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NADPH oxidase-mediated Rac1 GTP activity is necessary for non-genomic actions of the mineralocorticoid receptor in the CA1 region of the rat hippocampus

Fumiko Kawakami-Mori[1], Tatsuo Shimosawa [1,2], Shengyu Mu[1], Hong Wang[1], Sayoko Ogura[1,3] Yutaka Yatomi[2], and Toshiro Fujita[1]

[1]Department of Nephrology and Endocrinology, University of Tokyo Faculty of Medicine, Tokyo, Japan

[2]Department of Clinical Laboratory, University of Tokyo Faculty of Medicine, Tokyo, Japan

[3]Division of Laboratory Medicine, Department of Pathology and Microbiology, Nihon University of Medicine Faculty of Medicine, Tokyo, Japan

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Author contributions:

Prof. Fujita and Prof. Yatomi were contributed for funding. Dr. Ogura and Dr. Wang were for Rac1 pull down assay. Dr. Mu was for rat preparation. Dr. Shimosawa was contributed to the design of this work.

Contact information: Toshiro Fujita, MD, PhD

Department of Nephrology and Endocrinology, University of Tokyo Faculty of Medicine

7-3-1 Hongo Bunkyoku, 113-8655 Tokyo, Japan

fujita-dis@h.u-tokyo.ac.jp

Abstract

Mineralocorticoid receptors (MRs) in the central nervous system play important roles in spatial memory, fear memory, salt sensitivity, and hypertension. Corticosterone binds to MRs to induce presynaptic vesicle release and postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor aggregation, which are necessary for induction of long-term potentiation under psychological stress. On the other hand, cognitive dysfunction is an important problem clinically in patients with hypertension, diabetes, and cerebral infarction, and all of these conditions are associated with an increase in reactive oxygen species (ROS) generation. Oxidative stress has been shown to modify the genomic actions of MRs in the peripheral organs; however, there have been no reports until now about the relation between the non-genomic actions of MRs and ROS in the central nervous system. In this study, we investigated the relationship between ROS and the non-genomic actions of MR. We examined the non-genomic actions of MR by measuring the slope of the field excitatory postsynaptic potentials and found that ROS induced an additive increase of these potentials, which was accompanied by Rac1 GTP activation and ERK1/2 phosphorylation. An NADPH oxidase inhibitor, apocynin, blocked the non-genomic actions of MRs. A Rac1 inhibitor, NSC23766, was also found to block synaptic enhancement and ERK1/2 phosphorylation induced by NADPH and corticosterone. We concluded that NADPH oxidase activity and Rac1 GTP activity are indispensable for the non-genomic actions of MRs and that Rac1 GTP activation induces ERK1/2 phosphorylation in the brain.

Keywords: corticosterone, reactive oxygen species, field excitatory postsynaptic potentials

Introduction

The mineralocorticoid receptor (MR) is a member of the nuclear receptor superfamily mainly known for its actions in controlling sodium transport across epithelial cells, such as the epithelium of the distal portions of the nephrons and the colonic epithelium. The genomic actions of MRs have also been implicated in pathophysiological effects, such as inflammatory, fibrotic, and hypertrophic changes in the peripheral tissues. For example, MR activation has been shown to induce hypertrophy and fibrosis of the cardiomyocytes (17, 43). MR inhibition mitigates myocardial fibrosis and apoptosis by reducing oxidative stress without affecting systolic blood pressure (22). Interestingly, Yanomamo Indians, members of a Brazilian tribe with very low salt intakes and high serum aldosterone levels, are predisposed to neither hypertension nor arteriosclerosis (31). High salt loading in obese, spontaneously hypertensive rats induced the production of reactive oxygen species (ROS), which improved with the administration of an MR blocker (29). Rac1, which is downstream of the ROS production, induces actions of MRs independent of ligand binding (41). These findings suggest that organ damage associated with MRs might be independent of ligands, but closely related to ROS production.

The brain is one of the key targets of glucocorticoid actions (25, 39). MR expression is localized to specific regions and is important for cognitive memory, fear memory, and salt preference (3, 37, 40). Corticosterone (CS) is known to bind to both MRs and glucocorticoid receptors (GRs) (35). MRs have a 10-fold higher affinity (Kd=0.5-1 nM) for CS than GRs (Kd=2-5 nM) (6, 35), and under normal conditions, CS binds preferentially to the MR. With increases in the production of CS under conditions of stress, CS binds to low-affinity and membrane-bound MRs in the hippocampal region (33). MR activation can exert rapid non-genomic actions and

enhance long-term potentiation induction (1, 13), while blockade of MRs, which can pass through the blood-brain barrier (37), attenuates memory consolidation (2, 18). Meanwhile, GRs mediate slow, long-acting effects through transcriptional pathways (genomic actions), and long-term potentiation is conserved even after the levels of CS have returned to baseline, normal levels. GR actions also facilitate long-term depression by inducing sustained elevation of CS production (7, 8). Thus, MRs and GRs can cope with stress by coordinating memory formation through both non-genomic and genomic actions. Furthermore, it has recently been reported that cerebral ischemia induces MR expression and ROS production in the cortex and that MR blockers reduce ROS accumulation (32). MR expression has also been found to govern the fate of neurons in ischemic areas (23). Although a number of studies have shed light on the intracellular signaling associated with the non-genomic actions of MRs in the central nervous system (3, 19, 30), and some reports have shown ROS involvement in MR signaling in the peripheral organs, the relationship between the non-genomic actions of MR and ROS in the brain remains unknown.

The aim of this study was to determine whether acute increases in ROS production might modulate the non-genomic actions of MRs in the hippocampal region, and which MR signals might be affected by ROS.

Materials and Methods

Slice preparation

Experimental procedures involving live animals were conducted with the approval of the Tokyo University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (6-7 weeks of age) were decapitated under diethylether anesthesia. The decapitations were performed

at a fixed time (10:00-11:00 AM). After quickly isolating the hippocampi, transverse slices (350-μm thick) were prepared with a tissue slicer (Leica VT1000 S; Leica Microsistemas, Barcelona, Spain) in an ice-cold artificial cerebrospinal fluid solution composed of (mM): NaCl 119, KCl 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, glucose 11, $CaCl_2$ 2, and $MgCl_2$ 1.0 in pH 7.2 to 7.3 aerated with a 95% O_2 and 5% CO_2 mixture. The slices were incubated for at least 60 min in a humidified interface holding chamber at room temperature (22-28°C). After incubation, the slices were submerged in a recording chamber held by a platinum grid attached with nylon strings, and superfused (at 2 ml/min) with the artificial cerebrospinal fluid solution, as previously described (28).

Recording and data analysis

A bipolar tungsten electrode was placed in the CA1 (stratum radiatum), and synaptic responses were evoked at a stimulus frequency of 1/15 Hz. Field excitatory postsynaptic potentials (fEPSPs) in the CA1 region 200-400 μm distal to the stimulus site were recorded with a glass pipette filled with 2 M NaCl. The fEPSPs were recorded and filtered (4 kHz low-path filter) with an Axopatch 700A amplifier (Axon Instruments, CA), and digitized with pClamp 8 (Axon Instruments). All experiments were carried out at 25-27°C. All data were obtained in the presence of 500 nM actinomycin D to confirm the data as representing non-genomic actions.

Drug treatment and experimental procedure

In the electrophysiological experiment, hippocampal slices were preincubated with blockers for at least 30 min, except for NSC23766, a Rac1 inhibitor, which was applied to the slices 2 hr before the electrical stimulation; 1 μM NADPH was applied for 5 min and washed out thereafter, and 100 nM CS, 1 nM aldosterone (Ald), or 10 nM dexamethasone (Dex) was applied for 20 min.

Spironolactone (Spi), mifepristone (RU486), NADPH, 4-hydroxy TEMPO (Temp), actinomycin D, Dex, and Ald were obtained from Sigma-Aldrich (MO). CS was procured from Tocris (Bristol, UK) and BSA-conjugated CS was from Steraloids (PO). NSC23766 was purchased from Merck (Germany), EGF from Cell signaling (MA), apocynin (Apo) from WAKO (Japan), and eplerenone (Epl) from Pfizer (NY). We diluted DMSO at least 10000-fold when applying drugs to the slices, because it is known that 1000-fold DMSO does not change synaptic response.

Western blotting

Hippocampal slices were prepared as described above, and each sample for protein extraction was collected from three or four of these slices. The slices were stimulated with drugs by adding them to artificial cerebrospinal fluid aerated with a 95% O_2 and 5% CO_2 mixture. After stimulation, the samples were quickly frozen on dry ice, and the CA1 region was dissected. These samples were lysed in ice-cold magnesium-containing lysis buffer composed of 25 mM HEPES, 150 mM NaCl, 1% igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 25 mM NaF, and 1 mM $NaVO_4$, supplemented with a phosphatase inhibitor (Phosstop®, Roche, Basel, Switzerland). A 10-μg amount of total protein was separated by 10% SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride membranes. Western blot analysis was performed using rabbit polyclonal phospho ERK1/2 antibody and rabbit polyclonal ERK1/2 antibody (Promega, Madison,WI) as the primary antibodies.

Rac1 GTP pull-down assay

We used the Rac1 activation assay kit (Millipore, Billerica, MA), which leverages the affinity to the p21-activated-kinase-1 protein binding domain peptide, to measure the active form of Rac1, i.e., the GTP-bound form of Rac1, as previously reported (40, 41). Hippocampal slices were

lysed as described above, and 10 μg total protein was used to measure the total Rac1. To measure the GTP-bound form of Rac1, a 1-mg sample was incubated with 10 μg p21-activated-kinase-1 protein binding domain beads for 1 hr. After washing adequately with lysis buffer, the beads were boiled for 5 min in Laemmli sample buffer. The lysates were resolved by 15% SDS-PAGE, and the proteins were analyzed by immunoblotting with anti-mouse monoclonal Rac1 antibody.

Statistical analysis

Data were expressed as means ± standard error. Differences in values between groups were tested for significance by the *t*-test or ANOVA and the Tukey or Scheffe *post hoc* test. A probability level of <0.05 was accepted as evidence of statistical significance.

Results

Non-genomic actions of corticosteroids in CA1 synapses are mediated by MR

First, we confirmed that the non-genomic actions of corticosteroids in the CA1 region were mediated by MRs, as previously reported (19). CS rapidly increased the slope of the fEPSPs (124±2.3%, n=7, Fig. 1A and 1B), and this effect was blocked by the MR antagonists Spi (106±4.5%, n=6, Fig. 1A and 1B) and Epl (n=4, 96±6.4 %), but not by the GR antagonist RU486 (118±4.9%, n=5, Fig. 1A and 1B). Furthermore, the effect of 1 nM Ald (121±1.4%, n=5, Fig. 1C), 1/100 dose of CS, which is thought to be distributed in the hippocampal tissue (44) and binds selectively to the MR at this concentration, was comparable to the effect of CS, whereas 10 nM Dex did not significantly increase the slope of the fEPSPs (104±3.6%, n=4, Fig.

1C). To confirm that the non-genomic action of MRs is through membranous MR, we used membrane-impermeable CS (BSA-conjugated CS, 400 nM) and observed fEPSPs responses similar to CS (Fig. 7A).

NADPH oxidase activation has additive effects on the non-genomic actions of MRs

ROS have been reported to induce synaptic enhancement in the hippocampus (21). To determine whether this ROS action might influence the non-genomic actions of MRs, we simultaneously applied NADPH (1 μM) and CS (20 to 100 nM) and compared their effect with the effect of CS alone. NADPH alone increased the slope of the fEPSPs (138±12.0%, n=6, Fig. 2A and 2B), CS increased fEPSPs dose dependently (Fig. 2C), and coapplication of NADPH and CS increased the fEPSP slope markedly (1 μM NADPH and 100 nM CS 163±10.0%, n=6) as compared with CS application alone (Fig. 2B and 2C) at each concentration. Further, we examined the effect of another ROS mediator, EGF, on the non-genomic action of MR Fig. 8); 10 ng/ml EGF also enhanced the non-genomic action of MR. Therefore, it was indicated that ROS production enhanced non-genomic MR action. To clarify the additive effect of NADPH oxidase activity on the MR signaling, we examined the effect of Apo (100 μM, Fig. 3A, 3B, and 3E), a blocker of the NADPH oxidase. Surprisingly, Apo blocked the effect of both CS (102±4.0%, n=5, Fig. 3A) and Ald (107±6.4%, n=5, Fig. 3E). These findings indicated that the non-genomic actions of MR require ROS via NADPH oxidase.

MR and NADPH oxidase have an additive effect on ERK1/2 phosphorylation

The increase in presynaptic glutamate release in response to the non-genomic actions of the MR

has been reported to be mediated by ERK1/2 phosphorylation (19). We investigated whether ERK1/2 is also phosphorylated by MR activity and ROS, and whether MR and ROS might have an additive effect on ERK1/2 phosphorylation. Simultaneous application of CS and NADPH significantly increased the level of phosphorylation of ERK1/2 (1.88±0.33, n=7, $p<0.05$) in comparison with application of CS (1.29±0.13, n=7) or NADPH (1.44±0.17, n=7) alone (Fig. 4A). We investigated whether the ERK1/2 phosphorylation by CS and ROS could be suppressed by Spi, RU486, Apo, and the ROS scavenger Temp. ERK1/2 phosphorylation induced by CS was blocked by Spi, Apo, and Temp, but not by RU486 (Fig. 4C; CS, 1.32±0.06; RU486, 1.29±0.04; Spi, 1.04±0.04; Apo, 0.92±0.92; Temp, 0.81±0.04). Furthermore, Apo and Temp, but neither RU486 nor Spi, blocked the effect of NADPH (Fig. 4E; NADPH, 1.53±0.24; Spi, 1.64±0.23; Apo, 1.37±0.12; Temp, 1.24±0.27; n=7-9). These results indicate that the MR and NADPH oxidase actions share a common signaling pathway, and that NADPH activation is required for the non-genomic actions of the MR.

Rac1 activation is additively induced by MR and NADPH oxidase activation

We next investigated what signal underlies the need for NADPH oxidase-induced ROS for the non-genomic actions of MR. It is known that certain subfamilies of NADPH oxidase, namely NOX1 and NOX2, are regulated by small GTPase Rac1 (26, 27). It was recently reported that prolonged exposure to Ald also activates Rac1 and enhances ROS generation, probably by genomic actions, in vascular smooth muscle cells (15), and that Rac1 GTP enhances MR translocation to the nuclei of the renal podocytes via the p21-activated-kinase pathway (34, 40). We postulated that Rac1 activation is the key to the additive effect of ROS and MR signaling. We measured the changes in Rac1 GTP activity in the CA1 region of the hippocampus

immediately after the application of CS and NADPH. As shown in Figure 4B, application of CS or NADPH immediately increased the Rac1 GTP activities (CS: 1.29±0.13, n=8; NADPH: 1.44±0.17, n=8), and when both were applied concomitantly, an even greater degree of Rac1 GTP activation was observed (1.81±0.33, n=8, $p<0.05$). Rac1 GTP activation by CS was also reconfirmed using membrane-impermeable CS (BSA-CS, Fig.7B). We next investigated the effect of each blocker on the Rac1 GTP activation induced by CS and/or NADPH. Rac1 activation induced by CS was blocked by Spi, Apo, and Temp, but not by RU486 (CS, 1.27±0.07; RU486, 1.26±0.06; Spi, 1.03±0.07; Apo, 0.79±0.06; Temp, 0.65±0.08). Activation induced by NADPH was blocked by Apo and Temp, but not Spi. These results are comparable to ERK1/2 phosphorylation, confirming that the non-genomic actions of MRs require NADPH oxidase activity, and NADPH oxidase activation is at least in part via membranous MRs.

Rac1 activity is indispensable for synaptic enhancement mediated by the non-genomic actions of MR and NADPH oxidase activation

To determine whether Rac1 activation is required for synaptic enhancement induced by MR activity and ROS, we investigated the effect of a Rac1 inhibitor (NSC23766; NSC) that blocks the interaction between Rac1 and guanine nucleotide exchange factor (GEF), such as Tiam-1 (10), and might also block the synaptic enhancement. Since preincubation with NSC for 2 hr blocked the synaptic potentiation induced by both CS (1.01±0.08, n=6, Fig. 3C) and NADPH (0.94±0.11, n=5, Fig. 3D), the results indicate that Rac1 activation is also necessary for non-genomic MR signaling.

Rac1 GTP activation induces ERK1/2 phosphorylation

Next, we used a MEK inhibitor (U0126) and measured the Rac1 GTP activity to determine whether the Rac1 signal is upstream of ERK1/2. Preincubation with 20 μM U0126 for 30 min reduced the ERK1/2 phosphorylation level (Fig. 5B), but failed to block the Rac1GTP activation induced by CS (U0126 1.71±0.48 vs. U0126+CS 2.36±0.65, n=7, $p<0.05$, Fig. 5C). On the other hand, ERK1/2 phosphorylation induced by CS and/or NADPH was completely blocked by NSC (NSC, 1.00±0.01; NSC+CS, 0.98±0.09; NSC+NADPH, 0.94±0.11; n=6, Fig. 5A). Thus, our findings showed that CS rapidly activates Rac1 via MR activation, followed by ERK1/2 phosphorylation.

Discussion

The non-genomic action of MR is known to occur in various organs other than the brain, such as vascular smooth muscle cells, endothelial cells, and heart. It acts quickly and in a GRE-independent fashion when CS and/ or Ald level are elevated, and it is supposed to be responsible for the quick reaction under the stress (24). As previously noted, the genomic action of MR is associated with ROS (15). But little is known about the precise relationship of between non-genomic action of MR and ROS (42).

The results of this study demonstrate for the first time that NADPH oxidase activity is indispensable for the non-genomic actions of the MR, which is likely induced by membranous MR, and that it exerts an additive effect on the non-genomic actions of the MR in the hippocampus. We further demonstrate that Rac1 activation mediates both MR activation and ERK1/2 phosphorylation (Fig. 6).

The non-genomic actions of corticosterone mediated via the MR in the hippocampal region, affecting both pre- and postsynaptic responses, were recently clarified (5, 30). Synaptic vesicle release is enhanced by L-type Ca channels and is mediated by Src and ERK1/2 phosphorylation (4, 19). Postsynaptic K channel (I_A current) suppression and lateral diffusion and insertion of the postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor (8, 20, 30) are also enhanced. These actions might contribute to enhanced induction of long-term potentiation by CS under conditions of stress.

Since we evaluated the non-genomic actions of the MR by measuring fEPSPs, Rac1 GTP activity, and ERK1/2 phosphorylation ratio in slices of the CA1 region of the hippocampus, we could not separate the presynaptic effects from the postsynaptic effects. Paired-pulse ratio (PPR) usually reflects presynaptic release probability, but a postsynaptic I_A current would also change the PPR, and the PPR cannot indicate the release probability precisely. It has already been shown that ERK1/2 phosphorylation acts both pre- and postsynaptically (8, 30). Further, Rac1 GTP activation mediates PSD95 phosphorylation, which recruits surface AMPA receptors (20). Thus, the actions of the MR affect both pre- and post synaptic processes through Rac1 and ERK1/2 activation.

There is another possible mechanism by which ROS mediates the non-genomic actions of the MR: ROS may change the intracellular amounts of CS by increasing the activity of 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 (9). 11β-HSDs are enzymes that metabolize glucocorticoids, and consist of two isozymes, 11β-HSD 1 and 2. Expression of 11β-HSD2 is confined to certain areas of the brain, including the paraventricular nucleus and nucleus of the solitary tract (11, 45), where they contribute to salt preference (12, 40). In the hippocampus,

11β-HSD1 is dominantly expressed (37). 11β-HSD1 has both dehydrogenase and hydroxylase activity that is activated by $NADP^+$. Therefore, it is possible that local CS concentration (internal CS) might be increased by ROS via the 11β-HSD1 activation. However, in the present study, synaptic enhancement by NADPH was not blocked by Spi, and Apo preincubation, which may decrease the internal CS concentration through inhibition of 11β-HSD1, neither changed the synaptic response nor the ERK1/2 phosphorylation ratio (Fig. 9), and further Apo completely blocked the synaptic enhancement induced by additional CS application. These data indicate that the NADPH pathway is downstream of the MR and that alteration of 11β-HSD1 activation cannot explain the acute, additive effect of ROS and CS in our preparation.

We used CS as the main MR agonist in our preparation because relatively low levels of aldosterone cross the blood-brain barrier *in vivo* (6, 44), and CS is the main modulator of MR activity in the brain. In this study, both application of CS at 100 nM and Aldo at 1 nM, induced synaptic potentiation, consistent with previous reports (35). Further, we evaluated ERK1/2 phosphorylation of hippocmampus induced by intrapenetrial CS injection *in vivo* (Fig. 10). This suggests that physiological concentrations of corticosteroids can affect the synaptic intensity and cognitive functions.

Our study clarified that MR activation induces Rac1GTP activity (non-genomically, at least in part via membranous MR, Fig.7), and this pathway is mediated by NADPH oxidase. Additionally, we demonstrated that simultaneous exposure to CS and NADPH enhances synaptic response. Considering that NADPH activity induces glutamate toxicity (14), CS can also enhance glutamate toxicity via the NADPH pathway. Therefore, it is possible that excessive psychological stress and oxidative stress may cause glutamate toxicity in an additive manner. In conclusion, the present study revealed that non-genomic action of MR in the brain regulates

289 ROS and Rac1, and that the Rac1-ERK1/2 pathway is the intracellular signaling pathway of
290 MRs.
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Figure Legends

Figure 1. CS quickly increased fEPSPs via MR. A. Representative fEPSP traces in CA3-CA1 synapses. Exposure to 100 nM CS for 10 min increased the slope of the fEPSPs; 1 μM spironolactone (Spi) blocked this effect, while 500 μM RU486 did not. B. Time course of changes in the slope of the fEPSPs after CS application. C. Exposure to 1 nM Ald also potentiated the fEPSPs. Dex, which can bind to the GR, did not potentiate the fEPSPs.

Figure 2. NADPH and CS additively enhanced fEPSPs. A. Representative fEPSP traces after exposure to 1 μM NADPH and 100 nM CS + NADPH are shown. B. Exposure to NADPH for 5 min increased the slope of the fEPSPs. Simultaneous application of NADPH + 20-100 nM CS had an additive effect in increasing the slope of the fEPSPs.

Figure 3. Involvements of NADPH oxidase and Rac1 in non-genomic action of MR.A. Pretreatment with 100 μM Apo blocked the non-genomic actions of the MR. B. Apo also blocked the increase of the slope of the fEPSPs induced by 1 μM NADPH. C. Summary of the actions of CS, Ald, and NADPH in the presence of Apo. The fEPSPs (%) increased after the drug application. Effect of 50 μM NSC 23766 (Rac inhibitor) on the fEPSP amplitude following exposure to 100 nM CS (C) or 1 μM NADPH (D) F. Summary of the actions of CS, Ald, and NADPH in the presence of NSC23766.

Figure 4. A. ERK1/2 phosphorylation ratio after application of CS, NADPH, and NADPH + CS. B. Simultaneous stimulation with CS + NADPH significantly increased Rac1 GTP activity. C and D. The actions of CS were blocked by Spi, Apo, and Temp, but not by RU486. E and F.

NADPH actions were blocked only by Apo and Temp. (* $p<0.05$, C and D, significant difference was shown vs. CS application vs. NADPH application.)

Figure 5. A. Pretreatment with NSC23766 blocked ERK1/2 phosphorylation induced by CS and NADPH. MEK inhibitor (100 μM U0126) decreased the ERK1/2 phosphorylation ratio (B) but did not block Rac1 GTP activation by CS (C).

Figure 6. Schematic diagram of the non-genomic actions of MR and NADPH oxidase activity.Figure 7. Membranous MR induced not only fEPSPs increase but also Rac1 activity. A. The effect of 500 nM CS-BSA on field EPSPs. Membrane-impermeable CS quickly increased fEPSPs. B. Rac1 activation by 500 nM CS-BSA. Rac1 was also activated by CS-BSA.

Figure 8. Another ROS mediator, EGF increased fEPSPs. EGF (20 ng/ml) also quickly increased fEPSP slopes, and application both EGF and CS further increased fEPSP.

Figure 9. Changes in the ERK1/2 phosphorylation ratio when the hippocampal slices were preincubated with Spi or Apo. We rearranged the sample gel though they were run on the same gel. There were no significant differences between the groups. Changes of normalized fEPSPs after application of Spi or Apo. There were no significant changes.

Figure 10. The hippocampal ERK1/2 phosphorylation was evaluated *in vivo*. Ten minutes after intraperitoneal injection of 10 mg/kg CS, rats were immediately sacrificed and hippocampi were removed. PBS/DMSO was used as vehicle.

Fig.1 F Kawakami-Mori *et al.*

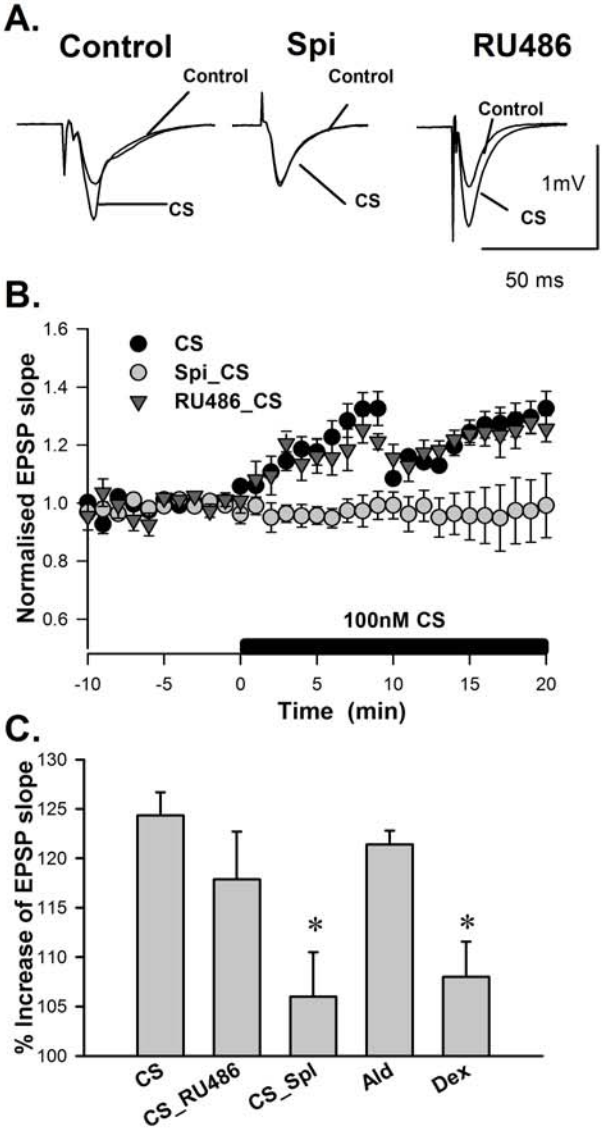
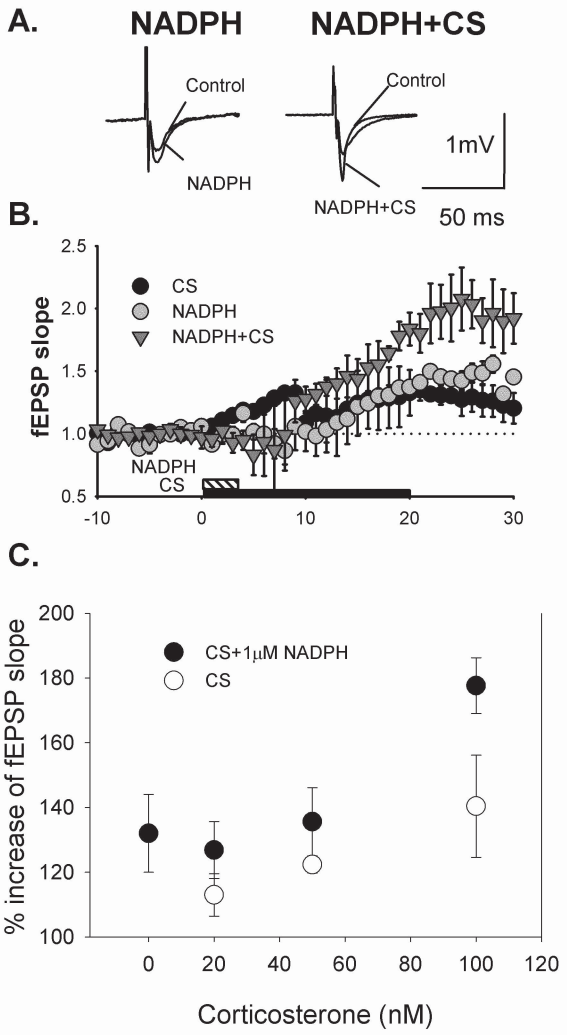


Fig. 2 F Kawakami-Mori *et al.*



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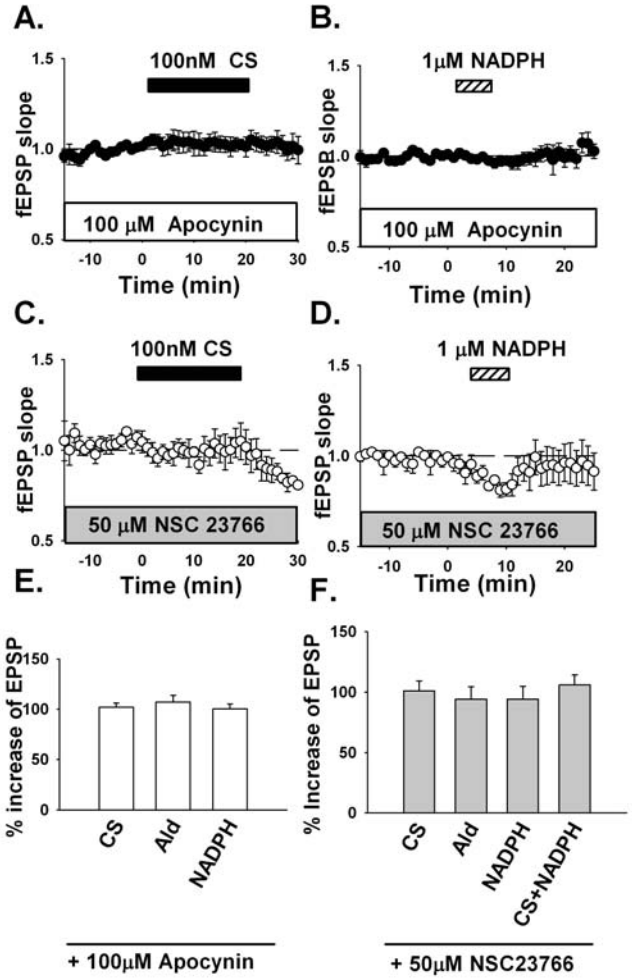

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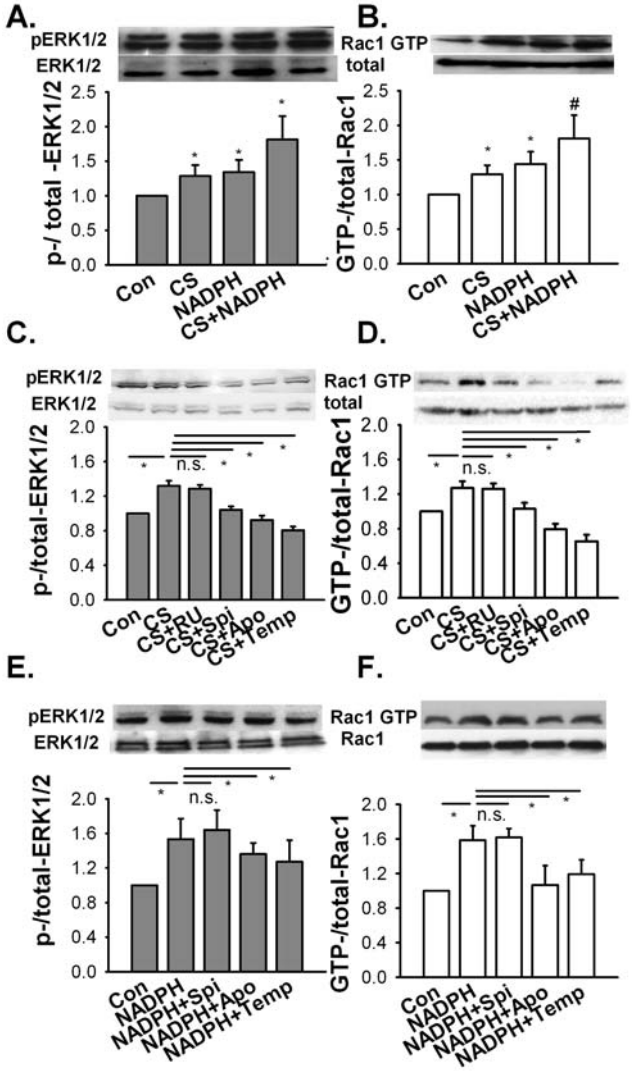

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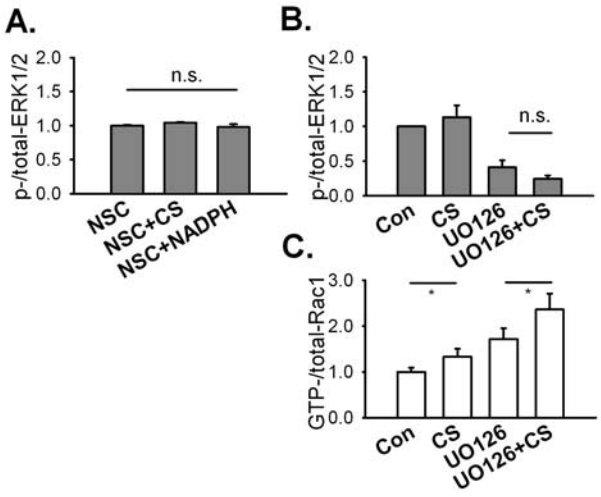

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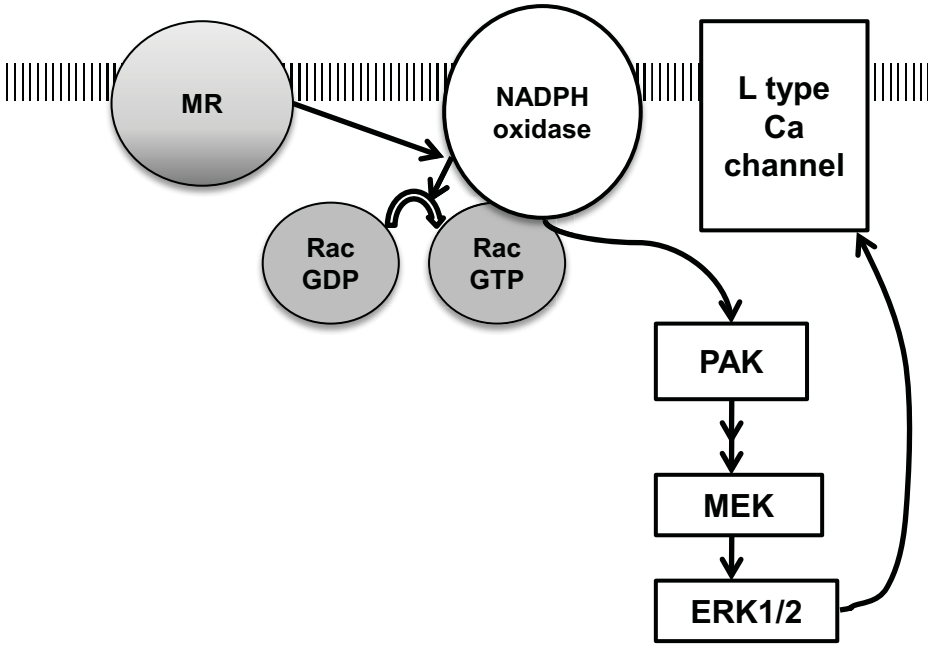

Fig. 7 Fumiko K Mori *et al.*

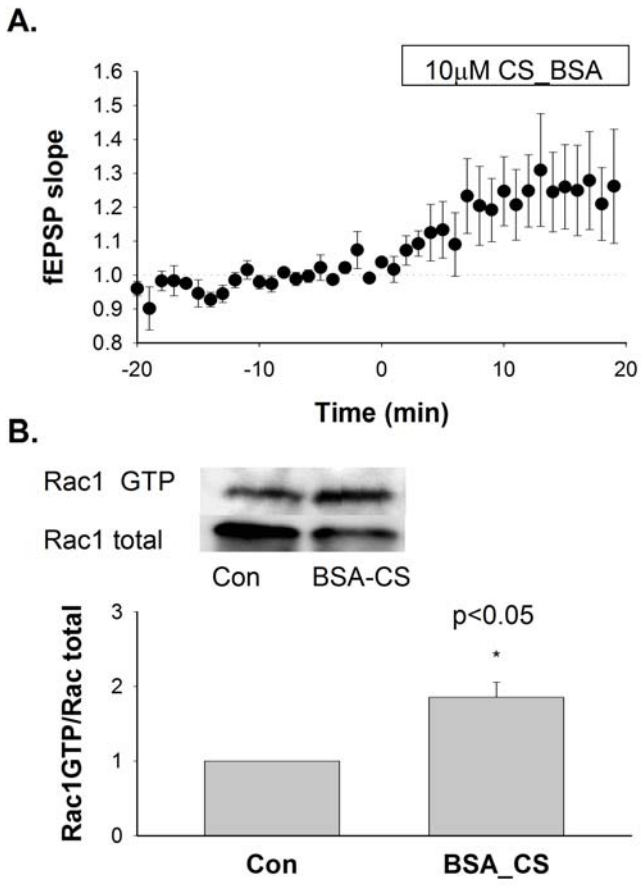

Fig. 8
Fumiko K Mori *et al.*

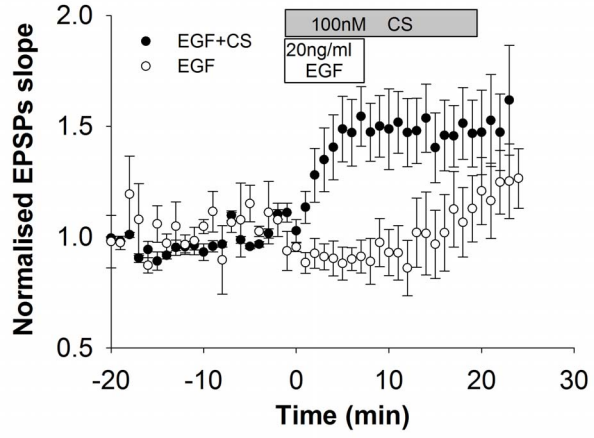

Fig. 9

F Kawakami-Mori *et al.*

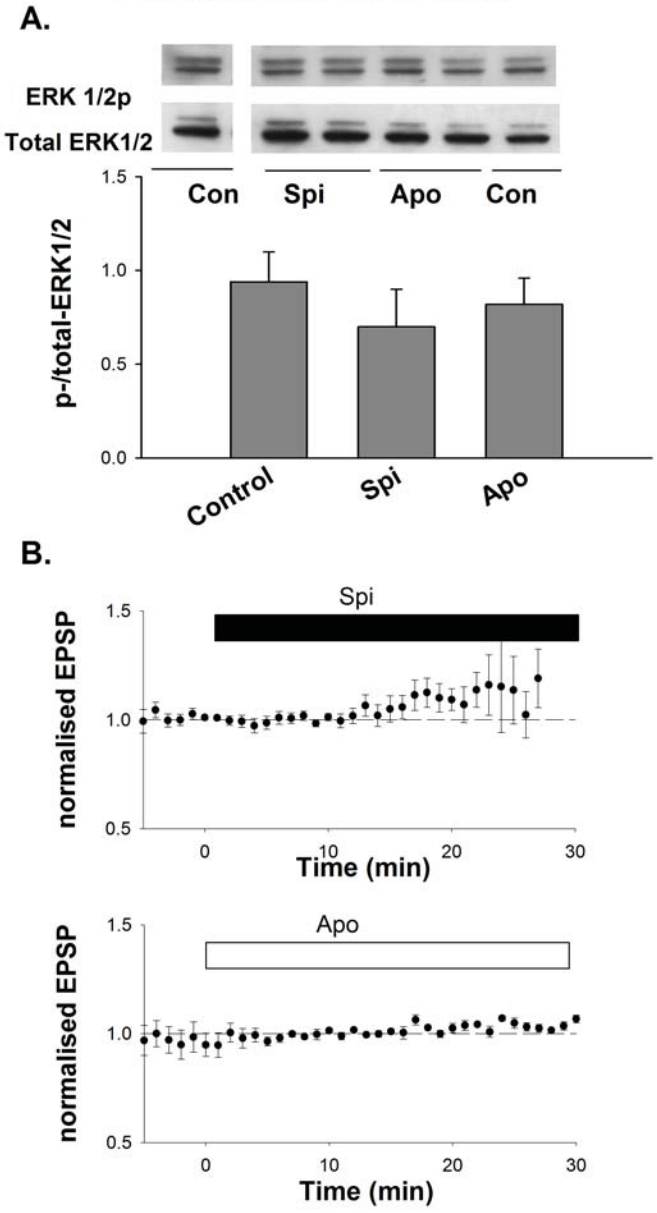

Fig. 10
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