin lihaskouristuksiin. Toisen osajulkaisun tulosten perusteella herkkyys epileptisiin kohtauksiin selittynee aivojen hippokampuksen kehitysvirheillä. Histologinen tutkimus osoitti, että osa hippokampuksen toiminnan kannalta tärkeistä soluista kuolee ennenaikaisesti pian syntymän jälkeen ja osa hermosolujen aksoneista ei saavuta kohdesolujaan. Samankaltaiset vahingot ihmisillä joko kehitysvammana tai trauman seurauksena aiheuttavat epileptisiä kohtauksia. Epileptisiä kohtauksia syntyy, kun hermoston aktiopotentiaaleja muodostuu hyvin tiheään, toisin kuin fysiologisissa tilanteissa.

Hippokampus toimii muisti- ja oppimiskeskuksena. Näissä toiminnoissa ei kuitenkaan havaittu poikkeavuuksia kolmannen osajulkaisun tutkimuksissa. Käyttäytymistä tutkivissa kokeissa kuitenkin havaittiin, että nor-1- poistogeeniset hiiret olivat hyvin pelokkaita. Ne jähmettyivät paikoilleen uudessa ympäristössä totutun häkin ulkopuolella ja vastustivat kiinniottamista. Tämä piirre kohtausherkkyyden ohella viittaa hippokampuksen toiminnan häiriöihin. Hippokampuksen lisäksi tumajyvänen on tärkeä pelkotiloja säätelevä aivojen alue. Sen vuoksi mahdollista tumajyväsen poikkeavaa toimintaa ei voida poissulkea pelkotilojen aiheuttajana nor-1-poistogeenisillä hiirillä.

Huolimatta siitä, että NR4A-perheenjäsenet ilmentyvät samoissa kudoksissa, niiden toiminta ja aktivaatio saattaa erota toisistaan samassakin solussa. Esimerkiksi tutkiessamme neljännessä osajulkaisussa tulehdusta välittävien molekyylien vaikutusta NR4A-geeniperheen ilmentymiseen, PGE_2 ja TNF-α lisäsivät ainoastaan *nurr1* geenin huomattavaa ilmentymistä.

Vaikka *nor-1* ilmentyy luustossa sikiönkehityksen aikana ja sen yliaktivoituminen ihmisillä johtaa luustosta metastoivaan syöpään, nor-1-poistogeenisten hiirten luut kehittyivät ja luutuivat normaalisti. Tämän perusteella Nor-1 ei vaikuta luuston kehitykseen hiirillä. Toinen mahdollisuus on, että Nurr1 tai Nur77 korvaa Nor-1-proteiinin toimintaa luustossa. Niiden ilmentymistä luustossa ei ole tähän mennessä selvitetty. Perheenjäsenten on kuitenkin havaittu korvaavan toisen proteiinin puuttumisen muutamissa eri kudoksissa, kuten lisämunuaisissa ja T-soluissa.

Tulokset vahvistavat Nor-1-proteiinin mahdollista osuutta syövän synnyssä (solujen jakautumisen säätelijä) sekä aivojen hippokampuksen kehitykseen tarvittavana tekijänä. Tutkimuksen perusteella voidaan selvittää Nor-1-proteiinin säätelemiä geenejä myöhemmissä tutkimuksissa ja verrata näitä esimerkiksi kondrosarkooma-syövässä aktivoituihin geeneihin.

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ORIGINAL PAPERS

I

The nuclear receptor nor-1 is essential for proliferation of the semicircular canals of the mouse inner ear

By

Ponnio, T., Burton, Q., Pereira, F. A., Wu, D. K. & Conneely, O. M.

Mol. Cell. Biol. 22: 935-945, 2002

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II

Nor-1 regulates hippocampal axon guidance, pyramidal cell survival and seizure susceptibility

By

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Manuscript

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III

Increased fear behavior in mice lacking nuclear receptor nor-1

By

Pönniö, T., Weeber, E. J., Sweatt, J. D. & Conneely, O. M.

Manuscript

IV

Activation of nuclear orphan receptor nurr1 transcription by NF-kB and cyclic adenosine 5′-monophosphate response element-binding protein in rheumatoid arthritis synovial tissue

By

McEvoy, A. N., Murphy, E. A., Ponnio, T., Conneely, O. M., Bresnihan, B., FitzGerald, O. & Murphy, E. P

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