MOLECULAR MECHANISMS UNDERLYING G PROTEIN DISTURBANCES IN BIPOLAR AFFECTIVE DISORDER: THE ROLE OF ADP-RIBOSYLATION PROCESSES

by

Stavroula Andreopoulous

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy, Department of Pharmacology, in the University of Toronto

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Molecular Mechanisms Underlying G Protein Disturbances in Bipolar Affective Disorder: The Role of ADP-ribosylation Processes
Ph. D Thesis- 2000
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ABSTRACT

This study examined whether the stimulatory G protein α subunit, αs, undergoes ADP-ribosylation in postmortem human brain, and whether disturbances in this pathway contribute to the elevated αs levels reported in bipolar affective disorder (BD). Endogenous and cholera toxin (CTX)-catalyzed [32P]ADP-ribosylated αs were characterized in postmortem temporal cortex by immunoprecipitation, and overlay comparisons of autoradiograms and Western blots of the [32P]ADP-ribosylated αs isoforms. Endogenous and CTX-catalyzed [32P]ADP-ribosylated αs in temporal, occipital and cerebellar cortices of BD, and age/postmortem delay-matched controls were then separated by SDS-PAGE and autoradiograms quantified by densitometry. αs protein levels were determined by Western blotting.

Two major endogenous [32P]ADP-ribosylated products (48 kDa and 45 kDa) were identified as αs-L and αs-S, respectively. Immunoprecipitation with αs specific antibody revealed a third endogenous [32P]ADP-ribosylated protein (39 kDa). Resolution by SDS-PAGE and limited protease digestion supported that this product corresponded to an αs-like protein, possibly a previously reported N-terminal truncated αs splice variant. Two major CTX-catalyzed [32P]ADP-ribosylated products were also identified as αs-L (52 kDa) and αs-S (45 kDa).
No differences were observed in either endogenous, and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{\text{+L}}$ in BD temporal cortex. However, $\alpha_{\text{+L}}$ immunolabeling was significantly elevated and correlated inversely with endogenously $[^{32}\text{P}]$ADP-ribosylated $\alpha_{\text{+L}}$ in this brain region suggesting reduced clearance of $\alpha_{\text{+L}}$ through the ADP-ribosylation pathway in BD temporal cortex. Reduced CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{\text{+S}}$ in BD temporal cortex also supports this interpretation. Moreover, lack of differences in endogenous $[^{32}\text{P}]$ADP-ribosylation of myelin basic protein (MBP) in temporal cortex of BD compared with controls suggests that elevations of $\alpha_{\text{+}}$ are not due to underlying disturbances in ADP-ribosyltransferase activity but rather may reflect changes in disposition or availability that are specific to $\alpha_{\text{+}}$.

Endogenous $[^{32}\text{P}]$ADP-ribosylation of the 39 kDa $\alpha_{\text{+}}$-like protein was reduced only in BD temporal cortex. This decrement correlated with lithium concentrations suggesting that lithium may modify the activity of specific ADP-ribosyltransferases in this region.

While the results of this study demonstrate that $\alpha_{\text{+}}$ is a substrate for ADP-ribosylation and that a 39 kDa $\alpha_{\text{+}}$-like protein is also expressed in human brain, the findings do not support the hypothesis that alterations in ADP-ribosyltransferase activity per se are responsible for the higher $\alpha_{\text{+}}$ levels seen in BD cerebral cortex regions.
ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my parents Constantine and Agne. I especially want to thank my mom for putting up with me and my mess for the last 5 years. I would not have accomplished anything in my life without you. I am what I am today because of you. Σ'αγαπώ πολύ. Thank you God for all that you’ve given me.

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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APAD</td>
<td>3-acetylpyridine adenine dinucleotide</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>β-AR</td>
<td>β-adrenoceptor</td>
</tr>
<tr>
<td>BD</td>
<td>bipolar affective disorder</td>
</tr>
<tr>
<td>BP-I</td>
<td>bipolar-I</td>
</tr>
<tr>
<td>BP-II</td>
<td>bipolar II</td>
</tr>
<tr>
<td>BZ</td>
<td>benzamidine</td>
</tr>
<tr>
<td>C</td>
<td>catalytic subunit of PKA</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3', 5' monophosphate</td>
</tr>
<tr>
<td>8-CPTcAMP</td>
<td>8-(4-chlorophenylthio)cAMP</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 3',5' monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>CRE binding protein</td>
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<tr>
<td>CTX</td>
<td>cholera toxin</td>
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<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (β-amoethylether)-N,N,N′,N′-tetraacetic acid</td>
</tr>
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<td>GAP-43</td>
<td>growth associated protein-43</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>Gpp(NH)p</td>
<td>guanylimidodiphosphate</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>GTPγS</td>
<td>guanosine 5′-O-(3-thiotriphosphate)</td>
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<td>INH</td>
<td>isonicotinic acid hydrazide</td>
</tr>
<tr>
<td>IP</td>
<td>iloprost</td>
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<tr>
<td>IP₃</td>
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<tr>
<td>K⁺</td>
<td>potassium</td>
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<tr>
<td>MAP-2</td>
<td>microtubule-associated protein-2</td>
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<tr>
<td>MARCKS</td>
<td>myristoylated alanine-rich C kinase substrate</td>
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<td>myelin basic protein</td>
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<td>MDD</td>
<td>major depressive disorder</td>
</tr>
<tr>
<td>MNL</td>
<td>mononuclear leukocyte</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl-maleimide</td>
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</table>
NO  nitric oxide
PDE  cyclic nucleotide phosphodiesterase
PGE₁  prostaglandin E₁
PIA  phenylisopropyl adenosine
PIP₂  phosphatidylinositol 4,5-biphosphate
PKA  protein kinase A
PKC  protein kinase C
PLA₂  phospholipase A₂
PLC  phospholipase C
PLCβ₁  phospholipase Cβ₁
PLCβ₂  phospholipase Cβ₂
PLCγ  phospholipase Cγ
PTX  pertussis toxin
R  regulatory subunit of PKA
SBTI  soybean trypsin inhibitor
SDS  sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRE  serum responsive element
SRF  serum-response factor
TCA  trichloroacetic acid
3D  three dimensional
TPA  tetradecanoyl-phorbol-13-acetate

TSH  thyroid stimulating hormone
CHAPTER I
OVERVIEW
1.1 Introduction

Bipolar Affective Disorder (BD) is a serious, chronic life-long illness with a life-time prevalence of about 1% (Weissman et al., 1988). It is an heterogenous disorder consisting of multiple subtypes, including bipolar I (BP-I) and bipolar II (BP-II), and generally involves recurring bouts of mania or hypomania, and depression (Goodwin and Jamison, 1990). In BP-I disorder, manic states are typically characterized by heightened mood, increased mental and goal directed activity, a higher energy level, markedly reduced need for sleep, impulsivity and psychotic symptoms, often including delusions of grandiosity but also paranoid or persecutory thinking (Winokur, 1984, Endicott et al., 1986, Black and Nasrallah, 1989). Patients diagnosed with BP-II disorder exhibit a history of at least one episode of hypomania and recurrent episodes of depression. In hypomania, the symptoms are less severe than those seen in mania and result in less pronounced alterations in mood, cognition and behaviour and no psychotic symptoms. In contrast, the depressive states in BD consist of depressed mood and behaviour indicated by a bleak and pessimistic outlook, feelings of despair and physical and mental lethary (i.e. psychomotor retardation), together with changes in sleep and eating patterns and/or recurrent thoughts of death or suicide (Carlson and Strober, 1979, Abrams and Taylor, 1980).

Evidence from family, genetic and psychobiological studies implicate a major role for biological factors in the genesis, expression and perpetuation of BD (Post and Ballanger, 1984, Merikangas et al., 1989, Gershon, 1990, Goodwin and Jamison, 1990). Of particular importance to the pathogenesis of this disorder, are the disturbances in genetic mechanisms and signal transduction processes, the latter of which involve a wide variety of intracellular
second messengers (Dubovsky et al., 1992, Manji, 1992, Hudson et al., 1993, Manji et al., 1995a,b. Warsh and Li, 1996, 1999, Li et al., 1999). Among the possible signaling disturbances considered particularly relevant to BD, considerable attention has focused on the importance of elevated levels and functionality (hyperfunction) of the stimulatory guanine nucleotide binding protein (G protein) α subunit (αs) in brain and mononuclear leukocytes (MNLs) from patients with this disorder (Schreiber et al., 1991, Young et al., 1991, 1993, 1994b, Manji et al., 1995a, Friedman and Wang, 1996, Mitchell et al., 1997, Avissar et al., 1997a, b). The mechanism(s) which accounts for the elevated levels of αs in postmortem brain and MNLs, and the functional implications of such alterations with respect to the pathophysiology of BD are poorly understood, however.

G protein levels may be regulated through multiple mechanisms, including gene expression, mRNA stability, as well as protein turnover and degradation (Green et al., 1992, Hadcock and Malbon, 1993). Recent findings regarding agonist-promoted regulation of G-protein α-subunit levels during receptor desensitization and downregulation highlight the critical significance of changes in G protein levels and functionality in the these processes (Mitchell et al., 1993, Mullaney et al., 1993). Such observations emphasize the possibility that the molecular diathesis which accounts for the enhanced levels and functionality of αs in BD may lie in disturbance(s) of the mechanisms coordinating production and/or turnover of the components of the membrane receptor-G protein-effector complexes. In the absence of altered αs messenger ribonucleic acid (mRNA) levels (Young et al., 1996), the higher αs immunolabeling in BD brain regions compared with controls may reflect an abnormality(ies) affecting the disposition and turnover of these critical signal transducing proteins. At least
four different processes may regulate, either alone or in combination, the degradation and/or turnover of specific G protein α subunits. These include: 1) agonist-promoted regulation of G protein turnover during receptor downregulation/desensitization; 2) cross-talk mechanisms; 3) the degradative mechanisms of ubiquitin and calpain proteolysis; and 4) adenosine diphosphate (ADP)-ribosylation. With respect to the latter, recent evidence suggests that ADP-ribosylation is an important process that regulates α, levels and function (Levis and Bourne, 1992) and may be altered in BD (Manji et al., 1995a, Nestler et al., 1995). Accordingly, the principal objective of this thesis was directed towards determining whether disturbances in this posttranslational mechanism contribute to the enhanced levels and hence functionality of α, as reported in this disorder.

In the following sections, recent literature is reviewed on those aspects of the structure and function of heterotrimeric G proteins pertinent to this thesis, and the G-protein mediated disturbances that have been implicated in the pathogenesis of BD. Subsequently, current understanding of biochemical processes that are known or implicated in regulating G protein α subunit levels and function are presented. These comprise a number of processes that affect α, including: agonist-induced regulation, effects of constitutive activation, lipid modifications, cytoskeletal interactions, cross-talk regulation, and the calpain and ubiquitin mediated degradative mechanisms. More importantly, evidence that α, turnover and degradation are modulated posttranslationally by ADP-ribosylation is discussed along with the potential role for this process in the pathophysiology of BD. This provides the essential framework for the development of the hypothesis tested in this thesis, namely that the observed elevations in α, levels in BD brain may result from a disturbance(s)
in the posttranslational modification of $\alpha$, by ADP-ribosyltransferases. A description of the research strategies employed to address this possibility is then presented, followed by the specific hypotheses tested.

1.2 G Proteins

1.2.1 Structure and Function

Heterotrimeric G proteins which are members of a larger guanosine triphosphatase (GTPase) superfamily. are comprised of $\alpha$, $\beta$ and $\gamma$ subunits. It is now well established that the trimeric G proteins play an important role in coupling cell surface receptors to intracellular effector systems (Gilman, 1987). Extensive homology and conserved three dimensional (3D) structure exist among the various subtypes of $\alpha$ (at least 20 isotypes, 39-52 kDa) (Strathmann et al., 1989, Bourne et al., 1991, Spiegel, 1991), $\beta$ (5 isotypes, 35-37 kDa) (Fong et al., 1986, Levine et al., 1990, Watson et al., 1994) and $\gamma$ (12 isotypes, 5-10 kDa) subunits (Gautam et al., 1989, 1990, Clali et al., 1992, Watson et al., 1994, Wilcox et al., 1994, Asano et al., 1998). Upon activation, $\alpha$ subunits can bind guanosine triphosphate (GTP) while $\beta\gamma$ subunits dissociate from $\alpha$ subunits as a tightly but noncovalently linked dimer, allowing each to interact with effector molecules. Hydrolysis of GTP to guanosine diphosphate (GDP) through the intrinsic GTPase activity of the $\alpha$ subunit terminates the cycle causing the reassociation of the heterotrimer to the inactive state. Both $\alpha$ subunits and $\beta\gamma$ dimers have the potential to regulate a variety of effector systems (Taussig et al., 1993) generating multiple downstream cellular signals that can affect a wide variety of processes including transcription (Migeon et al., 1994), vesicular transport (Helms, 1995) and ion channel regulation (Krapivinsky et al., 1995), among others.
I.2.2 α Subunits

α subunits range in size from 350 to 395 amino acids and molecular mass from 39 to 52 kDa (Gilman, 1987, Lochrie and Simon, 1988, Helper and Gilman, 1992) and exhibit extensive amino acid homologies (approximately 45-80% [Rens-Domiano and Hamm, 1995]). The highly conserved sequences among the various subtypes are involved in guanine nucleotide binding, interactions with the βγ dimer, and regulation of effector systems (Simon et al., 1991, Spiegel et al., 1992). The wide variety of α subunits identified and characterized have been grouped into 4 major classes based on amino acid homology and effector regulation as detailed in Table 1. Briefly, the class of α4 (which includes α4 and α_{olf}) is primarily involved in the stimulation of adenylyl cyclase (AC) (Gilman, 1984) while the αi class (including α1, α2, α3, α4, αβ and αγ) is responsible for receptor-mediated inhibition of AC, stimulation and inhibition of potassium (K') and of calcium (Ca^{2+}) channels, respectively, and modulation of cyclic guanosine 3',5'-monophosphate (cGMP)-phosphodiesterase (Sternweis and Robishaw, 1984, Gilman, 1987, Yatani et al., 1987a, 1987b, Van Dongen et al., 1988). The αq subfamily (αq, α11, α14 and α_{15/16}) is exclusively involved in the regulation of phospholipase C (PLC)β subtype, whereas the α12 class (α12 and α13) has been reported to affect Na^+/H^+ exchange (Dhanasekaran et al., 1994), activation of Rho (Hart et al., 1998, Katoh et al., 1998) and stimulation of c-Jun-NH2-terminal kinase (JNK) activity (Collins et al., 1996) (see Table 1).

II.2.2.1 αq

Of particular importance to this thesis is the stimulatory G protein α subunit, αq. The
Table 1 (Adapted from Hepler and Gilman, 1992, Spiegel, 1991)

Properties of Mammalian $\alpha$ Subunits

<table>
<thead>
<tr>
<th>G-Protein Subunit</th>
<th>Molecular Weight (kDa)</th>
<th>ADP-Ribosylation$^a$</th>
<th>Effect</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\alpha L}$</td>
<td>52</td>
<td>CTX</td>
<td>↑ AC</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>$G_{\alpha S}$</td>
<td>45</td>
<td>CTX</td>
<td>↑ Ca$^{2+}$ channels</td>
<td>olfactory, striatum, substantia nigra, nucleus accumbens</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>45/46</td>
<td>CTX</td>
<td>↑ AC</td>
<td></td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>41</td>
<td>PTX</td>
<td>↑ AC</td>
<td>brain</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>40</td>
<td>PTX</td>
<td>↑ K$^+$ channels</td>
<td>widely expressed</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>41</td>
<td>PTX</td>
<td></td>
<td>very low in CNS</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>39</td>
<td>PTX</td>
<td>↓ Ca$^{2+}$ channels</td>
<td>CNS/heart/1% of membrane protein</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>39</td>
<td>PTX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>39</td>
<td>PTX/CTX</td>
<td>↓ cGMP-specific phosphodiesterase</td>
<td>retina rod retina cone</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>40</td>
<td>PTX/CTX</td>
<td></td>
<td>tongue</td>
</tr>
<tr>
<td>$G_{\alpha I}$</td>
<td>41</td>
<td>PTX</td>
<td></td>
<td>brain/neuronal cells</td>
</tr>
</tbody>
</table>

$^a$Cholera toxin (CTX) and pertussis toxin (PTX) catalyzed ADP-ribosylation of the various $\alpha$ subunits: (↑) signifies stimulation whereas (↓) signifies inhibition.
Table 1 continued:

<table>
<thead>
<tr>
<th>G-Protein Subunit</th>
<th>Molecular Weight (kDa)</th>
<th>ADP-Ribosylation(^a)</th>
<th>Effect</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G_{4}, G_{11})</td>
<td>42/43</td>
<td>(\dagger) phospholipase C(\beta_1)</td>
<td>widely expressed including T cells</td>
<td></td>
</tr>
<tr>
<td>(G_{14})</td>
<td>41.5</td>
<td>(\dagger) phospholipase C(\beta_1)</td>
<td>spleen, lung, kidney, uterus, testis, bone marrow stromal cells</td>
<td></td>
</tr>
<tr>
<td>(G_{15,16})</td>
<td>43.5</td>
<td>(\dagger) phospholipase C(\beta_1)</td>
<td>haemopoietic lineage: spleen, thymus, bone marrow, B/T cells</td>
<td></td>
</tr>
<tr>
<td>(G_{12})</td>
<td>44</td>
<td>?</td>
<td>Na(^+)/H(^+) exchanger</td>
<td>widely distributed, low levels in intestine</td>
</tr>
<tr>
<td>(G_{13})</td>
<td>44</td>
<td>?</td>
<td>Na(^+)/H(^+) exchanger</td>
<td>activation of Rho, activation of JNK, widely expressed, high levels in eye, kidney, liver, lung, uterus, testis, platelets, low in brain</td>
</tr>
</tbody>
</table>

\(^a\)Cholera toxin (CTX) and pertussis toxin (PTX) catalyzed ADP-ribosylation of the various \(\alpha\) subunits; \((\dagger)\) signifies stimulation whereas \((\dagger)\) signifies inhibition.
α subunits of G, consist of 2 major isoforms, α_{+L} (long) and α_{+S} (short), with apparent molecular masses of 52 and 45 kDa, respectively. More recently, other α isoforms have been identified, including an extra long α, (XLα,) (Kehlenbach et al., 1994) and a novel α, mRNA that encodes for a N-terminal truncated α, (Ishikawa et al., 1990). The major α, isoforms (α_{+L} and α_{+S}) have been well characterized and are primarily involved in stimulating AC activity (Gilman, 1987) and opening of atrial Ca^{2+} channels (Schubert et al., 1989). The mRNAs encoding these long and short forms of α, are derived from a single precursor heterogeneous nuclear RNA (hnRNA) by alternative splicing. In humans, the gene for α, contains 13 exons (Kozasa et al., 1988). The short form results from the splicing out of a stretch of 45 bases corresponding to 15 amino acids encoded for by exon 3, after gene transcription. The difference among the 2 isotypes within each pair of α_{+L} and α_{+S} transcripts is the presence of an additional three codons 3' to the above mentioned 45 nucleotide strand (see Figure 1B, D; Bray et al., 1986).

As mentioned above, Kenlenbach et al. (1994) reported the existence of an extra long (XL) α, with an approximate molecular mass of 94 kDa. This protein was primarily localized in the postnuclear supernatant fractions derived from PC12 cells. Results from experiments that immunoprecipitated XLα, with antibodies raised against the C-terminal and an epitope encoded for by exon 2 of the α, gene, revealed XLα, to be highly homologous to α, containing all amino acids encoded for by exons 2-13 of the α, gene along with an extra N-terminal portion referred to XL. Transcription of XLα, mRNA starts at a site most likely located 5' of exon 1 accompanied by splicing of exon 1 (see Figure 1A; Kenlenbach et al., 1994). Localized to adrenal gland, brain and pituitary tissue, this protein is almost
The Various $\alpha_\text{i}$ Isoforms Generated from a Single Precursor $\alpha_\text{i}$ Gene

(A) $X_L\alpha_\text{i}$

(B) $\alpha_{L}$

(C) N-terminal truncated $\alpha_\text{i}$

Figure 1 (Adapted from Bray et al., 1986, Kozasa et al., 1988, Ishikawa et al., 1990)
exclusively detected in the trans-Golgi network membrane, is capable of binding GTP, and is modified posttranslationally by cholera toxin (CTX)-catalyzed ADP-ribosylation (Kenlenbach et al., 1994). In contrast to XLαs, Ishikawa et al. (1990) described an alternative novel αs mRNA that encodes for a truncated form of αs generated from transcription initiated at an alternative promoter site (see Figure 1C). This novel αs has an N-terminal deletion and does not undergo CTX catalyzed-ADP-ribosylation. Its functional relevance is still unknown (Ishikawa et al., 1990).

There is conflicting evidence as to whether there are functional differences between αsL and αsS. In the past, functional studies carried out with various recombinant αs-subtypes suggested no differences among the isoforms (Mattera et al., 1989). These splice variants were, however, synthesized in E. coli and may not have undergone co- or post-translational modifications necessary for differences to be observed. Walseth et al. (1989) demonstrated that higher passages of HIT cells (a clonal cell line of Syrian hamster pancreatic islet beta cells) resulted in increased αsS levels, and that this change was associated with increased isoproterenol (ISO). CTX and forskolin-induced cyclic adenosine 3', 5'-monophosphate (cAMP) formation. No changes in the levels of αsL were observed as a function of passage number. These findings suggested that the enhanced cAMP response was due to more efficient coupling of αsS, but not αsL, to AC. More recently, Seifert et al. (1998) studied the β2-adrenoceptor (β2AR)/Gαs interaction using receptor/G protein fusion proteins (β2ARGαsL and β2ARGαsS) heterologously expressed in Spodoptera frugiperda (Sf9) cells. They reported that the β2AR in β2ARGαsL displayed increased potency and intrinsic activity of partial agonists along with increased basal GTPase activity compared to the β2AR in
β2ARGαs (Seifert et al., 1998). These findings are in contrast to an earlier report by Jones et al. (1990), who showed that the rate of AC activation, in S49 lymphoma cell mutants lacking αs (cyc⁻) and PKA activity (kin⁻) transfected with cDNAs encoding for αs or αt, was slightly higher for αs in the presence of GTP-γ-S and ISO suggesting that αs may be activated more rapidly by agonist-bound receptor (Jones et al., 1990). The apparent discrepancies may result from different cell types (Sf9 vs. S49 cells) used which may also have different AC isotypes. Regardless, the observations support the notion that subtle structural differences between closely related G-protein α subunits can have important consequences for the functional properties of a G-protein linked receptor.

The relationship between the levels and function of αs has remained controversial. Hamacher et al. (1995) reported that the expression of high levels of αs protein in thyroid adenomas did not correlate with either basal or thyroid stimulating hormone (TSH)-promoted AC activity in these tumors. Similarly, overexpression of αs protein in NG108-15 cells had no effect on receptor regulation of AC activity (Mullaney et al., 1996). However, enhanced αs-mediated activation of AC was observed in C6 glioma cells chronically exposed to antidepressants (Chen and Rasenick, 1995a). These latter findings occurred without changes in αs protein levels suggesting that hyperfunctionality can also happen even in the absence of alterations in levels of α subunits (Chen and Rasenick, 1995b).

In contrast to the above data, there is recent evidence suggesting a relationship between the levels and function of αs. Thus, Young et al. (1993) reported that elevations of αs were accompanied by increased forskolin-stimulation of AC in BD postmortem brain compared with their age and postmortem-delayed matched controls. Moreover,
overexpression of \( \alpha_{s1} \) in HEK-293 cells resulted in stimulation of cAMP formation by prostaglandin E\(_1\) (PGE1), and alterations in downstream targets of the cAMP signalling cascade (i.e. increased phosphorylation of cAMP responsive element binding protein [CREB] and transcriptional activity of cAMP-dependent reporter gene [Yang et al., 1997]). Similarly, expression of recombinant \( \alpha_\text{s} \) in S49 cyc\(^{-}\) cells results in an increase in the proportion of \( \beta_2\text{AR-}\alpha_\text{s} \) complexes (Krumins and Barber, 1997). Full \( \beta_2\)-agonists also produced greater relative decreases in EC\(_{50}\) with increasing \( \alpha_\text{s} \) levels than partial agonists in this cell line (Krumins and Barber, 1997). Taken together, the above observations support the notion that increased levels of \( \alpha_\text{s} \) do, indeed, result in hyperfunctional \( \alpha_\text{s} \)-coupled AC mediated cAMP signaling, both at the receptor and G protein level.

1.2.3 \( \beta_\gamma \) Subunits

\( \beta \) subunits range in molecular mass between 35 to 44 kDa, and have highly conserved sequences (von Weizsäcker et al., 1992). \( \gamma \) subunits are low molecular mass proteins (5-10 kDa) that are tightly associated with \( \beta \) subunits. Together \( \beta_\gamma \) dimers play a critical role in \( \alpha \) subunit receptor interactions, inhibition of \( \alpha \) subunit activation, as well as in the regulation of a wide variety of effector systems (Birnbaumer et al., 1990, Kleuss et al., 1992, Spiegel, 1991). Studies have implicated the \( \beta_\gamma \) complex in the regulation of several effector enzymes, including inhibition of AC type I and stimulation of AC type II (Iyengar, 1993, Taussig et al., 1993) along with activation of PLC\( \beta \) isozymes (Camps et al., 1992, Wu et al., 1993) and PLA\(_2\) (Jelsema and Axelrod, 1987, Kim et al., 1989), in addition to stimulating ras-dependent activation of the mitogen-activated protein (MAP) kinase pathway (Crespo et al., 1994, Faure et al., 1994). Taken together, these observations show that branching of signal trans-
The branching of signal transduction may occur at several places in the cascade, including at the level of the receptor, G-protein (both α and βγ subunits) and further downstream. The multiplicity of these signaling components can confer diverse and selective signal regulation within a cell.
duction may also occur at the βγ level with the regulation of different effector systems by these dimers (see Figure 2).

1.2.4 Co- and Post-translational Modifications of G Proteins

As shown in Table 1, there are substantial data detailing co- and post-translational modifications of G protein subunits. A number of α subunits can undergo bacterial toxin-catalyzed ADP-ribosylation. αs and αi are particularly sensitive to CTX catalyzed ADP-ribosylation. CTX is an oligomeric protein (84 kDa) comprised of five identical B subunits and a single A subunit, consisting of A1 and A2 polypeptides linked together by a single disulfide bond (Gill, 1976, 1977). The B subunits bind to the oligosaccharide moiety of the monosialoganglioside GMI causing aggregation of the toxin to the cell surface facilitating the entry of the A subunit into the cell (Fishman and Atikkan, 1980, Osborne et al., 1982). Upon entering the cytoplasm, the bond between A1 and A2 are reduced by glutathione. The A1 moiety then combines with an ADP-ribosylation factor (ARF) (Kahn and Gilman, 1984) to yield an active complex with GTP that catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD+) to αs, releasing free nicotinamide (Gill and King, 1975, Moss et al., 1979). ADP-ribosylated αs in the presence of GTP then activates the catalytic subunit of AC resulting in cAMP formation. CTX activation is both temperature-dependent (Hansson et al., 1977) and thiol:protein-disulfide oxidoreductase sensitive (Moss et al., 1980). Furthermore, ARFs are believed to interact directly with the A1 moiety and are also modulated by various detergents such as sodium dodecyl sulfate (SDS) and Triton X-100 (Noda et al., 1988).

CTX is known to ADP-ribosylate αs,= on arginine 201 and αs,= on arginine 187. This
posttranslational modification renders \( \alpha_s \) constitutively active by inhibiting its intrinsic GTPase activity (Cassel and Selinger, 1977). Equally important is the finding that CTX catalyzed ADP-ribosylation of \( \alpha_s \) requires that the substrate be in the heterotrimeric and not dissociated state. Toyoshige et al. (1994) demonstrated that \( \alpha_s \), dissociated from \( \beta\gamma \) by MgCl\(_2\), was no longer ADP-ribosylated by CTX. In line with this observation, incorporation of \( \alpha_s \) into S49 cyc- membranes stripped of \( \beta\gamma \) did not result in any detectable CTX-catalyzed ADP-ribosylated \( \alpha_s \) (Rebois et al., 1997), similar to studies that employed guanosine 5'-O-(3-thiotriphosphate) (GTP\(_\gamma\)S)-activated \( \alpha, \) as substrate (Graziano et al., 1987). Subsequent addition of \( \beta\gamma \) into the S49 cyc- membranes resulted in a concentration-dependent increase in both CTX-catalyzed ADP-ribosylation of \( \alpha_s \) and stimulation of AC, suggesting that heterotrimeric \( \alpha_s\)-GTP\(_\gamma\)S-\( \beta\gamma \) is the preferred substrate for CTX-catalyzed ADP-ribosylation (Graziano et al., 1987). Taken together, the observations suggest that CTX is an important tool that can be used to assess the relative extent to which \( \alpha_s \) is in the heterotrimeric state and the functional activity associated with \( \alpha_s \).

Similar to CTX, pertussis toxin (PTX) is functionally comprised of A and B subunits; the A promoter possesses the enzymatic activity whereas the B subunits mediate binding of the complex to the cell surface and translocation of A into the cytoplasm. PTX modifies \( \alpha_s \), \( \alpha_{i1/2} \), and \( \alpha_i \) at the fourth cysteine residue from the C-terminal leading to uncoupling of the receptor from the G protein (Neer et al., 1984, Sternweis and Robishaw, 1984).

Several \( \alpha_i \) subunits including \( \alpha_v, \alpha_{\omega}, \alpha_{i1/2} \) also undergo cotranslational attachment of myristic acid at their N-terminal glycine residue. This modification is believed to play a role in membrane anchorage and the interactions with \( \beta\gamma \) (Jones et al., 1990, Mumby et al., 1990,
\( \alpha \) and \( \alpha_\gamma \) undergo posttranslational palmitoylation at the N-terminal cysteine residue via thioester-linkage, and this modification is thought to be the primary determinant of membrane attachment for these subunits (Degtyarev et al., 1993, Linder et al., 1993) while \( \beta \) subunits are not posttranslationally modified. \( \gamma \) subunits undergo posttranslational prenylation at the C-terminal cysteine residue. Prenylation also plays a role in anchoring \( \gamma \) subunits to membranes (Sanford et al., 1991) and increases the affinity of \( \beta \gamma \) dimers to \( \alpha \) subunits and effector enzymes such as AC type I and PLC\( \beta_2 \) (Asano et al., 1993, Sohma et al., 1993, Rahmatullah and Robishaw, 1994). These co- and post-translational modifications of G proteins are depicted in Figure 3.

### 1.3 G Proteins and Signal Transduction

#### 1.3.1 G Protein Mediated-cAMP Signaling

Extracellular signals (i.e. neurotransmitters, hormones) are transduced across the cell membrane via diverse plasma membrane receptors, of which the vast majority belong to the superfamily of G-protein coupled receptors (Watson and Arkinstall, 1994). The \( \beta AR \) is a prototypic heptahelical G-protein coupled receptor, of which multiple subtypes are known, which activate \( \alpha \), leading to stimulation of AC (with subsequent elevations in cAMP) and \( Ca^{2+} \) channels (Strader et al., 1989, Perkins, 1991).

ACs comprise a multigene family of proteins, structurally consisting of two-six transmembrane spanning helical segments with catalytical and regulatory sites located in the intracellular domains of the molecules. Nine distinct mammalian subtypes of ACs have been identified, some of which also have splice variants that may differ in their glycosylation and
Figure 3

Co- and Post-translational Modifications of G Protein α Subunits

Schematic representation of co- and post-translational modifications of G protein α subunits including: Palmitoylation of αs (1), CTX-catalyzed ADP-ribosylation of αs (2), Myristic acid attachment to αυ (3) and PTX-catalyzed ADP-ribosylation of αυ (4).
regulatory properties (Cali et al., 1996, Sunahara et al., 1996). As depicted in Table 2, the various AC subtypes can be modulated by a wide variety of regulators, which can either act alone or synergistically to stimulate and/or inhibit AC. For example, AC type II and IV activities can be stimulated by $\beta y$ subunits while AC type III is stimulated by Ca$^{2+}$/calmodulin (CaM) in the presence of $\alpha$. Furthermore, the activities of types V and VI can be modulated by protein kinase A (PKA) and protein kinase C (PKC) mediated-phosphorylation, thereby decreasing and increasing their activity, respectively (Kawabe et al., 1994, Iwami et al., 1995, Houslay and Milligan, 1997).

It has been suggested that cyclic nucleotide phosphodiesterases (PDEs), another large multigene family, play a major role in the modulation of cAMP levels and hence activation threshold of cAMP dependent processes (Bolger, 1994, Beavo, 1995, Conti et al., 1995, Manganiello et al., 1995). PDEs 1-4 are known to hydrolyze cAMP while PDE6 enzymes are specific for hydrolyzing cGMP (Mohamed et al., 1998). The structural heterogeneity of PDEs allows for different substrate specificities and susceptibilities to the action of selective stimulators and inhibitors. For example, PDE1 enzymes have two CaM-binding domains which mediate their activation by Ca$^{2+}$ (Rybalkin and Beavo, 1996). The PDE4 enzyme family is encoded by four genes generating a variety of isoforms by alternative splicing (Bolger, 1994, Conti et al., 1995). Three of these PDE4 subtypes (PDE4A, PDE4B and PDE4D) are highly expressed in brain, are involved in neuronal cAMP regulation (Engels et al., 1995, Iona et al., 1998) and can be differentially regulated by various therapeutic agents, including antidepressants (Ye and O'Donnell, 1996, Ye et al., 1997, Takahashi et al., 1999). Collectively, they represent integral components of G protein $\alpha$ subunit-mediated
Table 2 (Adapted from Houslay and Milligan, 1997)

Mammalian Adenylyl Cyclases (ACs)

<table>
<thead>
<tr>
<th>Type</th>
<th>Splice Variants</th>
<th>Ca²⁺</th>
<th>βγ</th>
<th>α_i</th>
<th>PKC</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>?</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>nc</td>
<td>↑ (+α_i)</td>
<td>nc</td>
<td>↑</td>
<td>?</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>↑ (+α_i)</td>
<td>nc</td>
<td>?</td>
<td>↑</td>
<td>?</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>nc</td>
<td>↑ (+α_i)</td>
<td>nc/↓</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>↓</td>
<td>nc</td>
<td>↓</td>
<td>nc/↑</td>
<td>↓</td>
</tr>
<tr>
<td>VI</td>
<td>2</td>
<td>↓</td>
<td>nc</td>
<td>↓</td>
<td>nc/↑</td>
<td>↓</td>
</tr>
<tr>
<td>VII</td>
<td>-</td>
<td>nc</td>
<td>↑</td>
<td>?</td>
<td>↑</td>
<td>?</td>
</tr>
<tr>
<td>VIII</td>
<td>3</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>IX</td>
<td>-</td>
<td>↓</td>
<td>nc</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

The multigene family of mammalian ACs. There are nine distinct AC subtypes: (↓) indicates significant increase in activity; (↑) indicates significant decrease in activity; (nc) signifies no change in activity; [↑ (+α_i)] indicates synergism with α_i.
signaling pathways, and are crucial to the modulation of intracellular cAMP and cGMP levels.

Resultant changes in intracellular cAMP levels affect PKA activity. Similar to ACs and PDEs, multiple PKA subtypes are expressed although they are functionally categorized in two main groups, type I and type II. In the inactivated state, PKA exists as a tetrametric holoenzyme comprised of a homodimer of regulatory (R) subunits and two catalytic (C) subunits, each binding to a R subunit. Each subunit is encoded for by a distinct gene and includes four R subunits (type I comprised of RIα, RIβ, while type II comprised of RIIα, RIIβ) and three catalytic (C) subunits (Ca, Cβ, Cγ) (McKnight et al., 1988, Beebe et al., 1990, Doskeland et al., 1993). Both R and C subunits have unique patterns of expression (Cadd and McKnight, 1989). For example, RI subunits are localized primarily in the cytosol while RII subunits tend to be found in the particulate fraction of neural homogenates (Ludvig et al., 1990, Glantz et al., 1992, Coghlan et al., 1995, Faux and Scott, 1996).

Binding of cAMP to R subunits induces a conformational change that results in dissociation of the active C subunits (Walsh and Van Patten, 1994). The various substrates for PKA include receptors, ion channels, structural proteins such as synapsins I and II (DeCamilli and Jahn, 1990, DeCamilli et al., 1990), microtubule-associated protein 2 (MAP-2) (Matus, 1988) and transcriptional factors (Meyer and Habener, 1993). With respect to the latter, when CREB is bound to a cAMP response element (CRE), a regulatory element in the promoter region of a number of genes, phosphorylation of a serine residue at position 133 by the C subunit of PKA results in modulation of the transcriptional activation of these genes (Gonzalez and Montminy, 1989, Meyer and Habener, 1993, Ghosh and Greenberg, 1995).
Phosphorylation of CREB can also be regulated through the G-protein mediated phosphatidylinositol second messenger system (see below) by both PKC and Ca\textsuperscript{2+}/CaM-dependent protein kinases (Meyer and Habener, 1993, Ghosh and Greenberg, 1995). A-kinase anchoring proteins (AKAPs) may also direct PKA holoenzymes to certain intracellular sites highlighting the notion that additional factors contribute to specificity in cellular responses (Dell’Acqua and Scott, 1997). Collectively, the multiplicity of the cAMP-signaling components (i.e. ACs, PDEs and PKAs) allow for intracellular targeting of various substrates that can confer diverse and selective signal regulation within a cell.

1.3.2 G protein Mediated-Phosphatidylinositol Signaling

The phosphoinositide second messenger system is another major G-protein linked cellular signal transduction process (Fisher, 1995). Hydrolysis of inositol phospholipid is catalyzed by PLCs which convert phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to inositol 1,4,5-triphosphate (IP\textsubscript{3}) and 1,2-diacylglycerol (DAG). IP\textsubscript{3} mobilizes Ca\textsuperscript{2+} from intracellular stores whereas DAG activates PKC (Berridge, 1984). Three major subgroups (\(\beta, \gamma, \delta\)) of PLC isozymes have been cloned and characterized (Rhee and Choi, 1992, Lee and Rhee, 1995). The PLC\(\beta\) isozymes (of which there are 4 subtypes) are activated by \(\alpha\) proteins (\(\beta_1,\beta_2,\beta_3,\beta_4\)) and the \(\beta\gamma\) complex of \(\alpha_\gamma(\beta_2,\beta_3,\beta_3)\) (Lee and Rhee, 1995, Exton, 1996, Katan, 1998). The \(\gamma\) isozymes (\(\gamma_1\), and \(\gamma_2\)) differ from the \(\beta\) subtypes in several crucial domains that are needed for their activation by growth factors and cytokines through receptors with intrinsic tyrosine kinase activity (Rhee and Choi, 1992, Lee and Rhee, 1995, Exton, 1996, Rhee and Bae, 1997). The \(\delta\) isozymes (4 have been identified) are differentially activated by Ca\textsuperscript{2+} (Essen et al., 1996, Katan, 1998).
As stated above, activation of receptors coupled to PLC also leads to DAG production which stimulates PKC, resulting in its translocation to the membrane and subsequent activation. PKC is highly enriched in brain, exists in both cytosolic and membrane fractions, is ubiquitously distributed and consists of at least 11 closely related isoforms categorized into conventional (α, β₁, β₁₁, γ: Ca²⁺-dependent and phorbol ester-sensitive), novel (δ, ε, η, θ, μ: Ca²⁺-independent and phorbol ester-sensitive) and atypical (ζ and λ: neither Ca²⁺-dependent nor phorbol ester-sensitive) subgroups (Nishizuka, 1988, Huang, 1989, Stabel and Parker, 1991, Hug and Sarre, 1993, Buchner, 1995). Activation of PKC is known to modulate many cellular functions including the release of several neurotransmitters through phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) and growth associated protein (GAP-43) (Degraan et al., 1990, Robinson, 1991). PKC also phosphorylates ion channels, AC subtypes (Morris et al., 1996, Sunahara et al., 1996) and transcription factors such as serum-response factors (SRFs), and regulates gene transcription through SRF binding to serum responsive elements (SRE) in target genes (Sheng et al., 1988).

1.3.3 G Protein Mediated-cAMP Signaling Disturbances in BD

In the past several years, compelling findings have been obtained from studies of postmortem brain and peripheral blood cells from BD patients (Hudson et al., 1993, Manji and Lenox, 1994, Manji et al., 1995a,b, Warsh and Li, 1996, Wang et al., 1997) supporting the notion that alterations in G protein mediated signal transduction processes (both cAMP and phosphatidylinositol signaling) play an important role in the pathophysiology of BD. Since these two signaling pathways subserve numerous neuronal functions and mechanisms,
it is not surprising that relatively minor alterations in these signal transduction systems could have important consequences for neuronal function. The following section focuses on recent evidence for one specific set of disturbances particularly relevant to the pathophysiology of BD, that is, those observations reporting elevated levels and functionality of $\alpha_\text{t}$. These are summarized in Table 3. The findings that lithium, the prototypic mood stabilizing agent used in the management of BD, exerts both direct and indirect effects at the G protein level (Manji et al., 1995a,b) provide another line of indirect pharmacological evidence implicating G protein abnormalities in BD. The modulatory effects of lithium on the posited altered functionality of the cAMP signaling cascade are also highlighted in the following section as well.

1.3.3.1 Postmortem Brain Studies

The possibility of postreceptor signal transduction disturbances in BD was first suggested by the findings of blunted $\beta$AR stimulated AC activity in MNLs from patients with unipolar and bipolar depression (Pandey et al., 1979, Extein et al., 1979, Mann et al., 1985, 1997). Evidence of a postreceptor signal transduction abnormality in BD was subsequently reported by Schreiber et al. (1991) who demonstrated increased agonist-stimulated binding of the stable GTP analogue, guanylyimidodiphosphate ($[^3]H$Gpp (NH)p) in MNL membranes from manic but not recovered BD patients compared with controls. The first direct evidence (see Table 3) demonstrating G protein disturbances in BD brain, however, came with the observations of higher $\alpha_{\text{t}}$-immunolabeling in frontal, temporal and occipital cortical regions of postmortem brain from BD subjects compared with nonpsychiatric controls (Young et al., 1991, 1993), findings that implicated elevated $\alpha$,
Table 3

Evidence for Gα, Mediated-cAMP Disturbances in BD from Postmortem Brain and Clinical Studies

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Disturbance</th>
<th>Implication</th>
<th>Authors</th>
</tr>
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<tbody>
<tr>
<td><strong>A) Postmortem BD Brain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) Frontal, temporal and occipital cortex</td>
<td>α&lt;sub&gt;i&lt;/sub&gt;-immunolabeling</td>
<td>Hyperfunctional α&lt;sub&gt;i&lt;/sub&gt;-coupled AC signaling</td>
<td>Young et al., 91, 93</td>
</tr>
<tr>
<td></td>
<td>Forskolin-stimulated AC activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iii) Frontal cortex</td>
<td>α&lt;sub&gt;i&lt;/sub&gt;-immunolabeling; noradrenergic, serotonergic and cholinergic agonist promoted binding of [35S]GTP&lt;sub&gt;γ&lt;/sub&gt;S</td>
<td>R- G protein coupling; heterotrimer state</td>
<td>Friedman and Wang, 96</td>
</tr>
<tr>
<td>iii) Cytosolic fractions from cortical regions</td>
<td>[3H]cAMP binding to PKA</td>
<td>R subunit abundance</td>
<td>Rahman et al., 97</td>
</tr>
<tr>
<td>iv) Temporal cortex (Cytosolic fractions)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; for cAMP-stimulated PKA activity; basal and maximal PKA activity</td>
<td>Enhanced PKA activity</td>
<td>Fields et al., 99</td>
</tr>
<tr>
<td><strong>B) Clinical Studies</strong></td>
<td></td>
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<tr>
<td>ii) MNLs from BD depressed patients</td>
<td>Blunted βAR stimulated AC activity in the absence of receptor changes</td>
<td>Possible postreceptor signal transduction disturbances</td>
<td>Mann et al., 85, 97</td>
</tr>
<tr>
<td>iii) MNLs from manic BD patients</td>
<td>Agonist stimulated binding of [3H]Gpp(NH)p</td>
<td>Altered G protein mediated signaling</td>
<td>Schreiber et al., 91</td>
</tr>
<tr>
<td>iii) MNLs in depressed BD patients</td>
<td>α&lt;sub&gt;i&lt;/sub&gt; and α&lt;sub&gt;l&lt;/sub&gt;-immunolabeling; blunted GTP&lt;sub&gt;γ&lt;/sub&gt;S and forskolin stimulated AC activity</td>
<td>Hyperfunctional α&lt;sub&gt;i&lt;/sub&gt; may be compensated for by elevations in α&lt;sub&gt;l&lt;/sub&gt;; Trait-dependent changes</td>
<td>Young et al., 94b, Warsh et al., 99</td>
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Table 3 continued:

Evidence for Go, Mediated-cAMP Disturbances in BD from Postmortem Brain and Clinical Studies

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Disturbance</th>
<th>Implication</th>
<th>Authors</th>
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<tbody>
<tr>
<td><strong>B) Clinical Studies</strong></td>
<td></td>
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<tr>
<td>iv) B-lymphoblasts from BD patients</td>
<td>agonist activated down-regulation of β-AR density; Recovery of response following lithium treatment</td>
<td>Disturbances in the coordinated regulation of βAR and α, interaction and function in BD</td>
<td>Kay et al., 94</td>
</tr>
<tr>
<td>v) MNLs from euthymic and manic BD subjects</td>
<td>α, immunolabeling</td>
<td>Hyperfunctional α, coupled AC signaling(?)</td>
<td>Manji et al., 95</td>
</tr>
<tr>
<td>vi) Platelets from both euthymic BP-I and BP-II patients</td>
<td>αL and αs immunolabeling; CTX-catalyzed ADP-ribosylated αL in BP-II only</td>
<td>Altered heterotrimeric state</td>
<td>Mitchell et al., 97</td>
</tr>
<tr>
<td>vii) Platelets in drug-free euthymic BD patients</td>
<td>cAMP-stimulated phosphorylation of a 22 kDa protein</td>
<td>Enhanced PKA activity</td>
<td>Perez et al., 95</td>
</tr>
<tr>
<td>viii) B-lymphoblasts from BD subjects</td>
<td>αs immunolabeling from BP-II</td>
<td>Trait-dependent changes</td>
<td>Enamghoreishi et al., 98</td>
</tr>
<tr>
<td>ix) MNLs from untreated manic and depressed BD patients</td>
<td>or α, and α, immunolabeling and agonist-stimulated [3H]Gpp(NH)p binding in manic or depressed BD patients, respectively</td>
<td>State-dependent changes</td>
<td>Avissar et al., 97a, 97b</td>
</tr>
<tr>
<td>x) Platelets in drug-free depressed and manic BD patients</td>
<td>PKA C subunit levels</td>
<td>State-dependent changes</td>
<td>Perez et al., 99</td>
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</table>
levels in the pathophysiology of this disorder. These immunolabeling changes were accompanied by increased forskolin-stimulated AC activity suggesting that higher levels of $\alpha$, result in hyperfunctional $\alpha$-coupled AC signaling in BD. More importantly, these changes were not likely attributable to extraneous factors such as postmortem delay, agonal status, subject age or antemortem lithium treatment (Young et al., 1993), occurred in the absence of altered $\beta$AR density (Young et al., 1994a) and were specific to BD (Young et al., 1993, Warsh and Li, 1996, Warsh et al., 1999).

Consonant with the $\alpha_{+4}$ immunolabeling findings (Young et al., 1991, 1993), Friedman and Wang (1996) also reported elevated $\alpha$, levels along with increased noradrenergic, serotonergic and cholinergic agonist-promoted binding of the stable GTP analogue, $[^{35}S]$GTP$\gamma$S, in postmortem frontal cortex from a small group of BD patients compared with controls. The latter observation supported the notion that receptor $\beta$AR-$\alpha$, coupling is enhanced in BD frontal cortex. In the same study, observations of increased heterotrimeric $\alpha$, as reflected by increased amounts of $\beta$ protein coimmunoprecipitated along with $\alpha$, broadened the spectrum of potential disturbances affecting the $\alpha$ subunit (Friedman and Wang, 1996). This finding suggested that posttranslational modifications and/or functional alterations affecting the heterotrimeric state of $\alpha$, might also be altered in BD.

The heightened cAMP signaling postulated to occur in BD might be expected to result in adaptive alterations in the activity of PKA, the downstream target of cAMP. In this regard, observations of reduced $[^3H]$cAMP binding to PKA in the cytosolic fractions across all brain regions of BD brain (Rahman et al., 1997) imply that there is a decrease in the abundance of the $R$ subunits of PKA, to which cAMP binds. Interestingly, chronic lithium
treatment has been shown to increase \(^{3}H\)cAMP binding and the R and C subunit levels of PKA in rat hippocampus and frontal cortex (Mori et al., 1998) highlighting the possibility that PKA may represent one of the target sites in the cAMP signaling cascade, underlying the therapeutic effect of lithium.

Since PKA activity is dependent on the stoichiometry of the abundance of R subunits relative to C subunits (Schwechheimer and Hofmann, 1977, Greenberg et al., 1987), reduced levels of R relative to C subunits would result in increased PKA activity at subsaturating cAMP concentrations and phosphorylation of its substrate proteins (Greenberg et al., 1987, Schwechheimer and Hofmann, 1977). In support of this notion, our laboratory (Warsh and Li, 1999, Fields et al., 1999) has found a reduced EC\(_{50}\) for cAMP-stimulated PKA activity and significantly elevated basal and maximal PKA activity in the cytosolic fraction of temporal cortex of BD brain compared with nonpsychiatric controls. Moreover, Perez et al. (1995) reported greater cAMP-stimulated phosphorylation of a 22-kDa protein in platelets from drug-free euthymic BD patients compared with age- and sex-matched healthy subjects. The enhanced cAMP-dependent endogenous phosphorylation may be due to the recently reported elevations in the levels of the C (but not R) subunit in platelets from untreated depressed and manic BD patients compared with untreated euthymic BD patients and healthy subjects (Perez et al., 1999). Collectively, these observations support the notion that hyperfunctional cAMP signaling occurs in BD leading to downstream alterations in PKA function in this illness.

During the course of this work, Dowalatshahi et al. (1999) reexamined G protein levels and AC activity in a larger cohort of BD, major depressive disorder (MDD) and
control subjects. In contrast to earlier studies, these authors did not find an effect of diagnosis on either $\alpha_1$ or $\alpha_2$ levels or function in temporal and occipital cortices from BD subjects. Neither was Dowalatshahi et al. (1999) able to replicate reported findings for MDD subjects. While the exact reason for this group’s failure to reproduce the earlier findings of elevated $\alpha_1$ levels and hyperfunctional $\alpha_1$-AC coupling remains to be clarified, several important clinical and demographic variables may have obscured the expected changes. For example, treatment with mood-stabilizing medications may account, in part, for the discrepancies since all of the patients in the above study were on several different mood stabilizers, including antidepressants. Furthermore, compared to the subjects included in this study, the larger cohort had longer postmortem delay times, were older in years of age, and more than half of the subjects had a documented history of comorbidity with alcohol abuse, variables that, within their own right, can affect G protein measures and functionality. Moreover, since almost 3/4 of the BD subjects in Dowalatshahi et al.’s study died by suicide, the mood-state of these patients may be an important confounding variable complicating the interpretation of their findings even further (Cowburn et al., 1994). Taken together, the lack of alterations in $\alpha_1$ level and function in BD subjects examined by Dowalatshahi et al. (1999) may reflect the influence of medications and mood state on these measures and emphasizes the importance of assessing these clinical factors when interpreting results of human postmortem brain studies.
1.3.3.2 Clinical Studies

The clinical relevance of the postmortem findings is difficult to discern clearly given the potential confounding effects of state dependent and extraneous (i.e. postmortem delay, medication, comorbidity) factors. Studies utilizing peripheral cells derived from living subjects, to an extent, provide a model in which these effects can be controlled for, and to test whether observed signal transduction disturbances are state or trait dependent. This is not possible with postmortem brain tissue.

One of the first lines of evidence suggesting that the aforementioned $\alpha$, immunolabeling alterations may be expressed peripherally in BD was the report of higher $\alpha$, and $\alpha$, immunolabeling in MNLs from depressed BD but not major depressive disorder (MDD) patients compared with age and sex-matched healthy subjects (Young et al., 1994b). Significantly elevated $\alpha_{4S}$ levels were also found in leukocyte membranes from BD patients by Manji et al., (1995a). In their report, BD patients were comprised of both treated euthymic and untreated, mostly manic, subjects. Significantly higher $\alpha_{sL}$ and $\alpha_{4S}$ levels also were found in platelets from both BP-I and BP-II patients who were euthymic at the time of study, compared with a matched group of healthy subjects (Mitchell et al., 1997). In the latter study, increased levels of the heterotrimeric form of $\alpha_{sL}$ were found in platelets of BP-II patients as measured by CTX-catalyzed ADP-ribosylation of the protein (Mitchell et al., 1997). Recently, elevations in $\alpha_{4S}$ immunolabeling have also been reported in Epstein-Barr virus-immortalized B-lymphoblasts obtained from BP-II but not BP-I subjects compared with healthy patients (Emamghoreishi et al., 1998). The observations that elevated $\alpha$, immunolabeling occurs independent of the state of illness in BD and is expressed in vitro in
cultured cells from BD patients, underscore the possibility that trait-dependent factors may contribute to the observed alterations in α₁ elevations and downstream signaling disturbances in this disorder.

In contrast, findings of lower α₁ and α₅ immunolabeling, and agonist-stimulated [³H]GppNHp binding to MNL membranes from patients with either major depression (Avissar et al., 1997a) or untreated bipolar disorder with a major depressive episode compared with healthy subjects (Avissar et al., 1997b) imply that state-dependent factors also contribute to the observed changes in G protein levels and function. This is further supported by the findings of statistically significant inverse correlations between Beck Depression Rating scores (which measure the severity of depression) and G protein α subunit levels and the degree of agonist-stimulated [³H]Gpp(NH)p binding (Avissar et al., 1997b). The same authors also reported higher MNL α₁ and α₅ immunolabeling in the manic phase and lower levels in the depressed phase in a group of untreated BD patients compared with healthy subjects (Avissar et al., 1997a), further supporting the view that the changes in G protein α subunit levels and function in these mood disorders is influenced by the state of illness.

That psychotropic medications may also influence the detection of underlying changes in membrane G protein levels that are related to the illness, further complicates the interpretation of peripheral blood cell G protein findings. For example, Mitchell et al. (1997) reported that platelet α₅₅ levels were significantly higher in platelets from patients receiving lithium only compared with those taking exclusively carbamazepine. Indeed, mean α₅₅ values in the latter group were quite similar to those in healthy comparison subjects,
suggesting a potential confounding effect of treatment with some (i.e. carbamazepine) but not other (i.e. lithium) mood stabilizing agents. The discrepancies in immunolabeling findings between the various studies (Young et al., 1994b, Manji et al., 1995a, Avissar 1997a, b, Mitchell et al., 1997) clearly suggest that state-dependent, medication and/or other extraneous factors act to confound changes reported in MNL α₁ levels in BD.

1.3.4 Summary

As summarized in Table 3, the aforementioned findings from postmortem human brain and clinical studies support the notion that postreceptor α₁-mediated cAMP signaling disturbances play an important role in the pathophysiology of BD. The focus of this thesis on the elucidation of the molecular mechanisms that cause the elevations and hyperfunctionality of α₁ does not mean to presume that only one biochemical abnormality underlies the pathophysiology of BD. Rather, multiple factors likely contribute to this disorder in addition to those considered above. This includes disturbances in the G-protein mediated phosphatidylinositol signaling pathway (Jope et al., 1996, Mathews et al., 1997), altered intracellular Ca²⁺ homeostasis (Dubovsky et al., 1991, Emamghoreishi et al., 1997), neuroanatomical changes (Drevets et al., 1997, Ongur et al., 1998, Soares and Mann, 1997) and genetic abnormalities (Berrettini, 1996, Gershon et al., 1998). In addition, extensive cross-talk exists between the various G-protein mediated signal transduction processes (Hill and Kendall, 1989, Liu and Simon, 1996, Dowd et al., 1997). While this work examines one particular posited pathophysiological alteration in BD which involves the G protein α subunit, α₁, future studies are needed to elucidate the degree and cause(s) of dysregulation among these multiple signaling systems.
1.3.5 The Molecular Mechanism Underlying $\alpha_i$ Immunolabeling Elevations in BD

1.3.5.1 $\alpha_i$ Gene and Altered Transcription

Irrespective of the influence of state as compared with trait factors on $\alpha_i$ levels and function, the mechanism(s) underlying the $\alpha_i$ subunit immunolabeling changes in BD remains to be elucidated. Genetic studies examining a dinucleotide repeat polymorphism in intron 3 or a biallelic polymorphism in exon 5 of the $\alpha_i$ gene have not revealed linkage between the $\alpha_i$ gene and BD (Ginns et al., 1992, Le et al., 1994, Ram et al., 1997). Similarly, no mutations have been identified in either the promoter or coding sequences of the $\alpha_i$ gene in a group of BD patients (Ram et al., 1997). These findings suggest that mechanisms other than mutations affecting the primary upstream regulatory elements of $\alpha_i$ gene account for the changes observed in BD.

Substantial evidence indicates that G protein $\alpha$ subunit levels can be regulated at the level of gene transcription (Hadcock and Malbon, 1993). However, the question of whether the higher levels of $\alpha_i$ are related to alterations in transcription has been addressed in only two studies to date. Young et al. (1996) reported no significant differences in $\alpha_i$ mRNA levels in the same cerebral cortical regions manifesting the elevation of $\alpha_i$ in BD brain compared with controls. Although the authors did not examine the mRNA levels of the each $\alpha_i$ isoform separately, the findings support the notion that the elevations of $\alpha_i$ observed in postmortem BD brain are not associated with concomitant changes in $\alpha_i$ mRNA levels. In contrast, Spleiss et al. (1998) reported markedly increased levels of $\alpha_i$ mRNA from neutrophils in drug-free and lithium-treated BP-I patients compared with their matched
controls. These changes were not observed in unmedicated or lithium-treated unipolar patients, highlighting the diagnostic specificity of these findings. \( \alpha_{12} \) mRNA levels were also increased, but only in lithium treated BP-I patients. However, in the absence of data on \( \alpha \), protein levels in the same cells caution is warranted in concluding prematurely that this accounts for the elevated \( \alpha_{s} \) protein levels found in other studies. Clearly, more work is needed to clarify the relationship and extent to which abnormalities in \( \alpha_{s} \) gene expression might account for elevations of this subunit in BD.

1.4 Biochemical Processes Regulating G Protein \( \alpha_{s} \) Subunit Levels

Levels of \( \alpha_{s} \) are also regulated by several important biochemical mechanisms posttranslationally (Levis and Bourne, 1992). Sensitization/desensitization of G protein-coupled receptors and cross-regulation between opposing receptor-effector systems also affect G protein levels (Hadcock et al., 1990, 1991, Hadcock and Malbon, 1993). In addition, specific G-protein subtypes are also the target for ubiquitin-dependent degradation (Madura and Varshavsky, 1994, Obin et al., 1994) and calpain proteolysis (Greenwood and Jope, 1994), providing yet additional means by which G-protein subunit levels may be modulated. Finally, cytoskeletal interactions and posttranslational modifications such as palmitoylation (Wedegaertner et al., 1993) and ADP-ribosylation can also affect the level of G proteins (Change and Bourne, 1989). The following discussion elaborates more fully on the role these posttranslational mechanisms play in regulating \( \alpha_{s} \) turnover/levels.

1.4.1 Agonist-Induced Regulation

A variety of evidence indicates that agonist-induced receptor activation modulates membrane G-proteins levels. For example, chronic treatment of rat adipocytes with
phenylisopropyl adenosine (PIA) or PGE₁ decreased immunoreactive levels of α₁₁-3, reduced adenosine-A₁ receptor density, and increased immunoreactive levels of α₂, but did not influence α₄ and α₅ mRNA levels (Longabaugh et al., 1989, Green et al., 1990, 1992). These changes were accompanied by the homologous and heterologous desensitization of the respective receptors, manifest in decreased sensitivity of PIA and PGE₁-induced inhibition of AC activity. In another paradigm, chronic ISO stimulation downregulated βAR and α₁ levels in rat ventricular myocardium (Kimura et al., 1993). Prior exposure to propanolol completely abolished these ISO induced effects (Kimura et al., 1993). In NG108-15 cells, sustained exposure to PGE₁ and/or iloprost decreased α₁ levels in a cAMP-independent manner, without altering mRNA or protein levels of α₁₂ and α₁₃ (McKenzie and Milligan, 1990. McKenzie et al., 1991. Aide and Milligan, 1993) or affecting translocation of α₁ from the membrane to the cytosol (McKenzie and Milligan, 1990). Similarly, sustained exposure of Chinese hamster ovarian cells expressing the human M1 muscarinic cholinergic receptor to carbachol produced a marked parallel decrease in αₙ11 levels and half-life (from 18 h to ~3 h), without inducing translocation of αₙ11 to the cytosol (Mitchell et al., 1993, Mullaney et al., 1993) or affecting αₙ11 mRNA levels. These reductions were likely mediated by alterations in mechanisms regulating protein turnover (Mitchell et al., 1993, Mullaney et al., 1993). Shah et al. (1995) also showed that prolonged treatment of αT3-1 gonadotroph cells with luteinizing hormone-releasing hormone decreased cellular levels of αₙ11 by increasing the degradation rate of the α-subunits without altering mRNA levels. The above findings clearly suggest that agonist-induced regulation of G proteins levels can occur at the posttranslational level, with or without accompanying receptor desensitization (Chambers
The mechanisms modulating α subunit levels in response to receptor stimulation are poorly understood, however. One possible mechanism may involve conformational change in α subunits elicited by agonists, rendering them more susceptible to degradation. For example, a mutant recombinant αs protein (G226A, preventing its dissociation from βγ) exhibited a much slower degradation rate than that seen for wild type αs when expressed in S49 cyc- cells, whereas constitutively active mutant αs (R201C) was degraded at a much faster rate (Levis and Bourne, 1992). In contrast, CTX catalyzed ADP-ribosylation increased the rate of degradation (reducing the half-life by approximately one third) for all three of these constructs. These findings suggest the conformational change secondary to CTX-catalyzed ADP-ribosylation and/or receptor activation (i.e. decreased GTPase activity) accelerates αs degradation (Levis and Bourne, 1992).

### 1.4.2 Effect of Constitutive Activation

Studies of the effects of CTX-catalyzed ADP-ribosylation on αs turnover have also provided insights into the regulation of αs levels. Chang and Bourne (1989) were the first to demonstrate that exposure of GH3 cells to CTX significantly decreased (74-95%) the amount of immunoreactive αs, suggesting that this covalent modification renders the protein more susceptible to accelerated degradation. Exposure of rat glioma C6 cells to CTX decreased membrane levels of αsL without altering mRNA levels and caused ~ 75% of the isoform to translocate to the supernatant (Milligan and Unson, 1989, Carr et al., 1990). Similarly, CTX reduced αsL levels in L6 skeletal myoblasts (Milligan et al., 1989), but in contrast to C6 cells, there was no cytoplasmic accumulation of αs, once again suggesting that
rapid degradation of αs occurs following CTX activation-induced release of αs from the membrane in this model. In mouse mastocytoma cells, CTX treatment enhanced iloprost (IP)-induced translocation of both isoforms of αs from the membrane to the cytosol and decreased specific [3H]-IP binding along with agonist-induced activation of AC (Negishi et al., 1992). In rat pineal, CTX treatment decreased the levels of both isoforms of αs (Babila and Klein, 1992, 1994), via cAMP-mediated mechanisms. In NG108-15 cells, CTX-induced ADP-ribosylation downregulated αs, αo and β1 subunits without altering their respective mRNA levels or affecting α-subunit translocation to the cytosol (Klinz and Costa, 1990, Maclead and Milligan, 1990). In the same cell line, IP decreased prostanoid receptor levels, along with those of αs (Donnelly et al., 1992, Kim et al., 1994). Nicotinamide, a feedback inhibitor of ADP-ribosylation (see below) blocked this reduction in αs, resulting in increased basal and IP-stimulated AC activity. Thus, extensive evidence suggests that ADP-ribosylation plays an important role in regulating the levels of αs, possibly by modifying its turnover due to constitutive activation, independent of second messenger mechanisms.

1.4.3 Lipid Modification

Posttranslational covalent lipid modifications may also play a role in receptor-activated modulation of membrane αs levels. Palmitoylation, a reversible lipid modification, provides membrane anchorage for αs (Linder et al., 1993, Parenti et al., 1993, Wedegaertner et al., 1993). Initially, it was suggested that activation of βAR lead to depalmitoylation of αs and subsequent translocation of αs from the membrane to cytosol without altering its turnover (Degtyarev et al., 1993, Mumby et al, 1994, Wedegaertner and Bourne, 1994). Thus, it was hypothesized that changes in the rate of palmitoylation during receptor
activation may regulate the cyclical acylation and deacylation, and consequently, membrane association and activity of this protein (Wedegaertner et al., 1993). Recently however, Huang et al. (1998) reported that depalmitoylated \( \alpha_i \) still maintains its membrane localization. Incubation of MA104 cell membranes with GTP\( \gamma \)S did not alter membrane association of \( \alpha \) subunits nor did the subsequent removal of palmitate from \( \alpha_i/\alpha_i \) change their localization. Despite the different findings, palmitoylation may still serve to regulate \( \alpha_i \) subunit interactions with other proteins/effectors, and modulate \( \alpha_i \)-signaling and its targeting to other degradative mechanisms (Wedegaertner et al., 1995).

1.4.4 Cytoskeletal Interactions

Tubulin is a major cytoskeletal protein implicated in modulating G protein function and neuronal effector responses (Rasenick and Wang, 1988, Yan and Rasenick, 1990, Lieber et al., 1993) possibly by regulating \( \alpha_i \) interactions with AC (Yan and Rasenick, 1990, Lieber et al., 1993). Microtubule formation requires both tubulin and MAP-2, the latter of which is modulated by Ca\(^{2+}/\)CaM-dependent phosphorylation (Shulman, 1984, Burns and Islam, 1986). Thus, the availability of tubulin for binding to and subsequent regulation of G proteins may be governed, in turn, by CaM and/or factors influencing intracellular Ca\(^{2+} \) homeostasis. Interestingly, disturbances of intracellular Ca\(^{2+} \) homeostasis have also been implicated in the pathogenesis of BD (Dubovsky et al., 1989, 1991, Warsh and Li, 1996, Emamghoreishi et al., 1997).

Similarly, caveolin has also been shown to functionally interact with G protein \( \alpha \) subunits (Chang et al., 1994). For example, activation of \( \alpha_i \) in MDCK cells abolishes its association with the caveolin-enriched fractions of membranes (Li et al., 1995). This
suggests that caveolin may serve to regulate αs subunit function, minimizing α subunit basal activity by holding the subunit in an inactive conformation (Li et al., 1995).

1.4.5 Cross-talk Regulation

G protein abundance also appears to be modulated by cross regulation via different second messenger pathways. For example, ISO treatment decreased αs levels and increased αs2 protein and mRNA levels in S49 lymphoma cells in a cAMP-dependent manner (Hadcock et al., 1990). On the other hand, persistent activation of the inhibitory AC pathway by PIA in DDT1MF-2 hamster vas deferens cells increased ISO-stimulated AC activity and β2-AR expression, effects that were completely abolished by PTX (Hadcock et al., 1991). These latter findings imply that, in these cells, persistent activation of the inhibitory AC pathway results in desensitization, along with an increased agonist-mediated sensitivity of the opposing stimulatory AC pathway (Hadcock et al., 1991). In another example, αs protein and mRNA levels were upregulated in thyroid cells cultured in the presence of TSH or forskolin, the effects of which were abolished by the PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA) (Dib et al., 1994). In line with these observations, Tasken et al. (1995) reported increases in αs, and αo mRNA levels in rat sertoli cells exposed to 8-(4-chlorophenylthio)cAMP (8-CPTcAMP, an activator of PKA) and TPA. The effects of TPA on αs mRNA were synergistic with cAMP-mediated increases, although surprisingly, there were no alterations in immunoreactive levels of αs. The above findings suggest that the production of second messengers (ie. cAMP, DAG, etc.) as a result of G protein activation may regulate the levels of G proteins. These processes appear to occur, in part, at the level of gene transcription, as for the case of αs, via the binding of the phosphorylated
transcriptional factor CREB to a CRE (Itoh et al., 1988, Berkowitz et al., 1989) as well as posttranslationally at the level of protein turnover. Similarly, TPA may mediate transcriptional regulation through stimulation of other transcription factors that may bind to α subunit genes. This raises the possibility that alterations in levels of α, seen in BD may be the result of cross regulation via different second messenger systems which may operate in concert with other processes to govern G protein levels.

1.4.6 Calpain-Dependent Proteolysis

The Ca\(^{2+}\)-dependent neural activated protease calpain also has the potential to modulate protein abundance (Suzuki and Ohno, 1990). Two major forms of calpain, μ-calpain and m-calpain (activated by μM and mM concentrations of Ca\(^{2+}\), respectively) cause limited degradation of membrane and cytoskeletal proteins (Murachi, 1984, Mellgren, 1987, Croall and Demartino, 1991, Lane et al., 1992, Sido et al., 1994). α subunits may serve as substrates for calpain-dependent proteolysis under physiological conditions, the relative susceptibility of the specific G protein to calpain being dependent upon the subtype (Greenwood and Jope, 1994). Incubation of rat cerebral cortical membranes with calpain decreased the levels of α, α, and α, among which α, is most susceptible to calpain-mediated proteolysis (Greenwood and Jope, 1994). Preincubation of membranes with 100 μM GTP-γ-S inhibited calpain-mediated proteolysis of α, This suggests that persistent activation of α subunits may prevent calpain degradation. Furthermore, lithium was without effect on calpain-mediated proteolysis of α, in the presence of GTP-γ-S whereas enhanced proteolysis was observed in the absence of the GTP analogue. This finding highlights the potential importance the functional activity of the G protein as a determinant of the effects of lithium.
on calpain-mediated degradation of α subunits. Interestingly, nitric oxide (NO) has been shown to reversibly inactivate both m- and μ-calpain at physiological and acidic pH's, respectively (Michetti et al., 1995). Since NO can also regulate ADP-ribosylation (see below), this compound has the potential to modulate α levels at various stages. In turn, alterations in the levels and/or activity of calpain may represent yet another possible mechanism by which α levels and function may be increased, as observed in BD.

1.4.7 Ubiquitin Modification

Degradation via this pathway involves targeting of the protein for covalent attachment of ubiquitin and subsequent degradation, with the release and recycling of free ubiquitin (Ciechanover. 1994). The targeting process involves recognition of the substrate protein based on the N-terminal amino acid (the "N-degron") and an internal lysine residue at which a multiubiquitin adduct is formed (Finley and Chau, 1991, Madura et al., 1993). The observations that Gpa1, the α subunit of a G-protein that regulates cell differentiation in *S. cerevisiae* is a substrate for ubiquitin-targeted degradation (Madura and Varshavsky, 1994) and that α, in retinal pigment epithelial cells is a substrate for ubiquitin-dependent proteolysis (Obin et al., 1994), suggest that these processes may participate in the metabolism of some G protein α subunits, as well. Ubiquitin-dependent degradation of G proteins is poorly understood, however, and the extent and specificity of this pathway with respect to α subtypes remain to be demonstrated.

1.4.8 The Posttranslational Process of ADP-Ribosylation

1.4.8.1 The ADP-ribosylation Cycle

ADP-ribosylation is a posttranslational modification catalyzed by ADP-
ribosyltransferases involving the transfer of ADP-ribose from NAD\(^+\) to specific protein acceptors with the production of free nicotinamide (see Figure 4). A number of different endogenous ADP-ribosyltransferases, either cytosolic or membrane bound, have been purified and characterized from a variety of vertebrate tissues such as turkey erythrocytes (Zolkiewska et al., 1994), skeletal and cardiac muscle (Kharadia et al., 1992), human myelin membrane (Boulias et al., 1995), rat brain (Matsuyama and Tsuyama, 1991) and adrenal gland (Fujita et al., 1995). Mono-ADP-ribosyltransferases catalyze the addition of one ADP-ribose moiety to a specific amino acid residue whereas poly-ADP-ribosyltransferases catalyze the addition of several moieties to a single amino acid residue. More importantly, endogenous ADP-ribosylation of substrate proteins has been demonstrated in rat cerebral cortical homogenates with the highest activity found in three regions: hippocampus, hypothalamus and cerebral cortex (Duman et al., 1991). Although CTX-catalyzed ADP-ribosylation is known to be dependent upon ARFs, which comprise a family of small GTP-binding proteins (Hong et al., 1994, Kahn et al., 1995), it is uncertain whether they participate in the action of endogenous ADP-ribosyl-transferases. To date, molecular cloning studies have identified a number of ARF subtypes occurring ubiquitously in various mammalian tissues (Kahn et al., 1991, Randazzo and Kahn, 1994, Terui et al., 1994) and indeed, several ADP-ribosyltransferases have been reported to be sensitive to ARFs (Matsuyama and Tsuyama, 1991, DeMatteis et al., 1994). It is possible that ARFs may be involved in the ADP-ribosylation of proteins endogenously, and therefore, along with ADP-ribosyltransferases, could play an important role in regulating \(\alpha\) subunit levels and subsequently signal transduction (Zhang et al., 1995). More importantly, the occurrence of
Figure 4: Depicted above is the ADP-ribosylation of the stimulatory α subunit, αs, on arginine 201. Mono-ADP-ribosylation is a reversible posttranslational modification: ADP-ribosyltransferases, bacterial toxins and NAD glycohydrolases (NADases) catalyze the forward reaction whereas ADP-ribosylhydrolases catalyze the reverse reaction (Matsuyama and Tsuyama, 1991, Zolkiewska et al., 1994). This posttranslational process involves the transfer of an ADP-ribose moiety from NAD⁺ to specific acceptor proteins yielding the production of free nicotinamide which can then act as a negative feedback regulator of this process. ADP-ribosyl-transferases/hydrolases can be stimulated and/or inhibited by a wide variety of agents including 3-acetylpyridine (APAD), dithiothreitol (DTT) and isonicotinic acid hydrazide (INH).
endogenous ADP-ribosyltransferases in many cells including neurons (Williamson et al., 1990) along with the fact that NO (a major inter/intra-cellular messenger) can modulate this posttranslational mechanism (Williams et al., 1992), supports the notion that endogenous ADP-ribosylation may be an important neuronal regulatory mechanism in the central nervous system (CNS).

I.4.8.2 Modulation of ADP-ribosylation

Increasing evidence suggests that α is a substrate for endogenous ADP-ribosyltransferase(s) and that a number of different agents have the potential to modulate the activity of this enzyme. For example, the agonist IP increased endogenous ADP-ribosylation of α, and decreased CTX-catalyzed ADP-ribosylation of this subunit in platelets (Molina y Vedia et al., 1989). In a different paradigm, sustained receptor stimulation of α in rat adipocytes by adenosine enhanced endogenous ADP-ribosylation of α, (Jacquemin et al., 1986). ISO or GTPγS-mediated stimulation of endogenous ADP-ribosylation of α, has also been reported in canine cardiac sarcolemma (Quist et al., 1994). Furthermore, nicotinamide reversed the IP-induced reduction of membrane α, in NG108-15 cells, resulting in increased CTX-catalyzed ADP-ribosylation of α, (Donnelly et al., 1992). Since α, can be a substrate for endogenous ADP-ribosyltransferase(s), this covalent modification may be one mechanism by which α, is targeted for degradation.

With respect to other agents capable of modulating ADP-ribosyltransferase activity, NO has been shown to increase ADP-ribosylation of endogenous substrates in rat cerebral cortical tissue (Duman et al., 1991, 1993, Tao et al., 1992, Williams et al., 1992). For example, enhanced ADP-ribosylation of α (involved in visual signal transduction) from
bovine rod outer segments occurs in the presence of sodium nitroprusside (NaNP) (Zoche and Koch, 1995).

Of great importance, is the recent observation of a link between endogenous ADP-ribosylation and downregulation of $\alpha_s$. C6 glioma cells exposed to 3-morpho-linosynomin hydrochloride (SIN-1, a NO donor) significantly decreased immunoreactive levels of $\alpha_{t}t$ and $\alpha_{t}s$, an effect that was blocked with a NO scavenger (Young et al., 1997b). This reduction was independent of alterations in $\alpha_t$ mRNA levels but was associated with significantly increased endogenous and CTX-catalyzed ADP-ribosylation of a 50 kDa protein, presumably $\alpha_{t}t$. These findings suggest that SIN-1 mediated downregulation of $\alpha_t$ occurs by increasing ADP-ribosylation of this protein.

Chronic lithium administration can also affect posttranslational processing of a wide variety of substrates. Nestler et al. (1995) demonstrated in vitro incubation of lithium inhibited endogenous ADP-ribosylation for all substrate proteins, including $\alpha_t$, in rat frontal cortex. This suggested that lithium may affect all, as opposed to a specific subset, of the endogenous ADP-ribosyltransferases. The inhibitory effect of lithium is hypothesized to involve antagonism of Mg$^{2+}$ binding sites that are present in certain ADP-ribosyltransferases (Tamir and Gill, 1988), similar to that observed in the effects of lithium on G proteins and AC (Manji et al., 1995a, b). Paradoxically, chronic in vivo lithium treatment increased endogenous ADP-ribosylation in the same tissue, possibly by upregulating certain ADP-ribosyltransferases (Nestler et al., 1995). Similarly, chronic exposure of C6 cells to lithium resulted in increased endogenous ADP-ribosylation of both a 52 kDa and 39 kDa band (presumably $\alpha_t$ and $\alpha_s$) (Young and Woods, 1996). In contrast, chronic valproic acid and
carbamazepine had little or no effect on the degree of endogenous ADP-ribosylation of αi and αi compared with untreated cells (Young and Woods, 1996). The regulation of endogenous ADP-ribosylation in brain by lithium supports the notion that disturbances in ADP-ribosylation could affect turnover or degradation rates of specific α subunits, as described above.

1.4.8.3 ADP-ribosylation in BD

During the course of this work, Young et al. (1997a) tested the possibility that chronic lithium treatment could regulate endogenous ADP-ribosylation in platelets from patients with BD. They reported significantly lower platelet endogenous ADP-ribosylation of a 39 kDa band (presumably αi) and a trend toward lower intensity of the 50 kDa band (presumably αi) in euthymic lithium-treated BD patients compared with unmedicated BD subjects, highlighting the inhibitory effect of lithium on ADP-ribosyltransferases in peripheral cells from BD patients. There were, however, no significant differences in endogenous ADP-ribosylation of αi and αi in both lithium-treated and unmedicated BD subjects as compared with control subjects (Young et al., 1997a). These findings are in contrast to those observed in previous studies in rat brain and C6 cells (Nestler et al., 1995, Young and Woods, 1996) and need to be interpreted with caution. Since many of the ADP-ribosyltransferases have not been characterized in various cell types, the different results in the above mentioned studies may be due to the presence of different tissue specific ADP-ribosyltransferases, ARFs and/or other substrates that are involved in this posttranslational mechanism. Purification of several mono-ADP-ribosyltransferases from rat brain and adrenal gland (Matsuyama and Tsuyama, 1991, Fujita et al., 1995) demonstrate differences
in molecular masses and differential regulation by ARFs, highlighting the heterogeneity of these enzymes.

The data, however, support the notion that potential disturbance(s) in ADP-ribosylation may occur in BD. In addition, this process represents one potential molecular target of action for lithium. Moreover, ADP-ribosylation regulates α subunit coupled signal transduction indirectly through the modulation of α subunit levels (Levis and Bourne, 1992) and uncoupling of the α subunit from the receptor (Manji et al., 1995b). Taken together, the findings support the posttranslational modification of α by ADP-ribosylation as a putative mechanism that may contribute to the increased α levels and pathophysiology of this disorder. Therefore, studies examining the role of ADP-ribosylation of α in BD may prove important with respect to the development of novel treatment strategies and therapeutic intervention in this disorder.

1.5 Objectives

The preceding discussion has detailed the substantial body of evidence pointing to disturbances in G protein levels and the cAMP signaling pathway in the pathophysiology of BD. In the absence of changes in α mRNA levels, elevated α levels and functionality in cerebral cortical regions of BD postmortem brain may occur secondary to alterations in mechanisms regulating the turnover of this α subunit. At least four different processes may regulate, either alone or in combination, the production and/or turnover of specific G proteins including: i) agonist-promoted regulation during receptor downregulation/desensitization and cross-talk mechanisms; ii) ubiquitin-dependent degradation; iii) Ca$^{2+}$-activated calpain proteolysis; and iv) ADP-ribosylation. These processes are depicted in Figure 5.
Figure 5: Schematic representation of biochemical posttranslational pathways that may regulate $\alpha_i$ protein levels and function. Agonist-induced receptor activation can modulate membrane $\alpha_i$ levels (1). Covalent lipid modifications such as palmitoylation may also modulate $\alpha_i$ levels and signaling (2). $\alpha_i$ signaling may in turn be regulated by tubulin binding (3). $\alpha_i$ abundance also appears to be modulated by cross regulation via different second messenger pathways (4) and by the Ca$^{2+}$-dependent neural activated protease calpain (5). It is not certain whether ubiquitin degradation of $\alpha_i$ occurs (6) but exists as one possible mechanism. More importantly, increasing evidence suggests that ADP-ribosylation plays an important role in the regulation of $\alpha_i$ levels (7), targeting the subunit for degradation. Both calpain and ADP-ribosyltransferases (ADP-R) can be regulated by NO.
Disturbances in one or more of these regulatory processes could alter synthesis and/or degradation rates governing G protein abundance, and are therefore important candidates to be considered in explaining the elevation in abundance and function of $\alpha$, in BD. Clarifying the nature and pathophysiological significance of potential mechanisms that may underlie the $\alpha$, disturbances would have important implications for pharmacological intervention.

In light of the experimental evidence demonstrating: 1) regulation of ADP-ribosylation by chronic lithium in rat brain (Nestler et al., 1995), C6 glioma cells (Young and Woods, 1996) and human platelets from BD patients (Young et al., 1997a); 2) ADP-ribosylation of $\alpha$, targets this subunit for removal from the membrane and subsequent degradation; 3) inhibition of this process can reverse the agonist-induced down regulation of $\alpha$, disturbance(s) in mono-ADP-ribosyltransferase and/or cofactors regulating its activity are prime candidates for exploration and characterization with respect to the cellular pathophysiology of BD.

Unfortunately, a major shortcoming of those studies that have shown that $\alpha$, is ADP-ribosylated by endogenous ADP-ribosyltransferases is that the identification of the ADP-ribosylated products has been based solely on molecular mass estimation (Williams et al., 1992). More importantly, very little is known about whether $\alpha$, is indeed a substrate for endogenous ADP-ribosylation in human brain, and the degree to which $\alpha$, is modified by this posttranslational process in this tissue. Accordingly, the principle objectives of this thesis are to demonstrate $\alpha$, isoforms serve as substrates for endogenous mono-ADP-ribosylation in postmortem human brain, to characterize the ADP-ribosylated products formed, and to determine whether there are differences in endogenous and CTX-catalyzed ADP-ribosylation
of α, in BD brain compared with age and postmortem-delayed matched controls. Since a case for altered heterotrimeric state of α, has been made in BD brain (Friedman and Wang, 1996) and CTX is an important tool that reflects the proportion of α, existing in the heterotrimeric state (Toyoshige et al., 1994), CTX-catalyzed ADP-ribosylation of α, will also be measured.

1.6 Research Plan

Postmortem brain studies have proven to be a particularly powerful strategy to reveal potential pathocellular disturbances in BD. Postmortem brain tissue was employed to delve further into the molecular basis of the G protein α, changes which have been described in BD. Although this preparation has some limitations (i.e. limited amount of tissue and potentially less control of extraneous factors such as the impact of medications, [Warsh and Li, 1999]), it does, however, permit for direct measurement of ADP-ribosylated α, in the disease state. Accordingly, the first phase of studies consisted of characterizing both endogenous and CTX-catalyzed ADP-ribosylated products in postmortem human temporal cortex using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation with α, antibodies, overlay comparisons between western blots and autoradiograms of immunodetected ADP-ribosylated α, isoforms, and limited protease digestion. Following this, a systematic exploration was conducted of the brain regional distribution of both endogenous and CTX-catalyzed [32P]ADP-ribosylation of α, in BD patients along with their age and postmortem delayed matched controls. The temporal and occipital cortical regions examined by Young et al. (1993) were used in this study since higher α+L immunoreactive levels and suggestion of hyperfunctional α,-AC coupled
response have previously been reported in these tissues. The cerebellum, in which no alterations in \( \alpha_1 \) level and function have been demonstrated, was used as a control region. Furthermore, whether observed alterations in ADP-ribosyltransferase activity are associated with a primary alteration in one or more mono-ADP-ribosyltransferase(s) or secondary to a disturbance in a cofactor or other regulatory input to this enzyme was also tested.

1.7 Hypotheses

The general hypothesis addressed in this doctoral thesis is that the observed elevations in \( \alpha_1 \) levels in BD brain result from a disturbance(s) in one or more of the regulatory mechanisms governing \( \alpha_1 \) subunit turnover/degradation. Hyperfunction of the cAMP signaling cascade in BD cerebral cortex would then occur consequent to the primary changes at this level of \( \alpha_1 \) regulation. As discussed in the preceding sections, there is increasing evidence implicating some disturbance at the level of mono-ADP-ribosyltransferase or some factor/process regulating this enzyme, over other posttranslational mechanisms, in the chain of cellular signaling alterations which occur in BD. This accounts for the in-depth focus on the posttranslational modification of \( \alpha_1 \) by ADP-ribosylation in postmortem human brain in this thesis. The specific hypotheses to be addressed include:

1) \( \alpha_1 \) is a substrate for the posttranslational modification of ADP-ribosylation in postmortem human brain;

2) endogenous mono-ADP-ribosyltransferase activity is selectively reduced in postmortem cerebral cortical regions from BD subjects showing elevated \( \alpha_1 \) immunolabeling compared with controls and;

3) this reduction is consequent to an alteration in mono-ADP-ribosyltransferase activity and not its regulation by cofactors or modulators.
CHAPTER II

MATERIALS AND METHODS
II. Materials

II.1 Materials and Chemicals

The following chemicals/materials were purchased from NEN-DuPont (Lachine, Que): $[^{32}\text{P}]\text{NAD}$ (specific activity: 30 Ci/mm), G-protein antisera RM/1 specific to $\alpha_{\text{1a&2}}$ (recognizing C-terminal peptide RMHLRQYELL), AS/7 specific to $\alpha_{\text{1a&2}}$ (recognizing C-terminal peptide KENLKDGLF) and GC/2 (recognizing N-terminal peptide GCTLSAEKALERS of $\alpha_{\alpha\beta\beta\beta}$).

Cholera toxin (CTX), ethyleneglycol-bis (β-aminoethyl)-$N,N,N',N'$-tetraacetic acid (EGTA), ethylenediamine-tetraacetic acid (EDTA), benzamidine (BZ), soybean trypsin inhibitor (SBTII), adenosine diphosphate (ADP)-ribose, thymidine, Triton X-100, MgCl$_2$, 5'-guanylylimidodiphosphate sodium salt (Gpp(NH)p), dithiothreitol (DTT), trichloroacetic acid (TCA), isonicotinic acid hydrazide (INH), 3-acetylpyridine adenine dinucleotide (APAD), phenylmethyl-sulfonylflouride (PMSF), leupeptin, aprotinin, antipain, pepstatin, Protein-A-agarose and N-ethyl-maleimide (NEM) were all purchased from Sigma Chemical Company (St. Louis, MO).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents including Tris, glycine, acrylamide, $N,N'$-methylene-bisacrylamide, SDS, $N,N',N'$-tetramethyl-ethylene-diamine (TEMED), ammonium persulfate, polyvinylidene difluoride (PVDF) membranes (0.2µm), horseradish peroxidase (HRP) conjugated-Protein A, prestained SDS-PAGE standards, protein assay dye (Bradford Reagent) and bovine serum albumin (BSA) were obtained from BioRad (Richmond, CA).

The following chemicals/materials were purchased from the suppliers as indicated:
α₉特定抗血清K-19是在合成肽（SC-385）对应α₉和α₉特定抗血清K-20是在合成肽（SC-835）对应α₁₁氨基酸110-119的α₉（Santa Cruz, CA）, 压敏免疫兔血清（Calbiochem, San Diego, CA), Staphylococcus aureus V-8蛋白酶（Boehringer Mannheim, Laval, Que）, 甘油醛-3-磷酸脱氢酶（GAPDH）（Sigma Chemical Co, St. Louis, MO), Mab-GAPDH 单克隆抗体（Biodesign International, Kennebunk, Maine), 提高化学发光（ECL）检测系统和Hyperfilm (Amersham, Oakville, ON)和Myelin Basic Protein (MBP, 纯化自牛脑) (Gibco BRL/Life Technologies, Burlington, ON).

II.2 Postmortem Brain Material

II.2.1 Subjects

尸检脑子通过一个网络的脑子库和协作的医学检查员提供了材料用于各种神经精神病学和神经退行性疾病研究的指导Dr. Stephen J. Kish of the Human Neurochemical Pathology Laboratory, Clarke Division, Center for Addiction and Mental Health, Toronto。纳入标准为: 1) 男性或女性>18岁在死亡时间; 2) 诊断为BD根据DSM-IIIR标准或无轴I精神病障碍作为确定由独立审查的可用医疗记录的两个研究精神科医生进行独立审查; 3) 神经学障碍确认由病理学和医学记录审查; 4) 尸检延迟(死亡到-80°C冷冻)少于36小时。乙醇和药物使用被了解。精神科治疗的历史是通过可用的医疗记录包括心理药物。
treatment (especially neuroleptics and lithium), electroconvulsive therapy (ECT), and institutionalization. Length of past treatment with lithium, time interval between lithium treatment and death, and recently obtained serum lithium levels were noted from medical records. Brain lithium concentrations were determined in all subjects by argon plasma emission spectroscopy (Rahman et al., 1997). Cause of death and details of the agonal state were noted in all cases.

II.2.2 Brain storage and dissection

Details of handling and dissection of postmortem brains were as previously described (Young et al., 1993). Briefly, at autopsy, whole brains (both left and right sides) were dissected from the crania and, after gross inspection, sectioned (2.5 mm thickness) coronally rostro-caudally. The following representative cortical sections were taken for sampling: frontal cortex at the caudal boundary of Brodmann's area 10 (includes prefrontal and orbito-frontal cortex); premotor cortex at the level of Brodmann's area 6 (includes the anterior pole of the temporal lobe inferiorly); at least two levels in the temporal lobe including hippocampus and amygdala (including areas 20, 21, 22). Cerebellum was blocked separately (maximum block dimensions 30 x 50 mm) as a comparison non-cortical region. A slice of the cerebellum was taken through the pole of the lateral cerebellar hemisphere. All other sections were blocked according to the regions of interest. Representative sections (frontal cortex; two levels in the temporal lobe including hippocampus and amygdala, diencephalon including hypothalamus, thalamus and parietal cortex; cerebellum; and occipital cortex) were taken for formalin fixation and subsequent neuropathological examination. Tissue blocks were rapidly frozen and stored (-80°C) until assay. Brain dissections were performed based
on visual landmarks and guided by a standard brain atlas (Riley, 1960) and Brodmann's classification (England and Wakely, 1991) by an experienced human brain neuroscientist (S. J. Kish) collaborating on this project. Determination of brain pH, an index of agonal status (Butterworth and Tennant, 1989) was performed as previously described (Young et al., 1993). Brain regions assayed for \(^{32}\)PADP-ribosylation included temporal (middle temporal gyrus, area 21, inferior temporal gyrus, area 20), occipital (lips of the calcarine sulcus, area 17) and cerebellar cortex.

II.3. Immunoassay of α, isoforms

II.3.1 Membrane Preparation

Briefly, portions of postmortem brain were sonicated in TME (50 mM Tris (pH 7.5) 1 mM EDTA and 2 mM MgCl\(_2\)) and centrifuged (12,000 x g, 10 min, 4°C). After centrifugation, the supernatants were discarded, membranes resuspended in TME, and protein concentrations determined (Bradford, 1976).

II.3.2 Gel electrophoresis and Immunoblotting

Aliquots of sample protein (7.5 µg) were prepared for SDS-PAGE and Western blotting as previously described (Young et al., 1993). Briefly, Laemmli buffer (40 mM Tris-HCl, pH 6.8, 1 mM DTT, 2% SDS) was added to each sample followed by heating for 5 min at 75°C. Then, 100 mM NEM was added and samples were incubated for another 15 min at 21°C. Samples were then diluted with gel-loading buffer (62.5 mM Tris-HCl, 3% SDS, 10% glycerol and 5% mercaptoethanol) and boiled for 3 min at 100°C to ensure complete denaturation and homogeneity of proteins throughout the sample.
\( \alpha_{\text{L}} \) and \( \alpha_{\text{S}} \) were electrophoretically separated on 10% polyacrylamide gels. Acrylamide stock solutions were comprised of 20 g acrylamide and 0.533 g bis acrylamide (37.5: 1) dissolved in 100 ml deionized water yielding a 20% acrylamide solution (2x). This was mixed in a 1:1 ratio with Lower Tris (2x concentrated, 0.75 M Tris, pH 8.8, 0.2% SDS) to prepare resolving gels. Polymerization was initiated with the addition of 10% ammonium persulfate (5 \( \mu \)l/ml) and TEMED (0.5 \( \mu \)l/ml). Stacking gels consisted of 4% acrylamide/0.11% bis acrylamide solutions, polymerized with 10% ammonium persulfate (5 \( \mu \)l/ml) and TEMED (1 \( \mu \)l/ml). These were prepared as follows: stock solutions of 8% acrylamide (8 g acrylamide, 0.21% bis acrylamide dissolved in 100 ml deionized water, 38:1, 2x concentrated) and Upper Tris (0.75 M Tris, pH 6.8, 0.2% SDS, 2x concentrated) were combined in a 1:1 ratio along with 10% ammonium persulfate and TEMED. Both resolving and stacking gels were cast in mini-PROTEAN II Dual Slab cells (0.75 mm thickness, BioRad, Richmond, CA) and allowed to polymerize for 50-55 min. Sample loading wells were formed with either 15-well or 10-well combs during stacking gel polymerization.

Gel lanes were first rinsed three times with Tris-glycine electrophoretic buffer comprised of 25% Tris-glycine and 10% SDS, pH 8.3. Prepared samples, along with molecular mass markers, were then loaded. Electrophoretic separation was carried out using Tris-glycine electrophoresis buffer at 200 V for approximately 1.5-2 hours or until molecular mass markers migrated pre-determined distances empirically found to optimally resolve \( \alpha \), isoforms. For \( ^{32}\text{P} \)ADP-ribosylated products, electrophoretic separation was carried out at 125 V for approximately 3 hrs.

Following SDS-PAGE, proteins resolved electrophoretically were transferred to
PVDF membranes (0.2 μm) using a semi-dry electrotransfer system (Trans Blot SD semi-dry electrophoretic transfer cell, BioRad, Richmond, CA). Gels and membranes were first equilibrated in transfer buffer, consisting of 48 mM Tris (pH 8.1), 39 mM glycine, 20% methanol and 0.375% SDS for 15 min. Prior to electrotransfer, excess moisture and air bubbles were removed from the gel and membrane ‘sandwiches’ by gentle compression with a test-tube. Semi-dry electrotransfer of proteins was carried out at 15 V for 36 min.

Following transfer, membranes were washed quickly 3 times with deionized water. Blots were then incubated with 5% BSA in phosphate buffered saline-Tween 20 (PBS-T, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, pH 7.5, with 0.5% Tween 20) for 1 hour at 21°C. Blots were then incubated overnight in plastic bags with primary antibody diluted in PBS-T (5 ml) and subsequently incubated with secondary antibody (Protein A-HRP, 1:3000) for 30 min at room temperature. For immunodetection, anti-Gα₃ (RM/1) and Mab-GAPDH were used at a dilution of 1:5000 and 1:500, respectively. Blots were washed thoroughly with PBS-T (3 x 5 min) prior to each incubation in plastic boxes. Immunoreactive proteins were detected by enhanced chemiluminescence using the ECL reagent kit (Amersham, Oakville, ON). Samples containing varying amounts of protein (5-40 μg) were included on each blot to ensure immunoreactive signals of sample proteins were within the linear range of detection and for normalization (to control for interassay variability). On all blots, samples were run in duplicate from patients paired with age and post-mortem delayed matched controls. This was done to control for the effect of inter-blot variance in G protein measures.

For detection of [³²P]ADP-ribosylated proteins, electrophoresed gels were laid on
blotting paper and dried for 2 hrs at 80°C under vacuum (99.9 kPa). Autoradiograms of radiolabelled proteins were obtained by exposure to Hyperfilm (18-72 hrs) at -70°C. For overlay comparisons, [32P]ADP-ribosylated proteins were electrotransferred overnight at 30 V. Subsequent blots were air dried and exposed to Hyperfilm as described above.

II.4 Assessment of [32P]ADP-ribosylation in Postmortem Human Brain

II.4.1 Endogenous [32P]ADP-ribosylation

Postmortem brain samples (20-25 mg) were slowly thawed on ice and homogenized with a hand held glass Teflon homogenizer, 20 times, in 2 vol. of ice cold buffer containing 20 mM Tris (pH 7.0), 1 mM EDTA, 0.5 mM BZ and 0.001% SBTII. Aliquots of homogenates (250 μg for samples, 100-400 μg from a reference pool, run to monitor assay linearity and to control for interassay variability) were incubated in 100 μl of 100 mM Tris-HCl (pH 7.5) buffer containing thymidine, 10 mM; EDTA, 1 mM; MgCl₂, 0.5 mM; Gpp(NH)p, 0.1 mM; DTT, 25 mM, 0.1% Triton X-100, and 2.5 μCi [32P]NAD. After incubation at 30°C for 60 min, the reactions were terminated with 3 μl TCA and after standing at 4°C for 15 min, the samples were centrifuged (15,000 x g, 10 min) and the supernatants discarded. Pellets were washed twice with 50 mM Tris (pH 8.0) buffer containing 3 mM BZ, 1 mM DTT, 6 mM MgCl₂, 1 mM EDTA, 5% sucrose and 1 μg/ml SBTII, resuspended in 100 μl 50 mM Tris-HCl containing 5% SDS, 50 mM DTT and heated (90°C, 5 min). Samples were then incubated with 10 μl of 100 mM NEM (15 min, 21°C) followed by 100 μl gel-loading buffer (250 mM Tris-HCl, [pH 6.8], 4% SDS, 5 mM EGTA, 3 mM EDTA, 10% mercaptoethanol, 10% glycerol) for 3 min (90°C) and prepared for SDS-PAGE as previously described (Young et al., 1993). Following electrophoresis, gels were
dried for 2 hours at 80°C under vacuum (99.9 kPa) and autoradiographed at -70°C.

II.4.2 CTX-catalyzed [$^{32}$P]ADP-ribosylation

CTX-catalyzed [$^{32}$P]ADP-ribosylation was assayed by a modification of previously described procedures (Tamir and Gill, 1988, Williams et al., 1992). After thawing as above, postmortem brain samples (10-15 mg) were homogenized with a hand held glass Teflon homogenizer, 20 times, in 5 vol. of ADP-ribosylation buffer containing 150 mM potassium phosphate, pH 7.0, 10 mM thymidine, 20 mM INH, 1 mM 3-APAD, 10 mM DTT, 0.1 mM Gpp(NH)p and 0.1% Triton X-100. CTX (2 mg/ml in 50% glycerol) was preactivated by incubating at 37°C for 15 min in 1.5 vol. of 15 mM sodium phosphate buffer (pH 7.5) containing 33 mM DTT and 0.83% SDS. The activated CTX was then diluted with 3 vol. of ADP-ribosylation buffer and added to sample preparations at a final concentration of 40 μg/ml. [$^{32}$P]ADP-ribosylation was performed using 30 μg for samples and 15-40 μg of control human cortical tissue for confirmation of assay linearity and normalization in 25 μl ribosylation buffer containing 20 mM ADP-ribose and 3 μCi [$^{32}$P]NAD. Samples were then incubated and reactions terminated as described above for endogenous activity. After washing, samples were subsequently processed for SDS-PAGE by incubating with 75 μl Tris-HCl containing 5% SDS, 50 mM DTT (90°C, 5min), followed by 7.5 μl NEM (15 min, 21°C) and 75 μl gel-loading buffer. Autoradiographs were quantified using densitometric image analysis (MCID Image Analysis System, St. Catherines, ON). Endogenous and CTX-catalyzed [$^{32}$P]ADP-ribosylation assays were established within the linear range of detection for both protein concentration and [$^{32}$P]incorporation with respect to time (see Results).
II.4.3 Endogenous $^{32}$PADP-ribosylation of Myelin Basic Protein (MBP)

Cortical regions demonstrating differences in $^{32}$PADP-ribosylation of $\alpha_s$ were reassessed for endogenous $^{32}$PADP-ribosylation of MBP (purified from bovine brain) to test the functional integrity of the mono-ADP-ribosyltransferases. Endogenous $^{32}$PADP-ribosylation of this substrate was determined as described above with the addition of MBP (300 $\mu$g, within the linear range of detection) prior to incubation of samples. $^{32}$P ADP-ribosylated MBP samples were electrophoretically resolved on 12.5% acrylamide/0.06% bisacrylamide gels. A range of MBP concentrations (100-400 $\mu$g) was included on each gel to monitor assay linearity as shown in Section III.

II.5 Characterization of the $^{32}$P ADP-Ribosylated Products in Human Brain

II.5.1 Molecular Mass Determination

Molecular weights of $^{32}$P ADP-ribosylated products were estimated using prestained molecular mass markers (New England BioLabs). For overlay comparisons, endogenous and CTX-catalyzed $^{32}$P ADP-ribosylated products were resolved by SDS-PAGE on 10% polyacrylamide gel and electrophoretically transferred overnight to PVDF membrane. Membranes with the $^{32}$P ADP-ribosylated proteins were first detected autoradiographically (18-72 hr exposure) followed by detection of $\alpha_s$ isoforms using anti-$\alpha_s$ (RM/1) and ECL. Identification of $^{32}$P ADP-ribosylated $\alpha_s$ was subsequently achieved by overlaying autoradiograms and immunoblots and aligning the two films with the aid of molecular mass markers as depicted in Figure 6.

II.5.2 Immunoprecipitation

Immunoprecipitation of $\alpha_s$ was performed as previously described with minor modi-
Overlay comparisons of Autoradiograms and Immunoblots of $^{32}$P]ADP-ribosylated $\alpha$.

**Figure 6:** Direct overlay comparisons were made between immunoblots and autoradiograms of the $^{32}$P]ADP-ribosylated proteins to further characterize these products. Briefly, endogenous and CTX-catalyzed $^{32}$P]ADP-ribosylated proteins were resolved by SDS-PAGE, electrophoretically transferred overnight onto PVDF membrane and then exposed to Hyperfilm. Membranes were then incubated with RM/1 antisera followed by Protein A-HRP and ECL detection. Test exposure of membranes to Hyperfilm (5-10 min) prior to ECL did not yield any $^{32}$P] radiolabel signal and hence did not contribute to the density of the immunolabeled bands detected.
fications (Levis and Bourne, 1992). Briefly, [³²P]ADP-ribosylated samples were pelleted (15,000 x g, 10 min) and solubilized in 500 μl immunoprecipitation buffer (0.5-1% Triton X-100, 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 8 μg/ml PMSF, 2 μg/ml each of leupeptin and aprotinin, 1 μg/ml of each antipain and pepstatin) on ice for 1 hr. Samples were then centrifuged (12,000 x g, 5 min), and supernatants transferred into new tubes and saved. Supernatants were then incubated with RM/l (1:500), AS/7 (1:500), GC/2 (1:500), K-19 (1:100) or K-20 (1:100) overnight at 4°C followed by Protein A-agarose precipitation of immune complexes for 1 hr. Control human temporal cortical sample was also precipitated in parallel with pre-immune serum. Immunoprecipitates were then pelleted by centrifugation (3,000 x g, 1 min) and washed twice with 50 mM Tris-HCL, pH (7.4) adjusted to 0.3% SDS and once with 0.05-0.1% NP-40. 10 mM Tris-HCl (pH 7.4). Immunoprecipitates were then suspended in Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 3% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue), boiled (3 min) and subjected to SDS-PAGE. Following electrophoresis, gels were dried and autoradiographed at -70°C.

II.5.3 One dimensional peptide mapping

Endogenous and CTX-catalyzed [³²P]ADP-ribosylated products were analyzed by one-dimensional peptide mapping as previously described (Cleveland et al., 1977, Duman et al., 1991). Briefly, bands corresponding to individual CTX-catalyzed and endogenous [³²P]ADP-ribosylated proteins were excised from wet 10% polyacrylamide gels that had been stored overnight at 4°C. Radiolabeled proteins were then subjected to SDS-PAGE once again on 15% polyacrylamide gels containing 4% stacking gels twice the normal depth
(approximately 4 cm). Gel slices were placed into the appropriate stacking wells filled with 125 mM Tris (pH 6.8) containing 1 mM EDTA, 2.5 mM DTT, 0.1% SDS (TEDS) using the extreme end of loading gel pipette tips and incubated for 10 min. Slices were first overlaid with 6 µl of TEDS containing 20% glycerol followed by 10 µl of TEDS containing 10% glycerol, 0.001% bromphenol blue and *Staphylococcus aureus* V-8 protease. A linear range of protease concentrations (50 ng-5 µg per lane) were used. Gels were run at 125 V until the bromphenol blue dye fronts had migrated approximately two-thirds of the distance into the stacking gels. Digestion was then carried out for 30 min. Following digestion, gels were electrophoresed until dye fronts reached the bottom of the resolving gels. Gels were then dried and autoradiographed overnight at -70°C.

II.6 Platelet and B-lymphoblast Preparation

Isolation of platelets and establishment of B-lymphoblast cell lines were performed as previously described (Emamghoreishi et al., 1997). Briefly, anticoagulated whole blood, collected in ACD vacutainer tubes by venipuncture from volunteer subjects, was centrifuged at 100 x g (20 min, 21°C) and the platelet-rich plasma removed, and platelets collected. Platelets were subsequently washed with RMPI-1640 (400 x g, 10 min), and frozen until the day of assay. The lower layer of sedimented red and white blood cells was mixed with RPMI-1640 (1:3 vol/vol). Eight ml of diluted cells was overlaid on 5 ml of 60% Percoll (Ficoll-Hypaque for B-cells) and centrifuged at 400 x g (30 min, 21°C). The MNL interface layer was transferred, pooled, diluted with an equal volume of RPMI-1640, and recentrifuged (400 x g, 10 min). The pellet was washed with RPMI-1640 and resuspended in the same medium. Cells for B-lymphocyte transformation and cell culture (at least 4 x 10^6 MNLs)
were resuspended in 1 ml RPMI-1640 containing L-glutamine (2 mM), pyruvate (1 mM), 20% fetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin. 0.5 ml of filtered supernatant from an Epstein-Barr virus-expressing B95-8 monkey kidney cell line culture and cyclosporine A (1.3 μg/ml) were added, and the cells incubated at 37°C in a 95% air/5% CO₂ humidified incubator for two weeks (Walls and Crawford, 1987). Once cell lines were established, cells were transferred and grown in suspension in the same medium in T-25 tissue culture flasks. The cells were ‘passaged’ every 2-3 days by adding an equivalent volume of fresh medium. At 12-15 passages, cells were sedimented (400 x g, 10 min), viabilities determined and cells subsequently harvested. Cell viability was greater than 95% as determined using trypan blue exclusion (Hunt, 1987). Following harvesting, cells were frozen at -70°C until the day of assay. Endogenous and CTX-catalyzed [³²P]ADP-ribosylation was performed as described above for postmortem brain tissue.

II.7 Protein Determination

Protein was determined using the BioRad Protein Assay Kit which is based on the method of Bradford (1976). Briefly, the dye reagent was diluted in deionized water (1:4) and filtered through a Whatman 2V filter. Serial dilutions of bovine serum albumin (BSA) were prepared and 10 μl of protein standard (0-400 μg/ml) and sample solutions were added to 200 μl of diluted dye in ELISA plates. After incubating for 30 min at room temperature with gentle stirring, the absorbances of samples were read at 595 nm using a microplate reader (Molecular Devices).

II.8 Video Densitometry

Autoradiograms and immunoblots of α₂ isoforms were analyzed using a MCID
Imaging Analysis System (St. Catherines, Ontario). Band densities were estimated based on relative optical densities (RODs), expressed as the log_{10} \left( \frac{1}{[\text{levels of image/256 gray scale levels}]} \right). Background levels were subtracted from all samples.

II.9 Statistical Analysis

All data are expressed as the means ± SD. Statistical comparisons were evaluated using parametric statistics after confirming homogeneity of variance. Differences in the dependent variables in postmortem brain studies (e.g. basal $[^{32}\text{P}]$ADP-ribosylated products, CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated products, $\alpha$, immunoreactivity levels) were assessed using two-way ANOVA with diagnosis and brain region as factors. Post hoc comparisons of cell means were assessed using the Tukey's test. Differences in $[^{32}\text{P}]$ADP-ribosylation of MBP in control vs. BD subjects were assessed using independent samples $t$-test. Correlations between biochemical and clinical dependent variables, such as age, severity ratings etc. were performed with the Pearson's Product Moment test with Bonferroni correction for experiment-wise error. Correlations between protein concentration, $[^{32}\text{P}]$ADP-ribosyltransferase activity, and $[^{32}\text{P}]$incorporation with respect to time were performed using linear regression analyses. Where curvature towards asymptotes was observed data were fitted to second-order polynomials by non-linear regression analyses. Statistical analyses were performed using Systat and SPSS statistical (release 7.0) software packages. Differences were considered statistically significant with $P < 0.05$ (two-tailed).
CHAPTER III

RESULTS
III.1 Results

III.1.1 Endogenous and CTX-catalyzed $^{32}$PADP-ribosylation in Postmortem Human Brain

As shown in Figure 7A, three major reaction products were obtained in autopsied human temporal cortex under the endogenous $^{32}$PADP-ribosylation conditions described, with estimated molecular masses of 48, 45 and 39 kDa, respectively. Two major protein bands with estimated molecular masses of 52 and 45 kDa, respectively, were radiolabeled in the presence of CTX in homogenates of postmortem temporal cortex (Figure 7B). Figure 7 also shows a number of higher (50.6 to 101 kDa) and lower (< 29 kDa) molecular mass $^{32}$PADP-ribosylated products under both endogenous and CTX-catalyzed assay conditions, but the levels for these products were very low. As the molecular sizes of the major $^{32}$PADP-ribosylated proteins identified were similar to those for G protein $\alpha$ subunits, including $\alpha_\alpha$, these more prominent radiolabeled bands were characterized further.

Since mono-ADP-ribosyltransferases can be affected by various compounds such as INH, APAD and DTT (Okazaki and Moss, 1996), the degree of endogenous and CTX-catalyzed $^{32}$PADP-ribosylation of the major substrate proteins was assessed under different assay conditions using these compounds. First, INH, APAD and ADP-ribose are known to inhibit NAD-glycohydrolase and mono-ADP-ribosyltransferase activity (Gill and Woolkalis, 1991, Matsuyama and Tsuyama, 1991). Inclusion of these compounds in the assay inhibited endogenous mono-$^{32}$PADP-ribosylation and enhanced CTX-catalyzed $^{32}$PADP-ribosylation (Figure 8). Thus 20 mM INH, 1 mM 3-APAD and 20 mM ADP-ribose were included in the assay buffer for CTX-catalyzed $^{32}$PADP-ribosylation but were omitted for endogenous $^{32}$PADP-ribosylation.
Assessment of Endogenous and CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylation in Postmortem Human Temporal Cortex

Figure 7: Assessment of endogenous and CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylation in postmortem human temporal cortex. A: Representative autoradiogram of duplicate endogenous \[^{32}\text{P}\]ADP-ribosylated substrate proteins. Samples were ADP-ribosylated with \[^{32}\text{P}\]NAD as described in Methods (Section II.4.1), subjected to SDS-PAGE, and gels dried and autoradiographed overnight at -70°C. B: Representative autoradiogram of duplicate CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylated substrate proteins. Samples were ADP-ribosylated with \[^{32}\text{P}\]NAD as described in Methods (Section II.4.2) and processed as described above for endogenous \[^{32}\text{P}\]ADP-ribosylation.
Effects of INH, APAD and ADP-ribose on Endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation in Postmortem Human Brain

Figure 8: Assessment of the degree of endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of the major substrate proteins under different assay conditions. A: Representative autoradiogram of endogenous $[^{32}\text{P}]$ADP-ribosylated proteins determined in duplicate samples of temporal cortex in the absence (-) and presence (+) of 20 mM INH, 1 mM 3-APAD and 20 mM ADP-ribose. Thymidine was included in all ADP-ribosylation assays to inhibit poly-ADP-ribosyltransferase activity. Samples were $[^{32}\text{P}]$ADP-ribosylated as described in Methods. B: Representative autoradiogram of CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation in the absence (-) and presence (+) of INH, APAD and ADP-ribose. Samples were processed as described above for endogenous $[^{32}\text{P}]$ADP-ribosylation.
As DTT has been shown to affect variably the activity of mono-ADP-ribosyltransferases, inhibiting some but stimulating others (Okazaki and Moss, 1996), the effect of varying DTT concentrations on the degree of $[^{32}\text{P}]$ADP-ribosylation was also examined. Previous studies measuring endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosyltransferase of proteins (including $\alpha_\text{h}$) in various tissues have employed 25 mM and 10 mM DTT, respectively, in their assay buffers (Tamir and Gill, 1988, Williams et al., 1992, Nestler et al., 1995). In this study, 25 mM concentrations generated a strong signal intensity for endogenous $[^{32}\text{P}]$ADP-ribosylation of the 39 kDa product (Figure 9A). However, maximum $[^{32}\text{P}]$radiolabeling of the 48 kDa and 45 kDa substrates in postmortem brain was not seen at 25 mM DTT, but rather at 10 mM and 1 mM DTT, respectively (Figure 9A). Thus, for comparison purposes, endogenous ADP-ribosylation was performed under two assay conditions: for the 48 and 45 kDa proteins using 10 mM DTT, and 25 mM DTT for measuring endogenous $[^{32}\text{P}]$ADP-ribosylation of the 39 kDa product. DTT did not appear to have any differential effect on the degree of CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation for each of the two major products as no linear incremental response was observed between 1-25 mM (Figure 9B). Again, since the majority of studies measuring CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation have employed 10 mM DTT, it was subsequently used for CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation experiments.

Varying the concentration of NAD (ranging between 2-25 $\mu$M) did not significantly affect the signal intensity obtained for the various endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated products in postmortem human temporal cortex (data not shown). For quantitative purposes, assay conditions were also established within a linear range of protein
Effects of Dithiothreitol (DTT) on \[^{32}\text{P}]\text{ADP-ribosylated Proteins Detected in Postmortem Human Brain}

\textbf{Figure 9:} Effect of dithiothreitol (DTT) on \[^{32}\text{P}]\text{ADP-ribosylated proteins detected and quantified in postmortem human brain.} A: Representative autoradiogram of the major endogenous \[^{32}\text{P}]\text{ADP-ribosylated proteins. Samples were ADP-ribosylated with \[^{32}\text{P}]\text{NAD and varying concentrations of DTT, electrophoresed on 10\% polyacrylamide gels and autoradiographed overnight at -70\^\circ\text{C.} \[^{32}\text{P}]\text{ADP-ribosylated proteins were densitized as described in Methods (Section II.8). Each lane contains 250 \mu g protein.} B: Representative autoradiogram of the major CTX-catalyzed \[^{32}\text{P}]\text{ADP-ribosylated proteins. Samples were processed and densitized as described above for endogenous \[^{32}\text{P}]\text{ADP-ribosylation. Each lane contains 40 \mu g protein.} Data are expressed as average and range for duplicate samples and are representative of 2 independent assays.}
Linear Range of Protein Concentrations for Both Endogenous and CTX-catalyzed $[^{32}P]$ADP-ribosylation in Postmortem Human Brain

Figure 10: Linear range of detection versus protein concentrations for both endogenous- and CTX-catalyzed $[^{32}P]$ADP-ribosylation in postmortem human brain. A: Representative autoradiogram of a linear range of protein concentrations (100-400 µg) for the major endogenous $[^{32}P]$ADP-ribosylated proteins. Samples were ADP-ribosylated with $[^{32}P]$NAD as described in Methods (Section II.4.1), electrophoresed on 10% polyacrylamide gels and autoradiographed overnight at -70°C. $[^{32}P]$ADP-ribosylated proteins were densitized as described in Methods (Section II.8). B: Representative autoradiogram of a linear range of detection versus protein concentrations (16-42 µg) for the major CTX-catalyzed $[^{32}P]$ADP-ribosylated proteins. Samples were processed and densitized as described above for endogenous $[^{32}P]ADP$-ribosylation. Data are expressed as average and range for duplicate samples and are representative of 23 independent assays.
Figure 11: Time course for endogenous (A, C) and CTX-catalyzed $[^{32}P]$ADP-ribosylation (B) of the $\alpha_i$ isoforms (A, B) and MBP (C). Postmortem human temporal cortex samples were $[^{32}P]$ADP-ribosylated for 30, 60, 90, 120 and 150 min. Data were quantified as described in Methods (III.8). Data are expressed as means and range for duplicate samples and are representative of two independent assays for each of endogenous, CTX-catalyzed $[^{32}P]$ADP-ribosylation and MBP condition.
concentrations for both endogenous (100-400 μg) and CTX-catalyzed (10-40 μg) \[^{32}\text{P}\]ADP-ribosylation (Figure 10A, B, respectively).

The relationship between time and appearance of endogenous and CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylated products of \(\alpha_{+L}\), \(\alpha_{+S}\) and the 39 kDa \(\alpha_\text{r}\)-like protein in postmortem human temporal cortex samples are depicted in Figure 11A and 11B, respectively. The radiolabeling of myelin basic protein (MBP) is also shown in Figure 11C. Endogenous \[^{32}\text{P}\]-incorporation of \(\alpha_{+L}\) was linear between 0 and 120 min, and up until 150 min for both \(\alpha_{+S}\) and for the 39 kDa \(\alpha_\text{r}\)-like protein. Radiolabeling of MBP was also linear with respect to time to at least 120 min. Furthermore, CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylation of \(\alpha_{+L}\) and \(\alpha_{+S}\) was linear for 90 min with saturation beginning to become evident at 150 min.

III.2 Characterization of \[^{32}\text{P}\]ADP-ribosylated Products in Postmortem Human Brain

III.2.1 Immunoprecipitation

The two major bands (52 and 45 kDa) obtained following CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylation of postmortem human temporal cortex proteins [Figure 12A(-)] corresponded to those expected for the long and short forms of \(\alpha_\text{r}\), respectively, based on electrophoretic migration and estimated molecular mass. Characterization of the major CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylated products was first undertaken by immunoprecipitation of radio-labeled samples with RM/1 (antisera specific to \(\alpha_\text{r}\)) immediately following \[^{32}\text{P}\]ADP-ribosylation. Immunoprecipitated isoforms of \(\alpha_\text{r}\) comigrated with the \[^{32}\text{P}\]ADP-ribosylated \(\alpha_\text{r}\) isoforms (Figure 12A i, compare with [+] and without [-] immunoprecipitation). Furthermore, similar migratory patterns were observed for the putatively identified CTX-catalyzed \[^{32}\text{P}\]ADP-ribosylated \(\alpha_\text{r}\) isoforms and those proteins that have been ADP-ribosyl-
Immunoprecipitation of Endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated $\alpha$, with RM/1

A

i

\[ \sim 52 \text{ kDa} \]

\[ \sim 45 \text{ kDa} \]

\[ \text{[}^{32}\text{P}] \text{ Unlabeled} \]

\[ - + \]

ii

\[ \sim 52 \text{ kDa} \]

\[ \sim 45 \text{ kDa} \]

\[ - + \]

Figure 12: Characterization of $[^{32}\text{P}]$ADP-ribosylated proteins detected in postmortem human brain. A:(i) Representative autoradiogram of CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated substrate proteins and immunoprecipitated radiolabeled $\alpha$, isoforms. Samples were ADP-ribosylated with $[^{32}\text{P}]$NAD in the presence of CTX as described in Methods (Section II.4.2). Immunoprecipitation of CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated proteins with RM/1 was performed immediately following $[^{32}\text{P}]$ADP-ribosylation. (+) and (-) indicate with and without immunoprecipitation, respectively. A:(ii) Representative autoradiogram and immunoblot of CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated substrate proteins. Samples were ADP-ribosylated with $[^{32}\text{P}]$NAD (-) and unlabeled NAD (+) as described in Methods (Section II.4.2). Samples ADP-ribosylated with unlabeled NAD (+) were subsequently detected with RM/1. Radiolabeled proteins (-) were subjected to autoradiograph. B: Representative autoradiograms of endogenous $[^{32}\text{P}]$ADP-ribosylated substrate proteins with (+) and without (-) immunoprecipitation.
lated in the presence of unlabeled NAD followed by immunodetection rather than immunoprecipitation with RM/1 (Figure 12Aii, compare with [+] and without [-] immunodetection). Taken together, these findings support the identity of the two major CTX-catalyzed \([^{32}\text{P}]\text{ADP-ribosylated products}\) (52 & 45 kDa) as \(\alpha_{+L}\) and \(\alpha_{+S}\), respectively.

As shown in Figure 12B (-), two of the three major endogenously \([^{32}\text{P}]\text{ADP-ribosylated proteins}\) (i.e. 48 and 39 kDa) in postmortem human temporal cortex were clearly immunoprecipitated with RM/1 antiserum, along with slight immunoprecipitation of the 45 kDa protein, suggesting their identity as \(\alpha_{-}\)-like products (Figure 12B, compare [-] and [+]). Interestingly, these proteins migrated faster than the major CTX-catalyzed \([^{32}\text{P}]\text{ADP-ribosylated products}\) (\(\alpha_{+L}\) and \(\alpha_{+S}\)). Incubation of solubilized temporal cortex membranes following endogenous \([^{32}\text{P}]\text{ADP-ribosylation}\) with anti-\(\alpha_{\text{olf}}\) (K-19) did not result in any detectable immunoprecipitation of an \(\alpha_{\text{olf}}\)-like product migrating at the expected mass of \(\approx 46\text{kDa}\) (data not known). Immunoprecipitation with anti-\(\alpha_{1/2}\) (AS/7) and anti-\(\alpha_{\text{a}}\) (GC/2) did not generate any signals different from that obtained with preimmune serum (Figure 13A). Both AS/7 and GC/2 show no cross-reactivity to the \(\alpha_{a}\) isoforms and likewise RM/1 to \(\alpha\), and \(\alpha_{\text{a}}\) (Figure 13B).

**III.2.2 Overlay Comparisons of Western Blots and Autoradiograms**

Direct overlay comparisons were made between immunoblots and autoradiograms of the \([^{32}\text{P}]\text{ADP-ribosylated proteins}\) to further characterize these products. Briefly, endogenous and CTX-catalyzed \([^{32}\text{P}]\text{ADP-ribosylated proteins}\) were resolved by SDS-PAGE, electrophoretically transferred overnight onto PVDF membranes and then exposed
Immunoprecipitation of Endogenous $^{32}$P-ADP-ribosylated Proteins with anti-$\alpha$, (RM/1 and K-20), anti-$\alpha_{II}$ (AS/7) and anti-$\alpha_{o}$ (GC/2)

Figure 13: (A) Representative autoradiogram of endogenous $^{32}$P-ADP-ribosylated proteins (ENDO) immunoprecipitated with various antibodies including anti-$\alpha$, (RM/1 and K-20), anti-$\alpha_{II}$ (AS/7), anti-$\alpha_{o}$ (GC/2) and preimmune serum (PI). Samples were ADP-ribosylated with $^{32}$P-NAD and immunoprecipitated as described in Methods (Section II.5.2). (B) Immunolabeling of $\alpha$, $\alpha_i$ and $\alpha_o$ with RM/1, AS/7 and GC/2, respectively. Both AS/7 and GC/2 show no cross-reactivity to the $\alpha_i$ isoforms and likewise RM/1 to $\alpha_i$ and $\alpha_o$ (Figure 13B).
Overlay Comparisons Between Western Blots and Autoradiograms of the $[^{32}\text{P}]{\text{ADP-ribose}}$ylated Proteins

**Figure 14:** Direct overlay comparisons between Western Blots and autoradiograms of the $[^{32}\text{P}]{\text{ADP-ribose}}$ylation substrate proteins. **A:** Representative autoradiogram of endogenous $[^{32}\text{P}]{\text{ADP-ribose}}$ylated substrates and immunodetected $\alpha_1$ proteins. Samples were endogenously $[^{32}\text{P}]{\text{ADP-ribose}}$ylated, subjected to SDS-PAGE and resolved proteins electrophoretically transferred overnight onto PVDF membrane. Membranes were first autoradiographed overnight at -70°C ($[^{32}\text{P}]$), then immunolabeled with RM/1 antisera and Protein A-HRP, and detected using the enhanced chemiluminescence (ECL) as described in Methods (Section II.5.1). **B:** Representative autoradiogram of CTX-catalyzed $[^{32}\text{P}]{\text{ADP-ribose}}$ylated substrates and immunodetected $\alpha_1$ proteins. Samples were $[^{32}\text{P}]{\text{ADP-ribose}}$ylated in the presence of CTX and processed as described above for endogenous $[^{32}\text{P}]{\text{ADP-ribose}}$ylation.
to Hyperfilm. Membranes were then incubated with RM/1 antisera followed by Protein A-HRP and then detected with ECL. Exposure of membranes to Hyperfilm (5-10 min) prior to ECL gave no $^{[32]}$P radiolabeled signal and hence did not contribute to the detected signal of the immunolabeled bands. Figure 14A (compare $^{[32]}$P and ECL) shows that the endogenous $^{[32]}$P ADP-ribosylated band migrating with an estimated mass of 48 kDa corresponds to the immunoreactively detected $\alpha_{+L}$, while that migrating at 45 kDa corresponds to immunolabeled $\alpha_{+S}$. A third, less abundant 39 kDa $\alpha_-$-like protein was also detected, which comigrated with the identified 39 kDa $^{[32]}$P ADP-ribosylated protein. Overlay comparisons of CTX-catalyzed $^{[32]}$P ADP-ribosylated products confirmed that these radiolabeled bands corresponded exactly with those immunolabeled with RM/1, i.e. $\alpha_{+L}$ and $\alpha_{+S}$ (Figure 14B, compare $^{[32]}$P and ECL).

At 10 mM DTT, the ratio of the signal intensities for the $\alpha_{+L}$:39 kDa endogenous $^{[32]}$P ADP-ribosylated proteins was approximately 1:1, whereas the ratio of the immunoreactive signals was approximately 4:1 (Figure 14A, compare $^{[32]}$P and ECL). In addition, the signal obtained from endogenous $^{[32]}$P ADP-ribosylation of $\alpha_{+S}$ was less intense (2:1 compared to 39 kDa protein) than that for its immunolabeled counterparts (1:2). Similarly, CTX-catalyzed $^{[32]}$P ADP-ribosylation of $\alpha_{+L}$,$\alpha_{+S}$ was approximately 1:1 which differed, once again, from the immunolabeling of the two isoforms. These observations suggest preferential labeling of the $\alpha_-$ isoforms for mono-ADP-ribosyltransferases and may reflect differential endogenous ADP-ribosylation of these proteins in postmortem human temporal cortex.
III.2.3.1 Endogenous [³²P]ADP-ribosylation with Protease Inhibitors

It was unlikely that the 39 kDa endogenous [³²P]ADP-ribosylated band represented a degradative product of either α₅L or α₅S since previous data has shown that α subunit protein levels are stable (i.e. either not altered or reduced [determined from two independent runs]) when homogenized in the presence of protease inhibitors. However, to conclusively rule out such a possibility, endogenous [³²P]ADP-ribosylation of sample homogenates was performed in the absence and presence of various protease inhibitors. As shown in Figure 15A (compare [-] & [+]), the 39 kDa endogenously [³²P]ADP-ribosylated protein was still present under the modified assay conditions.

III.2.3.2 Immunolabeling with Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)

To exclude the possibility that the 39 kDa [³²P]ADP-ribosylated product may have represented glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ≈39 kDa), an endogenously ADP-ribosylated protein recently described in rat hippocampus (Suzuki et al., 1997). ADP-ribosylated samples were immunolabeled with Mab-GAPDH + RM/1 and RM/1. As seen in Figure 15B (compare lanes 1, 2 and 3), RM/1 detected all three α, isoforms, while Mab-GAPDH detected a single major immunoreactive band migrating at approximately 36 kDa. GAPDH did not comigrate with the 39 kDa endogenously [³²P]ADP-ribosylated band. Moreover, the relative ratios of the α, isoforms were similar in the presence and absence of anti-GAPDH. Although it cannot be entirely ruled out that the 39 kDa α,–like protein band contains a very minute signal from GAPDH, it may be unlikely that this is the case (see Section III.2.4).
Endogenous $[^{32}P]ADP$-ribosylation with Protease Inhibitors and Immunolabeling with GAPDH

**Figure 15**: Endogenous $[^{32}P]ADP$-ribosylation of substrate proteins in postmortem human cortex and immunolabeling with GAPDH. 

**A**: Representative autoradiogram of endogenous $[^{32}P]ADP$-ribosylation in the presence (+) and absence (-) of protease inhibitors which included: leupeptin, SBTII, pepstatin A, antipain, calpain inhibitor II (10 μg/ml each), aprotinin, calpain inhibitor I (20 μg/ml each) and PMSF (25 μg/ml). Each lane contains 250 μg protein. 

**B**: Representative Western blot demonstrating immunolabeling of 5 ng GAPDH with anti-GAPDH and unlabeled ADP-ribosylated substrate products in human temporal cortex with anti-GAPDH + RM/1 (lanes 1,2), and RM/1 (lane 3).
Figure 16: Autoradiograms illustrating one-dimensional peptide maps of the major \[^{32}\text{P}\]\text{ADP-ribosylated} products in postmortem human brain. All samples were processed simultaneously with mass markers included on each gel for alignment. Samples were \[^{32}\text{P}\]\text{ADP-ribosylated} as described in Materials and Methods in the presence and absence of CTX. Gel bands corresponding to the 45 kDa CTX-catalyzed \[^{32}\text{P}\]\text{ADP-ribosylated} proteins (A), and the 39 kDa- (B) and the 45 kDa- (C) endogenous \[^{32}\text{P}\]\text{ADP-ribosylated} proteins were excised and subjected to proteolytic digestion using varying concentrations of \textit{Staphylococcus aureus} V8 protease (0-0.25 \mu g). The resulting gels were subsequently dried and autoradiographed at -70\degree C overnight. Arrows indicate the undigested (> 47.5 kDa) and major fragments observed at approximately 32.5, 25, 16.5, 6.5 and less than 6.5 kDa.
III.2.4 One Dimensional Peptide Mapping

To further characterize the 39 kDa α, like [32P]ADP-ribosylated protein, the [32P]ADP-ribosylated products detected in postmortem temporal cortex were subjected to one-dimensional peptide mapping. Preliminary experiments carried out on unlabeled ADP-ribosylated α, proteins revealed that following 30 min digestion, a maximal array of peptide fragments could be generated ranging from 47.5 to 6.5 kDa. Comparisons of peptide maps (autoradiograms were overlaid with the aid of molecular mass markers) using varying concentrations (0.01-0.25μg) of Staphylococcus aureus V-8 protease, which cleaves peptide bonds at the COOH-terminal of either aspartic or glutamic acid residues (Drapeau et al., 1972), generated a number of similar peptide fragments for both the 45 kDa α, s and 39 kDa α, like endogenous [32P]ADP-ribosylated proteins along with the CTX-catalyzed [32P]ADP-ribosylated α, s (Figure 16, compare lanes A, B & C). Major fragments were observed at approximately 32.5, 25, 16.5 and 6.5 kDa. All three [32P]ADP-ribosylated products generated the same fragments < 6.5 kDa. Analysis of the α, amino acid sequence predicts major fragments that would be generated by multiple site cleavage. However, direct comparisons of these estimated fragment masses to those generated by V8 proteolytic digestion was not possible since molecular mass estimations on 15% polyacrylamide gels cannot be accurately estimated.

III.3 Endogenous and CTX-catalyzed [32P]ADP-ribosylation in Human Platelets and Transformed B-lymphoblasts

For comparison purposes, endogenous and CTX-catalyzed [32P]ADP-ribosylation in both platelets and Epstein-Barr virus transformed B-lymphoblasts from human subjects were
Endogenous and CTX-catalyzed $^{32}$P-ADP-ribosylation of $\alpha_i$ isoforms in Postmortem Human Temporal Cortex, Platelets and Transformed B-lymphoblasts from Human Subjects

Figure 17: Representative autoradiogram of endogenous and CTX-catalyzed $^{32}$P-ADP-ribosylation of $\alpha_i$ isoforms in postmortem human temporal cortex (TC), platelets (P) and transformed B-lymphoblasts (BL) from human subjects. Samples $\alpha_i$ isoforms were ADP-ribosylated with $^{32}$P-NAD, resolved by SDS-PAGE, gels dried and autoradiographed overnight at -70°C as described in Methods (Sections II.4.1 and II.4.2). Assay buffers contained 10 mM DTT except where indicated in which case 25 mM DTT was used.
also examined to assess whether $\alpha_s$ is a substrate for ADP-ribosyltransferase in these tissues as well (Figure 17). It has been previously shown by immunolabeling assays that $\alpha_{s-L}$ is more abundant than $\alpha_{s-L}$ in both tissues (Stein et al., 1996, Emamghoreishi et al., 1998). In B-lymphoblasts, endogenous $^{32}$PADP-ribosylation (under the 10 mM DTT assay conditions) could only be detected for $\alpha_{s-L}$ and not for $\alpha_{s-S}$ (Figure 17, BL), while no signal was evident for either isoform under the 25 mM DTT assay conditions. In contrast, CTX-catalyzed $^{32}$PADP-ribosylation (under 10 mM DTT assay conditions only) was evident only for $\alpha_{s-S}$ in this tissue. Endogenous $^{32}$PADP-ribosylation of $\alpha_{s-L}$ was also more pronounced than $\alpha_{s-S}$ in human platelets under both 10 and 25 mM DTT assay conditions (Figure 17, P), whereas both isoforms underwent similar degrees of CTX-catalyzed $^{32}$PADP-ribosylation (10 mM DTT only). There was no endogenously $^{32}$PADP-ribosylated 39 kDa protein in either platelets or B-lymphoblasts under either 10 or 25 mM DTT assay conditions. Different mobility patterns were observed among the various tissues for both CTX-catalyzed $^{32}$PADP-riboslated $\alpha$, isoforms (Figure 17, TC, BL, and P), which may reflect cell-specific differences in brain and peripheral cells.

III.4 Assessment of Endogenous, and CTX-catalyzed $^{32}$PADP-ribosylation, and Immunolabeling in Postmortem Human Brain

III.4.1 Subject Characteristics

Demographic characteristics of the subjects from whom postmortem brain was obtained are shown in Table 4. Briefly, there were no significant differences ($P > 0.1$) in age (56 ± 8 vs. 60 ± 7 yrs) and time of death from autopsy (21 ± 3 vs. 16 ± 2 hr) between subjects diagnosed with BD compared with control subjects with no history of psychiatric
<table>
<thead>
<tr>
<th>Subject PMI</th>
<th>Age/Sex</th>
<th>Psychotropic Drugs</th>
<th>Cause of death</th>
<th>Temporal Cortex lithium (mM)</th>
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</thead>
<tbody>
<tr>
<td>BD&lt;sup&gt;a&lt;/sup&gt; 12</td>
<td>40/M</td>
<td>Lithium, haloperidol</td>
<td>Hepatic failure</td>
<td>0.079</td>
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<tr>
<td>C 14</td>
<td>48/M</td>
<td>None</td>
<td>Carcinoma</td>
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</tr>
<tr>
<td>BD 17</td>
<td>70/F</td>
<td>Lithium, benztpine, perphenazine</td>
<td>Carcinoma with erosion into the aorta</td>
<td>0.066</td>
</tr>
<tr>
<td>C 18</td>
<td>66/F</td>
<td>None</td>
<td>Myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>BD&lt;sup&gt;b&lt;/sup&gt; 17.4</td>
<td>80/M</td>
<td>Thioridazone</td>
<td>Pneumonia</td>
<td>0.129</td>
</tr>
<tr>
<td>C 17.5</td>
<td>92/F</td>
<td>None</td>
<td>Rectal carcinoma</td>
<td></td>
</tr>
<tr>
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<td>Haloperidol, lorazepam, maprotiline</td>
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<td>0.083</td>
</tr>
<tr>
<td>C</td>
<td>74/F</td>
<td>?</td>
<td>Myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>71/M</td>
<td>Lithium</td>
<td>Pneumonia</td>
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<tr>
<td>C</td>
<td>10</td>
<td>71/M</td>
<td>Pneumonia</td>
<td></td>
</tr>
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<td>BD</td>
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<td>Lithium, amitriptyline, codeine, chlordiazepoxide</td>
<td>Suicide, overdose of lithium, codeine</td>
<td>0.127</td>
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<tr>
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<td>Myocardial infarction</td>
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<tr>
<td>BD</td>
<td>27/F</td>
<td>Doxepin, amitriptyline</td>
<td>Suicide, overdose of doxepin, amitriptyline</td>
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<tr>
<td>C</td>
<td>30/M</td>
<td>?</td>
<td>Ruptured inferior vena cava</td>
<td></td>
</tr>
<tr>
<td>BD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42/F</td>
<td>Lithium, prochlorperazine, isocarboxazid, alprazolam</td>
<td>Suicide, hanging</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>58/F</td>
<td>Rectal carcinoma</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>94/F</td>
<td>?</td>
<td>Cerebrovascular accident</td>
<td>0.080</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>86/M</td>
<td>Arrhythmia</td>
<td></td>
</tr>
</tbody>
</table>

BD: bipolar disorder subject  
C: control subject  
PMI: postmortem delay interval, ND: not detected  
?: unknown  
<sup>a</sup>coexistent alcohol abuse  
<sup>b</sup>differential diagnosis of schizoaffective disorder  
<sup>c</sup>bipolar type II  

(Adapted from Rahman et al., 1997)
or neurological disorder. Five of the nine BD subjects had documented history of lithium use within 6 months prior to death while the remaining four did not, although previous lithium therapy could not be ruled out based on available medical records. Lithium levels measured in BD temporal cortex varied from 0.066 to 0.396 mM. Three of the patients with BD died by suicide (two by drug overdoses, one by hanging). Only one of the patients under 45 years of age at the time of death died from causes other than suicide. Furthermore, none of the control subjects died of suicide, neurological reasons, nor did any have a history of psychiatric disorder. The temporal, occipital and cerebellar (control region) cortices were used to assess the degree of both endogenous and CTX-catalyzed $^{32}$PADP-ribosylation of $\alpha_1$ isoforms.

III.4.2 Assessment of Endogenous and CTX-catalyzed $^{32}$P ADP-ribosylation, and Immunolabeling of $\alpha_{\text{L}}$ and $\alpha_{\text{S}}$ in Postmortem Human Brain under 10 mM DTT Assay Conditions

As previously reported (Andreopoulos et al., 1999, see also section III.1.1), DTT was shown to variably affect the radiolabeling of the endogenous $^{32}$PADP-ribosylated $\alpha_1$ isoforms. Increases in DTT concentration from 1 to 25 mM resulted in a linear increase in signal intensity for the 39 kDa product (Figure 9A). Maximum $^{32}$P radiolabeling of $\alpha_{\text{L}}$ and $\alpha_{\text{S}}$ substrates was seen at 10 mM DTT and 1 mM DTT, respectively (Figure 9A). As stated previously, for comparison purposes endogenous $^{32}$P ADP-ribosylation was performed under two separate assay conditions, employing either 10 or 25 mM DTT. As DTT did not have any differential effect on the degree of CTX-catalyzed $^{32}$P ADP-ribosylation for each
Representative Autoradiograms and Western Blots of Endogenous, and CTX-catalyzed $^{32}$P ADP-ribosylation, and Immunoreactivity of α, Isoforms in Temporal Cortex from BD and Control Subjects

Figure 18: Representative autoradiograms and Western blots showing endogenous (I) and CTX-catalyzed $^{32}$P ADP-ribosylation (II), and immunolabeling (III) of α, isoforms in postmortem temporal cortex from bipolar disorder (BD) and control (C) subjects. Samples were processed in duplicate for $^{32}$P ADP-ribosylation and SDS-PAGE as described in Methods (Sections II.4.1 and II.4.2). Endogenous $^{32}$P ADP-ribosylation (I) was performed with 10 mM DTT (Ia) (optimal ADP-ribosylation of α,L) and 25 mM DTT (Ib) (optimal ADP-ribosylation of the 39 kDa α,L-like protein).
Representative Autoradiograms and Western Blots of Endogenous, and CTX-catalyzed [\(^{32}\)P]ADP-ribosylation, and Immunoreactivity of \(\alpha_s\) Isoforms in Occipital Cortex from BD and Control Subjects

Figure 19: Representative autoradiograms and Western blots showing endogenous (I) and CTX-catalyzed-[\(^{32}\)P]ADP-ribosylation (II), and immunolabeling (III) of \(\alpha_s\) isoforms in postmortem occipital cortex from bipolar disorder (BD) and control (C) subjects. Samples were processed in duplicate for [\(^{32}\)P]ADP-ribosylation and SDS-PAGE as described in Methods (Sections II.4.1 and II.4.2). Endogenous [\(^{32}\)P]ADP-ribosylation (I) was performed with 10 mM (Ia) (optimal ADP-ribosylation of \(\alpha_{sL}\)) and 25 mM DTT (Ib) (optimal ADP-ribosylation of the 39 kDa \(\alpha_s\)-like protein).
Representative Autoradiograms and Western Blots of Endogenous, and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, and Immunoreactivity of $\alpha_s$ Isoforms in Cerebellum from BD and Control Subjects

**Figure 20:** Representative autoradiograms and Western blots showing endogenous (I) and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation (II), and immunolabeling (III) of $\alpha_s$ isoforms in postmortem cerebellum from bipolar disorder (BD) and control (C) subjects. Samples were processed in duplicate for $[^{32}\text{P}]$ADP-ribosylation and SDS-PAGE as described in Methods (Sections II.4.1 and II.4.2). Endogenous $[^{32}\text{P}]$ADP-ribosylation (I) was performed with 10 mM (Ia) (optimal ADP-ribosylation of $\alpha_s$) and 25 mM DTT (Ib) (optimal ADP-ribosylation of the 39 kDa $\alpha_s$-like protein).
of the two $\alpha_{4}$ isoforms, a 10 mM concentration was used for subsequent assay. Figures 18, 19 and 20 show representative autoradiograms and Western blots of endogenous, and CTX-catalyzed-[³²P]ADP-ribosylation (I and II), and immunoreactivity (III) of both $\alpha_{4}$ isoforms and the 39 kDa $\alpha_{4}$-like immunoreactive protein in temporal, occipital and cerebellar cortex from BD patients and control subjects.

Figure 21 summarizes the results of endogenous and CTX-catalyzed-[³²P]ADP-ribosylation under the 10 mM DTT assay conditions, and immunolabeling of $\alpha_{4-L}$ in postmortem temporal (n=8), occipital (n=9) and cerebellar cortex (n=6) of BD, and age and postmortem delay matched controls. Two-way ANOVA did not reveal significant main effects of diagnostic group (F=0.53 df=1, 45, $P=0.47$) or brain region (F=2.19, df=2, 45, $P=0.13$) in the degree of endogenous [³²P]ADP-ribosylation of $\alpha_{4-L}$ (Figure 21A). A significant main effect of brain region was observed for the degree of CTX-catalyzed ADP-ribosylation of $\alpha_{4-L}$ which was higher in temporal and occipital cortex compared to cerebellum (F=6.31, df=2, 45, $P=0.004$). There was no significant effect of group, however (F=0.64, df=1, 45, $P=0.43$) (Figure 21B). Significant main effects of diagnostic group (F=3.40, df=1, 45, $P=0.04$) and brain region (F=35.23, df=2, 45, $P<0.001$) were also found for $\alpha_{4-L}$ immunolabeling (Figure 21C). Post hoc analysis showed significantly higher $\alpha_{4-L}$ immunoreactivity in BD temporal ($t=3.41, df=7, P=0.01$) and occipital cortex ($t=2.85, df=8, P=0.02$) compared with control subjects.

With respect to $\alpha_{4-s}$ (Figure 22A), a significant main effect of region for the degree of endogenous [³²P]ADP-ribosylation was observed, which was significantly lower in temporal and occipital cortex (F=3.56 df=2, 45, $P=0.04$) compared to cerebellum but no
Regional Distribution of Endogenous, and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, and Immunolabeling of $\alpha_{\text{SL}}$ in Postmortem Temporal, Occipital and Cerebellar Cortices from BD and Control Subjects

Figure 21: Regional distribution of endogenous (A), and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation (B), and immunolabeling (C) of $\alpha_{\text{SL}}$ in postmortem human temporal (TC, $n=8$), and occipital (OC, $n=9$) cortex and cerebellum (CB, $n=6$) in BD patients compared with controls. Endogenous, and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, and immunolabeling of $\alpha_{\text{SL}}$ were performed as described in Methods (Section II). Levels of radio- and immunolabeled $\alpha_{\text{SL}}$ were quantified as described in Methods (Section II). Data represent means ± SD. $^\dagger P=0.004$ compared with the cerebellum. $^* P < 0.05$ compared with controls.
Regional Distribution of Endogenous, and CTX-catalyzed-[\textsuperscript{32}P]ADP-ribosylation, and Immunolabeling of \(\alpha_{\text{sS}}\) in Postmortem Temporal, Occipital and Cerebellar Cortices from BD and Control Subjects

**Figure 22:** Regional distribution of endogenous (A), and CTX-catalyzed-[\textsuperscript{32}P]ADP-ribosylation (B), and immunolabeling (C) of \(\alpha_{\text{sS}}\) in postmortem human temporal (TC, \(n=8\)), and occipital (OC, \(n=9\)) cortex and cerebellum (CB, \(n=6\)) in BD patients compared with controls. Endogenous and CTX-catalyzed-[\textsuperscript{32}P]ADP-ribosylation, and immunolabeling of \(\alpha_{\text{sS}}\) were performed as described in Methods (Section II). Levels of radio- and immunolabeled \(\alpha_{\text{sS}}\) were quantified as described in Methods (Section II). Data represent means \(\pm\) SD. \(\dagger P=0.04\) compared with the cerebellum. \(* P=0.053\) compared with controls.
difference between diagnostic groups (\(F=0.00, \text{df}=1, 45, P=0.99\)) existed. However, test of main effects revealed a trend towards reduction (\(F=3.5, \text{df}=1, 45, P=0.07\)) between diagnostic groups in CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylation of \(\alpha_{+5}\). Post hoc comparisons of group means showed that this change occurred in BD temporal cortex (\(\text{ROD}=0.48 \pm 0.05\) vs. \(0.61 \pm 0.06; t=2.32, \text{df}=7, P=0.053\)) compared with control subjects (Figure 22B). Interestingly, there were no significant effects of diagnostic group (\(F=0.12, \text{df}=1, 45, P=0.73\)) or brain region (\(F=1.29, \text{df}=2, 45, P=0.29\)) on \(\alpha_{+5}\) immunolabeling (Figure 22C).

### III.4.3 Assessment of Endogenous \(^{32}\text{P}\)ADP-ribosylation and Immunolabeling of the 39 kDa \(\alpha_{-}\)-like Protein

Figure 23A show the endogenous \(^{32}\text{P}\)ADP-ribosylation of the 39 kDa \(\alpha_{-}\)-like protein in assay buffer containing 10 mM DTT. ANOVA revealed significantly lower endogenous \(^{32}\text{P}\)ADP-ribosylated 39 kDa \(\alpha_{-}\)-like protein in temporal and occipital cortex (\(F=15.40, \text{df}=2, 45, P < 0.01\)) compared to cerebellum along with a trend toward a difference between diagnostic groups (\(F=3.19, \text{df}=1, 45, P=0.08\)) (Figure 23A). When assays were performed with 25 mM DTT (Figure 23B), there were significant effects of diagnosis (\(F=4.25, \text{df}=1, 45, P=0.04\)) and brain region (\(F=6.02, \text{df}=2, 45, P=0.005\)) on the degree of endogenous \(^{32}\text{P}\)ADP-ribosylation of the 39 kDa \(\alpha_{-}\)-like protein. Post hoc analysis revealed that endogenous \(^{32}\text{P}\)ADP-ribosylation of the 39 kDa \(\alpha_{-}\)-like protein was significantly lower (-30%) in BD temporal cortex compared with controls (\(\text{ROD}=0.62 \pm 0.07\) vs. \(0.91 \pm 0.09; t=4.27, \text{df}=7, P=0.004\)) but only showed a nonsignificant decrement in occipital (\(P=0.37\)) and cerebellar (\(P=0.77\)) cortex (Figure 23C). Endogenously \(^{32}\text{P}\)ADP-ribosylated \(\alpha_{-L}\) and \(\alpha_{+5}\)
Regional Distribution of Endogenous $^{32}$PADP-ribosylation and Immunolabeling of the 39 kDa $\alpha_2$-like Protein in Postmortem Temporal, Occipital and Cerebellar Cortices from BD and Control Subjects

**Figure 23:** Regional distribution of endogenous $^{32}$PADP-ribosylation (A, B) and immunolabeling (C) of the 39 kDa $\alpha_2$-like protein in postmortem human temporal (TC, n=8), and occipital (OC, n=9) cortex and cerebellum (CB, n=6) in BD patients compared with controls. Endogenous $^{32}$PADP-ribosylation was performed under 10 mM (A) or 25 mM DTT (B) assay conditions as described in Methods (Section II). Levels of the radio- and immuno-labeled 39 kDa $\alpha_2$-like protein were quantified as described in Methods (Section II). Data represent means ± SD. $^{\dagger}P<0.01$ compared with the cerebellum.

$^*P=0.004$ compared with controls.
were only weakly detectable at the protein concentrations (250 µg) used in these experiments and were not quantified. There was no difference in the immunolabeling of the 39 kDa α-like protein across these brain regions or between diagnostic groups (Figure 23C).

III.5 Relationship Between Endogenous $^{[32P]}$ADP-ribosylated $\alpha_{\gamma-L}$ and Immunolabeling of $\alpha_{\gamma-L}$ in BD Temporal cortex

Based on the observation of possible reductions in CTX-catalyzed $^{[32P]}$ADP-ribosylation of $\alpha_{\gamma}$ in BD temporal cortex, it was hypothesized that the higher $\alpha_{\gamma}$ levels may have offset the detection of reductions in endogenous $^{[32P]}$ADP-ribosylation, if they were relatively small. Therefore, the question of whether BD temporal cortical $\alpha_{\gamma-L}$ immunoreactivities correlated with the degree of its endogenous $^{[32P]}$ADP-ribosylation was assessed. Interestingly, a significant negative correlation ($r=0.83, n=8, P=0.012$) (Figure 24) was observed between endogenous $^{[32P]}$ADP-ribosylated $\alpha_{\gamma-L}$ and its immunolabeling in BD temporal cortex.

In contrast, no significant correlations were found between $\alpha_{\gamma-S}$ immunoreactivity and endogenous $^{[32P]}$ADP-ribosylation of $\alpha_{\gamma-S}$. Moreover, no other correlations were observed between either CTX-catalyzed $^{[32P]}$ADP-ribosylation of $\alpha_{\gamma-L}$ or $\alpha_{\gamma-S}$, and immunolabeling of the respective isoforms. Similarly, no correlations were observed in the occipital cortex between the immunoreactive levels of $\alpha_{\gamma-L}$, and the endogenous- or CTX-catalyzed $^{[32P]}$ADP-ribosylation of $\alpha_{\gamma-L}$. 
Relationship Between Endogenous $[^{32}\text{P}]$ADP-ribosylated $\alpha_{\text{L}}$ and Immunolabeling of $\alpha_{\text{L}}$ in BD Temporal Cortex

![Table and graph]

**Figure 24:** Correlation between endogenous $[^{32}\text{P}]$ADP-ribosylated $\alpha_{\text{L}}$ and immunolabeled $\alpha_{\text{L}}$. The linear regression line is shown, along with the 95% confidence intervals ($r = 0.83$, $n = 8$, *$P = 0.012$).
III.6 Relationship Between Lithium Concentration and $^{32}$P ADP-ribosylation of $\alpha_\gamma$ isoforms in BD Temporal Cortex

Since lithium is known to modify endogenous ADP-ribosylation (Nestler et al., 1995, Young and Woods, 1996), the relationship between lithium levels and changes in ADP-ribosylation of $\alpha_{+L}$, $\alpha_{+S}$ and the 39 kDa $\alpha_\gamma$-like protein in BD temporal cortex was determined. No significant correlations were found between either endogenous and CTX-catalyzed-$^{32}$P ADP-ribosylation of $\alpha_{+L}$, or $\alpha_{+S}$, and lithium levels (Figure 25, r<0.66, P>0.1 in all cases). However, there was a significant correlation between the endogenous $^{32}$P ADP-ribosylated 39 kDa $\alpha_\gamma$-like protein (under 25 mM assay conditions, percent change from controls) and lithium concentrations in BD temporal cortex (r=0.95, n=8, P=0.0002)(Figure 26).

III.7 Relationship Between Endogenous, CTX-catalyzed $^{32}$P ADP-ribosylation, and Immunolabeling of $\alpha_\gamma$ subtypes and Age and Postmortem Delay Intervals (PMI)

Figures 27 and 28 show scatter plot diagrams of the effects of age on endogenous and CTX-catalyzed $^{32}$P ADP-ribosylation, and immunolabeling of $\alpha_{+L}$, $\alpha_{+S}$ and the 39 kDa $\alpha_\gamma$-like protein. Similarly, Figures 29 and 30 show scatter plot diagrams of the effects of PMI on the above variables. All measures were stable with respect to age (Figs. 27, 28, r < 0.64, P > 0.16 in all cases) and postmortem delay intervals (Figure 29,30, r < 0.70, P > 0.1 in all cases) of the tissue samples used in this study (Table 5).
Relationship Between Lithium Concentration, and Endogenous and CTX-catalyzed $^{[32P]}$ADP-ribosylation of $\alpha_{\pm L}$ and $\alpha_{\pm S}$ in BD Temporal Cortex

**Figure 25:** Relationship between endogenous and CTX-catalyzed $^{[32P]}$ADP-ribosylated $\alpha_{\pm L}$, $\alpha_{\pm S}$ and the 39 kDa $\alpha_{\pm}$-like protein and lithium concentration in BD temporal cortex. The linear regression lines are shown.
Relationship Between the Endogenous \[^{32}P\]ADP-ribosylated 39 kDa \(\alpha_5\)-like Protein and Lithium Concentration in BD Temporal Cortex

**Figure 26**: Correlation between the endogenous \[^{32}P\]ADP-ribosylated 39 kDa \(\alpha_5\)-like protein and lithium concentration. The linear regression line is shown, along with the 95% confidence intervals \((r = 0.95, n = 8, *P = 0.0002)\). The correlation is still present after omission of the data point from a subject with high lithium level (see inset).
Effects of Age on Endogenous and CTX-catalyzed $^{32}$PADP-ribosylation and Immunolabeling of $\alpha_{+L}$, $\alpha_{+S}$ and the 39 kDa $\alpha_{+}$-like Protein

Figure 27: Relationship between endogenous $^{32}$PADP-ribosylation and CTX-catalyzed $^{32}$PADP-ribosylation, and immunolabeling of $\alpha_{+}$ subtypes, and age was assessed in temporal (TC) and occipital (OC) of BD subjects. No correlations were observed for patients or controls with respect to age (see Table 5).
Effects of Age on Endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation and Immunolabeling of $\alpha_{\text{L}}$, $\alpha_{\text{S}}$, and the 39 kDa $\alpha_{\text{L}}$-like Protein

Figure 28: Relationship between endogenous $[^{32}\text{P}]$ADP-ribosylation and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, and immunolabeling of $\alpha_{\text{L}}$ subtypes, and age was assessed in cerebellum of BD subjects. No correlations were observed for patients or controls with respect to age (see Table 5).
Effects of Postmortem Delay on Endogenous and CTX-catalyzed $[^{32}P]$ADP-ribosylation and Immunolabeling of $\alpha_{\varepsilon-L}$, $\alpha_{\varepsilon-S}$ and the 39 kDa $\alpha_{\varepsilon}$-like Protein

![Graphs showing the relationship between endogenous $[^{32}P]$ADP-ribosylation and CTX-catalyzed $[^{32}P]$ADP-ribosylation, and immunolabeling of $\alpha_{\varepsilon}$ subtypes, and postmortem delay interval (PMI) in temporal (TC) and occipital (OC) of BD subjects. No correlations were observed for patients or controls with respect to PMI (see Table 5).]

Figure 29: Relationship between endogenous $[^{32}P]$ADP-ribosylation and CTX-catalyzed $[^{32}P]$ADP-ribosylation, and immunolabeling of $\alpha_{\varepsilon}$ subtypes, and postmortem delay interval (PMI) was assessed in temporal (TC) and occipital (OC) of BD subjects. No correlations were observed for patients or controls with respect to PMI (see Table 5).
Effects of Postmortem Delay on Endogenous and CTX-catalyzed $^{32}$PADP-ribosylation and Immunolabeling of $\alpha_{\pm L}$, $\alpha_{\pm S}$ and the 39 kDa $\alpha_\tau$-like Protein

**CB**

![Figure 30: Relationship between endogenous $[^{32}\text{P}]$ADP-ribosylation and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, and immunolabeling of $\alpha_\tau$ subtypes, and postmortem delay interval (PMI) was assessed in cerebellum of BD subjects. No correlations were observed for patients or controls with respect to PMI (see Table 5).]
Table 5

Relationships Between Endogenous [\(^{23}\text{P}\)ADP-ribosylation and CTX-catalyzed [\(^{23}\text{P}\)ADP-ribosylation, and Immunolabeling of \(\alpha_x\) Subtypes, and Age and PMI in Temporal (TC), Occipital (OC) and Cerebellum (CB) of BD Subjects

1) AGE

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>OC</th>
<th>CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>(\alpha_{+L})</td>
<td>r=0.46, (P=0.2)</td>
<td>r=0.35, (P=0.4)</td>
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<td>[(^{23}\text{P})ADP-ribosylation</td>
<td>(\alpha_{+S})</td>
<td>r=0.59, (P=0.1)</td>
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<td>39 kDa</td>
<td>(\alpha_{-}\text{like})</td>
<td>r=0.52, (P=0.2)</td>
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<tr>
<td>CTX-catalyzed</td>
<td>(\alpha_{+L})</td>
<td>r=0.51, (P=0.2)</td>
<td>r=0.35, (P=0.4)</td>
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<td>[(^{23}\text{P})ADP-ribosylation</td>
<td>(\alpha_{+S})</td>
<td>r=0.25, (P=0.5)</td>
<td>r=0.24, (P=0.5)</td>
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<tr>
<td>Immunolabeling</td>
<td>(\alpha_{+L})</td>
<td>r=0.32, (P=0.4)</td>
<td>r=0.29, (P=0.4)</td>
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</table>

2) PMI

<table>
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<th>OC</th>
<th>CB</th>
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</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>(\alpha_{+L})</td>
<td>r=0.20, (P=0.6)</td>
<td>r=0.41, (P=0.3)</td>
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<tr>
<td>[(^{23}\text{P})ADP-ribosylation</td>
<td>(\alpha_{+S})</td>
<td>r=0.15, (P=0.7)</td>
<td>r=0.37, (P=0.3)</td>
</tr>
<tr>
<td>39 kDa</td>
<td>(\alpha_{-}\text{like})</td>
<td>r=0.22, (P=0.6)</td>
<td>r=0.03, (P=0.8)</td>
</tr>
<tr>
<td>CTX-catalyzed</td>
<td>(\alpha_{+L})</td>
<td>r=0.18, (P=0.6)</td>
<td>r=0.08, (P=0.8)</td>
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<td>[(^{23}\text{P})ADP-ribosylation</td>
<td>(\alpha_{+S})</td>
<td>r=0.48, (P=0.2)</td>
<td>r=0.62, (P=0.1)</td>
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<tr>
<td>Immunolabeling</td>
<td>(\alpha_{+L})</td>
<td>r=0.21, (P=0.6)</td>
<td>r=0.16, (P=0.7)</td>
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<tr>
<td></td>
<td>(\alpha_{+S})</td>
<td>r=0.44, (P=0.3)</td>
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<tr>
<td></td>
<td>39 kDa</td>
<td>r=0.52, (P=0.9)</td>
<td>r=0.43, (P=0.2)</td>
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</tbody>
</table>
III.8 Endogenous [$^{32}$P]ADP-ribosylation of Myelin Basic Protein (MBP)

The findings of a significant negative correlation between endogenous [$^{32}$P]ADP-ribosylation of $\alpha_{s-L}$ and its immunoreactive levels, and a trend towards a reduction in CTX-catalyzed [$^{32}$P]ADP-ribosylation of $\alpha_{s-S}$ suggested possible alterations in ADP-ribosyltransferases and/or cofactors regulating their activity in BD temporal cortex. To determine whether changes in [$^{32}$P]ADP-ribosylation in BD temporal cortex were specific to $\alpha_{s-L}$ and $\alpha_{s-S}$ or more generalized affecting other substrate proteins, the endogenous [$^{32}$P]ADP-ribosylation of MBP (a 21 kDa protein found in white matter [Boulis et al., 1995] that is known to undergo endogenous ADP-ribosylation), was determined in BD and control temporal cortex. If the changes in [$^{32}$P]ADP-ribosylation were specific to $\alpha_s$ (i.e. due to a disturbance(s) related to availability or structural change of $\alpha_s$, rendering it inaccessible to the addition of ADP-ribose), no differences in [$^{32}$P]ADP-ribosylated MBP would be expected in BD patients compared with controls. If differences in the degree of MBP [$^{32}$P]ADP-ribosylation in BD brain compared with controls were observed, it would likely reflect possible abnormalities associated with ADP-ribosyltransferases and/or cofactors. Therefore, exogenous MBP was added to postmortem temporal cortical homogenates and the samples subjected to ADP-ribosylation assay in the presence of 10 mM DTT. For quantitative purposes, assay conditions were also established within a linear range of protein concentrations for endogenous [$^{32}$P]ADP-ribosylation of MBP (Figure 31).

As shown in Figure 32, no significant differences were observed in endogenous [$^{32}$P]ADP-ribosylation of MBP between the two groups ($t=0.35$, df=14, $P=0.73$). Table 6 shows the data for endogenous ADP-ribosylation of $\alpha_{s-L}$, $\alpha_{s-S}$ and the 39 kDa $\alpha_s$-like protein.
in BD temporal cortex compared with controls assayed in the presence of MBP. ANOVA revealed no significant effects of diagnostic group for either $\alpha_{+L} \ (F=2.34, \ df=1, 15, \ P=0.15)$, $\alpha_{+S} \ (F=1.44, \ df=1, 15, \ P=0.25)$ and the 39 kDa $\alpha_{+}$-like protein $\ (F=0.00, \ df=1, 15, \ P=0.99)$, confirming the results obtained in the absence of MBP (see Sections III.4.2 and III.4.3).
Linear Range of Band Intensity Versus Protein Concentration for Endogenous
$[^{32}\text{P}]$ADP-ribosylation of Exogenous MBP Assayed in Postmortem Temporal Cortex Homogenates

**Figure 31:** Representative autoradiogram of a linear range of band intensity versus protein concentration (100-400 µg) for endogenous $[^{32}\text{P}]$ADP-ribosylated MBP. Samples were ADP-ribosylated in the presence of $[^{32}\text{P}]$NAD as described in Methods (Section II.4.3), electrophoresed on 12.5% polyacrylamide gels and autoradiographed overnight at -70°C.
Endogenous $[^{32}P]ADP$-ribosylation of MBP in Postmortem Temporal Cortex from BD and Control Subjects

Figure 32: Endogenous $[^{32}P]ADP$-ribosylation of MBP in postmortem human temporal cortex in BD patients (BD) compared with controls (C). Endogenous $[^{32}P]ADP$-ribosylation of MBP was performed as described in Methods (Section II.4.3). Levels of radiolabeled MBP were quantified as described in Methods (Section II.8). Data represent means ± SD for n=8 assayed in duplicate.
Table 6
Endogenous $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{v-L}$, $\alpha_{v-S}$ and the 39 kDa $\alpha_v$-like Protein in Postmortem Temporal Cortex From BD and Control Subjects Assayed With Exogenous Myelin Basic Protein (MBP)

<table>
<thead>
<tr>
<th>Substrate Protein</th>
<th>Subject</th>
<th>Endogenous $[^{32}\text{P}]$ADP-ribosylation (ROD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{v-L}$</td>
<td>C</td>
<td>0.58 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>BD</td>
<td>0.80 ± 0.36</td>
</tr>
<tr>
<td>$\alpha_{v-S}$</td>
<td>C</td>
<td>0.59 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>BD</td>
<td>0.77 ± 0.36</td>
</tr>
<tr>
<td>39 kDa</td>
<td>C</td>
<td>0.61 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>BD</td>
<td>0.61 ± 0.29</td>
</tr>
</tbody>
</table>

C: control. BD: bipolar disorder. Data are expressed as the mean ± SD for 8 subjects.
CHAPTER IV
DISCUSSION
The principle objectives of this thesis were first, to characterize the mono-ADP-ribosylated (both endogenous and CTX-catalyzed) products of α₁ isoforms in postmortem human brain and secondly, to determine whether in BD there were reductions in endogenous \[^{32}\text{P}]\text{ADP-ribosylation of } \alpha_1 \text{ in those brain regions demonstrating elevated } \alpha_1 \text{ immunolabeling, compared with age and postmortem-delayed matched controls. The third and final objective was to establish whether any observed differences in ADP-ribosylation could be ascribed specifically to reduced mono-ADP-ribosyltransferase activity or to other factors, such as altered cellular disposition or heterotrimeric state of } \alpha_1. \text{ For organizational purposes, the observations from each series of experiments are discussed separately along with the implications of the respective findings.}

IV. 1 Characterization of \[^{32}\text{P}]\text{ADP-ribosylated } \alpha_1 \text{ Isoforms in Postmortem Human Brain}

As discussed earlier, posttranslational modification of \( \alpha_1 \) by ADP-ribosylation occurs ubiquitously, being demonstrated in all organs/tissues of mammalian species studied to date. While it was expected that this would also be the case for human brain, there have been no previous direct demonstrations to support that this is so. In the characterization studies performed in this work, two major bands of 52 and 45 kDa, respectively, were identified in postmortem temporal cortex that underwent CTX-catalyzed \[^{32}\text{P}]\text{ADP-ribosylation. In comparison, three endogenous } \[^{32}\text{P}]\text{ADP-ribosylated polypeptides with masses of 48, 45 and 39 kDa, respectively, were identified. Immunoprecipitation of samples with antisera RM/1 specific to } \alpha_1 \text{ isoforms immediately following } \[^{32}\text{P}]\text{ADP-ribosylation revealed that}
immunoprecipitated α, isoforms comigrated with the tentatively identified major [32P]ADP-ribosylated products. Furthermore, overlay comparisons between Western blots and autoradiograms of the [32P]ADP-ribosylated proteins confirmed that these radiolabeled bands corresponded to those immunolabeled with RM/1, supporting the identity of the 52 kDa CTX-catalyzed and 48 kDa endogenous [32P]ADP-ribosylated bands as αs,t, and the 45 kDa endogenous and CTX-catalyzed [32P]ADP-ribosylated proteins as αs,s. The possibility that the 45 kDa endogenous [32P]ADP-ribosylated product was heterogenous and was also comprised of αolf could not be completely ruled out, however. Unfortunately, the signal generated by immunoprecipitating αolf with anti-αolf (K-19) was similar to preimmune serum. This may have been due to the inability of K-19 to immunoprecipitate αolf as efficiently compared to RM/1. Furthermore, since immunoprecipitation with anti-α, (AS/7) or anti-α, (GC/2) did not generate any signals different from that obtained with preimmune serum, the results obtained with these antibodies also did not support the identity of these bands as other potential G-protein α-subunits with similar molecular masses (39-41 kDa).

As shown in Figure 14, there are clear differences in the molecular mass of the endogenous [32P]ADP-ribosylated αs,t and αs,t, that were [32P]ADP-ribosylated by CTX (i.e. 52 kDa vs. 48 kDa). The discrepancy in migratory patterns between CTX-catalyzed and endogenous [32P]ADP-ribosylated products under similar electrophoretic conditions has been demonstrated in a variety of tissues (Williams et al., 1992, Elwardy-Mereza et al., 1994), and may be attributable to the number and specific amino acids (i.e. arginine, cysteine) that undergo endogenous as opposed to CTX-catalyzed ADP-ribosylation. While CTX is known to ADP-ribosylate Arg201 in αs,t and Arg187 in αs,s, it is not clear which and how many amino
acids are endogenously ADP-ribosylated in the α, subunits in human brain. Since ADP-ribosylation alters the charge of an amino acid, multiple sites of ADP-ribosylation could potentially affect SDS-binding, denaturation and hence, migratory patterns on polyacrylamide gels.

A novel substrate protein was also identified, which was immunoprecipitated with RM/1 and migrated as a 39 kDa α,−like protein as revealed by overlay comparisons between Western blots and autoradiograms of the endogenous [32P]ADP-ribosylated proteins. This 39 kDa α,−like [32P]ADP-ribosylated protein was not a degradative product of α, derived from the processing of the samples since inclusion of protease inhibitors in the homogenization and assay of samples did not reduce its abundance after [32P]ADP-ribosylation. One possibility that merited consideration was that the signal detected at 39 kDa represented [32P]ADP-ribosylated GAPDH, an endogenously ADP-ribosylated protein with similar molecular mass (Suzuki et al., 1997). However, immunolabeling with anti-GAPDH in postmortem human temporal cortex revealed a single major band migrating faster (≈36 kDa) than the 39 kDa α,−like protein found here, excluding the possibility that this product represented GAPDH.

To further characterize the 39 kDa α,−like protein, the [32P]ADP-ribosylated products were subjected to protease degradation. Proteolytic digestion of closely related proteins generates similar peptide fragments, while slight differences in polypeptide sequence result in different patterns of degradation (Cleveland et al., 1977). Preliminary experiments with non-radioactive excised ADP-ribosylated protein bands revealed that 30 min of V8 protease digestion provided optimal generation of multiple fragments. Comparisons of one-
dimensional peptide maps obtained with the 39 kDa $\alpha_\gamma$-like $^{32}$P-ADP-ribosylated protein to those from the 45 kDa endogenous and CTX-catalyzed $^{32}$P-ADP-ribosylated products suggest that the 39 kDa protein is likely to be a subtype of $\alpha_\gamma$, as revealed by the similar fragments generated at approximately 32.5, 25, 16.5 and 6.5 kDa, along with smaller fragments < 6.5 kDa in these proteins.

The possibility that more than two $\alpha_\gamma$ subtypes are expressed in cerebral cortex concurs with the findings of several earlier studies that have identified multiple immunolabeled and $^{32}$P-ADP-ribosylated $\alpha_\gamma$ isoforms (usually three). In rat myometrium, three bands were immunodetected with RM/I of apparent molecular masses of 47, 44, and 42 kDa (Elwardy-Merezak et al., 1994). Similarly, ovarian follicular membranes express immunolabeled and CTX-catalyzed $^{32}$P-ADP-ribosylated $\alpha_\gamma$-like proteins which migrated at 50, 48 and 45 kDa (Rajagopalan-Gupta et al., 1997). In each case, a third faster migrating $\alpha_\gamma$ immunoreactive species was evident. The basis for the different molecular size estimates between these studies may be due either to tissue specific differences and/or methodological differences in SDS-PAGE conditions used by the two groups. Collectively, however, these observations suggest the existence of a third distinct faster migrating $\alpha_\gamma$ isoform which in this work accounts for the 39 kDa $\alpha_\gamma$-like $^{32}$P-ADP-ribosylated protein.

One particularly interesting possibility is that the 39 kDa $^{32}$P-ADP-ribosylated $\alpha_\gamma$-like protein represents a N-terminal truncated form of $\alpha_\gamma$ as reported by Ishikawa et al. (1990). As described in the overview, these authors reported the expression of an $\alpha_\gamma$ mRNA transcript which encodes for a truncated form of $\alpha_\gamma$, resulting from initiation of transcription
at an alternative promoter in exon 2 of the αs gene. This protein is characterized by an amino
terminal deletion of 59 amino acids and the addition of 15 amino acids in exon 3. More
importantly, the truncated form of αs, when heterologously expressed in COS cells, did not
undergo CTX-catalyzed [32P]ADP-ribosylation. Since CTX-catalyzed ADP-ribosylation of
αs requires the G protein to be in the heterotrimeric state (Toyoshige et al., 1994), and the
N-terminal of α subunits is required to interact with βγ subunits, this amino terminal
truncated αs isoform may not serve as a substrate for CTX-catalyzed ADP-ribosylation
(Ishikawa et al., 1990). My findings that the 39 kDa [32P]ADP-ribosylated product was
evident only following endogenous, but not CTX-catalyzed [32P]ADP-ribosylation, are in
line with the latter observations.

It is not clear whether this 39 kDa novel αs isoform is functionally active. Ishikawa
et al. (1990) reported that the truncated αs isoform was still able to localize to the membrane
but could not bind GTPγS when expressed in COS cells. However, the latter authors
suggested that their inability to detect GTPγS binding was due to insufficient amount of
protein needed for the binding assay. Of note, Warner et al. (1996) clearly demonstrated that
an N-terminal modified αs could not mediate either hormone or GTP-γ-S stimulation of AC,
and was unable to undergo CTX-catalyzed [32P]ADP-ribosylation, further suggesting that the
N-terminal is essential for functional activity. Unequivocal identification that this 39 kDa
αs is an αs isoform, however, awaits the availability of antisera recognizing the N-terminal
domain of αs (within the first 50 to 60 amino acids) along with more conclusive techniques
such as microsequencing of the protein itself.
Since changes in the levels of $\alpha_s$ protein in BD have also been reported in peripheral cells (Young et al., 1994, Manji et al., 1995a, Mitchell et al., 1997, Avissar et al., 1997, Emamghoreishi et al., 1998), endogenous and CTX-catalyzed-$[^{32}\text{P}]\text{ADP-ribosylation}$ were examined in both platelets and Epstein-Barr virus transformed B-lymphoblasts for comparison, as well. Although ADP-ribosyltransferase activity has been previously reported in murine lymphoid cells including T cell lymphomas (Soman et al., 1991), this study, to my knowledge, is the first to demonstrate $[^{32}\text{P}]\text{ADP-ribosylation}$ of $\alpha_s$ in human B-lymphoblasts. The observation of a greater degree of endogenous $[^{32}\text{P}]\text{ADP-ribosylation}$ of $\alpha_{s-L}$ compared to $\alpha_{s-S}$ in both human platelets and B-lymphoblasts, despite the greater intensity of immunolabeling of $\alpha_{s-S}$ compared to $\alpha_{s-L}$, suggests that the two $\alpha_s$ isoforms display preferential labeling for this posttranslational modification in these two tissues. This is in contrast to results obtained in human brain tissue where endogenous ADP-ribosylation of $\alpha_{s-L}$ and $\alpha_{s-S}$ appear to reflect immunolabeling densities of the two subtypes. ADP-ribosylation of $\alpha_s$ on Arg$^{201}$ reduces its intrinsic GTPase activity rendering the protein constitutively active in the GTP-liganded state (Burns et al., 1982). Since constitutively active $\alpha_s$ degrades at a much faster rate than that of wild type- or heterotrimeric-$\alpha_s$ (Levis and Bourne, 1992), this raises the possibility that the turnover rate for $\alpha_{s-L}$ is faster than that of $\alpha_{s-S}$ leading to lower concentrations of $\alpha_{s-L}$ relative to $\alpha_{s-S}$ in platelets and B-lymphoblasts. However, other mechanisms may also contribute to the lower levels of $\alpha_{s-L}$ compared to $\alpha_{s-S}$, such as the differential regulation of splicing of the $\alpha_s$ gene and stability of each $\alpha_s$ mRNA. In a physiological context, such cell type specific differences in $\alpha_s$ isoform levels may
provide diversity of fine modulation of receptor-G-protein-effector responses in some peripheral cell types and in brain.

Regional differences in endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of both $\alpha_s$ isoforms in human brain is another notable finding of this study. For example, there was significantly greater CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{sL}$ in temporal and occipital cortex compared to cerebellum. It is interesting to speculate that the higher $\alpha_{sL}$ immunolabeling associated with the temporal and occipital cortices may be responsible, in part, for such a regional difference. However, the lack of a relationship between $\alpha_{sL}$ levels and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated $\alpha_{sL}$ argues against this notion. Moreover, the latter finding suggests that CTX-catalyzed ADP-ribosylation of $\alpha_s$ cannot be used reliably for quantification of $\alpha_s$ (Hamacher et al., 1995). Thus, other factors may account for the differences in CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{sL}$ across the regions. One possibility is that there may be proportionally more $\alpha_{sL}$ in the heterotrimeric state in temporal and occipital cortices. Alternatively, greater CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated $\alpha_{sL}$ in these brain areas may be a result of different enzymatic activities (i.e. ARF cofactors) present in each region.

If the higher CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation in temporal and occipital cortex compared to the cerebellum reflects an elevated proportion of $\alpha_{sL}$ in the heterotrimeric state, this finding may have functional relevance. For example, $\alpha_s$ incorporated into S49 cyc$^-$. membranes stripped of $\beta\gamma$ could not be ADP-ribosylated by CTX and stimulated AC poorly (Grazino et al., 1987, Rebois et al., 1987). However, incorporation of $\beta\gamma$ into S49 cyc$^-$.
membranes resulted in a dose-dependent increase in both AC stimulation and CTX-catalyzed ADP-ribosylation of \( \alpha_i \). Therefore, when more heterotrimeric \( \alpha_i \) is present, more can dissociate into free and active \( \alpha_i \), with subsequent stimulation of AC. There is also evidence to suggest that at least part of the pool of \( \alpha_i \) associated with AC may exist in the heterotrimeric state. In a study by Yeager et al. (1985), two peptides of 47 and 39 kDa copurified with AC from bovine brain membranes treated with Gpp[NH]p. The 47 kDa peptide was a substrate for CTX-catalyzed \(^{32}\)PADP-ribosylation indicating it was probably \( \alpha_i \), while the 35 kDa was likely G\( \beta \). Likewise, constitutively active \( \alpha_i \) (Q227L) is also a substrate for CTX-catalyzed ADP-ribosylation (Levis and Bourne, 1992) suggesting that it can form a heterotrimer with \( \beta \gamma \) in membranes in contrast to the expectation that constitutive activation would lead to dissociation of the heterotrimer and reduced CTX-catalyzed ADP-ribosylation (Graziano and Gilman, 1989, Masters et al., 1989, Osawa et al., 1990). Collectively, these observations argue for the possibility that increased heterotrimeric \( \alpha_i \) may play an important functional role in cortical regions showing greater CTX-catalyzed \(^{32}\)PADP-ribosylation of \( \alpha_i \).

Duman et al. (1991) also reported regional differences in the ADP-ribosylation of \( \alpha_i \) in seven regions from rat brain. In comparison with the findings found here for human brain, highest levels of endogenous \(^{32}\)PADP-ribosylation were observed in hippocampus, hypothalamus and cerebral cortex, with the lowest levels occurring in cerebellum (Duman et al., 1991). Similar to the findings of the above authors, Williams et al. (1992) also observed regional variations in the basal level of endogenous ADP-ribosylation of the major substrates in rat brain, with the lowest occurring in cerebellum and the highest in
hippocampus. Collectively, the differences in endogenous and CTX-catalyzed ADP-ribosylation among the various regions whether in rat or postmortem human brain may be explained by differential expression and functionality of ADP-ribosyltransferases and their cofactors, and different degrees of susceptibility of substrate to ADP-ribosylation, such as if the protein were already partially ADP-ribosylated or, in some other manner, less amenable to ADP-ribosylation. The potential physiological significance for such regional differences may provide for intricate control of protein levels and subsequent regulation of αs subunit-effector responses.

Lower endogenous [32P]ADP-ribosylation of αs and the 39 kDa αs-like protein were also observed in temporal and occipital cortex compared to cerebellum. Interestingly, there were slightly lower levels of αs and the 39 kDa αs-like protein in temporal and occipital cortices compared with cerebellum, as well. However, the differences in mean values of endogenous [32P]ADP-ribosylation of αs and the 39 kDa αs-like protein between the various regions were higher compared with the corresponding differences in immunolabeling values. This suggests that the lower immunoreactive levels of these two proteins could not solely account for such regional differences in endogenous [32P]ADP-ribosylation. Therefore, another possible explanation for this finding is that both αs and the 39 kDa αs-like protein might exhibit lower efficiency for this posttranslational mechanism in temporal and occipital cortex than in cerebellum. Alternatively, expression of different subtypes of ADP-ribosyltransferases and/or ARFs across the three brain regions may account for the lower endogenous [32P]ADP-ribosylation of the protein in temporal and occipital cortex. Moreover, endogenously [32P]ADP-ribosylated αs and the 39 kDa αs-like protein may be
cleared more rapidly once formed in the temporal and occipital cortices, and thus account for the lower $[^{32}\text{P}]/$incorporation in these two regions.

In summary, the results of the characterization study demonstrate for the first time that $\alpha_s$ is ADP-ribosylated both by endogenous and exogenous (i.e. CTX) ADP-ribosyltransferases in postmortem human brain (Andreopoulos et al., 1999), and that regional differences occur in the latter two processes. In addition, it has been shown that $\alpha_{\text{s-L}}$ and $\alpha_{\text{s-S}}$ serve as substrates for $[^{32}\text{P}]/$ADP-ribosylation in peripheral tissues such as platelets and B-lymphoblasts, as well as in postmortem human brain. Furthermore, a novel 39 kDa $\alpha_s$-like protein was also identified which undergoes endogenous but not CTX-catalyzed $[^{32}\text{P}]/$ADP-ribosylation (Andreopoulos et al., 1999). The different extent of ADP-ribosylation of each $\alpha_s$ isoform suggests that ADP-ribosyltransferases may display different affinities for the various $\alpha_s$ isoforms. This supports the notion that ADP-ribosylation may differentially regulate the levels of $\alpha_{\text{s-L}}$ and $\alpha_{\text{s-S}}$ by modulating the turnover/degradation of these isoforms leading to different functional outcomes with respect to effector stimulation (Seifert et al., 1998).

IV.2 Assessment of Endogenous and CTX-catalyzed-$[^{32}\text{P}]/$ADP-ribosylation of $\alpha_s$

Isoforms in Postmortem Human Brain from BD and Control Subjects

As discussed in the overview, the basis for higher $\alpha_s$ levels in postmortem human brain and peripheral blood cells from BD patients compared with controls cannot be explained by genetic mutations affecting the 5' regulatory regions of $\alpha_s$ or factors altering $\alpha_s$ gene expression. Furthermore, studies reporting linkage to markers on chromosomes 4
(Blackwood et al., 1996), 5 (Coon et al., 1993), 12 (Craddock et al., 1994, Barden et al., 1996), 21 (Straub et al., 1993, Detera-Wadleigh et al., 1997) and a vulnerability gene near the centromere of chromosome 18 (Paul et al., 1995, Stine et al., 1995, Berretti et al., 1997, McMahon et al., 1997) have not revealed any candidate $\alpha$-subunit genes, except for $\alpha_{oif}$ (Berretti et al., 1997). Taken together, these observations suggest that the increased levels of $\alpha$, in BD may be the result of disturbances in processes regulating $\alpha$, turnover/degradation at the posttranslational level. Evidence indicating that ADP-ribosylation can alter membrane levels of $\alpha$, and can be modulated by lithium, suggested that this posttranslational process merits more intense scrutiny to determine whether it contributes to the elevations in $\alpha$, levels as reported in cerebral cortical regions in postmortem human brain from BD subjects. Accordingly, endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, and immunolabeling of $\alpha$, were determined in temporal, occipital and cerebellar cortices from BP-I patients, and in age- and postmortem-delay matched controls for comparison.

IV.2.1 Temporal Cortex

Recent neuropathological and MRI studies suggest that the temporal lobe, a brain region already implicated in mood regulation (Johnson and Campbell, 1990, Starkstein et al., 1990) may be particularly affected in BD (reviewed in Warsh and Li, 1999). In this regard, increased temporal horn volume (Roy et al., 1998), reduced non-pyramidal cell number in the hippocampal CA2 region (Benes et al., 1998) and lower phosphomonoester level in both left and right temporal lobes (Deicken et al., 1995) have been reported in BD. Pronounced structural changes such as larger right anterior superior temporal gyrus and reduced left amygdala volume (Pearlson et al., 1997) and bilateral temporal volume reductions
(Altshuler et al., 1991) have also been reported. Furthermore, decreases in regional cerebral blood flow in the anterior superior temporal gyrus, left angular gyrus and anterior part of the insular cortex (Ito et al., 1996) have also been observed.

Evidence also implicates important biochemical changes in BD temporal cortex. For example, significantly decreased Na⁺-K⁺ ATPase α₂ subunit levels have been found in postmortem temporal cortex from BD patients compared with normal controls and schizophrenic patients (Rose et al., 1998). In the same cortical region, altered [³H]cAMP binding and PKA activity have been reported. These included findings of reduced [³H]cAMP binding accompanied by higher basal and maximal stimulated activity and significantly lower EC₅₀ for cAMP in the cytosolic fraction of temporal cortex of BD patients compared with matched controls (Rahman et al., 1997, Fields et al., 1999). Collectively, the localization of the changes found in the above studies to temporal cortex supports the view that this region may be an important pathogenic locus in BD.

Similar to previous reports demonstrating elevated α₅ immunolabeling in both postmortem BD brain (Young et al., 1993, Friedman and Wang, 1996) and peripheral cells from BD patients (Young et al., 1994, Manji et al., 1995a, Mitchell et al., 1997), significantly higher α₅ immunoreactivity in BD temporal cortex compared with control subjects was confirmed in the present study. Moreover, in the present work, α₅ immunolabeling was performed using area 20 of the temporal cortex due to limited tissue availability, whereas the elevated α₅ immunolabeling originally reported by Young et al. (1993) were found in area 21 of temporal cortex. Thus, this finding confirms and extends on earlier reports of higher α₅ immunolabeling in BD brain demonstrating that these changes occur more diffusely in
this cortical area. Area 20 from six BD subjects and area 21 from two patients were used for subsequent ADP-ribosylation studies.

Since inhibition of mono-ADP-ribosyltransferase activity leads to increased abundance of $\alpha_{\text{m}}$ as measured either by immunoblotting or CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation (Donnelly et al., 1992), it was hypothesized in this study that endogenously ADP-ribosylated $\alpha_{\text{mL}}$ levels would be reduced. However, no significant differences were found in the level of endogenous $[^{32}\text{P}]$ADP-ribosylated $\alpha_{\text{mL}}$ in temporal cortex of BD patients compared with control subjects. Several possibilities may explain the lack of reduction in endogenous $[^{32}\text{P}]$ADP-ribosylated $\alpha_{\text{mL}}$. Since $\alpha_{+}$ levels were already elevated in BD temporal cortex, this may have offset the detection of reductions in endogenous $[^{32}\text{P}]$ADP-ribosylation if they were relatively small. Indeed, an inverse relationship was found between temporal cortical $\alpha_{\text{mL}}$ immunoreactivities and the degree of endogenous $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{\text{mL}}$. This finding suggests that, at least in BD temporal cortex, increased $\alpha_{\text{mL}}$ is accompanied by decreased endogenous $[^{32}\text{P}]$ADP-ribosylation of this isoform, despite the fact that no statistically significant differences were found between BD and controls subjects. Alternatively, other cellular abnormalities affecting the disposition and clearance of $\alpha_{\text{mL}}$ may be responsible for the elevated $\alpha_{\text{mL}}$ levels found in this region, as will be discussed later.

CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{\text{mL}}$ also did not differ between BD patients and control subjects in temporal cortex despite the higher $\alpha_{\text{mL}}$ immunolabeling. As previously discussed, CTX requires both ARFs and $\alpha_{+}$ in its heterotrimeric form, to catalyze ADP-ribosylation of the $\alpha$ subunit. Thus, the lack of differences in CTX-catalyzed
\[^{32}\text{P}\]ADP-ribosylated $\alpha_{+L}$, despite the higher levels of $\alpha_{+L}$, implies that the proportion of heterotrimeric $\alpha_{+L}$ is reduced in BD temporal cortex compared with controls. In contrast, Friedman and Wang (1996) showed increased coimmunoprecipitable $\alpha_4$ isoforms with $\beta\gamma$ complexes in homogenates from BD frontal cortex in association with elevated $\alpha_4$ immunoreactive levels. This latter finding suggests that proportionately more $\alpha_4$ is in the heterotrimeric state and that the cellular disposition of $\alpha_4$ is thereby altered in BD, which differs from the observations found in this study. One possibility that may account for such a discrepancy is that only frontal cortex was examined in the latter study and this brain region may not be representative of changes that occur in BD temporal cortex. Furthermore, the possibility that the latter findings are a result of a sampling artifact because of the very small sample size ($n=5$) used in their study cannot be ruled out. Moreover, the authors did not report the variability associated with their immunoprecipitation values which does not allow for critical analysis of the data. Clearly, further work is required to clarify the heterotrimeric state of $\alpha_4$ in BD cortical regions (see Section IV.3).

As discussed earlier, CTX, an exogenous ADP-ribosyltransferase, requires ARFs for catalysis of ADP-ribosylation. Thus, alterations in ARF cofactors or their availability for CTX action could affect the degree to which the increased amount of $\alpha_{+L}$ undergoes CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation. However, the ability to distinguish differences between the diagnostic groups would be compromised if the two $\alpha_4$ subtypes show differential substrate affinity for this bacterial toxin-catalyzed ADP-ribosylation. For example, in S49 cyc$^-$ cells expressing recombinant $\alpha_4$ isoforms and S49 wild type cells, both $\alpha_{+L}$ and $\alpha_{+S}$ are sensitive to CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, but the short form shows preferential
CTX-catalyzed labeling (O’Donnell et al., 1991). If this also applies to neurons and glia, disturbances in the availability of ARFs needed for CTX-catalyzed ADP-ribosylation of αs subunits in BD temporal cortex would be more readily detectable by measuring CTX-catalyzed [32P]ADP-ribosylated αsS rather than αsL.

The lack of differences in CTX-catalyzed [32P]ADP-ribosylated αsL in the presence of elevated αsL immunolabeling in BD temporal cortex agrees with the findings of Mitchell et al. (1997). These authors showed that even though higher immunoreactive αsL levels were observed in platelets from BP-I patients, CTX-catalyzed [32P]ADP-ribosylated αsL was not different compared with healthy subjects. The latter observations also suggest that the proportion of αsL in heterotrimeric form is reduced in cells from BP-I patients. Of additional interest, the study also showed specific differences between BP-I and BP-II subtypes. Similar to the findings in BP-I patients, αsL immunolabeling was elevated in platelets from BP-II subjects but this elevation was accompanied by higher CTX-catalyzed [32P]ADP-ribosylation of the isoform compared with healthy controls. This may suggest that in BP-II patients proportionately more αsL occurs in platelets in the heterotrimeric state or alternatively, the increase in CTX-catalyzed [32P]ADP-ribosylated αsL may be indicative of the elevated protein levels. Such BD subtype-specific differences in the heterotrimeric state of αsL suggest that the mechanism(s) underlying the elevations of αs may be distinctive to the separate BD subtypes and that these changes may manifest themselves both in brain and platelets.
In contrast to \( \alpha_{s-L} \), there were no significant differences in the immunolabeling of \( \alpha_{s-S} \) in BD temporal cortex compared with control subjects, also confirming earlier observations (Young et al., 1993). Neither were there any differences between the two groups in endogenous \(^{32}\text{P}\)ADP-ribosylation of this \( \alpha_s \) isoform suggesting that endogenous ADP-ribosyltransferase activity is not altered in this brain region in BD. However, there was a reduction in CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylated \( \alpha_{s-S} \) in temporal cortex from BD patients compared with controls. Several possibilities may explain this reduction in CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylation of \( \alpha_{s-S} \). It is unlikely that reduced \( \alpha_{s-S} \) levels account for such a finding, since \( \alpha_{s-S} \) immunolabeling did not differ in BD patients compared with control subjects. Since CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylation is an irreversible process, disturbances in ADP-ribosylhydrolases that cleave ADP-ribose from \( \alpha_s \) could not interfere with the reaction and hence would not explain such a reduction. One likely possibility is that the above finding could reflect that a reduced fraction of \( \alpha_{s-S} \) occurs in the heterotrimeric state in BD temporal cortex. This may also apply to the finding that CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylated \( \alpha_{s-L} \) did not differ between BD and control subjects. Another prospect to entertain is that the trend towards reduction of CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylated \( \alpha_{s-S} \) may be related to disturbances in the levels/activity of ARF cofactors. It has been shown that modulation of ARF mRNA and protein levels by corticosterone treatment and adrenal gland removal increase and decrease, respectively, CTX-catalyzed ADP-ribosylation of \( \alpha_s \) in rat brain (Saito et al., 1989, Duman et al., 1990). Although the similar degree of CTX-catalyzed \(^{32}\text{P}\)ADP-ribosylation of \( \alpha_{s-L} \) in temporal cortex from BD compared with control subjects
seems to argue against this idea, differences between $\alpha_{+1}$ and $\alpha_{+5}$ in the efficiencies for CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation, as noted previously, could account for this discrepancy.

The experimental results comparing the amounts of endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylated products of $\alpha$, in postmortem temporal cortex of BD patients with controls were somewhat difficult to interpret since several possibilities existed to explain the cellular mechanisms producing these differences. That is, the differences could be explained by either a change in mono-ADP-ribosyltransferase activity and/or cofactors regulating its activity or an alteration in the disposition and/or heterotrimeric state of $\alpha$, affecting its accessibility to the enzyme. If the former were the case, then it would be expected that the activity of mono-ADP-ribosyltransferases would also be altered toward other substrates, such as MBP. MBP binds GTP at a single site, like the heterotrimeric G protein $\alpha$ subunits, and undergoes CTX-catalyzed ADP-ribosylation in the presence of NAD. Equally important, it can be ADP-ribosylated by an endogenous mono-ADP-ribosyltransferase in myelin membranes (Boulis et al., 1995). Secondly, MBP is primarily expressed in myelin containing fractions from white matter in brain (Braun et al., 1990, Enomoto and Asakawa, 1990, Boulis et al., 1995), with low amounts present in grey matter. Thus, spiking cortical grey matter homogenates with purified MBP would be akin to adding exogenous substrate as an internal standard to monitor the activity of the enzyme. Furthermore, this substrate should not be subject to dispositional effects limiting its access to the enzyme as it is exogenously added to the homogenates. In addition, its smaller molecular mass (approximately 21 kDa) allowed for complete separation and ease of quantification without the technical difficulties of overlap of the $[^{32}\text{P}]$ signal with any of the $\alpha$, isoforms. Finally,
MBP $[^{32}\text{P}]$ADP-ribosylation was linear with respect to time and protein concentration, and could provide a reasonable quantitative estimate of mono-ADP-ribosyltransferase activity towards the substrate.

Determination of endogenous $[^{32}\text{P}]$ADP-ribosylation of the exogenous MBP added to temporal cortex homogenates from BD patients and control subjects under similar assay conditions revealed no significant differences between the two comparison groups, however. This finding suggests that the results obtained with endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of the two $\alpha_4$ isoforms may be attributed to factors regulating their cellular disposition and/or heterotrimeric state that limit the access of $\alpha_4$ to mono-ADP-ribosyltransferase rather than to a defect(s) in ADP-ribosyltransferases themselves. In this regard, changes in ADP-ribosyltransferase activity, such as the modulatory effects seen with NO donor compounds, are known to affect the degree of the basal ADP-ribosylation of all major substrate proteins (Williams et al., 1992). While CTX and some endogenous ADP-ribosyltransferases require ARFs for catalysis of ADP-ribosylation (Matsuyama and Tsuyama, 1991), the enzyme catalyzing ADP-ribosylation of MBP may not. Therefore, the caveat must be raised that the inability to demonstrate differences in $[^{32}\text{P}]$ADP-ribosylation of MBP between BD patients and control subjects does not necessarily rule out potential disturbances in ARF levels and/or activity in BD temporal cortex.

**IV.2.2 Occipital Cortex**

Significantly higher $\alpha_{4-L}$ immunoreactivity was observed in BD occipital cortex compared with control subjects replicating earlier findings on these same subjects. There were, however, no differences in endogenous, and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation of
α<sub>α-L</sub> and α<sub>α-S</sub>, and in α<sub>α-S</sub> immunolabeling between the two groups. Moreover, α<sub>α-L</sub> immunoreactive levels did not correlate with those of endogenous [<sup>32</sup>P]ADP-ribosylated α<sub>α-L</sub> in BD occipital cortex as was observed in temporal cortex. This suggests that increased α<sub>α-L</sub> levels in this cortical region are not accompanied by decreased endogenous [<sup>32</sup>P]ADP-ribosylation of the isoform in this brain region.

The lack of differences in CTX-catalyzed [<sup>32</sup>P]ADP-ribosylation of α<sub>α-L</sub> in the presence of elevated α<sub>α-L</sub> immunolabeling could also be explained by the possibility that the proportion of heterotrimeric α<sub>α-L</sub> is reduced in this cortical region in BD, as well. Conceivably, however, the mechanism(s) which accounts for the possible reductions in the proportion of heterotrimeric α<sub>α-L</sub> in occipital cortex may be different from that in temporal cortex in BD. In support of regional differences in abnormalities in BD brain, significantly higher immunoreactive levels of α<sub>q11</sub> and moderate elevations of PLCβ<sub>1</sub> were found in BD occipital cortex compared with control subjects (Mathews et al., 1997). Altered α<sub>q11</sub> functional activity accompanied these changes, as demonstrated by reduced GTPγS-stimulated [<sup>3</sup>H]PI hydrolysis (Jope et al., 1996). These disturbances were not evident in either frontal or temporal BD cortex highlighting the uniqueness of such findings to BD occipital cortex. Unfortunately, the pathophysiological significance of such selective disturbances in this region at present is still unknown.

IV.2.3 Cerebellum

To confirm the specificity of the [<sup>32</sup>P]ADP-ribosylation findings to temporal cortex, endogenous and CTX-catalyzed [<sup>32</sup>P]ADP-ribosylation of α<sub>α</sub> isoforms were also measured
in cerebellum, a brain region in which no changes were observed in $\alpha_4$ levels in BD (Young et al., 1993). As expected, no differences were found between BD patients and control subjects in cerebellar endogenous, and CTX-catalyzed [$^{32}$P]ADP-ribosylation, and immunolabeling of $\alpha_{\text{s-L}}$ and $\alpha_{\text{v-s}}$. This also supports the selectivity of the [$^{32}$P]ADP-ribosylation changes to the temporal cortex in BD.

**IV.2.4 The 39 kDa $\alpha_\text{-like}$ Protein**

As presented in the Results (Section III), the effect of DTT was examined on endogenous [$^{32}$P]ADP-ribosylation of the $\alpha_\text{t}$ substrate proteins. Similar to earlier reports in which endogenous [$^{32}$P]ADP-ribosylation was measured (Williams et al., 1992, Young et al., 1997), 25 mM DTT gave a strong signal for the 39 kDa $\alpha_\text{-like}$ protein. However, in this study, the signal for $\alpha_{\text{s-L}}$ was more pronounced when assays were performed with 10 mM DTT. At this concentration of DTT, the endogenously [$^{32}$P]ADP-ribosylated 39 kDa $\alpha_\text{-like}$ protein was also still detectable and quantified by densitometry. Therefore, for comparison purposes and to ensure that any potential differences in ADP-ribosyltransferase activity were not missed, endogenous [$^{32}$P]ADP-ribosylation of the 39kDa $\alpha_\text{-like}$ protein was measured under both conditions (ie. 10 and 25 mM DTT).

Under assay conditions using 25 mM DTT, there was a significant reduction (30%) in this ADP-ribosylated product in temporal cortex of BD compared with control subjects. This finding suggests an alteration(s) in the ADP-ribosyltransferase pathway that is specific to the 39 kDa $\alpha_\text{-like}$ protein. For example, the reduction could result from a disturbance in an ADP-ribosyltransferase specific for the 39 kDa $\alpha_\text{-like}$ protein and/or cofactors needed
by that particular ADP-ribosyltransferase. Alternatively, the finding may reflect an altered cellular disposition of the protein itself rendering it inaccessible to ADP-ribosylation. It is unlikely, however, that the reduced endogenous $[^{32}\text{P}]\text{ADP-ribosylation}$ of the 39 kDa $\alpha_t$-like protein depends on the heterotrimeric state of the protein. Since no CTX-catalyzed $[^{32}\text{P}]\text{ADP-ribosylation}$ of the 39 kDa $\alpha_t$-like protein was detected, it does not appear to be able to form or maintain the necessary heterotrimeric state needed for CTX to catalyze its ADP-ribosylation in any of the cortical regions examined. This likely holds for endogenous ADP-ribosylation of the 39 kDa $\alpha_t$-like protein. Just as important, the above reduction in endogenous $[^{32}\text{P}]\text{ADP-ribosylation}$ of the 39 kDa $\alpha_t$-like protein highlights the uniqueness of such changes to the temporal cortex as no differences were observed between BD patients and controls subjects in occipital cortex and cerebellum. $[^{32}\text{P}]$ incorporation into $\alpha_{s-L}$ and $\alpha_{s-S}$ were only weakly detectable under these assay conditions and could not be reliably quantified simultaneously with the 39 kDa $\alpha_t$-like protein.

IV.2.4.1 Relationship Between Brain Lithium and ADP-Ribosylation of $\alpha_t$

Lithium is known to modify a variety of processes in brain and other tissues (Manji et al., 1995b) including ADP-ribosylation (Nestler et al., 1995). Both Nestler et al. (1995) and Young and Woods (1996) showed that chronic lithium treatment increases $[^{32}\text{P}]\text{ADP-ribosylation}$ of substrate proteins, in rat brain and C6 glioma cells, respectively, raising the possibility that lithium may modulate ADP-ribosyltransferase activity in postmortem BD brain as well. Although it is difficult to assess a direct effect of lithium on ADP-ribosylation in postmortem brain tissue, if such an effect exists, correlations should be observed between
brain lithium concentrations and the levels of $[^{32}\text{P}]$ADP-ribosylated $\alpha_i$ isoforms. However, there were no correlations found for $\alpha_{t-L}$ and $\alpha_{t-S}$ with respect to endogenous and CTX-catalyzed $[^{32}\text{P}]$ADP-ribosylation and lithium levels.

A significant positive correlation was observed in the present study, however, between the endogenous $[^{32}\text{P}]$ADP-ribosylated 39 kDa $\alpha_r$-like protein and lithium concentrations in BD temporal cortex. This observation suggests the possibility that lithium stimulates endogenous ADP-ribosylation of the 39 kDa $\alpha_r$-like protein in a concentration-dependent manner. It is tempting to speculate that this may reflect a possibly compensation for a defect in a particular ADP-ribosyltransferase specific for the 39 kDa $\alpha_r$-like protein. The absence of correlations between lithium concentration and endogenous $[^{32}\text{P}]$ADP-ribosylation of $\alpha_{t-L}$ and $\alpha_{t-S}$ also supports the notion that lithium may modulate a specific subtype of ADP-ribosyltransferases rather than affect the family of these enzymes in BD temporal cortex. Although lithium levels in BD temporal cortex were well below therapeutic concentrations in a number of the cases, six of the nine patients did have documented history of lithium use within a 6 month interval prior to death, and previous lithium therapy for the remaining three could not be ruled out. Since the effects of lithium are not immediately reversed upon withdrawal (Manji et al., 1995a), it is possible that modulation of ADP-ribosylation by lithium may have still persisted at autopsy.

As noted previously, a similar stimulatory effect of lithium was reported by Nestler et al. (1995) who observed that chronic administration of lithium to rats resulted in increased endogenous ADP-ribosylation of substrate proteins in vivo. Their observations suggested lithium exerts an effect on mono-ADP-ribosyltransferases in general as opposed to a specific
subset of mono-ADP-ribosyltransferases, at least in rat brain, since increased endogenous ADP-ribosylation was observed for all substrates. However, concurring with the finding of my study, Young and Woods (1996) also reported increased endogenous $[^{32}\text{P}]$ADP-ribosylation of a 39 kDa band following chronic lithium treatment in C6 cells, an effect which appeared to be more specific for a particular 39 kDa protein band, rather than for several other substrates.

The mechanism of action for lithium on endogenous ADP-ribosylation is not clearly understood. However, it is known that certain mono-ADP-ribosyltransferases possess Mg$^{2+}$-binding sites, which could be antagonized by lithium (Zolkiewska et al., 1992). It has also been shown that ADP-ribosyltransferases can be particularly sensitive to phospholipids (Matsuyama and Tsuyama, 1991). Since PIP$_2$ is reduced in platelet membranes from BD patients on lithium treatment compared with control subjects (Soares et al., 1999), this may be another indirect mechanism by which lithium affects ADP-ribosyltransferase activity. It would indeed be interesting to identify which and how many ADP-ribosyltransferases may be affected by lithium along with the mode of regulation in both human tissue and peripheral cells.

Young et al. (1997a) also reported that lithium could differentially affect ADP-ribosylation by significantly reducing endogenous $[^{32}\text{P}]$ADP-ribosylation of a 39 kDa protein band only in platelets from euthymic lithium-treated BD patients compared with unmedicated subjects. The identity of the 39 kDa band in platelets, however, was not characterized, but assumed to be $\alpha$, because of the estimated molecular mass (Young et al., 1997a). In the present study, there was no evidence of an endogenously $[^{32}\text{P}]$ADP-ribosylated
39 kDa protein in the human pool of platelets examined. In fact, only endogenous [³²P]ADP-ribosylated α⁺⁺ was detectable. The absence of a detectable endogenously [³²P]ADP-ribosylated 39 kDa protein may be due to methodological differences in platelet preparation or techniques employed in this study. The platelets used here consisted of a heterogeneous population of cells (i.e. pooled from 5 different subjects) and were frozen before assay. In addition, lower amounts of platelet protein were used in my study compared with the amounts used by the latter authors for SDS-PAGE. Furthermore, they used approximately twice the amount of [³²P]NAD (5 as compared with 2.5 µCi) along with a higher temperature and longer reaction time than the assay conditions used my experiments. Employing freshly isolated platelets, homogeneous population of cells, similar assay conditions and larger amounts of protein for electrophoretic separation would help to clarify the nature of endogenous [³²P]ADP-ribosylation of a 39 kDa protein band observed by others in platelets.

Due to limited tissue availability and in the absence of changes in the immunoreactive levels of the 39 kDa α⁺⁺-like protein, endogenous [³²P]ADP-ribosylation of MBP was not examined under the 25 mM DTT assay conditions. Although the significantly reduced endogenous [³²P]ADP-ribosylation of the 39 kDa α⁺⁺-like protein suggests some alteration(s) affecting one or more ADP-ribosyltransferase(s) may occur in BD temporal cortex, the pathophysiological significance of this modest difference in the face of limited changes in ADP-ribosylation of α⁺⁺ is uncertain. Moreover, since the reduction of the 39 kDa α⁺⁺-like protein correlates with lithium levels, it is possible that lithium is able to compensate partially for a selective defect in ADP-ribosyltransferase activity.
Finally, it must be considered whether ADP-ribosylation of $\alpha$, *in vitro* is affected by the preexisting degree of ADP-ribosylated $\alpha$, *in vivo*. Unfortunately, the true rate of *in vivo* endogenous ADP-ribosylation cannot be measured in brain and is not easily resolved by technical procedures. As shown by Philbert and Zwiers (1995), stability of the ADP-ribose bond decreases approximately 80% following prolonged exposure to constant temperature. Since all brain tissues have varying degrees of PMI, it is highly conceivable that this extraneous factor may influence the stability of the *in vivo* ADP-ribose bond to $\alpha$. Thus, if it were possible to measure the degree of ADP-ribose linkage to $\alpha$, in the *in vivo* state, it is certainly not an accurate reflection of already ADP-ribosylated $\alpha$. Therefore, addition of $[^{32}\text{P}]\text{NAD}$ allows for detection of this physiological process *in vitro*, providing a measure of mono-ADP-ribosyltransferase activity and the ability of $\alpha$, to undergo this posttranslational modification.

**V.3 Future Studies**

The findings of this study have raised the possibility that a potential disturbance(s) exists in the cellular disposition and/or heterotrimeric state of $\alpha_{\gamma\delta}$ and $\alpha_{\gamma}$, in BD temporal and occipital cortex. Further testing of this entails methodological and mechanistic considerations. For example, immunoprecipitation of $\alpha_{\beta\gamma}$ complexes from the temporal cortex of BD subjects would be the best way to confirm the possibility of altered heterotrimeric state of $\alpha$. However, this is contingent upon the availability of $\beta$ antibodies ($\beta_1$-$\beta_4$) with high immunoprecipitation efficiencies. Furthermore, at present, anti-$\beta_3$ is not commercially available and since it is not currently known what proportion of $\alpha$, is coupled
to each β subunit, there is a serious risk that the results of such immunoprecipitation assays would be inconclusive. Thus, this limitation taken together with the limited availability of the postmortem brain used in this study suggests other approaches are needed to examine alterations in the cellular disposition and/or availability of α.

Factors which are known to affect the cellular disposition and/or availability of α and warrant further investigation could include changes in its phosphorylation, palmitoylation and/or subcellular localization (Asterano et al., 1999). With respect to the latter, subcellular localization of α subunits (such as α4) to the trans-Golgi apparatus and secretory granules has been reported in a variety of tissues such as pancreas, liver and adrenal medulla (Maier et al., 1995, Denker et al., 1996, Asterano et al., 1999). Furthermore, immunoprecipitated tubulin has been shown to aggregate with α1 and α3 in rat cerebral cortical synaptic membranes (Yan et al., 1996). Incubation of the latter membranes with tubulin complexed with Gpp(NH)p has also been reported to inhibit AC activity (Yan et al., 1996). As discussed in the overview, inactive α, can associate with caveolin-enriched fractions (Li et al., 1995). Moreover, drug and physical treatments can alter the disposition and functional activity of α subunits. Thus, antidepressants such as amitriptyline, desipramine or ECT can enhance AC activity in rats and C6 cells by increasing the coupling between α and AC, as reflected by increases in the number of active α/AC complexes (Chen and Rasenick, 1995a, 1995b). Such alterations in the localization and cellular disposition of α in BD brain by one or more of these factors may lead to a fraction of α that is inaccessible to both endogenous and CTX-catalyzed ADP-ribosylation. This could then reduce the availability of α as a substrate for ADP-
ribosylation. Future studies involving subcellular fractionation of $\alpha$, to tease apart its intracellular localization/distribution could help in determining the degree of ADP-ribosyltransferase activity associated with each $\alpha$ fraction in BD temporal cortex.

Equally important, other posttranslational modifications such as N-terminal palmitoylation, may affect the cellular disposition and/or availability of $\alpha$. As discussed in the overview, palmitoylation serves to confer greater hydrophobicity to $\alpha$ subunits and enhances membrane interaction (Wedegaertner et al., 1993), although its exact role has not been fully elucidated. Bhrame et al. (1998) reported that in rat cortical membranes, receptor interactions with heterotrimeric $\alpha$, triggers its dissociation and depalmitoylation. The inability of $\alpha$, to undergo repalmitoylation once activated by the latter events could result in its targeting to the cytoplasm and other subcellular localizations. Therefore, reduced palmitoylation could serve to limit the availability of $\alpha$, to form a heterotrimer with $\beta\gamma$ subunits as depicted in Figure 33. Given the results of my investigations, a dynamic process such as palmitoylation would merit further exploration in understanding the basis for the changes in $\alpha$, observed in BD. However, examination of such processes is contingent upon the availability of a living cellular model which expresses the same G protein changes manifest in BD temporal cortex and presumably the same regulatory processes as neuronal/glia in this brain region. Unfortunately, to date, no such model has been unequivocally demonstrated.

Despite such an intriguing possibility, BD still remains a complex and multifaceted disorder. There may be more than one cellular mechanism(s) underlying the pathophysiological changes observed for $\alpha$, in this disorder (Li et al., 1999). As stated in the
overview. Other processes such as calpain- and ubiquitin-dependent degradative mechanisms may also warrant further investigation (see Figure 33) and can not be excluded as possible posttranslational processes that may account for the elevations of $\alpha_i$. However, clearance through the latter routes could also depend on the heterotrimeric state of $\alpha_i$ (Greenwood and Jope, 1994), and this may confound the interpretation of findings obtained through the latter two pathways. Taken together, priority should be given to the examination of mechanisms that can affect the disposition of $\alpha_i$ (such as palmitoylation) before assessing other potential processes.

IV.4 Conclusions

The results of this study have provided a number of new and important findings relevant to understanding the pathophysiology of BD, in particular the regulation/modulation of $\alpha_i$ protein levels by the posttranslational modification of ADP-ribosylation. First, this work is fundamental in demonstrating that $\alpha_i$ undergoes ADP-ribosylation in postmortem human brain. Characterization of both endogenous and CTX-catalyzed $^{[32P]}$ADP-ribosylated products of $\alpha_{+L}$ and $\alpha_{+S}$ by SDS-PAGE, immunoprecipitation with $\alpha_i$ antibodies and limited protease digestion confirms that $\alpha_i$ isoforms are indeed substrates for this process.

Of particular importance, is the identification of a novel, lower molecular weight $\alpha_i$-like protein in postmortem brain, which is also capable of undergoing endogenous $^{[32P]}$ADP-ribosylation. This protein is not a degradative product of $\alpha_i$ nor does the signal detected consist of $^{[32P]}$ADP-ribosylated GAPDH. One likely possibility is that the 39 kDa $^{[32P]}$ADP-ribosylated $\alpha_i$-like protein represents an N-terminal truncated form of $\alpha_i$. 
Alternative Posttranslational Mechanisms Regulating $\alpha_s$ Protein Levels

Figure 33: Schematic representation of alternative posttranslational pathways that may regulate $\alpha_s$ protein levels and function. Evidence from this study does not support the notion that ADP-ribosylation (1) plays an important role in the regulation of $\alpha_s$ levels in BD. The findings do, however, suggest that the cellular disposition and/or heterotrimeric state of $\alpha_s$ is altered. Processes that may affect $\alpha_s$ availability include lipid modifications such as palmitoylation (2) and regulation by tubulin and other subcellular localizations (3). Other mechanisms which may also be involved in modulating $\alpha_s$ levels include the degradative mechanisms of calpain (4) and ubiquitin (5) and cross regulation via different second messenger pathways (6).
It has been shown that $\alpha_s$ also undergoes endogenous and CTX-catalyzed [$^{32}\text{P}$]ADP-ribosylation in peripheral blood cells such as platelets, and for the first time, this has also been demonstrated to occur in human B-lymphoblast cell lines. Endogenous [$^{32}\text{P}$]ADP-ribosylation of $\alpha_{sL}$ in both human platelets and B-lymphoblasts is greater despite the fact that immunolabeling of the protein is markedly lower compared to $\alpha_{sS}$ suggesting preferential labeling of the two isoforms for ADP-ribosylation in these cells. This supports the notion that ADP-ribosylation may play a role in differentially regulating the levels of $\alpha_{sL}$ and $\alpha_{sS}$ (i.e. modulation of turnover) and hence affect functionality in human platelets and B-lymphoblasts.

This study is also the first to show differences in the degree of endogenous and CTX-catalyzed [$^{32}\text{P}$]ADP-ribosylation of the $\alpha_s$ isoforms among different regions in postmortem human brain. These differences include: decreased endogenous [$^{32}\text{P}$]ADP-ribosylation of both $\alpha_{sS}$ and the 39 kDa $\alpha_s$-like protein in temporal and occipital cortices compared to cerebellum; and increased CTX-catalyzed [$^{32}\text{P}$]ADP-ribosylation of $\alpha_{sL}$ in temporal and occipital cortices compared to cerebellum. Collectively, these observations suggest a differential expression of ADP-ribosyltransferases and/or cofactors within brain tissue.

This study also extends on the previously reported finding of elevated immunoreactive levels of $\alpha_{sL}$ in BD temporal and occipital cortex compared with controls demonstrating $\alpha_{sL}$ levels were also elevated in area 20 of BD temporal cortex, a subregion not previously examined. This indicates that changes in $\alpha_{sL}$ protein levels are likely widespread within the temporal cortex of BD patients.
The findings of a significant negative correlation between endogenous $^{[32P]}$ADP-ribosylated $\alpha_{\text{asL}}$ and its immunoreactive levels, together with reduced CTX-catalyzed $^{[32P]}$ADP-ribosylated $\alpha_{\text{asS}}$, suggest that the cellular disposition and/or heterotrimeric state of $\alpha$, may be altered in BD temporal cortex. Such abnormalities may render $\alpha$, less accessible to the process of ADP-ribosylation and perhaps, clearance mechanisms. The lack of differences in endogenous $^{[32P]}$ADP-ribosylation of MBP in BD temporal cortex compared with controls further supports that a disturbance in ADP-ribosyltransferase activity is unlikely the molecular mechanism underlying the elevated levels of $\alpha$, in BD brain.

Significant reductions in the endogenous $^{[32P]}$ADP-ribosylation of the 39 kDa $\alpha$, like protein in BD temporal cortex compared with controls suggests the possibility of a disturbance in a specific ADP-ribosyltransferase and/or cellular disposition for this 39 kDa $\alpha$, like protein in this cortical region. Moreover, the changes in the 39 kDa $\alpha$, like protein correlated with lithium concentrations, possibly reflecting that this mood stabilizer is also able to modulate the activity of some ADP ribosyltransferases in human brain. This finding concurs with observations that lithium modulates endogenous $^{[32P]}$ADP-ribosylation of a 39 kDa protein in platelets from BD subjects (Young et al., 1997a).
CHAPTER V

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