0.5, 1, 2, and 4 h with 10 nM DHT or 10 nM T. The enhancing effect on the total thorn density was approximately proportional to the incubation time, showing 2.6 (0.5 h), 3.0 (1 h), 3.2 (2 h), and 3.0 thorns/μm (4 h) in DHT-treatments, and 2.5 (0.5 h), 2.6 (1 h), 3.2 (2 h), and 3.1 thorns/μm (4 h) in T-treatments (Fig. 2A and B). Dose dependency was also examined after a 2 h incubation. In DHT-treatment group, the enhancing effect was most significant at 10 nM DHT (3.2 ± 0.2 thorns/μm) compared with 1 nM (2.5 thorns/μm) and 100 nM (3.1 thorns/μm) DHT. In T-treatment group, the enhancing effect was most significant at 10 nM T (3.2 ± 0.2 thorns/μm) compared with 1 nM (2.5 thorns/μm) and 100 nM (2.9 thorns/μm) T. Because a 2 h treatment with 10 nM DHT or 10 nM T was most effective for thorn-genesis, these incubation time and concentration were used in the following investigations unless specified. (Fig. 2C and D).

A 2 h treatment with DHT or T increased thorns from 2.2 thorns/μm (control, i.e. with no DHT or T) to 3.2 (10 nM DHT) or 3.2 thorns/μm (10 nM T). These results indicate that the enhancing effect of thorn-genesis by DHT and T is nearly identical. Blocking of androgen receptor (AR) by 1 μM hydroxyflutamide, a specific inhibitor of AR, completely abrogated the enhancing effect of DHT or T on the thorn density (2.2 or 2.2 thorns/μm, respectively). Washing DHT or T by treating slices with ACSF for another 2 h after DHT- or T-treatment abolished the effect of DHT or T (2.3 or 2.6 thorns/μm, respectively). (Fig. 3A and B).

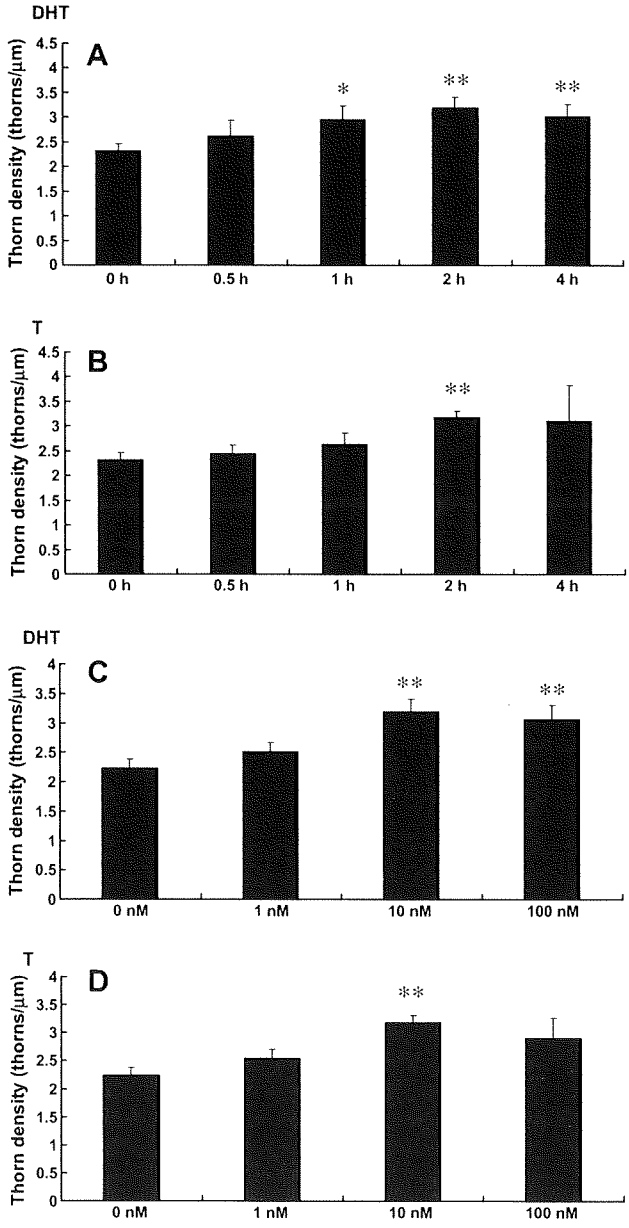

Fig. 2. Effect of androgens on the thorn density of CA3 neurons. (A and B) Time dependency of DHT and T. (A) No treatment with DHT (0 h), 0.5 h treatment (0.5 h), 1 h treatment (1 h), 2 h treatment (2 h), and 4 h treatment in ACSF with 10 nM DHT (4 h). (B) No treatment with T (0 h), 0.5 h treatment (0.5 h), 1 h treatment (1 h), 2 h treatment (2 h), and 4 h treatment in ACSF with 10 nM T (4 h). (C and D) Dose dependency of DHT and T. (C) A 2 h treatment in ACSF without DHT (0 nM), with 1 nM DHT (1 nM), with 10 nM DHT (10 nM), and with 100 nM DHT (100 nM). (D) A 2 h treatment in ACSF without T (0 nM), with 1 nM T (1 nM), with 10 nM T (10 nM), and with 100 nM T (100 nM). Vertical axis is the average number of thorns per 1 μm dendrite. Results are reported as mean ± SEM. The significance yielded $^{*}P < 0.05$, $^{**}P < 0.01$ to 0 h or 0 nM.

The effect of androgens was blocked by several kinase inhibitors

Next we investigated kinase signaling pathways involved in the androgen-induced thorn-genesis by using specific inhibitors for kinases. Blocking of Erk MAPK, by application of 25 μM U0126, abolished the DHT or T effect on the increase of the thorn density, resulting in 2.4 or 2.3 thorns/μm, respectively (Fig. 4A and B). Application of 10 μM SB203580, a p38 MAPK inhibitor, also prevented the effect by DHT or T resulting in 2.4 or 2.5 thorns/μm, respectively. Further, 1 μM KN-93, an inhibitor of CaMKII, reversed the effect of DHT or T (2.4 or 2.3 thorns/μm, respectively) (Fig. 4A and B). 1 μM cyclosporin A, an inhibitor of calcineurin (protein phosphatase 2B), abolished the effect of DHT or T (2.3 or 2.3 thorns/μm, respectively). On the other hand, 10 μM H-89, an inhibitor of protein kinase A (PKA), did not inhibit the enhancing effect but even increased the thorn density to 4.1 (DHT) or 4.1 (T) thorns/μm, respectively. A PI3 kinase inhibitor, 10 μM LY294002, also did not alter the increase of thorn density induced

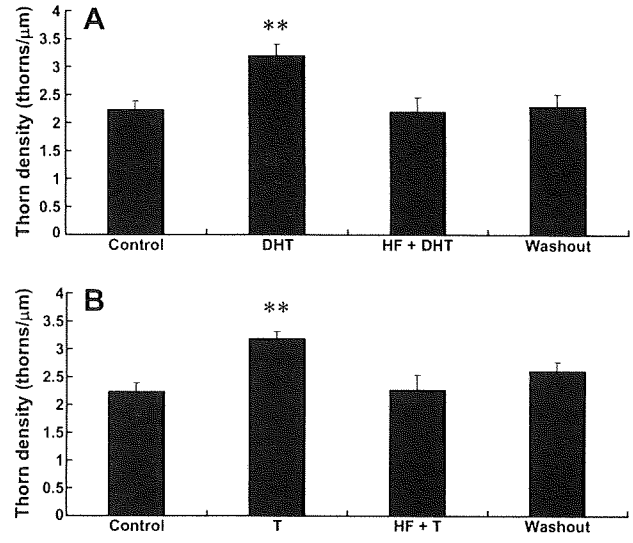

Fig. 3. Effect of receptor blocker on the thorn density in the CA3 neurons. (A) A 2 h treatment in ACSF without drugs (Control), with 10 nM DHT (DHT), with 10 nM DHT and 1 μM hydroxyflutamide (HF + DHT), and with 10 nM DHT followed by 2 h washout with ACSF (Washout). (B) A 2 h treatment in ACSF without drugs (Control), with 10 nM T (DHT), with 10 nM T and 1 μM hydroxyflutamide (HF + T), and with 10 nM T followed by 2 h washout with ACSF (Washout). Vertical axis is the average number of thorns per 1 μm. Results are reported as mean ± SEM. The significance yielded $^{*}P < 0.05$, $^{**}P < 0.01$.

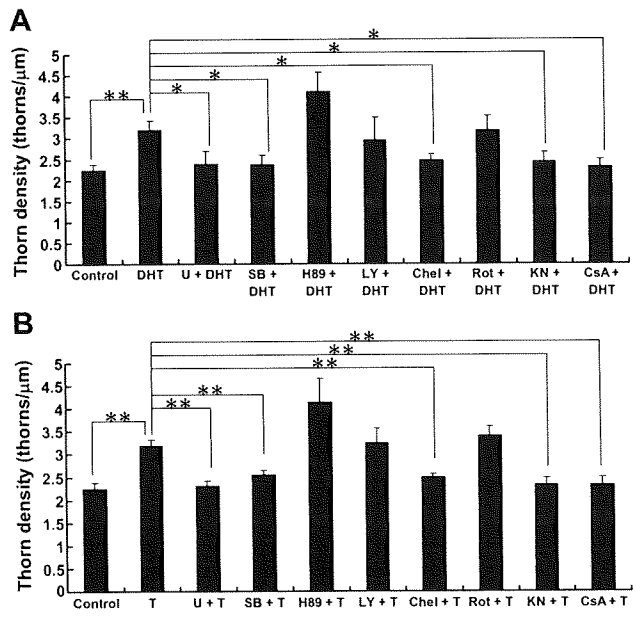

Fig. 4. Effect of kinase inhibitors on the changes in the density of thorns by DHT or T. (A) Effect of kinase inhibitors in the presence of DHT on the total thorn density in CA3 neurons. A 2 h treatment in ACSF without drugs (Control), with 10 nM DHT (DHT), with 10 nM DHT and 25 μM U0126 (Erk MAPK inhibitor) (U + DHT), with 10 nM DHT and 10 μM SB203580 (p38 MAPK inhibitor) (SB + DHT), with 10 nM DHT and 10 μM H-89 (PKA inhibitor) (H-89 + DHT), with 10 nM DHT and 10 μM LY294002 (PI3K inhibitor) (LY + DHT), with 10 nM DHT and 10 μM Chelerythrine (PKC inhibitor) (Chel + DHT), with 10 nM DHT and 5 μM Rottlerin (PKCδ inhibitor) (Rot + DHT), with 10 nM DHT and 1 μM KN-93 (CaMKII inhibitor) (KN + DHT), and with 10 nM DHT and 1 μM cyclosporin A (calcineurin inhibitor) (CsA + DHT). (B) Effect of kinase inhibitors in the presence of T on the total thorn density in CA3 neurons. A 2 h treatment in ACSF without drugs (Control), with 10 nM T (T), with 10 nM T and 25 μM (U + T), with 10 nM T and 10 μM SB203580 (SB + T), with 10 nM T and 10 μM H-89 (H-89 + T), with 10 nM T and 10 μM LY294002 (LY + T), with 10 nM T and 10 μM Chelerythrine (Chel + T), and with 10 nM T and 5 μM Rottlerin (Rot + T), with 10 nM T and 1 μM KN-93 (KN + T), and with 10 nM T and 1 μM cyclosporin A (CsA + T). Vertical axis is the average number of thorns per 1 μm. Results are reported as mean ± SEM. The significance yielded $^{*}P < 0.05$, $^{**}P < 0.01$.

by DHT or T (2.9 or 3.2 thorns/μm, respectively). When all subfamilies of protein kinase C (PKC) were blocked by 10 μM chelerythrine, a non-selective PKC subfamily inhibitor, the enhancing effect of DHT or T on the thorn density was considerably abrogated (2.5 or 2.5 thorns/μm, respectively). However, selective inhibition of PKCδ (by 5 μM rottlerin) did not suppress both DHT- and T-effect (3.2 and 3.4 thorns/μm, respectively).

Discussion

The current study demonstrated that the activation of AR by both DHT and T induced a rapid increase of thorns of thorny excrescences in CA3 pyramidal neurons of the adult male rat hippocampus. In addition, almost no difference was observed between DHT and T concerning thorn-genesis depending on kinase signaling. These results suggest that the conversion of T to estradiol is not significant within 2 h incubation with hippocampal slices. Androgen-induced changes have not been investigated in the stratum lucidum of the CA3, until the current study. An extremely concentrated distribution of thorny excrescences, as compared with sparse distribution of spines located in other regions, such as CA1, may have prevented detailed analysis of thorny excrescences by previous studies using Golgi staining methods [1,20]. We were able to analyze the number of thorns by the high-resolution image analysis of Lucifer Yellow-injected neurons, using deconvolution, and digital three-dimensional analysis. The rapid modulation of thorns observed in the current study is a novel phenomenon and is essential for consideration of the synaptic plasticity affected by androgen. There is much evidence that mossy fiber terminals originating from granule cells in the dentate gyrus provide excitatory inputs to CA3 neurons via thorny excrescences in the stratum lucidum [10,21–24]. Our data imply that DHT or T may significantly enhance the excitatory input to CA3 from dentate gyrus by increasing the density of thorns. Because the effect of T had been blocked by HF as well as that of DHT, we confirmed that T-effect was directly mediated by AR, not by its aromatase metabolite, estradiol. The expression of AR in the CA3 region has been demonstrated by immunoelectron microscopy as well as immunohistochemistry and *in situ* hybridization [25–27]. The intracellular distribution of AR has been found not only in the nuclei and cytoplasm but also in axon terminals and dendritic thorns [25], supporting the possibility that androgen-induced acute thorn-genesis may be mediated by extranuclear AR located in synapses.

It is important to compare the effect of androgen and estrogen on CA3 thorns. Our preceding study indicated that 1 nM estradiol application for 2 h significantly decreased the thorn density in hippocampal CA3 from 2.2 to 1.5 thorns/μm (by approximately 30%) [12]. On the other hand, current study revealed that androgens oppositely increased the thorns by approximately 50%. It is interesting that both androgen and estrogen activated Erk MAPK, but its outcomes on the thorn density are completely opposite. In this report, we demonstrated that androgen-induced thorn-genesis mediated by p38 MAPK, CaMKII, calcineurin, and PKC subfamilies, and that PKA, PI3K, and PKCδ were not included in the signaling. Such enzymes other than Erk MAPK may contribute to induce different effects of androgen and estrogen.

Over a decade, only a few reports have appeared regarding investigations of the effect of androgens on CA3 neurons. Neonatal castration has decreased the dendritic length, number of branches, and volume of neurons in adult hippocampal CA3, and androgens replacement has restored these deficits [28]. On the other hand, in the CA1 region, a number of investigations have been performed concerning the change in the spine density induced by androgens. Gonadectomy decreased the synapses in male rat hippocampus CA1, and the replacement of androgens by subcutaneous injection has restored the level of the total spine density after three days [3,29,30]. In addition to endocrine-derived hormones, we have recently demonstrated the endogenous *de novo* synthesis of androgens in male adult rat hippocampal neurons [13–15]. By using liquid chromatography tandem mass spectrometry, we have determined the concentrations of DHT and T in freshly isolated rat hippocampal slices to be 7 nM and 17 nM, respectively [4]. However, because the slices were incubated for 2 h in ACSF before exogenous androgen application, the concentration of androgens in the hippocampal slices decreased down to below 0.5 nM during the incubation. Accordingly, the increase of thorns after supplementation of 10 nM DHT or T in the current study may reflect restorative effect of DHT or T. These results imply that hippocampal neurons are exposed to local DHT and T, which might regulate synaptic plasticity rapidly.

References

[1] E. Gould, C.S. Woolley, M. Frankfurt, B.S. McEwen, Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood, J. Neurosci. 10 (1990) 1286–1291.
[2] C.S. Woolley, B.S. McEwen, Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat, J. Neurosci. 12 (1992) 2549–2554.
[3] C. Leranth, O. Petnehazy, N.J. MacLusky, Gonadal hormones affect spine synaptic density in the CA1 hippocampal subfield of male rats, J. Neurosci. 23 (2003) 1588–1592.

[4] Y. Hojo, G. Murakami, H. Mukai, S. Higo, Y. Hatanaka, M. Ogiue-Ikeda, H. Ishii, T. Kimoto, S. Kawato, Estrogen synthesis in the brain–role in synaptic plasticity and memory, Mol. Cell. Endocrinol. 290 (2008) 31–43.
[5] M. Ogiue-Ikeda, N. Tanabe, H. Mukai, Y. Hojo, G. Murakami, T. Tsurugizawa, N. Takata, T. Kimoto, S. Kawato, Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons, Brain Res. Rev. 57 (2008) 363–375.
[6] H. Ishii, T. Tsurugizawa, M. Ogiue-Ikeda, M. Asashima, H. Mukai, G. Murakami, Y. Hojo, T. Kimoto, S. Kawato, Local production of sex hormones and their modulation of hippocampal synaptic plasticity, Neuroscientist 13 (2007) 323–334.
[7] D.A. Henze, N.N. Urban, G. Barrionuevo, The multifarious hippocampal mossy fiber pathway: a review, Neuroscience 98 (2000) 407–427.
[8] M.R. Bennett, W.G. Gibson, J. Robinson, Dynamics of the CA3 pyramidal neuron autoassociative memory network in the hippocampus, Philos. Trans. R. Soc. Lond. B Biol. Sci. 343 (1994) 167–187.
[9] D.G. Amaral, A Golgi study of cell types in the hilar region of the hippocampus in the rat, J. Comp. Neurol. 182 (1978) 851–914.
[10] M.E. Chicurel, K.M. Harris, Three-dimensional analysis of the structure and composition of CA3 branched dendritic spines and their synaptic relationships with mossy fiber boutons in the rat hippocampus, J. Comp. Neurol. 325 (1992) 169–182.
[11] M.G. Stewart, H.A. Davies, C. Sandi, I.V. Kraev, V.V. Rogachevsky, C.J. Peddie, J.J. Rodriguez, M.I. Cordero, H.S. Donohue, P.L. Gabbott, V.I. Popov, Stress suppresses and learning induces plasticity in CA3 of rat hippocampus: a three-dimensional ultrastructural study of thorny excrescences and their postsynaptic densities, Neuroscience 131 (2005) 43–54.
[12] T. Tsurugizawa, H. Mukai, N. Tanabe, G. Murakami, Y. Hojo, S. Kominami, K. Mitsuhashi, Y. Komatsuzaki, J.H. Morrison, W.G. Janssen, T. Kimoto, S. Kawato, Estrogen induces rapid decrease in dendritic thorns of CA3 pyramidal neurons in adult male rat hippocampus, Biochem. Biophys. Res. Commun. 337 (2005) 1345–1352.
[13] Y. Hojo, T.A. Hattori, T. Enami, A. Furukawa, K. Suzuki, H.T. Ishii, H. Mukai, J.H. Morrison, W.G. Janssen, S. Kominami, N. Harada, T. Kimoto, S. Kawato, Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons, Proc. Natl. Acad. Sci. USA 101 (2004) 865–870.
[14] T. Kimoto, T. Tsurugizawa, Y. Ohta, J. Makino, H. Tamura, Y. Hojo, N. Takata, S. Kawato, Neurosteroid synthesis by cytochrome p450-containing systems localized in the rat brain hippocampal neurons: *N*-methyl-D-aspartate and calcium-dependent synthesis, Endocrinology 142 (2001) 3578–3589.
[15] K. Shibuya, N. Takata, Y. Hojo, A. Furukawa, N. Yasumatsu, T. Kimoto, T. Enami, K. Suzuki, N. Tanabe, H. Ishii, H. Mukai, T. Takahashi, T.A. Hattori, S. Kawato, Hippocampal cytochrome P450s synthesize brain neurosteroids which are paracrine neuromodulators of synaptic signal transduction, Biochim. Biophys. Acta 1619 (2003) 301–316.
[16] H. Mukai, N. Takata, H.T. Ishii, N. Tanabe, Y. Hojo, A. Furukawa, T. Kimoto, S. Kawato, Hippocampal synthesis of estrogens and androgens which are paracrine modulators of synaptic plasticity: synaptocrinology, Neuroscience 138 (2006) 757–764.
[17] H. Mukai, T. Tsurugizawa, M. Ogiue-Ikeda, G. Murakami, Y. Hojo, H. Ishii, T. Kimoto, S. Kawato, Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory, Neuroendocrinology 84 (2006) 255–263.
[18] G. Murakami, N. Tanabe, H.T. Ishii, M. Ogiue-Ikeda, T. Tsurugizawa, H. Mukai, Y. Hojo, N. Takata, A. Furukawa, T. Kimoto, S. Kawato, Role of cytochrome p450 in synaptocrinology: endogenous estrogen synthesis in the brain hippocampus, Drug Metab. Rev. 38 (2006) 353–369.
[19] H. Duan, S.L. Wearne, J.H. Morrison, P.R. Hof, Quantitative analysis of the dendritic morphology of corticocortical projection neurons in the macaque monkey association cortex, Neuroscience 114 (2002) 349–359.
[20] C.S. Woolley, E. Gould, M. Frankfurt, B.S. McEwen, Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons, J. Neurosci. 10 (1990) 4035–4039.
[21] D.G. Amaral, N. Ishizuka, B. Claiborne, Neurons, numbers and the hippocampal network, Prog. Brain Res. 83 (1990) 1–11.
[22] B.J. Claiborne, D.G. Amaral, W.M. Cowan, A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus, J. Comp. Neurol. 246 (1986) 435–458.
[23] D.G. Amaral, J.A. Dent, Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions, J. Comp. Neurol. 195 (1981) 51–86.
[24] T.H. Brown, D. Johnston, Voltage-clamp analysis of mossy fiber synaptic input to hippocampal neurons, J. Neurophysiol. 50 (1983) 487–507.
[25] N.E. Tabori, L.S. Stewart, V. Znamensky, R.D. Romeo, S.E. Alves, B.S. McEwen, T.A. Milner, Ultrastructural evidence that androgen receptors are located at extranuclear sites in the rat hippocampal formation, Neuroscience 130 (2005) 151–163.
[26] L. Xiao, C.L. Jordan, Sex differences, laterality, and hormonal regulation of androgen receptor immunoreactivity in rat hippocampus, Horm. Behav. 42 (2002) 327–336.
[27] J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa, Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus, Endocrinology 136 (1995) 3213–3221.
[28] C. Isgor, D.R. Sengelaub, Effects of neonatal gonadal steroids on adult CA3 pyramidal neuron dendritic morphology and spatial memory in rats, J. Neurobiol. 55 (2003) 179–190.
[29] E.G. Kovacs, N.J. MacLusky, C. Leranth, Effects of testosterone on hippocampal CA1 spine synaptic density in the male rat are inhibited by fimbria/fornix transection, Neuroscience 122 (2003) 807–810.
[30] N.J. MacLusky, T. Hajszan, J.A. Johansen, C.L. Jordan, C. Leranth, Androgen effects on hippocampal CA1 spine synapse numbers are retained in Tfm male rats with defective androgen receptors, Endocrinology 147 (2006) 2392–2398.