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The common inositol-reversible effect of mood stabilizers on neurons does not involve GSK3 inhibition, *myo*-inositol-1-phosphate synthase or the sodium-dependent *myo*-inositol transporters

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We previously showed that the mood stabilizers lithium, valproate (VPA), and carbamazepine (CBZ) have a common, inositol-reversible effect on the dynamic behavior of sensory neurons, suggesting that they all inhibit phosphoinositide (PIns) synthesis. We now report similar effects of the drugs in cortical neurons and show by mRNA analysis that these neurons do not express *myo*-inositol-1-phosphate synthase (MIP-synthase) or the sodium-dependent *myo*-inositol transporters (SMIT1 and SMIT2), but they do express the H^+/*myo*-inositol transporter (HMIT) mRNA and protein. We used glycogen synthase kinase-3 (GSK3) inhibitors and Western blotting of GSK3 targets to confirm that the common effects of the drugs on both sensory and cortical neuron growth cones are inositol-dependent and GSK3-independent. Moreover, the anti-convulsant drugs gabapentin and phenytoin do not mimic the mood stabilizers. These results confirm that the common inositol-reversible effect of mood stabilizers on neurons does not involve GSK3 and further show that the effects are independent of MIP-synthase and SMIT transporters.

Keywords: Sensory neurons; GSK3; Phosphoinositide signaling; Lithium; Valproate; Carbamazepine; Inositol; Transporters; MIP-synthase

Introduction

Bipolar disorder is a devastating psychiatric illness that affects about 1–2% of the world population. The mood-stabilizing drugs used to treat this illness target several intracellular signaling pathways and a better understanding of which targets are therapeutically relevant should shed light on the underlying causes of this illness and may also help in the development of more specific drugs. We showed previously that the three most often prescribed mood stabilizers—lithium, VPA, and CBZ—have a common effect on the dynamic behavior of rat sensory neuron growth cones. All three drugs inhibit the collapse and increase the spread area of growth cones (Williams et al., 2002). Moreover, both these effects are reversed by the addition of inositol to the culture medium, suggesting that all three mood-stabilizing drugs inhibit recycling of PIns, because this is the only known effect of inositol on intracellular signaling (Batty and Downes, 1995). Given the commonality of the drug effects in PIns signaling, we hypothesized that this pathway was the most likely therapeutic target for the drugs, and further, that defects in the regulation of PIns signaling may underlie bipolar disorder.

Lithium directly inhibits two key enzymes involved in PIns recycling, inositol monophosphatase (IMPase), and inositol polyphosphate 1-phosphatase (IPPase). Berridge et al. (1989), and later Batty and Downes (1995), suggested that these inhibitory effects on the PIns cycle may partly explain the therapeutic action of lithium. We recently showed that VPA directly inhibits the enzyme prolyl oligopeptidase (PO) (Cheng et al., 2005), which is also implicated in regulation of PIns metabolism, and we proposed that VPA inhibition of PO may partly explain the dual action of the drug in limiting mood swings to both mania and depression (Cheng et al., 2005). Both lithium and VPA treatment decrease brain inositol levels (by ~40% or less) in both rodents and humans (Silverstone et al., 2005), but it is not clear if this global decrease in inositol is relevant for their therapeutic action. Brain inositol levels are regulated by both transport from blood and by synthesis from glucose-6-phosphate to *myo*-inositol-1-phosphate by the enzyme MIP-synthase, whose activity is inhibited indirectly by VPA (Agam et al., 2002; Shaltiel et al., 2004). MIP-synthase expression in brain is confined to the vasculature (Wong et al., 1987), so it is unclear whether this enzyme contributes to the inositol-reversible effects of the mood stabilizers on neurons (Williams et al., 2002).

Another proposed target for the effects of mood stabilizers on inositol levels in the brain is the sodium-dependent *myo*-inositol transporter (SMIT) (van Calker and Belmaker, 2000). All three

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mood stabilizers affect high-affinity inositol transport in astrocytes by decreasing levels of mRNA encoding SMIT1 (Lubrich and van Calker, 1999). In the mature brain, the highest level of expression of SMIT1 is in the choroid plexus (Guo et al., 1997), although SMIT1 mRNA expression is increased in neural tissue following brain injury (Guo et al., 1997). There is a second sodium-dependent *myo*-inositol transporter, SMIT2, that is widely distributed in several tissues including the brain (Coady et al., 2002). Uldry et al. (2001) recently described an H^+-dependent *myo*-inositol transporter, HMIT, with a more restricted distribution that is expressed in several brain regions including neurons of the frontal cortex. Interestingly, HMIT is inserted into the neuronal plasma membrane from a vesicular compartment in an activity-dependent manner, suggesting that HMIT may be involved in regulating neuronal PIns synthesis (Uldry et al., 2004).

Lithium also inhibits GSK3 directly leading Klein and colleagues to suggest that GSK3 may instead be the therapeutically relevant target for lithium in mood stabilization rather than PIns signaling (Klein and Melton, 1996; O'Brien et al., 2004; Phiel and Klein, 2001). There are several reports that VPA also inhibits GSK3 either directly or indirectly (Chen et al., 1999; Hall et al., 2002; Kim et al., 2005; Werstuck et al., 2004, De Sarno et al., 2002), but there are other reports that VPA does *not* inhibit GSK3 either directly or indirectly (Jin et al., 2005; Phiel et al., 2001; Ryves et al., 2005; Williams et al., 2002). Another direct target for VPA is histone deacetylase (HDAC), whose inhibition results in changes in neuronal structure due to an increase in β-catenin mRNA and protein levels (Phiel et al., 2001).

Following our report on the inositol-reversible effects of mood stabilizers on rat sensory neuron growth cones, others have suggested that the effects of lithium and VPA on growth cones are instead mediated by inhibition of GSK3. For example, Owen and Gordon-Weeks (2003) reported that the GSK3 inhibitor SB-216763 and 10 mM lithium increased the growth cone spread area of chick sensory neurons. While studying the effects of Wnt signaling on neural development, Salinas and colleagues found that both lithium and VPA increased the spread area of growth cones of cerebellar mossy and granule neurons (Hall et al., 2000, 2002; Lucas and Salinas, 1997), but these drug effects were not reversed by inositol. Guidance cues such as semaphorins, which are involved in directing axons to or away from particular targets, can induce a dramatic 'collapse' of growth cones; the axons retract and do not elongate while semaphorin is present. Semaphorin 3A-induced 'collapse and retraction' behavior is dependent on activation of a pool of inactive GSK3 at the leading edge of the sensory neuron growth cones, which was elegantly demonstrated by Eickholt et al. (2002) using the GSK3 inhibitors SB-216763 and SB-415286 as well as 20 mM lithium. In discussing the evidence that the relevant therapeutic target of lithium in the treatment of bipolar disorder is either inhibition of GSK3 or inhibition of PIns signaling, O'Brien et al. (2004) make no distinction between semaphorin-induced 'collapse and retraction' and the cycles of dynamic 'collapse and spread' we described (Williams et al., 2002). In addition, O'Brien et al. (2004) comment that there may be additional functions of inositol other than the known effects on PIns recycling because addition of *myo*-inositol can reverse the developmental defects induced by dominant-negative GSK3β in *Xenopus* (Hedgepeth et al., 1997).

Our study on growth cones (Williams et al., 2002) is the only example of effects of all three mood stabilizers—lithium, VPA, and CBZ on a common intracellular signaling pathway in neurons. Given the importance of determining the therapeutically relevant targets of these drugs, we sought to clarify the above points and to further characterize this assay.

Results

In further experiments to test whether the growth cone assay is a useful model for mood stabilizing drug action, we labeled growth cones and scored them as collapsed or spread as illustrated in Fig. 1a. Using this assay, we confirmed our previous findings that lithium inhibits the frequency of collapse, and that this effect is reversed by the addition of 1 mM inositol to the tissue culture medium (Fig. 1b). To determine the specificity of this morphological assay, we then tested gabapentin (GPT) (50 μM) and phenytoin (PTN) (50 μM). There was no effect of either drug on collapse (Fig. 1c). These results show that two anti-convulsants that are not effective as anti-manic drugs (Yatham et al., 2002) do not induce inositol-reversible effects on sensory neuron growth

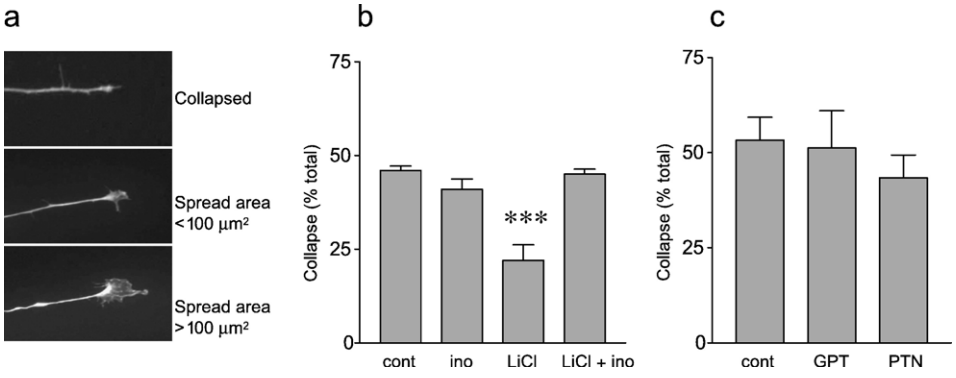

Fig. 1. (a) Micrographs showing a collapsed growth cone (top) and spread (middle and lower panels). Sensory explants were labeled with calcein and fluorescent images acquired with a ×40 objective using an Olympus BX51 microscope. (b) Lithium and inositol effects on growth cone collapse in sensory neurons. Histogram shows the effect of LiCl (3 mM) with/without inositol (1 mM) on the number of growth cones collapsed expressed as a percentage of total. In this and the following figures, data are presented as mean ± SEM. Significant change from control is indicated (one-way ANOVA and Fisher's LSD test, ***$P < 0.001$ in LiCl-treated explants). Results shown are from two independent experiments each in duplicate ($n = 4$). (c) Gabapentin (GPT) and phenytoin (PTN) do not mimic a mood stabilizer in sensory neuron growth cones. Histogram shows the percentage of growth cones collapsed in cultures treated with GPT 50 μM or PTN 50 μM. Results are from one representative experiment ($n = 3$ coverslips). This experiment was repeated three times with similar results.