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ORIGINAL ARTICLE

Widespread expression of liver receptor homolog 1 in mouse brain

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Abstract

OBJECTIVES: The distribution of Liver receptor homolog 1 (LRH-1) mRNA was studied in mice brain with the aim to establish whether this nuclear hormone receptor is expressed also in the brain in addition to liver and classical steroidogenic tissues.
METHODS: Expression of LRH-1 mRNA in juvenile (30 days old) and adult (60 days old) mouse brain was examined using non-radioactive in situ hybridization with digoxigenin labeled cRNA probes and with RT PCR using specific primers.
RESULTS: LRH-1 was strongly expressed throughout the brain. Semiquantitative RT PCR revealed very strong expression of LRH-1 mRNA in cerebrum in comparison to liver and testis, and in situ hybridization revealed that LRH-1 mRNA is uniformly expressed in most brain areas.
CONCLUSIONS: LRH-1 is strongly expressed throughout the mouse brain suggesting important roles for this transcription factor, although its precise roles in the CNS remain to be elucidated.

Introduction

Liver receptor homolog 1 (LRH-1, NR5A2), a member of orphan nuclear hormone receptor superfamily was initially identified in liver, where it has important roles in cholesterol transport and bile acid synthesis (reviewed in [9,28]). LRH-1 regulates expression of several genes that are involved either in cholesterol transport in liver or in production of bile acids such as SR-B1 [32], apolipoprotein A1 [7], cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1) [11,28]. Structurally, it is a close relative of Steroidogenic factor 1 (SF-1), another member of orphan nuclear receptor superfamily with important roles in development and function of endocrine system [9,25]. Both proteins can bind to the same hexameric elements [10] although they can not compensate for each other functionally as deletion of each gene is lethal, albeit at different times during development [6,20,24]. Expression patterns of both genes partially overlap, with LRH-1 expression being more widespread in comparison to SF-1 expression pattern. LRH-1 is expressed in liver, intestine, pancreas, preadipocytes as well as in steroidogenic tissues such as testis, ovary and

adrenal glands (reviewed in [9]). Interestingly, the expression of SF-1 and LRH-1 does not overlap in the ovary, where SF-1 is expressed mostly in theca cells and weakly in granulosa cells [14,22] while LRH-1 is abundantly expressed both in granulosa cells and in corpus luteum [8,13]. Similarly, both factors are differentially expressed in the testis. While both LRH-1 and SF-1 are expressed in the Leydig cells, SF-1 is also present in Sertoli cells while LRH-1 is expressed in pachytene spermatocytes and round spermatids [27]. Differential expression patterns between SF-1 and LRH-1 in the ovary and testis suggest different functions and several recent studies suggested that main function of LRH-1 might be a regulation of P450 aromatase expression both in the ovary and in the testis [13,27] and, furthermore, LRH-1 was also shown to regulate the expression of P450 aromatase in adipocytes [3,4]. P450 aromatase is responsible for converting C19 steroids into estrogens. This process is very important not only in gonads but also in the brain, where estrogens play extensive key roles in males and females. In the brain, peripherally produced testosterone is converted into estradiol by brain aromatase (reviewed in [5]). As both peripherally and locally produced steroids have important roles in development and function of many different areas of the brain, it is not surprisingly that P450 aromatase is also expressed in many areas of the brain [2] and references therein). However, brain expression of SF-1, one potential regulator of P450 aromatase expression at least in gonads [21], is limited to the ventromedial hypothalamus [15,34] and SF-1 therefore could not act as the main regulator of aromatase expression in different areas of the brain. Therefore LRH-1 could be a potential candidate for such a factor. In the present study, we examined the expression of LRH-1 in the mouse brain with the aim to determine whether LRH-1 expression overlaps with the reported distributions of either SF-1 or P450 aromatase.

Material and methods

Animals and tissue

Adult (60 days) and pre-/peripubertal (30 days) male and female inbred C57BL/6 mice were used in this study. Mice were bred in standard conditions and fed regular chow *ad libitum*. All animal experiments were done in accordance with ethical guidelines and EC council directive (86/609/EEC) and were approved by Veterinary commission of Slovenia. Mice were anaesthetized with mixture of ketamine (1.25mg/animal; Veyx-pharma, Schwarzenborn, Germany), Xylazine (0.125mg/animal; Chanelle, Lougrea, Ireland) and Acepromazine (0.025mg/animal; Fort Dodge Animal Health, Fort Dodge, Iowa. USA). While in deep anesthesia, mice were perfused with 4% buffered paraformaldehyde. Brains were dissected out, post-fixed in 4% paraformaldehyde overnight at room temperature and stored in 0.05M PBS at 4°C until use. Four brains were analyzed for each group, i.e., 60 days old male, 60 days old female, 30 days old male and 30 days old female (16 animals all together). For RNA extractions, mice were euthanized by carbon dioxide followed by cervical dislocation. Brains (separately cerebrum and cerebellum) and other tissues (testis, liver, gastrocnemius muscle) were immediately placed in TRIZOL (Invitrogen, Paisley, UK) and RNA was extracted following producer protocol. RNA was treated with RNAse free DNAse to remove traces of DNA. The amount of RNA was determined by spectrophotometer and RNA was stored at –80 °C until use. For in situ hybridization, eighty micrometers thick coronal brain sections were cut using Campden Vibroslice vibrating microtome (Campden instruments, Leicester, England) in RNAse free cold 0.01M PBS. Sections were placed in special baskets made from 2ml syringes with fine mesh glued to the bottom. Throughout the procedure, sections remained in the baskets and only baskets with sections were moved between the containers during different treatments.

Reverse transcription PCR

Primers for LRH-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were chosen based on a mouse cDNA sequences from genbank, gene accession numbers M81385 (LRH-1) and NM_001001303 (GAPDH). Primers for LRH were: 5' primer: 5'-CGACAGACTATGACAGAAGT-3', 3' primer: 5'-CGAGAATCAAGAGCTCAG-3' and for GAPDH 5' primer: 5'-GTTCCAGTATGACTCCACT-3' and 3' primer: 5'-GTGAAGACACCAGTAGACT-3'. Primers were amplifying 470 bp long DNA fragment from basepairs 910 to 1380 for LRH-1 and 170 bp long DNA fragment from basepairs 190 to 360 for GAPDH. RT PCR was performed using Promega (Madison, WI, USA) Access RT PCR kit and 100 ng RNA as a template. cDNA was synthesized with AMV reverse transcriptase and both 3' primers for 45 minutes at 48 °C. After cDNA synthesis step, Tfl DNA polymerase was added to the tubes and PCR was performed as follows: initial denaturation 3 minutes at 95 °C, followed by 35 cycles with 30" denaturation at 95 °C, 30" annealing at 54 °C and 1'30" elongation at 72 °C, followed by final elongation for 7' at 72 °C. Amplified products were loaded on a 2% agarose gel together with 100 bp DNA ladder (Promega).

cRNA probe synthesis

pBluescript plasmid containing 502 bp (nt 1369 – 1870, Genbank accession number M81385) long LRH-1 fragment [13] was donated by Margaret Hinshelwood. Cloned fragment corresponds to the ligand binding domain where homology between SF-1 and LRH-1 is only 63%, therefore preventing probe cross-reactivity with both mRNAs. Plasmid was linearized either by Bam HI or Sac I. cRNA probes were synthesized using T3 (antisense probe) and T7 (sense probe) RNA polymerases (Promega, Madison, Wisconsin, USA) and DIG RNA labeling mix (Roche, Mannheim, Germany) containing DIG-UTP. After digestion of remaining plasmid using RNAse free DNAse, cRNA probes were purified using Quiagen RNA spin columns (Quiagen, Hilden, Germany).

In situ hybridization

After cutting, sections were washed twice in 0.01 PBS (pH 7.4) at room temperature, followed by 10 minute digestion with proteinase K (10μg/ml; Sigma, Taufkirchen, Germany) in proteinase K buffer (0.1M Tris-HCl, pH 8, 0.05M EDTA; pH 8). After quick wash in TEA buffer (0.1M triethanolamine), sections were acetylated with acet anhydride in TEA buffer for 10 minutes at room temperature. Thereafter, sections were prehybridized with prehybridization buffer (Sigma) for 3–4 hours at 55 °C, followed by hybridization with 100 μl hybridization buffer (Sigma) containing ~50ng of probe per tissue section. Sections were hybridized overnight at 55 °C. Following day, sections were first washed in 2x SSC at room temperature 2x 5 minutes, followed by washes: 2x SSC at 55°C for 30 minutes, 0.5x SSC at 55 °C for 30 minutes, 2x SSC/30%formamide at 55 °C for 30 minutes and 2x 10 minutes 37 °C 2x SSC. Sections were then incubated with RNAse (20μg/ml; Promega) in RNAse buffer (0.5M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA, pH 8.0) for 30 minutes at 37 °C, washed in RNAse buffer at 37 °C for 30 minutes and transferred into washing buffer (Roche). Sections were blocked in blocking buffer (Roche) for 2 hours and incubated overnight at 4 °C with anti-digoxigenin antibodies (Roche), diluted 1:1000. Following day, sections were washed two times 15 minutes in washing buffer, 5 minutes in detection buffer (Roche) and incubated in NBT solution (Roche) for several hours. Sections were washed in PBS, mounted on slides and coverslipped using aqueous mounting media.

Photomicrographs

Photomicrographs were taken by Nikon microphot FXA and Nikon Coolpix5200 digital camera.

Results

Reverse transcription PCR

RT PCR amplification produced fragments of expected sizes for both LRH-1 and GAPDH (internal control). GAPDH fragments were of similar intensity

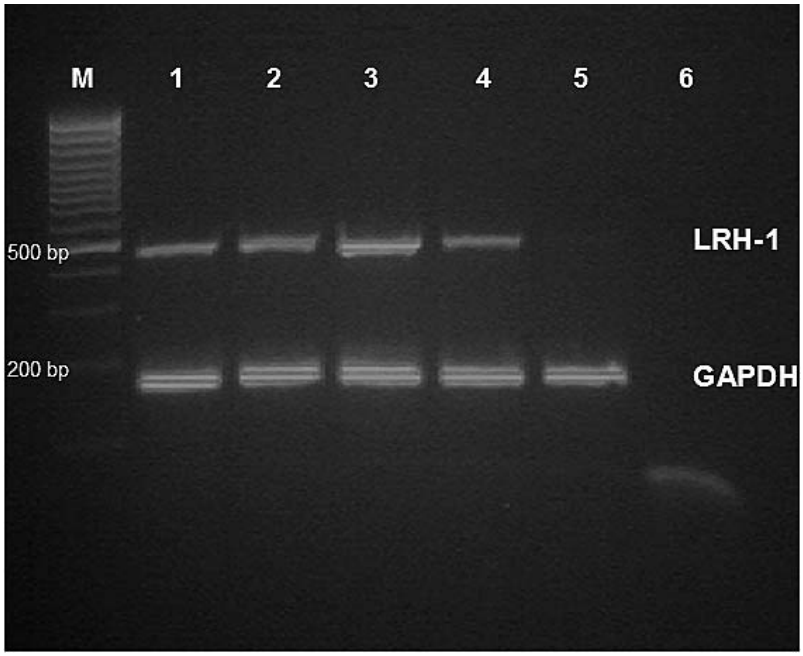

Figure 1: LRH 1 was detected in testis (1), liver (2), cerebrum (3) and cerebellum (4) using RT PCR and very weak, almost invisible band was also detected in RNA from muscle (5). GAPDH expression was at similar level in all five samples (lower band). 6 – control with no RNA, M – 100bp DNA ladder.

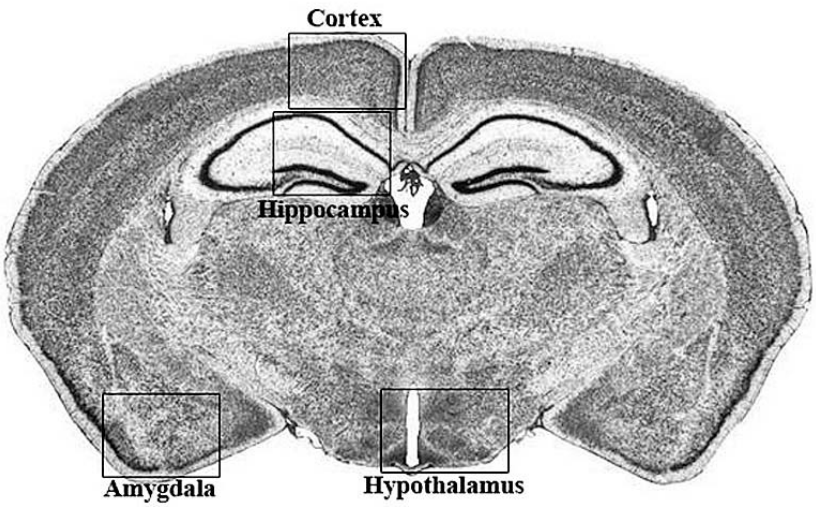

Figure 2: Coronal section of a Nissl stained murine brain through the caudal diencephalon. Labeled squares represent sections used in figure 3 at higher magnification.

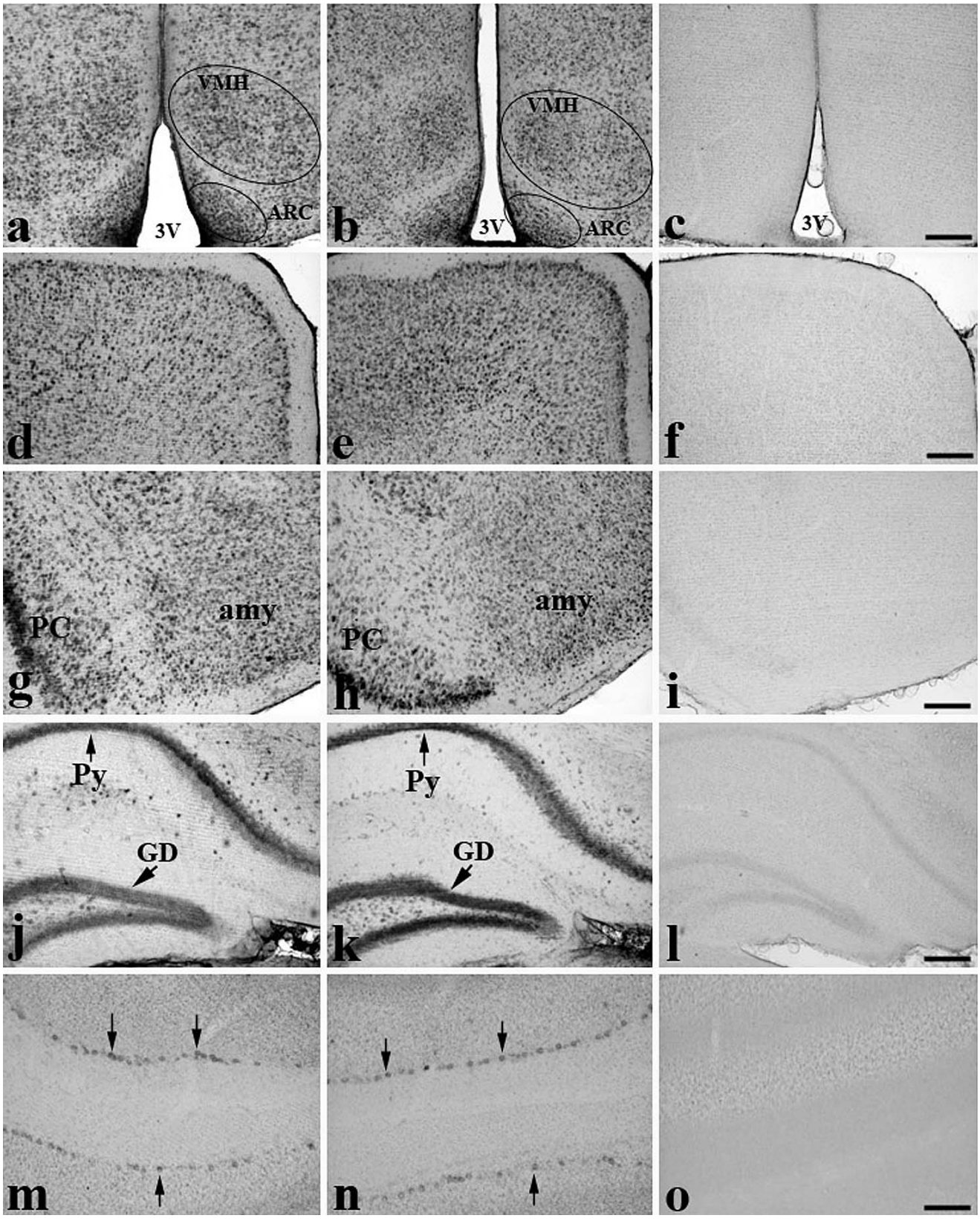

Figure 3: Coronal sections of 60 days old mouse brain probed with in situ hybridization probe for LRH-1. Panels **a, d, g, j** and **m** are from a representative male brain and panels **b, e, h, k** and **n** are from a representative female brain. Panels **c, f, i, l** and **o** are from sections reacted with the sense probe. Panels **a, b** and **c** show expression of LRH-1 in hypothalamus (VMH – ventromedial nucleus; ARC – arcuate nucleus; 3V – third ventricle), panels **d, e**, and **f** show cortex, panels **g, h**, and **i** amygdala (amy) and piriform cortex (PC), panels **j, k** and **l** hippocampus (**Py** – pyramidal cell layer, **GD** – gyrus dentatus) and panels **m, n** and **o** cerebellum with Purkinje cells (arrows).

in all samples. Interestingly, strongest expression of LRH-1 was found in cerebrum (diencephalon and telencephalon together). Levels of LRH-1 expression appear similar in testis, liver and cerebellum and there was also a very weak band in muscle, although this band was too weak to show on a photograph (*Figure 1*).

In situ hybridization for LRH-1 mRNA in adult brain

A low magnification image of a Nissl-stained coronal section shown in *figure 2* illustrates the regions shown for in situ hybridization using a probe for LRH. Hybridization using DIG labeled antisense probes revealed strong expression of LRH-1 mRNA throughout the brain. LRH-1 positive cells were found in hypothalamus (*Figure 3a, b*), cortex (*Figure 3d, e*), amygdala (*Figure 3g, h*), hippocampus (*Figure 3j, k*) as well as other parts of brain (olfactory bulbs, thalamus and others, not shown). LRH-1 was also strongly expressed in Purkinje cells in cerebellum (*Figure 3m, n*). Hybridization using sense probes did not produce any positive signal in any of the brain areas examined (*Figure 3c, f, i, l* and *o*). Although the results were not quantified and therefore minor differences could not be excluded, no obvious differences between male and female brain were observed and LRH-1 showed similar, widespread patterns of expression in 30 days old brains from mice of both sexes (not shown).

Discussion

In the present study, expression of LRH-1 mRNA in mouse brain was studied using both RT PCR and in situ hybridization approaches. By both methods, strong expression of LRH-1 mRNA was detected in most brain areas in addition to the liver, the site thought to be the main site of LRH-1 expression. LRH-1 is a member of orphan nuclear receptor family with close homology to SF-1 (reviewed in [9]). It was suggested that LRH-1, similar to SF-1, could regulate expression of some steroidogenic enzymes such as P450 aromatase, 11β-hydroxylase and 3β-hydroxysteroid dehydrogenase [12,26,27,39]. However, the expression pattern of LRH-1 only partially overlaps with the reported expression pattern of SF-1. Differential expression of SF-1 and LRH-1 implies different functions and it has been suggested that the main function of LRH-1 in steroidogenic tissues might be the regulation of P450 aromatase expression, enzyme responsible for converting C19 steroids into estrogens [13,27]. P450 aromatase is not only important for gonadal production of estrogens, but also has important roles in the brain. In males, peripherally produced testosterone is converted into estradiol in the brain and estradiol is responsible for many testosterone effects on the brain [2,5]. To enable conversion of testosterone to estradiol, P450 aromatase is expressed in many different areas of the brain. Although mRNA expression is relatively low, aromatase mRNA in situ [38], aromatase activity [29,36,37], and aromatase expression as determined by RT PCR was detected in may different parts of the mammalian brain including hypothalamus, preoptic area, amygdala, hippocampus, cortex and others [2,30]. In our study, LRH-1 mRNA expression was observed in most brain areas where P450 aromatase was previously detected, although expression of LRH-1 was much more widespread. Overlap with aromatase expression in certain areas could potentially suggest LRH-1 involvement in the regulation of aromatase gene expression also in the brain. However, more widespread expression of LRH-1 in comparison to aromatase expression would imply other functions of LRH-1 and even if it is involved in the regulation of aromatase expression, this would most likely require additional co-factors and modulators. Furthermore, aromatase expression was reported to be sexually dimorphic in hypothalamic and amygdalar regions [2,5,31]. We did not observe any obvious differences in LRH-1 expression between male and female brains, suggesting that either LRH-1 is not important for aromatase expression or it acts in a concert with other modulators and co-factors that might be expressed in sexually dimorphic manner, or we did not look early enough in development when aromatase activity is known to be greater in activity [36].

LRH-1 was also reported to regulate some other genes including members of the Oct family (POU domain genes). Cooney et al. [6] demonstrated that LRH-1 regulates Oct-4 in embryonic stem cells and dysregulation of Oct-4 expression in early embryos is responsible for early embryonic death of LRH-1 knockout mice [6,24]. Several members of Oct family including Oct-1, Oct-2, Oct-3, Oct-6 and Brn-5 [1,16,23,33] are also expressed in the murine brain. However, the pattern of expression of different POU domain genes is mostly restricted to particular parts of CNS or is developmentally regulated like Oct-6 [17]. Therefore, widespread expression of LRH-1 throughout the neuronal cells of the brain makes this possibility less likely, unless other transcription modulators are involved, that might precisely modulate transcriptional activity of LRH-1.

Although LRH-1 and SF-1 are structurally closely related, their pattern of expression differs considerably. While SF-1 expression is mostly limited to endocrine tissues that include gonads, adrenal glands, pituitary and ventromedial hypothalamus, LRH-1 is more widely expressed. Previous studies have demonstrated LRH-1 expression in liver, pancreas, intestine, adipose tissue, gonads and adrenal glands. In the current study, we have found LRH-1 expression in most areas of the brain that again differs considerably from SF-1 expression, limited to ventromedial nucleus of the hypothalamus (VMH). Although LRH-1 is also present in VMH, its expression is not limited to one particular area but is rather present in most neuronal cells of the brain including all hypothalamic nuclei. Besides aromatase, LRH-1 was reported to regulate expression of several other steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase [26] and 11β-hydroxylase [39], as well as expression of steroidogenic acute regulatory protein

(StAR) which plays a critical role in the initial steps of steroidogenesis [18]. Local production of steroid hormones is important for brain function and it is now well established that steroidogenic enzymes are expressed in several areas of the brain (reviewed in [35] along with the StAR gene, [19] necessary for the initial step in steroidogenesis. P450scc, P450c17, 3β-HSD and StAR are all expressed both in neurons and glial cells throughout the brain. Perhaps most striking similarity between LRH-1 expression and expression of steroidogenic enzymes is in cerebellum, where all genes are concomitantly expressed in Purkinje cells.

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