Journal of Neuroendocrinology
From Molecular to Translational Neurobiology

Journal of Neuroendocrinology **21**, 400–405

REVIEW ARTICLE

Activational and Organisational Effects of Gonadal Steroids on Sex-Specific Acetylcholine Release in the Dorsal Hippocampus

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Journal of Neuroendocrinology

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Acetylcholine (ACh) release in the dorsal hippocampus increases during stress, exploration or learning, exhibiting sex-specific 24-h release profile. We review the role of gonadal steroids on the ACh release in the dorsal hippocampus. In our studies, we found that male rats showed higher extracellular ACh levels than females, but gonadectomy decreased ACh levels in both sexes of rats and subsequently eliminated the sex difference. To examine the sex difference under comparable gonadal steroid levels, we implanted steroid capsules after gonadectomy. Oestradiol supplementation maintained circulating oestradiol to the levels in proestrous female rats, whereas testosterone capsules maintained circulating testosterone to the levels similar to intact male rats. Under comparable gonadal steroids levels, ACh levels were sex-specific. Testosterone replacement in orchidectomised rats clearly restored ACh levels, which were greater than ovariectomised testosterone-primed rats. Similarly, oestradiol replacement in ovariectomised rats successfully restored ACh levels, which were higher than orchidectomised oestradiol-primed rats. These results suggest sex-specific activational effects of gonadal steroids on ACh release. To further examine the organisational effect, female pups were neonatally treated with oil, testosterone, oestradiol, or dihydrotestosterone. These rats were bilaterally ovariectomised and a testosterone capsule was implanted at postnatal week 8. Neonatal treatment of either testosterone or oestradiol clearly increased ACh levels, whereas neonatal dihydrotestosterone treatment failed to change levels. These results suggest that: (i) gonadal steroids maintain the sex-specific ACh release in the dorsal hippocampus and (ii) neonatal activation of oestrogen receptors is sufficient to mediate masculinisation of the septo-hippocampal cholinergic system.

Key words: sex difference, circadian rhythm, androgen, oestrogen, learning and memory.

doi: 10.1111/j.1365-2826.2009.01848.x

Sex difference in higher brain functions

A number of studies have shown sex differences in spatial memory in various mammalian species, including humans (1, 2). Compared to females, males exhibit superior spatial memory function in many spatial tasks, such as a radial arm maze and the Morris water maze, and some spatial cognitive tasks, including mental rotation (3–13). Because spatial memory function is highly dependent on the hippocampus (14, 15), the results suggest a sex difference in the hippocampal function.

By contrast, females appear to excel in some neocortical functions. First, in a novel object recognition task, female rats were found to spend more time exploring the novel object than the familiar one compared to male rats (16), suggesting better visual object memory function in females compared to males. Because these functions are less dependent on the hippocampus and require the integrity of the medial prefrontal cortex, a sex-specific medial prefrontal cortical system may contribute to differences in performance (17–20). Second, in motor coordination tests, females appear to show better performance than males. Female rats achieved a higher score than male rats in rotarod tasks, the vertical pole task and the hanging wire task: female rats stayed on the pole or the wire lid longer than male rats (21, 22). In humans, women show better performance than men in some motor tasks, such as the insertion of pegs into rows of holes (23–25). Finally, in verbal recall tests, it is well documented that women perform better than men

(26). The sex difference may not depend on language or cultural background because an analysis of the Programme for International Student Assessment (PISA) in 40 countries worldwide consistently showed superior reading scores in girls (27).

Cholinergic neurones within the basal forebrain provide the major projection to the neocortex and hippocampus (28). Cortical regions receive cholinergic inputs mainly from the nucleus basalis magnocellularis or the diagonal band of Broca, whereas the hippocampus receives cholinergic inputs mostly from the medial septum and horizontal limb of diagonal band of Broca (28). Because the widespread cholinergic projections are necessary to maintain higher brain functions (29, 30), we hypothesised that a sex-specific acetylcholine (ACh) release in specific brain areas may underlie the difference. To estimate ACh release, we performed *in vivo* microdialysis study in freely-moving rats (31). Briefly, a microdialysis probe with semi-permeable membrane (1.0 mm in length) was inserted into a specific brain area via surgically pre-implanted guide cannula. We perfused inside of the membrane with artificial cerebrospinal fluid, and assayed ACh in dialysates using a high-performance liquid chromatography system. We found that females exhibited greater extracellular ACh levels in the medial prefrontal cortex (31) and the premotor/supplementary motor area (32), whereas males exhibited greater extracellular ACh levels in the dorsal hippocampus (33, 34). Moreover, recent stereological analyses showed that the number of choline acetyltransferase immunoreactive cells in the nucleus basalis magnocellularis was greater in females than males (31). Although the female superiority in neocortical functions is interesting, in this review, we focus on the sex difference in the ACh levels in the hippocampus.

Physiological role of ACh in the hippocampus

ACh appears to orchestrate major hippocampal functions. Behavioural studies have demonstrated that the extracellular ACh levels increase during learning (35–37) and are positively correlated with learning performance (38, 39). Because bilateral injections of scopolamine into the dorsal hippocampus impair spatial learning (40) and the encoding of contextual episodes (41), muscarinic ACh receptors appear to mediate the consolidation of both spatial and episodic memory. At the network level, ACh generates a theta rhythm (42) that modulates the induction of long-term potentiation (LTP) in hippocampal CA1 neurones (43). At the cellular level, both pyramidal and nonpyramidal neurones in the hippocampal CA1 area receive direct cholinergic afferents mediated by the muscarinic receptors (44–46). *In vitro* studies showed that bath application of carbachol, a cholinergic agonist, induces LTP in CA1 pyramidal neurones without electrical tetanus stimulus, suggesting that ACh in the hippocampus plays a principal role in the synaptic plasticity of the CA1 pyramidal neurones (47). A recent study further revealed that activation of muscarinic ACh receptors induces Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive stores to induce synaptic enhancement (48). ACh released in the hippocampus not only enhances synaptic plasticity via the M_1/M_2 receptors (49, 50), but also is responsible for neurogenesis in the dentate gyrus (51, 52). Therefore, it would be of interest to elucidate the molecular mechanisms underlying ACh regulation of hippocampus-dependent learning.

Sex difference in the ACh levels in the dorsal hippocampus

Our studies indicate a sex difference in the septo-hippocampal cholinergic system. Gonadally intact male rats consistently show a greater stress response of ACh release in the hippocampus compared with diestrous or proestrous female rats, suggesting that the response of the septo-hippocampal cholinergic system to environmental stimuli is sexually dimorphic (53). Moreover, we found a sex difference in the 24-h ACh release profile in the hippocampus in rats: the extracellular ACh levels in the hippocampus were consistently lower in female rats than in male rats (33). Although the ACh levels clearly showed a daily rhythm in female rats, females exhibited a smaller amplitude of daily change than males. However, the number of choline acetyltransferase immunoreactive cells in the medial septum or horizontal limb of diagonal band did not show a sex difference (34). Because the number of septo-hippocampal cholinergic neurones does not appear to be involved in the sex difference in ACh levels in the hippocampus, sex-specific neural circuits or substance(s) may affect the *in vivo* ACh release. We hypothesised that sex-specific ACh release in the hippocampus could be influenced by the neonatal organisational effects of testosterone and/or the activational effects of gonadal steroid hormones (54).

Activational effects of testosterone or oestradiol

For decades, neuroanatomical, neurochemical and behavioural evidence has suggested an activational effect of gonadal steroids on the septo-hippocampal cholinergic neurones (55–62), with little evidence presented in freely-behaving rats. To evaluate the sex difference under comparable levels of gonadal steroids, we implanted steroid capsules into gonadectomised rats. Oestradiol capsules maintained circulating oestradiol at the levels in proestrous female rats, whereas testosterone capsules maintained the circulating testosterone at the levels in intact male rats (54). Gonadectomy eliminated the sex difference with these rats by decreasing ACh levels. Testosterone replacement in orchidectomised rats clearly restored ACh levels, which were greater than in ovariectomised testosterone-primed rats. Conversely, oestradiol replacement in ovariectomised rats successfully restored the ACh levels, which were higher than in orchidectomised oestradiol-primed rats. Thus, the corresponding gonadal steroid hormone maintains the ACh levels in gonadectomised rats, suggesting sex-specific activational effects of gonadal steroids (Fig. 1).

Spontaneous behaviours increase the ACh levels

Acetylcholine levels episodically change with spontaneous movement (63–65). Levels increase during learning or exploratory behaviours (35–37), which stimulate electrical activity of cholinergic neurones in the basal forebrain (66). Moreover, voluntary running enhances neurogenesis, spatial learning and synaptic plasticity in

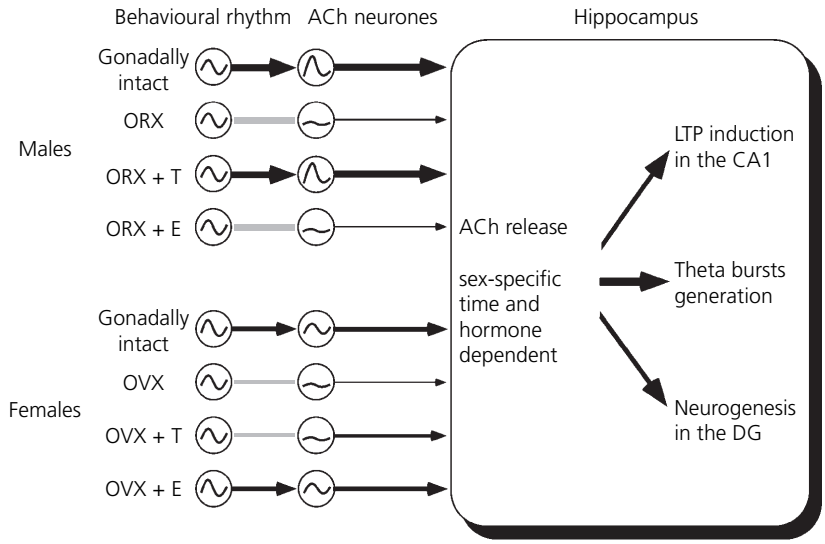

Fig. 1. A schema of hypothesis illustrating septo-hippocampal cholinergic systems. Acetylcholine (ACh) release in the dorsal hippocampus appears to be sex-specific, time-dependent and hormone-dependent. In male rats, testosterone increases coupling between the spontaneous locomotor activity and the cholinergic neurones. In orchidectomised (ORX) rats, testosterone replacement (+T), but not oestradiol replacement, maintains the coupling. In female rats, oestradiol increases coupling between the spontaneous locomotor activity and the cholinergic neurones. In ovariectomised (OVX) rats, oestradiol replacement (+E) maintains the coupling, whereas testosterone replacement (+T) failed to change the ACh release property to those seen in male rats. Moreover, neonatal treatment of testosterone or oestradiol fates the sex-specific activational effect, suggesting sexual differentiation of the septo-hippocampal cholinergic system. The released ACh appears to orchestrate major hippocampal functions (42–52). DG, dentate gyrus; LTP, long-term potentiation.

mice (67). By contrast, a restriction of exploratory behaviour reduces ACh levels (65) as well as spatial learning (68). Therefore, to analyse the precise effects of gonadal steroids on ACh levels, we simultaneously analysed ACh levels and spontaneous locomotor activity to determine the precise effect of gonadal steroids. We found that gonadectomy severely impaired ACh levels without changes in spontaneous locomotor activity.

Moreover, after gonadectomy the positive correlation between ACh levels and locomotor activity levels was no longer present, suggesting that hippocampal function may not always be activated at low gonadal steroid levels. Learning impairment in ovariectomised or orchidectomised rats (56, 57, 59, 60, 69) may be due to insufficient activation of hippocampus at the appropriate time. Because the replacement of sex-specific gonadal steroid restored the high positive correlation between ACh levels and activity levels, the correlation appears to depend on the presence of gonadal steroids. These results suggest that circulating gonadal steroids strengthen the coupling between spontaneous behaviours and ACh levels (Fig. 1).

Organisational effects of testosterone and oestradiol

As discussed earlier, testosterone replacement in ovariectomised rats failed to increase ACh levels to those seen in orchidectomised testosterone-primed rats. Similarly, oestradiol replacement was unable to restore ACh levels in orchidectomised rats. Moreover, oestradiol consistently increases *N*-methyl-D-aspartate receptor binding and spine density in the CA1 area of ovariectomised rats, although the treatment fails to increase these same parameters in orchidectomised rats (70, 71). These results suggest that sex-specific gonadal steroids are important for maintaining hippocampal functions. Based on our data, we hypothesised that the action of sex-specific steroids is due to neonatal sexual differentiation rather than the activational effects of gonadal steroids in adult rats. We found that neonatal androgenisation in females increased ACh levels to resemble that of normal males without affecting spontaneous activity levels. These results indicate an organisational effect of gonadal steroids on sex-specific ACh levels in behaving rats, and currently accepted theories of sexual differentiation.

Because testosterone can be aromatised to oestradiol in the forebrain, neonatal gonadal steroids activate both oestrogen and androgen receptors (72). In our study, both testosterone and oestradiol treatment in neonatal female pups masculinised ACh levels in adults, suggesting an oestrogen receptor-mediated masculinisation of septo-hippocampal cholinergic systems. These results are consistent with the previous finding that testosterone or oestradiol treatment in neonatal female pups improves their adult spatial performance, whereas neonatal gonadectomy in male pups causes decrements in performance (73). By contrast, dihydrotestosterone treatment failed to masclinise ACh levels. Although dihydrotestosterone has been classically considered as a prototypical androgen receptor agonist, a metabolite of dihydrotestosterone, 3β-diol, has higher affinity for oestrogen receptor β (74). Therefore, dihydrotestosterone and its metabolites may stimulate both androgen receptor and oestrogen receptor β, whereas oestradiol stimulates oestrogen receptor α and β. Considering the action of gonadal steroids and their metabolites, oestrogen receptor α may mediate the organisational effect on septo-hippocampal cholinergic system.

Conclusions

In summmary, we examined the activational and organisational effects of gonadal steroids on sex-specific acetylcholine release in the dorsal hippocampus. We found that: (i) male rats exhibited higher ACh levels in the dorsal hippocampus than females; (ii) the activational effects of gonadal steroids maintaining the ACh levels are sex-specific; and (iii) the organisational effects of gonadal steroids suggest oestrogen receptor-mediated masculinisation of the sex-specific activational effect.

Not only gonadal steroids, but also various environmental conditions appear to affect hippocampal learning performance or ACh levels. For example, if rats were reared in isolated condition after weaning, female rats exhibited a performance superior to that of male rats (3). In addition, exposure to an acute stressful event can enhance learning in male rats, whereas exposure to the same event impairs performance in females (75). We have reported that 4-day housing in a small cage attenuates spatial learning and ACh levels in the hippocampus in male rats (33, 65, 68), but not in females (33). Moreover, postpubertal feeding with powdered diet not only enhanced spatial learning, but also increased ACh levels in the hippocampus only in female rats (76). Considering the sex-specific influences of environmental conditions, it would be of interest to elucidate the interaction between gonadal steroids and environmental conditions.

Detailed information is available in our recent paper (77).

Acknowledgements

I greatly appreciate the commitee's invitation to present the US-Japan Symposium in Gifu, Japan.

Received: 12 November 2008,
revised 22 January 2009,
accepted 26 January 2009

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