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SEXUALLY DIMORPHIC EFFECTS OF THE *LHX7* NULL MUTATION ON FOREBRAIN CHOLINERGIC FUNCTION

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Abstract—It has been reported recently that mice lacking both alleles of the LIM-homeobox gene *Lhx7*, display dramatically reduced number of forebrain cholinergic neurons. In the present study, we investigated whether the *Lhx7* mutation affects male and female mice differently, given the fact that gender differences are consistently observed in forebrain cholinergic function. Our results show that in adult male as well as female *Lhx7* homozygous mutants there is a dramatic loss of choline acetyltransferase immunoreactive forebrain neurons, both projection and interneurons. The reduction of forebrain choline acetyltransferase immunoreactive neurons in *Lhx7* homozygous mutants is accompanied by a decrease of acetylcholinesterase histochemical staining in all forebrain cholinergic neuron target areas of both male and female homozygous mutants. Furthermore, there was an increase of M1-, but not M2-, muscarinic acetylcholine receptor binding site density in the somatosensory cortex and basal ganglia of only the female homozygous mutant mice. Such an increase can be regarded as a mechanism acting to compensate for the dramatically reduced cholinergic input, raising the possibility that the forebrain cholinergic system in female mice may be more plastic and responsive to situations of limited neurotransmitter availability. Finally, our study provides additional data for the sexual dimorphism of the forebrain cholinergic system, as female mice appear to have a lower density of M1-muscarinic acetylcholine receptors in the striatal areas of the basal ganglia and a higher density of M2-muscarinic acetylcholine receptors, in a number of cortical areas, as well as the striatal areas of the basal ganglia. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: acetylcholinesterase, choline acetyltransferase, muscarinic acetylcholine receptors, forebrain cholinergic neurons, gender differences, *Lhx7* knock out mice.

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Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; CA1-3, fields 1–3 of Ammon's horn; ChAT, choline acetyltransferase; CP, caudate-putamen; DB, diagonal band nucleus; DG, dentate gyrus; ER, estrogen receptor; FCN, forebrain cholinergic neuron; HDB, horizontal limb of diagonal band nucleus; IR, immunoreactive; mAChR, muscarinic acetylcholine receptor; MCPO, magnocellular preoptic nucleus; MS, medial septal nucleus; NAc, nucleus accumbens; NB, nucleus basalis; NGF, nerve growth factor; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RT, room temperature; SI, substantia innominata; TBS, Tris–HCl-buffered saline; TU, olfactory tubercle; VDB, vertical limb of diagonal band nucleus.

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The forebrain cholinergic system has been the subject of extensive investigations due to its role in cognitive functions and its impairment in neurodegenerative diseases, such as Alzheimer's disease (Whitehouse, 1998), and developmental disorders, such as Rett (Wenk and Hauss-Wegrzyniak, 1999) and Down's syndromes (Casanova et al., 1985; Coyle et al., 1986), which are accompanied by cognitive deficits. Based on their location and projection pattern, forebrain cholinergic neurons (FCNs) can be classified into interneurons and projection neurons. The cholinergic interneurons of the forebrain constitute a diffuse cellular network scattered in the striatal compartment of the basal ganglia, namely the caudate-putamen, CP; nucleus accumbens NAc; islands of Calleja, ICj; olfactory tubercle, TU. The cholinergic projection neurons which are located in the basal forebrain, are more densely packed and form contiguous nuclei along the anterior-posterior axis of the forebrain (i.e. medial septal nucleus, MS; vertical limb of diagonal band nucleus, VDB; horizontal limb of diagonal band nucleus, HDB; magnocellular preoptic nucleus, MCPO; nucleus basalis, NB; substantia innominata, SI) (Woolf, 1991). The basal forebrain cholinergic projection neurons send distal axons innervating the cortical mantle in a position-dependent manner: The most rostral ones project to allocortical areas, including the hippocampus, whereas the most caudal innervate the neocortex, including the somatosensory field (Bigl et al., 1982; Woolf et al., 1984; Woolf, 1991).

FCNs are born during embryogenesis in the ventral telencephalon, more specifically in the medial ganglionic eminence, the anterior entopeduncular and preoptic areas (Marin and Rubenstein, 2002). Although genetic and developmental mechanisms underlying the formation of FCNs are only beginning to be elucidated, recent studies have indicated that FCNs arise from progenitor cells expressing the homeobox gene *Nkx2.1* (Sussel et al., 1999; Marin et al., 2000). Furthermore, the LIM-homeobox gene *Lhx-7* has been shown to play a crucial role in the specification or differentiation of FCNs (Zhao et al., 2003; Mori et al., 2004; Fragkouli et al., 2005). In addition, it is well established that late stages of cholinergic differentiation are regulated by the neurotrophin nerve growth factor (NGF)—through binding to its high and low affinity receptors (TrkA and p75, respectively), both of which are expressed in FCNs (Yuen et al., 1996; Fagan et al., 1997; Chen et al., 1997). Interestingly, in rodents cholinergic projection neurons of the basal forebrain mature earlier in female than in male animals (Loy and Sheldon, 1987; Kornack et al., 1991; Ricceri et al., 1997), indicating that gonadal hormones may participate in their maturation,

possibly through an estrogenic modulation of the expression of NGF and its receptors (Gibbs et al., 1994, 1998; Pan et al., 1999; Ping et al., 2002).

In adult animals, significant gender differences in forebrain cholinergic function have been observed (Gibbs and Aggarwal, 1998; Rhodes and Rubin, 1999; McEwen, 2001), regarding nearly all cholinergic parameters, including cell size (Westlind-Danielsson et al., 1991) and cholinergic enzyme activity (Luine and McEwen 1983; Luine et al., 1986). Male and female rodents also differ in the sensitivity of both cholinergic interneurons and projection neurons to pharmacological manipulations (Miller, 1983; Witt et al., 1986) and lesions (Jonasson et al., 2004) and in the vulnerability of the basal forebrain cholinergic system during aging (Luine et al., 1986). Interestingly, with the exception of CP cholinergic interneurons, FCNs express estrogen receptors (ERs) (Shughrue et al., 2000; Miettinen et al., 2002) and estrogens have been shown to affect expression and activity of choline acetyltransferase (ChAT) (Gibbs et al., 1994; Miller et al., 1999; Pan et al., 1999; Gibbs, 2000; Granholm et al., 2002), high affinity choline uptake and acetylcholine release (O'Malley et al., 1987; Gibbs et al., 1997). Furthermore, estrogens induce expression of NGF and its receptors in the basal forebrain and/or in the target areas of basal forebrain cholinergic projection neurons, namely the hippocampus and neocortex (Gibbs et al., 1994, 1998; Pan et al., 1999; Ping et al., 2002).

It has been reported recently that mice deprived of the LIM-homeodomain protein LHX7 are characterized by a dramatic reduction in the number of both types of FCNs i.e. interneurons and projection neurons (Zhao et al., 2003; Mori et al., 2004; Fragkouli et al., 2005). However, these studies were either carried out with male mice, or did not discriminate between male and female animals. Given the sexually dimorphic nature of the forebrain cholinergic system, we investigated the possibility that loss of LHX7 function has a gender specific effect on the development of FCNs. Therefore, we examined the effect of an *Lhx7* targeted mutation on ChAT immunoreactivity, acetylcholinesterase (AChE) histochemistry and muscarinic acetylcholine receptor (mAChR) binding in adult male and female mice.

EXPERIMENTAL PROCEDURES

Animals

Lhx7 homozygous mutant ($Lhx7^{-/-}$) mice were generated as previously described (Fragkouli et al., 2005) and bred in a C57/BL6 genetic background. All efforts were made to minimize the number of animals used and their suffering. Heterozygous ($Lhx7^{+/-}$) mice were used for breeding, and animals of both genders reared in our laboratory were kept under standard conditions (24 °C, 12-h light/dark cycle, lights on at 8:00 a.m.) and received food and water *ad libitum*. Mice used in this study were virgin adult animals aged 3–5 months old. All animal experimentations were carried out in agreement with ethical recommendation of the European Communities Council Directive of 24 November 1986 (86/609/EEC).

ChAT immunohistochemistry

Animals were anesthetized with sodium pentobarbitone (Sagatal, 60 mg/ml, Rhone Merieux Ltd., UK) and perfused transcardially with cold saline (4 °C) followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4, 4 °C). Brains were removed, postfixed in the same fixative overnight at 4 °C, washed thoroughly with PBS, mounted in 3% agarose/PBS and cut into 30 μm thick coronal sections using a vibratome (Leica, Germany). For immunohistochemistry, brain sections were washed in Tris–HCl-buffered saline (TBS, pH 7.6), incubated for 15 min in 10% H_2O_2, 10% methanol in TBS and washed again in TBS. After permeabilization with 1% Triton X-100/TBS for 30 min, sections were blocked for 2 h at room temperature (RT) with 10% rabbit serum, 1% bovine serum albumin, 0.1% Triton X-100 in TBS and incubated overnight at RT with an antiserum specific for ChAT (goat polyclonal, Chemicon, USA) diluted 1:500 in a solution containing 1% rabbit serum, 0.1% bovine serum albumin, 0.1% Triton X-100 in TBS. The following day sections were thoroughly washed with TBS and incubated for 2 h at RT with biotinylated rabbit anti-goat IgGs (Dako Cytomation, Denmark) diluted 1:200 in a solution containing 1% rabbit serum, 0.1% bovine serum albumin, 0.1% Triton X-100 in TBS. After extensive washes with TBS, sections were incubated for 30 min at RT with an avidin–biotin complex (AB-HRP complex, Dako Cytomation) diluted 1:100 in TBS, washed thoroughly in TBS and developed with 3,3-diaminobenzidine, (DAB, Sigma-Aldrich, USA) 0.07% in 0.03% H_2O_2/TBS. Finally, sections were washed and mounted on silane-coated slides using Glycergel mounting medium (Dako Cytomation).

Cell counting. Coronal brain sections from wild-type (six males and eight females), $Lhx7^{+/-}$ (nine males and nine females) and $Lhx7^{-/-}$ (8 males and 10 females) mice were used for counting. ChAT-immunoreactive (IR) cells were counted independently and 'blindly' by two investigators using the image analysis program 'Image Pro Plus' (Media Cybernetics, USA), employing an unbiased systematic random approach: For each brain structure ChAT-IR cells were counted in a rostrocaudal series of equally spaced sections (every 300 μm, one section counted every 10 sections cut) starting from a section in which the brain structure first appeared in each animal—according to the mouse brain atlas (Paxinos and Franklin, 2001)—and spanning the full extent of the structure. In each section the cells in the entire nucleus/area were counted. The number of cells in each brain structure was calculated by multiplying the total number of cells in all the sections counted, by 10, the sampling distance. ChAT-IR cells in heterozygous and knockout animals were then expressed as a percent of the corresponding mean value of the wild type animals.

AChE histochemistry

Animals were anesthetized and decapitated and their brains were immediately removed, frozen in isopentane at −40 °C and stored at −80 °C for at least 24 h before sectioning. Brain tissue was cut into 10 μm thick coronal sections at −21 °C using a cryostat (Leica) and sections were collected on silane-coated slides, air-dried for 2 h and stored at −80 °C until further use. For histochemistry, cryosections were equilibrated at RT, postfixed in cold (4 °C) 4% PFA/PBS (pH=7.4), washed thoroughly in 0.1 M CH_3COONa (pH=5.6) and incubated for 2 h at 37 °C with the substrate acetylthiocholine iodine (1.73 mM, Sigma-Aldrich) in a reaction mixture containing 1 μM iso-OMPA (Sigma-Aldrich), 4 mM $C_6H_5Na_3O_7$, 3 mM $CuSO_4$, 0.1 mM $K_3Fe(CN)_6$ and 65 mM CH_3COONa (pH=5.6). To determine non-specific staining, adjacent sections were incubated with both the substrate and eserine (1 μM, Sigma-Aldrich). Samples were then washed thoroughly in 0.1 M CH_3COONa (pH=5.6), developed for 1 min in 1% Na_2S (pH=7.8), rinsed with 0.1 M $NaNO_3$ and incubated for 1 min in