

Glucocorticoid Regulates Parkin Expression in Mouse Frontal Cortex: Implications in Schizophrenia

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Abstract: Stress and glucocorticoid hormones, which are released into the circulation following stressful experiences, have been shown to contribute significantly to the manifestation of various psychiatric illnesses including schizophrenia and depression. Studies in rodents have reported dose and time dependent effects of glucocorticoids on the expression of proteins related to neuroplasticity. However, the mechanism(s) involved in the regulation of proteins by glucocorticoids are not clear. Ubiquitin ligases play important role in degradation, trafficking and stabilization of proteins. The present study investigated the effect of glucocorticoid on ubiquitin-proteasome system in mouse frontal cortex. A significant increase in mRNA and protein levels of parkin, an E3 ubiquitin ligase was found in cultured mouse primary cortical neurons following corticosterone treatment. An increase in parkin levels was also found in mouse frontal cortex *in vivo* following acute dexamethasone treatment. However, chronic treatment with corticosterone did not change parkin protein levels in mouse frontal cortex. Studies using postmortem brain samples from schizophrenia and control subjects indicated a significant increase in parkin protein levels in frontal cortex of schizophrenia subjects suggesting a response to increased stress conditions in schizophrenia. These findings suggest a possible role of parkin in the pathophysiology of stress-related psychiatric disorders.

Keywords: Parkin, glucocorticoid, cortex, schizophrenia, neurons.

INTRODUCTION

Stressful life events are considered as the major non-genomic factors contributing to the manifestation of various psychiatric illnesses [1]. Dysfunction of prefrontal cortical areas as a result of chronic stress is likely to contribute to the core symptoms of psychiatric disorders, such as attention deficit hyperactivity disorder, posttraumatic stress disorder, schizophrenia, and bipolar disorder [2, 3]. Moreover, alterations in Hypothalamic-Pituitary-Adrenal (HPA) axis dysfunction such as changes in glucocorticoid levels and glucocorticoid receptor function have been reported in subjects with schizophrenia [4, 5]. In addition, chronic glucocorticoid exposure has been shown to induce oxidative stress and mitochondrial dysfunction in rodents suggesting a possible relationship between oxidative stress and glucocorticoids [6, 7]. Although chronic stress has been shown to cause deleterious effects on neuronal function, recent studies in rodents indicate that acute stress is neuro-protective [8]. Chronic stress is known to induce alterations in the expression of many genes in the prefrontal cortex that are involved in synaptic plasticity, cell cycle progression and nuclear receptor signaling [9, 10]. These changes in proteins could result in impairments in neurodevelopment and cognitive functions often seen in subjects with psychiatric

illness such as schizophrenia [11]. However, the regulatory mechanism(s) involved in stress-induced changes in protein levels are poorly understood.

Ubiquitination is a post-translational modification of protein substrates by the covalent attachment of ubiquitin through a series of enzymatic reactions. Ubiquitination involves three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) [12, 13]. Although ubiquitination has been known to target proteins for degradation, recent studies have shown the role of ubiquitination in membrane trafficking [14] and receptor stabilization [15]. Depending on the number of ubiquitin moieties that are attached such as single ubiquitin moiety (mono-ubiquitination), multiple ubiquitin moieties (multi-ubiquitination) or polyubiquitination (through different chain topologies), the ubiquitination modulates the action of proteins in diverse cellular processes [16, 17]. Interestingly, a recent study has found changes in ubiquitin and ligases in postmortem brain samples from schizophrenia suggesting a possible role of ubiquitination pathway in the pathophysiology of schizophrenia [18].

In the present study, we have investigated the effects of glucocorticoids on ubiquitin ligases using cultured primary cortical neurons. We have also performed acute as well as chronic glucocorticoid treatment studies in mice *in vivo*. Furthermore, postmortem prefrontal cortex samples from subjects with schizophrenia and controls were used to understand the role of ubiquitin ligase in schizophrenia pathophysiology.

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MATERIALS AND METHODS

Animals

Adult and timed pregnant CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All experiments were done in compliance with Georgia Regents University (GRU) animal welfare guidelines. Animal experiments were consistent with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines as per Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Postmortem Samples

Prefrontal cortex samples from schizophrenia and control subjects were received from the Human Brain and Spinal Fluid Resource Center (Los Angeles, California, United States). Detailed description on the demographics of samples has been described elsewhere [19]. Confounding variables such as PMI, refrigeration interval, age at death, brain weight, duration of illness and gender did not show any significant difference between schizophrenia and control subjects. The brain samples were shipped frozen and stored at -80°C until analysis. Grey matter was removed from a 1.5–2.0 cm thick coronal slab of the frontal cortex anterior to the corpus callosum and the prefrontal cortex was dissected [20]. Prefrontal cortex tissue was homogenized in a tissue lysis buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 2 mM EGTA, 2 mM leupeptin, 1.5 mM pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin, and 2 mM dithiothreitol followed by centrifugation at 15,000 rpm for 15 min at 4°C . The supernatant was used for protein estimation by the bicinchoninic acid method (BCA Protein Assay Kit, Sigma).

Cerebral Cortical Neuronal Culture

Mouse cortical neurons were prepared as described previously [21]. Cerebral cortices from CD-1 murine embryos at E16 were aseptically dissected and plated at 3.5×10^5 cells per well on polyethyleneimine-coated 6-well plates. Neurons were cultured in Neurobasal medium supplemented with 2 mM L-glutamine, B27 and antibiotics (Invitrogen). On the third day *in vitro* (DIV3), the media was replaced with Neurobasal supplemented with B27 minus antioxidants, glutamine, and antibiotics. Treatment of neurons was conducted between DIV 4 and 6.

Drug Treatment

In Vitro Studies

Primary cortical neurons were treated with corticosterone (1 μM ; Sigma, St Louis, MO, USA) or vehicle (DMSO). The above corticosterone dose has been shown to induce neuroprotective in primary cortical neurons [8]. At the end of the treatments, cells were washed in Phosphate Buffered Saline (PBS) and lysed in ice-cold radioimmune precipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate and 1% Nonidet P-40) supplemented with protease inhibitor cocktail (Sigma) for immunoblotting. RIPA buffer provides efficient cell lysis and protein solubilization, but avoids

protein degradation and interference with immunoreactivity. Cell lysates were also collected for RNA extraction using a commercially available kit (SV RNA Isolation, Promega, Madison, WI), according to the manufacturer's instructions.

In Vivo Studies

Corticosterone and dexamethasone (Sigma) were dissolved in 0.45% hydroxypropyl- β -cyclodextrin (Sigma). In acute treatment studies, dexamethasone (5 mg/kg/day) was administered intraperitoneally to pups at postnatal day 4 and brains were collected at 0, 2, 4, 6 or 8 h following drug administration. In chronic treatment studies, corticosterone (35 $\mu\text{g}/\text{mL}$, equivalent to 5 mg/kg/day) was administered *ad libitum* in the drinking water for 7 weeks to adult male mice. The above dose and duration of treatment with corticosterone has been shown to induce anxiety and depression-like behaviors in mice [22, 23]. Control mice were administered 0.45% hydroxypropyl- β -cyclodextrin as vehicle. All animals were monitored for change in body weight and food intake daily as possible adverse effects of the treatment, and adjustments were made in the amount of drug depending upon the fluid consumed and weight of the animals. At the end of the corticosterone treatment period, mice were killed by decapitation, and the frontal cortex was dissected (2.34 mm anterior to bregma) according to the mouse brain stereotaxic coordinates [24]. The samples were lysed in RIPA buffer. After 15 min incubation on ice, the tissue extracts were centrifuged at 15,000 g for 15 min at 4°C and stored at -70°C . Protein content was estimated by the BCA method.

Immunoblotting

Protein samples (30–40 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then blocked for 1 h in PBS with the detergent Tween 20 and 5% non-fat milk or 5% BSA. The membranes were probed overnight with anti-parkin antibody (1:1000, Cell Signaling Beverly, MA, USA). Following washing, the membranes were incubated with secondary antibody for 1 h. We used enhanced chemiluminescence detection reagent kit (Amersham Biosciences) to detect the proteins. The intensity of the bands was quantified using densitometry software (Image J, NIH). The immunoblot data for parkin was corrected for corresponding β -tubulin (1:5000, Cell Signaling) or β -actin ((1:5000, Sigma) values.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

qRT-PCR was performed in a Mastercycler ep realplex 2 S (Eppendorf, Cepheid, Sunnyvale, CA) using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). The primers (Dharmacon, Lafayette, CO, USA) used were as follows: parkin (5-AAACCGGATGAGTGGT GAGT and 5-AGCTACCGACGTGTCCTTGT) and house-keeping gene, β -actin (5-TGTGATGGTGGGAATGGGT CAG and 5-TTTGATGTACGCACGATTTCC). Data were analyzed between vehicle and corticosterone treated samples. Primer specificity was confirmed by melting curve analysis and electrophoresis of PCR products on a 2% agarose gel to confirm the presence of a single band of the predicted size.

Mouse Ubiquitination Pathway PCR Array

The expression profile of 84 genes involved in the regulation of cellular proteins by the ubiquitin-proteasome system was examined in primary cortical neurons treated with vehicle or corticosterone for 24 h using Mouse Ubiquitination Pathway RT² ProfilerTM PCR Array (SA Biosciences) according to manufacturer's protocol. The array includes ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). The data were normalized to the average Ct value of five housekeeping genes.

Statistical Analyses

Data are expressed as mean±SE. One-way ANOVA with Bonferroni's multiple comparison test for post hoc analysis was performed in time-dependent studies. Student's t test was used to compare the difference between two groups. The level of statistical significance was set at $p < 0.05$.

RESULTS

Corticosterone Treatment Induces Changes in Ubiquitin-Proteasome System in Cortical Neurons

Primary cortical neurons at DIV5 were exposed to corticosterone (1 μ M) for 24 hour and the expression of 84 genes associated with ubiquitin-proteasome system was examined using PCR Array. We found significant changes in

a number of genes involved in ubiquitination pathway following corticosterone treatment (Fig. 1A). The expression of genes such as Fbxw10, Hecw2 and Park2 (parkin) were significantly increased, whereas genes such as Bard1, Herc6 and Ube2c were significantly downregulated following corticosterone treatment ($p < 0.05$). Since parkin has been shown to have neuroprotective role and glucocorticoid induces neuroprotective effects, we further studied the effect of glucocorticoids on parkin levels in our experiments. Next, we confirmed the increase in parkin levels found in PCR array by qRT-PCR. We found a significant increase in parkin mRNA levels in neurons following corticosterone exposure (Fig. 1B; $P < 0.05$).

Corticosterone Treatment Increases Parkin Protein Levels in Cortical Neurons

To determine whether corticosterone induces changes in parkin protein levels, we examined parkin protein levels in primary cortical neurons following corticosterone treatment for 3 or 24 h. A significant increase in parkin protein levels was found in neurons at 3 h (Fig. 2A; $P < 0.05$) and at 24 h (Fig. 2B; $P < 0.05$) following corticosterone treatment.

Acute, but not Chronic Glucocorticoid Exposure Increases Parkin Protein Levels in Mouse Frontal Cortex

In acute glucocorticoid treatment studies, pups were intraperitoneally administered with dexamethasone and the

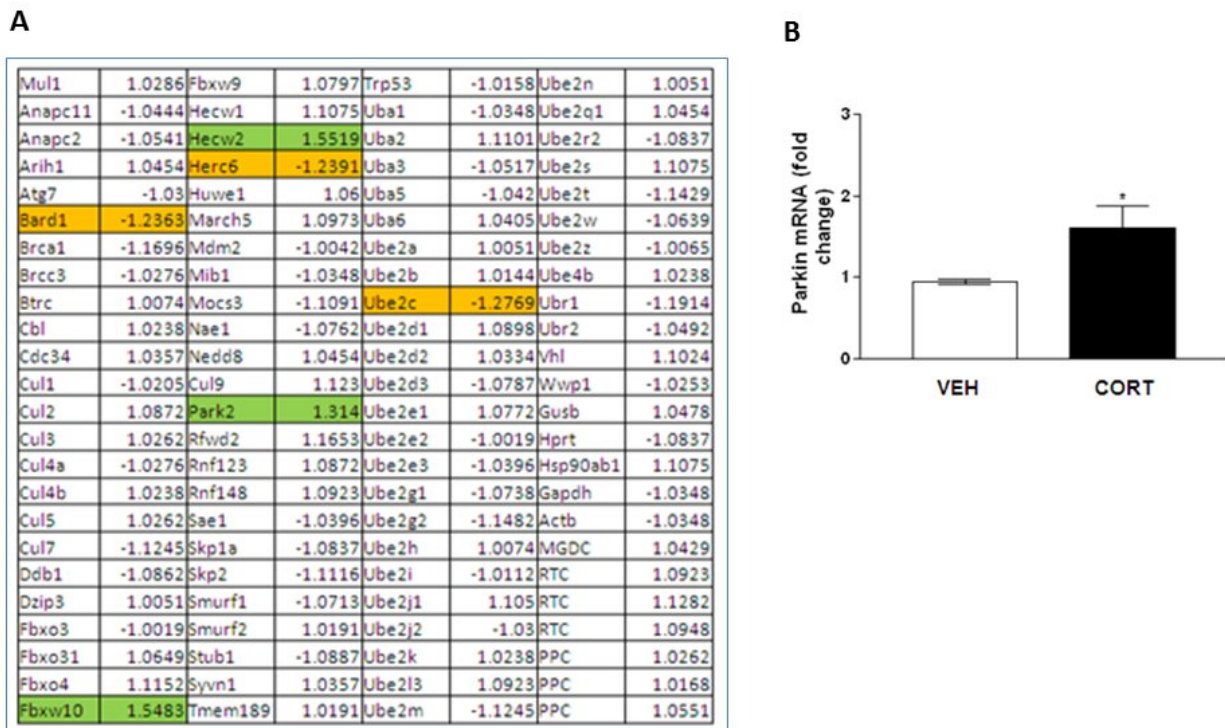


Fig. (1). Corticosterone treatment induces changes in the expression of genes associated with ubiquitin-proteasome system in cortical neurons. **(A)** Fold change in the expression of genes in primary cortical neurons following corticosterone treatment for 24 h as determined by PCR array. Significant ($p < 0.05$) increases in the fold change are highlighted in green and decreases are highlighted in yellow. $N = 3$ per group. **(B)** Parkin mRNA levels in primary cortical neurons following corticosterone (CORT) treatment for 24 h as determined by qRT-PCR. The graph represents fold change in normalized parkin mRNA levels. Values are mean \pm SE ($n = 3$). * $p < 0.05$ vs vehicle control (VEH).

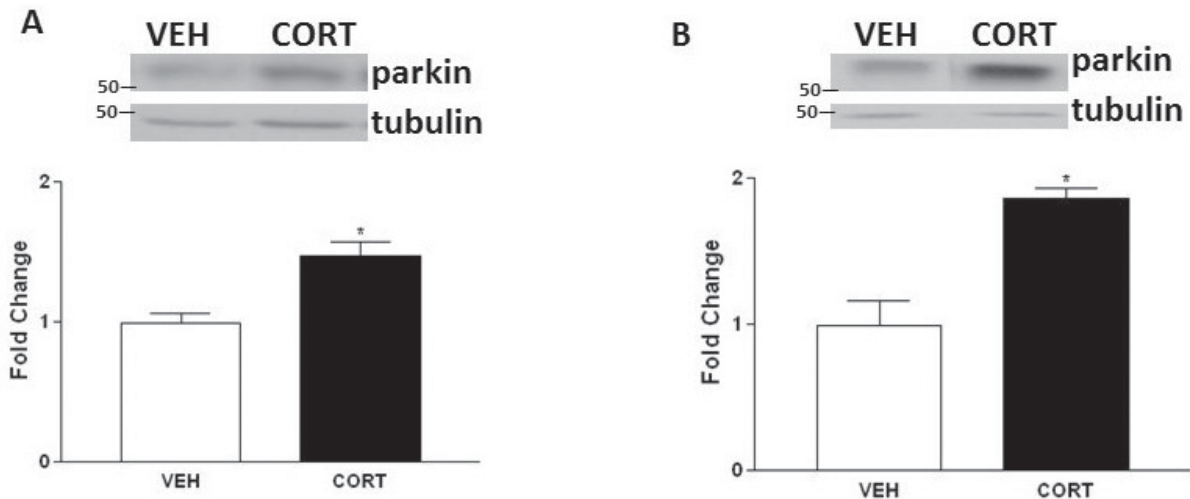


Fig. (2). Corticosterone treatment induces increase in parkin protein levels in cortical neurons. Parkin protein in primary cortical neurons following corticosterone (CORT) treatment for (A) 3 h or (B) 24 h as determined by immunoblot analysis. The upper panel shows representative autoradiogram of parkin and tubulin, and the lower panel represents fold change in normalized parkin protein levels. Values are mean ± SE (n=3). *p<0.05 vs vehicle control (VEH).

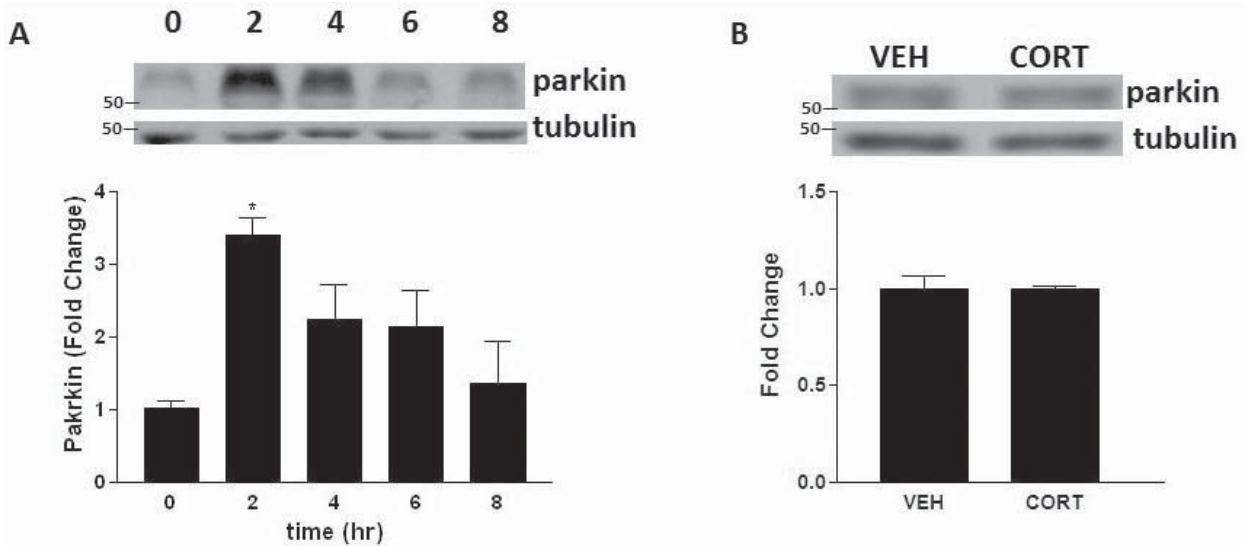


Fig. (3). Acute, but not chronic glucocorticoid treatment increases parkin protein levels in mouse frontal cortex. (A) CD-1 pups at postnatal day 4 were intraperitoneally administered with dexamethasone and the frontal cortex samples were collected 0, 2, 4, 6 or 8 h following drug treatment. Increase in parkin protein levels was detected in frontal cortex samples. The upper panel shows representative autoradiogram of parkin and tubulin, and the lower panel represents fold change in normalized parkin protein levels. Values are mean ± SE (n=3). *p<0.05 vs 0 hr as determined by one-way ANOVA. (B) Adult CD-1 male mice were administered corticosterone (CORT; 35 µg/mL, equivalent to 5 mg/kg/day) or vehicle (VEH) *ad libitum* in the drinking water for 7 weeks. No change in parkin protein levels was detected in frontal cortex samples following CORT treatment. The upper panel shows representative autoradiogram of parkin and tubulin, and the lower panel represents fold change in normalized parkin protein levels. Values are mean ± SE (n=6).

frontal cortex samples were collected 0, 2, 4, 6 or 8 h following drug treatment. A significant increase in parkin protein levels was found at 2 h following dexamethasone treatment (Fig. 3A; P<0.05). However, no significant change in parkin protein levels was found at 4, 6 or 8 h following dexamethasone treatment. In chronic glucocorticoid treatment studies, CD-1 male mice were treated with corticosterone or vehicle in the drinking water for 7 weeks. No significant difference was found in water intake or relative body weight gain during the experiment between the treatment groups (data not shown). Immunoblot analyses

showed no significant difference in parkin protein levels in the frontal cortex of corticosterone-treated mice as compared to vehicle-treated mice (Fig. 3B).

Increased Parkin Protein Levels in Prefrontal Cortex of Schizophrenia Subjects

Immunoblot analysis showed a significant increase in parkin protein levels in prefrontal cortex of subjects with schizophrenia as compared to controls (Fig. 4, p<0.05). Correlations between parkin and the confounding variables, such as brain weight, age at death, refrigeration interval, PMI

or duration of illness have been summarized in Table 1. We did not find any significant correlation between parkin and any of the confounding variables except age in control subjects.

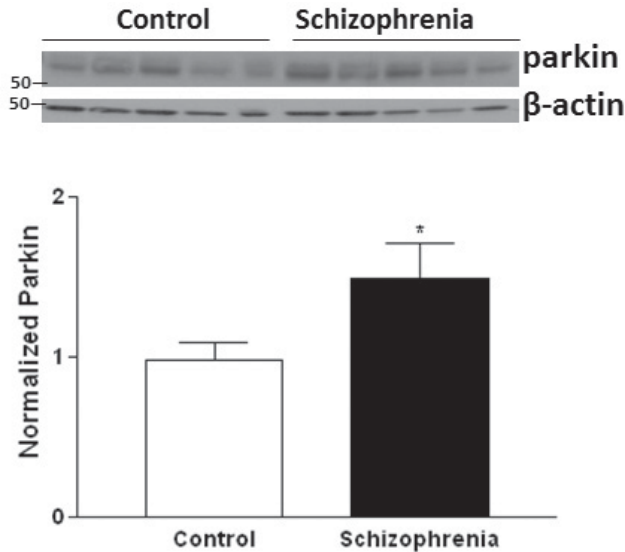


Fig. (4). Increased parkin protein levels in prefrontal cortex of schizophrenia subjects. The upper panel shows representative autoradiogram of parkin and actin, and the lower panel represents fold change in normalized parkin protein levels. Values are mean \pm SE (n=13 per group). * p <0.05 vs control subjects.

Table 1. Correlations between Parkin Protein Levels and Confounding Variables

Variable	Control		Schizophrenia	
	r^a	p^b	r	p
Age	0.693	0.008*	0.366	0.241
PMI	-0.453	0.120	-0.125	0.682
Brain wt	-0.075	0.807	0.355	0.257
Refrigeration interval	0.001	0.999	-0.113	0.882
Duration of illness			0.071	0.408

Pearson's product moment correlation shown for age, PMI, brain weight and refrigeration interval.

Spearman correlation shown for duration of illness.

^aCorrelation value.

^bSignificance.

DISCUSSION

Ubiquitin ligases are involved in the posttranslational modification of proteins and thereby controlling their stability and trafficking [25, 26]. A number of ubiquitin ligases have been identified in regulating the expression and signaling of proteins involved in neuronal functions such as synaptogenesis, axon guidance, and axon pruning [27-30]. Moreover, chronic stress leads to changes in the expression

of cellular proteins involved in neuroplasticity possibly *via* ubiquitination [18]. Here we demonstrate that glucocorticoid regulates parkin expression in cortical neurons *in vitro* and in frontal cortex *in vivo*. Furthermore, we found significantly higher levels of parkin in the prefrontal cortex of schizophrenia subjects. These observations suggest involvement of parkin in stress-related neuropsychiatric disorders.

Parkin is an E3 ubiquitin ligase and plays an important role in mitochondrial homeostasis, energy metabolism, mitochondrial function and oxidative stress [31, 32]. Mutations in the *parkin* gene (*parkin2*) have been shown to cause an autosomal recessive juvenile form of Parkinson's disease (AR-JP) [33]. Accumulating evidence indicate that parkin protects cells against cell death induced by various stressors including kainate, proteasomal inhibition, α -synuclein, ceramide, dopamine, and unfolded protein stress [34-39]. It has been shown that parkin mediates neuroprotection through activation of I κ B kinase (IKK)/nuclear factor κ B (NF- κ B) pathway [40]. NF κ B activation protects neurons by the up-regulation of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) [41]. Interestingly, acute glucocorticoid exposure is known to induce neuroprotective effect through TrkB signaling [8]. These studies suggest that the increase in parkin observed in neurons following acute glucocorticoid treatment in our study might represent a potential role of parkin in mediating the neuroprotective effects of glucocorticoids. However, chronic glucocorticoid treatment did not result in any significant change in parkin levels in mice. Earlier reports including studies from our laboratory have shown that corticosterone treatment for 7 weeks induces anxiety/depression-like behavior in mice [22, 23]. Moreover, a reduction in TrkB protein levels in frontal cortex has been found in corticosterone-treated mice [23]. However, we found a significant increase in parkin protein levels in the prefrontal cortex of subjects with schizophrenia. It is important to note that parkin is not only a stress-protective molecule, but also a stress-inducible protein [42]. It has been shown that parkin expression is increased following endoplasmic reticulum (ER) stress and mitochondrial stress, and the increased levels of parkin prevents ER stress-induced mitochondrial damage and cell death [42]. Also, the postmortem samples used in the present study were obtained from chronic schizophrenia subjects, and therefore, the effects of antipsychotic medications on stress and parkin levels cannot be excluded [43]. Thus, the increase in parkin levels observed in prefrontal cortex of schizophrenia subjects might represent a cytoprotective function of parkin in these subjects.

To the best of our knowledge, this is the first report on parkin levels in schizophrenia. We have used protein samples from prefrontal cortex of schizophrenia and normal subjects. We found that the age-related increase in parkin protein levels observed in control subjects was not present in schizophrenia. Accumulating evidence from neuroimaging and neurochemical studies has implicated the role of prefrontal cortex in the pathophysiology of schizophrenia [44]. A number of studies have found alterations in signaling pathways of neuroprotective molecules such as BDNF and vascular endothelial growth factor (VEGF) in the prefrontal

cortex of schizophrenia subjects [19, 21, 45]. Specifically, reductions in mRNA and protein levels of BDNF and its receptor TrkB have been found in the prefrontal cortex of subjects with schizophrenia [19, 46-49]. In addition, VEGF and its receptor, Flk1 levels were found lower in the prefrontal cortex of schizophrenia subjects as compared to controls [21, 45]. One of the mechanisms involved in neurotrophin-mediated neuroprotection is the inhibition of oxidative stress [49]. BDNF has been shown to protect neuronal cells against oxidative stress by preventing the accumulation of oxidative radicals and increasing antioxidant enzymes [50]. It is known that ubiquitin proteasomal system (UPS) is sensitive to oxidative stress. Mild oxidative stress has been shown to increase the rate of ubiquitination [51-53], whereas sustained physiologically relevant levels of oxidative stress is known to impair proteasome activity and reduce the degradation of ubiquitinated substrates in cells [54-56]. Accordingly, the loss-of-function parkin mutations results in the activation of c-Jun N-terminal kinase (JNK) leading to dopaminergic cell death, whereas the wild-type parkin protects against oxidative stress by inhibiting the JNK signaling [57, 58]. Moreover, parkin expression has been shown to be up-regulated by oxidative stressors suggesting a neuroprotective response of parkin to oxidative stress [59]. Alterations in oxidative stress markers are well documented in the pathophysiology of schizophrenia [60]. It would be interesting to study whether oxidative stress contributes to the altered expression of parkin in the frontal cortex of subjects with schizophrenia.

In summary, the present study provides initial evidence on the effects of glucocorticoids on parkin levels in frontal cortex. Our earlier study has found a significant increase in cortisol levels in prefrontal cortex and CSF samples from schizophrenia subjects using the same sample sets used in the current study [19]. Therefore, higher parkin levels observed in the current study are likely to be a response to stress conditions as reflected by higher cortisol levels in schizophrenia. However, additional studies with larger sample sizes are needed to establish the relationship between cortisol and parkin levels in schizophrenia subjects.

AUTHOR CONTRIBUTIONS

AP designed the research. CDP and AC performed the experiments. AP, CDP and AC analyzed the data. AP and CDP wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTEREST

We have no conflicts of interest in presenting this manuscript.

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