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EFFECTS OF NEONATAL HANDLING ON THE BASAL FOREBRAIN CHOLINERGIC SYSTEM OF ADULT MALE AND FEMALE RATS

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Abstract—Neonatal handling is an early experience which results in improved function of the hypothalamic–pituitary–adrenal axis, increased adaptability and coping as a response to stress, as well as better cognitive abilities. In the present study, we investigated the effect of neonatal handling on the basal forebrain cholinergic system, since this system is known to play an important role in cognitive processes. We report that neonatal handling results in increased number of choline-acetyl transferase immunopositive cells in the septum/diagonal band, in both sexes, while no such effect was observed in the other cholinergic nuclei, such as the magnocellular preoptic nucleus and the nucleus basalis of Meynert. In addition, neonatal handling resulted in increased M1 and M2 muscarinic receptor binding sites in the cingulate and piriform cortex of both male and female rats. A handling-induced increase in M1 muscarinic receptor binding sites was also observed in the CA3 and CA4 (fields 3 and 4 of Ammon's horn) areas of the hippocampus. Furthermore, a handling-induced increase in acetylcholinesterase staining was found only in the hippocampus of females. Our results thus show that neonatal handling acts in a sexually dimorphic manner on one of the cholinergic parameters, and has a beneficial effect on BFCS function, which could be related to the more efficient and adaptive stress response and the superior cognitive abilities of handled animals. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: early handling, sex differences, ChAT, AChE, M1 and M2 muscarinic receptors.

The basal forebrain cholinergic system (BFCS) consists of an extended continuum of subcortical neurons, forming densely packed cholinergic nuclei, which project to a variety of isocortical and allocortical areas, including the hippocampus. The contiguous nuclei of the BFCS along the anterior–posterior axis of the forebrain are the medial septal nucleus, MS; vertical limb of diagonal band nucleus, VDB; horizontal limb of diagonal band nucleus, HDB; magnocellular preoptic nucleus, MCPO; nucleus basalis of Meynert, NB; substantia innominata, SI (Woolf, 1991). The projection pattern of the basal forebrain cholinergic nuclei (BFCN) follows 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). The BFCS has been implicated in cognitive processes, since it is impaired in neurodegenerative diseases accompanied by various forms of dementia such as Alzheimer's (Whitehouse, 1998), Parkinson's, or Creutzfeldt-Jacob disease, Korsakoff's syndrome, progressive supranuclear palsy, olivopontocerebellar atrophy, dementia pugilistica (for review see Lucas-Meunier et al., 2003) and developmental disorders with cognitive deficits, such as Rett (Wenk and Hauss-Wegrzyniak, 1999) and Down syndrome (Casanova et al., 1985; Coyle et al., 1986). Furthermore, more than 20 years ago Bartus et al. (1982) first postulated the hypothesis of cholinergic geriatric and Alzheimer's disease memory dysfunction according to which the ageing-induced decline in cognitive abilities is accompanied by degeneration of the BFCS (Sarter and Bruno, 2004). Notably, the only drugs available for the treatment of dementia in humans, target this system. In addition, animal studies in rodents have demonstrated the importance of the BFCS in attentional processes and abilities for stimulus selection by showing that administration of cholinergic antagonists or lesions of the BFCS result in impaired performance in a wide variety of cognitive tasks [Morris Water maze (Buresova et al., 1986; Leanza et al., 1995; Waite et al., 1995), delayed nonmatching to position (Torres et al., 1994; Leanza et al., 1996), five-choice serial reaction time (McGaughy et al., 2002; Lehmann et al., 2003), radial arm maze (Sessions et al., 1998; Lehmann et al., 2003), passive avoidance (Torres et al., 1994; Leanza et al., 1995; Zhang et al., 1996), and social transmission of food preference (Berger-Sweeney et al., 2000; Vale-Martinez et al., 2002)] measuring different forms of learning and reference or working memory.

It is well known that early experiences, particularly the mother–infant interaction, influence brain development and have long-term consequences for adult behavior, including cognitive abilities. An experimental paradigm employed to study the effects of early experiences is "neonatal handling," which has been shown to alter the program-

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Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; BFCN, basal forebrain cholinergic nuclei; BFCS, basal forebrain cholinergic system; CA1-4, fields of Ammon's horn 1–4; ChAT, choline-acetyl transferase; ChAT-IR, choline-acetyl transferase immunoreactive; DB, diagonal band nucleus; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; mAChR, muscarinic acetylcholine receptor; MCPO, magnocellular preoptic nucleus; MS, medial septal nucleus; NB, nucleus basalis of Meynert; NDS, normal donkey serum; NGF, nerve growth factor; PFA, paraformaldehyde; PND, postnatal day; RT, room temperature; S.E.M., standard error of the mean; TBS, Tris–HCl-buffered saline.

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ming of hypothalamic–pituitary–adrenal (HPA) axis function, in such a way that the ability of the adult organism to respond, cope and adapt to stressful stimuli is increased (Levine, 1957; Ader and Grota, 1969; Hess et al., 1969; Meaney et al., 1991; Fernandez-Teruel et al., 2002). As a consequence, handled rats show less fear and anxiety in novel environments, more exploratory behavior, and lower emotionality (Meaney et al., 1991; Fernandez-Teruel et al., 1997; Vallée et al., 1997; Meerlo et al., 1999). Since adaptability and low anxiety levels are prerequisites for learning and memory, one would expect that neonatal handling would result in better cognitive abilities. Indeed it has been shown that neonatally handled rats perform better in a variety of learning paradigms and are protected from the ageing-associated loss of hippocampal pyramidal neurons and the consequent decline of spatial learning and memory (Meaney et al., 1988; Fernandez-Teruel et al., 1997).

In the present study we investigated the effect of neonatal handling on the BFCS in both male and female adult rats. Both sexes were studied since it has been previously shown that neonatal handling has a number of sexually dimorphic effects (Smythe et al., 1994; Papaioannou et al., 2002a,b; Park et al., 2003). Furthermore, there is a significant amount of experimental evidence that different aspects of BFCS function in rodents are sexually dimorphic. BFCS matures earlier in female than in male animals (Loy and Sheldon, 1987; Kornack et al., 1991; Ricceri et al., 1997). Furthermore, in adult animals, significant sex 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 cholinergic neurons to pharmacological manipulations (Miller, 1983; Witt et al., 1986) and lesions (Jonasson et al., 2004) and in the vulnerability of the BFCS during aging (Luine et al., 1986). In addition, in the rat, cognitive abilities have been shown to differ between males and females (Andrews, 1996).

Based on all the above, we investigated the effect of neonatal handling on: a) the number of choline-acetyl transferase (ChAT, the biosynthetic enzyme of acetylcholine) immunopositive cells in the nuclei of the BFCS; b) acetylcholinesterase staining (AChE, the degrading enzyme of acetylcholine) in the hippocampus, a projection area of the BFCS; and c) M1 and M2 muscarinic acetylcholine receptor (mAChR) binding sites in the target areas of the BFCN. Our hypothesis was that neonatal handling, acting in a sexually dimorphic way, would result in a functionally more effective BFCS.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats of both sexes reared in our laboratory were kept under standard conditions (24 °C; 12-h light/dark cycle, lights on at 8:00 a.m., three animals of the same sex per cage) and received food and water *ad libitum*. Virgin females were exposed to stud males and pregnancy was determined by the presence of sperm in the vaginal smear (day 0 of pregnancy). Prior to birth litters from each dam were randomly assigned to either the handled or non-handled category (six litters in each of the two categories). The average litter size (mean±standard error of the mean (S.E.M.): 8±1, range: 5–13) did not differ between non-handled and handled groups. Litters were not culled, since it has been shown that litter size within this range (5 to 18) does not affect maternal behavior (Champagne et al., 2003). The sex ratio did not differ among the litters employed in the different animal groups [average sex ratio (mean±S.E.M.): non-handled litters 1.08±0.13, handled litters 1.13±0.25]. The day of birth was defined as postnatal day 0 (PND0). Two cohorts of adult animals were used in this study: Cohort A was used for the determination of choline-acetyl transferase immunoreactive (ChAT-IR) cells (six male non-handled, six male handled, six female non-handled, six female handled; one animal of each sex from each litter employed); cohort B was used for the determination of AChE staining and M1 and M2 mAChR levels (six male non-handled, six male handled, six female non-handled, six female handled; one animal of each sex from each litter employed). All animal experimentations were carried out in agreement with ethical recommendation of the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

"Neonatal handling"

The neonatal handling protocol employed was as originally described by Levine (1957), which involves removal of the pups from the nest for 15 min daily during the neonatal period and placing them in a separate container, taking care to control for the pups' body temperature. In the present experiments "handling" was performed from the first PND (PND1) until weaning (PND22).

Specifically, every day between 9:00–10:00 a.m. mothers of the pups to be subjected to handling were removed from their home cages and temporarily placed separately into cages [the same cage for each mother every day for the duration (22 days) of handling]. Their pups were then removed and placed into plastic containers, lined with paper towels. After 15 min the pups, and then their mothers, were returned to their home cages. "Non-handled" pups were left undisturbed with their mothers in their home cage until weaning.

Tissue preparation

All animals were deeply anesthetized, decapitated and brains were isolated and frozen in −40 °C isopentane. Brain tissue was cut into coronal 20 μm sections for immunohistochemistry (animal cohort A) and histochemistry (one hemisphere from animals of cohort B) or 10 μm sections for mAChR *in vitro* binding (the other hemisphere from animals of cohort B), on a cryostat (Leica CM1900, Nussloch, Germany) at −17 °C, collected on silane-coated slides and stored at −80 °C until further processed.

ChAT immunohistochemistry

Sections were thawed, post-fixed with ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 1 h, washed in Tris–HCl-buffered saline (TBS, pH 7.6) (3×5 min), 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% normal donkey serum (NDS) (Chemicon, Temecula, CA, USA), 0.4% Triton X-100 in TBS and incubated overnight at RT with antiserum specific for ChAT (goat polyclonal, Chemicon) diluted 1:200 in a solution containing 4% NDS, 0.4% Triton X-100 in TBS. The following day sections were thoroughly washed with TBS (3×5 min) and incubated for 2 h at RT with biotinylated donkey anti-goat IgGs (Chemicon) diluted 1:200 in a solution containing 2% NDS in TBS. After extensive washes with TBS, sections were incubated for 45 min at RT with an avidin–biotin–