mediated Ca^{2+} signaling at 37 °C in cultured hippocampal neurons isolated from 3-day-old rats. Following a 20-min preincubation of neurons with 0.5–50 μM CORT in the absence of extracellular Mg^{2+}, the application of 100 μM NMDA induced an extremely prolonged Ca^{2+} elevation which was maintained over the experimental time range of 5–20 min. This prolonged $[Ca^{2+}]_i$ elevation was terminated either by the blocking of NMDA receptors with MK801 or by washout of CORT. The rapid effect of CORT was also investigated in hippocampal slices from adult male rats aged 3 months. NMDA stimulation at 300 μM induced a transient elevation in $[Ca^{2+}]_i$ which consisted of a rapid rise (which occurred within 10 s) followed by a slow decay to a plateau (approximately 60–70% of the maximal $[Ca^{2+}]_i$ rise), which was maintained within the experimental time range of 5 min. A 10-μM CORT perfusion for 20 min suppressed this NMDA-induced transient $[Ca^{2+}]_i$ elevation by enhancing the slow decay phase to 40–50% of the maximal $[Ca^{2+}]_i$ rise, although the initial rapid rise phase was unaffected (M. Harada and S. Kawato, unpublished results). These results suggested that the effects induced by CORT on the $[Ca^{2+}]_i$ signaling in cultured neurons from pups differ from those in neurons of slices from adult rats.

4.2. Electrophysiological investigations of the long-term potentiation

In the electrophysiological field potential measurements of the hippocampal slices, the long-term potentiation (LTP) of CA1 pyramidal neurons is observed as an approximate 1.5- to 1.6-fold increase in the initial slope of the excitatory postsynaptic potential (EPSP), which is attendant upon the high-frequency tetanic stimulation of Schaffer collaterals with 100 Hz for 1 s. The following experiments were performed using the hippocampal slices from young male Wistar rats, aged 4 weeks in the presence of a high concentration of Mg^{2+} (1 mM) at 30 °C.

4.2.1. Effect of PREGS

A 20-min preperfusion of hippocampal slices with 500 nM PREGS potentiated the induction of LTP, as judged from a significant increase in the EPSP slope (approximately 1.95-fold) (Fig. 7). Interestingly, even without tetanic stimulation in the presence of 1 mM Mg^{2+}, a 100-μM PREGS perfusion induced an immediate increase in both the slope and the peak magnitude of EPSP [the EPSP slope attained a peak of approximately 1.4 times the basal level after 20 min (N. Takata and S. Kawato, unpublished results)]. The LTP induction by PREGS is probably due to PREGS's reported ability to potentiate NMDA receptor-mediated Ca^{2+} currents [17,45]. Because PREGS has been shown to suppress the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type of glutamate receptors [16], the enhancement of LTP induction implies that the potentiation of NMDA receptors overcomes the suppression of AMPA receptors in synaptic signal transmission.

4.2.2. Effect of estradiol

The preperfusion of hippocampal slices with 0.0, 0.1, 1.0, 10 and 50 nM estradiol for 20 min reduced, in a dose-dependent fashion, the induction of LTP as indicated by an approximate 1.51-, 1.42-, 1.29-, 1.26- and 1.22-fold enhancement of the EPSP slope, respectively (see Fig. 7) (N. Yasumatsu and S. Kawato, unpublished results). This rapid suppressive effect by physiological concentrations of estradiol in hippocampal slices from 4-week-old rats is essentially the same as those described by Ito et al. [21].

4.2.3. Effect of CORT

A 20-min preperfusion of hippocampal slices with 10 μM CORT resulted in a significant suppression of the LTP induction, as observed by a reduction in the increase in the EPSP slope attendant upon a 100-Hz tetanic stimulation [i.e., from 1.51 ± 0.10 (control without CORT) to only 1.18 ± 0.04-fold (see Fig. 7)] (N. Yasumatsu and S. Kawato, unpublished results). These results imply that a high concentration of CORT (possibly secreted during stress) may acutely inhibit the synaptic signal transduction.

5. Possible pathway of steroidogenesis in the hippocampus

Taken in combination with previous reports, our results indicate that brain neurosteroid synthesis in the hippocampal neurons is likely to be catalyzed by the biotransformation of cholesterol to various steroids by the cytochrome P450-containing monooxygenase systems. This process is illustrated hypothetically in Fig. 1. The proposed process of neurosteroid synthesis is as follows. First, cholesterol is transported to the inner membrane of mitochondria along with StAR. In the mitochondria, cytochrome P450scc catalyzes the side-chain cleavage of cholesterol, resulting in the formation of PREG. PREG then reaches the microsomes (endoplasmic reticulum), where cytochrome P45017α catalyzes the conversion of PREG to DHEA. Following the transformation of DHEA to androstenedione by 3β-HSD, cytochrome P450arom catalyzes the conversion of androstenedione to testosterone. This is followed by a further transformation to 17β-estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD) (type 3). It appears likely that 17β-estradiol may also be formed by 17β-HSD (type 3) from estrone, which is converted from androstenedione by P450arom. Hydroxysteroid sulfotransferase converts PREG and DHEA to their sulfate forms, PREGS and DHEAS. Our study demonstrated the neuron-specific localization of P450scc, P45017α, P450arom and sulfotransferase in the hippocampus. The presence of mRNAs for 17β-HSD type 1 and type 3 has also been demonstrated in the human and rat hippocampus without a specification of cell type [48]. 17β-HSD type 1 and 17β-HSD type 3 catalyze the conversion of estrone to estradiol, and the conversion of androstenedione to testosterone [49,50]. It is possible that CORT is also a

member of brain neurosteroid synthesized in the hippocampus as illustrated in Fig. 1. In the microsomes, PREG is metabolized to progesterone by 3β-HSD [51]. Cytochrome P450c21 (CYP21) converts progesterone to deoxycorticosterone and deoxycortisol, which then reach the mitochondria, where P45011β (CYP11B) converts them to CORT and cortisol. Evidence supporting the synthesis of CORT from PREG is still fragmentary. Thus far, only the conversion of [^{3}H]-CORT from [^{3}H]-deoxycorticosterone has been reported [52]. Although the cell-specific localization of P450c21 and P45011β has not been demonstrated, the presence of the mRNAs for these P450s has been reported in the rat and human hippocampus [52–55].

It is important to consider whether the local concentration of brain neurosteroids is sufficiently high to allow action as local mediators. Dimensional conversion from picomoles per milligram protein to molars (moles per liter) was performed by considering that 10 mg wet weight of the hippocampal tissue contained 0.96 mg of protein. The concentration of PREGS detected in the hippocampus is then about 28 and 57 nM before and after the NMDA stimulation, respectively. The local concentration of PREGS in the pyramidal neurons is likely to be 10- to 20-fold higher than the bulk concentration of 57 nM, due to the relatively small volume of the P450-immunoreactive cells in the total hippocampus. These considerations suggest that the local concentration of PREGS could be as high as 0.6–1.2 μM. NMDA stimulation also increased the concentration of 17β-estradiol from 0.6 (basal) to 1.3 nM, which is estimated to correspond to a 13- to 26-nM local concentration within neurons. These levels are sufficient to allow PREGS and estradiol to act as local mediators that modulate LTP and NMDA receptors (this work, Refs. [20,21,56]). The concentration of PREGS and estradiol may change in a time-dependent fashion, due to further conversion to other steroids.

The physiological mode of action of brain neurosteroids could be as local mediators for brain neurons. However, the solid demonstration of local synthesis and action for 'neurosteroid' has been performed primarily in the peripheral glial cells and nerves. Sex steroids and DHEA had not been recognized as brain neurosteroids, because their endogenous synthesis had been poorly demonstrated in the brain especially in adult mammals. Only PREG(S), pregnenolone, allopregnenolone and allotetrahydrocorticosterone have been considered to be 'true' endogenously synthesized brain neurosteroids. Recently, progesterone has demonstrated to be another brain neurosteroid synthesized in the cerebellum [57]. Progesterone-induced dendrite growth of Purkinje neurons reported in the cerebellum is also indicative of the neurotrophic action of progesterone in the brain [58].

As reported in a number of studies over past decades, the absence of P45017α and its activity in the brain of adult rats has discouraged the investigation of the endogenous synthesis of sex steroids and DHEA [5–7]. Incubations of [^{3}H]-PREG(S) with brain slices, homogenates and microsomes, primary cultures of mixed glial cells, or astrocytes and neurons from rat and mouse embryos, had never produced a radioactive metabolite with the chromatographic behavior characteristic of [^{3}H]-DHEA [59]. In neonatal stage, however, the expression of mRNA for P45017α as well as an associated DHEA synthesis activity has recently been demonstrated in cultured cortical astrocytes and neurons [60,61]. Many attempts to demonstrate the immunohistochemical reactivity for P45017α in the adult rat brain had been unsuccessful [5]. The inability to detect mRNA for P45017α had been reported for both RNase protection assays and RT-PCR [6]. It was therefore generally concluded that the expression of mRNA for P45017α occurs only transiently, during rat embryonic and neonatal development [60–62], with the exception of one report that indicated its presence in the adult rat brain [53].

It has therefore been believed that DHEA and testosterone are supplied to the hypothalamus and the amygdala (where P450arom is expressed) via blood circulation, where they are converted to estradiol. The action of estradiol has been investigated mainly in female rats in relation to the estrous cycle as well as experimentally induced estrogen depletion and replacement (to modulate the estrogen level in blood circulation). Our elucidation of the NMDA-dependent machinery of estradiol synthesis (see description in Sections 3.1 and 3.2) [29,34], which begins with endogenous cholesterol and proceeds to estradiol and testosterone through DHEA, introduces essentially a new class of brain neurosteroids, with a new role in the process of signal transduction in the brain. This role is clearly different from their reproductive actions, as evinced by the observation of endogenous estradiol synthesis within the male brain.

Our ability to observe cytochrome P45017α in the adult rat hippocampus could be explained by the application of several experimental improvements. For example, to expose the antigens of P45017α, (1) we used fresh frozen slices instead of paraffin sections for immunostaining experiments, and (2) we used a slightly higher Triton X-100 concentration (0.5%). In the Western immunoblot experiments, we used very fresh microsome preparations, and included a careful treatment with protease inhibitors to suppress protease digestion of trace amounts of P45017α proteins, before gel electrophoresis. For the RT-PCR, probes were carefully designed using computer simulations, to ensure that the selected probe sequences were free from hairpin-loops.

So far, few studies have been reported which demonstrate the neuronal distributions of steroidogenic P450 'proteins' in the hippocampus. In the rat cerebellum, on the other hand, the neuronal localization of both P450scc and 3β-HSD has been demonstrated in Purkinje neurons and granule cells by immunohistochemistry or in situ hybridization [32,57]. PREGS has been observed to enhance the electrical activity of Purkinje neurons, an effect of which may be due to the suppression of GABA neurons. [32]. A significant amount of PREG (although a much lower amount of PREGS) has been observed in the rat cerebellum. A small level of neuronal expression of P450scc mRNA has

also been reported in other rat nervous systems, including neurons in the retinal ganglion, and sensory neurons in the dorsal root ganglia [6,27,53]. The NMDA-dependent synthesis of PREG(S) by P450scc has been reported in the rat retinal neurons [63,64]. The neural expression of P450s has been indicated in the frog brain [32,65].

6. Possible mechanisms of rapid modulation of signal transduction by brain neurosteroids

The combined results of recent experimental studies indicate that PREGS facilitates postsynaptic signal amplification, as illustrated in Fig. 8 [16,17,46]. In particular, an NMDA-gated Ca^{2+} influx triggers a cascade of steroidogenesis by StAR and P450scc [29]. This increases the production of PREG and PREGS, which in turn potentiates an NMDA receptor-mediated Ca^{2+} influx. By this means, PREGS facilitates the excitation of neurons at the postsynaptic level. The observation that the production of PREG and PREGS in the hippocampus was found to be enhanced by an approximate factor of 2 upon stimulation with NMDA strongly suggests the existence of positive feedback between NMDA receptor activation and the production of PREGS [29]. This possible rapid (<30 min) postsynaptic signal amplification through a "PREGS → NMDA receptor → Ca^{2+}" cycle could directly contribute to the LTP of hippocampal pyramidal neurons, during which PREGS acts as a mediator of the postsynaptic LTP induction. Our observation of the enhancement of LTP induction by PREGS in the CA1 pyramidal neurons strongly supports this hypothesis. The ability of a 500-nM PREGS perfusion to achieve potentiation of LTP induction is significant, because this concentration of PREGS is estimated to lie within the physiological, local concentration range typical of the hippocampus (0.6–1.2 μM). Before our study, there had been difficulties in explaining the physiological significance of the action of PREGS in the hippocampus, because PREGS requires micromolar concentrations (e.g., 20–100 μM) to display its potentiation effect [16,17,44,46].

The rapid action of estradiol on glutamate-mediated neuronal excitability was observed to be suppressive for 4-week-old rats (our results; Ref. [21]). Although the production of estradiol was enhanced by an NMDA-gated Ca^{2+} influx, this increase of estradiol appears to acutely suppress LTP induction. This suppressive action by estradiol may serve to prevent neurons from an overshoot excitation induced by the "PREGS → NMDA receptor → Ca^{2+}" cycle, because estradiol synthesis occurs much more slowly than PREGS (as judged from Fig. 1) (see Fig. 7). The effect of estradiol on hippocampal neurons is dependent on experimental conditions, and is also somewhat controversial. When a 1-s, 100-Hz tetanic stimulation was applied, a pretreatment of 0.1–50 nM estradiol suppressed the induction of LTP in hippocampal neurons taken from 4-week-old rats. In slices from adult rat (3 months old), on the other hand, an identical preperfusion with estradiol had no effect on the induction of LTP [21]. When theta-burst stimulation (e.g., five applications of 100 Hz for 200 ms each, in 10 s intervals) was applied to slices from an adult rat, a 20-min preperfusion with 0.1–10 nM estradiol

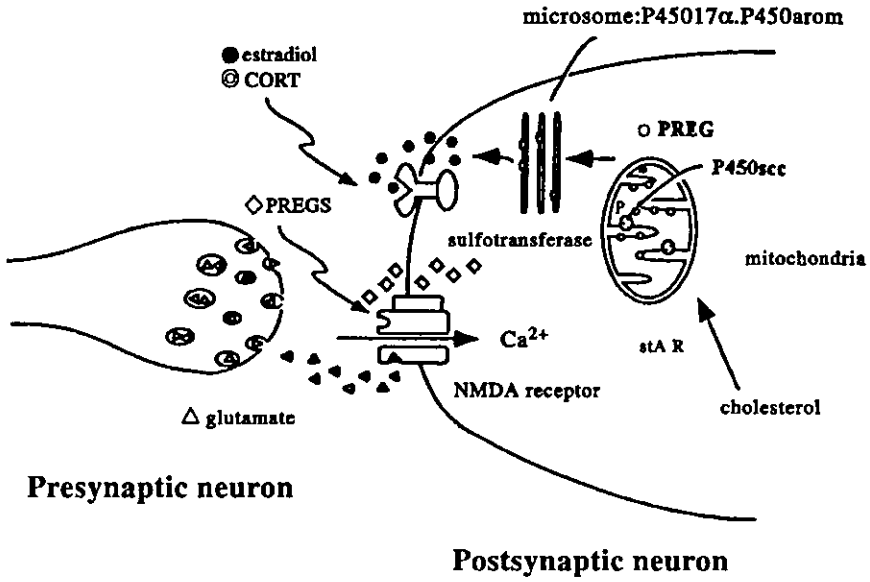

Fig. 8. Proposed postsynaptic signal amplification cascade mediated by PREGS in the hippocampus: NMDA-gating Ca^{2+} influx → StAR transports cytosolic cholesterol into mitochondria → P450scc converts cholesterol to PREG → conversion to PREGS → potentiation of NMDA receptor-mediated Ca^{2+} influx → StAR → P450scc → .... Possible modulation by PREGS, estradiol and CORT may be performed either by (1) direct binding to NMDA receptors or (2) binding to specific membrane receptors, followed by interactions with NMDA receptors. For illustrative purposes, the AMPA type of glutamate receptors is omitted.

was alternately effective at inducing LTP, as indicated by an approximate 2-fold enhancement of the slope and the peak magnitude of EPSP [20,56], or ineffective in inducing LTP as indicated by the near absence of either an enhancement or depression in the slope and the peak magnitude of EPSP (K. Ito, personal communication). In other reports, a 20-min preperfusion with 0.1–10 nM estradiol increased both the primed burst potentiation and the population spike amplitude [19]. It should be noted that we measure different types of responses when different types of stimulations are employed, for example, tetanic stimulation-induced LTP is essentially dependent on NMDA receptors, and theta-burst stimulation-induced LTP may be dependent on interneurons. Further experiments should therefore be performed to resolve the complicated effects of estradiol on neuron–neuron communication.

Although the intracellular signaling pathway, from estrogen receptors to NMDA receptors for rapid estradiol action, has not well been elucidated, the involvement of src tyrosine kinase has been indicated in the enhancement of LTP [56]. Estradiol has also been demonstrated to protect against the degradation of hippocampal NMDA receptors, upon only a 10-min exposure to estradiol, during which time MAP kinase-dependent protection of NMDA receptors occurred [56].

In this article, we focus on a consideration of the neurosteroid-induced modulation of NMDA receptors and LTP, because NMDA receptor-dependent LTP is likely to be the synaptic mechanism that implements memory. This view is supported by the observation that selective NMDA antagonists impair hippocampus LTP when delivered to the brain. Furthermore, the NMDA-dependent strengthening of CA1 synapses has been demonstrated to be essential for the acquisition and storage of spatial memory by transgenic mice in which the NMDA receptors in the CA1 pyramidal cells had been selectively deleted [66,67].

7. Putative membrane receptors for brain neurosteroids

Because classical nuclear steroid receptors mediate delayed genomic processes (which normally require hours to days), the rapid action (within 30 min) of brain neurosteroids may be mediated via novel membrane steroid receptors. The rapid enhancing effects of PREGS on NMDA receptors have been extensively studied in neurons from the hippocampus, cortex and hypothalamus [14,16,46]. The rapid modulation of PREGS has also been demonstrated for NMDA receptors expressed in *Xenopus* oocytes [46] and CHO cells [44]. It may therefore be deduced that PREGS could have specific binding sites on NMDA receptors. This hypothesis is further supported by the fact that no cytoplasmic/nuclear receptor has been observed for PREGS. PREGS is likely to have specific binding sites on GABA receptors as judged from investigations using electrophysiology and ligand binding assay [68,69]. There is also the possibility that novel membrane receptors (different from NMDA receptors) exist for estradiol [11,56,70–73]. This idea is supported by the observation that the rapid Ca^{2+} transients are induced by the application of 1–100 nM estradiol (alone) in both cultured rat hippocampal neurons (this work) and cultured dopaminergic neurons from the mouse embryonic midbrain [74].

The existence of putative surface CORT receptors also appears likely, and would serve to explain CORT's acute non-genomic effects [43]. This hypothesis is supported by studies which demonstrate that the immunoreactivity of antibodies against GR is associated with plasma membranes from hippocampal and hypothalamic neurons [75], and by reports that specific CORT binding to neuronal membranes may occur in several brain areas [76,77].

Further investigation is required to determine the primary and 3-D structure of these membrane steroid receptors.

8. Classical genomic effect of peripheral steroids

Brain neurosteroids act not only via rapid signaling pathways but also via classical cytoplasmic/nuclear steroid receptors. The concomitant classical genomic effect has been studied extensively in the past few decades. In the classical view of steroid hormone actions, steroids are considered to require binding to intracellular nuclear steroid receptors after reaching neurons via the circulation. Because activation of both the transcriptional and translational machinery of the cell is necessary to invoke classical steroid actions, a time-lag of hours to days must be present between the beginning of the steroid actions and their physiological consequences.

The chronic genomic effects of estradiol on synaptic plasticity have been extensively investigated. For example, the dendritic spine density in CA1 pyramidal neurons is sensitive to both naturally occurring estrogen fluctuations in rats [78], and experimentally induced estrogen depletion and replacement [79]. Recent evidence suggests that estrogens mediate these morphological changes by means of NMDA receptors. Estradiol increases the binding of NMDA agonist, as well as the NR1 levels in CA1 dendrites [80,81]. Moreover, estrogen-induced increases in dendritic spine density are blocked by NMDA receptor antagonists [82,83], and the electrophysiological properties of NMDA receptor-mediated transmission are altered by estrogens [20,84,85].

The administration of a 1-year therapy with 17β-estradiol for female patients of Alzheimer's disease following menopause has been shown to be very effective in improving their capacity for learning and memory [11]. Although this form of therapy requires the application of 17β-estradiol through blood circulation, the investigation of the signaling pathway induced by an endogenous paracrine supply of estradiol, which results in the modulation of neuron–neuron communication, may contribute to an understanding of this therapeutic effect.

With regard to its chronic genomic effects, CORT displays a so-called inverted U-shape type of modulation on neuronal excitability [86]. At endogenous low levels of CORT (0.5–1 nM in plasma), LTP was enhanced [87] in comparison to that observed in the absence of CORT following adrenalectomy. In stressful situations, a high level of CORT (1–10 μM), either produced in the hippocampus or supplied from the adrenal glands, may suppress LTP induction. Stress-induced increase in CORT secretion has been shown to produce neuronal cell damage [88,89]. The exogenous application of a high dose of CORT has also been shown to endanger the neurons in the hippocampus [90,91]. These chronic effects are also considered to be dependent on NMDA receptor-mediated Ca^{2+} conductance. Stress-elevated high levels of glucocorticoids have enhanced Ca^{2+} conductance. The blockage of NMDA receptors and the suppression of glutamate release are effective at inhibiting CORT-induced neuronal atrophy [92].

9. Conclusion and perspective

Brain neurosteroids could function as fourth generation neuromessengers in the brain, at least in the hippocampus. These substances are synthesized within the neurons and are responsible for the rapid modulation of neuron–neuron communication through neurotransmitter receptors. First-generation neuromessengers are neurotransmitters such as glutamate, GABA and acetylcholine. Second-generation neuromessengers are catecholamines such as dopamine and serotonin. Third-generation neuromessengers are neuropeptides such as enkephalin, vasoactive intestinal peptide, and substance P. In contrast with first- to third-generation neuromessengers, which are stored in synaptic vesicles and rapidly exocytosed from presynapses, brain neurosteroids are produced in mitochondria and microsomes, and are released relatively slowly by passive diffusion in neuronal cells. They then may diffuse to fill the interior of the neurons and, due to their amphipathic characters, may also reach other cells near to steroidogenic neurons, resulting in rapid modulation of neurotransmissions. In this sense, brain neurosteroids may serve as intracrine or paracrine modulators.

Several essential challenges must be addressed before we can claim to have a comprehensive understanding of this field. The first is a clear demonstration of the endogenous synthesis of brain neurosteroids in pure hippocampal neurons from the adult mammal. The second is a determination of the molecular structure of the accompanying membrane receptors, which may differ from those of nuclear receptors.

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Corticosterone acutely prolonged *N*-methyl-D-aspartate receptor-mediated Ca^{2+} elevation in cultured rat hippocampal neurons

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Abstract

This work reports the first demonstration that corticosterone (CORT) has a rapid and transient effect on NMDA receptor-mediated Ca^{2+} signaling in cultured rat hippocampal neurons. Using single cell Ca^{2+} imaging, CORT and agonists of glucocorticoid receptors were observed to modulate the NMDA receptor-mediated Ca^{2+} signals in a completely different fashion from pregnenolone sulfate. In the absence of steroids, 100 μM NMDA induced a transient Ca^{2+} signal that lasted for 30–70 s in 86.1% of the neurons prepared from postnatal rats (3–5 days old). After pre-treatment with 0.1–100 μM CORT for 10–20 min, NMDA induced extremely prolonged Ca^{2+} elevation. This prolonged Ca^{2+} elevation was terminated by the application of MK-801 and followed by washing out of CORT. The proportion of CORT-modulated neurons within the NMDA-responsive cells increased from 25.1 to 95.5% when the concentration of CORT was raised from 0.1 to 50 μM. Substitution of BSA-conjugated CORT produced essentially the same results. When hippocampal neurons were preincubated with 10 μM cortisol and 1 μM dexamethasone for 20 min, a very prolonged Ca^{2+} elevation was also observed upon NMDA stimulation. The CORT-prolonged Ca^{2+} elevation caused a long-lasting depolarization of the mitochondrial membrane, as observed with rhodamine 123. In contrast, incubation with 100 μM pregnenolone sulfate did not considerably alter the time duration of NMDA-induced transient Ca^{2+} elevation, but caused a significant increase in the peak amplitude of Ca^{2+} elevation in hippocampal neurons. These results imply that high levels of CORT induce a rapid and non-genomic prolongation of NMDA receptor-mediated Ca^{2+} elevation, probably via putative membrane surface receptors for CORT in the hippocampal neurons.

Keywords: acute effect, Ca^{2+} signals, corticosterone, glucocorticoids, NMDA receptor, hippocampus.

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Corticosterone (CORT) is a principal glucocorticoid synthesized in the rodent adrenal cortex and secreted in response to stress. There are a series of studies about the chronic and genomic effects of corticosteroids in the hippocampus (Reagan and McEwen 1997; Nair *et al.* 1998). The stress-induced increase of CORT secretion is known to produce neuronal cell damage. Exogeneous application of a high dose of CORT has been shown to elicit the neuronal atrophy in the hippocampus (Woolley *et al.* 1990). Rats exposed to restraint stress for 3 weeks exhibited neuronal atrophy identical to that seen in rats treated with a high-dose of CORT for 3 weeks (Watanabe *et al.* 1992). In addition to these classical genomic effects, which are actuated via intracellular steroid receptors, glucocorticoids act acutely on neuronal excitability (Landfield and Pitler 1984; Lupien and McEwen 1997). Stress levels of glucocorticoids have been demonstrated to acutely suppress within 20 min the long-term potentiation (LTP) of primed burst potentiation (Diamond *et al.* 1992).

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Abbreviations used: BSS, balanced salt solution; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; CORT, corticosterone; DMSO, dimethyl sulfoxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; GABA, γ-aminobutyric acid; LTP, long-term potentiation; MEM, minimum essential medium; PREGS, pregnenolone sulfate; n_i, the number of independent experiments.

The LTP of the population spike amplitude was also acutely (within 1 h) suppressed by a high concentration of glucocorticoids (Vidal *et al.* 1986; Diamond *et al.* 1992). CORT, injected just before the tetanic stimulation, was found to have an acute depressant effect on potentiation of both the field excitatory postsynaptic potentials (EPSPs) and the population spike amplitude in the dentate gyrus (Dubrovsky *et al.* 1993). Neurosteroids such as pregnenolone sulfate (PREGS), which are synthesized in the mammalian brain, have been observed to affect cell surface receptors very acutely (Paul and Purdy 1992; Baulieu 1997). PREGS' mode of modulation on NMDA receptors and γ-aminobutyric acid $(GABA)_A$ receptors has been extensively investigated (Wong and Moss 1994; Mienville and V.:ini 1989). PREGS potentiated the opening probability of NMDA receptors, but suppressed that of $GABA_A$ receptors, in cultured neurons (Wu *et al.* 1991; Irwin *et al.* 1992; Bowlby 1993; Fahey *et al.* 1995). In behavioral studies, the various influences of steroids on memory formation in the hippocampus have been attracting much attention. Peripheral steroids have been shown to have profound influences, either damaging or beneficial, on the memory performances exhibited in a wide variety of tasks (Vaher *et al.* 1994; Dohanich *et al.* 1994; Luine 1997; Oitzl *et al.* 1998). Steroids may influence memory processes by modulating LTP in the hippocampus, which has recently been shown to be inhibited by high stress-induced levels of glucocorticoids and to be enhanced by low endogenous levels (Pavlides *et al.* 1993, 1996; Rey *et al.* 1994; McEwen 1996, 1999; McEwen and Sapolsky 1995; Kim and Yoon 1998). Recently, stress hormones have been found to impair learning and memory function rapidly (within several tens of minutes), via unknown neuronal mechanisms (Mesches *et al.* 1999).

The rapid effects of CORT (appearing within 30 min) on NMDA-induced signal transduction have not been well elucidated in the cultured hippocampal neurons. We therefore examined the rapid effects of CORT on NMDA receptor-mediated Ca^{2+} signals in hippocampal neurons in comparison with those of the neurosteroid PREGS. CORT resulted in extreme prolongation of the time duration of NMDA receptor-mediated Ca^{2+} signals, resulting in Ca^{2+} neurotoxicity. On the other hand, PREGS did not change the time duration of Ca^{2+} signals, although the amplitude of NMDA-induced Ca^{2+} signals was increased. Agonists of glucocorticoid receptors such as dexamethasone and cortisol were investigated. These agonists also showed extremely prolonged NMDA receptor-mediated Ca^{2+} signals.

Materials and methods

Chemicals

Fura-2/AM was purchased from Dojindo Laboratory (Kumamoto, Japan). Cycloheximide was purchased from Wako pure chemicals (Osaka, Japan). PREGS, CORT, NMDA, nicardipine DNase I, cytosine arabinoside, rhodamine 123, MK-801, dexamethasone, cortisol and antineurofilament 200 kDa polyclonal antibodies were purchased from Sigma (St Louis, MO, USA). Dispase I was purchased from Boehringer Mannheim (Tokyo, Japan). Minimum essential medium (MEM) was from Gibco (Rockville, MD, USA). RU38486 and RU28632 were obtained from Roussel Uclaf (Romainville, France). All other chemicals were of the highest purity commercially available.

Preparation of rat hippocampal neurons and cell culture

Postnatal hippocampal tissue was taken from 3- to 5-day-old Wistar rats purchased from SLC (Japan). After exposure to an overdose of ether anesthesia, the rats were decapitated and the brains were removed using a sterile technique. The brains were placed in ice-cold MEM (–) (MEM supplemented with 2 mM glutamine, 22 mM glucose, 20 μM kanamycin and 26 mM $NaHCO_3$) which was continuously bubbled with 95% O_2–5% CO_2 gas. The hippocampi were then removed and rinsed once with ice-cold MEM (–). Hippocampal tissues were incubated in MEM (–) containing 3 U/mL of dispase I for 10 min at 37°C and triturated after the addition of 0.1% DNase I. The tissue was then filtered with a 100-μm cell strainer and suspended in MEM (–). The cell suspensions were centrifuged for 5 min at 120 *g*. Centrifugation was performed three times. Finally, neuronal cells were suspended in MEM (+) (MEM (–) supplemented with 10% heat-inactivated fetal calf serum), and seeded on poly L-lysine-coated glass-bottom dishes (35 mm, MatTek, Ashland, MA, USA) at the density of 10^6 cells/mL. Neurons were cultured for 7–10 days under 5% CO_2–95% ambient air at 37°C. Half of the culture medium was replaced every other day, in order to provide sufficient glucose for neuronal growth. The neuronal samples were treated on day 2, in culture with 10 μM cytosine arabinoside for 24 h, to prevent the glial proliferation. In the case of the preparation of embryonic neurons, E19–E20 rat pups were used. The culture medium and preparation methods were the same as those used for the postnatal neuron culture. The purity of cultured neuronal cells was determined by the immunostaining of the cells against neurofilament 200 kDa. More than 85 of cells were identified as neurons on day 7–10. All experiments using animals were conducted in accordance with the institutional guidelines.

Loading with fluorescent indicators

Measurement of cytoplasmic calcium concentration $[Ca^{2+}]_i$ was performed using the Ca^{2+}-sensitive indicator fura-2 (Grynkiewicz *et al.* 1985). Prior to Ca^{2+} signal measurements, neuronal cells were loaded for 30 min at 37°C with 5 μM fura-2/AM [from 1 mM stock solution in dimethyl sulfoxide (DMSO)] in the presence of 0.03% cremophore EL in 1 mL of a Mg^{2+}-free balanced salt solution (BSS), consisting of 130 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 5.5 mM glucose, 10 μM glycine and 10 mM Hepes (pH 7.3). Active mitochondria in hippocampal neurons were labeled with 0.2 μM rhodamine 123, a fluorescent dye which indicates mitochondrial membrane potential (Bellomo *et al.* 1991; Sureda *et al.* 1997) for 5 min at 37°C in BSS. After the neurons were labeled with fluorescent indicators, they were washed three times with BSS and incubated with chemicals for pre-treatment. Solutions of CORT, PREGS and thapsigargin were prepared at the appropriate dilution with BSS from the stock solution in DMSO, the final concentration of DMSO being less than 0.01% in each case.

Measurement of $[Ca^{2+}]_i$ and mitochondrial potential

Prior to the application of 100 μM NMDA, the neurons were incubated for 10–20 min at 37°C, in the presence of steroids in BSS. Control samples were preincubated in BSS alone for the same duration, at 37°C in the absence of steroids, before the application of NMDA. The neurons were perfused at a rate of 1.5 mL/min with O_2-bubbled BSS with or without steroids, NMDA and inhibitors. For fluorescence measurements, a digital fluorescence microscope system, consisting of an inverted microscope (Nikon TMD 300, Japan) equipped with a xenon lamp for excitation and a CCD camera (Hamamatsu Photonics C2400-77, Japan) was used. The inverted microscope is equipped with a temperature chamber which maintained the air around the sample at 37°C and high humidity, using a warm air-supplying system. In all measurements, from 12 to 24 neurons fell within the microscope field. For fura-2 measurements, the excitation wavelength varied discretely between 340 and 380 nm, every 1.15 s with a step motor. Fluorescence was measured above 520 nm with an IF excitation filter, a DM510 dichroic mirror, and a BA 520 emission filter. $[Ca^{2+}]_i$ in each cell was expressed as F_{340}/F_{380}, which is the ratio of the fluorescence intensity at 340 nm excitation (F_{340}) to that at 380 nm excitation (F_{380}) (Vergun *et al.* 1999). Fluorescence of rhodamine 123 was measured by the same microscope system with excitation at 470 nm and emission at 490 nm. Because rhodamine 123 is a single-wavelength dye and its initial fluorescence intensity may vary between cells, the fluorescence data sets were normalized by adjusting the resting fluorescence intensity to be 100%, to facilitate comparison. The fluorescence image analysis was performed with ARGUS-50 system (Hamamatsu Photonics, Japan). The acquired images were stored in a hard disk with 512 × 483 pixels resolution with 16-bit depth. For generation of the time dependent curves, data in each area of 10 × 10 pixels were averaged with a 2.3-s time resolution.

Statistical analysis

Statistical analysis was performed with the aid of Excel software (Microsoft, USA). Data expressed as mean ± SEM. An unpaired, two-tailed *t*-test, under the assumption of unequal variances, was utilized to test the significance of observed differences between groups. The number of independent experiments (n_i) was used to determine the parameters of *t*-distribution for the test.

Results

Characteristics of NMDA receptor – mediated Ca^{2+} signals

The response of cultured neurons to continuous NMDA exposure was characterized by a transient elevation in $[Ca^{2+}]_i$ followed by a decay to a plateau as NMDA receptors were inactivated (Fig. 1), as reported previously (Nicholls and Budd 1998; Nicholls *et al.* 1999; Vergun *et al.* 1999). It is noteworthy that the plateau level depended significantly on whether the hippocampal neurons were prepared from postnatal 3–5-day-old rats or from embryonic E19–E20 rats. Upon application of 100 μM NMDA in Mg^{2+}-free and 10 μM glycine-containing BSS, a complete decay of the Ca^{2+} elevation to the resting level was typically observed within 30–70 s, in the neurons which were prepared from postnatal 3–5-day-old rats and cultured for 7–10 days (94.6 ± 2.3% of the NMDA-responsive neurons, where the number of independent experiments was $n_i = 7$, and each contained

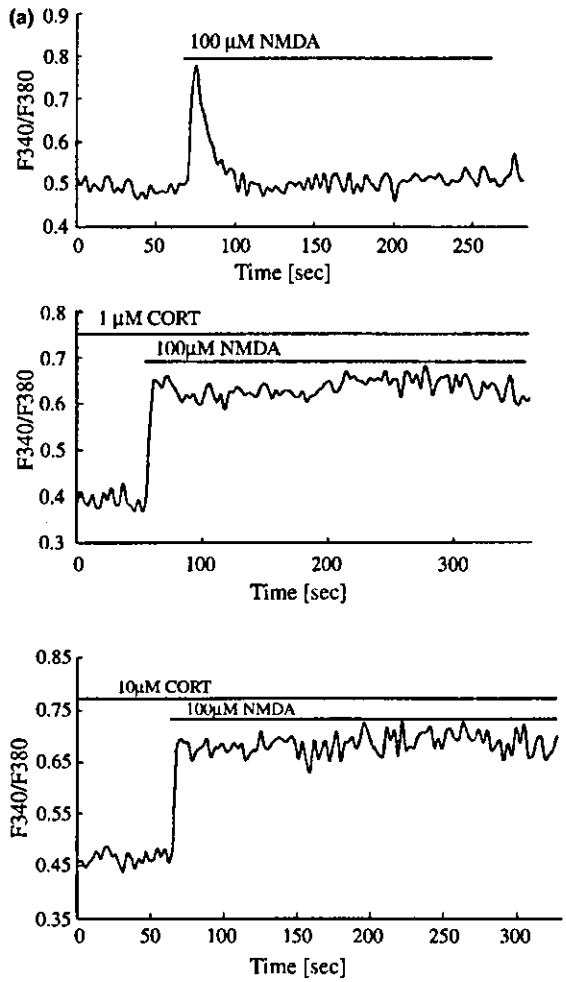

Fig. 1 Effect of corticosterone on the time course of NMDA-induced Ca^{2+} signals in the hippocampal neurons. (a) The time course of transient Ca^{2+} elevation induced upon application of 100 μM NMDA alone in a Mg^{2+}-free BSS (curve a). Pre-incubation with 1 μM (curve b) and 10 μM (curve c) CORT, before the application of 100 μM NMDA prolonged the Ca^{2+} elevation considerably. We performed between five and seven independent experiments (12–24 cells for each experiment) under each different condition and essentially the same results were obtained. The vertical scale (F340/F380) is the ratio of the fluorescence intensity of fura-2 excited at 340 nm and 380 nm, and the horizontal axis is the time in seconds. (b) Pseudo-colored images demonstrate that the neurons show a transient Ca^{2+} elevation upon 100 μM NMDA stimulation at $t = 55$ s (b1), Ca^{2+} signals in the presence of 1 μM CORT upon 100 μM NMDA stimulation at $t = 55$ s (b2). The ratio of fura-2 fluorescence is indicated with a color bar from blue (low $[Ca^{2+}]_i$) to red/white (high $[Ca^{2+}]_i$). Scale bar = 50 μm.

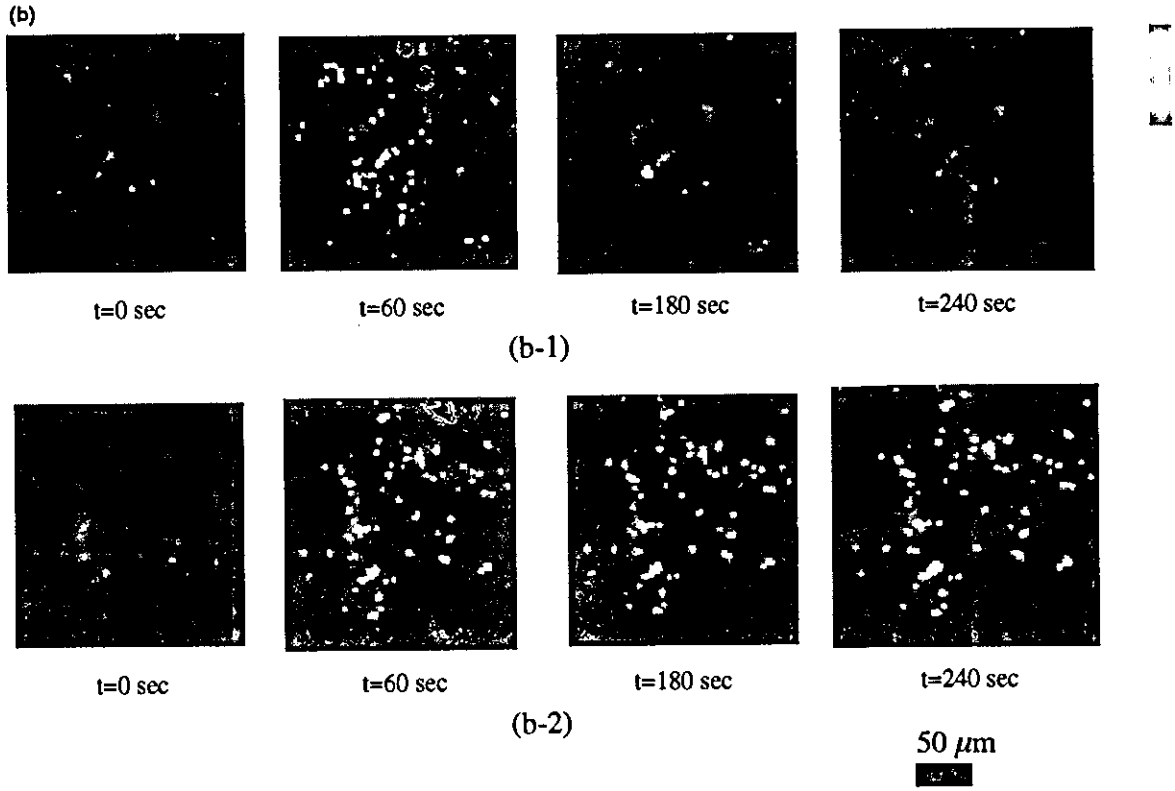

Fig. 1 (Continued)

12–24 neurons). On the other hand, the Ca^{2+} responses in most hippocampal neurons prepared from E19–E20 rats were not followed by a complete decay to resting levels after 100 μM NMDA stimulation, within the experimental time range of 300 s. $[Ca^{2+}]_i$ also remained at high plateau levels of approximately 74 ± 4% of the maximal peak values (76.5 ± 2.4% of the NMDA-responsive neurons; $n_i = 6$ experiments; 22–25 neurons/experiment). That the application of MK-801, alone induced no change in the neuronal $[Ca^{2+}]_i$ ($n_i = 3$) suggests that the MK-801 acted by blocking the NMDA-induced Ca^{2+} signal, rather than by directly inducing a change in $[Ca^{2+}]_i$.

Rapid effects of CORT on NMDA receptor-mediated Ca^{2+} signals

We examined the effect of CORT on hippocampal neurons prepared from postnatal 3–5 days and cultured for 7–10 days. Analyzed parameters of the Ca^{2+} signals are summarized in Fig. 2 and Table 1. Most of the postnatal neurons showed a rapid elevation in $[Ca^{2+}]_i$ upon application of 100 μM NMDA. The proportion of NMDA- responsive neurons {= 100 × [(the number of the NMDA-responsive neurons)/(the total number of the neurons)]} was 86.1 ± 3.6% ($n_i = 7$). The peak amplitude of $[Ca^{2+}]_i$ elevation, indicated as the increase in F_{340}/F_{380} from the resting level [$-(F_{340}/F_{380})$], was 0.26 ± 0.02 ($n_i = 5$). Most of the NMDA-responsive neurons showed a decrease in $[Ca^{2+}]_i$, towards the resting levels within 30–70 s, as described above.

After pre-treatment with 0.1–100 μM CORT for 20 min, an extremely prolonged $[Ca^{2+}]_i$ elevation was observed in a

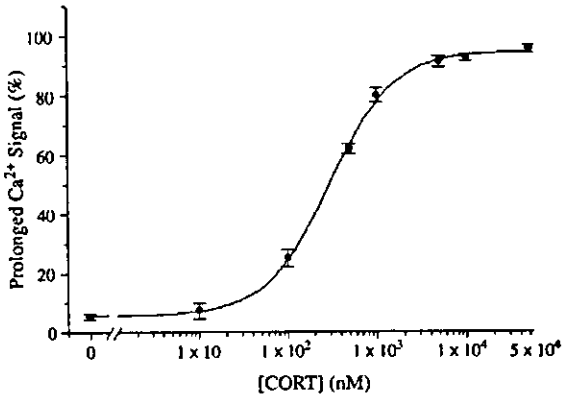

Fig. 2 Dose-dependency of the rapid prolongation effect of CORT on $[Ca^{2+}]_i$ elevation induced upon NMDA-stimulation. Vertical scale is the proportion of the neurons showing prolonged Ca^{2+} elevation [= 100 × (the number of neurons exhibiting prolonged Ca^{2+} elevation)/(the number of NMDA-responsive neurons)]. Horizontal axis indicates logarithmic concentration of CORT.

Table 1 Characteristics of Ca^{2+} signals in the postnatal hippocampal neurons stimulated by NMDA after short-term exposure to CORT or other modulators

Modulators	% Proportion of the of prolonged Ca^{2+} signals'[a] elevation[b]	Maximal amplitude $[Ca^{2+}]_i$
No modulator (NMDA alone) (n_i[c] = 7)	5.4 ± 1.1	0.26 ± 0.02
10 μM CORT (n_i = 7)	92.4 ± 1.2**	0.31 ± 0.04
10 μM CORT + 10 μM nicardipine (n_i = 5)	89.1 ± 2.2**	0.21 ± 0.02
10 μM CORT + 2 μM thapsigargin (n_i = 5)	94.2 ± 3.1**	0.24 ± 0.03
10 μM CORT + 10 μM cycloheximide (n_i = 5)	91.2 ± 3.3**	0.27 ± 0.02
1 μM CORT (n_i = 5)	80.3 ± 2.4**	0.28 ± 0.03
1 μM CORT + 10 μM nicardipine (n_i = 5)	79.8 ± 3.0**	0.23 ± 0.02
1 μM CORT + 2 μM thapsigargin (n_i = 5)	81.3 ± 2.5**	0.25 ± 0.01
1 μM CORT + 10 μM cycloheximide (n_i = 5)	79.4 ± 3.2**	0.29 ± 0.01
1 μM[d] BSA-CORT (n_i = 5)	78.3 ± 3.1**	0.27 ± 0.02
1 μM dexamethasone (n_i = 4)	94.2 ± 3.2**	0.28 ± 0.04
10 μM cortisol (n_i = 5)	88.4 ± 2.3**	0.27 ± 0.03
10 μM RU28362 (n_i = 4)	14.3 ± 3.3	0.22 ± 0.01
1 μM RU28362 (n_i = 4)	12.5 ± 3.0	0.25 ± 0.03
1 μM CORT + 1 μM RU38486 (n_i = 4)	84.3 ± 2.2**	0.25 ± 0.03
10 μM CORT + 10 μM progesterone (n_i = 5)	4.8 ± 1.3	0.27 ± 0.02
10 μM progesterone (n_i = 5)	5.8 ± 1.4	0.25 ± 0.03
100 μM PREGS (n_i = 5)	2.3 ± 0.8	0.36 ± 0.01**
100 μM PREGS + 10 μM progesterone (n_i = 3)	3.4 ± 2.2	0.34 ± 0.02**

After the preincubation of hippocampal neurons for 20 min with or without the modulators indicated in the table, neurons were stimulated with 100 μM NMDA. Between 12 and 24 neurons were analyzed in each independent experiment. Maximal amplitude of NMDA-induced $[Ca^{2+}]_i$ elevation is represented as the maximal increase in the ratio of fura-2 fluorescence intensities, excited at 340 nm and 380 nm [$\Delta(F_{340}/F_{380})$]. Values are means ± SEM. [a]The elevated $[Ca^{2+}]_i$ did not decay within the experimental time range of 300 s. [b]Maximal sustained amplitude in the case of prolonged Ca^{2+} signals, or peak amplitude in the case of transient Ca^{2+} elevation. [c]n_i is the number of independent experiments. [d]Approximately 20 CORT molecules per one BSA molecule. **Significantly larger than the value with no modulators ($p < 0.01$).

portion of the NMDA-responsive neurons upon the application of 100 μM NMDA (Figs 1 and 2). This sustained elevation of $[Ca^{2+}]_i$ did not decay in the presence of CORT, within the experimental time range of 300 s (Fig. 1). The proportion of CORT-modulated cells, which exhibited sustained $[Ca^{2+}]_i$ elevation increased with increasing CORT concentration, as shown by a sigmoidal dose-dependence curve (Fig. 2). The CORT dosage which yielded the half-maximal number of modulated cells (EC_{50}) was approximately 0.53 μM. The lowest effective dose was 0.1 μM. A dosage of 0.01 μM of CORT caused no significant effect. In particular, 1, 5, 10 and 50 μM of CORT induced the modulation in 80.3 ± 2.4% (n_i = 5), 91.2 ± 1.9% (n_i = 5), 92.4 ± 1.2% n_i = 7 and 95.5 ± 1.4% (n_i = 6) of treated neurons, respectively. Pre-treatment of the neurons by CORT had no significant effect on the proportion of NMDA responsive neurons, for example, remaining at 85.4 ± 3.4% (n_i = 7) with 10 μM CORT treatment. The presence of CORT did not cause an excessive change in the maximal amplitude of the $[Ca^{2+}]_i$ elevation.

The impact of inhibitors was generally investigated using 1–10 μM CORT, because concentrations in this range induced a prolonged $[Ca^{2+}]_i$ elevation in a sufficiently large proportion of neurons for the effects of the inhibitor to be precisely analyzed. The application of 10 μM MK-801 terminated the prolonged $[Ca^{2+}]_i$ elevation which was induced by 100 μM NMDA in the presence of 1 μM CORT, and a rapid $[Ca^{2+}]_i$ decay towards the resting level (n_i = 6; Fig. 3) was observed. Removal of CORT, by switching the perfusion solution from 1 μM CORT-containing BSS to a CORT-free BSS with 100 μM NMDA, also terminated the prolonged $[Ca^{2+}]_i$ elevation (Fig. 3). From these results, we conclude that the present effect of CORT was not due to the irreversible impairment of Ca^{2+} extrusion from the neurons. That the subsequent application of 100 μM NMDA (alone), after the removal of CORT, re-induced transient, typical NMDA-type $[Ca^{2+}]_i$ elevations (n_i = 5), also supports this conclusion.

Pre-treatment of neurons with 2 μM thapsigargin for 20 min, in the presence and absence of 1–10 μM CORT, did not change either the proportion of neurons that demonstrated a prolonged Ca^{2+} signal or the maximal amplitude of the NMDA-induced Ca^{2+} signal (see Table 1). This excludes the possibility that intracellular Ca^{2+} stores

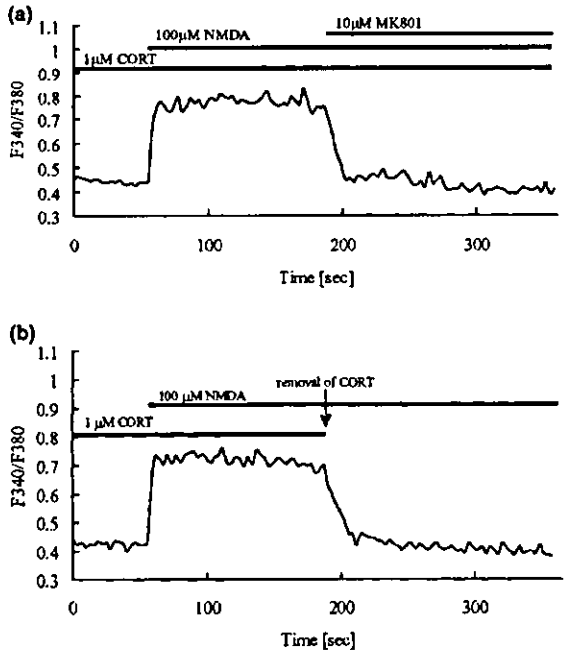

Fig. 3 Application of MK-801 or removal of CORT terminates the prolonged $[Ca^{2+}]_i$ elevation observed in the presence of CORT. (a) Change in the time course of Ca^{2+} signaling upon the application of 10 μM MK-801 during the prolonged Ca^{2+} signaling induced by 100 μM NMDA in the presence of 1 μM CORT. The vertical scale (F340/F380) is the ratio of the fluorescence intensity of fura-2, excited at 340 nm and 380 nm, and the horizontal axis is the time in seconds. (b) Change in the time course of Ca^{2+} signaling upon replacement of an external solution by a Mg^{2+}, CORT-free solution containing 100 μM NMDA. The prolonged $[Ca^{2+}]_i$ elevation was induced by 100 μM NMDA in the presence of 1 μM CORT.

significantly contributed to the prolonged $[Ca^{2+}]_i$ elevation induced by the CORT treatment. Pre-treatment with 10 μM nicardipine for 20 min did not significantly decrease the proportion of the neurons in which Ca^{2+} signals were prolonged by 1–10 μM CORT (see Table 1). This implies that a Ca^{2+} influx via L-type voltage sensitive Ca^{2+} channels was not necessary for the appearance of the present CORT effect. In order to activate all types of voltage-sensitive Ca^{2+} channels, 30–50 mM KCl in isotonic BSS was applied to the neurons. Upon the application of KCl alone, the neurons were depolarized, and a sustained $[Ca^{2+}]_i$ elevation was observed during the presence of KCl ($n_i = 3$). The KCl-induced Ca^{2+} signals observed in the absence and presence of CORT were not significantly different. The sustained level of $[Ca^{2+}]_i$ induced by 50 mM KCl was $\Delta(F_{340}/F_{380}) = 0.19 \pm 0.02$ (without CORT) and 0.20 ± 0.02 (with 1 μM CORT), respectively.

In the absence of NMDA, the application of CORT (alone) at 1–10 μM failed to induce a Ca^{2+} signal. This implies that the present CORT effect requires the activation of NMDA receptors. In order to examine whether new protein synthesis is involved in the present CORT effect, the neurons were subjected to a 40-min preincubation with cycloheximide, a protein synthesis inhibitor, prior to the NMDA application, and then the preincubation of neurons with CORT was additionally performed for 20 min prior to the NMDA application. The resulting concentrations of cycloheximide and CORT were maintained at the same level during the time course of the $[Ca^{2+}]_i$ measurements. When the neurons were coincubated with 10 μM cycloheximide and 1–10 μM CORT in this way, the NMDA application again induced a very prolonged $[Ca^{2+}]_i$ elevation in 85.1% of the neurons (see Table 1). This indicates that the prolongation by CORT did not require the synthesis of new proteins, which is necessary for the classical genomic effects of glucocorticoids, via intracellular steroid receptors.

Rapid effect of agonists and antagonist of glucocorticoid receptors on NMDA receptor-mediated Ca^{2+} signals

To examine whether the present rapid CORT effect appears via surface CORT receptors, membrane non-permeable BSA-conjugated CORT was substituted for CORT. After pre-treatment with 1 μM BSA-CORT (approximately 20 CORT molecules per BSA molecule) for 20 min, an extremely prolonged $[Ca^{2+}]_i$ elevation was observed in 78.3% of the NMDA-responsive neurons upon application of 100 μM NMDA (see Table 1). The maximal amplitude of NMDA-induced $[Ca^{2+}]_i$ elevation was the same for BSA-CORT and CORT. Dexamethasone, cortisol and RU 28362, which are known as potent agonists of glucocorticoid receptors were used to investigate the glucocorticoid effect on NMDA-induced $[Ca^{2+}]_i$ elevation (see Table 1). The pre-treatment of neurons with either 1 μM dexamethasone or 10 μM cortisol for 20 min induced an extremely prolonged $[Ca^{2+}]_i$ elevation upon NMDA stimulation, with essentially the same characteristics as that induced by CORT, with respect to both the proportion of neurons which demonstrated a prolonged $[Ca^{2+}]_i$ elevation, and the maximal amplitude of the $[Ca^{2+}]_i$ elevation. On the other hand, the preincubation with 1 or 10 μM RU28362 (a synthetic agonist of intracellular glucocorticoid receptor (GR, type-2 receptor)) had only a small effect on the prolongation of the NMDA-induced $[Ca^{2+}]_i$ elevation (see Table 1). The proportion of neurons which demonstrated a prolonged $[Ca^{2+}]_i$ elevation was 12.5 and 14.3% in the presence of RU28362, at 1 and 10 μM, respectively. The coapplication of 10 μM progesterone with 10 μM CORT completely prevented the modulating effect of CORT, while the pre-treatment of neurons with only 10 μM progesterone without CORT had no modulating effect on the $[Ca^{2+}]_i$ elevation induced by NMDA (see Table 1). The presence of an equimolar concentration of RU38486 (a synthetic antagonist of intracellular glucocorticoid receptors) with CORT did not significantly change the CORT-modulated, prolonged $[Ca^{2+}]_i$ elevation (see Table 1).

Effect of CORT on mitochondrial membrane potential upon NMDA stimulation

We examined the toxic effect of the prolonged $[Ca^{2+}]_i$ elevation induced by CORT and NMDA on mitochondria. Upon a 100-μM NMDA stimulation without CORT ($n_i = 4$), a transient decrease in the rhodamine 123 fluorescence was observed, which indicates that NMDA stimulation induced only a transient depolarization of the mitochondrial membrane (see Fig. 4). Pre-treatment with 1–10 μM CORT resulted in an extreme prolongation of the decreased phase in the fluorescence of rhodamine 123, upon 100 μM NMDA stimulation ($n_i = 5$). The time profile of the change of mitochondrial membrane potential coincided with that of the $[Ca^{2+}]_i$ elevation. It should be noted that when the mitochondrial membrane is depolarized, the sequestration of rhodamine 123 molecules within mitochondria is reduced, which results in a decrease in the fluorescence of rhodamine 123 in the concentration range of 0.1–1 μM (Sureda *et al.* 1997; Yang *et al.* 1997; Seo *et al.* 1999). In fact, the application of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), a mitochondrial uncoupler, decreased the fluorescence of rhodamine 123 ($n_i = 5$; Fig. 3).

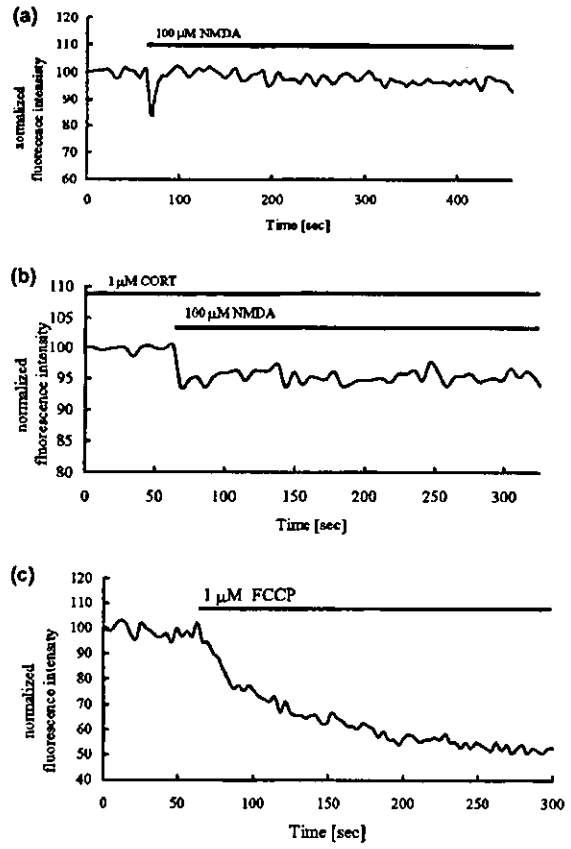

Fig. 4 Effect of CORT on the fluorescence of rhodamine 123, following NMDA stimulation in the hippocampal neurons. (a) The time course of rhodamine 123 fluorescence, following 100 μM NMDA application in the absence of CORT in a Mg^{2+}-free BSS. The initial fluorescence intensity was adjusted to 100%. The vertical scale is the normalized fluorescence intensity of rhodamine 123, and the horizontal axis is the time in seconds. (b) The time course of rhodamine 123 fluorescence upon 100 μM NMDA application in the presence of 1 μM CORT. (c) The time course of rhodamine 123 fluorescence upon the application of 1 μM FCCP.

PREGS-induced potentiation of NMDA receptor-mediated Ca^{2+} signals

By means of preincubation of the neurons with PREGS for 10–20 min, a significant potentiation was generated in the peak amplitude of the transient $[Ca^{2+}]_i$ elevation induced by NMDA (Fig. 5). In the presence of 100 μM PREGS, the peak amplitude of the $[Ca^{2+}]_i$ elevation was 0.36 ± 0.01 [expressed in terms of $\Delta(F_{340}/F_{380})$; $n_i = 5$, see Table 1]. The proportion of NMDA-responsive neurons was $92.1 \pm 2.2\%$ ($n_i = 7$) in the presence of PREGS. It should be emphasized that PREGS did not change the time duration of the NMDA-induced transient Ca^{2+} elevation (which lasted

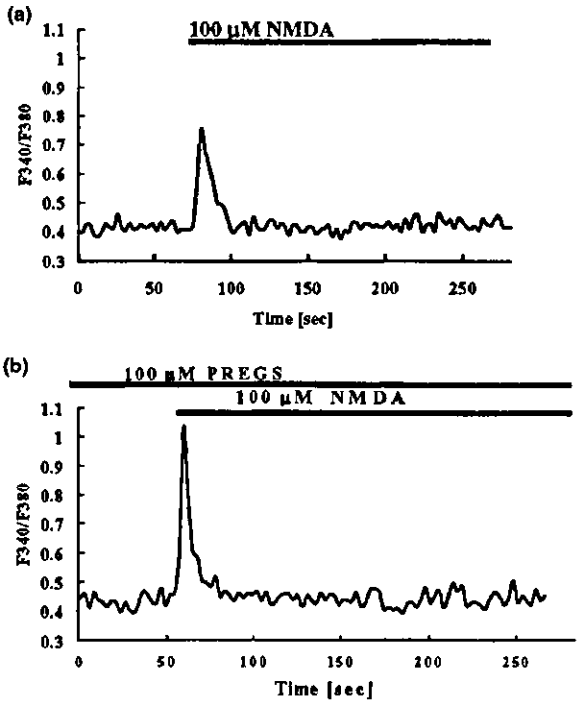

Fig. 5 Effect of pregnenolone sulfate on the magnitude of NMDA-induced Ca^{2+} signaling in the hippocampal neurons. The time course of transient $[Ca^{2+}]_i$ elevation in fura-2-loaded hippocampal neurons in the absence (a) and the presence (b) of 100 μM PREGS induced by the application of 100 μM NMDA in a Mg^{2+}-free BSS. The vertical scale F340/F380 is the ratio of fura-2 fluorescence excited at 340 nm and 380 nm, and the horizontal axis indicates the time in seconds.

for 30–70 s), although it did cause a significant increase in the mean peak magnitude of Ca^{2+} elevation. These results may correlate with the fact that PREGS increased the frequency of channel opening, although PREGS did not considerably enhance the mean duration of channel opening, or the current flow through a single NMDA receptor (Bowlby 1993; Wong *et al.* 1994).

Discussion

Characteristics of NMDA-induced Ca^{2+} signals in hippocampal neurons

Three distinct phases of Ca^{2+} dynamics (i.e. rise, decay, and plateau) were observed in the neurons, after NMDA application. This behavior may result from the difference in the time dependences of Ca^{2+} influx and extrusion. That the internal Ca^{2+} store did not contribute to the observed dynamics was established using thapsigargin (see Results and Table 1). In the rise phase, Ca^{2+} influx via the NMDA receptors exceeded Ca^{2+} extrusion. Conversely, in the following decay phase, Ca^{2+} extrusion exceeded Ca^{2+} influx via the NMDA receptors, probably due to an inactivation of the NMDA receptors as well as Ca^{2+} extrusion. In the plateau phase, influx and extrusion were balanced. Ca^{2+} influx depends on the activity of Ca^{2+}-permeable receptors/channels. Ca^{2+} extrusion, on the other hand, is sensitive to intracellular $[Ca^{2+}]_i$. It should be noted that Ca^{2+} extrusion may occur even before the inactivation of the NMDA receptors, because the rate of Ca^{2+} extrusion is a function of the level of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ plateau level following the rise and decay phases was the same as the initial resting level for the neurons obtained from postnatal 3–5-day-old rats, and 74% for the neurons obtained from embryonic E19–E20 rats (see Results). This difference in $[Ca^{2+}]_i$ decay may be due to differences in the level of Ca^{2+} extrusion by Ca^{2+}-ATPases or Ca^{2+} transporters (Garcia and Strehler 1999).

It was also confirmed that 10 μM MK-801, alone had no effect on $[Ca^{2+}]_i$, which suggests that MK-801 had no effect on either Ca^{2+} pumps or Ca^{2+} transporters.

Characteristics of the acute effect of glucocorticoids on NMDA-induced Ca^{2+} signals

Although there are many reports indicating that neurosteroids such as PREGS and dehydroepiandrosterone sulfate might rapidly alter the excitability of neurons via the modulation of ligand-gated receptors such as NMDA and $GABA_A$ receptors (Wu *et al.* 1991; Irwin *et al.* 1992; Bowlby 1993), the rapid effects of glucocorticoids and mineralocorticoids have not been well elucidated, particularly on transmitter-gated receptors (Joels 1997; Lupien and McEwen 1997). The present study demonstrates both the rapid and transient effects of CORT (within 20 min) on the NMDA receptor-mediated Ca^{2+} signaling, in postnatal hippocampal neurons at stress-induced high concentrations. The presence of CORT caused the extreme prolongation of NMDA receptor-mediated $[Ca^{2+}]_i$ elevation, resulting in a loss of mitochondrial membrane potential. Both dexamethasone (a strong agonist of glucocorticoid receptors) and cortisol (a dominant stress steroid in primates and cattle) caused similar acute prolongation effect on the NMDA receptor-mediated Ca^{2+} signals. On the other hand, PREGS increased the Ca^{2+} signal amplitude without essentially affecting the time duration of transient $[Ca^{2+}]_i$ elevation. Because CORT and PREGS, in the same concentration range, modulated NMDA receptor-mediated Ca^{2+} signaling very differently, the observed effects should not be non-specific due to, for example, the membrane-solvation of steroids or membrane-disordering by steroids. The existence of a specific mechanism of steroid-modulation for NMDA receptor-mediated Ca^{2+} signaling is also supported by our observations that progesterone did not affect NMDA-induced Ca^{2+} signaling, and that the coapplication of progesterone with CORT completely prevented the modulatory effect of CORT.

The blocking of NMDA receptors with MK-801 completely abolished Ca^{2+} signals, even in the presence of CORT. Because the net rate of change in $[Ca^{2+}]_i$ is equal to the difference between the influx and the extrusion of Ca^{2+}, the observed decay in the Ca^{2+} signal indicates that Ca^{2+} extrusion exceeded Ca^{2+} influx, after the blockage of Ca^{2+} influx via NMDA receptors by MK-801 (again, note that MK-801 itself did not change $[Ca^{2+}]_i$). Because (i) blocking L-type Ca^{2+} channels with nicardipine did not abolish the prolongation effect of CORT and (ii) the KCl-induced Ca^{2+} signal was not affected by CORT, voltage-sensitive Ca^{2+} channels are probably not involved in the CORT-induced prolongation of Ca^{2+} signals. These findings imply that the observed CORT effect is probably caused by modulation of the NMDA receptor-mediated Ca^{2+} influx.

The efficiency of these steroids on NMDA receptor-mediated Ca^{2+} signaling may not be lower than for $GABA_A$ receptors, as a PREGS concentration in excess of 1 μM was also necessary to modulate $GABA_A$ receptors. PREGS and dehydroepiandrosterone are known to have specific, high-affinity binding sites on $GABA_A$ receptors, whose modulation efficiencies are not altered by the agonists or antagonists of the benzodiazepine or barbiturate sites (Majewska *et al.* 1990; Demirgoren *et al.* 1991; Le Foll *et al.* 1997).

Possible mechanism of the acute glucocorticoid actions

According to the classical view of the steroid action, CORT is thought to penetrate into the cytoplasm of neurons, bind to intracellular receptors and induce genomic effects through new protein synthesis, resulting in the modulation of Ca^{2+} signals. The possibility of these classical genomic mechanisms can be excluded as an explanation for the present CORT effect however, for the following reasons. Cycloheximide, an inhibitor of protein synthesis, did not

abolish the effect of CORT on NMDA-induced Ca^{2+} signals. In addition, the present CORT effect was transient and immediately disappeared when CORT was removed (see Fig. 3), which is also distinct from the behavior expected from a genomic effect. The incubation time with CORT of 20 min is probably too short for the associated genomic effects via intracellular steroid receptors to appear (Sapolsky 1999), and even BSA-conjugated CORT was observed to induce a rapid prolongation of NMDA-induced Ca^{2+} signals without penetrating into the cytoplasm. The rapid CORT effect should therefore be due to a non-genomic effect via membrane surface receptors. This conclusion is further supported by the absence of a pronounced effect by specific agonists and antagonists of intracellular glucocorticoid receptors on the CORT-induced modulation of NMDA-induced Ca^{2+} signals. RU28362 (a synthetic agonist) caused no significant prolongation of NMDA-induced Ca^{2+} signals, and RU38486 (a synthetic antagonist) caused no significant inhibition of the CORT-induced prolongation of NMDA-induced Ca^{2+} signals.

The rapid decay of Ca^{2+} signals after the removal of CORT is probably due to a rapid inactivation of NMDA receptors, which occurred after the rapid release of CORT from its binding site. We used a 20-min preincubation for loading CORT, to account for likelihood that correct insertion of CORT to the binding sites on the cell surfaces probably requires a number of trial-and-error binding attempts, in contrast with the removal of CORT, which needs only one release process. Because the membrane non-permeable BSA-CORT was also observed to induce a rapid effect, it is likely that CORT binds to putative surface CORT receptors. Candidates for surface CORT receptors are unknown CORT receptors, NMDA receptors, and classical intracellular glucocorticoid receptors (e.g. GR, a type-2 receptor), which are bound to plasma membranes. This idea is supported by reports that the immunoreactivity of antibodies against GR was associated with plasma membranes from hippocampal and hypothalamic neurons (Liposits and Bohn 1993) and that specific CORT binding to neuronal membranes occurred in different brain areas with moderate affinity (K_d = 120 nM) (Towle and Sze 1983; Guo *et al.* 1995). Classical GRs are expressed in the cytoplasm of cultured hippocampal neurons (Packan and Sapolsky 1990). These glucocorticoid receptors are functional in genomic pathways (Nishi *et al.* 2001). Cultured hippocampal neurons seem to have both non-genomic corticosteroid action pathways and classical genomic pathways.

We speculate here on the existence of a possible protein kinase-dependent signaling pathway, from surface CORT receptors to NMDA receptors. CORT has recently been found to acutely and non-genomically modulate protein kinase C-dependent cascades via putative membrane binding sites (Qiu *et al.* 2001). It is also known that Ca^{2+}-dependent protein kinase C modulates NMDA receptor activation via two types of pathways: (i) the direct phosphorylation of NMDA receptors (NR1 subunit), or (ii) the indirect protein kinase C-dependent pathway, which involves the activation of other kinases (e.g. tyrosine kinases), and results in the phosphorylation of different NMDA receptor subunits. The direct pathway induces the NMDA receptor inactivation, while the indirect pathway activates NMDA receptors via the reduction of Ca^{2+} dependent inactivation (MacDonald *et al.* 2001). Taken together, our results could be explained by the binding of CORT to putative membrane glucocorticoid receptors, which induces the activation of the indirect protein kinase C-dependent pathways, resulting in the prolonged activation of the NMDA receptors. The decay of the prolonged Ca^{2+} elevation after CORT removal can be interpreted as the termination of this indirect protein kinase C-dependent process.

The counteracting effect of progesterone on the CORT action could be due to the competitive inhibition of CORT by progesterone at membrane glucocorticoid binding sites. Actually, progesterone has been demonstrated to bind to the membrane glucocorticoid binding sites with an affinity higher than that of dexamethasone (Guo *et al.* 1995). A sigma receptor-dependent pathway might be another possible candidate for the mechanism of the present counteracting effect of progesterone on the CORT action, because progesterone is known to suppress sigma receptor-mediated activation of the NMDA response in neurons (Bergeron *et al.* 1996; Maurice *et al.* 2001). If this is the case, CORT should act as an agonist of sigma receptors. This is, however, probably not the case, because (i) both CORT and progesterone are reported to inhibit the binding of sigma-1 agonist (e.g. SKF-10 047) to the sigma receptors (Su *et al.* 1990), and (ii) in contrast to the action of stress levels of CORT, most agonists of sigma receptors (sigma-1 and sigma-2 agonists) have antistress, antidepressant, and anti-amnesic actions (Maurice and Privat 1997; Maurice *et al.* 2001), indicating that CORT does not act as an agonist of sigma receptors.

It is also improbable that the present CORT effect on NMDA-induced Ca^{2+} signals is due to the reported inhibitory action of CORT on sigma receptors (Su *et al.* 1990), because progesterone, an antagonist of sigma receptors (Maurice *et al.* 2001), did not induce the prolongation of the NMDA-induced Ca^{2+} signals (see Table 1).

Neurophysiological consequences of the acute effect of glucocorticoids on NMDA-induced Ca^{2+} signal

Investigations of dose dependency showed that CORT was effective at a minimal dose of 0.1 μM and became considerably more effective in the range, 0.5–1 μM (Fig. 2). Rats which were subjected to immobilization-stress for 1 h showed high plasma CORT concentrations (e.g. 800 ng/mL (approximately 2 μM)), implying that CORT at 0.5–1 μM may be naturally secreted under stressful conditions

(Marinesco *et al.* 1999). The present CORT effect is therefore likely to be physiologically significant.

Demonstrations of rapid, non-genomic glucocorticoid action on neuronal excitability have accumulated in recent years (Lupien and McEwen 1997). In addition, extensive investigations have been conducted to investigate inhibitory effects on LTP-induction and learning, which result from chronic exposure (1–24 h) to high levels of glucocorticoids (McEwen 1996; Pavlides *et al.* 1996; Kim and Yoon 1998).

We have demonstrated further that the pre-treatment of 10 μM CORT for 20 min significantly suppresses the development of LTP in the CA1 region of the hippocampus upon high frequency tetanic stimulation with 100 Hz in 1 mM Mg^{2+} medium (Kawato *et al.* 2001). There are previous reports showing the CORT-induced suppression of the magnitude of LTP in the CA1 region and CA3 region, upon incubation with a high concentration of CORT and its agonists for 1 h (Kerr *et al.* 1994; Rey *et al.* 1994). In addition, because (i) prolonged Ca^{2+} elevation causes a permeability transition of the mitochondrial membranes, resulting in mitochondrial dysfunction (Nicholls *et al.* 1999), and (ii) the dysfunction of mitochondria is known to suppress LTP induction (Albensi *et al.* 2000), the CORT-induced suppression of LTP may be due, in part, to a dysfunction of the mitochondria induced by a prolonged decrease in the mitochondrial membrane potential.

We have recently discovered the neurosteroidogenic systems in pyramidal and granule neurons in the rat hippocampal formation, where synthesis of pregnenolone and PREGS from endogenous cholesterol is driven by Ca^{2+} influx through NMDA receptors (Kawato *et al.* 2000). CORT synthesis from [^{3}H]deoxycorticosterone has also been demonstrated in the rat hippocampus (Gomez-Sanchez *et al.* 1997). These novel observations led to the hypothesis that stress-induced CORT production may be performed not only in adrenal glands but also within the hippocampus, resulting in the acute and effective modulation of NMDA receptors and LTP.

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Research report

A selective increase in phosphorylation of cyclic AMP response element-binding protein in hippocampal CA1 region of male, but not female, rats following contextual fear and passive avoidance conditioning

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Abstract

Cyclic AMP response element-binding protein (CREB), a transcription factor on which multiple signal transduction pathways converge, has been implicated in long-term memory. We examined whether the sex difference in the performance of contextual fear or passive avoidance conditioning is associated with a change in the activation of CREB in the hippocampus, a neural structure important for long-term memory. The activation of CREB in different subregions within the hippocampus in male and female rats was determined immunohistochemically with an antibody that specifically recognizes the phosphorylated form of CREB (pCREB). With respect to the freezing time in contextual fear conditioning and the step-through latency in passive avoidance conditioning, male rats exhibited better performance than female rats. Phosphorylation of CREB (% pCREB) as revealed by the ratio of the pCREB-immunoreactive (pCREB-ir) cell number to the CREB-immunoreactive cell number was increased in the CA1 region, but not in CA3, CA4, or in the dentate gyrus following training for both types of conditioning in males. In females, such an increase in % pCREB was not found in any hippocampal subregion at any time after conditioning or by increasing the intensity of foot shock. Orchidectomy in males did not alter either the performance of contextual conditioning or conditioning-induced CREB phosphorylation in CA1. The close relationship between behavioral performance and CREB phosphorylation in the CA1 region suggests that hippocampal CREB is involved in the sex difference in some forms of learning and memory.

Theme: Neural basis of behavior
Topic: Learning and memory: physiology

Keywords: Sex difference; Cyclic AMP response element-binding protein; Hippocampus; Learning; Memory

1. Introduction

An increased intracellular level of the second messenger cyclic AMP after stimulation by hormones and neurotransmitters induces the expression of a variety of genes, indicating an important role of cyclic AMP in protein synthesis. The transcription factor cyclic AMP response element-binding protein (CREB) is activated by cyclic AMP-dependent protein kinase (PKA)-mediated phosphorylation on Ser133 [19] and binds to a consensus DNA sequence termed cyclic AMP response element (CRE) in promoter regions [55], leading to cyclic AMP-regulated expression of the genes for a wide range of proteins such as somatostatin, c-*fos*, brain-derived neurotrophic factor

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