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Research paper

Molecular characterization and analysis of the porcine *NURR1* gene

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Abstract

Orphan receptor NURR1 (also termed NR4A2) belongs to the nuclear receptor superfamily and functions as a regulatory factor of differentiation, migration, maturation and maintenance of mesencephalic dopaminergic neurons. NURR1 plays an important role in nigrostriatal dopamine neuron development and is therefore implicated in the pathogenesis of neurodegenerative diseases linked to the dopamine system of the midbrain.

Here we report the isolation and characterization of porcine *NURR1* cDNA. The *NURR1* cDNA was RT-PCR cloned using NURR1-specific oligonucleotide primers derived from *in silico* sequences. The porcine *NURR1* cDNA encodes a polypeptide of 598 amino acids, displaying a very high similarity with bovine, human and mouse (99%) NURR1 protein. Expression analysis revealed a differential *NURR1* mRNA expression in various organs and tissues. *NURR1* transcripts could be detected as early as at 60 days of embryo development in different brain tissues. A significant increase in *NURR1* transcript in the cerebellum and a decrease in *NURR1* transcript in the basal ganglia was observed during embryo development. The porcine *NURR1* gene was mapped to chromosome 15. Two missense mutations were found in exon 3, the first coding exon of *NURR1*. Methylation analysis of the porcine *NURR1* gene body revealed a high methylation degree in brain tissue, whereas methylation of the promoter was very low. A decrease in DNA methylation in a discrete region of the *NURR1* promoter was observed in pig frontal cortex during pig embryo development. This observation correlated with an increase in *NURR1* transcripts. Therefore, methylation might be a determinant of *NURR1* expression at certain time points in embryo development.

Keywords: DNA methylation; NURR1; Parkinson's disease; Pig; SNP; Transcription factor

1. Introduction

NURR1, also named NR4A2, is a member of the nuclear receptor superfamily of transcription factors [1–4]. This group of structurally related transcription factors is involved in programming developmental, physiological and behavioral responses to various chemical signals. NURR1 lacks a ligand-binding cavity and has been classified as a ligand-independent member of the steroid-thyroid hormone-retinoid receptor superfamily [5]. NURR1 is essential for the development and maintenance of dopaminergic neurons in the mesencephalon [3,6–9] and is also crucial for expression of a set of genes such as *SLC6A3*, *SLC18A2*, tyrosine hydroxylase (TH), dopamine transporter (DAT) and dopamine receptor D2 (DRD2), which are essential for development of DA neurons [9–11]. Furthermore, NURR1 knockout mice are not viable and display a dopaminergic neuron (DAN) deficiency in substantia nigra, indicating that NURR1 is responsible for differentiation, migration and maturation of DAN [6]. Also, an age-related decline in dopamine signalling was observed in

Abbreviations: CNS, central nervous system; DAN, dopaminergic neuron; DAT, dopamin transporter; DBD, DNA binding domain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NTD, N-terminal domain; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; SNP, Single nucleotide polymorphism; TSS, transcription start site; UTR, untranslated region.

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NURR1 heterozygous mice [12]. NURR1 also seems to play an important role in inflammation as a key transcriptional regulator of cytokine and growth factor action in several diseases, including age-related diseases (reviewed by McMorrow and Murphy 2011) [13].

The *NURR1* gene was cloned from mice [2,14] and humans [15,16]. The human *NURR1* gene spans approx. 6 kb and is organized into eight exons and seven introns and encodes a multidomain polypeptide composed of 598 amino acids and has a molecular mass of 66 kDa [15]. The first two exons are non-coding, covering the 5′UTR, and the translation start codon is localized in the third exon. The stop codon is found in the 5′ end of exon eight [16,17]. The human *NURR1* gene is localized on chromosome 2q22-q23 [14,16]. Several mutations in the *NURR1* gene, both in the promoter and the coding region, are associated with disorders displaying dopaminergic dysfunction, such as Parkinson's disease, schizophrenia and manic depression [8,18−25].

NURR1 is predominantly and highly expressed in the midbrain dopaminergic neurons [2,3]. At least 11 alternatively spliced transcript variants of human NURR1 have been described (Ensembl), but for the majority of these their biological validity has not been determined.

This study is the first to characterize the pig *NURR1* gene and its cDNA sequence. Since pigs and humans share significant similarities in the CNS [26], we believe that the pig is the best animal model for an investigation of the spatial and temporal expression of the *NURR1* throughout development. Similarities encompass both the size and anatomic characteristics of the cerebrum and cerebellum. Pigs have a gyrencephalic brain which is dominated by white matter and also with similar developmental peaks to those in humans [26]. We here describe the spatial and temporal *NURR1* expression and its possible function in pig organogenesis and development. Also, we present the methylation status of the gene body and the promoter of *NURR1*.

2. Materials and methods

2.1. Ethics statement

Housing of pigs and approval of experimental procedures have been described elsewhere [27]. The pigs were sacrificed by an intravenous injection with 30 mg/kg Pentobarbital.

2.2. Biological subjects

Eight different brain regions were included in this study: cerebellum, frontal cortex, occipital cortex, parietal cortex, temporal lobe, brain stem, hippocampus and basal ganglia. Samples from various peripheral organs were also selected. Biological samples were collected from two one-year old Danish Landrace pigs weighing 125−150 kg. Brain samples from the frontal cortex, cerebellum, brain stem, basal ganglia and hippocampus were collected from Danish Landrace developing embryos at 60, 80, 100 and 115 days of gestation. Three different brain samples (cerebellum, frontal cortex and brain stem) were collected at three time-points, i.e. at 50, 70 and 115 days of fetal development after transfer of cloned Yucatan embryos. Somatic cell nuclear transfer was used to perform the cloning, as described in Ref. [28]. Isolation of donor cells from cultured ear fibroblasts and transfer of cloned embryos was performed as described in Ref. [29].

2.3. Bioinformatic analyses

The ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to identify open reading frame of *NURR1*. Sequence analysis was performed using online software NCBI (http://ncbi.nlm.gov) and Expasy (http://expasy.org). The putative amino acid sequence was deduced using the Expasy translate tool (http://expasy.org/translate/). Homologues of NURR1 were retrieved from NCBI using blastx. ClustalW (http://www.genome.jp/tools/clustalw/) was used for sequence alignment. The theoretical pI (isoelectric point) and Mw (molecular weight) for NURR1 were estimated using Compute pI/Mw software (http://web.expasy.org/compute_pi/). Putative target sequences for microRNAs in the *NURR1* 3′UTR were identified by Target Scan Human 6.2 (http://targetsacn.org) and DIANA-microT-cds v5.0 (http://www.microrna.gr/microT-CDS.). The MatInspector and TFSEARCH program (http://molsun1.cbrc.aist.go.jp/htbin/nph-tfsearch) and the transfac database was used to identify transcription factor binding sequences.

2.4. Extraction of nucleic acids and cDNA synthesis

RNA was isolated from pig organs and tissues as previously described [30]. DNA was isolated from biological samples according to standard purification protocols [31]. Synthesis of the cDNA used for cloning was performed as previously described [32]. cDNAs used for expression analysis were synthesized from RNA isolated from various adult porcine organs and tissues, and from fetal brain tissues sampled at 60, 80, 100 and 115 days of gestation using random hexamer primers (Roche) and the manufacturer's protocol.

2.5. Cloning of the porcine NURR1 cDNA

In order to isolate the *NURR1* sequence encoding the porcine NURR1 protein, we screened in ENSEMBL Sus scrofa version 10.2 sequence database with of human and mouse *NURR1* sequences. A sequence similarity search was carried out with gapped alignment using NCBI Blastall with options blastn and minimum value 10^{-8}. The porcine cDNAs thus identified were used for design of oligonucleotide primers for cloning.

The porcine *NURR1* cDNA was RT-PCR cloned. The PCR reaction mix contained: 2.5 μl cDNA synthesized from RNA isolated from the frontal cortex and pituitary gland, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer NURR1-F and NURR1-R (Table S1) and 1U Phusion DNA polymerase (Finnzymes), in a total volume of 25 μl. PCR amplification was accomplished by employing the following program:

denaturation at 95 °C for 2 min, 10 cycles of touchdown (−0.5 °C per cycle) 95 °C for 20 s, 60 °C for 30 s, 72 °C for 45 s, followed by 25 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 45 s. The PCR program was concluded by an extension at 72 °C for 5 min. After completion of the PCR reaction, 25 μl of the amplification product was applied to a 1% agarose gel and visualized after electrophoresis by ethidium bromide staining. A PCR amplicon of approx. 1800 bp was isolated and eluted using the Qiaquick Gel Extraction kit from Qiagen. The eluted PCR product was cloned directly into the pCR TOPO 2.1 vector (Invitrogen) and sequenced in both directions.

2.6. Cloning of the porcine NURR1 genomic sequences

Porcine *NURR1* genomic sequences were PCR-amplified using genomic DNA isolated from Landrace pigs. The different oligonucleotide primer pairs used are shown in Table S1. The reaction mixture with a total volume of 25 μl contained: 50 ng DNA, 0.4 μM of primers, 0.4 mM dNTP, 0.02U Phusion DNA polymerase (Finnzymes) and 1 × Phusion buffer. The cycling conditions employed were: 95 °C for 2 min, 10 cycles at 95 °C for 20 s, 60 °C for 30 s with a touchdown of 0.5 °C/cycle and 72 °C for 45 s, followed by 25 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 1 min 30 s.

2.7. SNP analysis

Fourteen boars were examined for SNPs in exon 3 of *NURR1*. Exon 3 and flanking intron sequences were amplified by PCR. The primers used are listed in Table S1. The genomic *NURR1* fragment of exon 3 and flanking intron sequences were PCR-amplified, purified and sequenced as described previously [33]. The obtained sequences were analyzed by manual checking in Consed. The PCR was conducted in a total volume of 10 μL containing: 50 ng genomic DNA, 0.5 μM primers, 0.2 mM dNTP, 0.2U Phusion DNA polymerase (Finnzymes). The following cycling conditions were used: 95 °C for 30 s, 30 cycles at 95 °C for 5 s, 60 °C for 10 s, 72 °C for 15 s and a final extension at 72 °C for 5 min.

2.8. Methylation analysis of NURR1

The methylation status of *NURR1* was performed by library preparation, sequencing, mapping and analysis as described in Ref. [27]. For determination of the methylation percentage of specific genes or sequences from our methylome data file, we used Tabix [34]. Genomic DNA from different brain tissues of developing pig embryos was isolated and bisulfate-treated using the EZ DNA methylation kit (Zymo Research) following the manufacturer's instructions. Three primer-sets were used in pyrosequencing of bisulfite-treated DNA (Table S1). PCR amplification primers and sequencing primer were designed using the PyroMark Assay Design software (Qiagen). First-round PCRs were using bisulfate-treated DNA with forward and biotinylated reverse primers (Table S1) and using the Qiagen PyroMark PCR kit. Twelve microliters of PCR product were used for pyrosequencing applying the PyroMark Q24 system from Qiagen.

2.9. Expression analysis

Porcine *NURR1* mRNA expression was determined by Real-time RT-PCR analysis. The following porcine organs and tissues were included in the analysis: liver, prostate, lung, testis, spleen, tongue, cerebellum, occipital cortex, parietal cortex, temporal lobe, musculus longissimus dorsii, frontal cortex, heart and jaw muscle. The temporal expression analysis also included lung, basal ganglia, hippocampus, brain stem, cerebellum and frontal cortex sampled at 60, 80, 100 and 115 days of gestation. Three individual biological samples of each type of brain tissue and time in gestation were included, resulting in a total of 60 samples. Brain tissue samples were collected from three one-year old Danish Landrace pigs weighing 125−150 kg. Based on earlier observations [35] we used *GAPDH* as a reference gene in determination of *NURR1* mRNA expression. NURR1-specific primers were designed to span the exon 7-exon 8 junction using the EXIQON Human ProbeLibrary. Sequences of primers NURR1-RTF, NURR1-RTR, GAPDH-F, and GAPDH-R, used in the expression analysis are shown in (Table S1) The NURR1 primers generated an amplicon of 114 nucleotides. Probes (NURR1#37 from the human probe library and GAPDH, Table S1) were designed using either the ProbeFinder web tool (www.roche-applied-science.com) or the Primer Express software program (Applied Biosystems). The PCR primers and oligonucleotide probe, labeled with the fluorescent reporter SYBR Green or VIC, were designed with the Primer Express software program (Applied Biosystems) and the Probe finder web tool at default settings (www.probelibrary.com). Each reaction was performed in technical and biological triplicates. Real-time quantitative RT-PCR was performed as previously described [35]. Ethidium bromide-staining after real-time PCR confirmed specific amplification of the relevant PCR products (data not shown). Expression analysis data were analyzed using the analysis of variance (ANOVA) procedure of the Statistical Analysis Software (version 8.2; SAS Institute Inc. Cary, NC). The equality of *NURR1* expression levels between different times of gestation with different embryonic brain tissues was tested for statistical significance using the Relative Expression Software Tool (REST) [35]. The level of probability was set at $P < 0.03$ as statistically significant and 50,000 randomization steps were implemented in each comparison [35]. Data were analyzed using the analysis of variance (ANOVA) procedure of the Statistical Analysis Software (version 8.2; SAS Institute Inc. Cary, NC).

3. Results and discussion

3.1. Molecular characterization of the porcine NURR1 cDNA

The porcine *NURR1* cDNA sequence was RT-PCR cloned using RNA isolated from the frontal cortex and pituitary gland of a one-year-old Danish Landrace pig. The identity of the porcine *NURR1* cDNA was established by comparison of the deduced polypeptide sequence homology with human and other isolated *NURR1* sequences. The cloned *NURR1* cDNA (Fig. S1) consists of 1807 nucleotides and contains an open reading frame of 1797 bp, with a G + C content of 56%, plus three bp of a 5′-untranslated region and seven bp of a 3′-untranslated region. The PCR amplified *NURR1* cDNA lacks the 3′UTR region and part of the 5′UTR region, which explains the difference to the cDNA reported in Ensembl. The deduced NURR1 protein contains 598 amino acids with a calculated molecular mass of 66.5 kDa, and a pI of 8.1. Several domains and motifs characteristic of members of the nuclear receptor superfamily of transcription factors are found in the deduced porcine NURR1 amino acid sequence. The NURR1 protein contains three functional domains: (1) An N-terminal domain (NTD) responsible for transcriptional activation also called AF1 [36]. The AF1 domain in NURR1, shown as an underlined sequence in Fig. S1 is involved in regulating transcripts in a mitogen-activated protein kinase (MAPK)-dependent manner [36]. The AF1 NURR1 can be phosphorylated by the ERK2 kinase very close to the AF1 core [37]. (2) The middle part of NURR1 contains the DNA-binding domain (DBD) of 66–68 amino acids. This DNA-binding domain, which is common to all members of the nuclear receptor superfamily, is identified in the deduced pig NURR1 polypeptide (underlined as italic letters in Fig. S1). Maira et al. [38] demonstrated that NURR1 and other NR4A proteins bind to DNA at the NGFI-B response element (NBRE) sequence, 5′-AAAGGTCA-3′, as monomers. In addition, NURR1 also binds to the palindromic Nur77 response element sequence (5′-TGATATTTX$_6$AAATGCCA-3′) as a dimer. Furthermore, NURR1 and NUR77 form heterodimers with a retinoic acid receptor [39]. The DNA-binding domain comprises a cysteine-rich sequence of approx. 66 amino acids containing two type II zinc finger structural motifs. (3) Finally, the dimerization and putative ligand-binding domain are found in the carboxy-terminal part of NURR1 (double-underlined sequence in Fig. S1). The carboxy-terminal region of NURR1 contains two consensus regions that identify the protein as an authentic member of the steroid/thyroid receptor superfamily of transcription factors. The consensus regions are represented by 42 and 22 amino acids, respectively. Eight leucines contained in leucine zipper domains are found in these motifs of NURR1 (underlined bold characters in Fig. S1). Four leucines repeated every seventh amino-acid residue, characteristic of DNA-binding proteins, are separated by approx. 100 amino acids.

Amino acid sequence similarity between porcine NURR1 and its human, bovine and murine counterparts was determined by the Clustal method (Fig. 1). The encoded porcine NURR1 and other mammalian NURR1 proteins exhibited extremely high sequence identity (>99%). Only one amino acid difference was observed between the porcine and the human NURR1 protein, amino acid 238 being a serine residue in the porcine NURR1 sequence and a glycine in human, bovine and murine NURR1 proteins. The genomic *NURR1* sequences deposited in databases and all *NURR1* cDNA clones analyzed in our study contain an AGC codon encoding a serine residue at position 238. However, a non-synonymous A/G SNP (15:70676033) has been reported in the Ensembl database. The SNP changes the AGC codon to GGC, and results in a serine to glycine substitution at amino acids position 238. In conclusion, the A/A genotype resulting in a serine residue seem to be dominant in the pig breeds examined so far. Alignment with NURR1 sequences from other species also high sequence identities, e.g. *Xenopus* (91%) and zebrafish (85%). Because of the extremely high homology, the three-dimensional structure of porcine NURR1 must be very similar to that of its human counterpart.

3.2. Characterization of porcine NURR1 genomic sequence

To determine the genomic organization of the porcine NURR1 gene we performed a blast search in the *Sus scrofa* 10.2 sequence database using the porcine *NURR1* cDNA sequence. A 9.8 kb sequence covering the entire *NURR1* gene was retrieved (GenBank HQ738304). The structural organization of the *NURR1* gene is shown in Table 1. The genomic organization with eight exons separated by seven introns is similar to that of the human and mouse counterparts. The *in silico* data were confirmed by PCR cloning and sequencing of exons 1–7. An alignment of the genomic sequence with the coding region of porcine *NURR1* cDNA demonstrated that the exonic sequences matched 100%. Sequences of the exon/intron junctions and the size of each exon and intron are shown in Table 1. Exon-intron boundaries were estimated by alignment of the *NURR1* cDNA and the genomic sequences. This comparison revealed a total of eight exons with sizes ranging from 124 bp to 866 bp, the largest being exon 3 harbouring the ATG start codon. All exons of the porcine *NURR1* gene and the human *NURR1* gene have the same coding sequence length [14,17]. This is also the case for the mouse *NURR1* gene [2]. All the observed splice acceptor and donor sites were in accordance with the GT-AG rule. The introns of the porcine *NURR1* gene vary in size from 146 bp to 1690 bp and the lengths of the individual introns are remarkably identical to those of the human *NURR1* gene [17].

3.3. Sequence analysis of the 5′ flanking region of the porcine NURR1 gene

Using PCR we amplified a 5′ flanking 1942 bp sequence of the porcine *NURR1* gene also containing 3 bp of the 5′-untranslated region. Sequence analysis revealed this sequence as a putative *NURR1* promoter region. The nucleotide sequence of the ~2 kb genomic region was examined the presence of transcription factor binding sites. The analysis did not reveal a TATA or a CCAAT box in the 1939 bp 5′-flanking sequence of porcine *NURR1*. However, two GC-boxes were found close to the transcription start site at positions −109 and −116. Also, recognition sites for transcription factors R box element/Box4, GATA-1, CREB, CArG-like, SRY, MZF1, Sox5, NF-lap,

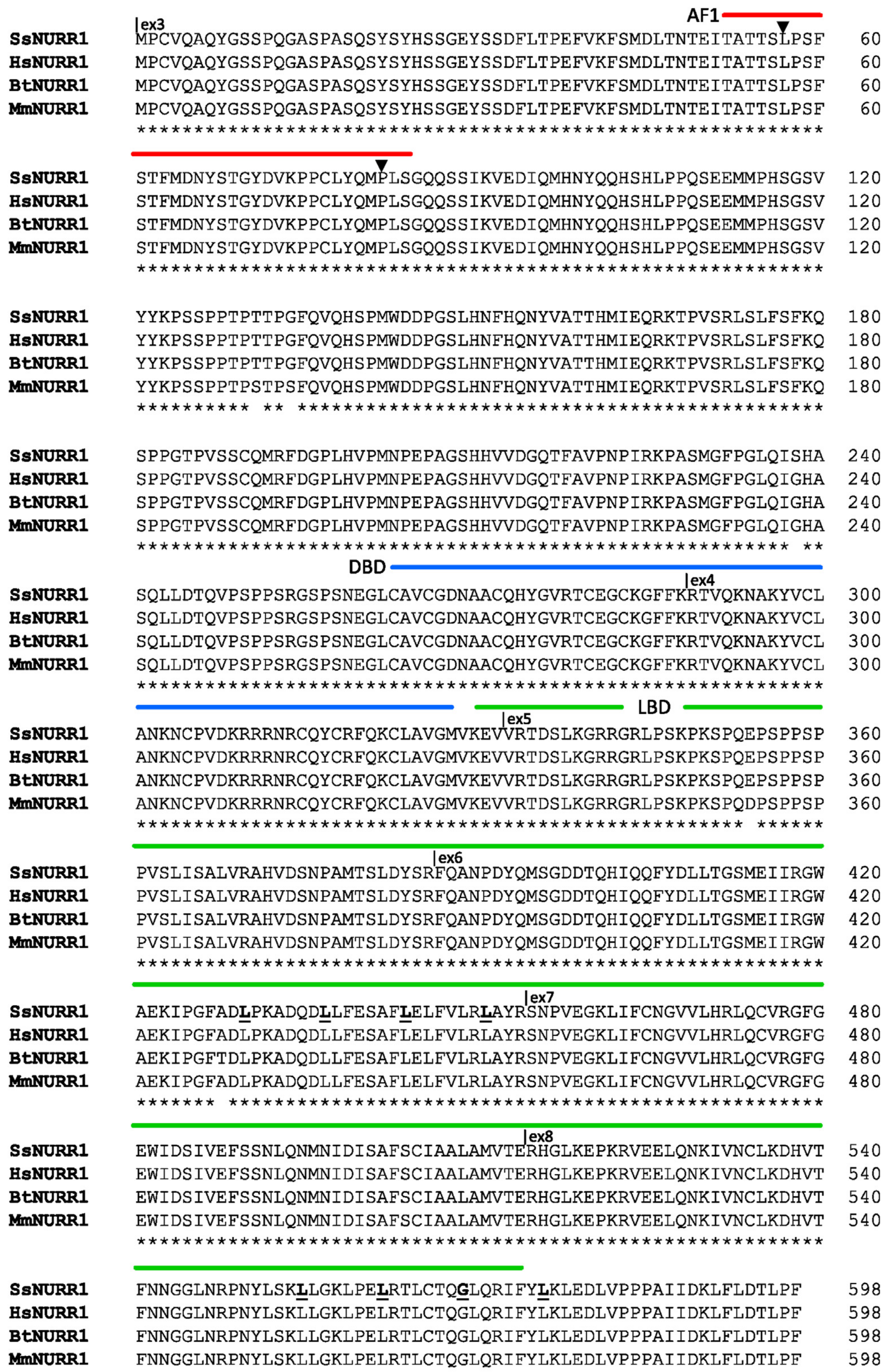

Fig. 1. Alignment of amino acid sequences of porcine NURR1 with NURR1 sequences from humans (GenBank ID: NM_006186), cows (GenBank ID: NM_001076208) and mice (GenBank ID: NM_0113613). Sequence alignment of was performed using the Clustal W program. The numbers represent the position of the amino acids in aligned protein sequences. Intron-exon boundaries are indicated by vertical lines. Identical amino acids in all sequences are shown by asterisks. The amino acids affected by two SNPs found in the porcine sequence are shown by filled arrowheads. Abbreviations for species names used: Ss = *Sus scrofa*; Hs = *Homo sapiens*; Bt = *Bos Taurus*; Mm = *Mus musculus*. The NURR1 protein contains an AF1 motif, a DNA binding (DBD) motif (residues 263–328), and a ligand binding (LBD) motif. Eight leucines repeated every seventh amino-acid residue, characteristic of DNA binding proteins, are separated by approx. 100 amino acids. Leucines are shown as bold underlined characters.

Table 1
Exon/intron structure of the porcine *NURR1* gene.

	Porcine size (bp)	5′-sequence	3′-sequence	Human size bp)
Exon1	236	ACATAAACA	AACTCCTAA	237
Intron 1	1690	GTGAGTAGG	CGGCTGAAG	1688
Exon 2	124	GGAGGAGAT	CGGCTGAAG	124
Intron 2	485	GTTAGTGCA	CATTTCCAG	479
Exon 3	866[a]	CCATGCCTT	TTCTTTAAG	866
Intron 3	777	GTGAGCCAG	CCTTTGCAG	789
Exon 4	130	CGCACGGTG	CCTTTGCAG	130
Intron 4	398	GTAGGTTGG	CCTTTGCAG	389
Exon 5	164	TGGTTCGAA	TATTCCAGG	164
Intron 5	890	GTAAGAAGC	AAATTCCAG	930
Exon 6	203	TTCCAGGCG	AGCATACAG	203
Intron 6	395	GTAATGGGG	TAATTGCAG	389
Exon 7	179	GTCCAACCC	TGGTCACAG	179
Intron 7	146	GTCAGTACT	TTTCTGCAG	149
Exon 8	257	AGAGACACG	CCTTTCTAA	1569[b]

[a] Contains the ATG start codon.
[b] Coding sequence and 3′ untranslated sequence.

CdxA and USF were identified in the 5′ flanking region of the *NURR1* gene (Fig. 2).

The porcine *NURR1* promoter is strongly homologous with its human and mouse counterparts [15,16] and the porcine and human *NURR1* promoter sequences were therefore compared by alignment of 600 nucleotides upstream of TSS.

A sequence identity of 82% between the porcine and the human *NURR1* promoter sequences was observed. The high degree of sequence conservation was observed in two separate regions. A nucleotide identity of 95% was observed in region −1 to −200 relative to the TSS. Recognition sites for the transcription factors CArG-like and CREB, found within this region, were completely conserved between the porcine and the human *NURR1* promoters. Similarly, two TGAC sequences are completely conserved in the pig, human and mouse *NURR1* promoter sequences [16]. Also, in this proximal region a potential recognition sequence for GATA-1 and two Sp1 sites were identified. A nucleotide identity of 82% was found within region −336 to −616 relative to the TSS. We speculate whether the high sequence similarity between human and porcine *NURR1* promoters indicates similar mechanisms for regulation of expression. The CREB sequence found in the *NURR1* promoter suggests that *NURR1* induction could be enhanced through the cAMP-mediated pathway.

3.4. Mapping of NURR1

We have used Blat software to localize the *NURR1* gene in the *Sus scrofa* 10.2 genome [40]. The *NURR1* gene was mapped to SsChr15:70,671,944-70677,289 (Table 1). The human and mouse *NURR1* genes have previously been mapped to chromosomes 2q22-23 and 2 of these species, respectively [14,16].

3.5. Evolutionary relationship of NURR1

The Clustal W method was used to investigate the evolutionary relationship of porcine NURR1 with homologues from other species. We included NURR1 polypeptide sequences from different species, including mammals, fish and an amphibian, in an unrooted phylogenetic tree constructed by the neighbor-joining method. The phylogenetic analysis, shown in Fig. S2, demonstrated that the phylogeny of NURR1 proteins from humans, pigs and cows were more closely related than to that of rodent NURR1. The mammalian NURR1 polypeptides clustered together in one clade, and the *Xenopus* was in a group by itself when compared to all other NURR1. The zebrafish NURR1 made its own clade distinct from mammalian and amphibian clades. The topology of the dendrogram was basically as expected from the classical taxonomy of the animal kingdom.

3.6. SNPs identified in the NURR1 gene

Investigation of the genetic variation in the porcine *NURR1* gene, using RT-PCR cloning, revealed two missense SNPs in exon 3. The SNPs were found in the RT-PCR amplicons of NURR1 from the frontal cortex and pituitary gland and were confirmed by results from RNAseq analyses [41]. A non-synonymous C/T SNP (nucleotide position 4765 in GenBank ID: HQ738304) was discovered in the AF1 domain (Fig. S3A). This SNP gives rise to a substitution from a leucine residue to a phenylalanine (L57F). Also, a missense C/A SNP (Fig. S3B) was identified in exon 3 (nucleotide position 4841 in GenBank ID: HQ738304). This non-synonymous C/A SNP substitutes a proline with a histidine residue (P82H) in the porcine NURR1 protein sequence. The SNP analysis was extended for the C/T SNP to estimate the genotype frequencies. The genotype of the C/T SNP was examined in genomic DNA isolated from a breed panel with14 unrelated Duroc boars [42]. The C allele seemed to be dominant in the tested animals as only two out of 12 analyzed boars were heterozygous C/T, whereas the rest were homozygous C/C. Also, the C/A SNP seems to be very rare; only two clones, homozygous for A/A, were identified by RT-PCR cloning of the porcine *NURR1*. Only the C/C genotype was detected in the boar panel. The obtained genotype frequencies for the two SNPs are only indicative and more precise values would need a larger breed panel with more individuals. None of the two SNPs found in this study have been identified in human *NURR1*. Since AF1 is important for its transcriptional activation, the two identified missense mutations might affect this function. We also performed a SNP analysis on exon 1 and 2 but did not identify any polymorphisms.

For the human *NURR1* gene a total of 69 exonic SNPs, among those 25 missense SNPs, were found in Ensembl. The distribution of variants found in the human *NURR1* gene was: exon 1: five 5′UTR SNPs, exon 2: five 5′UTR SNPs, exon 3: 14 synonymous SNPs and nine missense SNPs, exon 4: three synonymous SNPs, exon 5: one synonymous SNP and one missense SNP, exon 6: four missense SNPs, exon 7: three missense SNPs and exon 8: eight missense SNPs and 16 3′UTR SNPs. In addition, two intron splice variants were found 5′ in introns 4 and 5. It is very likely that some of the SNPs identified contribute to the many splicing variants of *NURR1*.

```
-1939 tttccctttcgttcgcgtggaatcgcttctgcccacagggcgctgacgctactcagctca -1956

-1955 tgctaataagctattcttcgccctcctcccctcaccccgctactcacacgcccaacacac -1895

-1894 tctctctctcacttcctggtttaatttttttctcgctctcgctagggcataactgagctg -1834

-1833 ctgcggctgcggcagcaatcgcaacatctgggggaaacttaaggtggtcacgtaggtttt -1773

-1772 ccgaggctgcggcacagaagagcctgcggcttctcggggcagccgcggggcatctgatgg -1712

-1711 caacgcccctccggcctccccagagcagaaggcgaagggagcaggcaaaggcgggtgtgc -1651

-1650 agattagagctgagcggctcaggaacccggagagttccaaggcgctttcggctggccagt -1590
          GATA-1              Sp1
-1589 actgggaacaccagggtgggggcgggggcaaaggggccccagcagactggaagctagcc -1529
            MZF1      Sp1
-1528 acgccgagcggggaggggcggttggaagtctccaggggccaaaagggggcgcttccgaac -1468

-1467 cctccctgccccggtgcctcccccagtctctccagagaagctccgaggcccgcccagctc -1407

-1406 cggcccagagacaggaaggggggttcgctcccgggagaagcggggtttctttgctagaaag -1346

-1345 cttcttgtgccctcagcgtggagggtggattgatttcccaagctgaatctgcggtgcctg -1285

-1284 gggcagccgcgggggtgcggcgagctcctcacactcgccgggcccctctcctgggagggc -1224

-1223 tggattcagagtacgaacgctttgctgagatgaagccctcacatccccacgtttagtctt -1163
                                                               CdxA
-1162 tacctcttggctctcagattgagactgcgaaagcccttgtgctgcagaggagggtcgatt -1102
                                 GATA-1
-1101 tatttccctaaaacttgtggaatggtatgagttgataggcacccaggagtatcagagtat -1041

-1040 gagagttggtaaaaggtgagactggagcacctttctgcagaaggcacacctgttgcccgc  -980

 -979 ggctcagctgcattatctgcaagaggaaagtgggcgacgcctaactttgacttttggggt  -919

 -918 ccccgtagggctagttcactgtctacccccagctctcctctcctctccccaggcactctg  -858
                USF
 -857 tcctccctcgcacgtgagcacttcagggaaagaagggaacacagaaaatgggtcctactc  -797

 -796 actcttctccactttggggtgtgtgtgtgcgtgtatgagagagggagagagagaatatgg  -736

 -735 gagggagggagagacggagggaggggggagaacacacacacacacacacacacacacacac  -675

 -674 acacacacacacaccagaggagtaaggccagggacaggaaaaagagaaaaggaggatgca  -614
              NF-kap
 -613 ctgggctagggaaatcccatcccaagtgcctcagccctctgaacatctcttgtcatggga  -553
                        Sox-5
 -552 catctgtattcgcttccgttaacaattgtgacaattgtgggtgggtgaggttacggcgcc  -492

 -491 gccaggcacaccccatctcgtgcgattccaggtgcgggctacctggcccatgtctgggtt  -431
                                        MZF1
 -430 cccttccgcccaggcttttgttgcaccctccccacgtgtgagcggccgagcagcagcag  -370
                   Sp1                                       Sp1
 -369 tggcgcgggggttgaggggggaggtgaggtgagatgtcgaggcgctgcgggctgcgctg  -309
                 R box            SRY
 -308 acttgagtggagaggtcacacctctttcggaaaaagaaaaaaaaaaaaaaaaaaaaaacc  -248
                                                               CREB
 -247 tagctggctaccaaggtgaaccctgaacggttcccaccttaaaatctgccctgctcgtga  -187
                CArG-like
 -186 cgtcaggtcggaaatataccaaagcgagcgccggccaggagtctggggagcgcggcggcg  -126
          GATA-1  Sp1 Sp1
 -125 cggcgattgggcggcgggccgctgacgcgcgctgacgcgcggagactttaggtgaatgtt   -65
                               AT-rich
  -64 ggcagcggcagcgcgagccacataaacaaaggcacattggcggccagggccagtccgccc    -4
                                                      Δ
         v
   -3 ggtGgc                                                          +3
```

Fig. 2. The nucleotide sequence of the promoter region of the porcine *NURR1* gene with 1939 nucleotides upstream of the TSS. The putative TSS (position 475) is shown by a plus (+) sign. The recognition sequences for known transcription factors, AP2, SP1, MBF-1, Pax8, and CdxA, identified by TransFac and MatInspector, are underlined and indicated by names. Also, a PSN-like sequence and a MOTIF5 conserved in NURR1 promoters of human, pig and *Monodelphis* (opossum) origin are indicated by underlining. The sequence was deposited to GenBank (HQ688299).

The missense mutation in exon 3 (709 C > G), leading to an amino acid substitution of serine to cysteine, affects the N-terminal region and is found in non-familial Parkinson's Disease (PD) patients [24,25,43].

In addition, three mutations in exon 3 have been associated with schizophrenia and bipolar disorder [44]. Two of these were missense mutations resulting in M97V (A/G) an H103R (A/G) [44]. Also, a three-nucleotide deletion was identified in ΔY122. Based on the nucleotide similarity it is possible that all the three human mutations in *NURR1* exon 3 could also be identified in the pig homologue.

Examination of the porcine *NURR1* gene in Ensembl revealed 24 exonic SNPs in total. The distribution of SNPs between exons was as follows: exon 1: two 5′UTR SNPs, exon 2: none, exon 3: three missense SNPs and six synonymous SNPs, exon 4: five synonymous SNPs, exon 5: none, exon 6: one missense, exon 7: two synonymous SNPs and exon 8: one missense SNP and three 3′UTR SNPs. None of the three SNPs identified in exon 3 in this study were found in the Ensembl data.

3.7. Identification of a potential microRNA recognition site in the 3′UTR of NURR1

Employing the miRanda search tool, we identified a recognition site for miR-132 in the 3′UTR of the porcine *NURR1* gene. The seed sequence, ACUGUU, is completely conserved within the *NURR1* 3′UTR of four species: pig, man, cow and mouse (data not shown). The localization within the 3′UTR of the recognition site was completely identical to that of the human counterpart. Recently, it was demonstrated that miR-132 regulates the differentiation of embryonic stem cells to dopamine neurons by directly targeting *NURR1* gene expression [45].

3.8. Spatial expression of NURR1

The spatial expression of *NURR1* mRNA was analyzed by quantitative real-time RT-PCR in organs and tissues isolated from three Danish Landrace pigs, aged 1–2 years. The expression analysis revealed particularly high *NURR1* expression levels in the liver, prostate, lung and testis; lower levels were found in the spleen, tongue and cerebellum, and little expression was detected in other brain tissues and the heart. Porcine liver showed the highest expression of total *NURR1* transcripts, which is approximately two-fold and 150-fold higher than in the prostate and parietal cortex, respectively (Fig. 3A). The various brain tissues from the occipital cortex, temporal lobe and frontal cortex demonstrated medium levels of *NURR1* mRNA expression. Jaw muscle contained negligible levels of *NURR1* mRNA expression. NURR1 is like other members of the NR4A subfamiliy, NUR77 and NOR1, expressed in high energy-demanding tissues including skeletal muscle, liver, heart, kidney, T-cells and brain [46,47]. A strong up-regulation of *NURR1* is seen in extreme obesity and a down-regulation is observed during differentiation of primary human preadipocytes [48]. *NURR1* is not only expressed in the brain but also in non-brain tissues such as the liver and osteoblasts [49]. Expression of *NURR1* mRNA can also be monitored in mouse pituitary cells and can be induced by CRF [50].

3.9. NURR1 mRNA expression in developing pig embryos

The developmental *NURR1* mRNA expression was also determined in pig embryos at 40, 60, 80, 100 and 115 days of gestation. Five different brain tissues as well as lung and heart tissues were included in the analysis. Based on earlier observations [35] we used *GAPDH* as a reference gene to determine *NURR1* mRNA expression. In this study we found a very uniform expression of *GAPDH* within the porcine brain tissues determined at different developmental stages. *NURR1* mRNA expression was detected in all five brain tissues at the developmental stages examined (Fig. 3C–G). *NURR1* transcript was found in the different brain areas of embryos as early as at 60 days of gestation. It is of notice that the mean standard deviation for *NURR1* expression is considerable, reflecting a high heterogeneity among pigs. The expression analysis revealed a 25-fold decrease in *NURR1* transcripts in the basal ganglia from day 40 of gestation to adult pig (Fig. 3C). A significant 3-fold increase was observed in the cerebellum between day 60 and adult (Fig. 3F).

In the basal ganglia the *NURR1* expression level was significantly higher at day 115 of gestation compared with day 60 in development ($P = 0.019$). In the other brain tissues examined, the hippocampus (Fig. 3D), brain stem (Fig. 3E) and frontal cortex (Fig. 3G), no significant changes were observed during embryo development. A 63-fold increase in *NURR1* mRNA, shown in Fig. 3B was demonstrated in lung tissue from day 40 to day 115 of development ($P = 0.001$). The rise in *NURR1* mRNA expression in lung continued to develop into adulthood. The *NURR1* transcript level was very low in heart tissue and did not change significantly during embryo development (data not shown). Three different brain tissues (cerebellum, frontal cortex and brain stem), isolated from embryos of cloned pigs, were included in the expression study. A six-fold increase ($P = 0.03$) in *NURR1* mRNA expression was observed in the cerebellum from day 60 of gestation to adult stage (Fig. 4A). A similar significant increase ($P = 0.019$) was seen in the cerebellum from normally fertilized embryos (Fig. 3F). No significant change in *NURR1* transcript was found in the frontal cortex and brain stem (Fig. 4B and C). The expression data, spatial and developmental, obtained for the porcine *NURR1* mRNA are of great value in future overexpression and loss-of-function experiments that could aid clarifying the role of NURR1. Collected evidence indicates that NURR1 plays an essential role in the maturation of midbrain dopaminergic progenitor cells. During development dopaminergic progenitor cell markers disappears and at birth all the dopamine markers examined are lacking in the NURR1 null mice [3,6]. Li et al. [51] have investigated the developmental expression of NURR1 protein in rat brain and

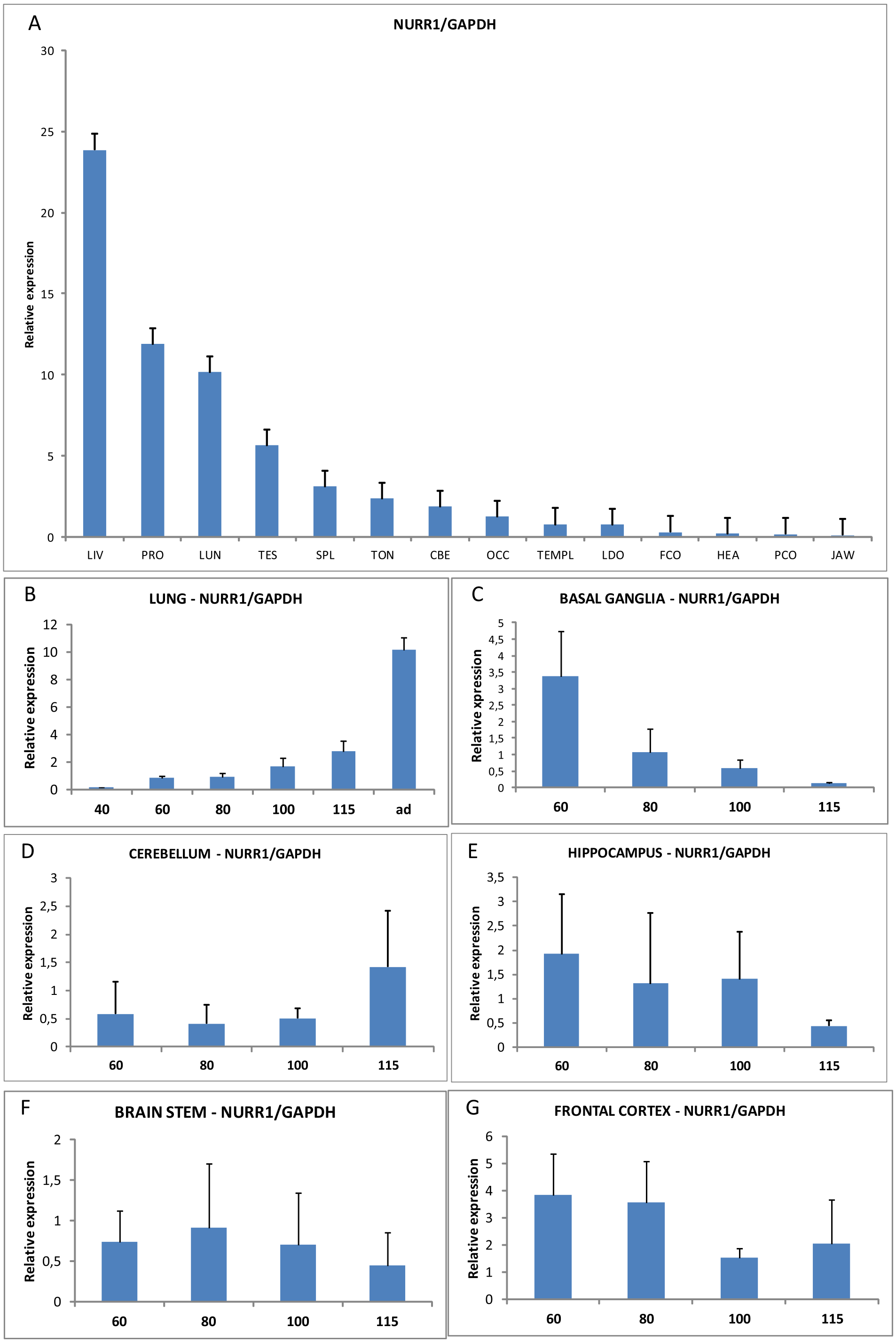

Fig. 3. Porcine *NURR1* mRNA expression, determined by quantitative real-time RT-PCR, in different organs and tissues (A). Abbreviations used: SPC, spinal cord; TEMPL, temporal lobe; BST, brain stem; OCC, occipital cortex; CBE, cerebellum; PCO, parietal cortex; FCO, frontal cortex; BLA, bladder; TES, testis; KID, kidney; SPL, spleen; PRO, prostate; LUN, lung, LDO, musculus longissimus dorsii; TON, tongue; HEA, heart; JAW, jaw muscle. Developmental *NURR1*mRNA expression, measured by real-time RT-PCR in various brain tissues isolated from developing pig embryos (C–G) and lung tissue from developing embryos (B). The expression of *NURR1*mRNA was normalized to the expression of the *GAPDH* gene. The presented values are the mean of triplicate determinations. CBE, cerebellum; COR, cortex; BSG, basal ganglia; BST, brain stem; HIP, hippocampus; LUN, lung.

spinal cord. The expression of NURR1 in the cerebral cortex reached a maximum 1–5 days after birth followed by a decrease as the cells matured, and even lower in the mature cerebral cortex. No NURR1-positive cells were found in the spinal cord after maturation. A study by Chu et al. [52] demonstrated a parallel age-related decrease of NURR1 and TH (tyrosine hydroxylase) in the substantia nigra. Hence, collected evidence suggests that NURR1 plays a regulatory role in the differentiation, migration and maturation of neurons in the rat central nervous system.

3.10. Methylation status of the NURR1 gene

The global methylation profiles of porcine brain (occipital cortex) and liver were determined by high throughput bisulfite sequencing on the Illumina HiSeq platform. Two one-year-old and unrelated Danish Landrace boars were used in this study [27]. Sequencing of bisulfite-converted pig genomic DNA yielded a dataset of 1926 and 1302 million reads, equal to 194.5 and 131.5 Gbp of paired-end sequence data for liver and occipital cortex, respectively. Mapping of reads and further analysis and determination of methylation levels and status was performed as described [30]. The methylation status for the *NURR1* gene body (5.3 kb), including the coding sequence and the 5′UTR and 3′UTR, was determined in porcine occipital cortex and liver. The analysis detected 4681 methylated CpG reads in the occipital cortex out of a total of 6481 reads, resulting in a methylation degree of 72% (Table 2). In the liver tissue 3273 methylated reads were found in a total of 7958 reads, yielding a methylation degree of 41%. In conclusion, the methylation was significantly higher brain tissue compared with liver. A 2 kb 5′ upstream region in the *NURR1* promoter was also examined for methylation. The analysis 259 methylated reads out of 9676 in occipital cortex, yielding a methylation degree of 2.6%, a value much lower than in the gene body. Only five reads out of 8138 reads were detected in liver tissue, resulting in a methylation degree of 0%, which is significantly lower than that found in the brain (using the chi-square test (P-value < 0.001)). To investigate the methylation levels of the individual CpG islands of the porcine *NURR1* promoter, approx. 2000 bp sequences upstream of the TSS were analyzed by Methyl Primer Express v 1.0 software (Fig. 5). Five CpG islands (GC content >60%) were detected in the promoter region of the porcine *NURR1* gene: Island E, 57–157 nucleotides upstream of the TSS; island D, 232–338 nucleotides upstream of the TSS; island C, 358–462 nucleotides upstream of the TSS; island B, 1138–1286 nucleotides upstream of the TSS and island A, 1539–1747 nucleotides upstream of the TSS (Fig. 5). Only CpG island B had a GC content higher than 70%. These islands may represent elements of epigenetic (methylation) control of *NURR1* transcription.

To investigate the methylation status in brain tissues during development we performed bisulfite sequencing of CpG islands B-E and found very low values for methylation (overall values <2%). However, in island D we observed slightly higher values. In addition, a Sp1 recognition sequence, containing a CpG site, was found in CpG island D. Also, a recognition sequence for an R-box element was observed in island D. The R-box element binds the transcription factors Rtg1p and Rtg3p, which are both basic helix-loop-helix/ leucine zipper proteins. Therefore island D was selected for further methylation analysis. The methylation status of five CpG sites located in CpG island D (Fig. 5) was determined in the frontal cortex from cloned pigs by bisulfite sequencing. A differential methylation degree was detected in the five CpG sites with values ranging from 1 to 20% (Fig. 6). A significant decrease was observed during embryo development from day 50 after surgical transfer of blastocysts to subsequent time points of analysis (Fig. 6). The significant decrease in methylation between days 50 and 70 occurred at all five CpG positions examined with the following P-values: CpG1, $P = 0.02$; CpG2, $P = 0.05$; CpG3. $P = 0.04$; CpG4, $P = 0.02$ and CpG5, $P = 0.018$. The methylation degree remained constant from day 70 to adulthood. The largest reduction in methylation degree was seen at CpG site three with a 10-fold decrease ($P = 0.04$).

Analysis of *NURR1* expression (Fig. 3B) and *NURR1* promoter methylation in frontal cortex samples from developing cloned pigs did not display any correlation between DNA hypermethylation and decreases in *NURR1* expression during the entire time-course. However, a decrease in DNA methylation of five CpG positions in the DNA from the frontal cortex correlated with an increase in *NURR1* transcripts from

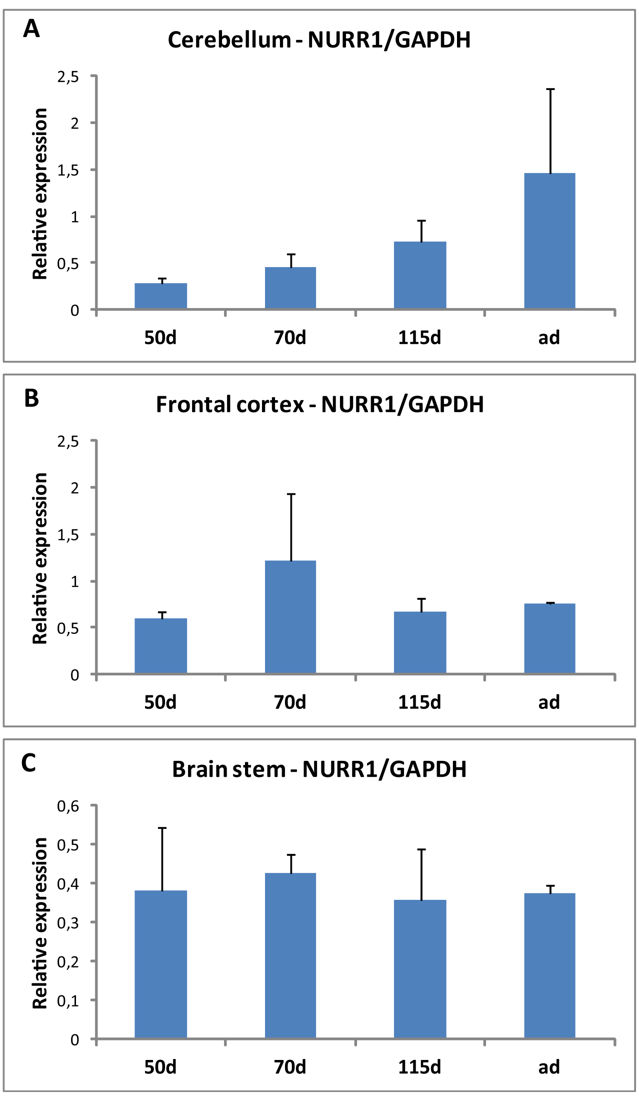

Fig. 4. Developmental expression of *NURR1* mRNA in cloned pigs. Real-time RT-PCR analysis of porcine *NURR1* mRNA in the cerebellum (CBE), frontal cortex (FCO) and brain stem (BST) from developing cloned embryos. The expression of *NURR1*mRNA was normalized to the expression of the *GAPDH* gene. The presented values are the mean of triplicate determinations.

Table 2
Methylation level of the porcine *NURR1* gene, including a 5-flanking sequence (pNURR1), in liver and brain (Sus scrofa 10.2).

Gene	Length (bp)	Chr	Start	End	Tissue	Methylated reads	Total reads	Methylation percentage (%)
NURR1	5345	15	70,671,944	70,677,289	Brain	4681	6481	72
					Liver	3273	7958	41
pNURR1	2059	15	70,679,281	70,681,340	Brain	259	9676	2.6
					Liver	5	8138	0

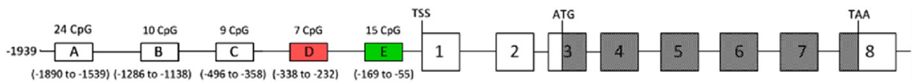

Fig. 5. Schematic representation (not drawn to scale) of the genomic organization of the porcine *NURR1*gene with exon-intron structure and with locations of CpG islands found in the *NURR1* promoter. CpG islands are marked with boxes and indicated by letters A to D. CpG islands B, C, D and E were subjected to bisulfite sequencing. The methylation status of island D (red box) was determined in DNA from normally fertilized pigs and cloned pigs.

day 50 to day 70. It therefore cannot be ruled out that changes in the degree of methylation of CpGs in other CpG islands determine the expression of NURR1. In future experiments we will extend our DNA methylation analyses to other brain tissues such as the cerebellum and brain stem.

Other studies indicate that DNA methylation of the *NURR1* gene could contribute to its regulation. Mill et al. [53] demonstrated a down-regulation of *NURR1*, the gene being hypermethylated in schizophrenic female brain samples. In a very recent study, Bordoni et al. [54] reported of increase in *NURR1* gene in expression in rat brain as a response to neonatal exposure to permethrin. They also reported that 44.4% of untreated offspring, produced from rats with early life exposure, have a similar variation in *NURR1* gene expression. Epigenetic modifications on *NURR1* possibly caused by permethrin exposure was studied in the F1 generation. Global DNA methylation profiling revealed that hypomethylation measured in the mothers exposed to permethrin during early life is transferred to the offspring, and that NURR1 is hypermethylated [54]. These data could indicate that changes in methylation of *NURR1* affect the expression of *NURR1*.

DNA methylation can affect regulation of gene expression in two opposing ways. Generally, in promoter regions, DNA methylation represses transcription. Opposing to this, in gene bodies, DNA methylation is associated with high levels of gene expression [55–59]. Several studies in humans, animals and plants have revealed that higher methylation levels are detected in exons compared with the flanking intron sequences [60–62]. Also, a study by Maunakea et al. [63] showed that 34% of all intragenic CpG islands are methylated in the human brain. Recently, it was shown that DNA methylation is significantly enhanced in included alternative spliced exons [64]. It is hypothesized that intragenic DNA methylation functions in exon definition to modulate alternative splicing by recruitment of the MeCP2 protein [65]. Several *NURR1* splicing variants have been identified in humans, mice and rats [14,17,65–68]. These variants of NURR1 are the result of alternative splicing of exons 3, 5 and 7. It is very likely that these also exist in the pig. However, currently we have no evidence of their existence. The high methylation level found in the pig brain could indicate that methylation is one of the determining factors in alternative splicing of *NURR1*. The higher methylation rate in the gene body of *NURR1* in the brain (72%) compared with the value for methylation seen in the liver of 41% correlates well with the general observation of higher splicing activity in the brain.

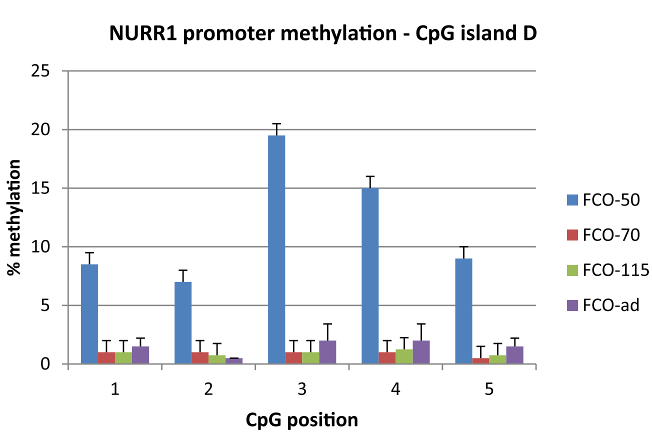

Fig. 6. Methylation of CpG positions in island D in cloned pigs. The methylation level was determined by bisulfite-sequencing. At least ten clones for each PCR product were sequenced. The presented values are the mean of triplicate determinations.

Bordoni et al. [54] reported a significant increase in *NURR1* gene expression in rats exposed to permethrin, a pesticide of the pyrethroid family. A similar increase in *NURR1* transcript was seen in one third of the offspring, indicating an intergenerational effect of exposure to permethrin. Interestingly, the authors also detected a reduction in global genome-wide DNA methylation in rats exposed early in life to permethrin. In contrast control rats displayed a hypomethylated genomic DNA. Unfortunately, the authors did not report on the methylation status of the NURR1 gene in rats exposed to permethrin.

4. Conclusion

The present study provides valuable molecular information about the porcine *NURR1* gene. The *NURR1* gene was cloned and characterized, and also two non-synonymous SNPs were identified. The spatial expression pattern of the *NURR1* transcript in pig organs and tissues was very similar to that described for human and rodents. In the developing pig fetal brain, *NURR1* mRNA was highly expressed in the cortex and basal ganglia at early embryonic stages and then declined in the later stages. In other tissues, such as the lung and cerebellum, increases in *NURR1* mRNA expression were observed during embryonic development. Together, these observations underline the importance of *NURR1* expression during development. Li et al. [53] studied *NURR1* expression during rat embryo development and found a decrease in expression during development and that *NURR1* transcripts are rare in the adult cortex. *NURR1* transcripts are only found in differentiating and migrating immature cells and absent in proliferating cells [53]. In conclusion, NURR1 very likely plays an important regulating role in the differentiation, migration and maturation of dopaminergic neurons in mammalian brain.

To investigate whether the differences in relative expression of *NURR1* are associated with differences in DNA methylation, we performed bisulfite sequencing of four regions upstream of the *NURR1* gene. The correlation between high DNA methylation in a discrete region of the *NURR1* promoter and very low expression of *NURR1* mRNA in the frontal cortex in the early stages (50 days) of pig embryo development indicated that DNA methylation could be a determining factor in transcriptional repression. Also, we found that a decrease in DNA methylation of five CpG positions in the DNA from the frontal cortex correlated with an increase in *NURR1* transcript from day 50 to day 70. Within this particular time-span the gyrencephalic structure develops. In conclusion, our studies show that certain CpG positions in the *NURR1* promoter region undergo dynamic changes in methylation during brain development. However, this was only investigated in the frontal cortex so studies of other brain regions remain to be done. The high degree of similarity in molecular characteristics between human and pig NURR1 supports the use of the pig as a good model to study PD. Elimination of *NURR1* gene function might create the genetic predispositions that elicit pathogenesis of PD. Transgenic *NURR1* knock-out pigs could contribute to the understanding of PD etiology. In future studies we intend to generate transgenic knock-out pigs with no or reduced expression of *NURR1*. Porcine Parkinson models may aid to clarify the underlying cellular and molecular mechanisms of disease initiation and promote the development of treatment therapies. A NURR1 knockout mouse was developed and characterized by Castillo et al. [14]. The *NURR1*-deficient mice, heterozygous NURR1 $^{(-/+)}$, did not recapitulate PD pathogenesis and exhibited no clinical symptoms. The NURR1-null mice were not viable and died within 12 h. A decrease in dopamine of around 90% was seen in these mice, although a moderate loss of dopaminergic neurons in the substantia nigra was seen [69]. Based on recent information about the role of NURR1 in neurogenesis and neuronal differentiation, NURR1 knock-out pigs could possibly serve as models for Parkinson's disease. Transgenic pigs with eliminated expression of *NURR1* could hopefully contribute to the understanding of normal brain function and PD etiology. Hence, in further studies we will use CRISPR-Cas technology to generate knock-out pigs with no expression of *NURR1*. We believe that the high resemblance in structure of the CNS and between pig and man, will make the pig the ideal model for various neurological diseases, including PD [70–72].

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biopen.2016.07.001.

Author contributions

Conceived and designed the experiments: KL, JM, LF and HC. Performed the experiments: LF and JM. Analyzed the data: KL, LF, JM and CB. Contributed reagents/materials/analysis tools: KL, HC and CB. Wrote the paper: KL.

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