only direct effects within the hippocampus but also indirect effects of estradiol via cholinergic or serotonergic neurons, projecting to the hippocampus (MacLusky et al., 2005).

Chronic effects of environmental chemicals on reproductive organs have been intensively investigated, primarily toxic effects of high dose environmental chemicals have been investigated about the development and functions of the reproduction systems (Al-Hiyasat et al., 2002; Fisher et al., 1999; Grote et al., 2004; Halldin et al., 2005). For example, treatment with BPA (37 mg/kg body weight/day for 18–25 days), DES (0.37 or 0.037 mg/kg/day for 18–25 days) and OP (150 mg/kg/day for 18–25 days) in neonatal stage has been reported to cause a decrease in epithelial cell height of the efferent ducts in the testis of young male rats (Fisher et al., 1999). Note that mg/kg high dosage may induce micromolar plasma concentration of environmental chemicals.

Human BPA exposure (at μg/kg low dosage) is concluded to be insufficient to elicit significant estrogenic responses in endocrine organs and gonads due to the low affinity of BPA for the cell nuclear estrogen receptors, ERα and ERβ, as well as weak bioactivity in standard tests of estrogenicity, such as the rat uterotrophic assay (Ashby, 2001; Degen et al., 2002; EC Scientific Committee on Food, 2002; Japan Ministry of the Environment's ExTEND 2005; U.S. Environmental Protection Agency (EPA) 1993). However, because μg/kg low dosage may induce nanomolar plasma concentration of endocrine disrupters, we emphasize that our investigations about the hippocampus imply that even nanomolar low dosage of endocrine disrupters could induce significant effects on memory processes.

REFERENCES

Al-Hiyasat, A.S., Darmani, H., Elbetieha, A.M., 2002. Effects of bisphenol A on adult male mouse fertility. Eur. J. Oral Sci. 110, 163–167.

Ashby, J., 2001. Increasing the sensitivity of the rodent uterotrophic assay to estrogens, with particular reference to bisphenol A. Environ. Health Perspect. 109, 1091–1094.

Balthazart, I., Ball, G.F., 2000. Fast regulation of steroid biosynthesis: a further piece in the neurosteroid puzzle. Trends Neurosci. 23, 57–58.

Baude, A., Nusser, Z., Molnar, E., McIlhinney, R.A., Somogyi, P., 1995. High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus. Neuroscience 69, 1031–1055.

Bi, R., Broutman, G., Foy, M.R., Thompson, R.F., Baudry, M., 2000. The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. Proc. Natl. Acad. Sci. U. S. A. 97, 3602–3607.

Brailoiu, E., Dun, S.L., Brailoiu, G.C., Mizuo, K., Sklar, L.A., Oprea, T.I., Prossnitz, E.R., Dun, N.J., 2007. Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. J. Endocrinol. 193, 311–321.

Chinta, S.J., Kommaddi, R.P., Turman, C.M., Strobel, H.W., Ravindranath, V., 2005. Constitutive expression and localization of cytochrome P-450 1A1 in rat and human brain: presence of a splice variant form in human brain. J. Neurochem. 93, 724–736.

Degen, G.H., Janning, P., Wittsiepe, J., Upmeier, A., Bolt, H.M., 2002. Integration of mechanistic data in the toxicological evaluation of endocrine modulators. Toxicol. Lett. 127, 225–237.

EC Scientific Committee on Food, 2002. Opinion of the Scientific Committee on Food on Bisphenol A. European Commission Health & Consumer Protection Directorate-General, Brussels. Available: http://europa.eu.int/comm/food/fs/sc/scf/out128_en.pdf.

Fisher, J.S., Turner, K.J., Brown, D., Sharpe, R.M., 1999. Effect of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ. Health Perspect. 107, 397–405.

Foy, M.R., Xu, J., Xie, X., Brinton, R.D., Thompson, R.F., Berger, T.W., 1999. 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J. Neurophysiol. 81, 925–929.

Fritschy, J.M., Weinmann, O., Wenzel, A., Benke, D., 1998. Synapse-specific localization of NMDA and GABA(A) receptor subunits revealed by antigen-retrieval immunohistochemistry. J. Comp. Neurol. 390, 194–210.

Gould, E., Woolley, C.S., Frankfurt, M., McEwen, B.S., 1990. Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. J. Neurosci. 10, 1286–1291.

Grote, K., Stahlschmidt, B., Talsness, C.E., Gericke, C., Appel, K.E., Chahoud, I., 2004. Effects of organotin compounds on pubertal male rats. Toxicology 202, 145–258.

Gu, Q., Moss, R.L., 1996. 17 Beta-estradiol potentiates kainate-induced currents via activation of the cAMP cascade. J. Neurosci. 16, 3620–3629.

Gu, Q., Korach, K.S., Moss, R.L., 1999. Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors Endocrinology 140, 660–666.

Halldin, K., Axelsson, J., Brunstrom, B., 2005. Effects of endocrine modulators on sexual differentiation and reproductive function in male Japanese quail. Brain Res. Bull. 65, 211–218.

Harrington, W.R., Sheng, S., Barnett, D.H., Petz, L.N., Katzenellenbogen, J.A., Katzenellenbogen, B.S., 2003. Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol. Cell. Endocrinol. 206, 13–22.

Hojo, Y., Hattori, T.A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H.T., Mukai, H., Morrison, J.H., Janssen, W.G., Kominami, S., Harada, N., Kimoto, T., Kawato, S., 2004. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. Proc. Natl. Acad. Sci. U. S. A. 101, 865–870.

Ishii, H., Shibuya, K., Ohta, Y., Mukai, H., Uchino, S., Takata, N., Rose, J.A., Kawato, S., 2006. Enhancement of nitric oxide production by association of nitric oxide synthase with N-methyl-D-aspartate receptors via postsynaptic density 95 in genetically engineered Chinese hamster ovary cells: real-time fluorescence imaging using nitric oxide sensitive dye. J. Neurochem. 96, 1531–1539.

Ito, K., Skinkle, K.L., Hicks, T.P., 1999. Age-dependent, steroid-specific effects of oestrogen on long-term potentiation in rat hippocampal slices. J. Physiol. 515 (Pt 1), 209–220.

Japan Ministry of the Environment's Perspectives on Endocrine Disrupting Effects of Substances—ExTEND 2005. Available: http://www.env.go.jp/en/chemi/ed/extend2005_full.pdf.

Kawato, S., 2004. Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint. Environ. Sci. 11, 1–14.

Kawato, S., Ogiue-Ikeda, M., Tanabe, N., Tsurugizawa, T., Hojo, Y., Mukai, H., 2007. Rapid modulation of long-term depression and spinogenesis by endocrine disrupters in adult rat hippocampus. Abstract, 4th International Meeting on Steroids and Nervous System, Torino.

Kishimoto, W., Hiroi, T., Shiraishi, M., Osada, M., Imaoka, S., Kominami, S., Igarashi, T., Funae, Y., 2004. Cytochrome P450 2D catalyze steroid 21-hydroxylation in the brain. Endocrinology 145, 699–705.

Komatsuzaki, Y., Murakami, G., Tsurugizawa, T., Mukai, H., Tanabe, N., Mitsuhashi, K., Kawata, M., Kimoto, T., Ooishi, Y., Kawato, S., 2005. Rapid spinogenesis of pyramidal neurons induced by

activation of glucocorticoid receptors in adult male rat hippocampus. Biochem. Biophys. Res. Commun. 335, 1002–1007.
Kretz, O., Fester, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., Prange-Kiel, J., Naumann, T., Jarry, H., Frotscher, M., Rune, G.M., 2004. Hippocampal synapses depend on hippocampal estrogen synthesis. J. Neurosci. 24, 5913–5921.
Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J.A., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138, 863–870.
Lee, H.K., Kameyama, K., Huganir, R.L., Bear, M.F., 1998. NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. Neuron 21, 1151–1162.
Lisman, J., 1989. A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc. Natl. Acad. Sci. U. S. A. 86, 9574–9578.
MacLusky, N.J., Luine, V.N., Hajszan, T., Leranth, C., 2005. The 17alpha and 17beta isomers of estradiol both induce rapid spine synapse formation in the CA1 hippocampal subfield of ovariectomized female rats. Endocrinology 146, 287–293.
McEwen, B., 2002. Estrogen actions throughout the brain. Recent Prog. Horm. Res. 57, 357–384.
McEwen, B.S., Alves, S.E., 1999. Estrogen actions in the central nervous system. Endocr. Rev. 20, 279–307.
Migaud, M., Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsay, M.F., Morris, R.G., Morrison, J.H., O'Dell, T.J., Grant, S.G., 1998. Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396, 433–439.
Miksys, S.L., Tyndale, R.F., 2002. Drug-metabolizing cytochrome P450s in the brain. J. Psychiatry Neurosci. 27, 406–415.
Milner, T.A., McEwen, B.S., Hayashi, S., Li, C.J., Reagan, L.P., Alves, S.E., 2001. Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. J. Comp. Neurol. 429, 355–371.
Milner, T.A., Ayoola, K., Drake, C.T., Herrick, S.P., Tabori, N.E., McEwen, B.S., Warrier, S., Alves, S.E., 2005. Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. J. Comp. Neurol. 491, 81–95.
Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashi, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Schaeffer, J.M., McEwen, B.S., Alves, S.E., 2003. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144, 2055–2067.
Monaghan, D.T., Holets, V.R., Toy, D.W., Cotman, C.W., 1983. Anatomical distributions of four pharmacologically distinct 3H-L-glutamate binding sites. Nature 306, 176–179.
Morohoshi, K., Yamamoto, H., Kamata, R., Shiraishi, F., Koda, T., Morita, M., 2005. Estrogenic activity of 37 components of commercial sunscreen lotions evaluated by in vitro assays. Toxicol. In Vitro 19, 457–469.
Mukai, H., Tsurugizawa, T., Murakami, G., Kominami, S., Ishii, H., Ogiue-Ikeda, M., Takata, N., Tanabe, N., Furukawa, A., Hojo, Y., Ooishi, Y., Morrison, J.H., Janssen, W.G., Rose, J.A., Chambon, P., Kato, S., Izumi, S., Yamazaki, T., Kimoto, T., Kawato, S., 2007. Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. J. Neurochem. 100, 950–967.
Murakami, G., Tsurugizawa, T., Hatanaka, Y., Komatsuzaki, Y., Tanabe, N., Mukai, H., Hojo, Y., Kominami, S., Yamazaki, T., Kimoto, T., Kawato, S., 2006. Comparison between basal and apical dendritic spines in estrogen-induced rapid spinogenesis of CA1 principal neurons in the adult hippocampus. Biochem. Biophys. Res. Commun. 351, 553–558.
Ogiue-Ikeda, M., Takata, N., Kawato, S., 2005. Non genomic rapid effects of endocrine disrupters on the rat hippocampus: multi-electrode dish analysis. Abstract PD-52, 8th Meeting of Japan Society of Endocrine Disrupters Research.
Orikasa, C., McEwen, B.S., Hayashi, H., Sakuma, Y., Hayashi, S., 2000. Estrogen receptor alpha, but not beta, is expressed in the interneurons of the hippocampus in prepubertal rats: an in situ hybridization study. Brain Res. Dev. Brain Res. 120, 245–254.
Pedram, A., Razandi, M., Levin, E.R., 2006. Nature of functional estrogen receptors at the plasma membrane. Mol. Endocrinol. 20, 1996–2009.
Pozzo-Miller, L.D., Inoue, T., Murphy, D.D., 1999. Estradiol increases spine density and NMDA-dependent Ca2+ transients in spines of CA1 pyramidal neurons from hippocampal slices. J. Neurophysiol. 81, 1404–1411.
Razandi, M., Pedram, A., Greene, G.L., Levin, E.R., 1999. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol. Endocrinol. 13, 307–319.
Reid, C.A., 2002. The role of dendritic spines: comparing the complex with the simple. Eur. J. Pharmacol. 447, 173–176.
Reid, C.A., Fabian-Fine, R., Fine, A., 2001. Postsynaptic calcium transients evoked by activation of individual hippocampal mossy fiber synapses. J. Neurosci. 21, 2206–2214.
Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., Prossnitz, E.R., 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307, 1625–1630.
Shibuya, K., Takata, N., Hojo, Y., Furukawa, A., Yasumatsu, N., Kimoto, T., Enami, T., Suzuki, K., Tanabe, N., Ishii, H., Mukai, H., Takahashi, T., Hattori, T.A., Kawato, S., 2003. Hippocampal cytochrome P450s synthesize brain neurosteroids which are paracrine neuromodulators of synaptic signal transduction. Biochim. Biophys. Acta 1619, 301–316.
Shughrue, P.J., Lane, M.V., Merchenthaler, I., 1997. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J. Comp. Neurol. 388, 507–525.
Simerly, R.B., Chang, C., Muramatsu, M., Swanson, L.W., 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J. Comp. Neurol. 294, 76–95.
Tanabe, N., Tsurugizawa, T., Komatsuzaki, Y., Mitsuhashi, K., Ogiue-Ikeda, M., Kimoto, T., Kawato, S., 2005. The endocrine disruptor Bisphenol A acutely induces changes of dendritic spine structures in rat hippocampal CA1 pyramidal neurons. Abstract PD-59, 8th Meeting of Japan Society of Endocrine Disrupters Research.
Tanabe, N., Kimoto, T., Kawato, S., 2006. Rapid Ca(2+) signaling induced by Bisphenol A in cultured rat hippocampal neurons. Neuro Endocrinol. Lett. 27, 97–104.
Teyler, T.J., Vardaris, R.M., Lewis, D., Rawitch, A.B., 1980. Gonadal steroids: effects on excitability of hippocampal pyramidal cells. Science 209, 1017–1018.
Thomas, P., Pang, Y., Filardo, E.J., Dong, J., 2005. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146, 624–632.
Tsurugizawa, T., Mukai, H., Tanabe, N., Murakami, G., Hojo, Y., Kominami, S., Mitsuhashi, K., Komatsuzaki, Y., Morrison, J.H., Janssen, W.G., Kimoto, T., Kawato, S., 2005. Estrogen induces rapid decrease in dendritic thorns of CA3 pyramidal neurons in adult male rat hippocampus. Biochem. Biophys. Res Commun. 337, 1345–1352.
Uchida, K., Suzuki, A., Kobayashi, Y., Buchanan, D., Sato, T., Watanabe, H., Katsu, Y., Suzuki, J., Asaoka, K., Mori, C., Arizono, K., Iguchi, T., 2002. Bisphenol-A administration during pregnancy results in fetal exposure in mice and monkeys. J. Health Sci. 48, 579–582.
U.S. EPA, 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. U.S. Environmental

Protection Agency. Available: http://www.epa.gov/scipoly/oscpendo/edspoverview/finalrpt.htm.

Vouimba, R.M., Foy, M.R., Foy, J.G., Thompson, R.F., 2000. 17beta-estradiol suppresses expression of long-term depression in aged rats. Brain Res. Bull. 53, 783–787.

Woolley, C.S., McEwen, B.S., 1992. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J. Neurosci. 12, 2549–2554.

Woolley, C.S., McEwen, B.S., 1994. Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. J. Neurosci. 14, 7680–7687.

Woolley, C.S., Gould, E., Frankfurt, M., McEwen, B.S., 1990. Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. J. Neurosci. 10, 4035–4039.

Woolley, C.S., Weiland, N.G., McEwen, B.S., Schwartzkroin, P.A., 1997. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. J. Neurosci. 17, 1848–1859.

Yang, S.N., Tang, Y.G., Zucker, R.S., 1999. Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. J. Neurophysiol. 81, 781–787.

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Estrogen synthesis in the brain—Role in synaptic plasticity and memory

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ABSTRACT

Estrogen and androgen are synthesized from cholesterol locally in hippocampal neurons of adult animals. These neurosteroids are synthesized by cytochrome P450s and hydroxysteroid dehydrogenases (HSDs) and 5alpha-reductase. The expression levels of enzymes are as low as 1/200–1/50,000 of those in endocrine organs, however these numbers are high enough for local synthesis. Localization of P450(17alpha), P450arom, 17beta-HSD and 5alpha-reductase is observed in principal glutamatergic neurons in CA1, CA3 and the dendate gyrus. Several nanomolar levels of estrogen and androgen are observed in the hippocampus.

Estrogen modulates memory-related synaptic plasticity not only slowly but also rapidly in the hippocampus. Rapid action of 17beta-estradiol via membrane receptors is demonstrated for spinogenesis and long-term depression (LTD). The enhancement of LTD by 1–10 nM estradiol occurs within 1 h. The density of spine is increased in CA1 pyramidal neurons within 2 h after application of estradiol. The density of spine-like structure is, however, decreased by estradiol in CA3 pyramidal neurons. ERalpha, but not ERbeta, induces the same enhancement/suppression effects on both spinogenesis and LTD.

1. Introduction

Sex hormones are synthesized in the gonads, and reach the brain via the blood circulation. In addition, the local endogenous synthesis of estrogens and androgens occurs in the mammalian brain, in areas such as the hippocampus (Kimoto et al., 2001; Kawato et al., 2002, 2003; Hojo et al., 2004; Kretz et al., 2004). A neurosteroid hypothesis was proposed by Baulieu's group in the 1980s, suggesting that pregnenolone (PREG), progesterone, and dehydroepiandrosterone (DHEA) may be endogenously synthesized in the brain due to the finding that PREG and DHEA has been found in the mammalian brain at concentrations greater than that in plasma (Corpechot et al., 1981; Baulieu, 1997). Because the concentration of PREG and DHEA does not decrease after adrenalectomy and castration, many experiments have been performed with the aim of demonstrating the *de novo* synthesis of DHEA within the brain (Corpechot et al., 1981; Robel et al., 1987).

Direct demonstration of steroidogenesis in the mammalian brain had, however, long been not successful over decades, due to the extremely low levels of steroidogenic proteins in the brain (Warner and Gustafsson, 1995). Therefore, sex steroids had not been considered to be brain-derived steroids, and rather thought to reach the brain exclusively via blood circulation after crossing the blood–brain barrier (Baulieu and Robel, 1998). This belief had been supported by many reports suggesting the absence of cytochrome P450(17α(DHEA synthase)) in adult mammalian brain (Le Goascogne et al., 1991; Mellon and Deschepper, 1993) and also by the observation of the complete disappearance of testosterone in the brain within 1 day after castration (Baulieu and Robel, 1998).

Neuromodulatory actions of gonadal sex hormones have been investigated in the hippocampus, because the hippocampus is attractive as a center of learning and memory (Woolley and

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CHO, Chinese Hamster Ovary; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DHEA, dehydroepiandrosterone; DPN, diarylpropionitrile; GPR30, G protein-coupled receptor 30; HSD, hydroxysteroid dehydrogenase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LTD, long-term depression; LTP, long-term potentiation; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MCF-7, human breast cancer cell line; NMDA, *N*-methyl-D-aspartate; PBR, peripheral benzodiazepine receptor; PSD, postsynaptic density; PREG, pregnenolone; PPT, propyl-pyrazole-trinyl-tris-phenol; MCF-7, human breast cancer cell line; StAR, steroidogenic acute regulatory protein.

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McEwen, 1994; Woolley, 1998; Foy et al., 1999; Pozzo-Miller et al., 1999; Bi et al., 2000; Shibuya et al., 2003). Many scientists had, however, not seriously considered that memory formation process might favor hippocampus-derived steroids rather than circulating gonadal steroids. Therefore, many investigations have been focused on the role of slow modulation by sex steroids on spinogenesis and electrophysiological properties, for example, upon s.c. injection of estradiol (in a time scale of days) (Woolley and McEwen, 1994; Woolley, 1998; Foy et al., 1999; Pozzo-Miller et al., 1999; Bi et al., 2000; Shibuya et al., 2003). The rapid effect of estrogen (within 1–2 h) is also observed on modulation of electrophysiological properties of the hippocampal slices (Teyler et al., 1980; Foy et al., 1999; Bi et al., 2000; Mukai et al., 2006a). These rapid modulations favor the hippocampus-derived steroids rather than circulating gonadal hormones which travel over the long distance until they reach the brain. A weak activity of sex steroid production in the hippocampus is sufficient for the local usage within small neurons (i.e., intracrine system). This intracrine system contrasts with the endocrine organs in which high expression levels of steroidogenic enzymes are necessary in order to supply steroids to many other organs via the blood circulation. For brain-derived sex hormones, the rapid modulation of synaptic plasticity and cognitive functions may be their essential functions.

2. Synthesis of estrogen in the hippocampus

In assay of brain steroidogenesis, it is essential to improve the sensitivity of measurements (e.g., immunostaining, Western blot as well as steroid metabolism assay) by 100–1000-fold of those in endocrine organs. Even RT-PCR method is necessary to be improved by specific primer pair design.

2.1. Neuronal localization of steroidogenic proteins

Which cells are steroidogenic in the hippocampus, neurons or glial cells? In earlier studies, glial cells were thought to be a major place for steroidogenesis, because the white matter including glial cells had been stained with anti-P450scc antisera, throughout the adult rat brain (Le Goascogne et al., 1987). However, this white matter staining of P450scc antisera is likely to be an artifact which is due to the nonspecific adsorption of the non-purified bovine antisera in rat hippocampus.

The role of neurons in steroid synthesis in mammalian brains had long been difficult to determine. The absence of P450(17α) in both neurons and glial cells had been believed due to the fact that many attempts to demonstrate the immunohistochemical reactivity in the rat brain had been unsuccessful for almost two decades

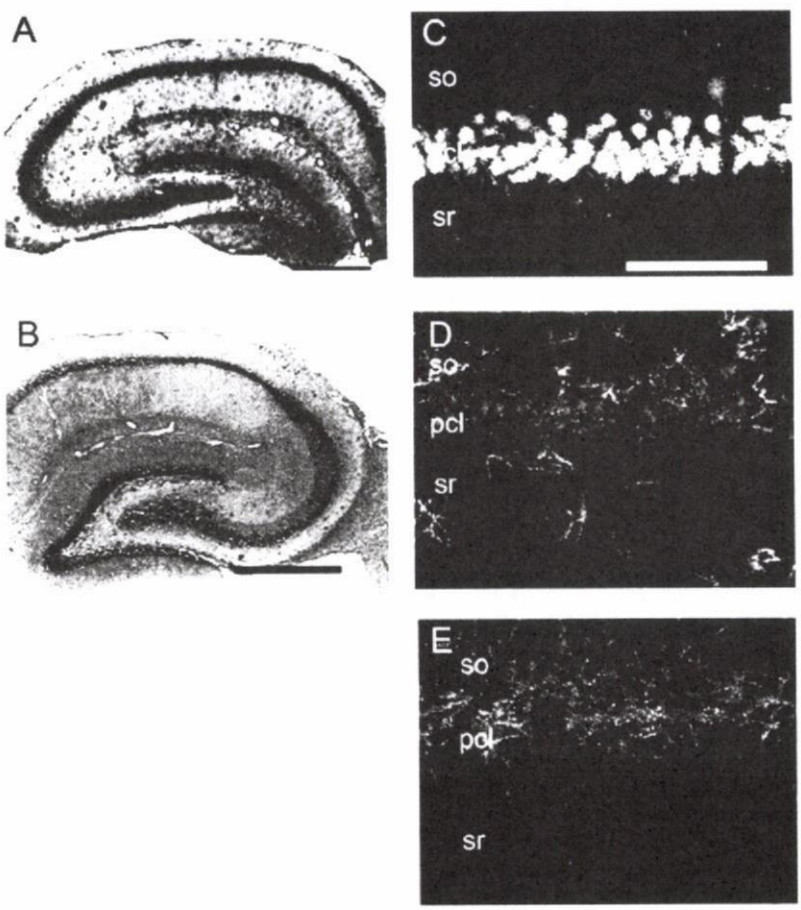

Fig. 1. Immunohistochemical staining of P450(17α) (A) and P450arom (B) in the coronal section of the adult male rat hippocampus. (C) Fluorescence dual staining of P450(17α) (green) and neuronal nuclear antigen, a marker for neurons (red). (D) Fluorescence dual staining of P450(17α) (green) and glial fibrillary acidic protein, a marker for astroglial cells (red). (E) Fluorescence dual staining of P450(17α) (green) and myelin basic protein, a marker for oligodendroglial cells (red). In (C)–(E) (CA1 region), superimposed regions of green and red fluorescence are represented by yellow. P450(17α) and P450arom are primarily expressed in neurons, although a weak expression of P450(17α) is associated with astroglial cells. pcl, pyramidal cell layer; so, stratum oriens; sr, stratum radiatum. Scale bar, 800 μm for A and B, and 120 μm for (C)–(E) (modified from Hojo et al., 2004).

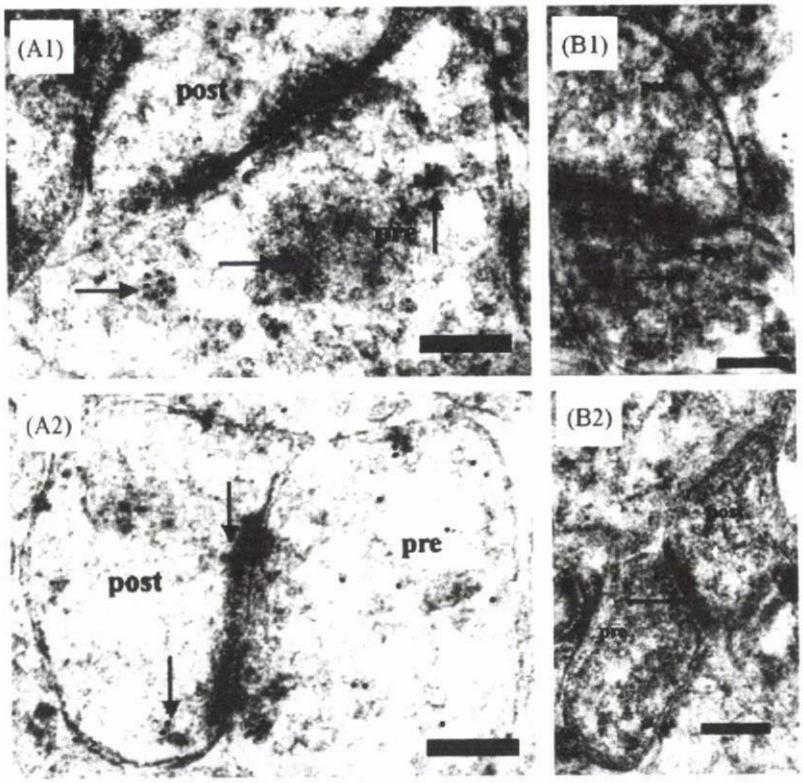

Fig. 2. Immunoelectron microscopic analysis of the distribution of P450(17α) (A1 and A2) and P450arom (B1 and B2) within axospinous synapses, in the stratum radiatum of the hippocampal CA1 region. Gold particles (indicated with arrows) were observed in the presynaptic region (A1 and B1), and the postsynaptic region (A2 and B2) of pyramidal neurons. Scale bar, 200 nm (modified from Hojo et al., 2004).

(Le Goascogne et al., 1991). We overcame difficulties of nonspecific immunostaining by using affinity column-purified antibodies (Shinzawa et al., 1988; Jakab et al., 1993) (instead of using non-purified antisera) in order to avoid cross-reaction with IgG with unknown proteins having similar antigen sequences, as well as using fresh frozen slices of hippocampus (instead of using paraffin sections). A significant localization of cytochromes P450scc, P450(17α) and P450arom was observed in pyramidal neurons in CA1–CA3, as well as in granule cells in the dentate gyrus (DG), by means of the immunohistochemical staining of hippocampal slices from adult (12 weeks) and developmental rats (Fig. 1) (Kimoto et al., 2001; Kawato et al., 2002; Shibuya et al., 2003; Hojo et al., 2004). The co-localization of immunoreactivity against P450s and NeuN (marker of neuron) confirmed the presence of P450s in these neurons (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). Astroglial cells were weakly stained with P450(17α) or P450scc antibodies, however, oligodendroglial cells were not stained significantly by these P450 antibodies (Fig. 1) (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). StAR was co-localized with P450s (Zwain and Yen, 1999a; Kimoto et al., 2001). These results imply that pyramidal neurons and granule neurons are equipped with complete steroidogenic systems which catalyze the conversion of cholesterol to PREG, DHEA, testosterone and estradiol. From a weak immunostaining of P450s in glial cells, the activity of neurosteroidogenesis in glial cells is probably much lower than that of neurons.

An immunoelectron microscopic analysis using postembedding immunogold method is very useful to determine the intraneuronal localization of P450(17α) in the hippocampal neurons of adult male rats. Surprisingly, we observed that both P450(17α) and P450arom were localized not only in the endoplasmic reticulum but also in the presynaptic region as well as the postsynaptic region of pyramidal neurons in the CA1 and CA3 regions and of granule neurons in DG (Fig. 2). These results suggest 'synaptic' synthesis of estrogens and androgens, in addition to classical microsomal synthesis of sex steroids.

The existence of these steroidogenic proteins was confirmed by Western immunoblot analyses (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). The molecular weights obtained for P450scc, P450(17α) and P450arom were identical to those obtained from peripheral steroidogenic organs. The relative levels of these P450s in the hippocampus were approximately 1/1000 (P450scc) and 1/300 (P450(17α) and P450arom) of that in the testis (P450scc and P450(17α)) and the ovary (P450arom), respectively.

2.2. mRNA expression of steroidogenic enzymes

From many molecular biological investigations (Warner and Gustafsson, 1995), the relative level of mRNA expressed in the hippocampus has been suggested to be lowest for cytochrome P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD), and highest for steroidogenic acute regulatory protein (StAR) and 5α-reductase, with those of P450(17α) and P450arom expressed at an intermediate level (Table 1).

The expression level of cytochrome P450scc (CYP11A1) mRNA is extremely low, preventing many scientists to believe the physiological significance of neurosteroid synthesis. P450scc is expressed in the brain is reported to be only 10^{-4} to 10^{-5} of that in the adrenal gland (Mellon and Deschepper, 1993; Sanne and Krueger, 1995). Our analysis showed approximately 1/50,000 for the P450scc level in the adult hippocampus as compared with that in the adrenal gland (Table 1) (Murakami et al., 2006b). As a result,

Table 1
Comparison of relative mRNA expression level for steroidogenic enzymes in the adult rat (12 weeks)

	Hippo[a]	Hypo	Adrenal/Testis/Ovary/Liver/Prostate
P450scc	1[b]	3	50,000 (Ad) 1000 (Te)
P450(17α)	1	3	300 (Te)
P450arom	1	3	300 (Ov)
17β-HSD (type 1)	1	3	200 (Ov)
17β-HSD (type 3)	1	3	300 (Te)
3β-HSD (type 1)	1	3	5,000 (Ov)
3β-HSD (types 2–4)	N.D[c].	D[d]	D[d] (Ov)
5α-Reductase (type 1)	1	2	5 (Li)
5α-Reductase (type 2)	1	1/3	200 (Pro)
ERα	1	5	15 (Ov)
ERβ	1	4	80 (Ov)

[a] Hippocampus (Hippo), hypothalamus (Hypo), adrenal gland (Ad), testis (Te), ovary (Ov), liver (Li), and prostate (Pro) are compared.

[b] The level in the hippocampus is normalized to be 1. The level of mRNA expression is approximate value obtained from semiquantitative RT-PCR analyses.

[c] 3β-HSD (types 2–4) were not detectable, even after 50 cycles of PCR amplification. For 3β-HSD (type 1), 40 cycles were used for PCR. For Reverse-Transcription, 200 ng of total RNAs were used for 3β-HSD (types 1–4), though 50 ng of total RNAs were always used for other steroidogenic enzymes examined.

[d] 3β-HSD (types 2–4) were expressed at roughly the same level as 3β-HSD (type 1).

the presence of P450scc mRNA could be demonstrated only by the RT-PCR method. The ribonuclease (RNase) protection assay for P450scc in the hippocampus, which was performed using a ^{32}P-labelled rat P450scc antisense riboprobe, however, yielded no detectable specific hybridization signals (Furukawa et al., 1998). On the other hand, StAR is most abundant, therefore, not only the PCR-amplification but also the RNase protection assay demonstrated the presence of StAR transcripts with an expression level of approximately 1/200 of the level in the adrenal gland (Furukawa et al., 1998; King et al., 2003).

The mRNAs for cytochrome P450(17α) (CYP17A) had not been detected in adult rat brain by either RT-PCR or RNase protection assays (Mellon and Deschepper, 1993). The expression of the mRNA for P450(17α) had been reported by many laboratories as only transient, occurring during rat embryonic and neonatal development (Compagnone et al., 1995; Zwain and Yen, 1999a,b). We overcame this difficulty by carefully choosing the sequence of primer pairs which have high specificity by minimizing Gibbs free energy upon recombination of a 3′-primer with cDNA, using computer calculation (Hojo et al., 2004). In the hippocampal tissues from adult male rats aged 3 months, we observed that P450(17α) transcripts expressed approximately 1/300, when compared with those expressed in the testis.

The role of P450arom (CYP19) (estrogen synthase) in the hippocampus had also not been easy to elucidate. Many studies had reported the absence of P450arom in the adult rat and mouse hippocampus. The significant expression of mRNA for P450arom in the pyramidal and granule neurons of the adult rat hippocampus, however, has recently been demonstrated using *in situ* hybridization (Wehrenberg et al., 2001). The level of the mRNA expression in the adult mouse hippocampus is approximately half of that in neonatal stages (Ivanova and Beyer, 2000). We observed the P450arom transcripts expressed approximately 1/300 (Hojo et al., 2004), as compared with those expressed in the ovary by using carefully designed primer pairs.

The presence of mRNAs for 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 3 is demonstrated in the human and rat hippocampus (Beyenburg et al., 2000). We investigated the expression level of mRNA transcripts for 17β-HSD (types 1–4) using carefully designed primer pairs in the hippocampus from adult male rats. The mRNA level of 17β-HSD transcripts observed was approximately 1/200, relative to the level in the ovary for 17β-HSD (type 1), and 1/300, relative to the testis for 17β-HSD (type 3), respectively (Hojo et al., 2004).

The localization in neurons of several steroidogenic enzymes is demonstrated by means of *in situ* hybridization. For example, mRNAs for both StAR and 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA (10^{-2} for StAR and 10^{-3} to 10^{-4} for 3β-HSD of the levels in the adrenal gland) have been observed to be localized along the pyramidal cell layer in CA1–CA3 and the granule cell layer in DG of rats (Furukawa et al., 1998; Ibanez et al., 2003). Though subtypes (types 1–4) of 3β-HSD had not been discriminated previously, our results showed only type 1 3β-HSD was expressed in the hippocampus and other subtypes 2–4 were not expressed (Table 1).

In embryonic and neonatal brains, glial cells have been considered to play an important role in steroidogenesis, as many reports have indicated the presence of mRNA for P450scc, P450(17α), 3β-HSD, and 17β-HSD in cultures of astrocytes and oligodendrocytes (Jung-Testas et al., 1989; Baulieu, 1997; Zwain and Yen, 1999a,b). Although similar levels of P450(17α) mRNA are expressed in both astrocytes and neurons in primary cell cultures from the brain of neonatal rats, a much lower metabolic activity is observed in neurons than astrocytes for the conversion of PREG to DHEA (Zwain and Yen, 1999a,b). The very low metabolic activity of neurons might be caused by cytosine arabinoside applied to cultures in order to suppress the proliferation of glial cells. These investigations are available on primary glial cell cultures which are easily prepared from embryonic and neonatal brains. However, information regarding the synthesis system of neurosteroids in 'adult' rat brain is not directly available from these cell culture studies, because we cannot culture adult neurons.

2.3. Synthesis of 17β-estradiol

A direct demonstration of the neuronal synthesis of PREG, DHEA, testosterone and 17β-estradiol in adult mammals is for the first time reported by our group (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). It had been assumed that testosterone is supplied to the male brain such as hypothalamus, via the blood circulation, where testosterone is converted to estradiol by P450arom (Baulieu, 1997; Baulieu and Robel, 1998). The absence of P450(17α) activity in the brain of adult mammals had been reported in a number of studies (Le Goascogne et al., 1991; Baulieu and Robel, 1998; Mensah-Nyagan et al., 1999; Kibaly et al., 2005). Incubations of ^{3}H-PREG with brain slices, homogenates and microsomes, primary cultures of mixed glial cells, or astrocytes and neurons from rat and mouse embryos, had failed to produce a radioactive metabolite ^{3}H-DHEA (Baulieu and Robel, 1998).

We succeeded in demonstration of the synthesis of DHEA, testosterone and estradiol in the adult (12 weeks) hippocampal slices by means of careful HPLC analysis (Kawato et al., 2002; Hojo et al., 2004). The purification of neurosteroids from very fatty brain tissues requires the combination of several sophisticated methods, which included purification with organic solvent, solid column chromatography, and normal phase HPLC (Wang et al., 1997; Kimoto et al., 2001; Hojo et al., 2004). The significant conversion from ^{3}H-PREG to ^{3}H-DHEA, from ^{3}H-DHEA to ^{3}H-androstenediol, ^{3}H-testosterone and ^{3}H-estradiol was observed after incubation with the slices for 5 h (Fig. 3) (Hojo et al., 2004). The rate of production for ^{3}H-estradiol from ^{3}H-testosterone was very slow, and the production rate of ^{3}H-dihydrotestosterone from ^{3}H-testosterone was much more rapid than that of estradiol. These activities were abolished by the application of specific inhibitors of cytochrome P450s. Surprisingly, ^{3}H-estradiol was extremely stable and not significantly converted to other steroid metabolites

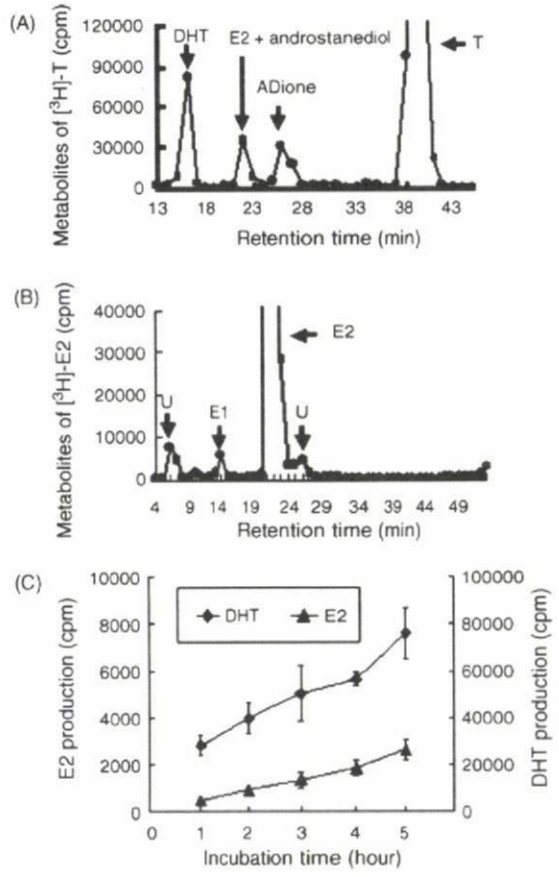

Fig. 3. HPLC analysis of steroid metabolism in adult rat hippocampal slices. (A) Profiles of [^{3}H]-T metabolites after incubation of slices for 5 h. (B) Profiles of [^{3}H]-E2 metabolites after incubation of slices for 5 h. (C) Time-dependent productions of E2 and DHT from [^{3}H]-T. Because the radioactive peaks of E2 and androstanediol were superimposed in (A), these fractions were re-analyzed to separate E2 and androstanediol by HPLC. The vertical axis indicates ^{3}H radioactivity (cpm). E2 was produced slowly but stably present, whereas DHT was rapidly produced and metabolized to androstanediol (modified from Hojo et al., 2004).

such as estrone. On the other hand, dihydrotestosterone was rapidly converted to 3α,5α-androstanediol.

We determined the concentration of DHEA and 17β-estradiol as well as PREG in hippocampal slices from adult male rats by means of RIA, after careful purification of steroids with normal phase HPLC. The basal concentrations of PREG, DHEA and estradiol, in the male rat hippocampus were approximately 18, 0.3 and 0.6 nM, which were 6–10 times greater than those typical of plasma (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). Recently we very much improved the determination of steroids by using liquid chromatography–tandem-mass spectrometry (LC–MS/MS) which has a high specificity and accuracy. A much higher value of 8 nM was obtained for estradiol (Kawato et al., 2007). The concentration of testosterone in the hippocampus (17 nM) was only slightly higher than that of circulating testosterone (15 nM) in male rats. To demonstrate the rapid net production of neurosteroids upon synaptic stimulation, the NMDA-induced production of PREG and estradiol was investigated in hippocampal slices (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). Upon stimulation with NMDA for 30 min, the hippocampal level of PREG and estradiol increased to approximately twofold of the basal levels. This implies that the NMDA-induced Ca^{2+} influx drives net production of PREG and estradiol. Estradiol synthesis is also demonstrated in cultured hippocampal slices from neonatal rats in the absence and presence of letrozole, an inhibitor of P450arom. After 4 days treatment with letrozole, the amount of estradiol released into the medium was significantly decreased (Kretz et al., 2004).

Interestingly, PREG sulfate and DHEA sulfate have been reported to be absent in the rat brain as measured by direct mass spectrometric analysis, although cholesterol sulfate is present (Higashi et al., 2003; Liu et al., 2003; Liere et al., 2004). In many previous publications, PREG sulfate or DHEA sulfate had been determined indirectly, i.e., measuring PREG or DHEA after solvolysis of water-soluble fractions which may contain some lipoidal derivatives of PREG, different from sulfated steroids (Corpechot et al., 1981; Baulieu, 1997; Liere et al., 2000; Kimoto et al., 2001; Liu et al., 2003). Because numerous publications have reported that sulfated steroids are important participants in neuromodulation, these results merit careful consideration (Wu et al., 1991; Vallee et al., 1997; Baulieu and Robel, 1998).

Is the local concentration of brain neurosteroids sufficiently high to allow action as local mediators? Based on RIA determination, the concentration of estradiol detected in the hippocampus was only about 0.6 nM (basal) and 1.3 nM after the NMDA-stimulation, respectively (Hojo et al., 2004). However, from accurate LC–MS/MS analysis, basal level of estradiol was determined to be roughly 8 nM (Hojo et al., 2006). This level is sufficient to allow estradiol to act as local mediators that modulate synaptic plasticity (Gu and Moss, 1996; Foy et al., 1999; Ito et al., 1999; Bi et al., 2000; Shibuya et al., 2003).

Functional differences for estradiol produced from circulating testosterone and estradiol produced from hippocampus-derived testosterone may be differences in the time-dependence of their levels. Brain is filled with circulating testosterone (for male), or estradiol (for female) whose level slowly changes depending on the circadian rhythm, while the endogenous synthesis of estradiol (for both male and female) is a transient event depending on neural activity (Hojo et al., 2004).

3. Modulation of synaptic plasticity by estrogen

3.1. Spinogenesis

Brain-derived estradiol may rapidly modulate several different types of synaptic plasticity of neurons. One is spinogenesis, and another one is synaptic transmission such as LTD or LTP. Spinogenesis includes not only spine-synapses (spines forming synapses) but also free spines (spines without forming synapses), whereas LTD and LTP probe the characteristics of preformed synapses. Modulation of spinogenesis is essential action of estrogen in memory processes, involving production of new spines that creates sites for new neuronal contacts. We demonstrated that dendritic spines were rapidly modulated upon estradiol application, using single spine analysis of Lucifer Yellow-injected neurons in hippocampal slices from adult male rats (3 months) (Komatsuzaki et al., 2005; Tsurugizawa et al., 2005; Mukai et al., 2006b; Murakami et al., 2006a). Following a 2-h treatment with estradiol in the stratum radiatum of CA1 region, the treated dendrites have significantly more spines at 1 nM estradiol (1.31 spines/μm) than dendrites at 0 nM estradiol (0.85 spines/μm) (Fig. 4) (Mukai et al., 2007). Propyl-pyrazole-tris-phenol (PPT, ERα agonist) (Harrington et al., 2003) induced a significant enhancement of the spine density to 1.20 spines/μm. However, diarylpropionitrile (DPN, ERβ agonist) (Harrington et al., 2003) increased the spine density only slightly (0.95 spines/μm). Blocking of ERα by ICI 182,780 completely suppressed the enhancing effect of estradiol on the spine density. Blocking of phosphorylation of Erk MAP kinase by PD98059 or U0126 completely prevented the estradiol-induced spinogenesis

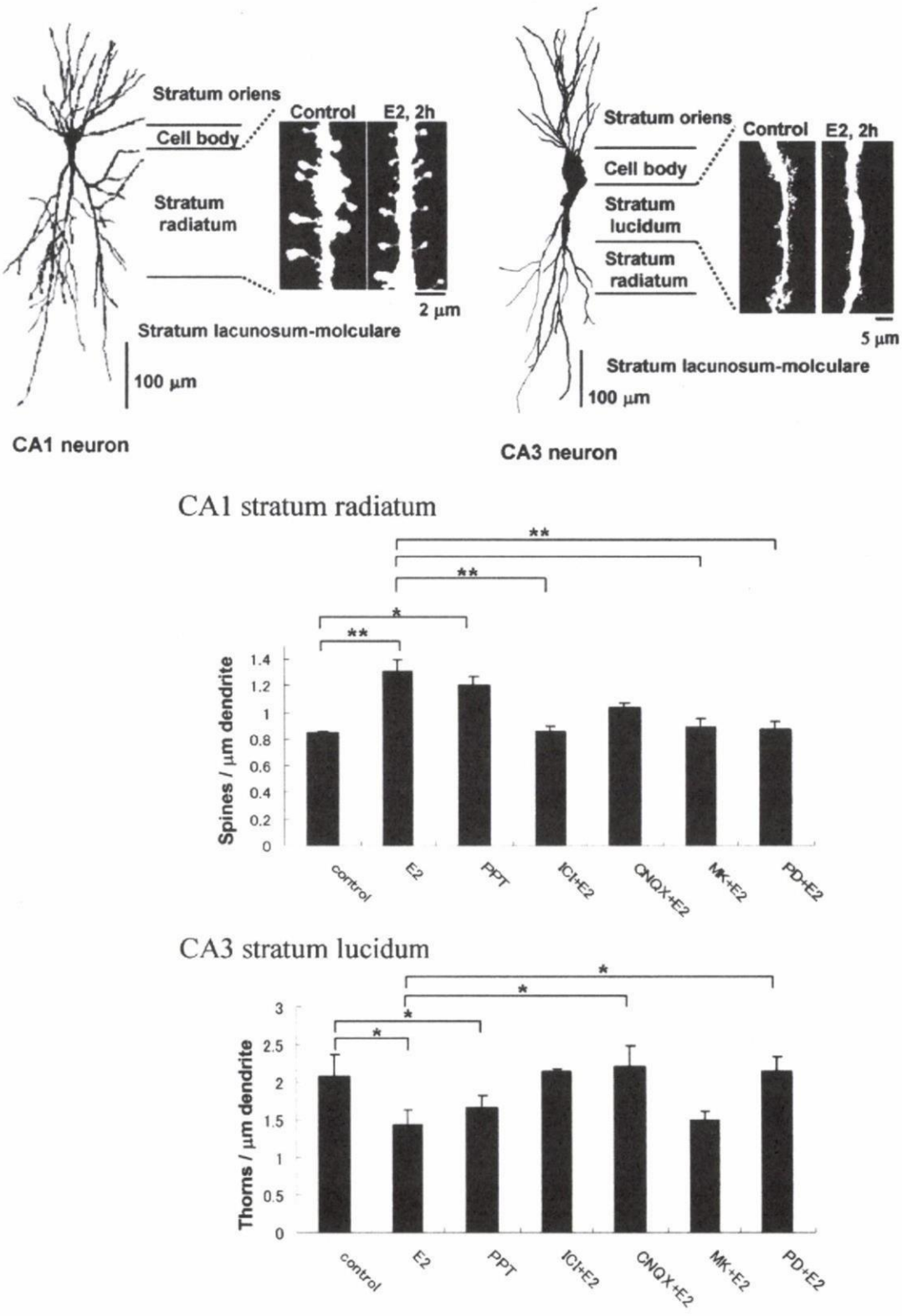

Fig. 4. Changes in the density and morphology of spines in CA1 or thorns in CA3 pyramidal neurons upon treatments of 17β-estradiol (E2) and drugs in hippocampal slices from adult male rats. Spines/thorns were analyzed along the dendrites of pyramidal neurons. Upper left panel (CA1 neuron), a whole image of Lucifer Yellow-injected pyramidal neuron, vertical bar 100 μm. (Control and E2) Maximal intensity projections onto XY plane from z-series confocal micrographs, showing spines along the dendrites in stratum radiatum without drugs (Control) or with 1 nM E2 (E2). Horizontal bar 2 μm. Upper right panel (CA3 neuron), a whole image of Lucifer Yellow-injected pyramidal neuron. Vertical bar 100 μm. (Control and E2) Maximal intensity projections onto XY plane from z-series confocal micrographs, showing thorns in the stratum lucidum without drugs (Control) or with 1 nM E2 (E2). Horizontal bar 5 μm. Lower panel (CA1 stratum radiatum), effect of drug treatments on the total spine density of CA1 neurons

(Murakami et al., 2006a). Taken together, the enhancement of the spine density is probably induced by activation of Erk MAP kinase via estradiol and ERα at the basal low Ca^{2+} concentration of around 0.1–0.2 μM in resting neuronal synapses (Ishii et al., 2007). When the Ca^{2+} concentration in spines was further decreased by blocking NMDA receptors with MK-801, the enhancing effect by estradiol was completely suppressed. Function of estradiol-bound ERα therefore needs the basal level of Ca^{2+} concentration of around 0.1–0.2 μM. The morphological changes in CA1 spines occurred by 2-h estradiol treatments. In control slices (0 nM estradiol), the relative population of spines was approximately 24% for mushroom spine, 62% for thin spine, 1% for filopodium, and 13% for stubby spine. Upon 1 nM estradiol treatment, the density of thin spine was selectively increased, from 0.57 spines/μm to 0.97 spines/μm, while the density of mushroom and stubby was not significantly altered.

The spine density is not always increased but in some cases decreased by the estradiol treatment. The estradiol-induced spinogenesis is region specific and heterogeneous. In fact, in CA3 pyramidal neurons, the total density of thorns of thorny excrescences (spine-like postsynaptic structures in the stratum lucidum of CA3, having contacts with mossy fiber terminals originated from granule cells) decreased dramatically to approximately 70% upon a 2-h treatment of 1 nM estradiol (Fig. 4) (Tsurugizawa et al., 2005). PPT significantly decreased the density of thorns from 2.19 to 1.66 thorns/μm, but DPN did not significantly change the density of thorns (Fig. 4). Blocking of Erk MAP kinase by PD98059 completely prevented the estradiol-induced decrease of thorns. Taken together, in the stratum lucidum of CA3, the decrease of the thorn density is probably induced by activation of Erk MAP kinase by estradiol-bound ERα at the basal Ca^{2+} concentration of around 0.1–0.2 μM. When the Ca^{2+} concentration was decreased to below the basal level by blocking AMPA receptors with CNQX, or by changing the outer medium to Ca^{2+}-free ACSF, the supression effect of estradiol was completely inhibited (Fig. 4). These results suggest that the decrease of thorns requires the basal Ca^{2+} concentration which is kept by spontaneous postsynaptic Ca^{2+} fluctuation via voltage-activated calcium channels depending upon AMPA receptor-mediated spontaneous voltage fluctuations, because the spontaneous Ca^{2+} influx within thorny excrescences occurs mainly via voltage-activated calcium channels (Monaghan et al., 1983; Baude et al., 1995; Fritschy et al., 1998; Reid et al., 2001; Reid, 2002). Note that blocking of NMDA receptors by MK-801 did not prevent the estradiol-induced decrease of thorns. This may be due to much smaller contribution of NMDA receptors to the spontaneous Ca^{2+} influx within thorns than that of voltage-activated calcium channels. Function of ERα therefore needs the basal level of Ca^{2+} concentration around 0.1–0.2 μM.

We always use isolated hippocampal slices in order to examine the direct effect of estradiol on glutamatergic neurons within slices. Results from *in vivo* investigations using whole rat may reflect not only direct but also indirect effects of estradiol on glutamatergic neurons via cholinergic or serotonergic neurons, projecting to the hippocampus (Leranth et al., 2000; MacLusky et al., 2005).

The rapid effect of estrogens has also been observed *in vivo*. Leranth and co-workers have demonstrated that the estradiol (60 μg/kg) increases the spine-synapse density due to synaptic rearrangements in ovariectomized adult rats as rapid as after 30 min of estradiol injection using electron micrographic analysis (MacLusky et al., 2005). On the other hand, the slow genomic effects (1–4 days) of estradiol on spine plasticity, have been extensively investigated *in vivo* from the view point of estrogen replacement therapy. For example, supplement of estrogens in ovariectomized adult female rats (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; MacLusky et al., 2005), increase the density of spines in the stratum radiatum of CA1 pyramidal neurons, resulting in recovery of spines to the level of intact rat. These effects of enhancement in spinogenesis have also been observed as rapid as at 4.5 h after s.c. injection of estrogen (MacLusky et al., 2005). *In vitro* investigations have also shown that spine density in CA1 increases following several days' treatment of cultured hippocampal slices with exogenous estradiol (Pozzo-Miller et al., 1999). The contribution of hippocampus-derived estradiol has been reported by Rune and co-workers who demonstrated that the suppression of endogenous estradiol synthesis by letrozole treatments for 4 days significantly decreased the density of spines, spine-synapses, spinophilin (spine marker) and synaptophysin (presynaptic marker) in the stratum radiatum of the CA1 region in cultured slices (Kretz et al., 2004). No increase in the density of spines, spine-synapses and spinophilin expression was seen after exogenous application of 100 nM estradiol to the medium of slice cultures that had not been treated with letrozole. Application of 100 nM estradiol, however, induced rescue effect which restored the synaptophysin expression that had been once decreased by letrozole.

3.2. Modulation of long-term depression (LTD) and long-term potentiation (LTP)

Estradiol-induced modulation of LTD or LTP occurs only in pre-existent synapses, because newly generated spines by estradiol treatments do not form new synapses within 2 h, as judged from no increase in the baseline magnitude of excitatory postsynaptic potential (EPSP) signal during 2 h of estradiol perfusion (Mukai et al., 2007).

Evidence is emerging that estradiol exerts a rapid influence (0.5–1 h) on synaptic transmission of hippocampal slices from adult rats (3 months), as demonstrated by electrophysiology (Teyler et al., 1980; Gu and Moss, 1996; Foy et al., 1999; Ito et al., 1999; Shibuya et al., 2003).

In memory processing, not only LTP (memory forming mechanism) but also LTD is essential. Mutant mice, which show enhanced LTP and suppressed LTD, have shown impaired learning of Morris water maze (Migaud et al., 1998). This suggests that LTD may be required to "correct" wrong memories formed by initial LTP processes, which store not only correct information but also wrong information. We found that LTD was very sensitive to 17β-estradiol treatments in hippocampal slices from adult male rats. We demonstrated, for the first time, a significant rapid enhancement of LTD by 1–10 nM estradiol perfusion in CA1, CA3 and DG (Fig. 5) (Mukai et al., 2007). Recordings were performed using 64 planar multielectrodes particularly arranged to stimulate the Schaffer collaterals in the stratum radiatum of CA1, the recurrent collateral fibers in the stratum radiatum of CA3, and the medial perforant pathways in the molecular layer of DG. LTD was induced pharmacologically by the transient application (3 min) of 30 μM NMDA. This LTD was

in the stratum radiatum. Vertical axis is the average number of spines per 1 μm of dendrite. A 2-h treatment in ACSF without drugs (Control), with 1 nM E2 (E2), with 100 nM PPT (PPT), with 1 nM E2 and 1 μM ICI 182,780 (ICI + E2), with 1 nM E2 and 20 μM CNQX (CNQX + E2), with 1 nM E2 and 50 μM MK-801 (MK + E2), with 1 nM E2 and 50 μM PD98059 (PD + E2). Statistical significance (*$p < 0.05$). (CA3 stratum lucidum) Effect of drug treatments on the average number of thorns per 1-μm dendritic segment. A 2 h treatment in ACSF without estradiol (Control), with 1 nM estradiol (E2), with 100 nM PPT (PPT), with 1 nM E2 and 1 μM ICI 182,780 (ICI + E2), with 1 nM estradiol and 20 μM CNQX (CNQX + E2), with 1 nM estradiol and 50 μM MK-801 (MK + E2), with 1 nM E2 and 20 μM PD98059 (PD + E2) (modified from Mukai et al., 2007; Murakami et al., 2006a,b; Tsurugizawa et al., 2005).

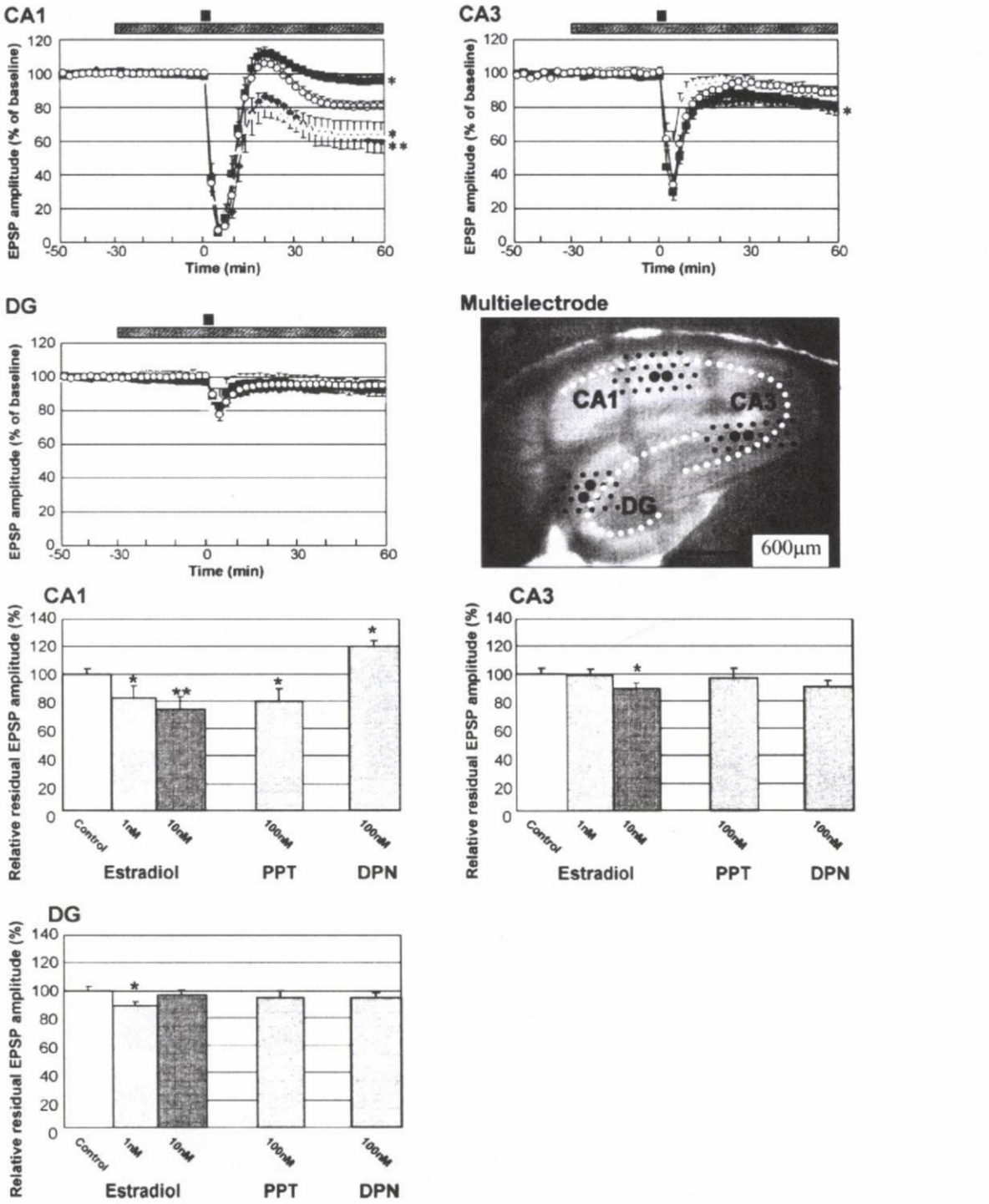

Fig. 5. Rapid modulation of LTD by 17β-estradiol and agonists in hippocampal slices from adult male rats. (Upper CA1, CA3 and DG) Time dependence of maximal EPSP amplitude in CA1 (CA1), CA3 (CA3) and DG (DG). Estradiol concentration was 0 nM (open circle), 10 nM (red closed diamond), 100 nM PPT (yellow closed triangle) and 100 nM DPN (blue closed square), respectively. (Multielectrode) Custom-made 64 multielectrode probe (MED64, Panasonic, Japan) with the hippocampal slice. Stimulation (red circle) and recording (blue circle) electrodes are indicated. Here, 100% EPSP amplitude refers to the EPSP value at $t = 40$ min prior to NMDA stimulation, irrespective of the test condition. LTD was induced by 30 μM NMDA perfusion at time $t = 0$–3 min (closed red bar above the graph). Hatched bar above the graph indicates period of time during which estradiol was administered. The LTD enhancement was reproducibly observed in more than 90 slices out of 100 slices. (Lower CA1, CA3 and DG) Comparison of modulation effect of LTD by 17β-estradiol and agonists in the CA1 (CA1), CA3 (CA3) and DG (DG) of hippocampal slices. Vertical axis is relative EPSP amplitude at $t = 60$ min, where EPSP amplitude at $t = 60$ min of the control slice without drug application is taken as 100%. From left to right, 17β-estradiol (Estradiol), PPT and DPN at indicated concentrations. Note that co-perfusion of 1 μM ICI with 10 nM 17β-estradiol did not suppress the enhancing effect of LTD by estradiol (data not shown). The significance of the estradiol effect was confirmed at 60 min via statistical analysis using ANOVAs (*$p < 0.05$; **$p < 0.01$) (modified from Mukai et al., 2007).

induced by the activation of phosphatase due to a moderate Ca^{2+} influx through NMDA receptors (Lee et al., 1998). LTD is effectively induced by the transient application of NMDA to adult hippocampus, whereas low-frequency stimulation cannot induce LTD in adult slices. Low-frequency stimulation can induce LTD in slices from animals younger than 2 weeks. The plateau EPSP amplitude at 60 min after NMDA application was 80.4% (CA1), 88.8% (CA3) and 95.1% (DG), respectively. A 30 min pre-perfusion of 10 nM estradiol significantly enhanced LTD resulting in the residual EPSP amplitude of 59.7% (CA1), 79.1% (CA3) and 92.2% (DG) (Fig. 5) (Mukai et al., 2007). Investigations using specific estrogen agonists indicated that the contribution of ERα (but not ERβ) was essential to these estradiol effects. PPT at 100 nM exhibited a significant LTD enhancement in CA1, while DPN did induce a suppression of LTD in CA1, implying that the contribution of ERβ was opposite to that of ERα in the estradiol effect on LTD. Taken collectively, estradiol-bound ERα may activate phosphatase at the moderate Ca^{2+} concentration of around 0.7–1 μM induced upon 30 μM NMDA application (Lisman, 1989), and facilitated dephosphorylation of AMPA receptors may induce enhancement of LTD. On the other hand, estradiol-bound ERα is not functional in LTP modulation at the transiently high Ca^{2+} concentration of around 5–12 μM under tetanic stimulation (Lisman, 1989; Yang et al., 1999; Mukai et al., 2006b; Ogiue-Ikeda et al., 2008), because phosphorylation of AMPA receptors by CaM kinase II is a dominant process at the high Ca^{2+} concentration.

The enhancement of LTP has been occasionally observed by 1–10 nM estradiol in CA1 pyramidal neurons. In this case, a baseline increase by 20–30% has always been observed upon the onset of 10 nM estradiol perfusion in the initial slope of EPSP, which has been attendant upon a further increase to approximately 160% upon high-frequency tetanic stimulation of Schaffer collaterals of hippocampus from adult rat (3 months) (Foy et al., 1999; Bi et al., 2000; Kawato, 2004). However, without this 20–30% baseline increase in EPSP slope (before the tetanic stimulation), the enhancement of LTP by estradiol is not apparent, with regard to the pure tetanic stimulation-induced LTP. In other words, the magnitude of pure tetanic stimulation-induced LTP (approximately 130%) is nearly the same between the presence and the absence of 10 nM estradiol (Ito et al., 1999; Sato et al., 2004). It should be noted that in 3–4 weeks puberty rats, 10 nM estradiol even suppressed LTP-induction down to the same level as that for adult rats (Ito et al., 1999; Shibuya et

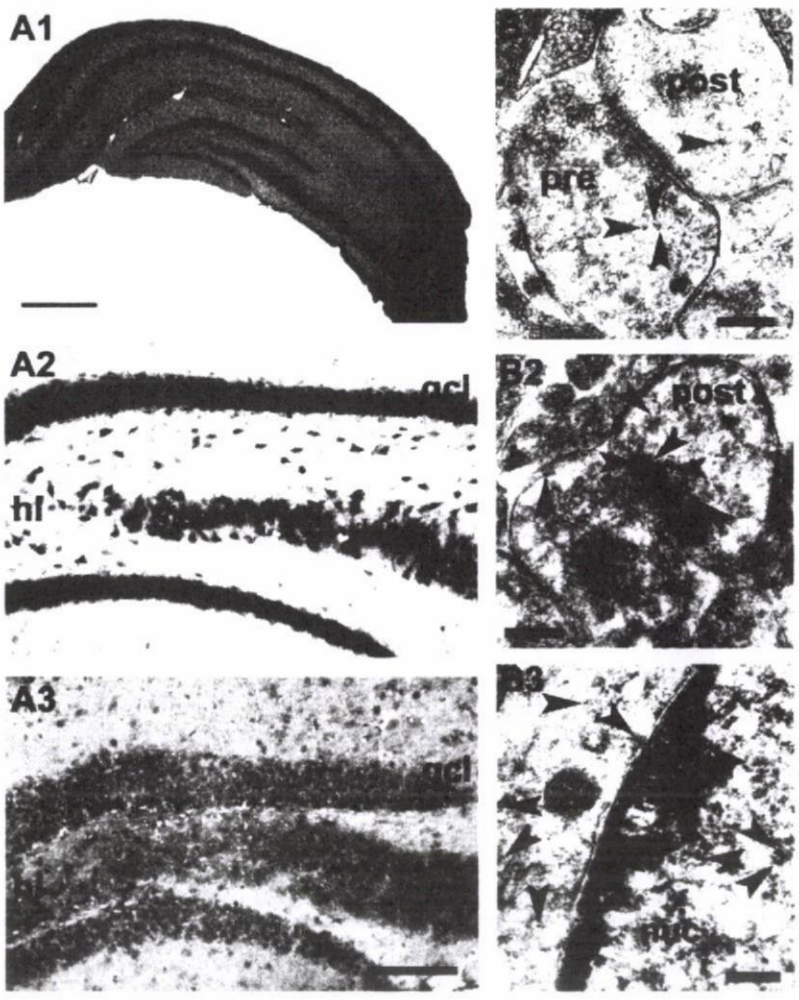

Fig. 6. (A) Immunohistochemical staining of ERα with RC-19 antibody in the hippocampal slices from adult male rat (A1 and A2) and adult male ERα KO mouse (A3). (A1) Coronal section of the whole hippocampal formation; (A2) DG; (A3) DG of ERα KO mouse. gcl, granule cell layer; hl, hilus. Scale bar, 500 μm for A1, and 200 μm for A2 and A3. (B) Immunoelectron microscopic analysis of the distribution of ERα within axospinous synapses, in the stratum radiatum of the hippocampal slices from adult male rat. (B1) Gold particles (arrowheads) were localized in the pre- and postsynaptic regions. (B2) In dendritic spines, gold particles were associated with PSD regions. (B3) Gold particles were also localized in the nuclei. pre, presynaptic region; post, postsynaptic region; scale bar, 200 nm (modified from Mukai et al., 2007).

al., 2003). Estradiol effects on LTP are strongly dependent on the age of rats.

3.3. Synaptic estrogen receptors

What is the receptor of 17β-estradiol that mediates rapid actions (1–2 h) on synaptic plasticity in the hippocampus? Putative synaptic membrane estrogen receptors remain poorly defined. Many attempts have been made to identify membrane estrogen receptors. At the present stage, the most probable candidates for synaptic estrogen receptors may be ERα, ERβ and GPR30.

Classical nuclear-type receptors ERα and ERβ are candidates for synaptic estrogen receptors. Because ICI do not suppress estradiol-induced rapid modulation of electrophysiological properties such as LTD, LTP, and kinate-induced currents, classical estrogen receptors are suggested to be not involved in these modulations (Gu and Moss, 1996). However, these results do not eliminate the possibility that ERα and ERβ could drive these synaptic transmissions. ICI has been indicated to display its effect by inhibiting dimerization of ERα and ERβ. If dimerization processes are not involved in rapid modulation of electrophysiological phenomena, then ICI cannot block these phenomena. On the other hand, rapid enhancement of spinogenesis via ERα was significantly blocked by ICI (Fig. 4) (Mukai et al., 2007), therefore dimerization processes occur for synaptic ERα in spinogenesis.

We identified the membrane estrogen receptor ERα localized in the spines of hippocampal pyramidal and granule neurons by means of immunoelectron microscopic analysis as well as Western blot analysis using affinity-column purified anti-ERα antibody RC-19 (C-terminal antibody) (Mukai et al., 2007). Attention must be paid that non-purified ERα antisera often react significantly with unknown proteins, resulting in wrong staining different from real ERαl distribution. A post-embedding immunogold electron microscopic analysis demonstrated the synaptic localization of ERα in the glutamatergic neurons in CA1, CA3 and DG (Fig. 6). ERα was also localized in the nuclei. Western blot analysis demonstrated that ERα (67 kDa) and Erk MAP kinase were tightly associated with postsynaptic density fractions (PSD) (Fig. 7). On the other hand, ERα was not expressed at dendritic raft (Fig. 7). Because the estradiol-induced modulation of LTD and spine density appeared so rapidly in the time range of 1–2 h, the synaptic ERα observed at PSD or postsynaptic compartments probably plays an essential role in driving rapid processes. Interestingly, a significant accumulation of ERα at PSD was observed by a 3-min stimulation with 30 μM NMDA used for the LTD induction, implying that ERα may be dynamically movable in spines (Fig. 7). Note that specific binding of purified RC-19 antibody to real ERα (67 kDa) in the hippocampus was verified using MALDI-TOF mass-spectrometric analysis of RC-19 reacted proteins as well as the absence of reactivity of RC-19 with ERα KO mice hippocampus (Mukai et al., 2007). These analyses are essential in the hippocampus, because we found that non-purified MC-20 antisera, frequently used in previous investigations, often reacted with 62 kDa unknown proteins in the brain and did not significantly react with real ERα (67 kDa) (Mukai et al., 2007). AS409, another frequently used antisera did mainly react with unknown proteins different from real ERα (Mukai et al., 2007). Non-purified antisera may largely react with proteins having amino acid sequences similar to the real antigen in the hippocampus in which extremely low level of ERα is expressed as compared with that in the ovary. Surprisingly, ERα antisera are often examined for their reactivity only in endocrine organs such as the ovary in which ERα is highly expressed. Therefore, staining of interneurons and no staining of primary neurons with non-purified antisera (such as MC-20 or AS409) probably do not show real ERα distribution in the hippocampus. Antisera should be purified before application to the hippocampus.

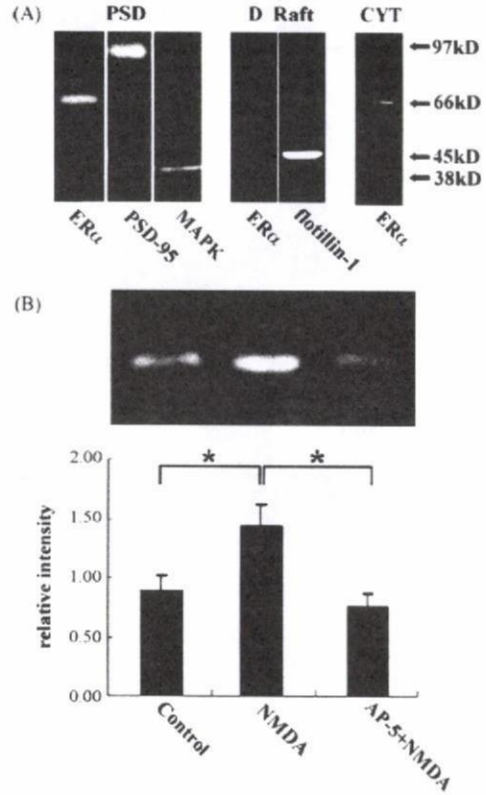

Fig. 7. Western blot analysis of ERα in male rat hippocampal neurons. (A) Blot of ERα in postsynaptic density (PSD), dendritic raft (D Raft) and cytoplasm (CYT). From left to middle, blot of PSD fractions with RC-19 IgG (ERα), PSD-95 IgG (PSD-95), and Erk MAP kinase IgG (MAPK). From middle to right, blot of DR with RC-19 (ERα) and flotillin-1 IgG (flotillin-1). At rightmost lane, blot of CYT with RC-19 (ERα). The amount of protein applied was 20 μg for each lane, except for left most PSD lane in which 60 μg protein was applied in order to improve the signal to noise ratio. (B) NMDA-induced accumulation of ERα at PSD. PSD fractions were blotted with RC-19 after stimulation for 3 min with 0 μM NMDA (Control), 30 μM NMDA (NMDA), 30 μM NMDA plus 200 μM AP-5 (AP-5 + NMDA). The relative intensity of the blots was illustrated below (*$p < 0.05$). The amount of protein applied was 20 μg for each lane. The amount of PSD-95/mg of protein in each lane was not changed by the NMDA stimulation. A 3-min stimulation with 30 μM NMDA is the condition used to induce LTD. An elongated stimulation for 30 min with 30 μM NMDA did not further increase the accumulation of ERα in PSD (modified from Mukai et al., 2007).

ERα knock-out mice may be useful to investigate the participation of ERα in modulation of synaptic plasticity. However, so far no data are available for real ERα knock-out mice. Electrophysiological investigations are performed by using knock-down mice (not knock-out mice) by Moss and co-workers (Gu and Moss, 1996; Gu et al., 1999). They have reported no essential contribution of ERα to estradiol-induced rapid enhancement of the kainate currents of CA1 neurons. They reach this conclusion due to the observation of very small difference in estradiol effect on the kainate currents between wild-type and ERα-Neo knock-down mice which have been constructed by the method of Neomycin insertion into exon 1 (the previously named exon 2) (Couse et al., 1995). It should be noted that in Neomycin-insertion ERα-Neo knock-down mice, N-terminal-modified ERα (61 kDa) is expressed (Couse et al., 1995; Kos et al., 2002; Pendaries et al., 2002). Because the N-terminal-modified ERα is demonstrated to be still active on estradiol binding and drives genomic processes (Couse et al., 1995; Kos et al., 2002; Pendaries et al., 2002), the participation of ERα in electrophysiological properties of the CA1 cannot be excluded from their

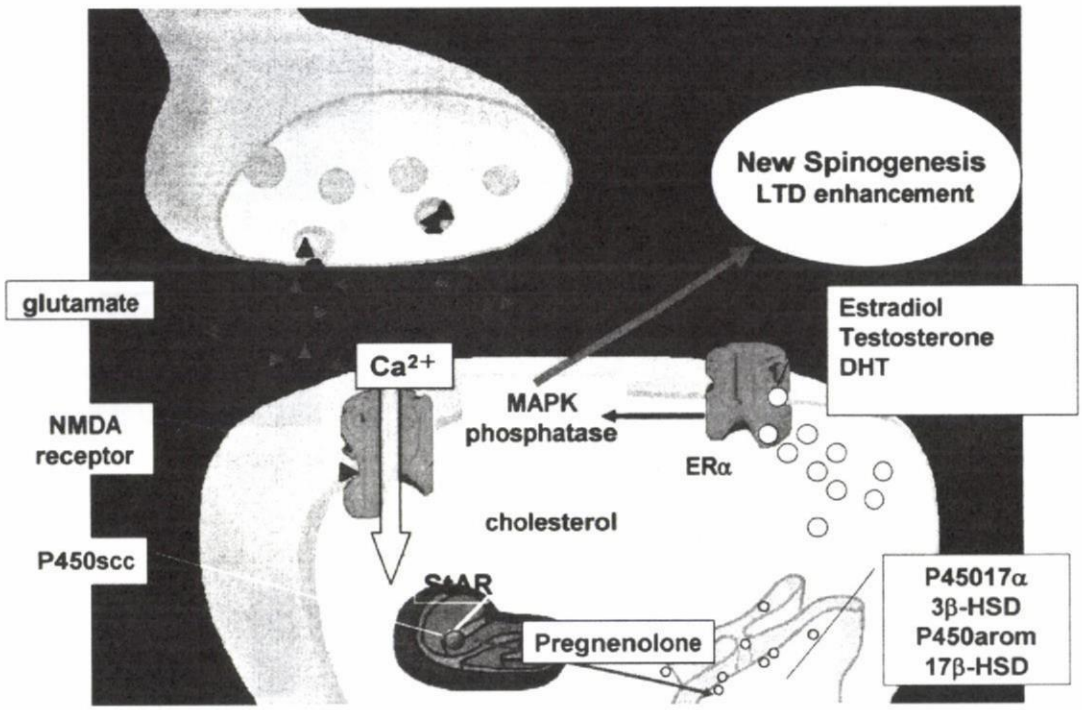

Fig. 8. Schematic illustration for the synaptic synthesis of sex hormones (synaptocrinology mechanisms), and the modulation of the synaptic plasticity of neurons by estradiol. StAR and P450scc are present in the mitochondria. P450(17α), 3β-HSD, 17β-HSD and P450arom are localized at the membranes in the synaptic compartments, in addition to endoplasmic reticulum which drive intracrinology mechanisms. The site of rapid action for estradiol is synaptic ERα. Synaptic ERβ might also function. The site of delayed action for estradiol is ERα present in cytoplasm and nuclei. Only NMDA-type glutamate receptor is illustrated, and AMPA-type glutamate receptor is omitted for clarity.

investigations. Therefore, it is necessary to investigate real ERα knock-out mice which are, for example, deleted in the whole exon 2 of the mouse ERα gene (Dupont et al., 2000). Note that nomenclature of ERα exon changes recently, and the current exon 1 and exon 2 (Kos et al., 2002; Pendaries et al., 2002) correspond to the previous exon 2 and exon 3, respectively (Dupont et al., 2000).

ERβ has been reported to associate with membranes in genetically expressed CHO cells and MCF-7 cells (Razandi et al., 1999; Pedram et al., 2006). Several investigations of immunostaining of ERβ have suggested extranuclear expression of ERβ including dendritic appearance in the hippocampal principal neurons (Milner et al., 2005). ERβ is, however, not yet identified as synaptic membrane receptors. Subcellular immunostaining patterns of these reports might reflect relatively minor expression of ERβ and major expression of unknown proteins, due to multiple reactivity of non-purified ERβ antisera to several unknown proteins in Western blot analysis of hippocampal tissues. The purity of commercially available ERβ antisera may be worse than that of ERα antisera as judged from Western blot analysis.

Recently transmembrane G-protein coupled estrogen receptor GPR30 has been identified in the plasma membrane of SKBR3 breast cancer cells that lack ERα and ERβ (Thomas et al., 2005) as well as in endoplasmic reticulum membranes of COS7 after genetic expression of GPR30 fused with green fluorescent protein (Revankar et al., 2005). Because expression of GPR30 has also been observed in the hippocampal neurons (Brailoiu et al., 2007), further investigations may reveal its contribution to rapid estradiol modulation of synaptic plasticity.

4. Synaptocrinology and intracrinology

Based on experimental observations, we illustrate in Fig. 8, a hypothetical model for the synaptic synthesis of brain steroid (synaptocrine mechanisms) and the modulation of the synaptic plasticity of neurons by brain steroid. Brain steroid synthesis proceeds in the following manner. First, glutamate release from the presynapse induces a Ca^{2+} influx through the NMDA receptors. The Ca^{2+} influx drives StAR (Kimoto et al., 2001) to transport cholesterol into the mitochondria, where P450scc converts cholesterol to PREG. The conversion of 'PREG → DHEA → androstenediol → testosterone → estradiol, or testosterone → dihydrotestosterone → androstanediol' is performed at spines in addition to endoplasmic reticulum by P450(17α), 3β-HSD, 17β-HSD, P450arom, 5α-reductase and 3α-HSD. Produced estradiol binds to synaptic ERα and drives signaling pathway including kinases (such as Erk MAP kinase) or phosphatases, finally resulting in modulation of AMPA receptors or NMDA receptors. Note that brain steroids are synthesized also in endoplasmic reticulum and mitochondria in cell body of neurons. Genomic pathway via nuclear ERα receptors also functions in delayed estradiol effects such as neuroprotection, spinogenesis, keeping homeostasis, etc. (intracrine mechanisms).

References

Baude, A., Nusser, Z., Molnar, E., McIlhinney, R.A., Somogyi, P., 1995. High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus. Neuroscience 69, 1031–1055.

Baulieu, E.E., 1997. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. Recent Prog. Horm. Res. 52, 1–32.

Baulieu, E.E., Robel, P., 1998. Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. Proc. Natl. Acad. Sci. U.S.A. 95, 4089–4091.

Beyenburg, S., Watzka, M., Blumcke, I., Schramm, J., Bidlingmaier, F., Elger, C.E., Stoffel-Wagner, B., 2000. Expression of mRNAs encoding for 17beta-hydroxisteroid dehydrogenase isozymes 1,2,3 and 4 in epileptic human hippocampus. Epilepsy Res. 41, 83–91.

Bi, R., Broutman, G., Foy, M.R., Thompson, R.F., Baudry, M., 2000. The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple

effects of estrogen in hippocampus. Proc. Natl. Acad. Sci. U.S.A. 97, 3602–3607.
Brailoiu, E., Dun, S.L., Brailoiu, G.C., Mizuo, K., Sklar, L.A., Oprea, T.I., Prossnitz, E.R., Dun, N.J., 2007. Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. J. Endocrinol. 193, 311–321.
Compagnone, N.A., Bulfone, A., Rubenstein, J.L., Mellon, S.H., 1995. Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. Endocrinology 136, 5212–5223.
Corpechot, C., Robel, P., Axelson, M., Sjovall, J., Baulieu, E.E., 1981. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc. Natl. Acad. Sci. U.S.A. 78, 4704–4707.
Couse, J.F., Curtis, S.W., Washburn, T.F., Lindzey, J., Golding, T.S., Lubahn, D.B., Smithies, O., Korach, K.S., 1995. Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. Mol. Endocrinol. 9, 1441–1454.
Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., Mark, M., 2000. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development 127, 4277–4291.
Foy, M.R., Xu, J., Xie, X., Brinton, R.D., Thompson, R.F., Berger, T.W., 1999. 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J. Neurophysiol. 81, 925–929.
Fritschy, J.M., Weinmann, O., Wenzel, A., Benke, D., 1998. Synapse-specific localization of NMDA and GABA(A) receptor subunits revealed by antigen-retrieval immunohistochemistry. J. Comp. Neurol. 390, 194–210.
Furukawa, A., Miyatake, A., Ohnishi, T., Ichikawa, Y., 1998. Steroidogenic acute regulatory protein (StAR) transcripts constitutively expressed in the adult rat central nervous system: colocalization of StAR, cytochrome P-450SCC (CYP XIA1), and 3beta-hydroxysteroid dehydrogenase in the rat brain. J. Neurochem. 71, 2231–2238.
Gould, E., Woolley, C.S., Frankfurt, M., McEwen, B.S., 1990. Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. J. Neurosci. 10, 1286–1291.
Gu, Q., Moss, R.L., 1996. 17 beta-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. J. Neurosci. 16, 3620–3629.
Gu, Q., Korach, K.S., Moss, R.L., 1999. Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. Endocrinology 140, 660–666.
Harrington, W.R., Sheng, S., Barnett, D.H., Petz, L.N., Katzenellenbogen, J.A., Katzenellenbogen, B.S., 2003. Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol. Cell. Endocrinol. 206, 13–22.
Higashi, T., Sugitani, H., Yagi, T., Shimada, K., 2003. Studies on neurosteroids. XVI. Levels of pregnenolone sulfate in rat brains determined by enzyme-linked immunosorbent assay not requiring solvolysis. Biol. Pharm. Bull. 26, 709–711.
Hojo, Y., Nakajima, K., Nakanishi, H., Higo, S., Ishii, H., Mukai, H., Morrison, J.H., Janssen, W.G., Kominami, S., Harada, N., Kimoto, T., Kawato, S., 2006. Synthesis brain steroids and localization of P450s in the hippocampal neurons. In: Proceedings of the 20th IUBMB International Congress of Biochemistry and Molecular Biology, pp. 2PA–311.
Hojo, Y., Hattori, T.A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H.T., Mukai, H., Morrison, J.H., Janssen, W.G., Kominami, S., Harada, N., Kimoto, T., Kawato, S., 2004. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. Proc. Natl. Acad. Sci. U.S.A. 101, 865–870.
Ibanez, C., Guennoun, R., Liere, P., Eychenne, B., Pianos, A., El-Etr, M., Baulieu, E.E., Schumacher, M., 2003. Developmental expression of genes involved in neurosteroidogenesis: 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase in the rat brain. Endocrinology 144, 2902–2911.
Ishii, H., Tsurugizawa, T., Ogiue-Ikeda, M., Asashima, M., Mukai, H., Murakami, G., Hojo, Y., Kimoto, T., Kawato, S., 2007. Local production of sex hormones and their modulation of hippocampal synaptic plasticity. Neuroscientist 13, 323–334.
Ito, K., Skinkle, K.L., Hicks, T.P., 1999. Age-dependent, steroid-specific effects of oestrogen on long-term potentiation in rat hippocampal slices. J. Physiol. 515 (Pt 1), 209–220.
Ivanova, T., Beyer, C., 2000. Ontogenetic expression and sex differences of aromatase and estrogen receptor-alpha/beta mRNA in the mouse hippocampus. Cell Tissue Res. 300, 231–237.
Jakab, R.L., Horvath, T.L., Leranth, C., Harada, N., Naftolin, F., 1993. Aromatase immunoreactivity in the rat brain: gonadectomy-sensitive hypothalamic neurons and an unresponsive "limbic ring" of the lateral septum-bed nucleus-amygdala complex. J. Steroid Biochem. Mol. Biol. 44, 481–498.
Jung-Testas, I., Hu, Z.Y., Baulieu, E.E., Robel, P., 1989. Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. Endocrinology 125, 2083–2091.
Kawato, S., 2004. Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint. Environ. Sci. 11, 1–14.
Kawato, S., Hojo, Y., Kimoto, T., 2002. Histological and metabolism analysis of P450 expression in the brain. Methods Enzymol. 357, 241–249.
Kawato, S., Yamada, M., Kimoto, T., 2003. Brain neurosteroids are 4th generation neuromessengers in the brain: cell biophysical analysis of steroid signal transduction. Adv. Biophys. 37, 1–48.
Kawato, S., Ogiue-Ikeda, M., Tanabe, N., Tsurugizawa, T., Hojo, Y., Mukai, H., 2007. Rapid modulation of long-term depression and spinogenesis by endocrine disrupters in adult rat hippocampus. In: Proceedings of the 4th International Meeting Steroids and Nervous System, Torino.
Kibaly, C., Patte-Mensah, C., Mensah-Nyagan, A.G., 2005. Molecular and neurochemical evidence for the biosynthesis of dehydroepiandrosterone in the adult rat spinal cord. J. Neurochem. 93, 1220–1230.
Kimoto, T., Tsurugizawa, T., Ohta, Y., Makino, J., Tamura, H., Hojo, Y., Takata, N., Kawato, S., 2001. Neurosteroid synthesis by cytochrome p450-containing systems localized in the rat brain hippocampal neurons: *N*-methyl-D-aspartate and calcium-dependent synthesis. Endocrinology 142, 3578–3589.
King, S.L., Marks, M.J., Grady, S.R., Caldarone, B.J., Koren, A.O., Mukhin, A.G., Collins, A.C., Picciotto, M.R., 2003. Conditional expression in corticothalamic efferents reveals a developmental role for nicotinic acetylcholine receptors in modulation of passive avoidance behavior. J. Neurosci. 23, 3837–3843.
Komatsuzaki, Y., Murakami, G., Tsurugizawa, T., Mukai, H., Tanabe, N., Mitsuhashi, K., Kawata, M., Kimoto, T., Ooishi, Y., Kawato, S., 2005. Rapid spinogenesis of pyramidal neurons induced by activation of glucocorticoid receptors in adult male rat hippocampus. Biochem. Biophys. Res. Commun. 335, 1002–1007.
Kos, M., Denger, S., Reid, G., Korach, K.S., Gannon, F., 2002. Down but not out? A novel protein isoform of the estrogen receptor alpha is expressed in the estrogen receptor alpha knockout mouse. J. Mol. Endocrinol. 29, 281–286.
Kretz, O., Fester, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., Prange-Kiel, J., Naumann, T., Jarry, H., Frotscher, M., Rune, G.M., 2004. Hippocampal synapses depend on hippocampal estrogen synthesis. J. Neurosci. 24, 5913–5921.
Le Goascogne, C., Robel, P., Gouezou, M., Sananes, N., Baulieu, E.E., Waterman, M., 1987. Neurosteroids: cytochrome P-450scc in rat brain. Science 237, 1212–1215.
Le Goascogne, C., Sananes, N., Gouezou, M., Takemori, S., Kominami, S., Baulieu, E.E., Robel, P., 1991. Immunoreactive cytochrome P-450(17 alpha) in rat and guinea-pig gonads, adrenal glands and brain. J. Reprod. Fertil. 93, 609–622.
Lee, H.K., Kameyama, K., Huganir, R.L., Bear, M.F., 1998. NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. Neuron 21, 1151–1162.
Leranth, C., Shanabrough, M., Horvath, T.L., 2000. Hormonal regulation of hippocampal spine synapse density involves subcortical mediation. Neuroscience 101, 349–356.
Liere, P., Akwa, Y., Weill-Engerer, S., Eychenne, B., Pianos, A., Robel, P., Sjovall, J., Schumacher, M., Baulieu, E.E., 2000. Validation of an analytical procedure to measure trace amounts of neurosteroids in brain tissue by gas chromatography–mass spectrometry. J. Chromatogr. B: Biomed. Sci. Appl. 739, 301–312.
Liere, P., Pianos, A., Eychenne, B., Cambourg, A., Liu, S., Griffiths, W., Schumacher, M., Sjovall, J., Baulieu, E.E., 2004. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain. J. Lipid Res. 45, 2287–2302.
Lisman, J., 1989. A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc. Natl. Acad. Sci. U.S.A. 86, 9574–9578.
Liu, S., Sjovall, J., Griffiths, W.J., 2003. Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography–electrospray mass spectrometry. Anal. Chem. 75, 5835–5846.
MacLusky, N.J., Luine, V.N., Hajszan, T., Leranth, C., 2005. The 17alpha and 17beta isomers of estradiol both induce rapid spine synapse formation in the CA1 hippocampal subfield of ovariectomized female rats. Endocrinology 146, 287–293.
Mellon, S.H., Deschepper, C.F., 1993. Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Res. 629, 283–292.
Mensah-Nyagan, A.G., Do-Rego, J.L., Beaujean, D., Luu-The, V., Pelletier, G., Vaudry, H., 1999. Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. Pharmacol. Rev. 51, 63–81.
Migaud, M., Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsay, M.F., Morris, R.G., Morrison, J.H., O'Dell, T.J., Grant, S.G., 1998. Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396, 433–439.
Milner, T.A., Ayoola, K., Drake, C.T., Herrick, S.P., Tabori, N.E., McEwen, B.S., Warrier, S., Alves, S.E., 2005. Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. J. Comp. Neurol. 491, 81–95.
Monaghan, D.T., Holets, V.R., Toy, D.W., Cotman, C.W., 1983. Anatomical distributions of four pharmacologically distinct ^{3}H-L-glutamate binding sites. Nature 306, 176–179.
Mukai, H., Takata, N., Ishii, H.T., Tanabe, N., Hojo, Y., Furukawa, A., Kimoto, T., Kawato, S., 2006a. Hippocampal synthesis of estrogens and androgens which are paracrine modulators of synaptic plasticity: synaptocrinology. Neuroscience 138, 757–764.
Mukai, H., Tsurugizawa, T., Ogiue-Ikeda, M., Murakami, G., Hojo, Y., Ishii, H., Kimoto, T., Kawato, S., 2006b. Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory. Neuroendocrinology 84, 255–263.
Mukai, H., Tsurugizawa, T., Murakami, G., Kominami, S., Ishii, H., Ogiue-Ikeda, M., Takata, N., Tanabe, N., Furukawa, A., Hojo, Y., Ooishi, Y., Morrison, J.H., Janssen, W.G., Rose, J.A., Chambon, P., Kato, S., Izumi, S., Yamazaki, T., Kimoto, T., Kawato, S., 2007. Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. J. Neurochem. 100, 950–967.
Murakami, G., Tsurugizawa, T., Hatanaka, Y., Komatsuzaki, Y., Tanabe, N., Mukai, H., Hojo, Y., Kominami, S., Yamazaki, T., Kimoto, T., Kawato, S., 2006a. Comparison between basal and apical dendritic spines in estrogen-induced rapid spinogenesis of CA1 principal neurons in the adult hippocampus. Biochem. Biophys. Res. Commun. 351, 553–558.

Murakami, G., Tanabe, N., Ishii, H.T., Ogiue-Ikeda, M., Tsurugizawa, T., Mukai, H., Hojo, Y., Takata, N., Furukawa, A., Kimoto, T., Kawato, S., 2006b. Role of cytochrome p450 in synaptocrinology: endogenous estrogen synthesis in the brain hippocampus. Drug Metab. Rev. 38, 353–369.

Ogiue-Ikeda, M., Tanabe, N., Mukai, H., Hojo, Y., Murakami, G., Tsurugizawa, T., Takata, N., Kimoto, T., Kawato, S., 2008. Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons. Brain Res. Rev. 57, 363–375.

Pedram, A., Razandi, M., Levin, E.R., 2006. Nature of functional estrogen receptors at the plasma membrane. Mol. Endocrinol. 20, 1996–2009.

Pendaries, C., Darblade, B., Rochaix, P., Krust, A., Chambon, P., Korach, K.S., Bayard, F., Arnal, J.F., 2002. The AF-1 activation—function of ERalpha may be dispensable to mediate the effect of estradiol on endothelial NO production in mice. Proc. Natl. Acad. Sci. U.S.A. 99, 2205–2210.

Pozzo-Miller, L.D., Inoue, T., Murphy, D.D., 1999. Estradiol increases spine density and NMDA-dependent Ca^{2+} transients in spines of CA1 pyramidal neurons from hippocampal slices. J. Neurophysiol. 81, 1404–1411.

Razandi, M., Pedram, A., Greene, G.L., Levin, E.R., 1999. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol. Endocrinol. 13, 307–319.

Reid, C.A., 2002. The role of dendritic spines: comparing the complex with the simple. Eur. J. Pharmacol. 447, 173–176.

Reid, C.A., Fabian-Fine, R., Fine, A., 2001. Postsynaptic calcium transients evoked by activation of individual hippocampal mossy fiber synapses. J. Neurosci. 21, 2206–2214.

Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., Prossnitz, E.R., 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307, 1625–1630.

Robel, P., Bourreau, E., Corpechot, C., Dang, D.C., Halberg, F., Clarke, C., Haug, M., Schlegel, M.L., Synguelakis, M., Vourch, C., et al., 1987. Neuro-steroids: 3 beta-hydroxy-delta 5-derivatives in rat and monkey brain. J. Steroid Biochem. 27, 649–655.

Sanne, J.L., Krueger, K.E., 1995. Expression of cytochrome P450 side-chain cleavage enzyme and 3 beta-hydroxysteroid dehydrogenase in the rat central nervous system: a study by polymerase chain reaction and in situ hybridization. J. Neurochem. 65, 528–536.

Sato, S., Osanai, H., Monma, T., Harada, T., Hirano, A., Saito, M., Kawato, S., 2004. Acute effect of corticosterone on *N*-methyl-D-aspartate receptor-mediated Ca^{2+} elevation in mouse hippocampal slices. Biochem. Biophys. Res. Commun. 321, 510–513.

Shibuya, K., Takata, N., Hojo, Y., Furukawa, A., Yasumatsu, N., Kimoto, T., Enami, T., Suzuki, K., Tanabe, N., Ishii, H., Mukai, H., Takahashi, T., Hattori, T.A., Kawato, S., 2003. Hippocampal cytochrome P450s synthesize brain neurosteroids which are paracrine neuromodulators of synaptic signal transduction. Biochim. Biophys. Acta 1619, 301–316.

Shinzawa, K., Ishibashi, S., Murakoshi, M., Watanabe, K., Kominami, S., Kawahara, A., Takemori, S., 1988. Relationship between zonal distribution of microsomal cytochrome P-450s (P-450(17)alpha,lyase and P-450C21) and steroidogenic activities in guinea-pig adrenal cortex. J. Endocrinol. 119, 191–200.

Teyler, T.J., Vardaris, R.M., Lewis, D., Rawitch, A.B., 1980. Gonadal steroids: effects on excitability of hippocampal pyramidal cells. Science 209, 1017–1018.

Thomas, P., Pang, Y., Filardo, E.J., Dong, J., 2005. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146, 624–632.

Tsurugizawa, T., Mukai, H., Tanabe, N., Murakami, G., Hojo, Y., Kominami, S., Mitsuhashi, K., Komatsuzaki, Y., Morrison, J.H., Janssen, W.G., Kimoto, T., Kawato, S., 2005. Estrogen induces rapid decrease in dendritic thorns of CA3 pyramidal neurons in adult male rat hippocampus. Biochem. Biophys. Res. Commun. 337, 1345–1352.

Vallee, M., Mayo, W., Darnaudery, M., Corpechot, C., Young, J., Koehl, M., Le Moal, M., Baulieu, E.E., Robel, P., Simon, H., 1997. Neurosteroids: deficient cognitive performance in aged rats depends on low pregnenolone sulfate levels in the hippocampus. Proc. Natl. Acad. Sci. U.S.A. 94, 14865–14870.

Wang, M.D., Wahlstrom, G., Backstrom, T., 1997. The regional brain distribution of the neurosteroids pregnenolone and pregnenolone sulfate following intravenous infusion. J. Steroid Biochem. Mol. Biol. 62, 299–306.

Warner, M., Gustafsson, J.A., 1995. Cytochrome P450 in the brain: neuroendocrine functions. Front. Neuroendocrinol. 16, 224–236.

Wehrenberg, U., Prange-Kiel, J., Rune, G.M., 2001. Steroidogenic factor-1 expression in marmoset and rat hippocampus: co-localization with StAR and aromatase. J. Neurochem. 76, 1879–1886.

Woolley, C.S., 1998. Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. Horm. Behav. 34, 140–148.

Woolley, C.S., McEwen, B.S., 1992. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J. Neurosci. 12, 2549–2554.

Woolley, C.S., McEwen, B.S., 1994. Estradiol regulates hippocampal dendritic spine density via an *N*-methyl-D-aspartate receptor-dependent mechanism. J. Neurosci. 14, 7680–7687.

Woolley, C.S., Gould, E., McEwen, B.S., 1990. Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res. 531, 225–231.

Wu, F.S., Gibbs, T.T., Farb, D.H., 1991. Pregnenolone sulfate: a positive allosteric modulator at the *N*-methyl-D-aspartate receptor. Mol. Pharmacol. 40, 333–336.

Yang, S.N., Tang, Y.G., Zucker, R.S., 1999. Selective induction of LTP and LTD by postsynaptic $[Ca^{2+}]i$ elevation. J. Neurophysiol. 81, 781–787.

Zwain, I.H., Yen, S.S., 1999a. Dehydroepiandrosterone: biosynthesis and metabolism in the brain. Endocrinology 140, 880–887.

Zwain, I.H., Yen, S.S., 1999b. Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. Endocrinology 140, 3843–3852.

Why Public Health Agencies Cannot Depend on Good Laboratory Practices as a Criterion for Selecting Data: The Case of Bisphenol A

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Background: In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (FDA) and a counterpart in Europe, the European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by Good Laboratory Practices (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world.

Objectives: We reviewed differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. We examined the methods and results in the GLP studies that were pivotal in the draft decision of the U.S. FDA declaring BPA safe in relation to findings from studies that were competitive for U.S. National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes.

Discussion: Although the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the U.S. NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodologic flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that GLP yields valid and reliable scientific findings (i.e., "good science"). Their rationale for favoring GLP studies over hundreds of publically funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely, independent replication, and use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research.

Conclusions: Public health decisions should be based on studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

Key words: bisphenol A, endocrine disruptors, FDA, Food and Drug Administration, GLP, good laboratory practices, low-dose, nonmonotonic, positive control. *Environ Health Perspect* 117:309–315 (2009). doi:10.1289/ehp.0800173 available via *http://dx.doi.org/* [Online 22 October 2008]

Regulatory agencies in the United States and the European Union (EU) have justified the decision to declare the estrogenic chemical bisphenol A (BPA) safe at current levels of human exposure based on a few studies conducted using Good Laboratory Practices (GLP). In contrast, these agencies have rejected for consideration in their risk assessment of BPA hundreds of laboratory animal and mechanistic cell culture studies conducted by academic and government scientists reporting harm at very low doses of BPA. These studies were rejected primarily because they were not conducted using GLP. We suggest that decisions based on this logic are misguided and will result in continued risk to public health from exposure to BPA, as well as other manmade chemicals.

GLP is a federal rule for conducting research on the health effects or safety testing of drugs or chemicals submitted by private research companies for regulatory purposes. The GLP outlines basic guidelines for conducting scientific research, including the care and feeding of laboratory animals, standards for facility maintenance, calibration and care of equipment, personnel requirements, inspections, study protocols, and collection and storage of raw data (Goldman 1988). These regulations were developed in response to widespread misconduct by private research companies; this misconduct was possible because their data usually do not go through the rigorous, multistage scientific review that is normal for academic data funded by federal agencies and published in the peer-reviewed literature. The lack of these safeguards from academic science had enabled fraud. The U.S. Food and Drug

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Administration (U.S. FDA) first issued rules for GLP in 1978 after a 2-year federal investigation into sloppy laboratory practices of a number of private research companies (Lublin 1978; Markowitz and Rosner 2002). What began as serious concerns about poor quality research expanded into a criminal investigation of Industrial Bio-Test (IBT), one of the largest private laboratories at the time and a subsidiary of Nalco Chemical Company. In response to the federal investigation, the U.S. Environmental Protection Agency (EPA) demanded that 235 chemical companies re-examine the > 4,000 tests conducted by the laboratory. In 1983, three men from IBT were found guilty of deliberating doctoring data and were sentenced to prison (Lublin 1978; Markowitz and Rosner 2002). The fraudulent practices of IBT brought into question 15% of the pesticides approved for use in the United States. That same year, the U.S. EPA issued similar GLP rules for regulatory testing.

Both the U.S. FDA (2008a) and European Food Safety Authority (ESFA 2006) have recently published documents demonstrating that their decision to continue to declare BPA safe at current exposure levels was based primarily on the results of a few industry-funded studies that followed GLP guidelines. These decisions stand in stark contrast to the decisions concerning the potential risks to human health reached by a panel of 38 experts at a U.S. National Institutes of Health (NIH)-sponsored conference, who published The Chapel Hill Consensus Statement (vom Saal et al. 2007), as well as five review articles (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007a; Vandenberg et al. 2007a; Wetherill et al. 2007). These peer-reviewed articles covered approximately 700 articles concerning BPA and represented a comprehensive review of the literature as of the end of 2006. In addition, the U.S. FDA draft decision contradicted the conclusions reached by the National Toxicology Program (NTP), which had spent 2 years investigating this question (NTP 2008). An important role of the NTP is to advise the U.S. FDA about the science relating to toxic chemicals in food, but in an unusual move, the U.S. FDA chose to release its draft report before the release of the final report on BPA by the NTP and without indicating who at the U.S. FDA was involved in preparing the draft report (U.S. FDA 2008b). At a hearing on 16 September 2008 regarding the draft report on BPA, the U.S. FDA announced that their goal was to have a subcommittee of the U.S. FDA Science Board complete a review of the draft decision by the end of October 2008. This would presumably also involve review by the subcommittee members of the approximately 1,000 articles relating to BPA.

We believe that the methods employed in chemical industry–sponsored GLP studies are incapable of detecting low-dose endocrine-disrupting effects of BPA and other hormonally active chemicals. Detecting endocrine-disrupting effects at low doses of chemicals such as BPA requires sophisticated and modern assays and analyses that have been developed in advanced, usually federally funded laboratories over the past decade. This is especially apparent when one examines what is now known about functional effects of BPA on a wide range of end points (Richter et al. 2007a; Welshons et al. 2006; Wetherill et al. 2007). These end points include those mediated by recently discovered estrogen response pathways initiated in human and animal cell membranes (nonclassical or alternative estrogen response mechanisms), which multiple laboratories have shown to be equally sensitive to BPA and estradiol in terms of activating effects in human and animal cells at low picomolar through low nanomolar concentrations (Alonso-Magdalena et al. 2008; Wetherill et al. 2007; Wozniak et al. 2005; Zsarnovszky et al. 2005).

The effects of BPA documented in these studies include a diverse array for which there are no data from GLP studies because the end points have not been examined: altered metabolism related to metabolic syndrome (Alonso-Magdalena et al. 2005, 2006, 2008; Ropero et al. 2008); altered adiponectin secretion (Hugo et al. 2008), which is a condition predicting heart disease and type 2 diabetes (Lang et al. 2008); altered epigenetic programming leading to precancerous lesions of the prostate (Ho et al. 2006); differential growth patterns in the developing prostate (Timms et al. 2005); abnormal growth, gene expression, and precancerous lesions of the mammary glands (Soto et al. 2008); and adverse effects on the female reproductive system, including uterine fibroids, paraovarian cysts, and chromosomal abnormalities in oocytes (Newbold et al. 2007; Susiarjo et al. 2007). There is also a large literature on neuroanatomic, neurochemical, and behavioral abnormalities caused by low doses of BPA (Leranth et al. 2008; Richter et al. 2007a), which also are not capable of being detected by current GLP studies conducted for regulatory purposes because of their out-of-date assays.

The approaches used by academic and government scientists to study the potential health hazards of BPA contrast sharply with those still used by the chemical industry that are relied on by regulatory agencies in the United States and Europe, including the two studies identified by both the U.S. FDA and European Food Safety Authority (EFSA) as central to the decision to declare BPA safe at current human exposure levels (Tyl et al. 2002, 2008a). By using outdated and insensitive assays that were supposed to have been replaced by a new battery of screens and tests by 2000 [as mandated by the U.S. Congress in 1996 in the Food Quality Protection Act (1996), but which has, as yet, still not occurred], these studies conducted using GLP fail to find any adverse effects.

Reliability and Validity

Reliability and validity are separate issues, although in the experimental research described here, validity and reliability basically refer to research that is credible. Golafshani (2003) noted that "reliability" refers to the extent to which results are consistent over time and are an accurate representation of the total population under study. Of central importance is that the results of a study must be reproduced under a similar methodology to be considered to be reliable. "Validity" refers to whether the research measures what it was intended to measure, and valid findings are considered to be true. In other words, reliability is determined by whether the results are replicable, whereas validity is assessed by whether the methods used result in finding the truth as a result of the investigator actually measuring what the study intended to measure.

Use of GLP in Regulatory Decision Making

Despite strong evidence of aberrations caused by low doses of BPA in animals exposed during fetal and neonatal life in studies conducted by the world's leading academic and government experts in the fields of endocrine disruption, endocrinology, neurobiology, reproductive biology, genetics, and metabolism, a relatively small number of studies reporting no adverse effects at low doses of BPA have continued to be promoted by the chemical industry and used by regulatory agencies (e.g., Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2002, 2008a). According to the U.S. FDA, these are accepted because they used GLP (U.S. EPA 2008), with the implication that studies not employing GLP are not reliable or valid (U.S. FDA 2008a).

GLP does not guarantee reliability or validity of scientific results. Unfortunately, although GLP creates the semblance of reliable and valid science, it actually offers no such guarantee. GLP specifies nothing about the quality of the research design, the skills of the technicians, the sensitivity of the assays, or whether the methods employed are current or out-of-date. (All of the above are central issues in the review of a grant proposal by an NIH panel.) GLP simply indicates that the laboratory technicians/scientists performing experiments follow highly detailed U.S. EPA requirements [or in the EU, Organization for Economic Co-operation and Development (OECD) requirements] for record keeping, including details of the conduct of the

experiment and archiving relevant biological and chemical materials (U.S. EPA 2008).

These record-keeping procedures in GLP were instituted because of widespread misconduct being committed by commercial testing laboratories (described above). These fraudulent results were possible because contract laboratory studies used in the regulatory process are rarely subject to the checks and balances that peer-reviewed, replicated scientific findings undergo. Without that acid test of reliability (replication by other independent scientists), other procedures were needed. Hence GLP was implemented, despite its severe limitations.

NIH-funded research subject to more stringent reviews than GLP. Although few NIH-funded investigators adhere to GLP-mandated record keeping, the procedures of GLP are actually surpassed by the procedures required for NIH-funded science published in peer-reviewed journals. NIH-funded studies pass through three phases of peer review that are far more challenging than GLP requirements. First, the principal scientists must have demonstrated competence to conduct the research, and experimental methods, assays, and laboratory environment must involve use of state-of-the-art techniques to be competitive for NIH funding. Second, results are published in peer-reviewed journals, with detailed evaluations by independent experts examining all aspects of the study. And third, the findings are challenged by independent efforts to replicate; for example, the initial findings concerning the stimulating effects of estrogenic chemicals on the mouse prostate (Nagel et al. 1997; vom Saal et al. 1997) were independently replicated and extended by Gupta (2000), which led to an editorial identifying "initial results confirmed" (Sheehan 2000).

Typically, within a laboratory, interesting findings are also followed by subsequent publications extending the prior findings; examples include the findings of BPA effects on β cells in the mouse pancreas (Alonso-Magdalena et al. 2005, 2006, 2008) and the effects of estrogenic chemicals and drugs on the developing mouse prostate that followed earlier findings (described above) from this same group (Timms et al. 2005; Richter et al. 2007b). In particular, independent replication by competent, respected scientists is the main criterion of acceptance of the findings as having been demonstrated to be reliable and having been validated by virtue of coming to the same conclusion using a variety of sophisticated techniques in multiple publications.

An important criticism of the approach taken by the U.S. FDA in its assessment of the now approximately 1,000 articles on BPA is that it appears to have made no attempt to connect the dots between replicated studies; instead, the U.S. FDA appears to have assessed each study without regard to whether it had been confirmed by other studies.

Thus, collectively, many phases used to verify the reliability and validity of NIH-funded published research have been completely ignored by the U.S. FDA, whereas industry-funded GLP research is rarely, if ever, subject to these central requirements and yet is accepted by regulatory agencies as reliable and valid.

The U.S. FDA's misguided gold standard. In this light, the U.S. FDA's reliance upon GLP as the gold standard is scientifically misguided. Furthermore, U.S. FDA administrators are ignoring published critiques of the GLP studies it considers reliable and valid, such as the study by Tyl et al. (2002) and two coordinated studies conducted at the same time by Ashby et al. (1999) and Cagen et al. (1999). Each was an industry-funded study conducted using GLP. Each was harshly criticized in peer-reviewed publications by academic scientists and government panels [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2007; NTP 2001; vom Saal and Hughes 2005; vom Saal and Welshons 2006]. Yet, the U.S. FDA and EFSA panels still assert that these studies represent the gold standard in toxicologic research.

Specifically, the studies of Cagen et al. (1999) and Ashby et al. (1999) were recently rejected by the NTP CERHR panel on BPA as unusable for consideration in its evaluation of the health hazards posed by BPA (CERHR 2007). Both the Ashby et al. (1999) and Cagen et al. (1999) studies reported finding no effect of their positive control [the estrogenic drug diethylstilbestrol (DES)] on any outcome, although these failures were not acknowledged by the authors in either article. In experimental science, the failure of a positive control to show an effect indicates the experiment failed, which is the conclusion reached by the CERHR panel (CERHR 2007).

The Tyl et al. 2002 study, which the U.S. FDA still accepts as a major study for determination of the safety of BPA (U.S. FDA 2008a, 2008b), was criticized by an NTP panel that met in 2000 to examine the low-dose issue (NTP 2001), as well as in subsequent publications (vom Saal and Hughes 2005; vom Saal and Welshons 2006), for using an insensitive rat (the CD-SD rat) that requires extremely high doses (≥ 50 μg/kg/day) of the potent estrogenic drug ethinylestradiol to show effects such as those examined in the study by Tyl et al. (2002). This dose of ethinylestradiol is > 100 times higher than the approximately 0.3 μg/kg/day used by women in oral contraceptives. The fact that Tyl et al. (2002) adhered to GLP did not protect them from using insensitive animals. This led the NTP (2001) to state:

> Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine-active agents of concern (i.e., responsive to positive controls), not on convenience and familiarity.

Thus, when reviewed by other scientists, three prior major GLP studies of BPA have been found to be so flawed as to be useless for guiding regulatory agencies in decision making. A new GLP study has now been published by Tyl et al. (2008a). Close examination of this study also reveals fatal flaws which render it useless for regulatory purposes, even though it conforms to GLP.

Examples of Flaws Ignored by the U.S. FDA and EFSA in a Recent GLP Study of BPA

In summary, the flaws in Tyl et al. (2008a) are as follows:

- The high dose required for the positive control (estradiol) to cause an effect means the system used by Tyl et al. (2008a), at least in her laboratory, is relatively insensitive to exogenous estrogens and thus inappropriate for studying low-dose effects of estrogenic compounds such as BPA. The lack of response to low doses of estradiol or BPA in the Tyl laboratory is puzzling, in that the strain of mice used in these experiments (the CD-1 mouse) has been reported in > 20 other peer-reviewed publications to show adverse effects in response to very low doses of BPA (vom Saal 2008), as well as many other studies showing low-dose effects in response to the natural hormone estradiol, the estrogenic drugs ethinylestradiol and DES, and to other estrogenic chemicals.
- Tyl et al. (2008a) used insensitive, out-of-date protocols and assays that are incapable of finding many of the adverse effects reported by more sophisticated studies conducted by independent NIH-funded scientists as well as scientists funded by government agencies in other countries.
- In the specific case of testing for changes in prostate weight, Tyl et al. (2008a) reported an abnormally high prostate weight for control animals that exceeds by > 70% the prostate weights reported by other studies for animals of the same strain and similar age (e.g., Gupta 2000; Ruhlen et al. 2008). This suggests that the dissection procedures for the prostate in the Tyl laboratory included other nonprostatic tissues in the weight measurements, rendering them unusable for studying weight changes in the prostate in response to BPA or estradiol; neither chemical showed any effect on the selected end points, which directly contradicts other findings concerning opposite effects of low and high doses of estrogen on the prostate (Putz et al. 2001; Timms et al. 2005; vom Saal et al. 1997).

Aberrant insensitivity of CD-1 mouse to estrogens. Tyl et al. (2008a) used estradiol as a positive control. It was fed to female mice before and during pregnancy and lactation at 80–220 μg/kg/day; after weaning, estradiol was fed to offspring at doses of 80–100 μg/kg/day. Estradiol was used as a positive control because BPA is a man-made endocrine-disrupting estrogenic chemical.

Many published findings reporting effects of very low doses of positive control estrogens and BPA in CD-1 mice demonstrate that the CD-1 mouse was somehow rendered insensitive in the test system used by Tyl et al. (2008a). The fact that a dose of 100–200 μg/kg/day estradiol was necessary to show an effect of the positive control predicts that Tyl et al. (2008a) should not detect effects of BPA < 10–100 mg/kg/day, far above the low-dose range relevant to human exposures that was supposedly of interest.

For nuclear estrogen receptor–mediated effects via regulation of gene activity (nuclear estrogen receptors are transcription factors whose activity is regulated by binding to estrogen), prior studies have typically shown a 1,000-fold lower activity for BPA relative to estradiol or potent estrogenic drugs, including DES and ethinylestradiol. For example, Richter et al. (2007b) reported an increase in androgen receptor gene activity to estradiol at 1 pM (0.28 pg/mL) in fetal CD-1 mouse prostatic mesenchyme cells in primary culture, and the same response was found for BPA at 1,000 pM (228 pg/mL); the *in vitro* response to estradiol was predicted by the response of the prostate to increasing free serum estradiol from 0.2 to 0.3 pg/mL in male mouse fetuses via estradiol administration to the mother (vom Saal et al. 1997). Other research showed that a significant effect on development of the male reproductive system in CF-1 mice occurred at a maternal dose of 0.002 μg/kg/day ethinylestradiol (Thayer et al. 2001), similar to effects observed with 2–20 μg/kg/day BPA (vom Saal et al. 1998). The research of Honma et al. (2002) showed accelerated puberty in CD-1 (ICR) mice at a DES dose of 0.02 μg/kg/day (the positive control), and the same response to BPA occurred at 20 μg/kg/day, again revealing a 1,000-fold difference between the positive control estrogen and BPA.

There are many other examples of findings where a higher dose of BPA was required to cause the same effect as the positive control estrogen (estradiol, ethinylestradiol, or DES) in studies where the effects were mediated by the classical nuclear estrogen receptors, in contrast to the more recently discovered rapid signaling estrogen response system where BPA and these positive control estrogens have equal potency, as described above. In summary, CD-1 mice have been used by a large number of academic and government investigators and have been reported in peer-reviewed publications to be sensitive to positive control estrogens within the range of human sensitivity based on *in vivo* and *in vitro* studies via the classical estrogen receptor α–mediated response mechanism. The CD-1 mouse is the animal model that has been used by the U.S. National Institute of Environmental Health Sciences (NIEHS) for decades, because it is considered the best animal model for predicting the effects of developmental exposure to estrogen in humans (Newbold 1995; Newbold et al. 2007).

The failure of traditional toxicologic studies conducted by Tyl et al. (2008a, 2008b) to detect the wide range of adverse effects of even relatively high doses of BPA or of low doses of estradiol that have been reported in numerous studies by academic and government scientists provides evidence that the GLP protocols established long ago by regulatory agencies to determine the toxicity of chemicals are inappropriate for detecting the endocrine-disrupting activities of chemicals such as BPA. Indeed, this was the premise of the congressional mandate in the Food Quality Protection Act (1996) for the U.S. EPA to establish a new set of assays for endocrine-disrupting chemicals, although this process has been systematically delayed and is > 8 years behind the congressionally mandated date of 2000 to have these new assays validated.

Citing Tyl et al. (2008a), the EFSA report on BPA (EFSA 2006) stated that "the positive control substance, 17β-estradiol, resulted in reproductive and developmental toxicity." This report failed to acknowledge that only a very high dose of the positive control was sufficient to elicit effects and that this meant that the experiments conducted in the Tyl laboratory were for some reason very insensitive to any estrogen and thus inappropriate for use in a study to examine low-dose estrogenic effects of BPA.

Based on the preliminary report released by the U.S. FDA regarding BPA (U.S. FDA 2008a), it appears that the U.S. FDA has followed the lead of the EFSA in its lack of understanding of the importance of the dose of the positive control estrogen required to cause adverse effects. The consequence is that the U.S. FDA has relied primarily on the study of Tyl et al. (2008a, 2008b), with the result that the U.S. FDA has assured Americans that BPA is safe at current human exposure levels.

Several factors might account for the insensitivity of the CD-1 mouse in the Tyl et al. studies (2008a, 2008b) conducted at Research Triangle Institute (RTI), a testing facility that conducted these (as well as previous) studies funded by the American Chemistry Council. One possibility is that the diet used in these studies may have interfered with the results. The feed used by Tyl et al. (2008a) in this experiment (Purina 5002) has been shown by others to interfere with responses to exogenous estrogenic chemicals, blocking adverse effects documented on other diets. For example, a number of years ago, Thigpen et al. (2003) at the NIEHS recommended against the use of Purina 5002 in studies of endocrine-disrupting chemicals. Tyl et al. (2008a) measured some specific phytoestrogens in Purina 5002 feed by chemical analysis; however, in a report on NIH-sponsored meetings on this subject, Heindel and vom Saal (2008) pointed out that this is an insufficient control for total dietary estrogenic contaminants that can disrupt studies involving the effects of estrogenic chemicals.

A second possibility is that there are strain differences in sensitivity developed in the CD-1 mouse sold by the various Charles River Laboratories located in different regions. We consider this unlikely, because most laboratories regularly replace their CD-1 mouse breeder stock from Charles River Laboratories, and practices there make it unlikely that the sensitivity of this outbred stock to estrogens has changed dramatically over a very short period of time. Also, because RTI, where the Tyl studies were conducted, is very near the laboratories of the NIEHS, it is likely that the CD-1 mice used by these two programs were purchased from the same breeding facility.

Use of insensitive, out-of-date protocols and assays. Another serious concern about the two recent studies by Tyl et al. (2008a, 2008b) is the experimental approach used, thus raising questions about the validity of the studies. The study design used by Tyl et al. (2008a, 2008b) has been superseded by advances in both experimental design and analytical tools developed by NIH-funded scientists (and their counterparts in Europe and Asia) since the mid-1990s. The methods used by Tyl et al., primarily wet weight changes of tissues, gross histologic changes, and developmental landmarks such as vaginal opening, were established procedures by the 1950s. Thus, a major limitation of the Tyl studies is the failure to measure more meaningful and sensitive end points in order to detect the effects of low-dose BPA exposure, which are often not macroscopic in nature. Indeed, in 2001, the director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA stated that the inconclusive results concerning effects of BPA on reproductive toxicology can only be solved by understanding the mechanisms (Triendl 2001). With current GLP standards it is not possible to study mechanisms because they still rely on out-of-date assays.

As one example of a comparison between the approach by Tyl et al. (2008a) and independent government-funded academic scientists, extensive research has been conducted by Soto et al. (2008) and by other independent academic and government scientists