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REVIEW ARTICLE

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Rapid nongenomic modulation by neurosteroids of dendritic spines in the hippocampus: Androgen, oestrogen and corticosteroid

G. Murakami[1] | Y. Hojo[2] | A. Kato[3] | Y. Komatsuzaki[4] | S. Horie[5] | M. Soma[6] | J. Kim[6] | S. Kawato[3,5,6]

[1]Department of Liberal Arts, Faculty of Medicine, Saitama Medical University, Iruma, Saitama, Japan

[2]Department of Biochemistry, Faculty of Medicine, Saitama Medical University, Iruma, Saitama, Japan

[3]Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro, Tokyo, Japan

[4]Department of Physics, College of Science and Technology, Nihon University, Chiyoda, Tokyo, Japan

[5]Department of Urology, Graduate School of Medicine, Juntendo University, Hongo, Tokyo, Japan

[6]Department of Cognitive Neuroscience, Faculty of Pharma-Science, Teikyo University, Itabashi, Tokyo, Japan

Correspondence
Suguru Kawato, Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro, Tokyo, Japan.
Email: kawato@bio.c.u-tokyo.ac.jp

Abstract

Memories are stored in synapses that consist of axon terminals and dendritic spines. Dendritic spines are postsynaptic structures of synapses and are essential for synaptic plasticity and cognition. Therefore, extensive investigations concerning the functions and structures of spines have been performed. Sex steroids and stress steroids have been shown to modulate hippocampal synapses. Although the rapid modulatory action of sex steroids on synapses has been studied in hippocampal neurones over several decades, the essential molecular mechanisms have not been fully understood. Here, a description of kinase-dependent signalling mechanisms is provided that can explain the rapid nongenomic modulation of dendritic spinogenesis in rat and mouse hippocampal slices by the application of sex steroids, including dihydrotestosterone, testosterone, oestradiol and progesterone. We also indicate the role of synaptic (classic) sex steroid receptors that trigger these rapid synaptic modulations. Moreover, we describe rapid nongenomic spine modulation by applying corticosterone, which is an acute stress model of the hippocampus. The explanations for the results obtained are mainly based on the optical imaging of dendritic spines. Comparisons are also performed with results obtained from other types of imaging, including electron microscopic imaging. Relationships between spine modulation and modulation of cognition are discussed. We recognise that most of rapid effects of exogenously applied oestrogen and androgen were observed in steroid-depleted conditions, including acute slices of the hippocampus, castrated male animals and ovariectomised female animals. Therefore, the previously observed effects can be considered as a type of recovery event, which may be essentially similar to hormone replacement therapy under hormone-decreased conditions. On the other hand, in gonadally intact young animals with high levels of endogenous sex hormones, further supplementation of sex hormones might not be effective, whereas the infusion of blockers for steroid receptors or kinases may be effective, with respect to suppressing sex hormone functions, thus providing useful information regarding molecular mechanisms.

KEYWORDS

androgens, cortisol/corticosterone, glucocorticoids, membrane/nuclear receptors, neuroactive steroids, oestrogens, steroids

1 | INTRODUCTION

The hippocampus is a centre for learning and memory, as well as a target of age-dependent cognitive impairment, including Alzheimer's disease. Synaptic modulations via treatment with oestrogen and androgen are essential for understanding the molecular mechanisms of hormone replacement therapy. The hippocampus is also a target of stress because this area is vulnerable to stress-induced neuroendocrine responses. These events are associated in part with changes in hippocampal synaptic structures such as dendritic spines.

In the rat and mouse hippocampus, in addition to the slow/genomic actions of sex steroids, the rapid action (eg, occurring between 30 and 120 minutes) of sex steroids has been a target of a number of electrophysiological investigations, including long-term potentiation (LTP)[1-3] and dendritic spine analyses.[4,5]

The possibility of rapid signalling mechanisms of sex steroids via multiple kinases, including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), was suggested on the basis of several investigations.[5-8] However, the contributions of many other essential kinases that participate in the modulation of synaptic plasticity have not been extensively studied. These important kinases are protein kinase A (PKA), protein kinase C (PKC) and LIM domain kinase (LIMK), which all are essential for the regulation of the synaptic plasticity.[9,10]

The candidates of membrane receptors, triggering the rapid effects of sex steroids, may be classic nuclear receptors, localised on the synaptic membrane, as indicated by the results of many recent investigations.[11-15] These synaptic classic receptors comprise: androgen receptor (AR), oestrogen receptor (ER) and progesterone receptor (PR). Synaptic membrane localisation of these receptors might be performed via the palmitoylation of receptors.

Rapid synaptic modulation by sex steroids in the hippocampus may be involved in physiological events because androgen and oestrogen are locally and rapidly synthesised.[3,14,16–22] As a result of local synthesis, the in vivo levels of dihydrotestosterone (DHT), testosterone, oestradiol (E_2) and progesterone (PROG) in the adult hippocampus are higher than those in the plasma, as determined by mass spectrometric analysis.[23,24] Therefore, hippocampal sex steroids (ie, hippocampus-synthesised steroids plus plasma steroids) play a much more important role in synaptic modulation than only plasma sex steroids.

Rapid nongenomic steroid signalling is also observed for corticosterone (CORT) in hippocampal acute stress reactions, in addition to slow/genomic processes.[25,26] Therefore, a comparison between the actions of sex steroids and CORT would be interesting in hippocampal spinogenesis.

In this mini review, we focus on the kinase-dependent signalling mechanisms involved in the rapid modulation of dendritic spinogenesis by sex steroids (DHT, testosterone, E_2, PROG) and stress steroids (CORT) in adult male rat hippocampal slices. Because investigations of spine modulations were also performed on cultured neurones, in vivo hippocampus and the female hippocampus, we compare all these results and provide explanations about the similarities and discrepancies between them. We then consider the role of classic steroid receptors as synaptic (membrane) steroid receptors in these rapid effects.

Finally, possible relationships between neurosteroid-induced modulations of cognitive function and spines are discussed.

2 | RAPID MODULATION OF DENDRITIC SPINES BY ANDROGEN, OESTROGEN, PROG AND CORT IN THE HIPPOCAMPUS

Investigations of the rapid effects of androgen (DHT and testosterone), E_2, PROG and CORT on the modulation of the dendritic spine density and morphology have been performed using several different methods, such as electron micrographic analysis of spine-synapses,[27,28] optical microscopic analysis of Golgi-stained spines[10,29] and confocal microscopic imaging of fluorophore-stained spines.[15,30,31] Rapid progress in high-resolution fluorescence imaging with confocal laser microscopy and molecular biological tools has dramatically advanced our understanding of the complex structure-function relationships in the synapses of central spiny neurones.

Sex steroid effects are most significant on CA1 glutamatergic neurones and particularly secondary branches of the apical dendrites in the stratum radiatum. Therefore, we describe mostly the CA1 results that play an essential role in spatial memory. We should also take into account that basal levels of neurosteroids are very low (<0.5 nmol L^{-1}) in acute slices[32,33] because almost all steroids are leaked to the outer medium after 2 hours of incubation in artificial cerebrospinal fluid (steroid free). Therefore, the application of exogenous steroids has some recovery effects for acute slices as a result of the elevation of steroid levels.

3 | ANALYSIS OF SPINE DENSITY AND SPINE HEAD MORPHOLOGY: MAINLY FROM FLUORESCENCE MICROSCOPIC STUDIES AND GOLGI-STAINING STUDIES

To investigate the synaptic modulation of neurosteroids in acute slices of rat and mouse hippocampus, single spine imaging is often performed for fluorophore-injected neurones. The three-dimensional images are taken from sequential Z-series scans with a confocal laser scan microscope. The density of spines, as well as the head diameter, is analysed using computer-assisted software, such as NUEROLUCIDA (MBF Bioscience, Williston, VT, USA) or SPISO-3D (http://kawato-glia.sakura.ne.jp/proj.html) (software mathematically calculating the geometrical parameters of spines).[34] Not only total spine density changes, but also morphological changes (spine head diameter changes) occur as a result of neurosteroid treatment. In many fluorescence confocal images, most spines (>90%) have distinct heads in the adult hippocampus with high-resolution confocal imaging; therefore, classification on the basis of spine head diameter could be used to characterise morphological changes (Figure 1).[15,30] From electron microscopic analysis, in the adult rat hippocampus (approximately 3-4 month), filopodia spines

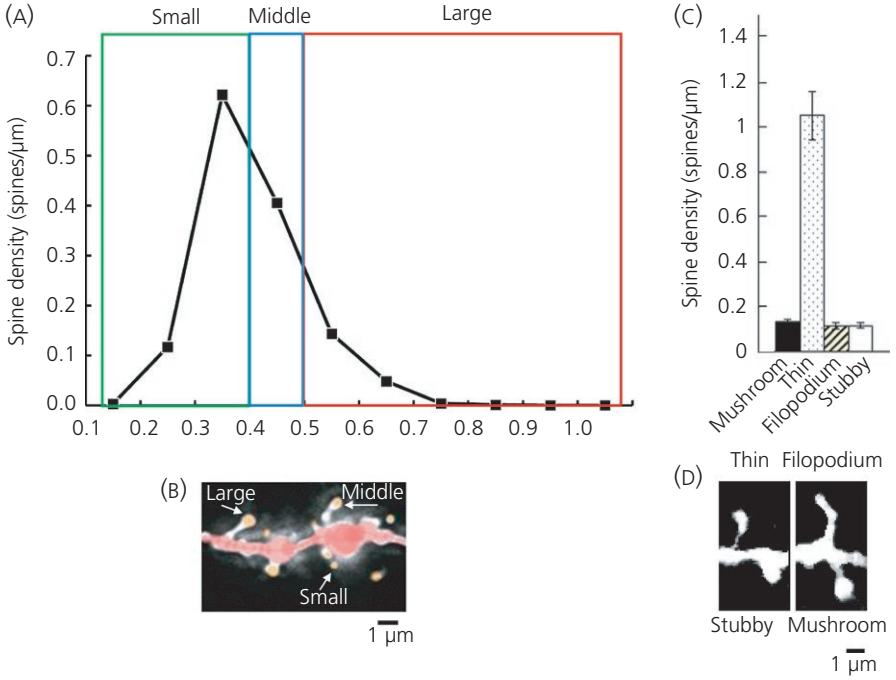

FIGURE 1 Classification of dendritic spines. (A) A typical head diameter distribution of spines with clear heads, obtained from fluorescence confocal images of oestradiol (E_2)-treated acute hippocampal slices, and analysed with mathematical software SPISO-3D.[34] Three subclass classification is shown together. Small-head spine (0.2-0.4 μm), middle-head spine (0.4-0.5 μm) and large-head spine (>0.5 μm). Filopodium and stubby spines are not included, because they do not have clear heads. (B) Max-XY projection of a confocal image. (C) Mushroom, thin, filopodium and stubby classification of the same E_2-treated slices as (A). Thin (0.2-0.6 μm) and mushroom (>0.6 μm) have head structures, whereas filopodium and stubby do not have clear head structures. (D) Typical shapes of mushroom, thin, filopodia and stubby

represent a very minor population (<1%)[35] and the stubby spine population comprises no more than 10%. Although, historically, spines with distinct heads have often been classified into two categories, including thin spines (with head diameter <0.6 μm) and mushroom spines (with head diameter >0.6 μm), these two classes are not sufficient to distinguish complicated neurosteroid-induced changes. For example, the effects of some kinase inhibitors cannot be clearly observed when only intermediate sized spines (intermediate size between thin and mushroom) are changed.

For statistical analysis of fluorescence confocal images, the classification of the spines (which have clear heads) into three categories is useful with respect to their head diameters: 0.2-0.4 μm as small-head spines, 0.4-0.5 μm as middle-head spines and 0.5-1.2 μm as large-head spines.[34] Small-, middle- and large-head spines may be significantly different regarding the number of AMPA receptors and therefore these three types of spines may have different efficiencies with respect to signal transduction. The number of AMPA receptors (including GluR1 subunit) in the spine increases as the surface area of postsynapse increases, whereas the number of NMDA receptors (including NR2B subunit) may be relatively constant.[36]

Here, not only fluorophore-stained spines, but also Golgi-stained spines are compared together in terms of the essential effects of neurosteorids. From Golgi-staining experiments of hippocampal slices, many stubby and filopodia spines (which do not have clear heads) were observed in addition to mushroom and thin spines (which have clear heads and necks) and research has mainly focused on changes in the density of mushroom and thin spines upon the application of neurosteroids.[37] In some cases of Golgi-staining, stubby and filopodia spines occupied almost 40% of the total spines, respectively.[37] Currently, we cannot explain well why there are so many stubby and filopodia spines in Golgi-stained spines, nor why these spines comprise less than 10% of the population in fluorophore-stained spines. One possible reason might be the low resolution of the Golgi-stained spines; therefore, the necks of spines might not be visible, leading to more stubby spines being observed.

3.1 | Androgen (DHT and testosterone) effects

In acute hippocampal slices of adult male rats, the total spine density in CA1 region was significantly increased after 2 hours of incubation with 10 nmol L^{-1} DHT and testosterone, respectively, from the control density of approximately 1.00 spines μm^{-1} (Figure 2).[30,31] These applied concentrations of DHT and testosterone had values similar to the concentrations of endogenous hippocampal DHT and testosterone in vivo.[23] On analysing the head diameter, differences in the effects of DHT and testosterone were shown (Figure 2). After 2 hours of treatment with DHT, the densities of middle- and large-head spines were significantly increased, whereas the density of small-head spines was not significantly altered.[30] Upon treatment with testosterone, the density of small-head spines was significantly increased, whereas the densities of middle- and large-head spines were not significantly changed. Testosterone may be converted to E_2 by cytochrome P450arom.[16] However, within 2 hours of incubation of slices with testosterone, any possible contribution of E_2 converted from testosterone did not occur because the addition of letrozole (an inhibitor of P450arom) did not suppress the testosterone-induced spine increase.[30,31] During the recovery incubation of acute slices, the spine density was decreased because of leakage of sex steroids from slices; therefore, treatments with DHT and testosterone recovered and rescued the level of spine density.

Although rapid spine modulation via in vivo androgen treatment has not been studied extensively, the rapid increase in CA1 spines was observed 0.5-2 hours after s.c. injection of testosterone (750 μg kg^{-1}) in castrated male rats.[29]

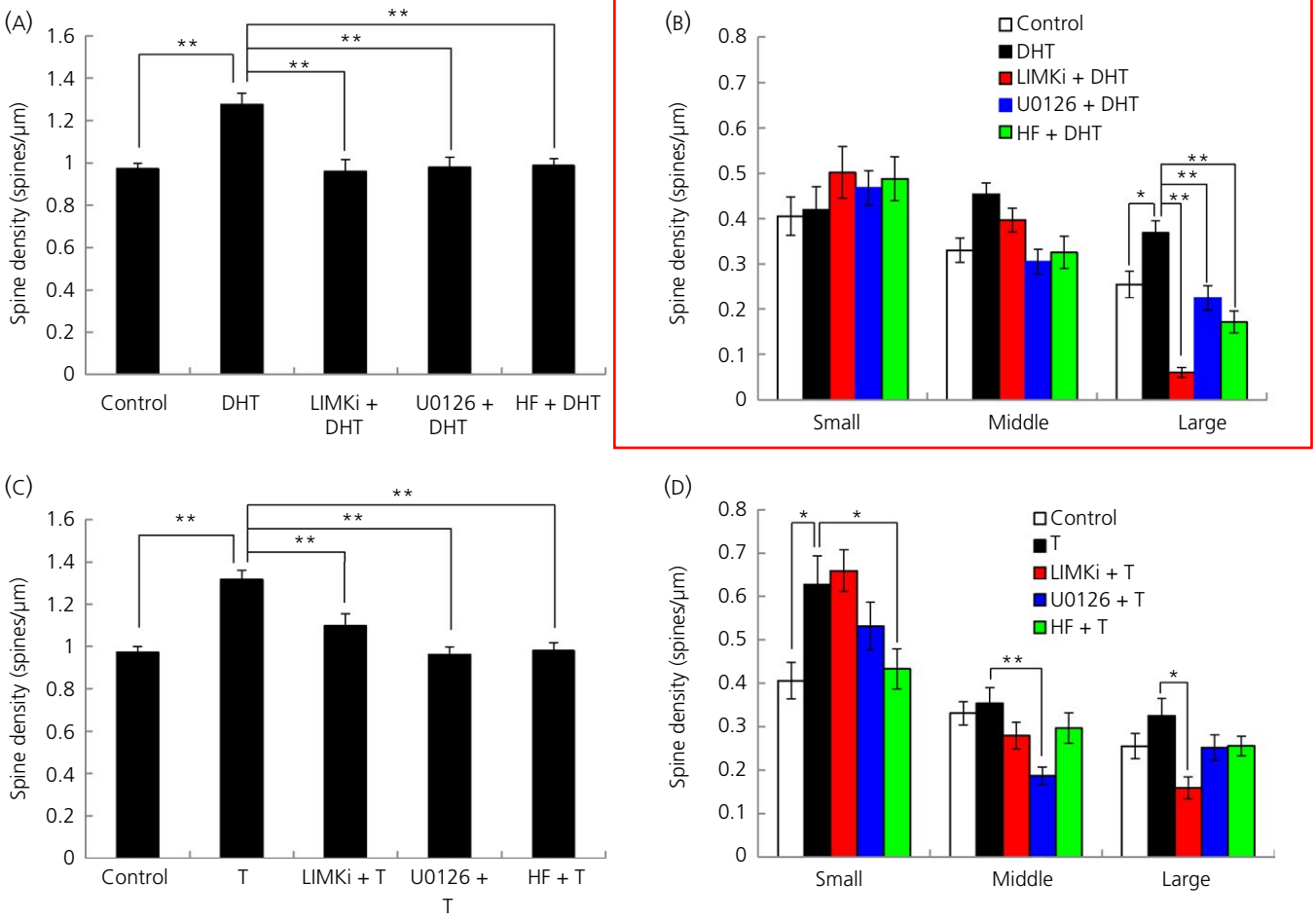

FIGURE 2 Effects of 2 h of treatment with 10 nmol L^{-1} dihydrotestosterone (DHT) and 10 nmol L^{-1} testosterone (T), with/without blockers of kinases and receptors on the spine density. (A) DHT effect on total density. (B) DHT effect on density of three subtypes of spines. (C) Testosterone effect on total density. (D) Testosterone effect on density of three subtypes of spines. LIMKi, U0126 and hydroxyflutamide (HF) are inhibitors of LIM domain kinase (LIMK), extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase (MAPK) and androgen receptor (AR), respectively. Statistical significance, $^{*}P < .05$, $^{**}P < .01$ vs DHT or testosterone treated. Raw data are in Hatanaka et al. [30]

Apart from rapid androgen effects, slow/genomic androgen effects in vivo (within several days) on spine-synapses obtained from electron microscopic analyses have been studied extensively.[38] Castration decreases spine-synapses in male rat hippocampus as a result of a significant decrease of DHT and testosterone in the hippocampus;[23] therefore, the replacement of DHT and testosterone recovered the level of spine-synapse density of CA1 neurones after several days of treatment.[27,39]

3.2 | E_2 effects

In acute hippocampal slices of adult rats, the total spine density in CA1 was significantly increased via E_2 treatment for 2 hours (Figure 3).[13,15] The increase effect was observed when the E_2 level was more than 1 nmol L^{-1}, whereas 0.1 nmol L^{-1} of E_2 did not affect the spine density, probably because the basal level of E_2 in slices is approximately 0.5 nmol L^{-1}.[32,33] Examining the head diameter, treatment with 1 nmol L^{-1} E_2 for 2 hours increased only the density of small-head spines, whereas the densities of middle- and large-head spines were not significantly altered (Figure 3).[15] An increase in the applied E_2 level from 1 to 10 nmol L^{-1} did not increase the total spine density further, although it enlarged spine heads by increasing the middle-head spines. Therefore, the head-diameter of newly formed spines might be proportional to the E_2 concentration applied.

Numerous investigations have been performed regarding the rapid E_2 effects on spines, in addition to isolated slices. In vivo E_2 treatment via s.c. injection rapidly increased (within 15-40 minutes) the spine density in CA1 hippocampal neurones of ovariectomised (OVX) mice.[10,29,40,41] The injection of E_2 rapidly (~30 minutes) increased the spine-synapse density as a result of synaptic rearrangements in OVX female rats.[42] In these in vivo investigations, because OVX surgery decreased spines, we can conclude that E_2 recovered the spine density.

Although it is not a hippocampal study, in primary cultured cortical neurones, E_2 application increased the spine density after 30 minutes and the spine density returned to control levels at 60 minutes.[43]

To completely understand what occurs in gonadectomised rats and mice upon sex steroid supplementation, we must know the exact levels of sex steroids in the hippocampus. Accurate determination can be achieved only when we use freshly prepared hippocampal

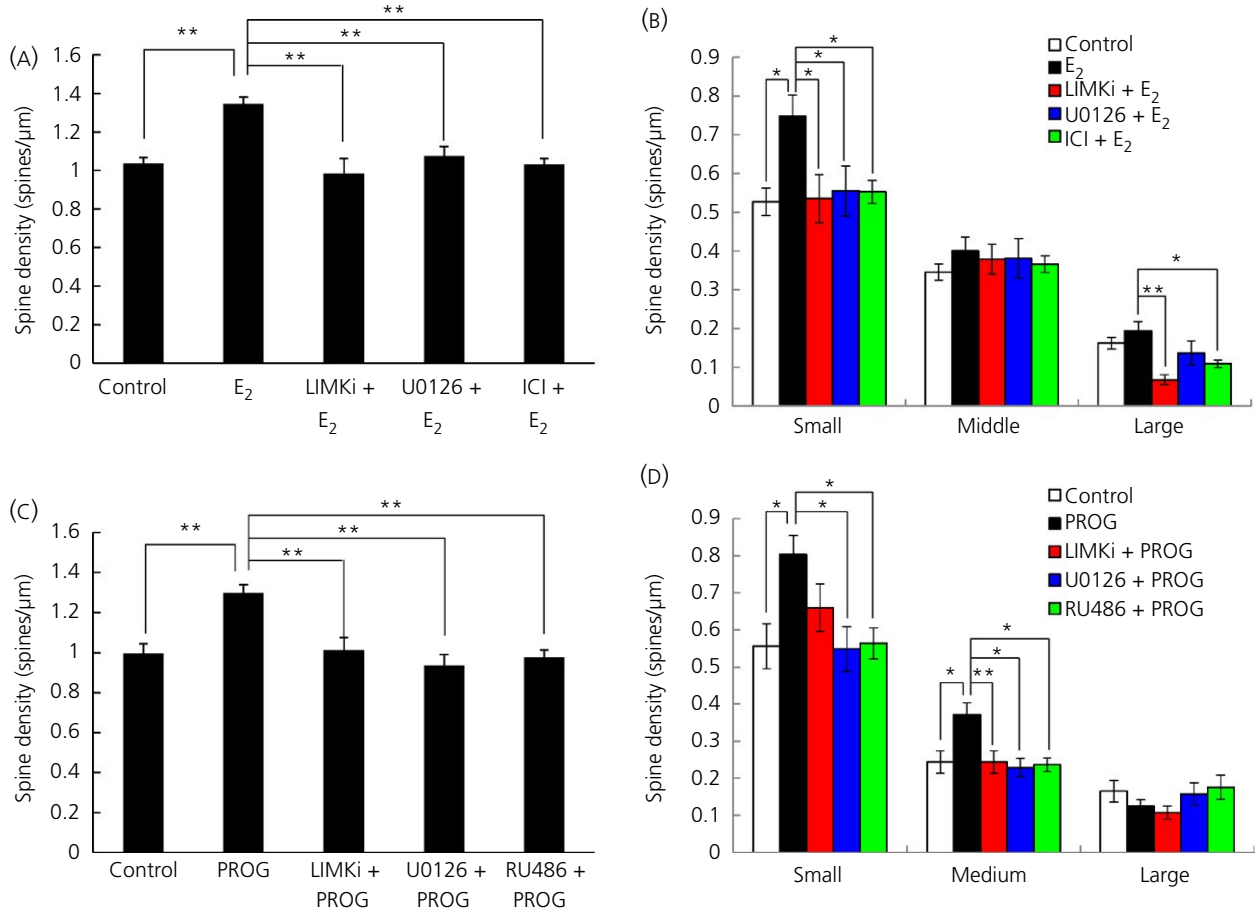

FIGURE 3 Effect of 2 h of treatment with 1 nmol L^{-1} oestradiol (E_2), 100 nmol L^{-1} progesterone (PROG) with/without blockers of kinases and receptors on the spine density. (A) E_2 effect on total density. (B) E_2 effect on density of three subtypes of spines. (C) PROG effect on total density. (D) PROG effect on density of three subtypes of spines. ICI182,780 (ICI) and RU486 are inhibitors of oestrogen receptor (ER) and progesterone receptor (PR), respectively. Statistical significance, $^*P < .05$, $^{**}P < .01$ vs E_2 treated. Some of the raw data are in Hasegawa et al. [15] and Kato et al. [45]

homogenates with anti-oxidation treatments because E_2 is particularly unstable as a result of oxidation of the 3-OH group of the phenol ring during extraction, high-performance liquid chromatography purification and quantitative mass-spectrometric analysis.[23] However, this still appears to present technical difficulties for many laboratories. Ovariectomy of female rats decreased the hippocampal E_2 and testosterone levels down to the level of oestrus stage (~0.5 nmol L^{-1});[24] however, surprisingly, castration of male rats did not decrease the hippocampal E_2 levels at all but, instead, decreased the hippocampal testosterone significantly (down to 20-30%) and decreased the hippocampal DHT level to almost zero.[23] Therefore, E_2 supplementation in OVX female animals showed recovery effects with a good reproducibility; however, low-dose E_2 supplementation in castrated male animals may not always show recovery effects. Indeed, E_2 injection (approximately 30 μg kg^{-1} for 2 days) did not show any effect in castrated male rats, although DHT and testosterone injections (approximately 1.5 mg kg^{-1}) induced significant recovery effects.[27,44] From our consideration, no effects as a result of low-dose E_2 injection should occur because of the barrier of such a high endogenous level of E_2 in the male hippocampus (2-10 nmol L^{-1} with individual variation and approximately 7 nmol L^{-1} on average) even at 2 weeks after castration.[23] Therefore, the injection of low-dose E_2 could not always overcome the endogenous E_2 level in the castrated male hippocampus. This high endogenous level of E_2 may be a result of local E_2 synthesis in the castrated rat hippocampus. Interestingly, however, a single s.c. injection of low dose E_2 (approximately 20 μg kg^{-1}) did demonstrate a spine increase in castrated male rats that were fed with low phytoestrogen chow, probably because of decrease in the endogenous E_2 level.[29] Nevertheless, to obtain a significant increase in spines, we must inject a sufficiently high dose of E_2 to castrated male animals (eg, 200 μg kg^{-1} E_2).

3.3 | PROG effects

PROG is an essential female sex hormone regulating the oestrus cycle. Therefore, a possible role of PROG in rapid spine modulation was examined. In acute hippocampal slices of adult male rats, treatment with 100 nmol L^{-1} PROG for 2 hours significantly increased the total spine density in CA1 (Figure 3).[24] The applied high concentration of PROG (100 nmol L^{-1}) was chosen based on the endogenous concentration.

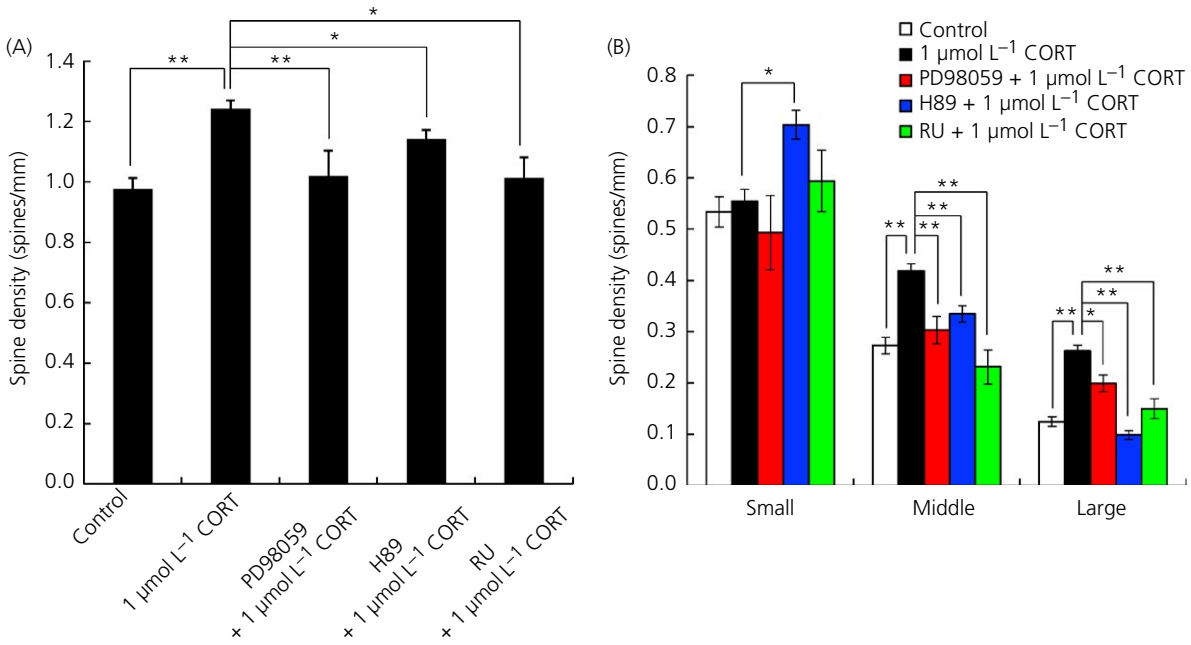

FIGURE 4 Effect of 1 h of treatment with 1 μmol L^{-1} corticosterone (CORT) with/without blockers of kinases and receptors on the spine density. (A) Total density. (B) Density of three subtypes of spines. PD98059, Chel and RU486 are inhibitors of extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and glucocorticoid receptor (GR), respectively. Statistical significance, $^{*}P < .05$, $^{**}P < .01$ vs CORT treated. Some of the raw data are in Komatsuzaki *et al*. [26]

From head diameter analysis, treatment with 100 nmol L^{-1} PROG increased the density of small-head and middle-head spines.[45] It is known that applied PROG might be metabolised to allopregnanolone, which could modulate $GABA_A$ receptor, leading to a spine increase.[46] Therefore, RU486, a PR antagonist, was applied to investigate this possibility. Because RU486 completely suppressed the spine increase, the possibility of allopregnanolone action is excluded in acute slices undergoing 2 hours of incubation of PROG (Figure 3B).

3.4 | CORT effects

The effect of acute stress on the hippocampus is caused by the rapid elevation of plasma CORT. As a model of acute stress, hippocampal acute slices from adult rats were treated with 1 μmol L^{-1} CORT for 1 hour and then the total spine density was significantly increased (Figure 4).[26] Unlike the effects seen for sex steroids, a CORT-induced increase in spines occurred after 1 hour of incubation (ie, shorter than 2 hours). Dose-dependency analysis showed that the spine-increase effects were already significant when applying 200 and 500 nmol L^{-1} CORT. In the analysis measuring head diameter, 1 hour of incubation with CORT enlarged the spine head in a dose-dependent manner between 100 and 1000 nmol L^{-1}.[26] The large- and middle-head spines were significantly increased only with 1 μmol L^{-1} CORT. Lower level CORT (100 and 200 nmol L^{-1}) increased only small-head spines but not middle- and large-head spines. Head diameter modulation is therefore dependent on the CORT concentration.

Rapid CORT effects were also demonstrated in vivo. Acute stress (ie, placing on an elevated platform for 1 hour) increased the density of long-thin and mushroom spines in CA1.[37]

4 | MECHANISMS OF THE RAPID SPINE INCREASE DEPENDING ON PROTEIN KINASES: MAINLY FROM FLUORESCENCE MICROSCOPIC STUDIES AND GOLGI-STAINING STUDIES

With respect to the most probable rapid nongenomic signalling cascade in the neurosteroid-induced spine increase, investigations have been performed to analyse the involvement of diverse kinase networks that are essential for controlling synaptic plasticity by use of selective inhibitors of kinases in acute hippocampal slices. The involvement of many kinases was demonstrated, including LIMK, extracellular signal-regulated kinase (Erk) MAPK, p38 MAPK, PKA, PKC and PI3K, in the rapid spinogenesis induced by neurosteroids, such as androgen, oestrogen and corticosteroid in the hippocampal CA1 region.[5,10,15,30]

LIMK and cofilin are good candidates for signal proteins in the neurosteroid-induced actin reassembly leading to spinogenesis via the pathway: LIMK → phosphorylation of cofilin → actin polymerisation → spine increase.[15,47-49] In addition to cofilin, MAPK and cortactin cascade may couple with PKA and PKC via the pathway of PKC → Raf1 → MAPK, PKA → B-Raf → MAPK → phosphorylation of cortactin → actin polymerisation → spine increase. PI3K and phosphatase (calcineurin) may also be involved in neurosteroid-induced modulation.

The above cascades could be confirmed by experiments using selective blocking of each kinase. Selective blockers could abolish the spine-increase action of these neurosteroids and reverse total spine density to the control level. The analysis of spine head diameter is useful because only observing the total spine density is not sufficient to explain the complex kinase effects.

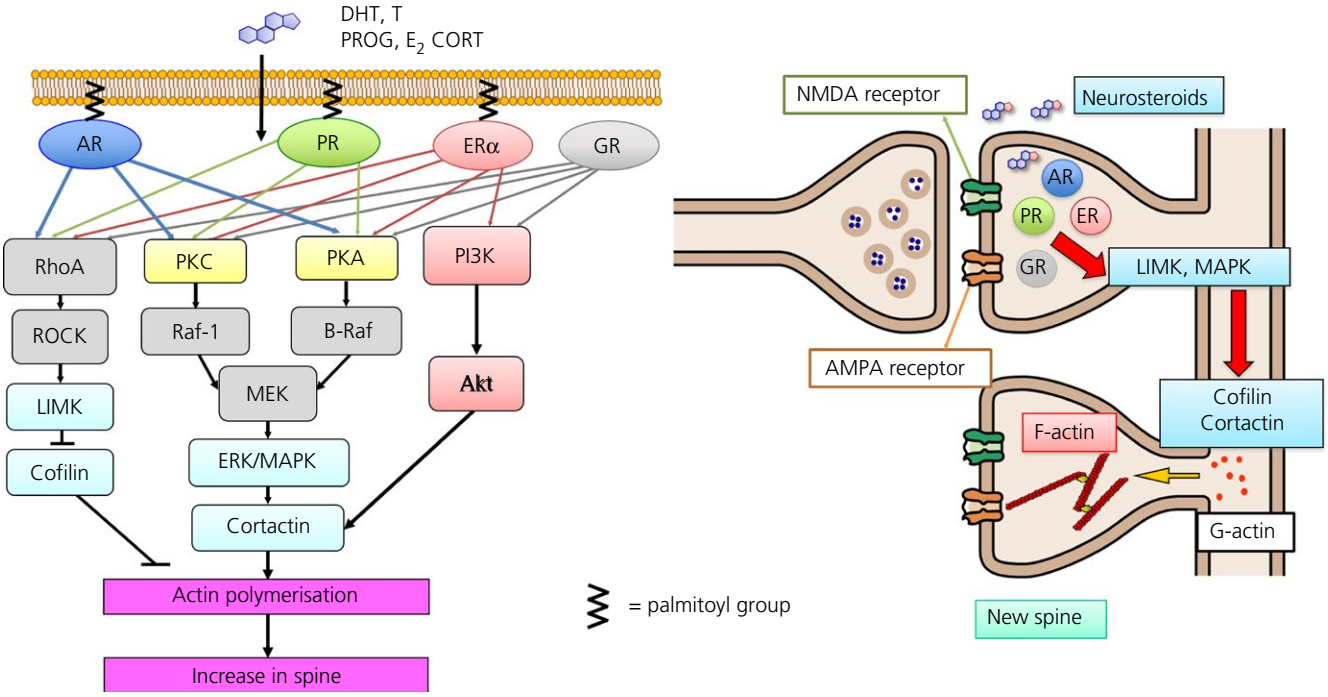

FIGURE 5 Kinase-mediated signalling for rapid effects of sex steroids and corticosterone (CORT) on spinogenesis. Dihydrotestosterone (DHT), testosterone (T), oestradiol (E_2), progesterone (PROG) and CORT bind to synaptic receptors [oestrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR) and glucocorticoid receptor (GR)] within spines. Then, various kinases [LIM domain kinase (LIMK), protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)] are activated. Phosphorylation of cofilin suppresses actin depolymerisation, leading to new spine formation. Phosphorylation of cortactin induces actin polymerisation, leading to new spine formation

4.1 | Androgen signalling

In acute hippocampal slices of male rats, increases in the total spine density and three head diameter subpopulations (small-, middle- and large-heads) via DHT- and testosterone-treatments were suppressed by the blocking of kinases, including LIMK, Erk MAPK, p38 MAPK, PKA and PKC (Figures 2 and 5). Blocking PI3K, however, did not affect the DHT- and testosterone-induced increase in total spine density.[30] Figure 2 shows representative blocking effects of Erk MAPK and LIMK.

To our knowledge, in vivo studies of kinase signalling in androgen-induced spine modulation have not been reported.

4.2 | E2 signalling

In acute hippocampal slices of male rats, application of inhibitors of LIMK, Erk MAPK, p38MAPK, PKA and PKC blocked individually the spine increase effects from E_2 (Figures 3 and 5).[15] Note that the blockade of PI3K suppressed the spine increase caused by E_2, which is distinctive compared to the DHT- and testosterone-induced spine increase. Figure 3 shows representative blocking effects of Erk MAPK and LIMK.

In vivo studies also showed that E_2 infusion directly into the hippocampus increased the spine density in CA1 neurones within 30 minutes by activation of Erk MAPK in OVX female mice.[10] Apart from the hippocampus, in cultured cortical neurones, the E_2-induced increase in spine density at 30 minutes was accompanied by the phosphorylation of Erk MAPK and this spine increase was blocked by inhibition of Erk MAPK.[43]

4.3 | PROG signalling

Application of inhibitors of LIMK and Erk MAPK blocked the increase in spines by PROG in acute hippocampal slices of male rats (Figures 3 and 5).[45] To our knowledge, in vivo studies have not been reported regarding kinase signalling with respect to PROG actions.

4.4 | CORT signalling

In acute slices, the application of inhibitors of Erk MAPK, PKA, PKC and PI3K prevented the increase in total spine density and the three head diameter subpopulations induced by 200-1000 nmol L^{-1} CORT (Figures 4 and 5).[26]

PKA activation was observed in vivo after infusion of CORT into the medial prefrontal cortex (mPFC),[50] although hippocampus investigations were not reported.[25]

4.5 | Other kinases/phosphatases

In addition to the kinases described above, calcineurin, a phosphatase, also participated in neurosteroid-induced rapid spinogenesis for all of

the sex steroids and CORT examined. In all the cases examined above, however, blocking Jun kinase (JNK) showed no suppression of the neurosteroid-induced increase in spine density.[15,26,30]

It should be noted that these kinase inhibitors alone did not significantly affect total spine density, indicating that the observed inhibitory effects are not the result of simple nonspecific blockade.

5 | SYNAPTIC (MEMBRANE) RECEPTORS FOR NEUROSTEROID ACTION

The synaptic receptor of neurosteroids must evidently be identified to explain the mechanism of rapid (1-2 hours) spinogenesis that is induced by neurosteroids in the hippocampus.

5.1 | ARs

The involvement of classic AR in rapid action was observed by suppressing androgen-induced spinogenesis using hydroxyflutamide (HF), a specific inhibitor of AR in acute slices of rat hippocampus (Figures 2 and 5).[30] AR immunoreactivity was localised in CA1 neurones with optical microscopic analysis and the extranuclear localisation of AR (in dendrites and spines) was also observed with electron microscopic analysis.[51] The subcellular distribution of AR was also examined by western immunoblot analysis using PG-21, an anti-AR antibody. The AR protein band was observed in the postsynaptic density (PSD) fraction, as well as in cytoplasmic and nuclear fractions, which indicates that AR localised in the PSD could participate in androgen-induced spinogenesis.[30]

Inhibition of NMDA receptors by MK-801 significantly suppressed the DHT-induced increase in the spine density, suggesting that AR possibly localises close to NMDA receptors (ie, within spines).[30]

In vivo studies have shown that intrahippocampal administration of an AR antagonist, flutamide, can increase anxiety-like behaviour in intact male rats and DHT-replaced castrated male rats.[52] These results suggest AR involvement in anxiety, which may influence learning behaviour. Note that castration increased anxiety-like behaviour, whereas DHT-replacement reversed it.

On the other hand, from spine analyses, a possibility of non-AR membrane receptors has been proposed and investigated because AR antagonist HF did not suppress the DHT-induced increase in spine-synapses with electron microscopic analysis.[38,53] However, the putative non-AR membrane receptor has not yet been purified or cloned, preventing further definitive characterisation.[54]

DHT may be converted to androstanediol by 3α-hydroxysteroid-dehydrogenase and might modulate $GABA_A$ receptors, leading to synaptic changes.[55] This possibility in acute hippocampal slices within 2 hours of incubation was excluded because HF, an AR antagonist, completely suppressed the DHT-induced spine increase (Figure 2).

5.2 | E2 receptors

Judging from many investigations, the most probable candidates for synaptic (membrane) oestrogen receptors are classic nuclear type receptors (ERα, ERβ). The blocking of classic ER by ICI182,780 (ICI), a specific antagonist of ERα and ERβ, completely abrogated the enhancing effect of E_2 on the spine density in acute hippocampal slices, suggesting that rapid effect of E_2 on spinogenesis is mediated by ER (Figures 3 and 5).[15] The involvement of ERα and ERβ in rapid spinogenesis was further demonstrated by introducing oestrogen receptor agonists. ERα agonist, (propyl-pyrazole-trinyl) tris-phenol (PPT), unlike ERβ agonists such as (4-hydroxyphenyl)-propionitrile (DPN), rapidly increased the spine density in hippocampal neurones in CA1 in male rats [15] and OVX female mice.[40] These results support the exclusive involvement of ERα in rapid signalling. An ER knockout (KO) mice study further confirmed the involvement of ERα in rapid signalling.[56] Treament for 2 hours with E_2 preferably increased the density of middle-head spines in hippocampal slices of wild-type mice. The oestradiol-induced increase in middle-head spines was observed in ERβKO mice (which express ERα) but not in ERαKO mice. These results indicate that ERα is necessary for oestrogen-induced spinogenesis, whereas ERβ is not essential for this oestrogen-induced spinogenesis.

On the other hand, there are contrasting reports that indicate ERβ participation. The rapid administration of DPN but not PPT enhanced object and place recognition memory in OVX female rats.[4] Treatment with WAY-200070, an ERβ agonist, increased spine density and spine size, as well as PSD-95 accumulation in membrane regions, within 30 minutes in cultured cortical neurones.[57] Regarding slow genomic effects, treatment with WAY-200070 for 2 days improved performance in a hippocampus-dependent radial arm maze task in OVX rats.[58] An increase in the mushroom type of spines in the dentate gyrus but not in CA1 was observed in these OVX rats. Currently, however, we cannot explain these discrepancies between ERα signalling and ERβ signalling.

The expression of ERα in glutamatergic neurones in rat and mouse hippocampus is clearly demonstrated by immunostaining with purified antibody RC-19. ERα is localised not only in nuclei/cytoplasm, but also in pre- and postsynapses, as indicated by an immunogold electron microscopic analysis.[13] Expression of ERβ in pre- and postsynapses was also observed by immunogold electron microscopic analysis.[59] An association of ERα with PSD was observed by western blotting of PSD fractions, implying the synaptic membrane binding of ERα.

In cultured cells of peripheral origin, some populations of ERα and ERβ are plasma membrane-bound and they are anchored via palmitoylation.[11] Therefore, membrane binding of ERα and ERβ might also occur in neurones.[13,14,17,59]

E_2-induced increases in spine density are blocked by NMDA receptor antagonists,[13,60,61] suggesting that ER is possibly localised close to NMDA receptors (ie, within spines).

Interestingly, in cultured female hippocampal neurones, ERα and ERβ were translocated to the membrane via complex formation with metabotropic glutamate receptors (mGluR).[62] Palmitoylation of ERα and ERβ also played a role in the translocation of ERα and ERβ to the membrane of the female hippocampus. These ER/mGluR complexes phosphorylate cAMP response element-binding protein very rapidly (approximately 1 minute) upon E_2 binding.[62]

Another candidate for synaptic oestrogen receptor is G protein coupled receptor (GPR) localised in the cellular membrane. GPR30/

GPER was expressed in the membrane of endoplasmic reticulum but not in the plasma membrane.[63] GPR30/GPER may not participate in E_2-induced spine modulation because of the low binding affinity of GPR30 with E_2 and no rapid E_2 signalling was seen in SKBR-3 cells (ER negative, GPR30/GPER positive).[64-66] By contrast to ERα and ERβ, GPR30/GPER agonist G-1 did not activate Erk MAPK but did activate JNK; it also enhanced object recognition and spatial memory performance.[66] Therefore, GPR30/GPER signalling appears to occur very differently compared to ERα-induced and ERβ-induced signalling.

5.3 | PROG receptors

Blocking of PR by PR inhibitor (RU486) completely abolished the enhancing effect from 100 nmol L^{-1} PROG on the total spine density (Figures 3 and 5).[45] Therefore, classic PR is involved in PR-induced spinogenesis. The spine localisation of PR was observed via immunoelectron microscopic analysis.[67] Although RU486 is an inhibitor for both PROG and CORT, because basal levels of CORT and PROG were below 0.5 nmol L^{-1} in acute slices, RU486 only suppresses exogenous PROG binding to PR in these experiments.

Novel membrane-associated progesterone receptors (mPR) have also been investigated. Although immunostaining of mPR and its molecular biology studies showed the expression of mPR, the PROG-dependent signalling of mPR has not been clearly demonstrated.[68]

5.4 | CORT receptors

Blocking of glucocorticoid receptor (GR) by GR inhibitor (RU486) completely abolished the enhancing effect from 1 μmol L^{-1} CORT on the total spine density (Figures 4 and 5).[26] Therefore, classic GR is involved in the CORT-induced spinogenesis. Dexamethasone, a GR agonist, rapidly increased spines within 1 hour, further supporting the involvement of GR in CORT-induced spinogenesis.[69] GR localisation within spines was found by electron microscopic analysis.[2,70] Although RU486 is an inhibitor for both CORT and PROG, because basal levels of CORT and PROG were below 0.5 nmol L^{-1} in acute slices, RU486 only suppresses exogenous CORT binding to GR in these CORT-effect experiments. Blockade of NMDA receptors leads to the suppression of the CORT-induced increase in spine density, suggesting that GR is possibly localised close to NMDA receptors (ie, within spines).

Evidence for involvement of membrane GR in rapid nongenomic signalling in neurones has accumulated,[25,71] although the mechanisms of translocation of GR to the membrane are not clear. By contrast to AR, ERα/ERβ and PR, GR may not be palmitoylated.[72]

6 | RELATIONSHIPS BETWEEN NEUROSTEROID-INDUCED MODULATIONS OF COGNITION AND SPINES

Rapid modulation of spines by sex steroids has a close relationship with rapid changes in learning and memory. Recently, many observations support rapid E_2 effects with respect to the cognition of OVX rats.[9,73] These review papers showed that E_2 rapidly enhanced memory consolidation within approximately 1 hour. Subcutaneous infusion of E_2 for 40 minutes improved learning and memory processes, such as object recognition and object replacement, in OVX female mice.[41] The same study group reported that the injection of a selective agonist for ERα (PPT) but not for ERβ (DPN) rapidly (approximately 1 hour) increased dendritic spines and improved learning in OVX female mice.[40] Subcutaneous injection of E_2 rapidly (30-60 minutes) induced not only hippocampus-dependent memory performance, but also spine increases in the hippocampus in OVX female rats.[29] In OVX female rats, relatively rapid E_2 effects (approximately 4 hours after s.c. injection) were observed on object recognition and object placement tests.[74] The spine-synapse increase was observed much earlier at 30 minutes after s.c. injection of E_2.[42] Recognition memory was rapidly increased by s.c. treatment of E_2 30 minutes before the initiation of the behavioural analysis, which is sufficient to increase basal spine density in OVX rats.[75]

Why is it that OVX rats been used in these studies? It is likely that, as a result of OVX treatments, the hippocampal level of E_2 decreases,[24] leading to a decrease in both cognitive performance and spine density; accordingly, E_2 infusion can recover these decreases. On the other hand, infusion of E_2 into gonadally intact females may not induce a further increase in the spine density or an improvement in cognition. By contrast, hippocampal infusion of ERα/ERβ blockers might effectively impair cognition and decrease in spines.

In addition, rapid actions of E_2 are also involved in reproductive behaviours, such as maternal behaviours or lordosis, which are under the control of hypothalamus, in OVX mice and rats.[76,77]

Concerning PROG effects on cognition, intrahippocampal infusion of PROG in OVX female mice enhanced object recognition memory consolidation and very rapid MAPK phosphorylation (approximately 5 minutes) was involved in this enhancement process.[78]

Androgen may also show rapid improving effects on cognition in castrated male rats and mice; however, the experimental reports are poor compared to the effects of E_2.

The rapid enhancement of spatial memory was observed at 0.5-2 hours after testtosterone injection (750 μg kg^{-1}) in castrated male rats.[29] Rapid androgen effects (1-2 hours) on anti-anxiety behaviour in vivo as a result of hippocampal infusion of DHT have been observed in castrated male rats.[52] Intrahippocampal administration of an AR antagonist can increase anxiety-like behaviour in DHT-replaced castrated male rats and gonadally intact male rats.[52] Subcutaneous testosterone injection not only increased anti-anxiety behaviour, but also enhanced cognitive performance in castrated mice.[79] It is likely that castration increased anxiety-like behaviour as a result of a decrease in hippocampal DHT and testosterone;[23] therefore, DHT-replacement could reverse this. By contrast to rapid effects, slow genomic effects of testosterone and DHT on cognition have been studied extensively. In vivo treatments with DHT capsules for 5 months showed an improvement of spatial memory performance in castrated male mice.[80] Testosterone injection improved spatial memory in castrated male rats, when testosterone injection was given every day from 7 days prior to water maze test.[81]

By contrast to sex steroids, acute stress caused the impairment of cognition. Acute stress (placing on an elevated platform for 1 hour) impaired spatial memory retrieval for short-term memory on the object placement task (hippocampus-dependent), associated with CORT elevation in plasma.[37] This effect of acute stress on hippocampus-dependent memory task was associated with an increase in the density of long-thin and mushroom spines in CA1.[37] Infusion of CORT into mPFC at 60 minutes before the test impaired working memory in a PKA-dependent manner.[50]

7 | CONTRIBUTION OF LOCAL HIPPOCAMPAL SYNTHESIS OF NEUROSTEROIDS

Adult rat and mouse hippocampi locally synthesise sex steroids.[16,20] The expression of mRNAs and proteins of steroidogenic enzymes, including cytochromes P450 (17α), P450arom and 17β-hydroxysteroid dehydrogenase, was demonstrated.[16,19,23,82,83] Immunohistochemical analysis combined with immunogold electron microscopic analysis showed that these enzymes are expressed in glutamatergic neurones in CA1, CA3 and the dentate gyrus, and were partly localised in pre- and postsynapses. The production of DHT, testosterone, E_2, PROG and CORT was demonstrated by metabolism analysis of radioactive substrates. These results show that hippocampal neurones are equipped with a full set of enzymes to perform the synthesis of neurosteroids from cholesterol.

The rapid modulations of spines, as discussed in the current review, may match that of locally synthesised steroids. For example, hippocampal neurosteroid synthesis (including pregnenolone and E_2 synthesis) was a neural activity-dependent rapid process (within 30 minutes) that is triggered by Ca influx via NMDA receptors.[3,16,19] On the other hand, the change in the level of circulating sex hormones is probably very slow, mainly depending on the circadian rhythm or oestrus cycle. It should be noted that the rapid manipulation of plasma sex steroid levels, with injection and infusion after castration and ovariectomy, can rapidly change hippocampal sex steroid levels because considerable amounts of sex steroids penetrate into the hippocampus by crossing the blood-brain barrier.[23,24] Therefore, sex steroid injection/replacement after castration and ovariectomy has been a useful method for in vivo investigations of the rapid modulation of spines and cognition by sex steroids.

As a result of local neurosteroid synthesis, the levels of sex steroids are higher in the hippocampus than in the plasma.[23] For example, in the male rat, sex steroid levels are approximately 7 nmol L^{-1} (E_2), 18 nmol L^{-1} (testosterone) and 6 nmol L^{-1} (DHT) in the hippocampus and approximately 0.01 nmol L^{-1} (E_2), 14 nmol L^{-1} (testosterone) and 0.6 nmol L^{-1} (DHT). The female rat sex steroid levels are 0.5-4 nmol L^{-1} (E_2) and 1-2 nmol L^{-1} (testosterone) in the hippocampus and 0.01-0.1 nmol L^{-1} (E_2) and 0.02-0.1 nmol L^{-1} (testosterone) in the plasma.[24] These variations in female are dependent on oestrus stages. Interestingly, OVX reduced the hippocampal E_2 level down to almost the same level as that in the dioestrus stage, although this was not reduced to zero level, implying that E_2 is synthesised in the female hippocampus.[24] Therefore, direct determination of local steroid levels in hippocampal slices is very important because the spine density rapidly changes, dependent on the rapid change in the local sex steroid levels.

Because the male/female hippocampus in vivo contains high levels of local E_2 and androgens,[23] the hippocampus infusion of inhibitors against steroid synthase (eg, letrozole or finasteride) in rats in vivo may be very effective with respect to the rapid alteration of spines, which could provide useful evidence regarding the hippocampal synthesis of sex steroids.[84] Slow hippocampal synthesis of sex steroids has been observed after repetitive i.p. injection of letrozole, a P450arom inhibitor, for 1-7 days in the female hippocampus from LTP measurements.[85]

On the other hand, in isolated acute slices, all of the steroids are depleted (resulting in <0.5 nmol L^{-1}) after 2 hours of recovery incubation of slices that were prepared by dissection and vibratome-slicing of the hippocampus.[2,32] As a result, exogenously applied DHT, testosterone, E_2 and PROG concentrations (>1 nmol L^{-1}) were above the local steroid levels (<0.5 nmol L^{-1}), thereby demonstrating the significant effects on spinogenesis and LTP.

8 | SUMMARY AND FUTURE DIRECTIONS

To our surprise, although the physiological functions of steroids are very diverse (ranging from sex hormones to stress hormones), the nongenomic signal pathways show a large overlap. Kinase-driven signalling is used for nongenomic action of sex hormones and stress hormones. Spine-localised classic steroid receptors (AR, ER, PR and GR) trigger kinase-driven rapid signalling, leading to spine modulation. These receptors may anchor to the synaptic membranes via palmitoylation because these steroid receptors have a conserved sequence for palmitoylation.[11,86] The palmitoylation of essential many synaptic proteins, including PSD-95, is shown to be an important mechanism for spine formation in the hippocampus.[87,88] However, the membrane localisation of GR does not appear to be mediated via palmitoylation and therefore further clarification is necessary.[72]

Because sex steroids and stress steroids are observed to drive essential kinases that regulate synaptic plasticity, neurosteroids could play important roles in memory performance. Although rapid E_2 effects (in vitro and in vivo) have been extensively studied and well clarified, the rapid effects of androgens and stress steroids need much more investigation to enable a deeper understanding of the common or differing molecular mechanisms of these neurosteroidal actions. As one additional example of the CORT effect, circadian cycle-dependent oscillation of CORT (3-30 nmol L^{-1}) induced the cyclic rise and fall of spine density and these changes were a result of kinase-dependent signalling, including MAPK, LIMK, PKA and PKC.[48]

The interactions between rapid/nongenomic signalling and slow/genomic signalling via classic steroid receptors comprise another interesting topic for investigation.[12]

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID


S. Kawato http://orcid.org/0000-0001-7024-2660

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