

## ORIGINAL ARTICLE

# Acute and chronic stress differentially regulate cyclin-dependent kinase 5 in mouse brain: implications to glucocorticoid actions and major depression

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Stress activates the hypothalamic–pituitary–adrenal axis, which in turn increases circulating glucocorticoid concentrations and stimulates the glucocorticoid receptor (GR). Chronically elevated glucocorticoids by repetitive exposure to stress are implicated in major depression and anxiety disorders. Cyclin-dependent kinase 5 (CDK5), a molecule essential for nervous system development, function and pathogenesis of neurodegenerative disorders, can modulate GR activity through phosphorylation. We examined potential contribution of CDK5 to stress response and pathophysiology of major depression. In mice, acute immobilized stress (AS) caused a biphasic effect on CDK5 activity, initially reducing but increasing afterwards in prefrontal cortex (PFC) and hippocampus (HIPPO), whereas chronic unpredictable stress (CS) strongly increased it in these brain areas, indicating that AS and CS differentially regulate this kinase activity in a brain region-specific fashion. GR phosphorylation contemporaneously followed the observed changes of CDK5 activity after AS, thus CDK5 may in part alter GR phosphorylation upon this stress. In the postmortem brains of subjects with major depression, CDK5 activity was elevated in Brodmann's area 25, but not in entire PFC and HIPPO. Messenger RNA expression of glucocorticoid-regulated/stress-related genes showed distinct expression profiles in several brain areas of these stressed mice or depressive subjects in which CDK5-mediated changes in GR phosphorylation may have some regulatory roles. Taken together, these results indicate that CDK5 is an integral component of stress response and major depression with regulatory means specific to different stressors, brain areas and diseases in part through changing phosphorylation of GR.

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## INTRODUCTION

Unpredicted short- and long-term stressful events have a significant influence over our daily activities.<sup>1–3</sup> The physiological response to stress includes integration of neural and humoral information at multiple levels in the brain and periphery, with the hypothalamic–pituitary–adrenal (HPA) axis playing a major role in coordination of central and peripheral components.<sup>4</sup> Glucocorticoids secreted from the adrenal cortex in response to stress and following activation of the HPA axis, not only facilitate appropriate behavioral responses to stress, but also generate adaptive responses in intermediary metabolism, feeding, immunity and reproduction.<sup>1,5</sup> Stress-induced increase in glucocorticoid secretion is normally curtailed through a negative feedback mechanism functional in the HPA axis to maintain overall homeostasis.<sup>2</sup> However, glucocorticoid secretion is sometimes unrestrained and maladaptive if the stress stimulus exceeds threshold of individual quality, intensity and/or chronicity, leading to development of an array of adverse effects, such as mood alteration, induction of anxiety and cognitive dysfunction, as well as immune suppression, osteoporosis and central obesity-associated insulin resistance and hyperlipidemia.<sup>2,5,6</sup>

The glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily, mediates actions of circulating glucocorticoids in local tissues.<sup>6,7</sup> GR is expressed virtually in all organs and tissues including the central nervous system, and acts as a ligand-dependent transcription factor for these steroids.<sup>8,9</sup> Upon binding to ligand glucocorticoids, GR changes transcriptional rates of glucocorticoid-responsive genes positively and negatively by communicating with specific DNA sequences located in their promoter regions and/or with numerous transcriptional regulatory molecules, including other transcription factors, transcriptional cofactors and the RNA polymerase II.<sup>6</sup> GR consists of three subdomains, N-terminal, middle DNA-binding and C-terminal ligand-binding domain.<sup>9</sup> Transcriptional activity of GR is regulated through a number of posttranslational modifications, including phosphorylation, acetylation, SUMOylation and ubiquitylation, to adjust its diverse actions to needs of local organs and tissues.<sup>6</sup>

The cyclin-dependent kinase 5 (CDK5) is a serine/threonine kinase and a member of the cyclin-dependent kinase family.<sup>10,11</sup> Like other family members, CDK5 is activated through formation of a heterodimer with its partner molecules, such as p35 and p39.<sup>12,13</sup> CDK5 is essential for neuronal development and

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survival.<sup>12–18</sup> In addition to these physiologic actions, aberrant CDK5 activation has a pivotal role in the pathogenesis of neurodegenerative disorders, such as Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease.<sup>10</sup> In the latter condition, aberrantly activated CDK5 contributes to the hyperphosphorylation of the cytoskeletal protein tau and subsequent neurodegeneration.<sup>19</sup> Activated CDK5 may also contribute to stress-induced depression-like behavior in rodents, in line with our previous suggestion that stress may trigger pathological processes shared by Alzheimer's disease and major depression.<sup>20–23</sup>

We previously reported that CDK5 phosphorylates multiple serine residues of the human GR, such as serines 203, 211 and 213 (serines 212, 220 and 224 of mouse GR) located in its N-terminal domain, and modulates GR-induced transcriptional activity in rat primary neuronal cells.<sup>7,24</sup> We therefore examined the CDK5 activity and its protein levels in prefrontal cortex (PFC) and hippocampus (HIPPO) of the mice exposed to acute or chronic stress, as well as levels of phosphorylated GR and messenger RNA (mRNA) expression of 10 glucocorticoid-responsive and/or stress-related genes. We further examined these parameters in postmortem brains of the subjects with major depression. Our results suggest that acute and chronic stress differentially regulate CDK5 activity/expression in a brain area-specific fashion, which may further contribute to pathophysiology of stress-triggered major depression.

## MATERIALS AND METHODS

### Animal treatments and sampling of brain tissues

All animal procedures used in this manuscript were approved by the Government of Upper Bavaria, and were accordance with European Union Directive 2010/63/EU. Male mice of the C57Bl6/6NCrI strain, aged 12–14 weeks were maintained (three to four per cage) under standard conditions (21 °C; 12:12 light–dark cycle, lights on at 0600 h) at least for 2 weeks before the experiments. Mice were then randomly assigned to one of the following experimental groups: unstressed control (control,  $n=10$  for each time point), acute stress (AS,  $n=8$  for each time point), chronic unpredictable stress (CS,  $n=10$  for each time point) or acute corticosterone-injected (CORT injection or CI,  $n=10$  for each time point). The CI group received a single intraperitoneal injection of corticosterone (20 mg kg<sup>-1</sup> in 40% cyclodextrin). Mice of the AS group were immobilized for 1 h in a 50 ml Falcon tube (with ventilation holes).<sup>25</sup> Mice of the AS and CI groups, and their corresponding untreated controls were killed at 0, 1, 3 and 24 h after the treatment for obtaining blood and brain samples. The CS paradigm consisted of exposure to two different pairs of stressors either in the subjective day or in subjective night for 1 week, and then the same cycle was repeated for 4 weeks.<sup>25–27</sup> The following seven pairs of stressors were applied during 1-week cycle: (Day 1) 1-h cage placement with orbital shaking (100 r.p.m.)+12-h damp bedding; (Day 2) 30-min immobilization in a 50 ml Falcon tube (as explained above for AS)+12-h placement in a tilted (45°) cage; (Day 3) 1-h exposure to white noise with overcrowding by placing four animals in a plastic box (10 × 10 × 5 cm) with ventilation holes +1-h cage placement with orbital shaking (100 r.p.m.); (Day 4) 1-h immobilization in a 50 ml Falcon tube+12-h placement in a tilted (45°) cage; (Day 5) 1-h exposure to white noise and overcrowding (see above) +12-h damp bedding (see above); (Day 6) 1-h cage placement with orbital shaking (100 r.p.m.)+12-h placement in a tilted (45°) cage; and (Day 7) 1-h immobilization in a 50 ml Falcon tube+24-h lights on (no dark period). Animals exposed to CS were killed on Day 29 by decapitation, which was carried out during the daily period of light, between 0800 and 1000 h. Trunk blood was collected at the time of euthanasia and serum corticosterone levels were measured in the harvested sera by using a commercial radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA, USA). Brains were rapidly excised from the skull just after euthanasia, and PFC and HIPPO were dissected out, snap-frozen and stored at –80 °C until assays were performed (see below).

### Human brain samples

Thirty-three cryopreserved human brain samples of particular brain areas (13 HIPPO, eight PFC, four inferior frontal gyrus and eight Brodmann's area 25 (BA25)) obtained from eight deceased patients (five males and three

females) with major depression and 14 control subjects (eight males and five females) without any psychiatric disorders but with other disorders, such as cancer, heart failure and chronic obstructive pulmonary disease, were donated from the Netherlands Brain Bank (Amsterdam, The Netherlands) and from the Human Brain and Spinal Fluid Resource Center of the West L.A. Healthcare Center (Los Angeles, CA, USA). Medication records were not available. The mean ages of disease and control subjects were 67.5 ± 5.09 (s.e.) and 74.7 ± 3.50, respectively ( $P=0.244$ , not significant).

### Brain sample preparation for CDK5 kinase assays and western blots

Cryopreserved brain samples were crushed in a mortar and homogenized by using a dounce homogenizer and the NE-PER Nuclear and Cytoplasmic Extraction Kit (PIERCE Biotechnology, Rockford, IL, USA). Protein concentrations were measured by using the BCA Protein Assay Kit (PIERCE Biotechnology). Protein extracts were stored at –80 °C until use.

### CDK5 kinase assay

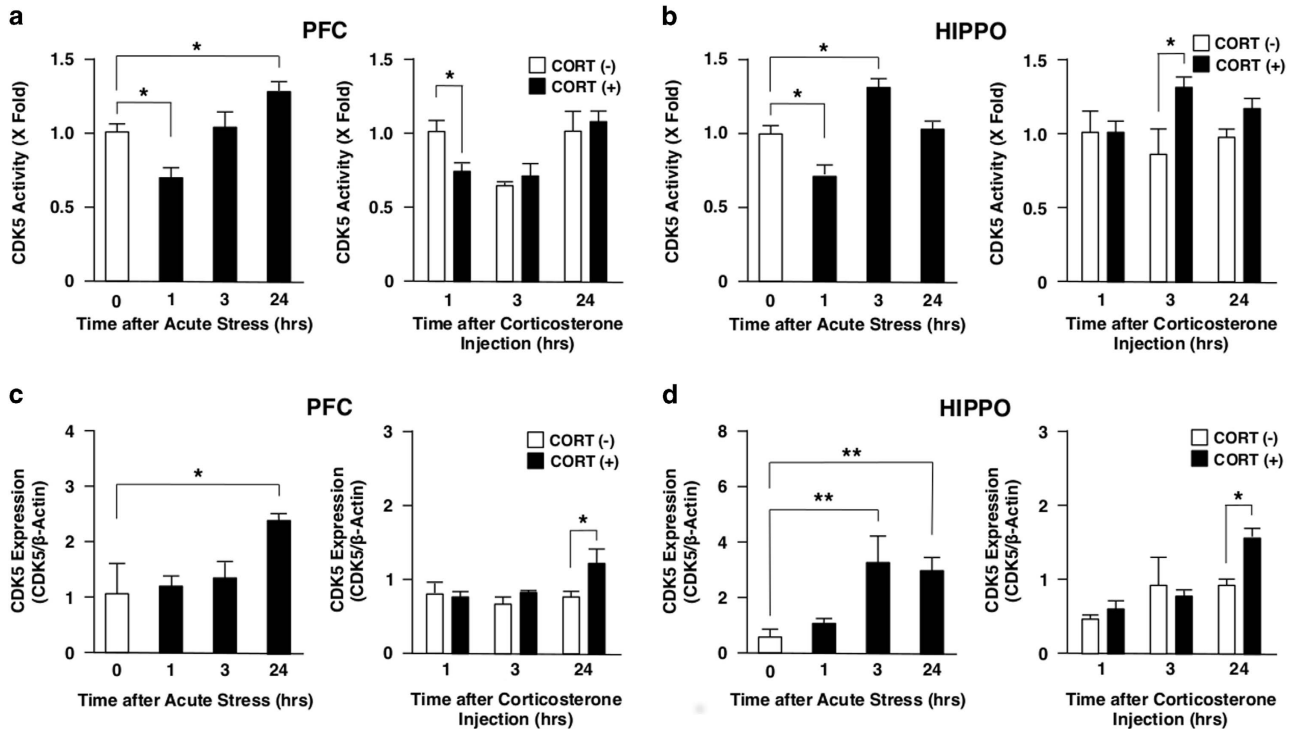
CDK5 kinase assays were performed as previously described.<sup>28</sup> Briefly, homogenized brain samples were incubated overnight at 4 °C with the anti-CDK5 antibody (C8; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the antibody-CDK5 complex was precipitated with the Protein A-Sepharose beads for 2 h at 4 °C. Immunoprecipitates were then washed three times with the lysis buffer, and then once with the kinase buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM MgCl<sub>2</sub>, 10 mM sodium fluoride and 1 mM sodium orthovanadate. CDK5 kinase assays were performed in the same buffer containing 1 mM DTT, 0.1 mM ATP and 0.185 MBq of [ $\gamma$ -<sup>32</sup>P]ATP with 20 ng of histone H1 as a control substrate. The reaction was performed in a final volume of 50  $\mu$ l, and was incubated for 60 min at 30 °C, stopped subsequently by addition of 10% SDS sample buffer followed by heating at 95 °C for 5 min. Twenty-five microliter aliquots of the reaction mixture were plotted on a Whatman p80 paper, washed, dried and were placed in a scintillation vial for counting.

### Western blot

Western blots were performed for evaluating expression levels of CDK5, total GR and the GR phosphorylated at serine 211 (human) or 220 (mouse). Western blots were also conducted for  $\beta$ -actin as an internal control. Proteins were separated on 4–12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA), and were transferred to a nitrocellulose membrane. After transfer, the membrane was incubated with the appropriate primary antibody obtained from Santa Cruz Biotechnology (anti-GR, CDK5 and  $\beta$ -actin antibody) or the anti-GR antibody specifically reacting to the GR phosphorylated at serine 211 (human) or 220 (mouse) (Cell Signaling Technology, Danvers, MA, USA), followed by incubation with the horseradish peroxidase-conjugated secondary antibody. The blotted proteins were then detected with the Amersham ECL Detection Reagents (GE Healthcare Bio-Science, Piscataway, NJ, USA). Blotted membranes were used repeatedly for different antibodies by stripping bound antibodies with the Restore western blot stripping buffer (Thermo Fisher Scientific, Waltham, MA, USA). The optical density of bands was measured using the Image J software (National Institutes of Health, Bethesda, MD, USA). The band density was first corrected with the background density obtained from proximity of the target band in X-ray films. The optical density of proteins of interest was further normalized against those for  $\beta$ -actin (also adjusted with the same procedure) to obtain relative expression levels. Relative phosphorylated GR levels were calculated by correcting band density of phosphorylated GR with that of total GR.

### SYBR Green real-time PCR analysis for mRNA expression

Total RNA was purified from frozen brain samples using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA). Complementary DNA was synthesized using the TaqMan Reverse Transcription Reagents and oligo-dT as a primer (Applied Biosystems, Foster City, CA, USA). PCR was then performed in the 7500 Real-time PCR system (Applied Biosystems), as previously described.<sup>29,30</sup> Amplification was performed in a three-step cycle: 95 °C for 10 min, then 40 cycles of the reaction consisting of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min, followed by a melting curve analysis. Ten target genes, which are reported to be glucocorticoid-responsive and/or regulated by stressful stimuli (glucocorticoid-responsive/stress-related genes) were the arginine



**Figure 1.** Acute stress and corticosterone injection differentially regulate CDK5 activity and protein expression in mouse PFC and HIPPO. Mice were immobilized in a 50 ml Falcon tube for 1 h or injected intraperitoneally with corticosterone (CORT, 20 mg kg<sup>-1</sup>) and were killed at 0, 1, 3 and 24 h after the treatment. CDK5 activity (a and b) and protein expression (c and d) were examined respectively with the CDK kinase assay and with the western blot using anti-CDK5 antibody. Bars represent mean ± s.e. values of fold kinase activity against baseline (the condition at time 0 for AS, and the condition at 1 h in the absence of CORT for CORT injection) and the protein levels of CDK5. \**P* < 0.05, \*\**P* < 0.01, compared with the conditions indicated. HIPPO, hippocampus; PFC, prefrontal cortex.

vasopressin (AVP),<sup>31</sup> brain-derived neurotrophic factor (BDNF),<sup>32,33</sup> corticotropin-releasing hormone (CRH),<sup>31,34</sup> FBJ murine osteosarcoma viral oncogene homolog (*cFos*),<sup>35</sup> *FOSB/ΔFOSB*,<sup>36</sup> 5-hydroxytryptamine (serotonin) receptor 1A (*HTR1A*),<sup>37</sup> inhibitor of DNA-binding 3 (*ID3*),<sup>38</sup> nischarin,<sup>39</sup> nuclear speckle splicing regulatory protein 1 (*NSRP1*),<sup>40</sup> protein phosphatase 1 regulatory subunit 10 (*PPP1R10*)<sup>41</sup> and serum/glucocorticoid-regulated kinase 1 (*SGK1*).<sup>42</sup> Primer pairs used in SYBR Green real-time PCR were designed so that the sequence spanning between a forward and a reverse primer contains at least one intron. Their sequences are listed in Supplementary Table 1. C<sub>t</sub> values of the examined genes were normalized with those of the ribosomal protein large P0 (*RPLP0*) gene, and fold changes were obtained by using the comparative C<sub>t</sub> method.<sup>24</sup>

#### Statistical analysis

For all the experiments/measurements, sample sizes were chosen to achieve enough statistical power. Experiments/measurements were repeated at least three times. Randomization and blinding procedures were not used for animal studies. All the data are presented as means ± s.e. in figures. Statistical analysis was performed by using the Student's *t*-test with two-tailed value and one-way analysis of variance with Bonferroni correction in the GraphPad PRIZM Version 5 (GraphPad Software, San Diego, CA, USA). The data variance was similar between the groups compared in each statistical analysis. Statistical significance was set at *P* < 0.05.

## RESULTS

### Acute and chronic stress differentially modulate CDK5 activity in mouse prefrontal cortex and hippocampus

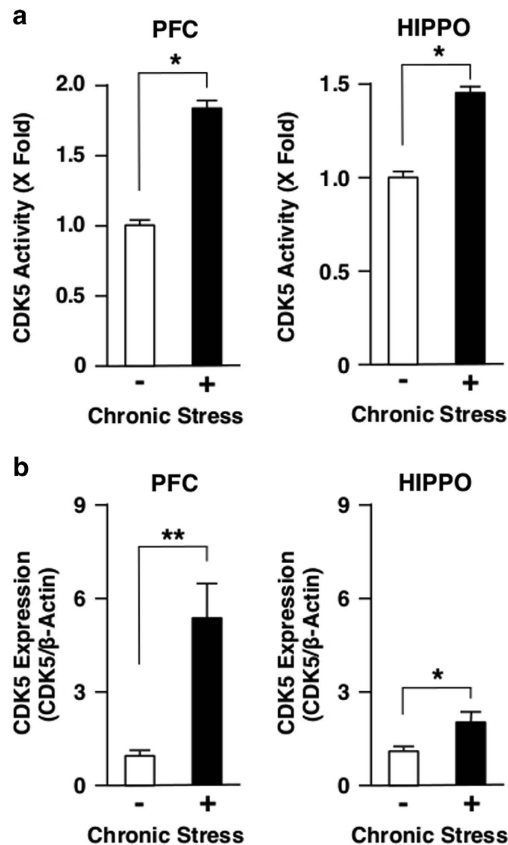
To evaluate impact of acute and chronic stress on CDK5 kinase activity, we focused on two brain areas, the PFC and the HIPPO, because they are both major targets of stress,<sup>1</sup> and are implicated in mood, emotion and cognitive functions.<sup>43,44</sup> Since brain has a strong compensating activity and duration of stress significantly

influences direction and magnitude of response,<sup>2</sup> we used two stress stimuli, the acute (AS) and chronic stress (CS): practically, we respectively used 1 h immobilization and chronic unpredictable stress. The latter consists of four cycles of 7-day stress treatment in which two different types of stressors were applied each day. Since these stressors stimulate the HPA axis and increase serum corticosterone levels,<sup>2</sup> we also injected this steroid intraperitoneally to examine the effect of systemic glucocorticoid elevation on these brain regions. Indeed, 1 h immobilization AS significantly increased serum corticosterone concentrations after 1 h, which returned to the basal levels in 24 h (Supplementary Figure 1a). Corticosterone injection (20 mg kg<sup>-1</sup>) markedly increased serum levels of this steroid, which also returned to the basal levels after 24 h, although vehicle injection itself mildly increased corticosterone levels as well (Supplementary Figure 1b).

Both in PFC and in HIPPO, AS developed a biphasic effect on the CDK5 activity: it suppressed the CDK5 activity 1 h after the exposure, while it reciprocally increased after 24 h in PFC (Figure 1a) and after 3 h in HIPPO (Figure 1b). CORT injection also suppressed the CDK5 activity 1 h after the injection in PFC (Figure 1a) and increased it after 3 h in HIPPO (Figure 1b), indicating a potential cause–effect relationship between elevated CORT secretion and the early changes in CDK5 activity observed upon exposure to AS.

We measured CDK5 protein levels using western blots to address the mechanisms underlying alteration of the CDK5 activity by AS. We found elevation of CDK5 protein levels 24 h and 3 h after exposure to AS in PFC and HIPPO, respectively. CDK5 protein levels mildly elevated only at a 24-h time point after CORT injection both in PFC and HIPPO (Figures 1c and d). These results indicate that alteration in the CDK5 protein levels may partly account for its elevated kinase activity observed at later time





**Figure 2.** Chronic stress differentially regulates CDK5 activity and protein expression in mouse PFC and HIPPO. Mice were treated with chronic unpredictable stress for 28 days. CDK5 activity (**a**) and protein expression (**b**) were examined, respectively, with the CDK5 kinase assay and with the western blot using anti-CDK5 antibody. Bars represent mean  $\pm$  s.e. values of fold kinase activity against baseline (the condition in the absence of chronic stress) and the protein levels of CDK5 in the presence (closed bars) or absence (open bars) of chronic stress. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the conditions indicated. HIPPO, hippocampus; PFC, prefrontal cortex.

points upon AS, while the effects of AS on CDK5 protein expression were only partially mediated by secreted CORT.

We next examined impact of CS to the CDK5 activity in mouse PFC and HIPPO. CS markedly increased the CDK5 activity both in these brain regions (Figure 2a), indicating that chronic exposure to stress is a strong stimulator of this kinase activity. CS also increased CDK5 protein levels both in PFC and HIPPO (Figure 2b).

CDK5 may phosphorylate GR upon acute stress

We previously reported that CDK5 modulates GR-induced transcriptional activity through phosphorylation of this receptor.<sup>24</sup> Thus, we examined the effect of acute or chronic stress on the phosphorylation of GR in PFC and HIPPO, using western blots with the antibody specifically reacting to the GR phosphorylated at serine 220, the residue known to be phosphorylated by CDK5.<sup>24,33</sup> AS increased GR phosphorylation 24 h after the exposure in PFC, while it suppressed GR phosphorylation after 1 h and increased after 3 h in HIPPO (Figures 3a and b). CORT injection increased GR phosphorylation after 1 h in PFC, while the injection increased it after 1 h and 3 h in HIPPO (Figures 3a and b), consistent with a well-known fact that GR is phosphorylated just after binding to ligand.<sup>45</sup> These results indicate that the elevation of CDK5 activity by AS, respectively, observed after 24 h and 3 h in PFC and HIPPO may account for the

elevation of GR phosphorylation in these areas. AS reduced CDK5 activity at 1 h time point in PFC and HIPPO, whereas CORT injection increased the phosphorylation at the same time point. Thus, mixture of these opposing activities observed upon AS might have caused the mild changes in the GR phosphorylation at early time points upon exposure to this stress. In contrast to AS, CS reduced GR phosphorylation in PFC but not in HIPPO (Figure 3c), while CDK5 activity was elevated in these brain areas (Figure 2b). Thus, unknown mechanisms appear to be functional in the regulation of GR phosphorylation following exposure to CS.

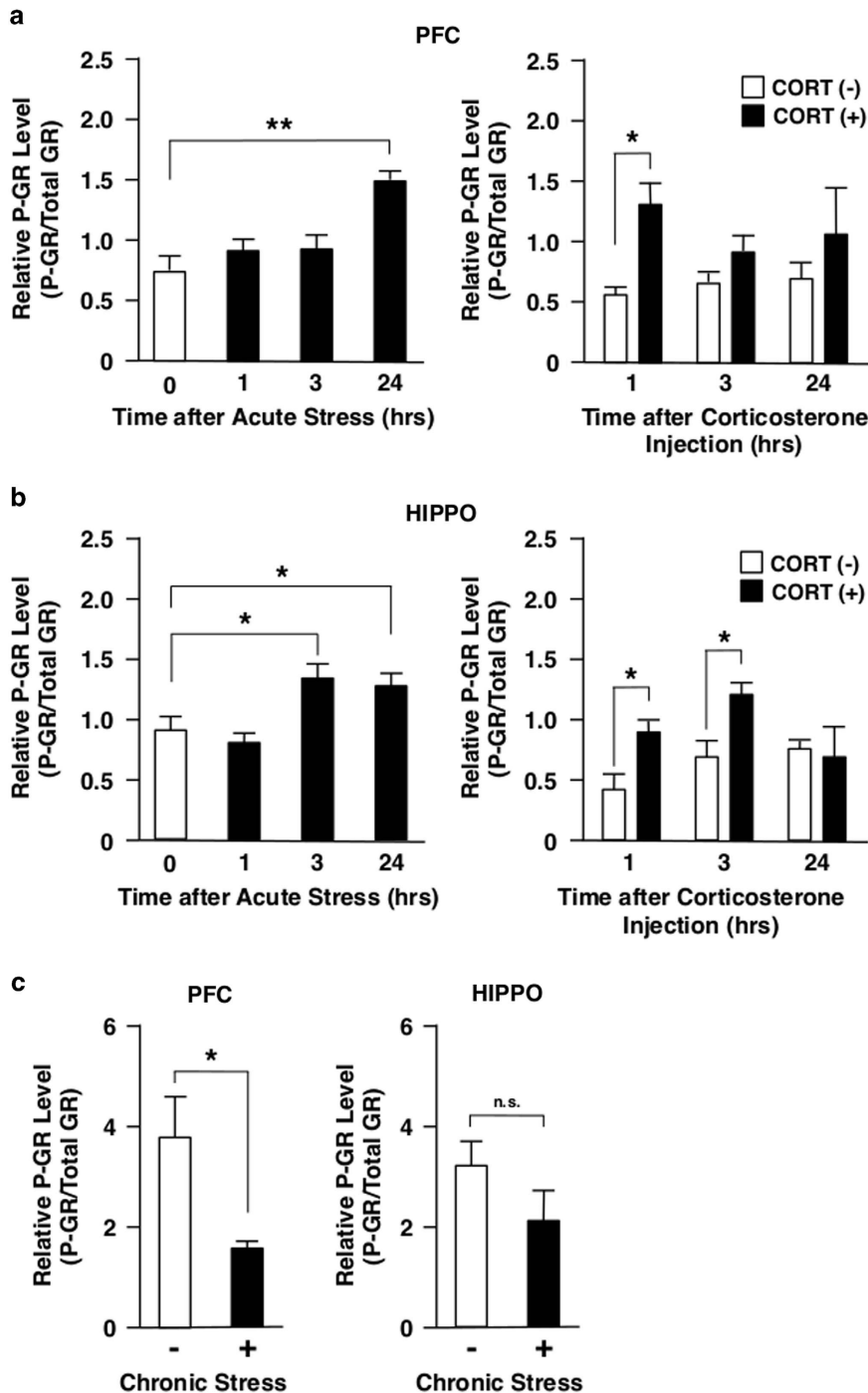
Distinct mRNA expression profiles of the glucocorticoid-responsive/stress-related genes in acute and chronic stress

We examined mRNA expression profiles of a panel of glucocorticoid-responsive/stress-related genes in PFC and HIPPO upon exposure to AS, CS or CORT injection to examine whether there is a link between their expression and the observed alteration in the CDK5 kinase activity, its protein levels and phosphorylation of GR upon these stimuli. Indeed stimulation/suppression of transcript expression is a primary function of ligand-activated GR, while we showed in the current analysis that CDK5 in some part participated in the phosphorylation of GR upon AS.

Among the glucocorticoid-responsive/stress-related genes that we used, CORT injection increased in PFC *Id3* mRNA levels after 24 h and *Avp* mRNA levels after 3 and 24 h (Figure 4a), whereas it increased in HIPPO *Id3* mRNA abundance after 3 and 24 h (Figure 4b). These results suggest that *Id3*, and possibly *Avp* in PFC, is the gene responsive to the elevation of circulating corticosterone in these brain areas, although several other genes, such as *Bdnf*, *Crh* and *Sgk1*, are reported to respond to glucocorticoids *in vivo* or *in vitro*.<sup>33,34,42,46</sup> We then examined mRNA expression of all these glucocorticoid-responsive/stress-related genes in PFC and HIPPO of the mice exposed to AS or CS. Results of the genes demonstrating statistically significant changes after exposure to AS are shown in Figures 4c and d. We also displayed results of the all-examined genes in Supplementary Figures 2a and b for convenience of comparison. AS markedly increased mRNA expression of *Avp*, *Bdnf*, *FosB/ΔFosB*, *Nischarin* and *Rgd*, and suppressed *cFos* and *Ppp1r10* mRNA expression in PFC, whereas it increased *Crh* mRNA abundance and suppressed *Avp*, *Id3*, *FosB/ΔFosB* and *Nischarin* mRNA expression in HIPPO. These results suggest that the effect of AS on the mRNA expression of these genes is brain region-specific. Since the changes of mRNA expression profiles observed upon AS were completely different from those observed after CORT injection (particularly expression of *Id3* and *Avp*), some regulatory mechanisms in addition to the elevation of circulating corticosterone are functional in the former case. In contrast to the effect of AS and CORT injection, CS stimulated mRNA expression of *Htr1a* and *cFos* in PFC and that of *FosB/ΔFosB* in HIPPO (Figures 4e and f), indicating that CS has quite different regulatory activity on the mRNA expression of glucocorticoid-responsive/stress-related genes compared with AS.

CDK5 activity was elevated in BA25 of depressive subjects

Since chronic stress is associated with pathophysiology of major depression and GR is a potential target of anti-depressant drugs,<sup>22,47,48</sup> we measured CDK5 activity in PFC and HIPPO of postmortem brains of subjects with major depression (Figure 5a). In addition, we measured CDK5 activity in two specific subregions of the PFC implicated in mood and symptoms related to mood disorders, namely the subgenual anterior cingulate cortex (BA25) and the inferior frontal gyrus (corresponding to BA44, 45 and 47) to avoid a possibility of overlooking the phenomena specific to these brain areas.<sup>49–52</sup> We found that the CDK5 activity was elevated in BA25 of these depressive subjects, while the activity in their PFC, HIPPO and inferior frontal gyrus was similar to that of control subjects. Our ability to detect the change in BA25 most



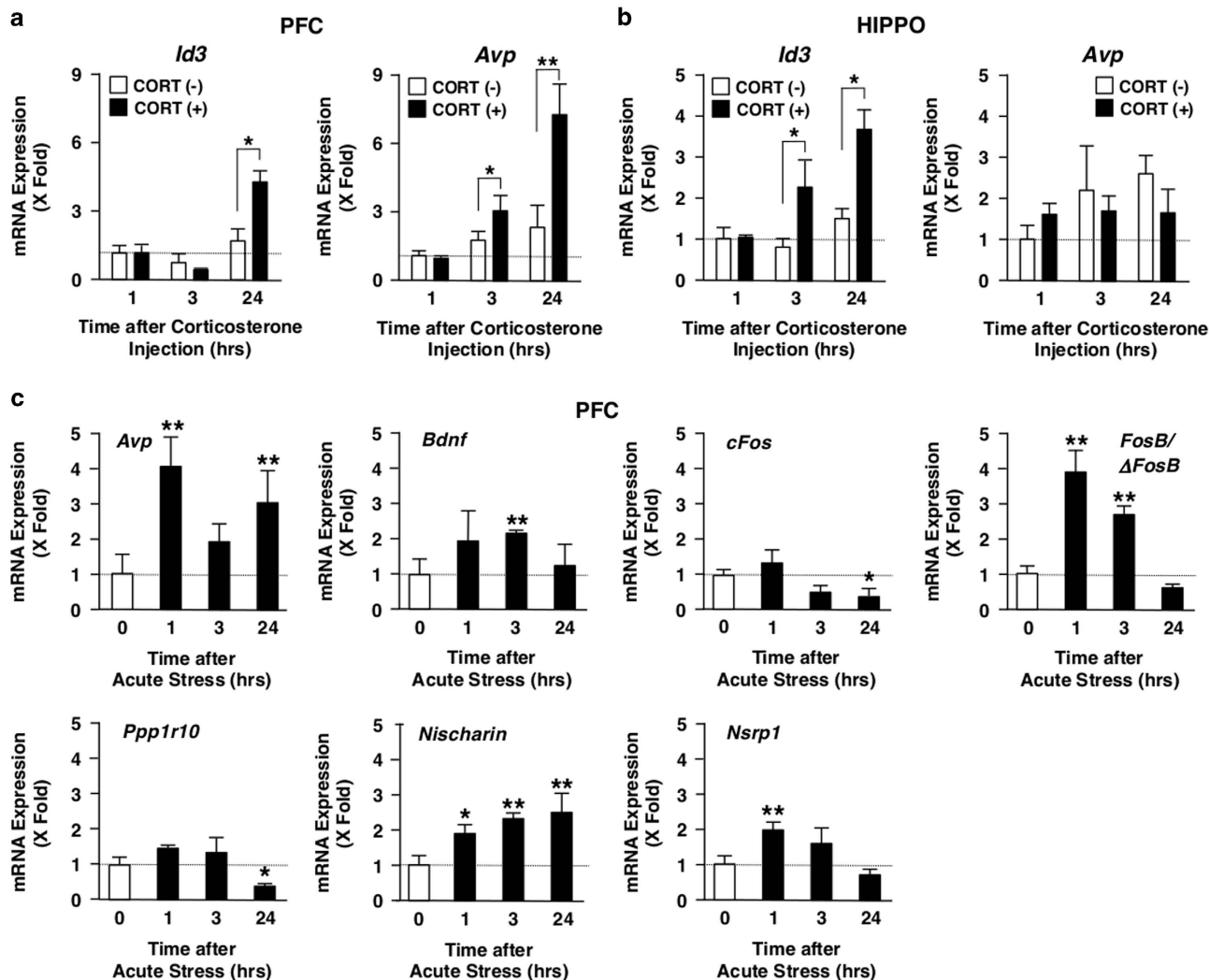
**Figure 3.** Acute stress, corticosterone injection and chronic stress differentially regulate phosphorylation of GR at serine 220 in mouse PFC and HIPPO. Mice were either immobilized in a 50 ml Falcon tube for 1 h (acute stress), injected with corticosterone (CORT, 20 mg kg<sup>-1</sup>) intraperitoneally (**a** and **b**) or treated with chronic unpredictable stress for 28 days (**c**) and their PFC and HIPPO were obtained at the time points indicated. The GR phosphorylated at serine 220 (P-GR) was examined with western blots using a specific antibody for this phosphorylated GR and relative P-GR levels were calculated by correcting band density of P-GR with that of total GR. Bars represent mean  $\pm$  s.e. values of relative P-GR levels. \* $P < 0.05$ ; \*\* $P < 0.01$ ; n.s., not significant, compared with the conditions indicated. GR, glucocorticoid receptor; HIPPO, hippocampus; PFC, prefrontal cortex.

likely reflects its 'small size' and 'dilution effects' when whole PFC was analyzed.

Due to little availability of brain samples of these subjects, we were able to examine CDK5 protein levels and GR phosphorylation only in HIPPO. CDK5 protein levels were increased in depressive

patients compared with control subjects, while GR phosphorylation decreased in this brain area (Figures 5b and c).

We further examined mRNA expression of some glucocorticoid-responsive/stress-related genes in HIPPO and BA25 of post-mortem brain samples of depressive patients and control subjects. mRNA expression of most of the genes successfully examined was



**Figure 4.** mRNA expression of glucocorticoid-responsive/stress-related genes in mice injected with corticosterone, or exposed to acute or chronic stress. **(a and b)** Corticosterone injection stimulates *Avp* and *Id3* mRNA expression, respectively, in mouse PFC and HIPPO. Mice were injected intraperitoneally with corticosterone (CORT, 20 mg kg<sup>-1</sup>), and were killed at 0, 1, 3 and 24 h after the treatment. mRNA expression of indicated genes was measured with the SYBR Green real-time PCR using their specific primers. Bars represent mean  $\pm$  s.e. values of fold mRNA expression of *Avp* and *Id3* in PFC **(a)** and HIPPO **(b)** in the presence (closed bars) or absence (open bars) of CORT injection. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the conditions indicated. **(c and d)** Acute stress differentially regulates mRNA expression of stress-related genes in mouse PFC and HIPPO. Mice were immobilized in a 50 ml falcon tube for 1 h, and were killed at 0, 1, 3 and 24 h after the treatment. mRNA expression of indicated genes was measured in PFC **(c)** and HIPPO **(d)** with the SYBR Green real-time PCR using their specific primers. Bars represent mean  $\pm$  s.e. values of fold mRNA expression of indicated genes in PFC **(c)** and HIPPO **(d)**. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the conditions indicated. **(e and f)** Chronic stress differentially regulates mRNA expression of glucocorticoid-responsive/stress-related genes in mouse PFC and HIPPO. Mice were treated with chronic unpredictable stress for 28 days, and were killed after the treatment. mRNA expression of indicated genes was measured in PFC **(e)** and HIPPO **(f)** with SYBR Green real-time PCR using their specific primers. Bars represent mean  $\pm$  s.e. values of fold mRNA expression of indicated genes of stressed mice to that of control unstressed mice in PFC **(e)** and HIPPO **(f)**. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the condition without chronic stress. HIPPO, hippocampus; mRNA, messenger RNA; PFC, prefrontal cortex.

suppressed in HIPPO (*SGK*, *ID3*, *BDNF*, *cFOS*, *FOSB/ΔFOSB* and *RGD*) and in BA25 (*SGK*, *ID3*, *BDNF*, *FOSB/ΔFOSB* and *RGD*) of the patients compared with control subjects (Figures 5d and e).

## DISCUSSION

Stress is a well-known trigger of major depression and has been more recently implicated in the pathogenesis of cognitive disorders, including Alzheimer's disease.<sup>53</sup> These pathological conditions are believed to reflect exposure of the brain to high levels of circulating glucocorticoids.<sup>2</sup> Since we previously reported that CDK5 regulates GR transcriptional activity by phosphorylating this receptor,<sup>24</sup> we examined in the current study the potential

role of CDK5 in stress response by focusing on mouse PFC and HIPPO. We also used several postmortem brain tissues including those of PFC and HIPPO from human subjects with major depression. Our present work indicates a potentially important role of CDK5 in the molecular mechanisms underlying stress response and major depression. They show that CDK5 activity is differentially regulated by stress in different brain areas, with duration of stress being a key variable.

We found that acute stress caused a biphasic change in CDK5 activity initially suppressing but increasing afterwards in PFC and HIPPO. The mechanism(s) underlying this characteristic change is (are) not known, but glucocorticoid secreted in response to acute stress may participate in this regulation as CORT injection caused

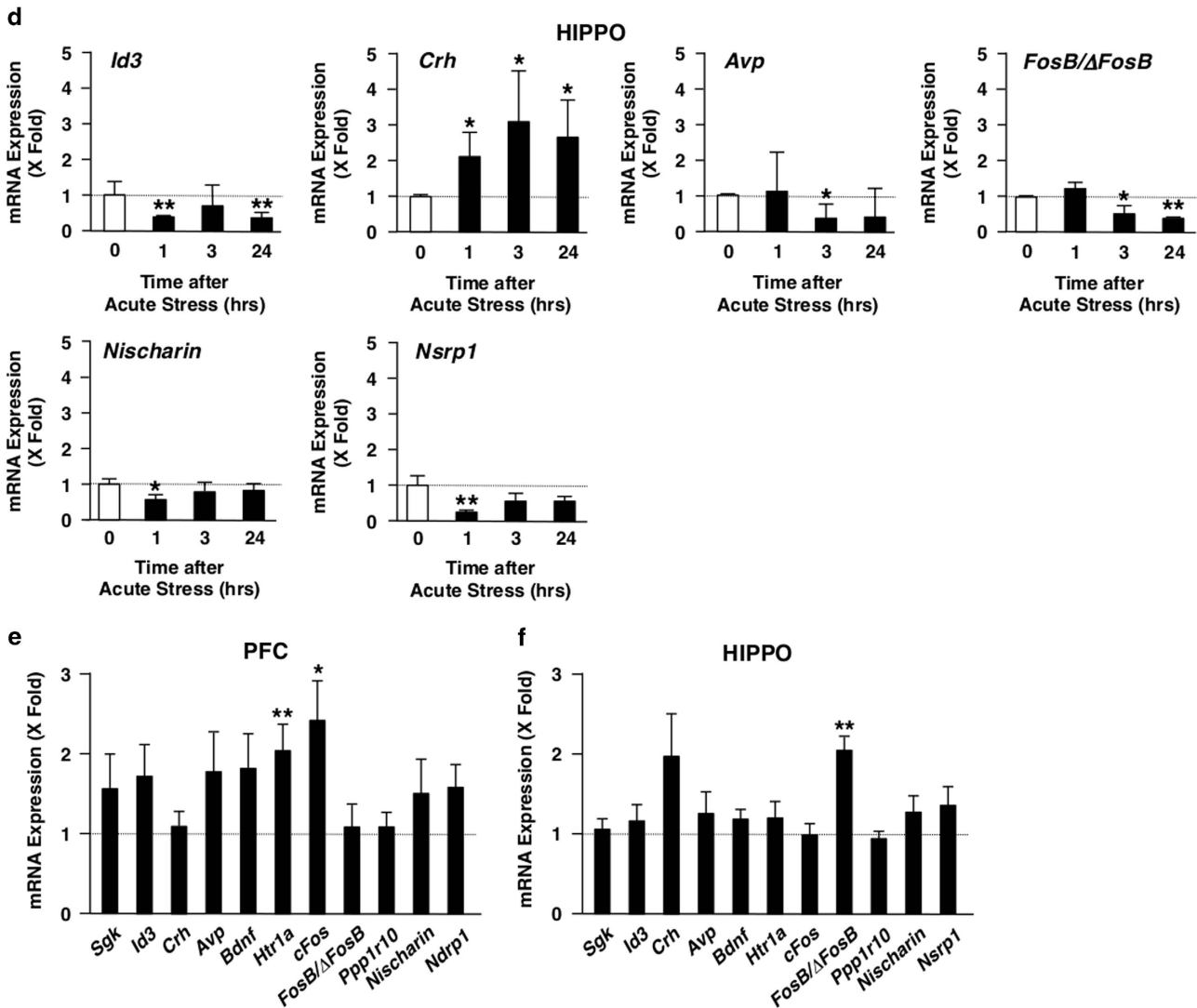


Figure 4. Continued.

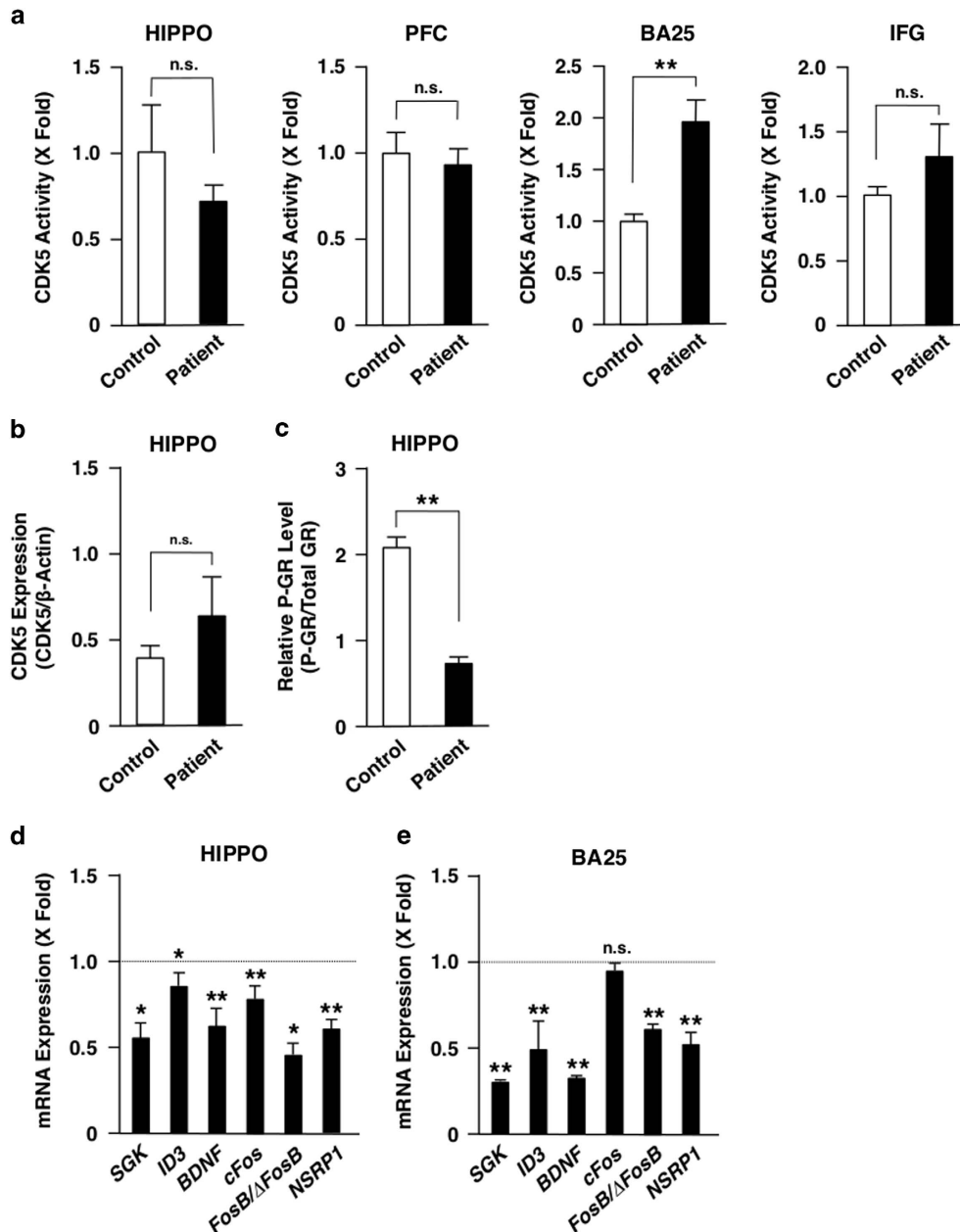
in some part similar changes. Acute stress-induced biphasic change in CDK5 activity further indicates presence of yet unknown biological pathway(s) that regulate(s) this kinase activity in a compensative fashion: the pathway through changing the CDK5 protein levels may be one potential mechanism based on our results, while those through phosphorylation of CDK5 itself, such as by Wee1 and Myt1, as well as through alteration in the level of CDK5 activators, such as p25, p35 and p39,<sup>13</sup> might also have roles.

We found that chronic stress increased the CDK5 kinase activity and its protein levels in PFC and HIPPO. These results support an idea that CDK5 is a component of adaptive response against chronic stress, consistent with previous reports obtained in microarray studies.<sup>54,55</sup> Our results may also provide mechanistic explanation to the known link between chronic stress and cognitive impairment, and further, Alzheimer's disease. Indeed, individuals in chronic stress are more likely to develop cognitive impairment than those without stress, and exposure to chronic stress is a potential risk factor for the development of Alzheimer's disease in later life.<sup>53,56,57</sup> Mice under chronic mild stress significantly impairs spatial memory and increases tau phosphorylation in hippocampus, while CDK5 is essential for adult hippocampal neurogenesis required for memory consolidation.<sup>56,58</sup> Thus, our results are in line with the emerging hypothesis that

major depression and Alzheimer's disease lie in a common trajectory and that the two diseases may share common mechanisms.<sup>20-23</sup>

Since chronic stress is associated with major depression,<sup>47,48</sup> we examined the CDK5 kinase activity in PFC and HIPPO in postmortem brains of patients with major depression. This analysis failed to reveal any differences between depressed and control subjects in these brain areas, while increased CDK5 activity was found in BA25. The latter finding is important because BA25, a subregion of the PFC, is implicated in major depression as well as treatment response.<sup>49-51</sup> However, comparing or extrapolating data obtained in the mouse study to those found in the human study suffers some limitations to be considered; First, these species sometimes respond differently to stressors.<sup>59</sup> Second, sampling procedures were different between them. Last, we used male mice in the animal study but enrolled both male and female subjects in the human study. It should be noted that stress appears to exert different effects on male and female rodent behavior,<sup>60</sup> and that men and women may differ in their vulnerability to depression and expression of depressive symptoms.<sup>61</sup>

We examined influence of stress-activated CDK5 on the glucocorticoid/GR actions by evaluating levels of the GR phosphorylated at serine 211 (human) or 220 (mouse), known targets of CDK5. In our animal experiments, CDK5 appeared to



**Figure 5.** Depressive patients show variable CDK5 activity, its protein expression, GR phosphorylation and mRNA expression of glucocorticoid-responsive/stress-related genes in some brain areas. **(a)** Depressive patients demonstrate altered CDK5 activity in BA25, but not in PFC, HIPPO and IFG. The CDK5 activity was evaluated in HIPPO, PFC, BA25 and IFG of deceased patients with major depression or deceased control subjects. Bars represent mean  $\pm$  s.e. values of fold kinase activity in depressive patients (closed bars) or in control subjects (open bars; mean values of control subjects as '1'). \*\* $P < 0.01$ , n.s., not significant, compared with the conditions indicated. **(b)** and **(c)** Depressive patients have unaltered CDK5 protein levels but reduced GR phosphorylation in HIPPO. Levels of the CDK5 protein **(b)** and the GR phosphorylated at serine 211 (P-GR) **(c)** were evaluated in HIPPO of deceased patients with major depression or deceased control subjects. Bars represent mean  $\pm$  s.e. values of relative CDK5 protein and P-GR levels in depressive patients (closed bars) or in control subjects (open bars). \*\* $P < 0.01$ ; n.s., not significant, compared with the conditions indicated. **(d)** and **(e)** Depressive patients demonstrate altered mRNA expression of glucocorticoid-responsive/stress-related genes in HIPPO and BA25. mRNA expression of the indicated genes was measured in HIPPO **(d)** and BA25 **(e)** of deceased patients with major depression and deceased control subjects with SYBR Green real-time PCR using their specific primers. Bars represent mean  $\pm$  s.e. values of fold mRNA expression of indicated genes of depressive patients to those of control subjects. \* $P < 0.05$ , \*\* $P < 0.01$ ; n.s., not significant, compared with control subjects. GR, glucocorticoid receptor; HIPPO, hippocampus; IFG, inferior frontal gyrus; mRNA, messenger RNA; PFC, prefrontal cortex.

participate in the phosphorylation of GR upon acute stress, indicating that CDK5 functions as a kinase for phosphorylating GR not only in *in vitro* cellular conditions but also in *in vivo* animals.<sup>24</sup> We, however, did not see a correlation between CDK5 activity and GR phosphorylation in chronic stress, suggesting that this condition is much more complicated than acute stress in terms of

GR phosphorylation. There are several kinases and phosphatases whose activity is regulated by exposure to stress, whereas several other cyclin-dependent kinases, p38 MAPK, c-jun N-terminal kinases and AMK-activated protein kinase share the same GR phosphorylation sites with CDK5.<sup>7</sup> Chronic psychosocial stress is known to alter phosphorylation of these GR sites in PFC and



HIPPO.<sup>62–65</sup> Further, the regulatory component of protein phosphatase 1, a known phosphatase for GR, were upregulated in the raphe nucleus of patients with major depression.<sup>66,67</sup> It is thus possible that these kinases/phosphatases participate in the phosphorylation and/or dephosphorylation of GR under chronic stress.

Upon exposure to acute stress, genes, such as *Avp*, *Bdnf*, *FosB/ΔFosB*, *Id3*, *Nsrp1* and *Ppp1r10*, demonstrated a biphasic change in their mRNA expression in PFC and/or HIPPO, while CDK5 activity and GR phosphorylation also demonstrated in response to acute stress a biphasic profile similar or mirrored to the mRNA expression of these genes. This coincidence may suggest that CDK5 influenced in some degree their mRNA expression through phosphorylation of GR. Alternatively, CDK5/GR phosphorylation may share upstream regulatory pathway(s) with these genes.

In the mice under chronic stress, *Htr1a* and *cFos* mRNA were highly induced in PFC, while *FosB/ΔFosB* mRNA was elevated in HIPPO; a distinct set of genes compared with those induced by acute stress or CORT injection. Moreover, mRNA expression of most of the genes examined in human subjects was suppressed in HIPPO and BA25 of depressive subjects. These results thus indicate that acute, chronic stress and depression are quite different conditions in regard to mRNA expression of glucocorticoid-responsive and/or stress-related genes, and suggest presence of gene-regulatory mechanisms unique to each of these stimuli/disease, consistent with previous reports using the microarray analysis.<sup>68–70</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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