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Region-specific alterations in glucocorticoid receptor expression in the postmortem brain of teenage suicide victims

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Amygdala;
Teenage suicide;
Postmortem brain

Summary
Introduction: Abnormal function of the hypothalamic–pituitary–adrenal (HPA) axis has been implicated in the pathophysiology of depression and suicide. The purpose of this study was to test the hypothesis that the reported dysregulation of the HPA axis in suicide may be related to a disturbed feedback inhibition caused by decreased corticoid receptors in the brain. We therefore determined the protein and gene expression of glucocorticoid (GR) and mineralocorticoid receptors (MR) in the postmortem brain of teenage suicide victims and matched normal controls.
Methods: Protein and mRNA expression of GR (GR-α and GR-β) and MR and the mRNA expression of glucocorticoid-induced leucine zipper (GILZ), a target gene for GR were determined by immunolabeling using Western blot technique and the real-time RT-polymerase chain reaction (qPCR) technique in the prefrontal cortex (PFC), hippocampus, subiculum, and amygdala obtained from 24 teenage suicide victims and 24 teenage control subjects.
Results: We observed that protein and gene expression of GR-α was significantly decreased in the PFC and amygdala, but not in the hippocampus or subiculum, of teenage suicide victims compared with normal control subjects. Also, the mRNA levels of GR inducible target gene GILZ was significantly decreased in PFC and amygdaloid nuclei but not in hippocampus compared with controls. In contrast, no significant differences were observed in protein or gene expression of MR in any of the areas studied between teenage suicide victims and normal control subjects. There was no difference in the expression of GR-β in the PFC between suicide victims and normal controls.
Conclusions: These results suggested that the observed dysregulation of the HPA axis in suicide may be related to a decreased expression of GR-α and GR inducible genes in the PFC and amygdala of teenage suicide victims. The reason why GR receptors are not dysregulated in the hippocampus or subiculum, presumably two sites of stress action, are not clear at this time.

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1. Introduction

Suicide, which is a major public health concern, is a leading cause of death in the United States (Goldsmith et al., 2002; WHO, 2001). It is an even larger problem in teenagers, ranking as the second or third leading cause of death in the adolescent population. While there is some understanding of the neurobiology of adult suicide, the neurobiology of teenage suicide, whose characteristics may be slightly different from those of adult suicide (Brent et al., 1993; Pfeffer, 1989), is relatively unclear and understudied.

Depression and stress are among the major risk factors for suicidal behavior. Abnormalities of the hypothalamic–pituitary–adrenal (HPA) axis have been consistently reported in patients with major depressive disorder (MDD) (Holsboer, 2000) and in patients who completed suicide (Lester, 1992). Whereas about 50% of patients with MDD have an abnormal dexamethasone suppression test (DST), almost all those MDD patients who eventually completed suicide had an abnormal DST (Coryell and Schlesser, 2001). This suggests an even stronger association between an abnormal HPA axis and suicide.

Although the HPA axis has been found to be abnormal in depression and suicide, the mechanism underlying this abnormality is not clearly understood. The dysregulation of HPA axis function observed in depression and suicide is believed to be at least in part due to a disturbed feedback inhibition by endogenous corticoids (Holsboer, 2000; Nemeroff, 1996; Pariante and Miller, 2001). In humans, cortisol serves as a negative feedback regulator of HPA axis by its action on two types of receptors known as glucocorticoid (GR) and mineralocorticoid (MR) receptors in the brain and the pituitary (Bao and Swaab, 2010; Jacobson, 2005). It is believed that this impaired HPA axis negative feedback in depression may be related to altered GR and/or MR function.

To test the hypothesis that an abnormal HPA axis observed in depression and suicide is related to altered sensitivity of GR, several investigators have determined the function or the sensitivity of GR in depression by determining its sensitivity in peripheral cells, such as lymphocytes (Pariante and Miller, 2001). Although not always consistent, these studies do indicate altered sensitivity of GR in the peripheral cells of depressed patients. However, these studies did not demonstrate if a similar abnormality or decrease in GR sensitivity exists in the brain of depressed or suicidal subjects. Therefore, it is important to examine if a dysregulated HPA axis in depression or suicide is related to altered GR or MR in the brain. Although there are several studies of other components of the HPA axis in the human postmortem brain, such as CRF (Nemeroff, 1996), there are very few studies of GR or MR in the postmortem brain of suicide or depressed subjects (Mason, 1959; Matheson et al., 1971; Perlman et al., 2004; Redgate and Fahringer, 1973; Webster et al., 2002) and no studies in teenage suicide.

To examine if altered HPA axis in suicide, as reported by other investigators (Coryell and Schlesser, 2001), is related to changes in corticoid receptors in the brain, we determined the protein and gene expression of GR-α, GR-β, and MR in the prefrontal cortex (PFC), hippocampus, subiculum, and amygdala of teenage suicide victims and matched control subjects, as these brain areas have been implicated in the pathophysiology of depression and suicide and in the regulation of HPA axis (see Gold et al., 2002). In order to examine the functional consequence of abnormal GR, we also measured the protein and mRNA expression of a GR target gene known as glucocorticoid-induced leucine zipper protein (GILZ) in the different areas of postmortem brain. This target gene of GR (i.e., GILZ) is not only involved in apoptosis but also affects innate and adaptive immunity (Ayroldi and Riccardi, 2009; Beaulieu and Morand, 2011).

2. Methods

2.1. Acquisition of human postmortem brain samples

Brain tissue was obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center, Baltimore, Maryland, in collaboration with the Office of the Chief Medical Examiner of the State of Maryland. Tissue samples were obtained from 24 teenage suicide victims and 24 teenage control subjects (Table 1). Amygdala tissue was available for only 10 suicide victims and 10 normal control subjects. Toxicological data were obtained by analysis of urine and blood samples from these subjects. All procedures were approved by the University of Maryland Institutional Review Board and written consents were obtained from family members. None of the subjects included in this study had any gunshot wounds to the head.

2.2. Dissection of different brain areas

Brain regions were dissected by using the ''punch'' technique (Palkovits, 1973). First, the brains were sliced with about 15–20 mm thick coronal sections. These thick sections that contained the investigating areas were further cut into 1–2 mm thin sections, than the actual brain areas were punched out with special punch needles with 10–20 mm inside diameters or small tissue blocks were cut out with a Graefe knife. All of the procedures had been performed on frozen (0 °C to −5 °C) brain sections.

The samples of prefrontal cortex include the dorsolateral prefrontal cortex from the superior frontal gyrus (Brodmann area 9; BA-9). Coronal sections through the entire amygdala from both sides were alternatively used: one section used as the entire amygdala with its seven nuclei, while the central (CeA) and medial (MeA) amygdaloid nuclei were individually micropunched from the alternate amygdalae sections.

Two separate areas have been cut out from the hippocampal cortex: the hippocampal CA areas together with the dentate gyrus and the subiculum.

2.3. Diagnostic method

At least one family member and/or a friend, after giving written informed consent, underwent an interview based on the Structured Clinical Interview for DSM-IV (SCID) (First et al., 1997). The interviews were done by a trained psychiatric social worker. Two psychiatrists independently reviewed the write-up from this interview, as well as the

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Table 1 Characteristics of teenage suicide and normal control subjects.

Patient no/sex/age (y)/race	PMI (h)	Brain pH	Cause of death	Drug toxicity	Psychiatric diagnosis
Control group[a]					
1/M/19/B	6	6.14	GSW	None	Normal
2/M/16/B	6	6.54	GSW	None	Normal
3/M/16/B	8	5.64	GSW	None	Normal
4/M/19/B	12	5.9	GSW	None	Normal
5/M/13/W	NA	5.19	Accident	None	Normal
6/M/17/B	11	6.52	GSW	None	Normal
7/M/16/W	10	5.42	Stabbing	None	Normal
8/M/17/B	10	5.9	GSW	None	Normal
9/M/13/B	22	6.07	GSW	None	Normal
10/M/14/B	18	5.73	GSW	None	Normal
11/M/18/B	27	6.09	Drowning	None	Normal
12/M/16/W	21	5.97	Accidental hanging	None	Normal
13/F/18/W	35	5.99	Multiple injuries	None	Normal
14/F/17/B	26	6.23	Multiple injuries	None	Normal
15/F/19/W	30	6.2	Cardiac arrhythmia	None	Normal
16/F/18/B	16	6.6	MVA	None	Normal
17/M/13/B	20	6.6	Drowning	None	Normal
18/M/19/B	16	6.6	Congenital heart disease	None	Normal
19/F/16/W	24	6.71	Myocarditis	None	Normal
20/M/15/W	16	6.5	Cardiac arrhythmia	None	Normal
21/M/15/W	21	6.37	PE/DVT	Cyclobenzaprine	Normal
22/F/16/W	20	6.89	MVA	None	Normal
23/F/13/B	23	6	Hanging	Not yet done	
24/M/18/W	19	5.80	Complications of Morbid Obesity	Morphine (Rx) and bupivicaine	Normal
Suicide group[b]					
1/F/15/W	7	5.48	GSW	Ethanol	Alcohol abuse
2/M/20/W	32	6.41	Hanging	Ethanol	Alcohol abuse
3/M/12/B	10	5.91	Hanging	None	Major depression
4/M/15/W	11	5.33	Asphyxia	None	Major depression
5/F/15/W	17	5.58	Drug overdose	Imipramine, desipramine	Major depression, Hyperactivity attention deficit disorder
6/M/15/W	27	6.08	GSW	Pseudoephedrine, phenylpropanolamine	Adjustment disorder, Major depression
7/M/18/W	17	6.3	Hanging	None	Major depression — single episode
8/M/19/W	18	6.2	CO intoxication	Ethanol, CO	Major depression, Ethanol abuse, Polysubstance abuse
9/F/15/W	20	6.59	Hanging	None	Major depression — single episode, Ethanol abuse
10/M/17/W	23	6.66	Hanging	Ethanol	Major depression — single episode, Ethanol abuse
11/M/13/W	18	6	Hanging	Ritalin	Hyperactivity attention deficit disorder
12/F/17/W	25	5.55	Drug overdose	Verapamil	Adjustment disorder
13/F/16/W	33	6.61	GSW	None	Adjustment disorder
14/M/16/W	24	6.81	Hanging	None	Adjustment, Conduct disorders
15/F/15/W	21	6.48	Hanging	None	Adjustment disorder with depressed mood
16/F/15/W	20	6.1	Hanging	None	Borderline personality disorder

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Table 1 (*Continued*)

Patient no/sex/age (y)/race	PMI (h)	Brain pH	Cause of death	Drug toxicity	Psychiatric diagnosis
17/M/19/W	15	6.9	GSW to chest	Fluoxetine	Dissociative disorder, substance abuse (kind unclear), PTSD
18/F/15/W	11	6.44	Drug overdose	Phenylpropanolamine Cholorophenylamine, Codeine, Salicylate, Acetaminophen	No mental disorder
19/M/17/A	7	5.9	GSW	Ethanol	No mental disorder
20/M/16/H	20	6.17	Hanging	None	No mental disorder
21/F/16/W	18	6.31	GSW	Amitriptyline	No mental disorder
22/M/15/W	16	6.18	Hanging	None	No mental disorder
23/M/14/B	22	6.44	Hanging	None	No mental disorder
24/F/17/W	24	6.49	Diphenhydramine overdose	Diphenhydramine, citalopram	Dx (not enough info)

Abbreviations: M, male; F, female; B, black; W, white; A, Asian; H, Hispanic; ACSVD, atherosclerotic cardiovascular disease; GSW, gunshot wound; MVA, motor vehicle accident; NA, not available; PE/DVT, pulmonary embolism, deep vein thrombosis; and PMI, postmortem interval.
[a] Mean $\pm$ SD age was 16.29 $\pm$ 2.03 years; PMI, 18.13 $\pm$ 7.67 h; and brain pH, 6.15 $\pm$ 0.43, 17 male, 7 female.

SCID that was completed from it, as part of their diagnostic assessment of the case. Diagnoses were made from the data obtained in this interview, medical records (if available) from the case, and records obtained from the Medical Examiner's office. This was done for all subjects (i.e., normal controls, non-suicide and suicide cases). The collection of clinical data for the control and patient subjects was identical, and the procedure for the diagnosis of normal control subjects was the same as for all the other depressed and/or suicide subjects. The diagnoses from two psychiatrists were compared and discrepancies were resolved by means of a consensus conference. There was a consistent reliability of diagnosis with kappa >0.9.This has been found to be a very accurate way to make diagnoses (Ramirez Basco et al., 2000). Control subjects were verified as free from mental illness using these consensus diagnostic procedures.

2.4. Determination of protein levels of GR and MR using Western blot technique

The detailed Western blot procedure has previously been published by us (Dwivedi et al., 2003). Briefly, protein samples (30 μg protein) were loaded onto 7.5% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel for GR and MR. The gels were run and transferred electrophoretically to an enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham, Arlington Heights, IL). The blots were incubated overnight at 4 °C with primary polyclonal anti-GR-α, anti-GR-β (Thermo Scientific Pierce, Fisher Scientific Company, LLC, Hanover Park, Illinois) at a dilution of 1:500 or anti-MR antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:1000. The membranes were extensively washed with TBST and exposed to ECL autoradiography film. The same nitrocellulose membrane was stripped and re-probed with β-actin antibody (Sigma Chemical Co., St. Louis, MO). The bands on the autoradiogram were quantified using the Loats Image Analysis System (Loats Associates, Inc., Westminster, MD), and the optical density of each sample was corrected by the optical density of the corresponding β-actin band. The values are represented as a percent of the control.

2.5. Determination of mRNA levels of GR and MR using real-time PCR

2.5.1. RNA extraction and reverse transcription

Total RNA was extracted from 100 mg of tissue using the TRIZOL reagent as per the manufacturer's instructions (Invitrogen, USA). RNA yield and quality was determined by absorbance at 260 nm using NanoDrop®ND-1000 and using Agilent Bioanalyzer 2100. Only samples showing 28S/18S ratios >1.2 and RIN ≥7.0 were included.

2.5.2. Relative real-time PCR

Expression levels of mRNA were determined using a two-step real-time RT-PCR (qPCR) method. One ug of total RNA was reverse transcribed using 50 ng random hexamers, 2 mM dNTP mix, 10 units ribonuclease inhibitor, 50 mM Tris—HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, and 200 units MMLV-reverse transcriptase (Invitrogen) in a final reaction volume of 20 μl. Reverse transcription was performed at 37 °C for 60 min, and enzyme was denatured at 70 °C for 15 min. The cDNA was stored at −20 °C.

Real-time PCR was performed with MX3005p sequence detection system (Agilent) using pre-designed Taqman gene expression assays (Applied Biosystems, Foster City, CA) targeting GR-α, GR-β, GILZ and MR along with two housekeeping genes β-actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), shown in Table 1. The stability and optimal number of housekeeping genes was determined using geNORM version 3.4 (PrimerDesign Ltd, UK) according to the manufacturer's instructions (Vandesompele et al., 2002). This comparison identified ACTB and GAPDH as the most stable housekeeping genes for this cohort. PCR efficiency after 5-log dilution series of pooled cDNA was similar for all housekeeping and target genes. For each primer/probe set, qPCR reaction is carried out using 10 μl of cDNA (diluted 1:10) in 1X TaqMan

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Universal PCR Master Mix (Applied Biosystems) as per manufacturer's instructions. Each qPCR plate included a "no reverse transcriptase" and "no template" control to eliminate non-specific amplification and each sample is assayed in triplicate.

For qPCR gene expression analysis, raw expression data (C_t) are normalized to the geometric mean of the two housekeeping genes. Outliers were excluded if the normalized (delta C_t) values were greater than 2 standard deviations from the group mean. Relative expression levels, reported as fold change, were determined by $2^{-(\Delta\Delta Ct)}$ method, as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859) and ΔC_t values are used for further statistical analysis.

2.5.3. TaqMan primer/probes used for qPCR assays

	TaqMan accession	Probe location (exon boundary)	Assay function
ACTB	Hs99999903_m1	1—1	House Keeping (HK)
GAPDH	Hs99999905_m1	3—3	HK
GR-α (NR3C1)	Hs00353740_m1	4—5	Target Gene
GR-β (NR3C1)	Hs00354508_m1	8—9	Target Gene
GILZ (TSC22D3)	Hs00608272_m1	2—3	Target Gene
MR (NR3C2)	Hs01031809_m1	7—8	Target Gene

2.6. Statistical analysis

The data analyses were performed using the SAS 9.2 statistical software package. We used unpaired t-test to compare the differences in protein and mRNA levels of GR-α and MR between suicide victims and normal control subjects without adjusting for any covariates like age, gender, postmortem interval (PMI) or brain pH.

There were more blacks in the control group ($n = 14$) compared with the suicide group ($n = 2$). We therefore compared all measures between black and non-black controls and did not find significant differences in any of the measures between the groups.

To examine whether the difference in protein or mRNA expression levels of GR-α and MR were affected by age, PMI, gender or brain pH, we determined the relationship between the levels of outcome variables and PMI, age, gender, and brain pH by Pearson product moment correlation analysis. Although we did not observe any significant effects of age or gender, we observed a significant effect of PMI and brain pH on GR-α and MR protein expression levels in PFC. We then employed a fixed-effect model with four covariates, namely age, PMI, gender, and brain pH, to analyze the difference in the outcome variable between control subjects and suicide victims. We observed that the results from unpaired t-test and fixed-effect model were similar. However, the comparisons of the outcome variables between normal control subjects and suicide victims are described using the fixed-effect model. Comparisons between the groups were also performed using the Wilcoxon—Mann—Whitney test. The Wilcoxon—Mann—Whitney test is a non-parametric analog to the independent samples t-test and can be used when one does not assume that the dependent variable is a normally distributed interval variable.

The statistical differences between subgroups of suicide victims (with and without mental disorders) and controls were evaluated by one-way analysis of variance (ANOVA), followed by post hoc analysis, using Bonferroni's correction.

3. Results

The demographic and clinical characteristics of the teenage suicide victims ($n = 24$) and control subjects ($n = 24$) were previously published by us (Ren et al., 2013) and are shown in Table 1. The age range of the teenage suicide victims was from 12 to 20 years. There were no significant differences in the mean age, PMI, and brain pH between the suicide victims and the normal control subjects.

3.1. mRNA levels of GR-α in the PFC, hippocampus, subiculum, and amygdala of teenage suicide victims and normal control subjects

We first determined the RIN value for all the samples to examine the quality of RNA, and we found that the mean RIN was 7.15 ± 0.39 for normal controls and 7.21 ± 0.41 for teenage suicide victims. These are acceptable RINs for the mRNA expression studies. We then compared the levels of mRNA determined with qPCR, as described in Section 2 between the normal controls and suicide victims. When we compared the mRNA levels of GR-α in the PFC (BA-9), we found that the mean mRNA levels of GR-α in the PFC was significantly decreased in teenage suicide victims compared with matched normal control subjects as shown in Fig. 1A, but was not significantly different in the hippocampus and subiculum of teenage suicide victims compared with controls (Fig. 1 A). We also found that the mRNA levels of GR-α were significantly decreased in the amygdala of teenage suicide victims compared with normal control subjects (Fig. 1A).

Since we observed a decrease in the mRNA expression of GR-α in the amygdala, we also examined if this decrease was specific to amygdaloid nuclei. We therefore determined mRNA expression of GR-α in the CeA and MeA nuclei of the teenage suicide victims and normal controls. We found that the GR-α expression was significantly decreased in CeA but not in the MeA of teenage suicide victims compared with controls (Fig. 2A).

3.2. mRNA levels of GR-β in the PFC of teenage suicide victims and normal control subjects

We determined the mRNA expression of GR-β in the PFC, hippocampus, and amygdala of teenage suicide victims and normal controls. Of the two GR isoforms, GR-α is predominantly expressed, especially in the brain. Whereas we were able to quantitate GR-β mRNA expression in the PFC, GR-β was almost undetectable in the hippocampus and amygdala. However, we found that there was no significant difference in the PFC mRNA expression of GR-β between suicide victims and normal controls, as shown in Fig. 3A.

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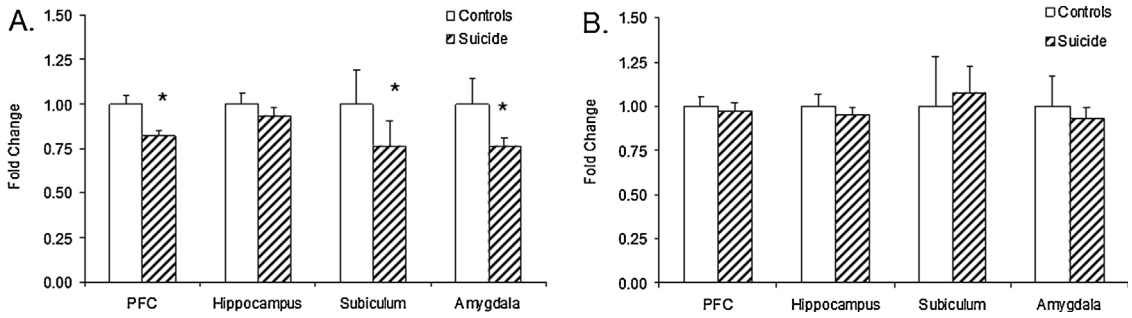

Figure 1 (A) The mRNA expression levels of GR-α in the postmortem brain of teenage suicide victims and teenage normal control subjects. The mean mRNA expression of GR-α in the prefrontal cortex (BA-9) of control subjects (1.00 ± 0.05) and suicide victims (0.82 ± 0.03), in the hippocampus of control subjects (1.00 ± 0.06) and suicide victims (0.94 ± 0.05), in the subiculum of control subjects (1.00 ± 0.19) and suicide victims (0.77 ± 0.14), and in the amygdala of control subjects (1.00 ± 0.14) and suicide victims (0.77 ± 0.05). (B) The mRNA expression levels of MR in the postmortem brain of teenage suicide victims and teenage normal control subjects. The mean mRNA expression of MR in the prefrontal cortex (BA-9) of control subjects (1.00 ± 0.05) and suicide victims (0.95 ± 0.04), in the subiculum of control subjects (1.00 ± 0.28) and suicide victims (1.08 ± 0.28) and in the amygdala of control subjects (1.00 ± 0.17) and suicide victims (0.93 ± 0.06). The data are shown as fold change in mRNA levels. Values are fold change ± SEM *$p < 0.05$.

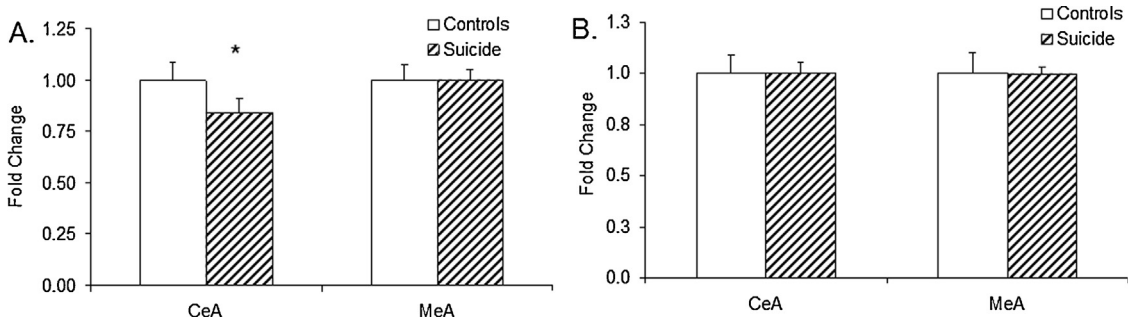

Figure 2 (A) The mean mRNA expression levels of GR-α in the central amygdaloid nucleus (CeA) of teenage normal controls subjects (1.00 ± 0.09) and teenage suicide victims (0.84 ± 0.07) and in the medial amygdaloid nucleus (MeA) of normal controls subjects (1.00 ± 0.08) and suicide victims (1.00 ± 0.05). (B) The mean mRNA expression levels of MR in the central amygdaloid nucleus (CeA) of teenage normal control subjects (1.00 ± 0.09) and teenage suicide victims (1.00 ± 0.05) and in the medial amygdaloid nucleus (MeA) of normal controls (1.00 ± 0.10) and suicide victims (1.00 ± 0.03). The data are shown as fold change in mRNA levels. Values are fold change ±SEM. *$p < 0.05$.

3.3. mRNA levels of GILZ in the PFC, hippocampus and amygdala of teenage suicide victims and normal control subjects

We determined the mRNA levels of GILZ in the PFC, hippocampus and amygdaloid nuclei in teenage suicide victims and normal control subjects. When we compared the mean mRNA expression of GLIZ between teenage suicide victims and normal control subjects, we found that the mRNA expression levels of GILZ were significantly decreased in the PFC of teenage suicide victims compared with normal controls (Fig. 4) The mRNA expression of GILZ was not significantly different in the hippocampus of teenage suicide victims compared with normal control subjects. When we compared the mRNA levels of GILZ in the amygdaloid nuclei, we found that mRNA expression of GILZ was significantly decreased in the CeA of the suicide victims compared with normal control subjects. However, there were no significant differences in the mRNA expression of GILZ in the MeA of suicide victims compared with normal control subjects (Fig. 4). These studies of the mRNA expression of GILZ thus show that the changes in GILZ were very similar to those observed in the mRNA expression of GR-α in the postmortem brain.

3.4. mRNA expression levels of MR in the PFC, hippocampus, subiculum, and amygdala of teenage suicide victims and normal control subjects

We determined the mRNA expression of MR in the PFC, hippocampus, subiculum, and amygdala (Fig. 1B), and the amygdaloid nuclei (Fig. 2B), and we found that there were no significant differences in the mRNA levels of MR in the PFC, hippocampus, subiculum, amygdala, or amygdaloid nuclei (CeA and MeA) between teenage suicide victims and normal control subjects, as shown in Fig. 1B and Fig. 2B.

3.5. Protein expression level of GR-α in the PFC, hippocampus, and amygdala of teenage suicide victims and normal control subjects

In order to examine if the changes in GR-α mRNA expression observed in the PFC and amygdala of teenage suicide victims

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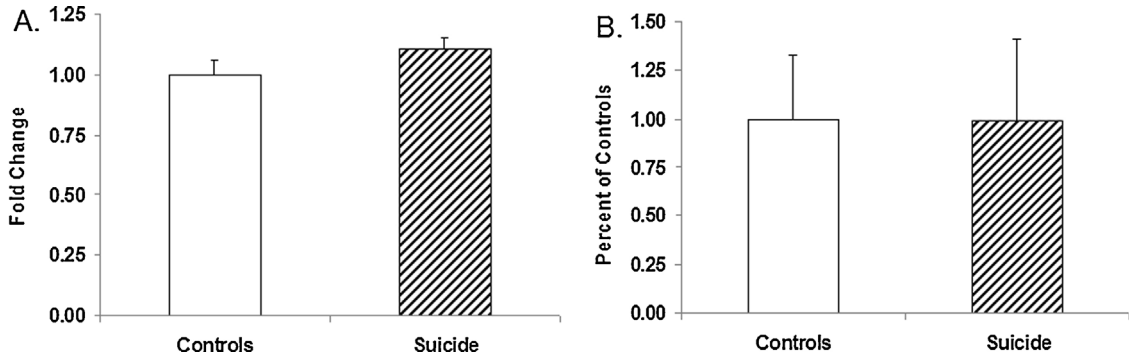

Figure 3 (A) The mRNA expression levels of GR-β in the postmortem brain of teenage suicide victims and teenage normal control subjects. The mean mRNA expression of GR-α in the prefrontal cortex (BA-9) of control subjects (1.00 ± 0.10) and of suicide victims (1.10 ± 0.11). The data are shown as fold change in mRNA levels. Values are fold change ± SEM. (B) The mean protein expression levels of GR-β in the prefrontal cortex (BA-9) of teenage suicide victims (n = 24) and teenage normal control subjects (n = 24). The data are shown as percent of controls. Values are mean ± SD.

are also associated with altered protein expression, we determined the protein expression levels of GR-α in the PFC, hippocampus, and amygdala of teenage suicide victims and normal control subjects, using the specific anti-GR-α antibody. Representative GR-α immunoblots of 2 normal controls and 2 suicide victims are shown in Fig. 5. We found that the protein expression levels of GR-α were significantly decreased in the PFC and amygdala, but not in the hippocampus of teenage suicide victims compared with normal control subjects (Fig. 6A).

3.6. Protein expression levels of GR-β in the PFC of teenage suicide victims and normal controls subjects

We also determined the protein expression levels of GR-β using a specific antibody and the representative immunoblots of 2 normal controls and 2 suicide victims are shown in Fig. 5. GR-β migrated at 90 kDa and there appears to be no significant differences between the two groups. When we compared the mean GR-β protein expression we found that there were no significant differences in the PFC between teenage suicide victims and normal control subjects (Fig. 3B). The protein expression of GR-β in the hippocampus and amygdala was almost undetectable.

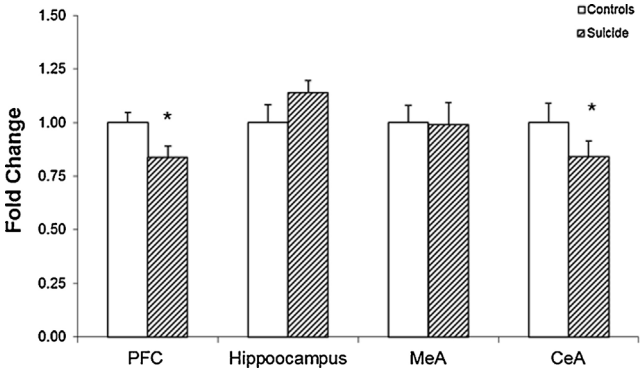

Figure 4 The mRNA expression levels of GILZ in the postmortem brain of teenage suicide victims and teenage normal control subjects. The mean mRNA expression of GILZ in the prefrontal cortex (BA-9) of control subjects (1.00 ± 0.05) and suicide victims (0.84 ± 0.06), in the hippocampus of control subjects (1.00 ± 0.08) and suicide victims (1.14 ± 0.05), in the central amygdaloid nucleus (CeA) of control subjects (1.00 ± 0.09) and suicide victims (0.84 ± 0.07), in the medial amygdaloid nucleus (MeA) of control subjects (1.00 ± 0.08) and suicide victims (0.99 ± 0.10). The data are shown as fold change in mRNA levels. Values are fold change ±SEM. *$p < 0.05$.

3.7. Protein expression levels of MR in the PFC, hippocampus, and amygdala of teenage suicide victims and normal control subjects

We compared the protein expression levels of MR between teenage suicide victims and matched normal control subjects in the three brain areas. As shown in Fig. 6B, there were no significant differences in the protein expression levels of MR between teenage suicide victims and normal control subjects in PFC, hippocampus, or amygdala. These results suggested that similar to mRNA expression, the protein expression of MR

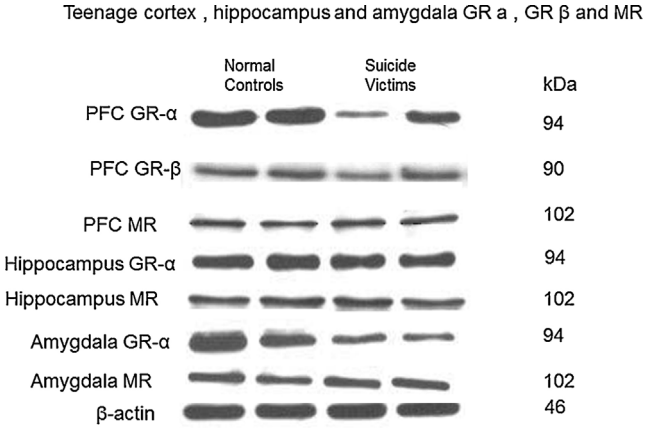

Figure 5 Representative Western blots showing the immunolabeling (cytosol fraction) of GR-α, GR-β, MR, and β-actin in the prefrontal cortex (BA-9), hippocampus and amygdala of two normal control subjects and two suicide victims. kDa indicates kilodaltons.

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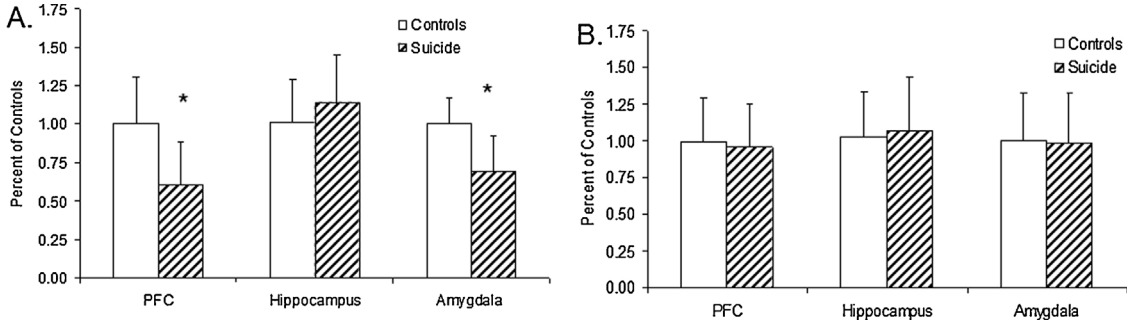

Figure 6 (A) The protein expression levels of GR-α in the postmortem brain of teenage suicide victims and teenage normal control subjects. The mean protein expression levels of GR-α in the prefrontal cortex (BA-9) ($n = 24$), hippocampus ($n = 24$), and amygdala ($n = 10$). (B) The protein expression levels of MR in the postmortem brain of teenage suicide victims and teenage normal control subjects. The mean protein expression levels of GR-α in the prefrontal cortex (BA-9) ($n = 24$), hippocampus ($n = 24$), and amygdala ($n = 10$). The data are shown as percent of control. Values are mean ± SD. *$p < 0.05$.

is not significantly different in teenage suicide victims in any of the brain areas we studied.

Protein expression of GR-α, GR-β, and MR was not determined in subiculum, CeA or MeA because of the small amount of the tissue.

3.8. Effect of diagnosis and race

In order to examine if the changes in protein/mRNA levels of GR-α, GR-β or MR in different brain region were related to presence of specific mental disorders or were specific to suicide, we compared the expression of GR-α, GR-β, and MR protein and mRNA levels in different diagnostic groups using ANOVA. The main diagnostic categories in teenage suicide group were subjects with no mental disorders ($n = 6$), those with primary diagnosis of major depression ($n = 7$), and those with adjustment or conduct disorder ($n = 5$). The number of subjects in other groups, such as alcohol abuse ($n = 2$), attention deficit hyperactivity disorder ($n = 1$), borderline personality disorder ($n = 1$) or dissociative disorder ($n = 1$) were too small for a meaningful comparison. We therefore compared the GR-α, GR-β, and MR in the different brain region using ANOVA in subjects with no mental disorders, subjects with major depression and subjects with adjustment disorder. We found that there were no significant differences between these diagnostic groups either in the protein or mRNA levels of GR-α, or MR in any of the brain region we studied. Also, there were no significant differences between these diagnostic groups in protein or mRNA levels of GR-β in the PFC. These results suggest that changes in GR-α or GR-β in PFC, and GR-α in the amygdala or amygdaloid nuclei found in the teenage suicide victims were not related to diagnosis.

3.9. Effect of suicide method and toxicology

We examined the effect of the suicide method on protein and mRNA expression of GR-α, GR-β, and MR in teenage suicide victims. Out of the 24 suicide victims 19 committed suicide by violent means and 5 by non-violent means, such as drug overdose. The mean protein and mRNA levels of GR-α, GR-β or MR in suicide victims using non-violent means were not significantly different from those suicide victims who used violent means for suicide.

We then examined the effect of antidepressants and alcohol presence on the protein and mRNA expression of GR-α, GR-β, and MR in the suicide victims. Out of the 24 subjects, 5 had presence of ethanol and 4 had presence of antidepressants. The exclusion of the subjects with antidepressants or alcohol did not change any of our findings and the mean expression levels of GR-α, GR-β or MR in the antidepressant group or the alcohol group were also not significantly different from those suicide victims who had no presence of antidepressants or ethanol in their blood at the time of death. Thus, this suggested that the presence of antidepressants or alcohol had no significant effect on the overall results.

4. Discussion

In this study, we determined the protein and mRNA expression of GR-α and MR, and mRNA expression of GILZ, a GR target gene, in the PFC, hippocampus, subiculum, and amygdala of teenage suicide victims and matched normal control subjects. We also determined the protein and mRNA expression of the other isoform of GR receptor, known as GR-β, in the PFC of suicide victims and normal controls. GR-β expression was almost absent in the hippocampus and amygdala. Our observation that GR-β is almost undetectable in the hippocampus and amygdala is consistent with previous observations of DeRijk et al. (2003) who observed very low abundance of GR-β in human hippocampus. In order to examine the functional consequences of changes in the GR we also determined the mRNA expression of GR target gene known as GILZ in the PFC, hippocampus, and amygdala of suicide victims and normal controls. We found that the protein and mRNA expression of GR-α was significantly decreased in the PFC and amygdala, but not in the hippocampus or subiculum of teenage suicide victims compared with matched control subjects. There was no significant difference in the mRNA expression of GR-β in the PFC between suicide victims and normal control subjects. We didn't find any significant differences in the protein or mRNA expression of MR in the PFC, hippocampus or amygdala of teenage suicide victims compared with normal control subjects. We also observed that among the amygdaloid nuclei the gene expression of GR-α was significantly

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decreased in the CeA but not in the MeA in the suicide victims compared with controls. Again, the gene expression of MR was not significantly different between the suicide victims and normal controls in the CeA or MeA.

Two isoforms of human GR have been identified which are known as GR-α and GR-β and they originate from the same gene by alternative splicing of the GR primary transcript (Hollenberg et al., 1985; Oakley et al., 1996). Of the two isoforms, GR-α isoform is more predominantly expressed specifically in the brain and shows steroid binding activity. In the absence of a ligand, GR-α resides primarily in the cytoplasm of cells and is held inactive by its binding to heat shock proteins. Upon hormone binding, GR-α is phosphorylated, dissociates from heat shock proteins and subsequently translocates to the cell nucleus where it mediates either trans-activation or trans-suppression, trans-repression of target genes. The mechanism of gene activation is mediated through binding of the GR homodimers to specific glucocorticoid elements on the promoter region of the target genes. GR-β, on the other hand, acts as a dominant negative inhibitor of GR-α activity through a mechanism that involves the formation of transcriptionally impaired GR-α, GR-β heterodimers.

Pujols et al. (2002) determined the expression of GR-β and GR-α in several human tissues, including the inflammatory cells and the brain. They found the highest expression of the GR-β in the skeletal muscles, lung, and liver and the lowest expression in the brain. In order to examine the role GR-α or GR-β play in the brain, we determined the mRNA expression levels of both the GR-α and GR-β in the PFC, hippocampus and amygdala where we were able to quantitate the mRNA expression levels of GR-β in the PFC and the expression levels of GR-β in the hippocampus and in the amygdala is almost absent. Our observation that GR-β is not detectable is hippocampus is similar to the report by DeRijk et al. (2003) in the human hippocampus. Alt et al. (2010) also determined the expression levels of GR-α and GR-β in the human limbic system and found that GR-α is the predominant (83%) while GR-β was barely detectable (almost 0.02%). They also found in MDD the total GR levels where unchanged although GR-α was decreased in the amygdala and the cingulate gyrus.

The inhibition of corticotropin-releasing factor (CRF) release is regulated by GR-MR in the paraventricular nucleus (PVN) (Marques et al., 2009). However, there is ample evidence to suggest that hypothalamic control of CRF release is also regulated by GR/MR distal to the hypothalamus, such as the amygdala, hippocampus, and PFC (Herman et al., 2003). Since these areas are also involved in emotion, stress, and anxiety (see review by Gold et al., 2002), which are major risk factors for suicide, it was important to examine the status of GR in these brain areas in suicide victims.

Whereas we found a decrease in GR-α expression in PFC and amygdala, we did not find changes in GR-α expression in hippocampus or subiculum of teenage suicide victims compared with controls. Since the hippocampus is a major site for stress regulation (Bremner et al., 1995; Sapolsky et al., 1986), this finding was contrary to our expectations.

There may be two possible explanations for the absence of GR-α changes in the hippocampus of teenage suicide victims. Stress-related changes in the levels of corticosteroids have been implicated in hippocampal atrophy in PTSD (Bremner et al., 1995) and depression (Sheline et al., 2003). While Bremner et al. (1995) observed reduction in left hippocampal volume in adults with childhood trauma and recent diagnosis of PTSD, De Bellis et al. (1999) did not find changes in hippocampal volume in children with PTSD. Teicher et al. (2002) also did not observe changes in hippocampal volume in sexually abused children. One possible explanation of this discrepancy between adults and adolescents may be that the stress of PTSD exerts a gradual effect on hippocampal morphology, so that stress effects on the hippocampus cannot be observed in children or adolescents, but only in adults. This may be one of the reasons why we did not observe changes in GR-α in the hippocampus of teenage suicide victims.

Another reason for the lack of any changes in GR-α in the hippocampus may be that changes in the GR-α are not related to suicide but may change as a result of early life trauma. In an epigenetic study, McGowan et al. (2009) observed lower mRNA expression of GR (NR3C1) in hippocampi of suicide victims with a history of childhood abuse, but not in non-abused suicide victims, suggesting that GR mRNA may be specifically decreased in abused adult suicide victims. In another study in primates, Arabadzisz et al. (2010) found reduced mRNA levels for both GR and MR in the hippocampus of those subjects with early life stress compared with control subjects.

Glucocorticoids mediate their effect through transcriptional regulation of GR target genes (Ayroldi and Riccardi, 2009; Beaulieu and Morand, 2011). One of these target genes mediating the effects of GR is GILZ which was originally identified in 1977 during a study of genes transcription induced by glucocorticoids and responsible for glucocorticoids-mediated apoptosis (D'Adamio et al., 1997). GILZ mediates several GC functions which include cytokine production, apoptosis, cell proliferation and the immune function (Delfino et al., 2004). GILZ is a target gene activated by GR activation and its expression is shown to be disrupted in the hippocampus as a result of hypercortisolemia in the rat (Sarabdjitsingh et al., 2010). That GILZ may play a role in depression was shown by a study of Frodl et al. (2012) who observed that those MDD patients who had reduced expression of GILZ had a smaller hippocampus volume, probably related to over-exposure to cortisol. Since we observed a decreased in the mRNA and protein expression of GR in some areas of postmortem brain of teenage suicide victims we also examined if decreased GR may also be related to its functional consequence, i.e., the transcription of target genes. We therefore determined the GILZ expression in different brain areas of teenage suicide victims as described in the Section 3.

The results were interesting because GILZ expression was found to be lower in the PFC and CeA, but not in the hippocampus or MeA. These results were very similar to what we have observed with the GR expression showing that the GR abnormalities in the postmortem brain of teenage suicide victims may be associated with its abnormal function, as shown by the decreased expression of GILZ in those areas of the brain where GR levels was found to be decreased.

There are several studies which suggest that amygdala activates HPA axis (Gray et al., 1989). For example, electrical

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stimulation of amygdala promotes biosynthesis and release of corticosterone in rats, rabbits and monkeys (Mason, 1959; Matheson et al., 1971; Redgate and Fahringer, 1973). Localized stimulation/lesion paradigms implicate CeA, MeA and basolateral subnuclei in the control of ACTH release (Herman et al., 2003). Both, CeA and MeA have rich connections with brainstem structures innervating the PVN through the nucleus of solitary tract (NST), as shown by Schwaber et al. (1982). We therefore examined if GR and MR receptors are altered in these nuclei. Our observation of decreased GR receptors in CeA, but not in MeA is intriguing. A possible reason for this may be that CeA has richer connections to PVN and may be more involved in CRF release. However, the precise reason is unclear.

The dysregulation of the HPA system or its hyperactivity is one of the most consistent finding in depression and suicidal behavior (Pariante and Lightman, 2008). It is based on the observation of non-suppression of the DST and abnormal CRF-ACTH test results in depressed patients (Holsboer, 2000). Also, there is some evidence to suggest that a dysregulated HPA axis may be a stronger predictor of suicidal behavior in depressed patients (Coryell and Schlesser, 2001; Lester, 1992). The reasons for the hyperactive or dysregulated HPA axis are not clear, but it is believed that this dysregulation may at least in part be due to an altered feedback inhibition by the endogenous glucocorticoids mediated by binding to their receptors: GR and MR (de Kloet et al., 2005).

Two types of adrenal steroid receptors, known as GR and MR, have been identified in rodent and human brain (Reul and de Kloet, 1985; Seckl et al., 1991). GR has a high affinity for synthetic glucocorticoid dexamethasone and a lower affinity for cortisol or corticosterone (Reul and de Kloet, 1985; Sanchez et al., 2000). Because of the high affinity of MR for cortisol, these receptors are generally fully occupied at basal cortisol levels. However, GR are occupied when cortisol levels are increased, e.g., in stress or illness. Thus, GR get activated mostly during high concentrations or increase in cortisol levels, and may thus mediate the feedback effect of high levels of cortisol, such as seen with stress or depression. It is therefore believed that GR are important in the regulation of stress response when endogenous levels of cortisol are high and therefore may play a role in stress-related disorders, such as depression or suicide.

The studies of GR and MR in human postmortem brain of subjects with mood disorders and suicide are few and are limited to specific brain areas. Lopez et al. (1998) determined GR and MR in various subfields of hippocampus of 6 suicide victims and 6 controls. They found a significant decrease in MR mRNA in CA3, but not in other subfields. However, mRNA for GR was not significantly different between suicide and control subjects in any subfield. Perlman et al. (2004) found that GR mRNA was reduced in the basolateral nuclei of amygdala in schizophrenia and bipolar disorder subjects, but not in subjects with major depression. Webster et al. (2002) found region-specific decrease in GR mRNA in postmortem brain of subjects with major depression, bipolar illness and schizophrenia. Our studies are comparable to those of Lopez et al. (1998) who studied suicide victims and, similar to our findings, did not find changes in GR in hippocampus of suicide victims.

There are some studies of MR in postmortem brain from MDD or suicide subjects. Klok et al. (2011) determined transcript levels of MR and GR in five areas of postmortem brain from MDD and control subjects. They found decreased MR and GR expression in the hippocampus, inferior frontal gyrus and cingulate gyrus, but not in the amygdala or nucleus accumbens. On the other hand, Medina et al. (2013) found decreased expression of MR in the anterior but not posterior hippocampus of MDD subjects compared with controls. However, we did not find any significant differences in MR expression in any of the brain areas we studied. The reason for this discrepancy is unclear but may be related to different populations being studied — teenage suicide in our case and adult MDD subjects studied by Klok et al. (2011) and Medina et al. (2013).

Other evidence of the important role GR plays in regulating the HPA axis and its involvement in depression is derived from the studies of knockout mice. Mice with deficient GR have been shown to exhibit depression-like behavior (Boyle et al., 2005; Chourbaji et al., 2008).

In summary, we observed that the mRNA and protein expression of GR, but not MR, is decreased in the PFC and amygdala of teenage suicide victims compared with control subjects. That decrease GR is associated with decreased expression of GR target gene GILZ in the areas where GR expression is decreased suggests a decreased functional consequence of GR in suicide. This observation is consistent with the suggested involvement of GR in altered HPA feedback mechanism. Previous studies, although few, have examined GR mRNA or protein expression in some areas of the brain in adult depression or suicide subjects. This comprehensive study of GR and MR suggests a dysregulation of HPA feedback mechanism in teenage suicide that may be related to changes in GR in specific brain areas and specific amygdaloid nuclei.

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Conflict of interest

All authors declare that they have no financial interests or potential conflicts of interest related directly or indirectly to this work.

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