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Co-Application of Corticosterone and Growth Hormone Upregulates NR2B Protein and Increases the NR2B:NR2A Ratio and Synaptic Transmission in the Hippocampus

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المعالجة المشتركة بهرمون النمو و هرمون الكورتيكوستيرون يزيد من بروتين (NR2B) و نسبة (NR2B:NR2A) ويؤدى الى زيادة سرعة انتقال الإشارات العصبية عبر نقاط الاشتباك العصبى في الحصين

غاده محمود وأيمن عامر

ABSTRACT: ***Objectives:*** This *in vitro* study aimed to investigate the possible mechanism underlying the protective effect of growth hormone (GH) on hippocampal function during periods of heightened glucocorticoid exposure. ***Methods:*** This study was conducted between January and June 2005 at the Joan C. Edwards School of Medicine, Marshall University, in Huntington, West Virginia, USA. The effects of the co-application of GH and corticosterone (CORT) were tested at different concentrations on the field excitatory postsynaptic potentials (fEPSPs) of the hippocampal slices of rats in two different age groups. Changes in the protein expression of N-methyl-D-aspartate receptor (NMDAR) subunits NR1, NR2B and NR2A were measured in hippocampal brain slices treated with either artificial cerebrospinal fluid (ACSF), low doses of CORT alone or both CORT and GH for three hours. ***Results:*** The co-application of CORT and GH was found to have an additive effect on hippocampal synaptic transmission compared to either drug alone. Furthermore, the combined use of low concentrations of GH and CORT was found to have significantly higher effects on the enhancement of fEPSPs in older rats compared to young ones. Both GH and CORT enhanced the protein expression of the NR2A subunit. Simultaneous exposure to low concentrations of GH and CORT significantly enhanced NR2B expression and increased the NR2B:NR2A ratio. In contrast, perfusion with CORT alone caused significant suppression in the NR1 and NR2B protein expression and a decrease in the NR2B:NR2A ratio. ***Conclusion:*** These results suggest that NMDARs provide a potential target for mediating the GH potential protective effect against stress and age-related memory and cognitive impairment.

Keywords: Growth Hormone; Corticosterone; Biological Stress; Postsynaptic Potentials; N-Methyl-D-Aspartate Receptors; Hippocampus; Neuronal Plasticity; Western Blotting.

الملخص: الهدف: هذه الدراسة تهدف للتحقق من آلية التأثير الواقي لهرمون النمو (GH) على وظيفة الحصينخلال فترات التعرض المتزايد لهرمون الجلوكورتيكويد. **الطريقة:** أجريت هذه الدراسة في الفترة ما بين يناير و يونيو 2005. تم اختبار المعالجة المشتركة بهرمون النمو و هرمون الكوتيكوستيرون (CORT) بجرعات مختلفه على سرعة انتقال الإشارات العصبية عبر نقاط الاشتباك العصبي في الحصين على فئتين عمريتين لفئران التجارب. درسنا التعبيرالبروتيني عن مستقبلات NMDAR (NR1، NR2B، و NR2A) في شرائح الحصين التى يتم معالجتها لمدة ثلاث ساعات بهرمون الكوتيكوستيرن أو هرمون الكوتيكوستيرون مع هرمون النمو في آن واحد أو السائل السحائى الاصطناعي. **النتائج:** وجدنا أن إضافة هرمون النمو لهرمون الكوتيكوستيرن له تأثير أقوى على زيادة سرعة انتقال الإشارات العصبية عبر نقاط الاشتباك العصبي فىالحصين فى جميع الجرعات التى استخدمت مقارنة باستخدام كل هرمون على حدا. وعلاوة على ذلك ، وجدنا أن الجمع بين استخدام جرعة منخفضة من هرمون النمو و هرمون الكوتيكوستيرن لها آثار أعلى بكثير على تعزيز انتقال الإشارات العصبية في الفئران الكبيرة فى السن مقارنة مع صغارالسن. أظهرنا أن كلا من هرمون النمو و هرمون الكوتيكوستيرن يزيد من البروتين NR2A. كما أثبتنا أن العلاج المشترك لجرعات منخفضة من هرمون النمو و الكوتيكوستيرون يزيد من بروتين NR2B ويؤدى إلى زيادة نسبة NR2A/NR2B مقارنة بهرمون الكوتيكوستيرن لوحدة . وكذلك وجدنا أن العلاج بهرمون الكورتيكوستيرون لوحدة يسبب نقص كبير في بروتينات NR1 و NR2B وانخفاض في نسبة NR2A/NR2B. **النتيجة:** تدل نتائج هذا البحث على أن هرمون النمو له تأثيروقائي من خلال تأثيره على مستقبلات NMDAR ضد ضعف الذاكرة و الإدراك المتعلقة بتقدم العمر و التوتر النفسي والعصب.

مفتاح الكلمات: هرمون النمو؛ كوتيكوستيرن؛ التوترالنفسي و العصبي؛ الإشارات العصبية المنتقلة عبر نقاط الاشتباك العصبي؛ مستقبلات ن ميثيل أسبارتيت؛ الحصين؛ تقوية الوصلات العصبية؛ لطخة وسترن.

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Advances in Knowledge

- *The perfusion of hippocampal brain slices in vitro with corticosterone (CORT) for a short period of time was found to enhance the synaptic transmission of the hippocampus. This result did not change significantly when the duration of treatment was extended.*
- *An additive effect on hippocampal synaptic function was observed when using low doses of growth hormone (GH) and CORT, resulting in the enhanced protein expression of the NR2A subunit of the N-methyl-D-aspartate receptors.*
- *Additionally, exposure to low doses of GH and CORT was demonstrated to reverse the CORT-induced downregulation of the NR2B subunit protein expression as well as the decrease in the NR2B:NR2A ratio.*

Application to Patient Care

- *The results of this study could explain the mechanism underlying the previously reported benefits of long-term GH treatment on memory and cognitive brain function among the elderly.*
- *A possible mechanism is also suggested regarding the beneficial effect of GH in combating memory deficits associated with anxiety disorders.*

Stress causes large amounts of glucocorticoids (GCs) to be released; these affect the brain and, in particular, the hippocampus.[1] Corticosteroids released during stress induce memory retrieval impairment; however, Moisan *et al.* found that this could be abolished by lowering the blood GC pool in corticosteroid-binding globulin knockout mice and restored by an infusion of corticosterone (CORT) in the hippocampus.[2] The early exposure of the immature hippocampus to a traumatic experience is known to be associated with post-traumatic stress disorder in humans, although there is no recall of the trauma in most cases.[3]

Previous studies have indicated that growth hormone (GH) therapy has a protective effect on cognitive brain function.[4,5] An earlier study demonstrated that GH had a beneficial effect on synaptic transmission in the *Cornu Ammonis* 1 (CA 1) area of rat hippocampi.[4] Ageing and repeated stress have been demonstrated to damage the hippocampus, a vulnerable structure of the central nervous system.[6] Research has shown that the overexpression of GH can reverse a stress-induced decrease in both the acquisition and long-term storage of fear memories, thus enhancing the ability of the hippocampus to combat stress.[5]

This study therefore aimed to investigate the possible beneficial effects of the co-application of GH and CORT at low concentrations on hippocampal synaptic transmission, compared to CORT alone. The effects of this co-exposure were tested at different concentrations on the field excitatory postsynaptic potentials (fEPSPs) in hippocampal slices from rats of two different age groups. A previous study proved that N-methyl-D-aspartate receptor (NMDAR) antagonist 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid impaired both long-term potentiation (LTP) and long-term depression (LTD) in the hippocampal dentate *gyrus* of rats.[7] It was therefore the hypothesis of the current study that CORT would modulate hippocampal synaptic function through the NMDARs. To this end, the change in protein expression of the NMDAR subunits NR1, NR2B and NR2A were tested in hippocampal brain slices treated with either artificial cerebrospinal fluid (ACSF), low concentrations of CORT alone or both CORT and GH.

Methods

This study was conducted between January and June 2005 at the Joan C. Edwards School of Medicine, Marshall University, in Huntington, West Virginia, USA. Male adult Sprague-Dawley rats were obtained from Hilltop Lab Animals Inc. (Scottdale, Pennsylvania, USA) and divided into two groups. The first group consisted of rats aged 2.5–3 months while the second group was made up of rats aged 15–18 months.

Rats were sedated using a mixture of 95% carbon dioxide (CO_2) and 5% oxygen (O_2) administered by inhalation in a closed container. The skull was opened and the brain was removed and submerged in chilled oxygenated (95% O_2 and 5% CO_2) low calcium and high magnesium ACSF composed of 124.0 of mM sodium chloride (NaCl), 26.0 mM of sodium bicarbonate, 3.0 mM of potassium chloride, 0.5 mM of calcium chloride, 5.0 mM of magnesium sulfate and 10.0 mM of glucose. The preparation of the hippocampal slices, synaptic stimulation and recording of field potentials were performed using a previously described method.[4]

In order to study the interactions of GH and CORT on synaptic transmission, hippocampal slices from the younger rats were divided into four groups: group 1 was perfused with GH only (at a concentration of 0.1, 0.5 or 2.0 nM) for two hours; group 2 was perfused with CORT only (at 0.5, 5.0 or 30.0 nM) for two hours; group 3 was perfused with both CORT and GH (either CORT at 0.5 nM plus GH at 0.1 nM, CORT at 5.0 nM plus GH at 0.5 nM or CORT at 30.0 nM plus GH at 2.0 nM) starting with CORT alone in the first hour and both hormones in the second hour, and group 4 was

perfused with both CORT and GH (CORT at 5.0 nM plus GH at 0.5 nM) starting with GH alone in the first hour and both hormones in the second hour. There were approximately five or six rats in each group. To test if any additive effect could be gained by applying CORT to slices pretreated with GH, hippocampal slices from older rats were divided into two groups: group 1 was perfused with GH alone (0.5 nM) for two hours and group 2 with GH (0.5 nM) for one hour followed by GH (0.5 nM) plus CORT (5.0 nM) for another hour. There were eight rats in each group. Rat CORT and recombinant rat GH were obtained from Cell Sciences Inc. (Canton, Massachusetts, USA).

Low impedance (3–4 MΩ) glass micropipettes filled with standard ACSF were placed into the *stratum radiatum* of area CA1 of the hippocampi to record the extracellular potentials. The signals were amplified (at a gain of 1,000) and filtered (0.1–3,000 Hz) using a DAM50 Bio-Amplifier (World Precision Instruments, Inc., Sarasota, Florida, USA). The signals were then digitised at 10 kHz using a NI 5102 digitiser (National Instruments Corp., Austin, Texas, USA) and stored on a personal computer. Postsynaptic potentials were evoked by delivering constant voltage stimuli through a bipolar stimulating electrode placed into the *stratum radiatum* of the rat hippocampal slices. Stimuli were delivered at 15-second intervals. A 15-minute baseline recording of the postsynaptic potentials using standard ACSF was taken before the application of any drug. The postsynaptic potentials evoked while using standard ACSF were quantified by measuring the slope of the linear portion of the initial response. Any changes in synaptic response caused by the drug treatment were expressed as a percentage of the change from the average baseline response prior to treatment.

The effects of CORT application alone and the possible interaction of CORT and GH on the protein expression of NMDAR subunits were examined by Western blotting. Hippocampal tissue was removed from the animals, sliced and incubated for one hour in ACSF. Hippocampal slices obtained from the same animal were separated into three interface chambers. The first chamber contained ACSF alone and served as a control, the second chamber contained ACSF and CORT (5.0 nM) and the third chamber contained ACSF, CORT (5.0 nM) and GH (1.0 nM). Each group was incubated for three hours. Immediately after the incubation period, the three groups were placed in dry ice, frozen and stored at –80 °C. To isolate hippocampal protein, slices were homogenised in a protein lysis buffer composed of 1% octylphenoxypolyethoxyethanol, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M of NaCl, 0.01 M of sodium phosphate (pH 7.2), 2 mM of ethylenediaminetetra-acetic acid (EDTA) and a 1% protease inhibitor cocktail. The hippocampal tissue proteins were then sonicated for <10 second bursts. Following this, they were centrifuged at a speed of 14,000 × g for 20 minutes at 4 °C. The supernatant solution was obtained and the total protein was estimated using the Bradford method.[8]

Equal amounts of total protein (200 µg) from the hippocampal slices treated with CORT, GH or both and the control hippocampal slices were separated on 8% polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes (Micron Separations Inc., Westborough, Massachusetts, USA).[9] The membranes were incubated for one hour at room temperature in 5% non-fat dry milk in a tris tween saline (T-T-S) solution (0.5% polysorbate 20, 10.0 mM of tris hydrochloride buffer solution [pH 8.0], 150.0 mM of NaCl and 0.2 mM of EDTA) to block non-specific binding sites.[10] The membranes were then incubated with a primary antibody overnight at 4 °C. To correct for possible loading differences, blots were probed with a primary antibody against neuron-specific enolase (NSE). Next, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) for one hour at room temperature. Both primary and secondary antibodies were diluted in 5% non-fat dry milk in T-T-S solution. The blots were washed again and proteins were detected using Amersham-Pharmacia ECL Prime Western Blotting Detection Reagents (General Electric Healthcare, Little Chalfont, Buckinghamshire, UK). X-ray autoradiograms were scanned and their images were saved.

The following primary antibodies were used: anti-NSE (AB951, Merck Millipore, Darmstadt, Germany) at a dilution of 1:6,000; anti-NMDAR1 (60021A, BD Biosciences Pharmingen, San Diego, California, USA) at a dilution of 1:1,000; anti-NMDAR2A (AB1555P, Merck Millipore) at a dilution of 1:500, and anti-NMDAR2B (N38120, BD Biosciences Transduction Laboratories, Franklin Lakes, New Jersey, USA) at a dilution of 1:2,000.

Data were collected and analysed using the Windows Whole Cell Program (WinWCP), Version 5.01 (University of Strathclyde, Glasgow, Scotland, UK). The western blotting bands representing different NMDAR protein expressions were quantified for their relative densities using the gel analysis densitometry tool from ImageJ, Version 1.31c (National Institutes of Health [NIH], Bethesda, Maryland, USA).[11–13] Additional analysis was completed using GraphPad Prism, Version 5 (GraphPad Software Inc., La Jolla, California, USA) and Origin® Graphing and Analysis software, Version 5.0 (OriginLab Corp., Northampton,

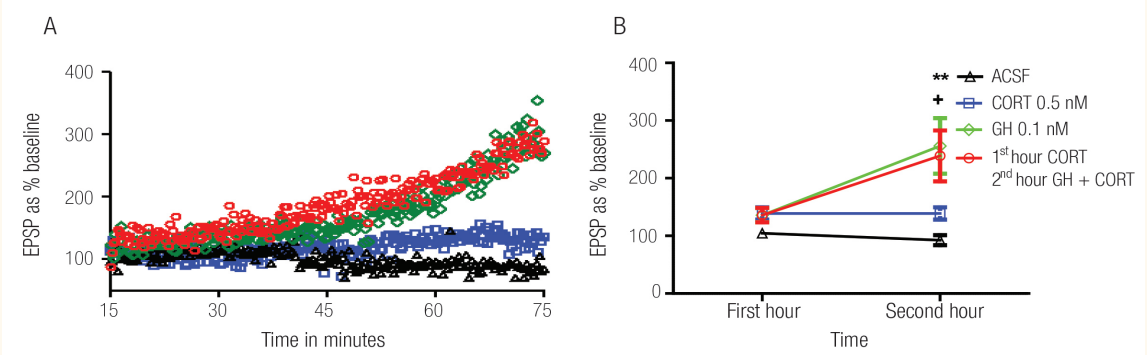

Figure 1 A & B: The effect of the co-application of corticosterone (CORT) at 0.5 nM and growth hormone (GH) at 0.1 nM in hippocampal slices pretreated with CORT (0.1 nM). **A:** Results of a one-hour treatment with either artificial cerebrospinal fluid (ACSF), CORT, GH or CORT plus GH. **B:** Average field excitatory postsynaptic potentials recorded over 10 minutes at the end of the first or second hour of treatment compared to ACSF (n = 5).

CORT = corticosterone; GH = growth hormone; ACSF = artificial cerebrospinal fluid; EPSP = excitatory postsynaptic potentials.

***P <0.01 for GH plus CORT compared to CORT; +P <0.05 for the second hour of treatment compared to the first hour.*

Massachusetts, USA). Statistical significance was assessed using paired and unpaired t-tests, as appropriate, and a *P* value of <0.05 was considered significant. Data were compared among the groups using a two-way analysis of variance (ANOVA) with the Bonferroni *post hoc* test.

All experimental procedures in this study were done in accordance with the NIH's Guide for the Use and Care of Laboratory Animals.[14] This study was approved by the Institutional Animal Care & Use Committee of the Joan C. Edwards School of Medicine at Marshall University.

Results

The effect of the co-application of CORT (0.5 nM) and GH (0.1 nM) in hippocampal slices pretreated with CORT are shown in Figures 1A and B. The application of CORT caused a significant enhancement of the fEPSPs compared to the baseline recordings or the control slices perfused with standard ACSF for 1–2 hours. The continuous treatment of CORT for the second hour did not demonstrate any significant changes to the enhanced fEPSPs compared to the first hour. No significant differences were found between exposure to GH for two hours or CORT plus GH for two hours. Perfusion with GH plus CORT in the second hour caused a significant additional enhancement of the fEPSPs caused by CORT alone for 1–2 hours. A significant interaction was found by the two-way ANOVA. The interaction accounted for 15.71% of the total variance ($F = 3.76$; df [3, 16]; $P = 0.032$). The treatment groups accounted for 27.78% of the total variance after adjusting for matching ($F = 6.82$; df [3, 16]; $P = 0.004$). Time accounted for 12.49% of the total variance after adjusting for matching ($F = 8.97$; df [1, 16]; $P = 0.009$).

The effect of the application of CORT (5.0 nM)

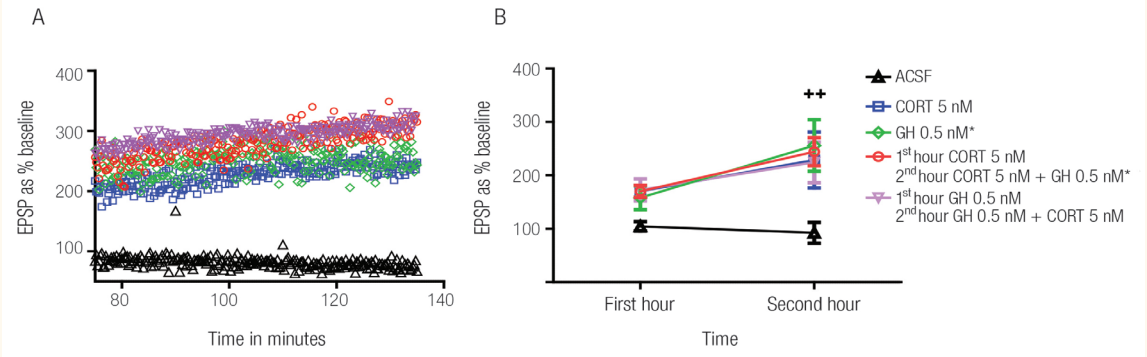

Figure 2 A & B: The effect of the co-application of corticosterone (CORT) at 5.0 nM and growth hormone (GH) at 0.5 nM in hippocampal slices pretreated with CORT or GH alone. **A:** Results of a one-hour treatment with artificial cerebrospinal fluid (ACSF), CORT, GH or CORT plus GH after pretreatment with GH or CORT. **B:** Average field excitatory postsynaptic potentials recorded over 10 minutes at the end of the first or second hour of treatment compared to ACSF (n = 5).

CORT = corticosterone; GH = growth hormone; ACSF = artificial cerebrospinal fluid; EPSP = excitatory postsynaptic potentials.

++P <0.01 for the second hour compared to the first hour in cases of GH or both GH and CORT in slices pretreated with CORT.

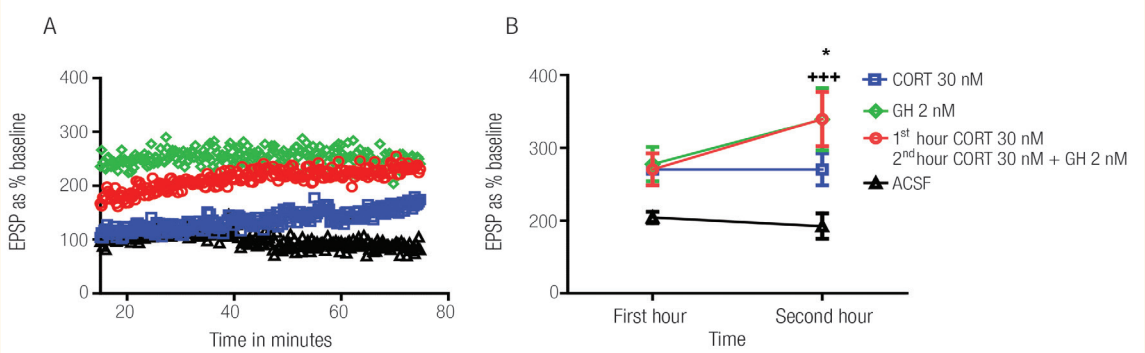

Figure 3 A & B: The effect of the co-application of corticosterone (CORT) at 30.0 nM and growth hormone (GH) at 2.0 nM in hippocampal slices pretreated with CORT. **A:** Results of a one-hour perfusion with artificial cerebrospinal fluid (ACSF), CORT, GH or CORT plus GH (0.5 nM). **B:** Average field excitatory postsynaptic potentials recorded over 10 minutes at the end of the first or second hour of treatment as compared to ACSF (n = 6).

CORT = corticosterone; GH = growth hormone; ACSF = artificial cerebrospinal fluid; EPSP = excitatory postsynaptic potentials.

**P <0.05 for CORT plus GH compared to CORT alone; +++P <0.01 for the second hour of treatment compared to the first hour.*

and GH (0.5 nM) on hippocampal slices primed with either drug alone was as follows. Treatment with either CORT, GH or CORT plus GH after pretreatment with GH or CORT alone caused a significant enhancement of the fEPSPs compared to the baseline recordings or the control slices (*P* <0.01). The continuous perfusion of the hippocampal slices with CORT for the second hour did not cause any significant changes in the enhanced fEPSPs compared to the first hour. The continuous perfusion of hippocampal slices with GH for the second hour had a moderately significant effect on enhancing fEPSPs compared to the first hour (*P* <0.01). Co-exposure to GH and CORT in the second hour caused a significant additional enhancement of the fEPSPs caused by CORT alone in the first hour (*P* <0.01). Conversely, co-exposure to GH and CORT in the second hour did not cause a significant additional enhancement of the fEPSPs caused by GH alone for 1–2 hours [Figures 2A & B]. In this regard, a non-significant interaction was found by the two-way ANOVA. The interaction accounted for 5.38% of the total variance (F = 1.51; df [4, 20]; *P* = 0.238). The treatment groups accounted for 28.35% of the total variance after adjusting for matching (F = 3.87; df [4, 20]; *P* = 0.017). Time accounted for 11.75% of the total variance after adjusting for matching (F = 13.14; df [1, 20]; *P* <0.005).

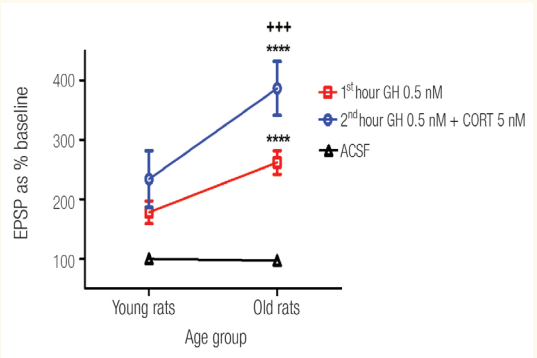

Figure 4: The effect of the co-application of corticosterone (CORT) and growth hormone (GH) on the field excitatory postsynaptic potentials (fEPSPs) of young and older rats. The average fEPSPs recorded over 10 minutes at the end of the first or second hour of treatment were compared to artificial cerebrospinal fluid (*P* <0.01), baseline recordings, the first hour in old rats only and old rats compared to young rats (n = 8).

CORT = corticosterone; GH = growth hormone; ACSF = artificial cerebrospinal fluid; EPSP = excitatory postsynaptic potentials.

*+++P <0.005 for the old rats compared to baseline recordings; ****P <0.001 for the old rats compared to young rats.*

The effect of CORT (30.0 nM) and GH (2.0 nM) in hippocampal slices pretreated by CORT was also observed. CORT caused a significant enhancement of the fEPSPs compared to the baseline recordings or the control slices (*P* <0.05). The continuous perfusion of hippocampal slices with CORT for the second hour did not cause any significant changes in the enhanced fEPSPs compared to the first hour. No significant differences were found between the perfusion of GH for two hours and CORT plus GH. Perfusion with GH plus CORT in the second hour caused a significant additional enhancement of the fEPSPs caused by CORT alone for 1–2 hours (*P* <0.05) [Figures 3A & B]. In this case, a significant interaction was found by the two-way ANOVA. The interaction accounted for 5.59% of the total variance (F = 9.17; df [3, 20]; *P* <0.001). The treatment groups accounted for 33.79% of the total variance after adjusting for matching (F = 4.27; df [3, 20]; *P* = 0.018). Time accounted for 3.81% of the total variance after adjusting for matching (F = 18.74; df [1, 20]; *P* <0.001).

The study also attempted to determine if the additive effect of CORT and GH in young adult rats would be seen in older rats as well. In the older rats

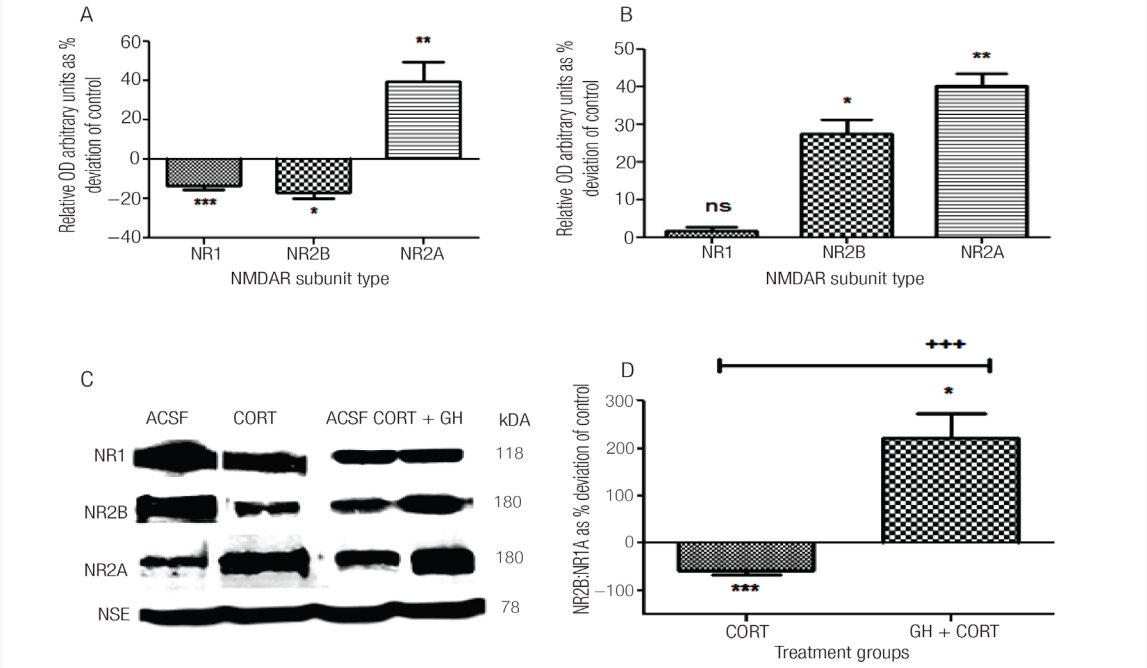

Figure 5 A–D: The effect of the co-exposure of corticosterone (CORT) and growth hormone (GH) on protein expression of N-methyl-D-aspartate receptor subunits in hippocampal brain slices. **A:** Effect of CORT (5.0 nM) perfusion for three hours (n = 9). **B:** Effect of CORT (5.0 nM) plus GH (1.0 nM) perfusion for three hours (n = 7). **C:** Sample western blot of the NR1, NR2A, NR2B subunits and neuron specific enolase. **D:** NR2B:NR2A ratio of protein expression in hippocampal slices which had been perfused with either artificial cerobrospinal fluid, CORT or CORT plus GH for three hours.

NMDAR = N-methyl-D-aspartate receptor; OD = optical density; ns = not significant; ACSF = artificial cerebrospinal fluid; CORT = corticosterone; GH = growth hormone; NSE = neuron specific enolase.

$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$ compared to the control; $^{+++}P<0.005$ compared to CORT-treated slices.

only, the effect of co-exposure to a low dose of CORT (5.0 nM) and GH (0.5 nM) in the second hour on the enhanced fEPSPs by GH alone in the first hour was significantly higher than baseline recordings and when compared to the first hour. The same was true in old rats compared to young rats in both the first and second hour [Figure 4]. A significant interaction was found by the two-way ANOVA. The interaction accounted for 6.33% of the total variance ($F = 3.67$; df [2, 21]; $P = 0.043$). The treatment groups accounted for 47.10% of the total variance after adjusting for matching ($F = 26.05$; df [2, 21]; $P < 0.001$). The rats' age accounted for 9.46% of the total variance after adjusting for matching ($F = 10.97$; df [1, 21]; $P < 0.005$).

The effect of CORT and GH on the protein expression of NMDAR subunits in hippocampal slices was also investigated. To test for the role of NMDARs in mediating the additive effect of GH and CORT on enhancing hippocampal synaptic transmission, hippocampal slices were perfused with ACSF alone, with CORT (5.0 nM) alone or with CORT plus GH (1.0 nM) in standard ACSF for three hours. The protein expressions of NMDAR subunits NR1, NR2A, NR2B and the NR2B:NR2A ratio were then sought. Three hours of treatment with CORT significantly decreased the expression of the NR1 and NR2B subunits and increased the NR2A subunit when compared to the control slices [Figures 5A & C]. Three hours of treatment with CORT plus GH significantly increased the expression of NR2A and NR2B and did not change the NR1 protein expression compared to the control slices [Figures 5B & C]. The exposure of hippocampal slices to CORT alone caused significant suppression in the NR2B:NR2A ratio compared to the control slices [Figure 5D]. Co-exposure of hippocampal slices to CORT plus GH for three hours significantly increased the NR2B:NR2A ratio when compared to the control slices as well as CORT exposure alone [Figure 5D].

Discussion

A normal ageing-induced decrease in GH is associated with a decrease in the learning and memory functions of the hippocampus and its glutamatergic function in both rats and humans.[15] A previous study found that GH has a beneficial effect on hippocampal synaptic function.[4] In addition, another study found that adrenal

steroids given at low to moderate doses over several hours enhanced synaptic and memory functions, while higher doses had the opposite effect.[16] The current study aimed to determine whether co-exposure to GH and CORT had any additive or subadditive effect on hippocampal synaptic transmission and if this effect differs with age. The results demonstrated that there is an additive effect on hippocampal fEPSPs in cases of co-exposure to GH and CORT when compared to CORT alone; this response was both concentration- and age-dependent. The additive effect was significantly higher in the older rats than in the younger rats, suggesting a protective effect of GH against stress, especially in old age. The beneficial effect of GH in both young and old rats is supported by a previous study which found that acute GH treatment improved spatial learning among both young and old rats in a radial maze.[17] In agreement with the current study's findings, Molina *et al*. reported that both GH and insulin-like growth factor-1 enhanced the fEPSPs of *in vitro* CA1 hippocampal slices to a similar extent in both young and older rats, mediated via a postsynaptic mechanism.[15]

While both GH and CORT have been evidenced to enhance synaptic transmission upon short-term exposure, the question remains as to how this additive effect affects the long-term storage of information. This is related to how much calcium enters the postsynaptic cells and for how long. A high calcium signal that only lasts seconds causes the activation of protein kinases and the insertion of more glutamate receptors by exocytosis, favouring LTP; in comparison, a low calcium signal for minutes causes the activation of phosphatases and an internalisation of the synaptic glutamate receptors by endocytosis and LTD.[18–20] NMDARs are the major route of calcium entry into the postsynaptic neurons. Corticostrerone enhances glutamate release and the overactivation of NMDARs, causing excitotoxicity.[21]

Molina *et al*. observed that GH had a direct effect on restoring age-induced NMDAR-mediated changes in the basal synaptic functions of the hippocampus in older rats.[15] The effects of co-exposure to GH and CORT on the protein expression of NMDARs compared to CORT alone were investigated in the current study. This revealed that perfusing hippocampal slices with CORT alone significantly increased the expression of NR2A, decreased NR2B and NR1 protein expression and decreased the NR2B:NR2A ratio. These results are supported by those of a previous study which found that implanting mice with a 21-day release CORT formulation (20 mg/kg/day) caused a decreased protein expression of postsynaptic density protein 95, NR1 and synaptopodin in the postsynaptic membranes.[22] Chronic restraint stress has been reported to induce a loss of dendritic spines and NMDAR subunits in cultured hippocampal CA1 neurons.[23] In addition, both acute and chronic stressors have been shown to inhibit neuronal synthesis and reduce cell survival through a mechanism that involves NMDARs in the dentate *gyrus*.[24]

However, the results of the present study found that the co-exposure of hippocampal slices to a low concentration of both CORT and GH for three hours significantly increased the NR2A and NR2B subunit expression compared to control slices. It also increased the NR2B:NR2A ratio, which was significantly suppressed by exposure to CORT alone, while no change in the NR1 protein expression was obtained. Although the NR1 subunit is obligatory for channel function, the NR2 composition plays an important functional modulatory role, affecting channel kinetics and pharmacology.[25]

The beneficial effects of the upregulation of NR2B on memory and synaptic transmission have been proven by several previous studies. One of these reported a reduction in synaptic plasticity due to a lower NR2B:NR2A ratio through a calcium/calmodulin-dependent protein kinase II-dependent mechanism.[26] Another study showed an enhancement of NR2B expression by the administration of D-cycloserine; this enhanced retention and the recall of fear extinction memories in the dentate *gyrus* as well as the CA1 and CA3 areas of the hippocampus.[27] Müller *et al*. found that NR2B upregulation enhances LTP in the CA1 synapses of the hippocampus, while NR2B inhibition in tissue from epileptic animals significantly increased epileptic activity and susceptibility to hyperexcitability.[28] Additionally, chronic restraint stress, acute CORT treatment or GC receptor agonists have been demonstrated to seriously deteriorate the contextual fear memory function of the hippocampus through an effect that involves protein transcription.[29,30] An acute and chronic stress-induced rise of GCs in the prefrontal cortex and hippocampus has been shown to cause changes in glutamate transmission and the cognitive functions that underlie stress-induced mental illnesses.[31]

In view of these findings and those of the literature, the ability of GH to reverse the effect of CORT through maintaining NR1, upregulating NR2B and increasing the NR2B:NR2A ratio may be greatly important in the management of age-related disorders in memory function. Furthermore, the use of GH may also have implications in the management of stress-related disorders, particularly depression and anxiety disorders. Further *in vitro* as well as *in vivo* studies are needed to demonstrate other mechanisms that

may explain the beneficial effects of GH in combating stress.

Conclusion

The results of this study demonstrated an additive effect of the co-exposure of low concentrations of GH and CORT on hippocampal synaptic transmission. This beneficial effect was significantly greater in older rats in comparison to younger rats, suggesting a possible protective effect of GH against age-related stress and memory impairment. Although short-term treatments of low concentrations of CORT for two hours enhanced the fEPSPs in the CA1 area of the *in vitro* rat hippocampi, it decreased NR2B expression, which is known to increase synaptic excitability and susceptibility to epilepsy, especially in old age. The co-exposure to both GH and CORT reversed these effects by the upregulation of NR2B and by increasing the NR2B:NR2A ratio, which is suppressed by CORT alone. The present study also demonstrated the direct role of GH in reversing CORT-related changes in the NMDAR subunits component of the hippocampus.

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References

1. Xiao L, Feng C, Chen Y. Glucocorticoid rapidly enhances NMDA-evoked neurotoxicity by attenuating the NR2A-containing NMDA receptor-mediated ERK1/2 activation. Mol Endocrinol 2010; 24:497–510. doi: 10.1210/me.2009-0422.
2. Moisan MP, Minni AM, Dominguez G, Helbling JC, Foury A, Henkous N, et al. Role of corticosteroid binding globulin in the fast actions of glucocorticoids on the brain. Steroids 2014; 81:109–15. doi: 10.1016/j.steroids.2013.10.013.
3. Poulos AM, Reger M, Mehta N, Zhuravka I, Sterlace SS, Gannam C, et al. Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. Biol Psychiatry 2014; 76:306–14. doi: 10.1016/j.biopsych.2013.10.007.
4. Mahmoud GS, Grover LM. Growth hormone enhances excitatory synaptic transmission in area CA1 of rat hippocampus. J Neurophysiol 2006; 95:2962–74. doi: 10.1152/jn.00947.2005.
5. Vander Weele CM, Saenz C, Yao J, Correia SS, Goosens KA. Restoration of hippocampal growth hormone reverses stress-induced hippocampal impairment. Front Behav Neurosci 2013; 7:66. doi: 10.3389/fnbeh.2013.00066.
6. Afadlal S, Polaboon N, Surakul P, Govitrapong P, Jutapakdeegul N. Prenatal stress alters presynaptic marker proteins in the hippocampus of rat pups. Neurosci Lett 2010; 470:24–7. doi: 10.1016/j.neulet.2009.12.046.
7. Medvedev NI, Popov VI, Rodriguez Arellano JJ, Dallérac G, Davies HA, Gabbott PL, et al. The N-methyl-D-aspartate receptor antagonist CPP alters synapse and spine structure and impairs long-term potentiation and long-term depression induced morphological plasticity in dentate gyrus of the awake rat. Neuroscience 2010; 165:1170–81. doi: 10.1016/j.neuroscience.2009.11.047.
8. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–54. doi: 10.1016/0003-2697(76)90527-3.
9. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350–4.
10. Spinola SM, Cannon JG. Different blocking agents cause variation in the immunologic detection of proteins transferred to nitrocellulose membranes. J Immunol Methods 1985; 81:161–5. doi: 10.1016/0022-1759(85)90132-2.
11. Humphries BJ, Newton RU, Abernethy PJ, Blake KD. Reliability of an electrophoretic and image processing analysis of human skeletal muscle taken from m.vastus lateralis. Eur J Appl Physiol Occup Physiol 1997; 75:532–6. doi: 10.1007/s004210050200.
12. Melrose J, Shen B, Ghosh P. Affinity and Western blotting reveal homologies between ovine intervertebral disc serine proteinase inhibitory proteins and bovine pancreatic trypsin inhibitor. Proteomics 2001; 1:1529–33. doi: 10.1002/1615-9861(200111)1:12<1529::AID-PROT1529>3.0.CO;2-J.
13. Sage D, Unser M. Easy Java Programming for Teaching Image Processing. From: www.bigwww.epfl.ch/publications/sage0101.html Accessed Jan 2014.
14. National Institutes of Health. Guide for the Care and Use of Laboratory Animals: Eighth edition. From: www.grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf Accessed: Aug 2014.
15. Molina DP, Ariwodola OJ, Weiner JL, Brunso-Bechtold JK, Adams MM. Growth hormone and insulin-like growth factor-I alter hippocampal excitatory synaptic transmission in young and old rats. Age (Dordr) 2013; 35:1575–87. doi: 10.1007/s11357-012-9460-4.
16. Joëls M. Corticosteroid effects in the brain: U-shape it. Trends Pharmacol Sci 2006; 27:244–50. doi: 10.1016/j.tips.2006.03.007.
17. Ramis M, Sarubbo F, Sola J, Aparicio S, Garau C, Miralles A, et al. Cognitive improvement by acute growth hormone is mediated by NMDA and AMPA receptors and MEK pathway. Prog Neuropsychopharmacol Biol Psychiatry 2013; 45:11–20. doi: 10.1016/j.pnpbp.2013.04.005.
18. Bear MF. Mechanism for a sliding synaptic modification threshold. Neuron 1995; 15:1–4. doi: 10.1016/0896-6273(95)90056-X.
19. Lisman JE, McIntyre CC. Synaptic plasticity: A molecular memory switch. Curr Biol 2001; 11:R788–91. doi: 10.1016/S0960-9822(01)00472-9.
20. Manabe T. [Molecular mechanisms for memory formation]. Brain Nerve 2008; 60:707–15.
21. Armanini MP, Hutchins C, Stein BA, Sapolsky RM. Glucocorticoid endangerment of hippocampal neurons is NMDA-receptor dependent. Brain Res 1990; 532:7–12. doi: 10.1016/0006-8993(90)91734-X.
22. Cohen JW, Louneva N, Han LY, Hodes GE, Wilson RS, Bennett DA, et al. Chronic corticosterone exposure alters postsynaptic protein levels of PSD-95, NR1, and synaptopodin in the mouse brain. Synapse 2011; 65:763–70. doi: 10.1002/syn.20900.

23. Pawlak R, Rao BS, Melchor JP, Chattarji S, McEwen B, Strickland S. Tissue plasminogen activator and plasminogen mediate stress-induced decline of neuronal and cognitive functions in the mouse hippocampus. Proc Natl Acad Sci U S A 2005; 102:18201–6. doi: 10.1073/pnas.0509232102.

24. Nacher J, McEwen BS. The role of N-methyl-D-asparate receptors in neurogenesis. Hippocampus 2006; 16:267–70. doi: 10.1002/hipo.20160.

25. Yoshimura Y, Ohmura T, Komatsu Y. Two forms of synaptic plasticity with distinct dependence on age, experience, and NMDA receptor subtype in rat visual cortex. J Neurosci 2003; 23:6557–66.

26. Barria A, Malinow R. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 2005; 48:289–301. doi: 10.1016/j.neuron.2005.08.034.

27. Ren J, Li X, Zhang X, Li M, Wang Y, Ma Y. The effects of intra-hippocampal microinfusion of D-cycloserine on fear extinction, and the expression of NMDA receptor subunit NR2B and neurogenesis in the hippocampus in rats. Prog Neuropsychopharmacol Biol Psychiatry 2013; 44:257–64. doi: 10.1016/j.pnpbp.2013.02.017.

28. Müller L, Tokay T, Porath K, Köhling R, Kirschstein T. Enhanced NMDA receptor-dependent LTP in the epileptic CA1 area via upregulation of NR2B. Neurobiol Dis 2013; 54:183–93. doi: 10.1016/j.nbd.2012.12.011.

29. Yun J, Koike H, Ibi D, Toth E, Mizoguchi H, Nitta A, et al. Chronic restraint stress impairs neurogenesis and hippocampus-dependent fear memory in mice: Possible involvement of a brain-specific transcription factor Npas4. J Neurochem 2010; 114:1840–51. doi: 10.1111/j.1471-4159.2010.06893.x.

30. Furukawa-Hibi Y, Yun J, Nagai T, Yamada K. Transcriptional suppression of the neuronal PAS domain 4 (Npas4) gene by stress via the binding of agonist-bound glucocorticoid receptor to its promoter. J Neurochem 2012; 123:866–75. doi: 10.1111/jnc.12034.

31. Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: The impact of stress and glucocorticoids on glutamate transmission. Nat Rev Neurosci 2011; 13:22–37. doi: 10.1038/nrn3138.