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Behavioral Reversal of Lithium Effects by Four Inositol Isomers Correlates Perfectly with Biochemical Effects on the PI Cycle:

Depletion by Chronic Lithium of Brain Inositol Is Specific to Hypothalamus, and Inositol Levels May be Abnormal in Postmortem Brain from Bipolar Patients

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The inositol depletion hypothesis of lithium (Li) action has been criticized, because depletion of inositol after chronic Li treatment has not been reproducible, effects of inositol to reverse Li-induced behaviors occurred also with epi-inositol, a unnatural isomer, and because inositol is ubiquitous in brain and hard to relate to the pathogenesis of affective disorder. Therefore, we review our studies showing that lithium depletion of brain inositol occurs chronically in the hypothalamus, a region not previously examined; that behavioral effects of four different inositol isomers including epi-inositol correlate perfectly with their biochemical effects; and that inositol in postmortem human brain is reduced by 25% in frontal cortex of bipolars and suicides as compared with controls. Because inositol in postmortem brain is reduced and not increased in bipolar patients, the relationship between inositol, lithium, and affective disorder is complex.
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KEY WORDS: *Inositol; Hypothalamus; Epi-inositol; Myo-inositol; Bipolar disorder; Frontal cortex*

Lithium (Li^+) has therapeutic and prophylactic effects on both the manic and depressive phases of bipolar affective disorder; however, the mechanism of Li's therapeutic action is not clear. Several biochemical actions have been attributed to Li^+ (Wood and Goodwin 1987), but none has been incontrovertibly associated with its effects on behavior or mood.

Li^+ inhibits the dephosphorylation of four of the inositol monophosphates as well as two inositol biphosphates, thereby increasing brain levels of inositol monophosphate and two biphosphates and reducing levels of myo-inositol (Allison et al. 1976; Allison et al. 1980; Honchar et al. 1989; Sherman et al. 1981, 1985b). These effects are attributable to Li^+'s inhibition of inositol monophosphatase (Hallcher and Sherman 1980; Moscovich et al. 1990) and inositol polyphosphate 1-phosphatase (Inhorn and Majerus 1987). In rats, lithium chloride (LiCl), 10 meq/kg, reduced brain inositol levels by 30% and increased inositol monophosphate levels 20-fold after 6 h (Allison et al. 1976, 1980) and 40-fold after 24 h (Sherman et al. 1985b). Chronic administra-

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tion of Li^+ has been reported to reduce brain inositol levels (Sherman et al. 1985b), but others have not replicated this critical finding (Jope and Williams 1994) (see below).

Various biological effects of Li^+ can be reversed by addition of myo-inositol in vitro (Kofman and Belmaker 1993). To ascertain if Li^+'s inhibition of inositol monophosphatase is relevant to its therapeutic effects in patients with affective disorders, it is critical to demonstrate that behavioral effects of Li^+ are also reversed by myo-inositol. Because myo-inositol does not easily penetrate the blood–brain barrier when injected systemically (Spector and Lorenzo 1975), it is preferable to inject myo-inositol directly into the brain. ICV myo-inositol was found to reverse inhibition of rearing in rats induced by an acute injection of Li^+ (Kofman and Belmaker 1993).

INOSITOL REVERSES Li-PILOCARPINE SEIZURES

One of the most robust behavioral effects of Li^+ is that normally subconvulsant doses of muscarinic agonists will induce limbic seizures in rats pretreated with lithium (Honchar et al. 1983). Induction of Li^+-pilocarpine seizures is concomitant with a reduction in cortical myo-inositol levels and an elevation of inositol monophosphate, which is about 10-fold greater than the effects elicited by either Li^+ or pilocarpine alone (Sherman et al. 1985a, 1986). Tricklebank et al. (1991) reported that ICV injections of myo-inositol prolonged the latency to seizures elicited by ICV or systemic Li^+ in mice. We found similar results in rats (Kofman et al. 1993).

Surgery and Injection

Twenty-eight male Sprague–Dawley rats were implanted with guide cannulae in the lateral ventricle using standard stereotaxic procedures under pentobarbital anesthesia. Coordinates for the cannula placement were 0.8 mm posterior to bregma, 1.4 mm lateral to midline, and 5.0 mm below skull surface. Rats were randomly divided into three groups and injected ICV with myo-inositol or artificial cerebrospinal fluid (CSF) via an injection cannula attached with polyethelene tubing to a 100-μl Koehln microsyringe. As a control, the inositol stereoisomer, L-chiro-inositol was injected in a third group of rats. Myo- and L-chiro-inositol were injected in a dose of 10 mg/40 ul, and CSF was injected in a volume of 40 ul. Injections were made manually over a period of 2 minutes, and the injection cannula was left in place for 1 min before being replaced by the stylet. Then rats were injected with LiCl, 3 meq/kg in a volume of 15 cc/kg IP. Twenty-four hours later, they were reinjected ICV with the same drug they had received the previous day, and 30 min later, they were injected with pilocarpine, 30 mg/kg, sc, or 20 mg/kg sc.

Behavioral Observations

The animals were rated for signs of seizure according to a modified version of the scale used by Patel et al. (1988) once every 5 minutes for 75 minutes. The scoring was as follows: 0 = no response; 1 = gustatory movements and/or fictive scratching; 2 = tremor; 3 = head bobbing; 4 = forelimb clonus; 5 = rearing, clonus, and falling. In addition, the latency to attain forelimb clonus (score 4) was recorded for each rat. The observer was blind to the treatment condition.

Brains were removed and frozen for biochemical analysis and confirmation of cannula site. The frozen brains were cut at the cannula site, and those subjects that did not have cannulae in the lateral ventricle were excluded from behavioral and biochemical analysis.

Results

Latency to clonus was significantly prolonged by myo-inositol (41.2 min) as compared to artificial CSF (19.5 min) or L-chiro-inositol (16.9 min), $F = 10.48$, $p = .00074$), in rats treated with lithium and pilocarpine 30 mg/kg. Post-hoc Scheffe tests indicated that there was a significant difference between myo-inositol and L-chiro-inositol ($p < .002$) and between myo-inositol and artificial CSF ($p = .005$).

The seizure score was analyzed by Kruskal–Wallis test at each time point. There was a significant difference between the three groups at 20, and 30 to 45 min (Figure 1A) for 30 mg/kg pilocarpine.

The latency to exhibit clonus in rats treated with Li^+ and pilocarpine, 20 mg/kg ($F = 28.35$, $p < .00001$) significantly increased with myo-inositol. Post-hoc Scheffe tests indicated that this difference was significant when myo-inositol was compared to vehicle ($p < .00002$) and to L-chiro-inositol ($p < .00002$); using Kruskal–Wallis, there was a significant effect of myo-inositol at 25 to 50 min and 60 to 75 min (Figure 1B).

Eight of 16 rats treated with myo-inositol did not attain seizure scores of 4 (clonus) or higher during the 75-min observation period (and until sacrifice); whereas, only one of the 14 vehicle-treated rats did not exhibit clonus. All the rats treated with L-chiro-inositol had clonic seizures. A chi-square test for the number of animals that did not reach stage 4 indicated a significant difference between myo-inositol and vehicle groups ($p < .01$) and between myo-inositol and L-chiro-inositol groups ($p < .004$). There was no significant difference between the vehicle and L-chiro-inositol groups (Table 1). There was no effect of myo-inositol in Li^+-free rats injected with pilocarpine, 200 mg/kg (data not shown).

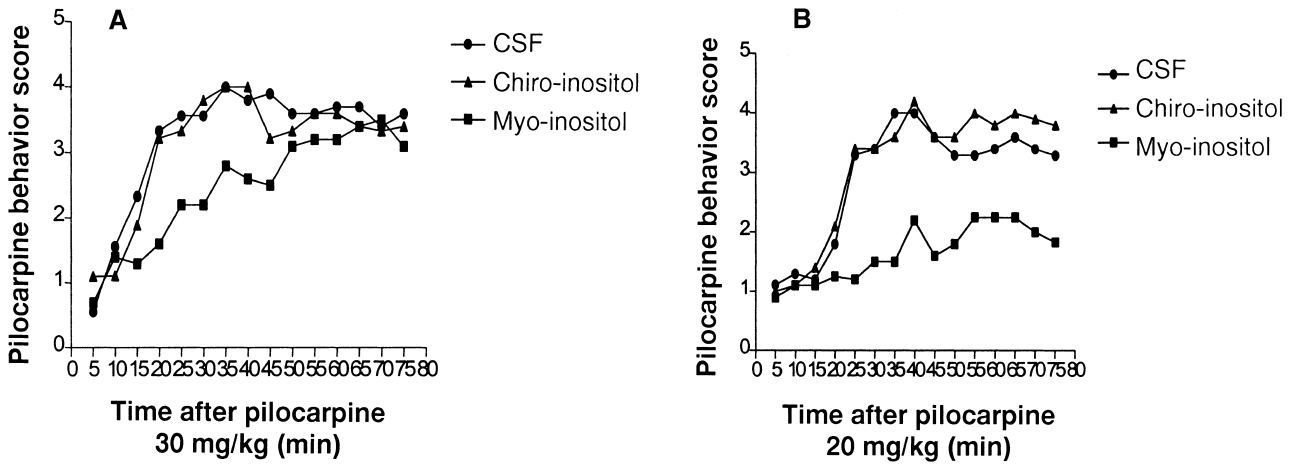

Figure 1. Score representing the intensity of pilocarpine-induced behaviors (ordinate) as described in the text. Abscissa represents the time in minutes following the injection of pilocarpine. **(A)** 30 mg/kg pilocarpine. Kruskal–Wallis tests indicated a significant effect of myo-inositol at 20 min ($H = 9.55$, $p < .01$), 30 min ($H = 12.42$, $p < .005$), 35 min ($H = 8.06$, $p < .02$), 40 min ($H = 12.69$, $p < .002$), and 45 min ($H = 12.97$, $p < .002$). **(B)** 20 mg/kg pilocarpine. Kruskal–Wallis tests showed a significant effect of myo-inositol at 25 min ($H = 14.28$, $p < .001$), 30 min ($H = 13.97$, $p < .001$), 35 min ($H = 16.31$, $p < .0005$), 40 min ($H = 9.6$, $p < .01$), 45 min ($H = 14.48$, $p < .001$), 50 min ($H = 9.51$, $p < .01$) and 55 min ($H = 7.25$, $p < .05$), 65 min ($H = 7.68$, $p < .05$), 70 min ($H = 8.69$, $p < .05$) and 75 min ($H = 9.38$, $p < .01$).

These results suggest that myo-inositol depletion is critical to the development of Li^+-pilocarpine seizures. The ICV injections of myo-inositol elevated cortical myo-inositol levels and simultaneously attenuated and prevented Li^+-pilocarpine seizures. The fact that seizures induced by a high dose of pilocarpine alone were not attenuated by myo-inositol suggests that the myo-inositol reversal is specific to the Li^+ effect.

THE EPI-INOSITOL PROBLEM

However, Williams and Jope (1995) found that the stereoisomer epi-inositol, which was not incorporated into PI, also prevents lithium–pilocarpine seizures. This unexpected effect of a stereoisomer, which, in contrast to myo-inositol, did not reverse either the teratogenic effect of lithium in xenopus oocytes (Busa and Gimlich 1989) or the suppression of neuronal firing of suprachiasmatic nucleus cells by lithium in vitro (Mason and Biello 1992), suggested that the mechanism of the reversal of behavioral effects of lithium by myo-inositol may be unrelated to the metabolism of PI-derived second messengers. Moreover, Williams and Jope (1995) reported that myo-inositol delayed, but did not prevent seizures induced by administration of pilocarpine to rats treated with chronic dietary lithium, raising the possibility that myo-inositol may not be relevant to chronic behavioral effects of lithium.

Table 1. Number of Rats Exhibiting Clonic Seizures (Score 4) in Rats Treated with 3 meq/kg Li^+ IP and 20 mg/kg pilocarpine SC 24 h Later

ICV Treatment	No Seizure	Seizure	Total
Artificial CSF	1	13	14
L-*chiro*-Inositol	0	12	12
Myo-Inositol	8	8	16

Chi-square for *myo*-inositol vs. vehicle = 6.53, $p < .01$.
Chi-square for *myo*-inositol vs. *chiro*-inositol = 8.40, $p < .004$.

Following the report by Williams and Jope (1995), we conducted a series of studies in an attempt to define the conditions under which the epi- and myo-inositol isomers reversed lithium–pilocarpine seizures (Patishi et al. 1996b). Three questions were raised: (1) Is epi-inositol effective following acute and chronic lithium treatment? (2) Can a low dose of myo-inositol, combined with an inactive stereoisomer of inositol, effectively attenuate lithium–pilocarpine seizures? Because epi-inositol could conceivably be converted to myo-inositol and may contain up to 10% of the active isomer (WR Sherman, personal communication), it was hypothesized that a low dose of myo-inositol, coadministered with a biologically inactive isomer, may be equally effective in reducing the effects of lithium. Myo-inositol is sequestered in various pools, and it has been estimated that only about 15% of cellular myo-inositol is involved in second messenger synthesis (Fain and Berridge 1979; Sherman 1991). Conceivably, under conditions of lithium-induced inositol depletion, the "false" inositol, might stimulate the release of myo-inositol from a hitherto "inactive" pool. (3) Is myo-inositol effective following chronic lithium, as has been previously reported for acute lithium treatment?

We (Patishi et al. 1996b) were able to replicate the results of Williams and Jope (1995), showing that epi-

inositol is also effective in attenuating lithium–pilocarpine seizures. Both myo-inositol and epi-inositol, also effectively prevented lithium-pilocarpine seizures following chronic lithium (Table 2).

Epi-inositol ICV attenuated seizures after both acute and chronic lithium. After acute lithium, 4/6 control rats, but only 1/7 rats treated with myo-inositol, and 0/7 rats treated with epi-inositol had seizures. Following chronic lithium, epi-inositol also reduced the number of rats showing clonus, preventing seizures in 3 of 8 rats ($c^2 = 3.69$, $p = .055$). All of the control rats had seizures. The latency to onset of clonus was significantly longer in epi-inositol-treated rats (59.2 ± 8.04 min, mean ± SD) than in control rats (27 ± 11.67 min) ($t = 5.38$, $p < .0005$). The serum lithium levels were 1.24 ± 0.5 mmol/l for the epi-inositol group and 1.22 ± 0.4 mmol/l for the control group.

The inactive steroisomer L-chiro-inositol, adulterated with a low dose of myo-inositol, did not affect lithium–pilocarpine seizures. Two of 12 rats treated with 9 mg l-chiro-inositol and 1 mg myo-inositol and 2/10 rats treated with vehicle did not have behavioral seizures. This difference was not statistically significant ($c^2 = 0.04$). The latency to the onset of seizures was 33 ± 15.53 min (mean ± SD) in the control rats and 42.1 ± 12.71 min in the inositol-treated rats.

ICV myo-inositol prevented lithium–pilocarpine seizures following chronic dietary lithium. After chronic lithium treatment, only two of seven rats pretreated with ICV myo-inositol had lithium–pilocarpine seizures; whereas, all seven of the vehicle-treated rats showed seizures at a mean latency of 22.14 ± 8.49 (mean ± SD) minutes. The reduction in the incidence of clonus by myo-inositol was significant ($c^2 = 7.78$, $p < .01$).

The finding that epi-inositol effectively blocked the onset of seizures in rats treated with acute or chornic lithium, followed by 20 mg/kg pilocarpine replicates the findings reported by Williams and Jope (1995). However, in contrast to their findings, we (Patishi et al. 1996b) found that both myo- and epi-inositol isomers were effective when rats were treated with chronic lithium using a lower dose of pilocarpine and a longer interval between the administration of myo-inositol and pilocarpine than they used. It is likely that the absence of blockade of chronic lithium–pilocarpine seizures by myo-inositol reported by Williams and Jope, can be attributed to differences in the dose of pilocarpine (30 vs. 20 mg/kg) and the interval between administration of ICV inositol and pilocarpine (30 min vs. one h). Williams and Jope found an increased latency to onset of seizures in the myo–inositol-treated group following chronic lithium, similar to that reported by our group using a higher dose (30 mg/kg) of pilocarpine and acute lithium (Kofman et al. 1993).

Although these data support the relevance of inositol depletion to chronic lithium treatment, it was unclear why the epi-inositol stereoisomer would be as effective as myo-inositol. Epi-inositol does not seem to be a substrate for phosphatidylinositol synthase (Benjamins and Agranoff 1969) nor, unlike myo-inositol, does it reverse teratogenic effects of lithium (Busa and Gimlich 1989) or lithium-induced suppression of suprachiasmatic nucleus cell firing in vitro (Mason and Biello 1992). It was, therefore, critical to re-examine the claim of Williams and Jope (1995) that epi-inositol is biochemically inactive in the PI cycle. To avoid the confounding effects of exogenous inositols on the specific activity of radiolabeled myo-inositol, the turnover of the PI cycle was determined by measuring levels of tritiated cytidine monophosphorylphosphatidate ([^{3}H]CMP-PA) in intact Chinese hamster ovary cells or cross-chopped slices of rat cerebral cortex (Richards and Belmaker 1996). These tissues were incubated in physiological buffer containing [^{3}H]cytidine in the absence or presence of increasing concentrations of myo-, epi-, L-chiro-, and scyllo-inositol for 60 min at 37°C. Carbachol and lithium (1 and 10 mM final concentrations, respectively) were then added, and the incubation continued for 30 min. Incor-

Table 2. Summary of the Effect of ICV *myo*-Inositol and *epi*-Inositol Following Various Regimens of Lithium Administration and 20 mg/kg Pilocarpine

Lithium Regimen	ICV Treatment	No. Animals with Clonus	Mean + SD Latency to Onset of Clonus (min)
Acute 3 meq/kg	Vehicle	4/6	NA
	epi-inositol	0/7	
	myo-inositol	1/7	
Chronic dietary—21 days	Vehicle	8/8	27 ± 11.67
	epi-inositol	5/8	59.2 ± 8.04
Acute 3 meq/kg	Vehicle	8/10	33 ± 15.53
	90% L-*chiro*- + 10% *myo*-inositol	10/12	42.1 ± 12.71
Chronic dietary—21 days	Vehicle	7/7	22.14 + 8.49
	myo-inositol	2/7	NA

NA—Latencies are not shown in cases where the number of rats showing seizures is small.

poration of radioactivity into cell membranes was quantified by scintillation spectrometry. In CHO cells, the accumulation of [^{3}H]CMP-PA was 60 ± 10 times basal (n = 13) in the presence of carbachol plus lithium. Neither scyllo-inositol (up to 10 mM) nor L-chiro-inositol (up to 30 mM) had an effect on the stimulated formation of [^{3}H]CMP-PA (Figure 2); whereas, 25 mM scyllo-inositol slightly inhibited the response (<20%). In contrast, both myo-inositol and epi-inositol concentration-dependently inhibited the accumulation of [^{3}H]CMP-PA induced by carbachol plus lithium (Figure 2). Full inhibition was attained with 10 mM myo-inositol; whereas, epi-inositol at 30 mM inhibited response by 83 ± 4% (n = 6). Myo-inositol was slightly but significantly ($p < .001$, unpaired t-test) more potent than epi-inositol: EC_{50} values were 1.9 ± 0.2, and 6.1 ± 0.6 mM, respectively (n = 6 for each). Although a smaller stimulation of [^{3}H]CMP-PA by carbachol plus lithium was found in rat cerebral cortex cross-chopped slices (9.8 ± 1.2 times basal, n = 3), the same order of activity was observed for the four inositol isomers in the two tissues (Richards and Belmaker 1996). Thus, there is a precise correlation between the effects of the four inositol isomers on PI turnover, as measured by their effects on the accumulation of [^{3}H]CMP-PA in two tissues, with their effects on lithium–pilocarpine-induced seizures. Both in vitro and in vivo, L-chiro- and scyllo-inositol had little or no effect; whereas, epi-inositol was less potent but almost as effective as myo-inositol. The perfect rank order of activity of these four inositol isomers in biochemical and behavioral tests strongly suggest a common basis of action.

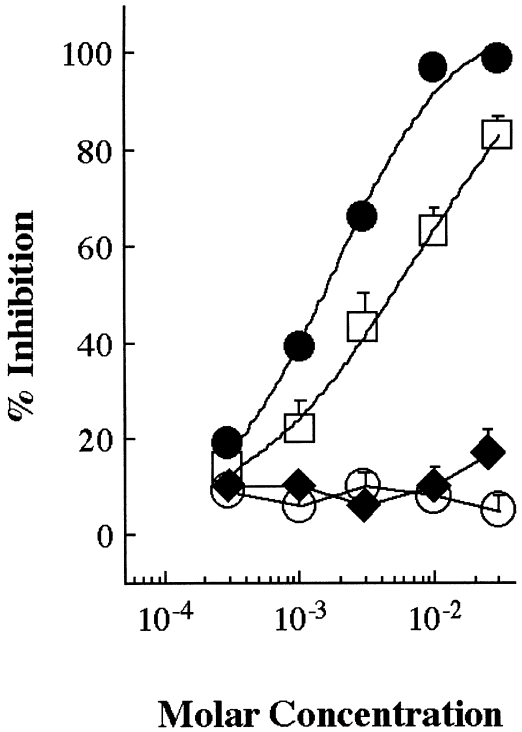

Figure 2. Inhibition of stimulated [^{3}H]CMP-PA formation in CHOm3 cells by myo-inositol (●), epi-inositol (□), scyllo-inositol (◆) (n = 6 for each) and L-chiro-inositol (○, n = 3). Values are means ± SEM for (n) experiments. (From Richards and Belmaker 1996).

ANATOMICAL SPECIFICITY OF INOSITOL DEPLETION BY LITHIUM

Although attractive and seminal, the "inositol depletion hypothesis" has been questioned on the grounds that, even with toxic doses of lithium, the brain's depletion of inositol is only small and might be restricted to acute treatment. Furthermore, although in vitro and ex vivo experiments indeed showed an attenuation of agonist-stimulated inositol phosphate formation in brain slices of lithium-treated rats, these effects were apparently caused by an exaggerated depletion of inositol in the slice system and were not observed in other species, in which the supply of inositol is not as readily compromised (Jope and Williams 1994). It has been argued, however, that a lithium-induced depletion of inositol might be limited to selected brain areas or even cells that might be particularly vulnerable to this effect because of restricted inositol supply and/or increased activity of the inositol phospholipid second messenger system (Nahorski et al. 1991).

We (Lubrich et al. 1997) have, therefore, measured the effect of both acute and chronic treatment with lithium on the inositol content in various areas of rat brain. The results indicate that after chronic treatment, a lithium-induced decrease in inositol is significantly evident in the hypothalamus (Lubrich et al. 1997).

Rats were fed chow containing 0.2% Li chloride for 21 days or regular feed. Separate bottles of water and 0.9% NaCl solution were provided ad libitum for both groups of animals. After 21 days, animals were sacrificed by decapitation, the fresh brains were dissected into five regions, Li levels were measured in carotid blood and were found to be 0.79 ± 0.16 mM. Samples were stored at −70°C until assay by high-performance liquid chromatography (HPLC) analysis blind to the treatment of each sample.

All reagents were purchased from Merck (Darmstadt) with the exception of glucose oxidase and pyridine from Sigma (Munich). Pyridine was kept dry by storing over a molecular sieve 0.4 nm (Merck, Darmstadt). Solvents for the mobile phase and chloroform for dissolving the derivates were all HPLC grade. All other chemicals were analytical grade. A Waters HPLC system of Waters 501 pump and a Waters 486 UV detector set at 254 nm were used for all HPLC separations. As column, a 10-μ porasil column (4.6 × 300 mm) (Grom/Herrenberg-Kayh) was used. The frozen tissue was

thawed and roughly homogenized with a spatula, while kept on ice. To an aliquot of 5 to 15 mg of each sample 500 μl of deionized water was added, then vortexed for 10 s. The samples were deproteinated by heating to 95°C for 5 min. After vortexing once more, the homogenates were centrifuged at 13,000 *g* for 30 min at 5°C. The entire supernatant was transferred to 2-ml plastic tubes with 20 units glucose oxidase. The tubes were then incubated for 40 min at 37°C in a shaking water bath. Aliquots of 50 μl of each sample were transferred to glass tubes (Assistent/Sondheim-Roehn) containing 0.2 μl epi-inositol as internal standard. The probes were evaporated in a vacuum centrifuge (Speed vac 200, Bachofer/Reutlingen), followed by addition of 200 μl of a freshly prepared solution of 20% (w/v) p-nitrobenzoyl chloride in pyridine to each sample. After mixing, samples were incubated for 90 min at 60°C. The reaction was stopped by adding a few drops of deionized water until a solid pellet formed. Immediately, 2 ml of chloroform were added, and the pellet was dissolved by vortexing vigorously. Six extractions were performed with twice 2 ml deionized water, twice 2 ml 5% NaHCO3 (w/v), and twice 3 ml 1N HCl. Following each extraction, the samples were centrifuged for 1 min to separate the phases, and the aqueous phase was eliminated. Twenty μl of the organic phase were injected into a 10-μ porasil column, using hexane-chloroform-acetonitrile (10:3:2) and water 0.1% as mobile phase. The flow rate was 1.5 ml/min. The integrator was calibrated using pure standards at several concentrations between 0 and 1.5 μg for myo-inositol and 0 and 0.5 μg for epi-inositol. UV detection was at 254 nm. Retention times were about 12 min for myo-inositol and 14 min for epi-inositol, respectively. Each sample was analyzed in duplicate.

After chronic dietary administration of LiCl, a significant reduction of inositol was found only in the hypothalamus ($p < .01$) whereas no effect was found in the other brain areas (Table 3). This reduction was significant even after Bonferroni correction for multiple testing (five areas). No (Sherman et al. 1981; Hirvonen and Savolainen 1991; Whitworth and Kendall 1989), or only minute (Sherman et al. 1985a), reductions of the inositol content in various rat brain areas have been found after chronic treatment with lithium given either as daily injection [3.5 or 2.5 mEq/kg, (Sherman et al. 1981; Hirvonen and Savolainen)] or in the diet (Sherman et al. 1985; Withworth and Kendall 1989), and dietary lithium was even reported to increase cortical inositol by 30% (Withworth and Kendall 1989). In agreement with most reports, we found no alteration of inositol after chronic lithium treatment in the brain areas examined, with the exception of the hypothalamus. This region has, to our knowledge, never been previously examined after chronic treatment, but a 26% reduction of inositol was found 6 hours after acute injection of 10 mEq/kg lithium (Sherman et al. 1986).

The effect observed in the hypothalamus is interesting, because this region has been implicated in the neurobiology of affective disorders. Previous work from our groups has shown that the hypothalamus, the brain region with the highest basal inositol content (Table 3), accumulates inositol given intraperitoneally more efficiently than other brain regions, particularly the cortex, the region with the lowest basal inositol content (Patishi et al. 1996c).

INOSITOL IN POSTMORTEM BRAIN

Lithium reduces brain inositol levels (Allison and Stewart 1971), and considerable research has focused on the role of inositol in the mechanism of action of Li (Kofman and Belmaker 1993). Somewhat paradoxically, Barkai et al. (1978) reported that inositol is reduced in CSF in both bipolar and unipolar depressed patients. Levine et al. (1993) gave inositol to 11 unipolar-resistant depressed patients, with dramatic results in seven of the 11. Levine et al. (1995) then performed a controlled double-blind study of 28 depressed patients with 12 gm of inositol or placebo for 4 weeks. Inositol treatment reduced Hamilton Depression Scale Scores (HDS) significantly more than placebo. We (Shimon et al. 1997) were able to measure inositol and inositol monophosphatase, the enzyme that forms inositol in brain, in postmortem brain specimens from patients with bipolar affective disorder, in suicides, and in normal controls.

Brain specimens in the National Institute of Mental Health (NIMH) brain collection were obtained at autopsy from the Washington, DC Medical Examiner's office. Blood and urine samples were collected at the same time for toxicological analysis and for neuroleptic level determination. None of the subjects had measurable serum levels at the time of death. All brains were screened by a neuropathologist for confounding neurological conditions (none were found in the specimens used in this study). Psychiatric diagnosis was determined by independent review of medical records by at least two psychiatrists. After collection from autopsy, the brain tissue was dissected into 1-cm coronal slabs,

Table 3. Effect of Chronic Lithium on Brain Inositol Levels (mmol/kg wet weight)

	Control	Li-Treated
Hypothalamus	4.37 ± 1.37	3.46 ± 0.96*
Cortex	2.31 ± 0.66	2.23 ± 0.86
Cerebellum	3.08 ± 1.1	2.79 ± 0.8
Caudate	2.65 ± 0.89	2.66 ± 0.79
Hippocampus	3.12 ± 1.01	2.82 ± 0.73

Effects of chronic (3 weeks) dietary treatment with lithium (0.2% LiCl w/w) on the level of inositol in five brain regions. Student's *t*-test was significant for hypothalamus ($p = .004$). Chronic oral lithium lowers inositol by 27%.

which were individually frozen in isopentane cooled with dry ice (−40°C). Tissue blocks were stored at −70°C until dissection for this study. Postmortem interval (PMI) was defined as the time from death until removal of the brain into the frozen state. Lithium was measured in bipolar patient and suicide victim brains by flame emission spectroscopy and was undetectable in all, except for three bipolar patients with levels of 0.35, 0.48, and 0.23 mmol/kg wet weight.

Human brain free myo-inositol levels were analyzed as trimethylsilyl (TMS) derivatives by gas–liquid chromatography, as previously described by Allison et al. (1976), with minor modification. Samples of tissue (approximately 50 mg) were dissected from the various brain areas, weighed, extracted in 0.5 ml of boiling water containing 400 μg mannitol for 5 min, the denatured tissue was spun down, and 250μl: 1 supernatant lyophilized (3 h Speed Vac SC 110); silylation of the dried sample was carried out with 200μl: 1 of a mixture of pyridine: bis(trimethylsilyl) trifluoroacetamide: chlorotrimethylsilan 10:2:1 (v/v/v) for 24 h at room temperature. 2μl: 1 aliquots were chromatographed on a 6-ft column packed with 3% SE-30 on 80/100 mesh gas chrome Q (Supelco), using a Carlo Erba SCU 600 gas chromatograph with a hydrogen flame ionization detector. The oven temperature was isothermal at 220°C, and the carrier gas was nitrogen with a flow of 120 ml/min. The TMS derivatives of mannitol and myo-inositol had retention times of 7 and 11 min, respectively. Under these conditions, quantitation was performed with the use of TMS derivatives of standard myo-inositol under the same conditions and with mannitol as an internal standard. Standard curves were run daily, and linearity was verified at the beginning and periodically during the processing of the samples.

Enzyme activity was measured (Hallcher and Sherman 1980) in brain homogenates obtained by the addition of 2.0 ml of homogenization buffer (50 mM Tris-HCl pH 8.5, 150 mM KCl, 0.5 mM EDTA and 0.1 mM EGTA) to 0.1 g of tissue. Brain homogenization was carried out in a cell disrupter followed by 15 min centrifugation at 7,500 *g* and 4°C. The reaction mixture contained in a final volume of 210μl: 1, 0.7 mM inositol-1-phosphate, 50 mM Tris-HCl pH 7.8, 250 mM KCl, 3 mM $MgCl_2$ and 90μl: 1 enzyme preparation. Incubation was carried out for 1 hour at 37°C and then 10μl: 1 of 100% solution tricholoroacetic acid (TCA) were added to stop the reaction. The mixture was centrifuged for 15 min at 7,500 *g* and 4°C. Inorganic phosphate in the supernatant was determined spectrophotometrically (Taussky et al. 1953). To distinguish inositol monophosphatase activity from nonspecific phosphatases, we use the fact that inositol monophosphatase is inhibited by Li. The reaction was carried out in the absence and in the presence of 30 mM Li, each tube in triplicate. The enzyme activity was calculated as the difference between the values in the presence and in the absence of Li. Enzyme activity is calculated per mg protein, assayed according to Lowry et al. (1951).

All samples were assayed at least twice (of the triplicate tubes), and the results presented are the average of the replicates. Assays were performed in a balanced design so that each run included samples from each clinical group and controls and all available brain areas.

To evaluate possible effects of postmortem decay on brain inositol and inositol monophosphatase, rats were

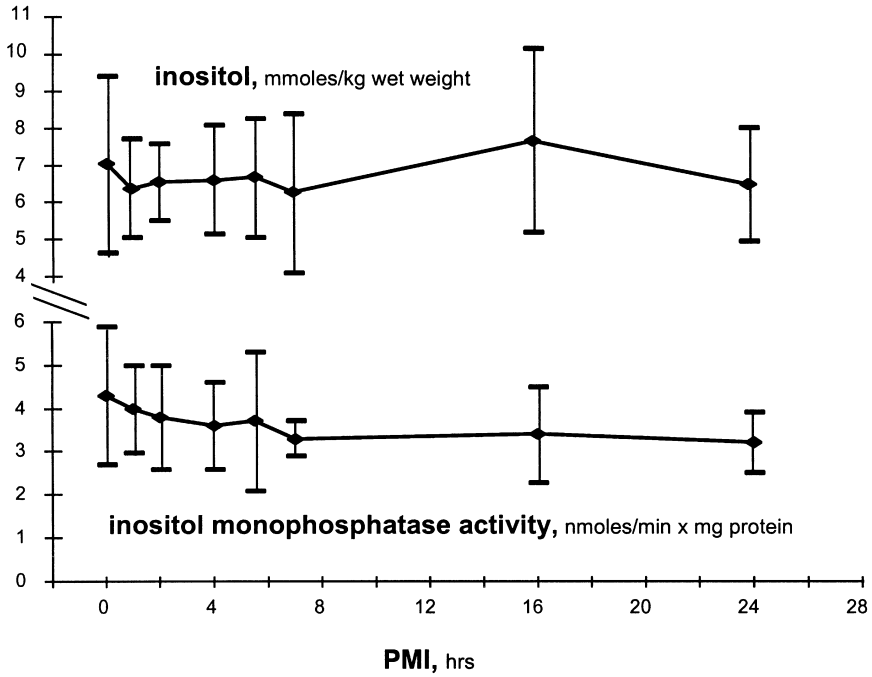

Figure 3. The effect of postmortem interval (PMI) on rat brain inositol monophosphatase activity and inositol levels (x ± SD, N = 7–8 at each time point).

killed by decapitation, and the heads were left out at room temperature for 0, 1, 2, 4, 6, 7, 16, or 24 hrs. After the specified times, the brains were removed from the skulls and frozen at −70°C, until assay as above for inositol and inositol monophosphatase. All assays were performed in a balanced design so that each run included one sample from each time point.

Figure 3 shows inositol levels and inositol monophosphatase levels up to 24 h after decapitation in rat brain. There was no significant effect of PMI up to 24 h on rat brain inositol or inositol monophosphatase activity.

Table 4 contains the demographic characteristics of the sample. Table 5 demonstrates the inositol results. Analysis of variance (ANOVA) shows a significant difference between the groups for frontal cortex (F = 3.83, df 2/25, p = .035), but not for occipital cortex (F = 1.8, df 2/24, ns) or cerebellum (F = .03, df 2/21, ns). Post-hoc t-test for frontal cortex shows suicides significantly lower than controls (p = .01) and bipolars significantly less than controls (p = .01). Omitting subjects with unknown PMI or PMI greater than 30 hours, ANOVA shows a significant difference for frontal cortex (F = 4.24, df 2/21, p = .03) and post-hoc t-test is significant for normals vs. bipolars ($p < .05$), and normals vs. suicides (p = .01).

Nonparametric analysis shows significant group difference with the Kruskall–Wallis ANOVA for frontal cortex (2, H = 6.3, p = .04, N = 28). Bipolars are lower than normals in frontal inositol with Kruskall–Wallis (1,

Table 4. Demographic Data

	Bipolars	Suicide	Normal
Run 1			
Age[a]	47	86	58
Sex	F	F	F
PMI[b]	16.5	22.0	29.75
Run 2			
Age	49	65	68
Sex	F	M	M
PMI	28.0	19.75	19.75
Run 3			
Age	36	34	84
Sex	F	F	M
PMI	24	20.5	18.0
Run 4			
Age	51	55	47
Sex	F	F	M
PMI	17.25	24.0	24.0
Run 5			
Age	83	46	54
Sex	F	F	M
PMI	46.0	28.0	22.0
Run 6			
Age	64	29	39
Sex	F	M	F
PMI	Unknown	21.0	41.5
Run 7			
Age	50	52	46
Sex	M	F	F
PMI	21.25	15.0	22.5
Run 8			
Age	31	77	34
Sex	M	M	M
PMI	Unknown	26.5	14.0
Run 9			
Age		41	47
Sex		M	M
PMI		7.0	24.5
Run 10			
Age		51	59
Sex		F	F
PMI		21.0	17.0

[a] Age in years.
[b] PMI in hours.

Table 5. Inositol Levels (mmole/kg wet weight)

Frontal			
Run	**Bipolar**	**Suicide**	**Normal**
A	8.18	6.48	11.6
B	7.05	6.35	10.4
C	8.82	5.75	10.5
D	4.90	6.02	7.02
E	4.35	4.37	8.85
F	2.27	5.23	6.55
G	5.90	7.80	4.05
H	5.75	5.40	11.4
I	——	8.45	9.03
J	——	12.1	8.05
x ± SD	5.90 ± 2.12	6.74 ± 2.21	8.74 ± 2.39
Occipital			
Run	**Bipolar**	**Suicide**	**Normal**
A	11.2	10.8	7.00
B	7.24	5.55	6.35
C	6.95	5.30	8.15
D	5.55	5.77	9.25
E	——	5.40	12.5
F	1.77	7.65	6.55
G	8.10	9.25	10.8
H	6.05	3.15	16.5
I	——	9.65	9.48
J	——	6.66	4.20
x ± SD	6.69 ± 2.84	6.91 ± 2.37	9.08 ± 3.53
Cerebellum			
Run	**Bipolar**	**Suicide**	**Normal**
A	10.55	8.90	5.55
B	5.0	4.35	4.45
C	——	5.55	5.30
D	——	5.57	8.30
E	——	6.91	11.2
F	——	5.55	13.80
G	8.00	15.5	8.35
H	5.35	5.00	7.15
I	——	13.15	8.05
J	——	6.85	4.75
x ± SD	7.22 ± 2.58	7.72 ± 3.73	7.69 ± 2.98

$N = 18$; H = 4.93; $p = .026$), and suicides are lower than normals in frontal inositol with Kruskall–Wallis (1, $N = 20$; H = 3.86; $p < .05$). Omitting subjects with unknown PMI or PMI greater than 30 hours, Kruskall–Wallis ANOVA is even more significant (2, $N = 24$, H = 7.5, $p = .02$). Kruskall–Wallis for these data shows bipolar patients' frontal inositol lower than controls, (H, 1 = 3.7; $N = 14$; $p = .053$), and suicides' frontal inositol is lower than controls (H, 1 = 6.4; $N = 19$; $p = .011$).

A parametric covariance analysis of frontal inositol levels with PMI as covariant was performed. ANOVA group difference after covariance was significant, F = 3.7, df 2/22, $p = .04$). PMI correlated with frontal inositol ($r = -.39$, $N = 26$, $p = .046$). Excluding the two subjects with PMI greater than 30 hours, correlation of PMI and frontal inositol declined to $r = -.13$ (ns).

There was no significant correlation of age with frontal ($r = -.06$), occipital ($r = -.16$), or cerebellum ($r = -.24$) inositol levels. There was no significant difference in frontal cortex inositol between women (6.7 ± 2.7) and men (7.9 ± 1.9), in occipital cortex between women (7.1 ± 3.1) and men (8.3 ± 2.9), or in cerebellum between women (7.4 ± 2.3) and men (8.4 ± 3.7). In occipital cortex, PMI correlates with inositol, $r = -.27$ (ns) and in cerebellum, $r = -.23$ (ns).

Table 6 shows the inositol monophosphatase results. Inositol monophosphatase activity in these human brain cortex samples is less than one-third the activity in rat cortex (Patishi et al. 1996a), see Figure 3. Inositol monophosphatase activity does not correlate with PMI ($r = -.16$, frontal; $r = -.17$, occipital; and $r = -.13$, cerebellum). Inositol monophosphatase activity does not correlate with inositol levels (frontal $r = .11$, ns; occipital $r = -.11$, ns; cerebellum $r = -.15$, ns). Inositol monophosphatase activity correlates with age in frontal cortex ($r = .24$, ns) in occipital cortex ($r = .51$, $p = .01$) and in cerebellum ($r = .51$, $p = .01$). There was no difference in inositol monophosphatase activity between suicides, bipolar disorder, and normal control groups by ANOVA.

These data suggest that bipolar affective disorder and suicide victims may have a reduction in frontal cortical inositol levels as compared with controls, with a similar but statistically nonsignificant trend in occipital cortex. Cerebellum inositol shows no difference between the clinical groups. Interestingly, cerebellum also shows no increase in inositol-1-phosphate after Li treatment (Allison et al. 1976), suggesting that cerebellum inositol may be less involved in neuronal PI-linked signal transduction and more related to osmolyte function. A recent study found that exogenously administered inositol is taken up by cortex, hippocampus, and hypothalamus but not by cerebellum (Patishi et al. 1996c).

Inositol levels in the normal samples, about 9 mM, agree well with in vivo results by magnetic resonance spectroscopy (Gruetter et al. 1992). Dixon et al. (1992) reported that primate brain inositol levels are two times higher than those in the rat. The standard deviation of inositol levels in these postmortem samples is similar in controls and in suicides and bipolar patients, suggesting that postmortem degradation or agonal illness in a subgroup of patients are not critical factors in the reduced inositol levels. Moreover, the standard deviation is similar to that in freshly decapitated rat cortex (Agam et al. 1994; also see Figure 3).

There were no significant differences between clinical groups in inositol monophosphatase activity. Inositol monophosphatase has previously been reported to be elevated in red blood cells (RBC) of schizophrenic

Table 6. Inositol Monophosphatase Activity (nmol/min × mg protein)

Frontal			
Run	**Bipolar**	**Suicide**	**Normal**
A	1.899	1.298	1.547
B	1.170	0.713	0.939
C	0.467	1.599	2.038
D	2.168	2.917	0.508
E	0.648	0.723	1.275
F	0.804	1.834	0.923
G	0.818	1.631	0.388
H	0.411	0.789	1.435
I	——	0.632	1.034
J	——	0.688	0.722
x ± SD	1.047 ± 0.655	1.282 ± 0.734	1.081 ± 0.503
Occipital			
Run	**Bipolar**	**Suicide**	**Normal**
A	3.655	1.888	1.753
B	2.058	2.233	1.413
C	0.315	2.184	3.961
D	0.726	1.466	1.743
E	——	0.844	1.316
F	2.157	0.770	1.246
G	0.802	0.854	1.164
H	0.392	2.317	0.627
I	——	1.052	0.713
J	——	2.046	1.313
x ± SD	1.458 ± 1.222	1.566 ± 0.636	1.524 ± 0.931
Cerebellum			
Run	**Bipolar**	**Suicide**	**Normal**
A	1.902	1.837	1.446
B	0.771	1.990	1.110
C	——	0.297	1.280
D	——	0.387	0.895
E	——	0.542	0.364
F	——	0.653	0.528
G	0.469	0.281	0.275
H	0.224	0.227	1.163
I	——	0.370	0.285
J	——	0.804	0.581
x ± SD	0.841 ± 0.741	0.738 ± 0.645	0.792 ± 0.439

patients by 35% (Zilberman-Kaufman et al. 1992). Inositol monophosphatase varies in RBC between individuals by a full order of magnitude (Agam and Livne 1989), and the variability in human brain between individuals is thus similar. Inositol monophosphatase activity increases with age (Patishi et al. 1996), as does the activity of monoamine oxidase (MAO), also a neurotransmitter-related catabolic enzyme (Murphy et al. 1977). It is noteworthy that human brain inositol monophosphatase exhibits region differences as observed for rat brain inositol levels (Table 3). IMPase activity was lowest in cerebellum, highest in occiptal cortex, and medium in frontal cortex (Table 6).

Inositol levels and inositol monophosphatase activity do not clearly decline in the first 24 h postmortem, either in the rat experiment or in the human brain covariance analysis. Inositol is not metabolizable in brain (Sherman 1991), and this argues against postmortem effects as the cause of the finding of reduced frontal inositol levels. Inositol phosphates that could be metabolized to inositol postmortem are present in very small concentrations as compared to inositol (Sherman 1991) and are unlikely to be able to explain the clinical difference. These brain samples have also been studied for other biochemical factors (HK Manji, personal communication) including G protein α subunits and PKC isozymes, especially the PKC γ, which is sensitive to proteolytic degradation, and have been found to have robust levels compared with rat cortex, with no correlation with PMI and no suggestion of postmortem degradation.

High-dose acute Li is reported to lower inositol in rat brain cortex, but therapeutically equivalent acute doses or chronic Li have smaller and less consistent effects (Sherman et al. 1985a). Therapeutic doses of Li lower cortical inositol by about at most 10%, and toxic doses by at most about 30% (Agam et al. 1994). Thus, Li overdoseage is not the likely cause of the 25% reduction of frontal cortical inositol in these patients.

The pathophysiological implications of low frontal cortex inositol are unclear. Phosphatidylinositol (PI) synthase may not be saturated at physiological levels of 10 mM inositol (Ghalayini and Eichberg 1985). Several intracellular pools of inositol may exist (Bersudsky et al.

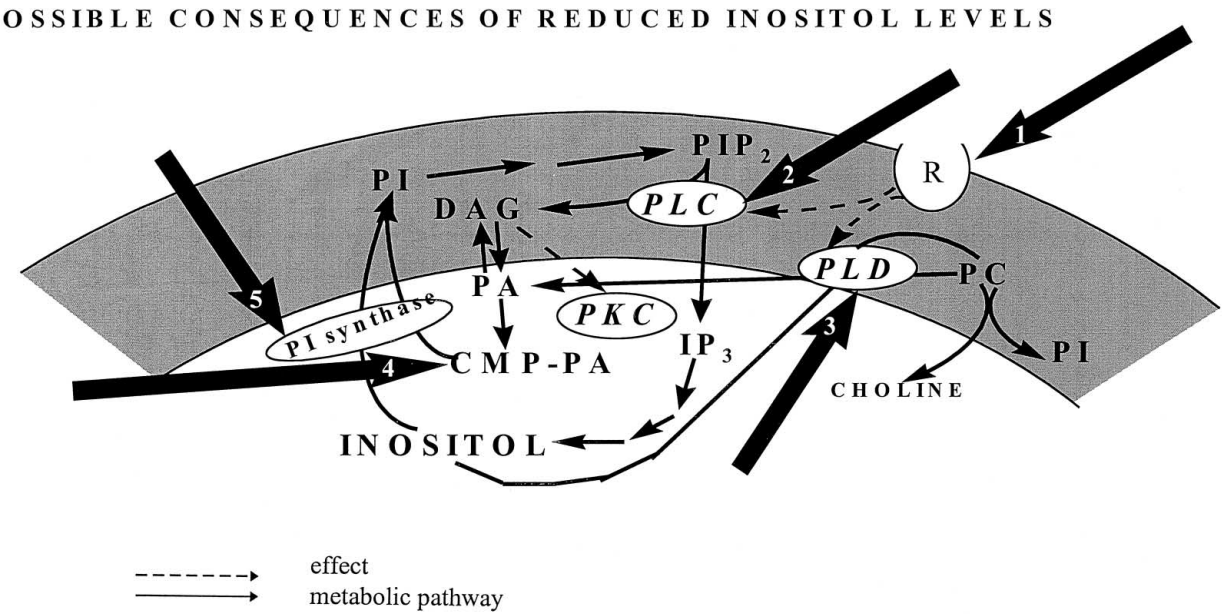

Figure 4. Description of the sites of possible effect of reduced inositol levels (depicted by the bold arrows) according to their numbers: 1. In the absence of exogenous inositol, 5-HT induces heterologous desensitization of the facilitated NMDA depolarization in response to 5-HT agonists (Rahman and Neuman 1993); suggested mechanism: receptor sequestration; 2. In systems with low inositol level, coupling of PLC and PI synthase stimulation is controlled by intracellular inositol concentration. Depletion of inositol attenuates accelerated PI resynthesis driven by agonist-activation of PLC (Batty and Downs 1995); 3 (a). In response to agonists, in addition to PI hydrolysis by PLC, PLD cleaves PC to produce PA, which is dephosphorylated to DAG (Nishizuka 1992); (b) PLD also exchanges the choline moiety in PC with free inositol to produce PI, which may be further cleaved by PLC to produce more DAG. Inositol depletion may attenuate these pathways, decrease DAG levels, resulting in deficient PKC activation; 4. Reduced inositol levels result in increased CMP-PA accumulation because of a deficiency in inositol available to combine with CMP-PA (Godfrey 1989), which may lead to DAG accumulation and subsequent PKC desensitization; 5 (a) In cells with low inositol levels, PI synthase is possibility unsaturated (Km for inositol 4.6 mM, Ghalayini and Eichberg 1985), decreasing PI levels; (b) Reduced inositol levels may relieve repressed gene expression of INO1 (inositol-1-P synthase) and FAS_1 and FAS_2 (fatty acid synthase) via ICRE (inositol/choline responsive elements) existing in yeast genes (Lopes and Henry 1991; Schuller et al. 1992) resulting in elevated PI resynthesis; this may also exist in mammals.

1994), and the reduction in frontal cortex may be specific to a pool critical for neuronal second messenger function. Batty and Downes (1994, 1995) have reported that coupling of PLC and PI synthase requires high enough inositol levels. If "normal" inositol levels do regulate PI concentration and phospholipase C activity, which breaks down PI in response to receptor stimulation, then low inositol levels could cause functionally deficient responses to one or more receptors linked to PI.

Inositol functions as an important brain osmolyte (Thurston et al. 1989) as well as a second messenger precursor. Hyponatremia lowers brain inositol, and if suicides or affective patients were more likely to be hyponatremic for several days before death than normals, this could artifactually lower brain inositol in the non-neurotransmitter-related pool. Agonal diseases (Hardy et al. 1985) in these patients cannot be ruled out as a cause of the finding. Diabetic ketoacidosis (Kreis and Moss 1992) and Alzheimer's disease (Miller et al. 1993) have been reported to raise brain inositol. Hepatic encephalopathy markedly reduces brain inositol (Haussinger et al. 1994). Postmortem brain studies have inherent methodological limitations (Palmer et al. 1988). Brain inositol may be measured in vivo by magnetic resonance spectroscopy (MRS). Gruetter et al. (1992) and Deicken et al. (1995) have reported phosphomonoester data consistent with our postmortem findings.

CONCLUSION

Inositol is a simple compound present in high concentration in brian, and it is difficult in this age of increasing specificity of receptor-active psychotropics, for many psychopharmacologists to see it as a potential therapeutic mechanism. However, L-dopa was similarly difficult to accept as a precursor requiring gram dosages for effectiveness, and it is still not clear how exogenous L-dopa is therapeutic in Parkinson's disease, where cell death leads to massive changes in specific innervation. The fact that lithium lowers inositol and bipolar patients have low frontal cortical inositol would seem to be a self-contradictory hypothesis. However, Figure 4 illustrates several different points in the PI cycle where Li-induced decrease in inositol levels or psychopathology-associated reduction in inositol levels could affect the functioning of the cycle. The effects are complex and could be opposing in different brain areas, in different subcellular compartments, and under different baseline conditions.

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