Decreased GRK3 but not GRK2 expression in frontal cortex from bipolar disorder patients

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Abstract

Overactivation of G-protein-mediated functions and altered G-protein regulation have been reported in bipolar disorder (BD) brain. Further, drugs effective in treating BD are reported to up-regulate expression of G-protein receptor kinase (GRK) 3 in rat frontal cortex. We therefore hypothesized that some G-protein subunits and GRK levels would be reduced in the brain of BD patients. We determined protein and mRNA levels of G-protein subunits and GRK3 in post-mortem frontal cortex from 10 BD patients and 10 age-matched controls by using immunoblots and real-time RT–PCR. There were statistically significant decreases in protein and mRNA levels of G-protein subunits and of GRK3 in BD brain but not a significant difference in the GRK2 level. Decreased expression of G-protein subunits and of GRK3 may alter neurotransmission, leading to disturbed cognition and behaviour in BD.

Key words: Bipolar disorder, brain, G-protein β subunit, G-protein γ subunit, GRK2, GRK3.

Introduction

G-protein-coupled receptors (GPCRs) are regulated by G-protein-coupled receptor kinases (GRKs). GRKs are a family of serine/threonine kinases involved in the homologous desensitization of agonist-activated GPCRs (Krupnick & Benovic, 1998; Palczewski et al. 1991). GRKs phosphorylate the agonist (endogenous ligand)-activated receptors (Pitcher et al. 1998), leading to uncoupling of the activated receptor from further stimulation of its G protein (Pitcher et al. 1998).

Hundreds of different GPCRs are regulated by seven types of GRKs (Gainetdinov et al. 2004). GRK1 and GRK7 are found only in the retina. GRK4, GRK5 and GRK6 are not activated by the G-protein subunit βγ, whereas GRK2 and GRK3 are activated by G-protein subunit βγ and are translocated from the cytosol to the membrane by this subunit (Gainetdinov et al. 2004; Koch et al. 1993; Pitcher et al. 1992; Premont et al. 1995). GRK3 is abundantly expressed in several brain regions including cortex, hippocampus, and ventral striatum, suggesting an important role for the GRK3 gene in the modulation of neurotransmission in these regions (Arriza et al. 1992; Erdtmann-Yourlitis et al. 2001). GRK3 regulates several GPCRs, including the adrenergic (Carman & Benovic, 1998), cholinergic, muscarinic (Willetts et al. 2001), dopaminergic (Tiberi et al. 1996), histaminergic (Shayo et al. 2001), and corticotropin-releasing factor receptors (Dautzenberg et al. 2001, 2002).

Several studies have suggested alteration in G proteins, GPCRs, and in their responses in mood disorders. The changes include: (1) increased G-protein Gas subunit in post-mortem brain (Friedman & Wang, 1996; Young et al. 1993); (2) elevated [35S]GTPγS binding to platelet membrane Gas, Gai, and Gαq/11 subunits (Hahn et al. 2005); (3) increased serotonergic, dopaminergic and muscarinic receptor-mediated coupled responses (Dilsaver, 1986; Friedman & Wang, 1996; Pantazopoulos et al. 2004), (4) overactivated serum phospholipase A2 (PLA2) activity (Noponen et al. 1993); and (5) elevated forskolin-stimulated cAMP formation in bipolar disorder (BD) post-mortem brain (Young et al. 1993). Several pre-clinical studies also indicate that G proteins are differentially attenuated by mood stabilizers (lithium or carbamazepine) and antidepressant treatments (Avissar & Schreiber, 1992a,b; Avissar et al. 1988).
Some studies have demonstrated alterations in GRKs in BD due to a single nucleotide polymorphism in the promoter region of the GRK3 gene (Barrett et al. 2003) and a decrease in GRK3 protein level in lymphocytes (Niculescu et al. 2000). In contrast, the mood stabilizers lithium and carbamazepine, when given chronically to rats to produce therapeutically relevant concentrations, were reported to up-regulate GRK3 but not GRK2 in the frontal cortex (Ertley et al. 2007). This effect could have arisen from up-regulation of the G-protein \( \beta \) subunit if lithium and carbamazepine were to act through this mechanism (Jakobsen & Wiborg, 1998).

Thus, studies imply overactivation of various GPCRs and alteration of GRK3 in BD. We therefore hypothesized that the post-mortem brain of BD subjects would have decreased G-protein \( \beta \) and \( \gamma \) subunits and decreased GRK3 but not GRK2 protein and mRNA levels. To test this hypothesis, we determined G-protein \( \beta \) and \( \gamma \) subunits and GRK2 and GRK3 protein and mRNA levels in post-mortem frontal cortex from BD patients and age-matched controls. We also measured protein and mRNA levels of neuron-specific enolase (NSE), a marker of post-mortem tissue integrity in the absence of acute injury (Dautzenberg et al. 2001; Nogami et al. 1998; Preece & Cairns, 2003).

We examined the frontal cortex because studies indicate structural, metabolic, and signalling abnormalities in the frontal cortex of bipolar patients (Buchsbaum et al. 1986; Lopez-Larson et al. 2002; Lyoo et al. 2004; Rajkowska, 2002; Rubinsztein et al. 2001; Suhara et al. 1992).

### Material and methods

**Human post-mortem brain samples**

Frozen post-mortem human frontal cortex (Brodmann area 9) was provided by the Harvard Brain Tissue Resource Center (McLean Hospital, Belmont, MA) under PHS grant number R24MH068855. This study was approved by the institutional review boards of the McLean Hospital and the Office of Human Subjects Research (OHSR) of NIH no. 4380. The study was performed on tissue from 10 BD patients and 10 age-matched controls. Table 1 summarizes the age, post-mortem interval, the reported cause of death, and medication taken at the time of death. The pH of the frozen brain samples was measured by the method of Harrison et al. (1995). The age (yr) (control 43 ± 3.5 vs. BD 49 ± 7.2), post-mortem interval (h) (control 27 ± 1.5 vs. BD 21 ± 3.0) and brain pH (control 6.6 ± 0.16 vs. BD 6.7 ± 0.09) did not differ significantly between the two groups.
groups, whereas the BD patients were exposed to various psychotropic medications.

**Preparation of cytosolic and membrane fractions**

Cytosolic and membrane extracts were prepared from post-mortem frontal cortex of BD and control subjects as previously described (Dwivedi et al. 2000). Briefly, frontal cortex tissue was homogenized in a homogenizing buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EGTA, 5 mM EDTA, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulphonyl fluoride, 0.2 U/ml aprotinin, and 2 mM dithiothreitol, using a Polytron homogenizer. The homogenate was centrifuged at 100,000 g for 60 min at 4 °C. The resulting supernatant 1 (S1) was the cytosolic fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (w/v) Triton X-100. The suspension was kept at 4 °C for 60 min with occasional stirring and then centrifuged at 100,000 g for 60 min at 4 °C. The resulting supernatant 2 (S2) was the membrane fraction. Protein concentrations in membrane and cytosolic fractions were determined with Bio-Rad protein reagent (Bio-Rad, USA). The membrane and cytosolic fractions were characterized with specific markers for cadherin and tubulin, respectively.

**Western blot analysis**

Cytosolic or membrane extracts (75 µg) were separated on a 10–20% SDS–polyacrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. Membrane blots were incubated overnight with primary antibody for the G-protein β3 subunit (1:200), G-protein γ subunit (1:200) (Millipore, USA), NSE (1:10,000) and cadherin (1:200) (Abcam, USA). Both membrane and cytosolic blots were incubated with primary antibody for GRK2 or GRK3 (1:200) (Abgent, USA) in TBS buffer containing 5% non-fat dried milk and 0.1% Tween-20, followed by HRP-conjugated secondary antibody (1:1,000) (Bio-Rad). Blots were visualized and quantified after correcting for β-actin as previously described (Rao et al. 2005). Before starting the immunolabelling experiments with the samples, the procedure was standardized using 10–200 µg protein. We found that the optical density of the bands varied linearly with concentrations up to 100 µg protein, and 75 µg total protein was used in the present study as previously described (Ertley et al. 2007).

**Total RNA isolation and real time RT–PCR**

Total RNA was isolated from post-mortem frontal cortex of control and BD patients using an RNeasy lipid tissue kit (Qiagen, USA). Briefly, tissue was homogenized in Qiagen lysis solution and total RNA was isolated by phenol–chloroform extraction. cDNA was prepared from total RNA according to the manufacturer’s instructions using a high-capacity cDNA archives kit (Applied Biosystems, USA). RNA integrity number (RIN) was measured using a Bioanalyzer (Agilent 2100 bioanalyzer, USA). RIN values (mean ± S.E.M.) were control 6.9 ± 0.4 and BD 7.15 ± 0.5. cDNA was generated in a thermal cycler using 5 µg total RNA and a mixture of multiscribe reverse transcriptase (50 U/µl), random primers (10×), and dNTPs (25×). Expression of G-protein β3, G-protein γ, GRK2, GRK3 and NSE was determined using specific primers and probes for G-protein β and G-protein γ, GRK2, GRK3 and NSE purchased from TaqMan Gene Expression Assays (Applied Biosystems) consisting of a 20× mix of unlabelled PCR primers and Taqman minor groove binder (MGB) probe (FAM dye-labelled). The fold change in gene expression was determined using the ΔΔCt method (Livak & Schmittgen, 2001). Data were expressed as the relative level of the target gene (G protein, GRK and NSE) in the BD frontal cortex normalized to the level of the endogenous control (β-globulin) and relative to the controls (calibrator), as previously described (Rao et al. 2005). All experiments were carried out twice in triplicate with 10 independent samples per group.

**Statistical analysis**

Data were expressed as mean ± S.E.M. When two groups were compared (control and BD), statistical significance was determined using an unpaired two-tailed t test. When three groups were compared (control, BD and BD treated with lithium or BD patients that died by suicide), statistical significance was determined using a one-way analysis of variance and a Bonferroni’s multiple comparison test. Statistical significance was set at p < 0.05.

Statistical significance of differences was calculated using a two-tailed unpaired t test performed between control, BD and BD with lithium treatment or BD with suicide. Pearson correlations were made between age, post-mortem interval and pH of the frontal cortex, and mRNA levels of G-protein subunits and GRKs in post-mortem brain from controls and BD patients, separately. Statistical significance was set at p < 0.05.

**Results**

**Decreased protein and mRNA levels of G-protein β3 and γ subunits**

Figure 1(a, b) shows that mean protein levels of G-protein β3 and G-protein γ were decreased...
Fig. 1. Mean G-protein $\beta$ (a) and G-protein $\gamma$ (b) protein (with representative immunoblots) in control ($n = 10$) and bipolar disorder (BD) frontal cortex ($n = 10$). Data are ratios of optical densities of G-protein subunits to $\beta$-actin, expressed as percent of control. mRNA levels of G-protein $\beta$ (c) and G-protein $\gamma$ (d) in post-mortem control ($n = 10$) and BD ($n = 10$) frontal cortex, measured using real time RT–PCR. Data are levels of G-protein $\beta$ subunits in the BD patients normalized to the endogenous control ($\beta$-globulin) and relative to control level (calibrator) using the $\Delta \Delta C_T$ method (mean $\pm$ S.E.M., ** $p < 0.01$).

Fig. 2. Representative immunoblots of GRK2 and GRK3 protein levels in membrane (a, d) and cytosol (b, e) in frontal cortex of controls ($n = 10$) and bipolar disorder (BD) patients ($n = 10$). Data are ratios of optical density of GRK2 and GRK3 to $\beta$-actin, expressed as percent of control, and compared using a two-tailed, unpaired t test (mean $\pm$ S.E.M., * $p < 0.05$, ** $p < 0.01$). Bar graphs of membrane/cytosol ratios of GRK2 (c) and GRK3 (f) in frontal cortex of controls and BD patients (mean $\pm$ S.E.M., * $p < 0.05$).
significantly, by 37% ($p<0.01$) and 33% ($p<0.01$), respectively, in BD compared to control frontal cortex. Further, mean mRNA levels of G-protein $\beta_3$ and $\gamma$ subunits were significantly decreased by 45% ($p<0.01$) in BD brain compared to control brain (Fig. 1c, d).

### Decreased protein and mRNA levels of GRK3

Compared to control brain, there was no significant difference in the mean protein level of membrane GRK2 in bipolar brain (Fig. 2a). However, there was a significant decrease (43%, $p<0.01$) in the mean protein level of membrane GRK3 (Fig. 2d). There was no significant difference in the cytosolic GRK2 or GRK3 protein levels (Fig. 2b, e). The ratio of membrane/cytosol was significantly decreased for GRK3 but not for GRK2 (Fig. 2c, f) ($p<0.05$). The decreased GRK3 was associated with a significant decrease in the GRK3 mRNA level (Fig. 3a). However, there was no significant difference in GRK2 mRNA (Fig. 3b). Mean mRNA and protein levels of NSE did not differ significantly between BD and control brain (Fig. 3c, d). Using Bonferroni’s multiple comparison test between control, BD and BD with lithium treatment showed a significant decrease in GRK3 protein ($F=8.68$, d.f. 2, 21, $p=0.01$) and mRNA ($F=25.38$, d.f. 2, 21, $p=0.001$) expression compared to control subjects. Bar graph is ratio of optical density of NSE protein to that of $\beta$-actin, expressed as percent of control (mean ± S.E.M., *** $p<0.001$).

**Fig. 3.** mRNA levels of GRK3 (a), GRK2 (b) and NSE (c) in post-mortem control ($n=10$) and bipolar disorder (BD) ($n=10$) frontal cortex, measured using real time RT–PCR. Data are levels of GRK and NSE in the BD patients normalized to the endogenous control ($\beta$-globulin) and relative to control level (calibrator), using the ΔΔCt method. Mean neuronal-specific enolase (NSE) (d) protein in post-mortem frontal cortex from control and BD subjects. Bar graph is ratio of optical density of NSE protein to that of $\beta$-actin, expressed as percent of control (mean ± S.E.M., *** $p<0.001$).

**Fig. 4.** GRK3 (a) protein and (b) mRNA levels in frontal cortex from control, bipolar disorder (BD) and BD + lithium treatment groups. Groups were compared using Bonferroni’s multiple comparison test ($F=8.68$, d.f. 2, 21, ** $p=0.01$) ($F=25.38$, d.f. 2, 21, *** $p=0.001$).
in BD; but no significant change in BD with lithium treatment (Fig. 4).

Correlation data with brain variables
Pearson correlations between mRNA and protein levels in BD brain treated separately on the one hand, and post-mortem interval, age and pH on the other, were all statistically insignificant ($p > 0.05$) (Table 2). Mean values of the three parameters did not differ significantly between control and BD patient groups.

Discussion
The present study demonstrates significant decreases in protein and mRNA levels of G-protein $\beta$ and $\gamma$ subunits and of membrane GRK3 in post-mortem frontal cortex of BD compared to control subjects. There was no significant group difference in the protein or mRNA level of GRK2 or NSE.

GPCR overactivation has been associated with increased G$_a$s and heterotrimeric G-protein subunit levels in platelets and in post-mortem brain from BD patients (Friedman & Wang, 1996; Manji & Lenox, 2000; Mathews et al. 1997; Vawter et al. 2000). Overactivation of G protein and G-protein-coupled mediated functions by serotonin (Friedman & Wang, 1996), increased muscarinic (Dilsaver, 1986; Tollefsen & Senogles, 1983), and dopaminergic receptors (Pearlson et al. 1995; Wong et al. 1997) have also been reported in post-mortem BD brain. These GPCRs can be coupled to multiple effectors including cytosolic phospholipase A$_2$ (Barak et al. 2003; Basselin et al. 2003, 2005a; Bhattacharjee et al. 2005; Felder, 1995; Felder et al. 1990), phospholipase C (Mathews et al. 1997), and adenylylate cyclase (Young et al. 1993).

Taken together, these studies suggest overactivation of G-protein-mediated neurotransmission in BD. Drugs (lithium and carbamazepine) that are effective in the manic phase of BD have been reported to reduce the G-protein levels in rat brain (Jakobsen & Wiborg, 1998) and in PC12 cells (Li & Jope, 1995). Two weeks of lithium and valproate treatment also reduced PKC activation and receptor–G-protein coupling in platelets of bipolar manic patients (Hahn et al. 2005). Furthermore, lithium, and carbamazepine, when administered for 4–6 wk to rats to produce therapeutically relevant plasma levels, reduced signal transduction involving arachidonic acid and cytosolic PLA$_2$ activation, coupled via G-proteins to dopaminergic D$_2$ and serotonergic receptors (Basselin et al. 2005a, b; 2008). In the present study, we found a decrease in the protein and mRNA levels of G-protein $\beta$ and $\gamma$ subunits in post-mortem frontal cortex from BD patients. This may be due to the altered heterotrimeric complex expression.

GRK activation is a highly regulated process that can be measured in terms of expression level and intrinsic activity but also by subcellular compartmentalization of the GRKs (Penn et al. 2000). GRKs are

| Table 2. Probabilities and Pearson correlations ($r^2$) between brain mRNA/protein levels and subject age, post-mortem interval (PMI) and brain pH |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| mRNA | G-protein $\beta$ | G-protein $\gamma$ | GRK2 | GRK3 | Protein | G-protein $\beta$ | G-protein $\gamma$ | GRK2 | GRK3 |
| Controls | | | | | | | | | |
| Age (yr) | 0.17 | 0.63 | 0.06 | 0.82 | 0.90 | 0.62 | 0.52 | 0.71 |
| $r^2$ | 0.21 | 0.02 | 0.39 | 0.00 | 0.00 | 0.03 | 0.05 | 0.01 |
| PMI (h) | 0.29 | 0.78 | 0.08 | 0.64 | 0.54 | 0.59 | 0.34 | 0.71 |
| $r^2$ | 0.13 | 0.00 | 0.32 | 0.02 | 0.04 | 0.03 | 0.11 | 0.01 |
| pH | 0.36 | 0.23 | 0.66 | 0.74 | 0.52 | 0.59 | 0.22 | 0.24 |
| $r^2$ | 0.10 | 0.16 | 0.02 | 0.01 | 0.05 | 0.03 | 0.17 | 0.16 |
| Bipolar disorder | | | | | | | | |
| Age (yr) | 0.81 | 0.49 | 0.84 | 0.06 | 0.24 | 0.14 | 0.28 | 0.21 |
| $r^2$ | 0.00 | 0.05 | 0.00 | 0.36 | 0.16 | 0.24 | 0.13 | 0.18 |
| PMI (h) | 0.49 | 0.56 | 0.09 | 0.83 | 0.50 | 0.06 | 0.96 | 0.73 |
| $r^2$ | 0.24 | 0.04 | 0.31 | 0.00 | 0.05 | 0.38 | 0.00 | 0.01 |
| pH | 0.39 | 0.44 | 0.71 | 0.53 | 0.27 | 0.07 | 0.07 | 0.25 |
| $r^2$ | 0.09 | 0.07 | 0.01 | 0.05 | 0.14 | 0.34 | 0.34 | 0.15 |
located in the cytosol, become activated, and then are translocated to the membrane. Of all the GRKs, GRK2 and GRK3 have a carboxy-terminal domain that binds to G-protein βγ subunits (Daaka et al. 1997; Gainetdinov et al. 2004; Koch et al. 1993). The βγ subunits, released from receptor-activated G proteins, are responsible for translocating the GRK to membrane from cytosol (Daaka et al. 1997).

The present study demonstrated a significant decrease in membrane GRK3 protein in frontal cortex of BD patients but no significant difference in the cytosolic GRK3 protein level. A significant decrease in membrane/cytosol GRK3 ratio was observed, suggesting decreased translocation of GRK3 from cytosol to membrane. Because GRK3 is activated by the G-protein subunits βγ (Koch et al. 1993), the observed decrease in membrane GRK3 might be secondary to decreased expression of the G-protein βγ subunits. In contrast, Shaltiel and co-workers did not find a significant difference in lymphocyte GRK3 mRNA levels obtained from BD patients (Shaltiel et al. 2006). Further, there was no significant change in GRK2 expression or in the membrane/cytosol ratio of GRK2 protein expression level. Brain levels of GRK2 are reported to be increased in the prefrontal cortex of depressed patients and lowered in patients who received antidepressant therapy (Grange-Midroit et al. 2003). Lack of change in expression of GRK2 in frontal cortex of BD patients suggests that such a change may be selective to major depression and not associated with BD pathology. The decrease in GRK3 membrane protein expression in frontal cortex of BD patients might be due to the decreased mRNA level. There was no significant change in the mRNA levels of GRK2, consistent with no change in its protein levels. Compared to earlier animal and clinical studies on lymphocytes, the present study did not find a statistical difference between the lithium-treated subgroup or suicide subgroup compared to the whole patient group regarding GRK3 or G-protein subunit expression (Ertley et al. 2007; Jakobsen & Wiborg, 1998; Shaltiel et al. 2006). This may be due to smaller size samples; further studies are needed to explain influences of those confounded factors.

The present study supports the hypothesis that decreased GRK3 protein translocation from cytosol to membrane contributes to overactivation of GPCRs in BD. Other studies have demonstrated a polymorphism in the promoter region of the GRK3 gene in BD patients but not in schizophrenia patients (Yu et al. 2004). GRK3 protein levels were decreased in lymphocytes of BD patients (Niculescu et al. 2000), and studies have repeatedly demonstrated an association of the GRK3 gene polymorphism in BD patients (Barrett et al. 2003, 2007).

The BD patients had been exposed to a variety of drugs not experienced by the control subjects, which may have confounded the results. Because of this exposure, we are not sure that our findings were not related to drug exposure, or that they are specific to BD. Future studies should examine G proteins and GRK expression in brains from patients with schizophrenia (to control for roughly comparable drug exposure), unipolar (primary major) depression, or Alzheimer’s disease (to test for disease specificity) (Benes, 2007).

In summary, post-mortem frontal cortex from BD patients compared to controls showed a decrease in G-protein βγ subunits and membrane GRK3 protein and mRNA levels but not a significant difference in GRK2 levels. These decreases may impair homologous desensitization and thereby induce the reported GPCR supersensitivity of D2 and other GPCRs in BD patients.

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Statement of Interest

None.

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