

Tipping the scales: lessons from simple model systems on inositol imbalance in neurological disorders

Running title: Lessons on inositol imbalance from simple models

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Abstract

Inositol and inositol-containing compounds have signalling and regulatory roles in many cellular processes, suggesting that inositol imbalance may lead to wide-ranging changes in cellular functions. Indeed, changes in inositol-dependent signalling have been implicated in various diseases and cellular functions such as autophagy, and these changes have often been proposed as therapeutic targets. However, few studies have highlighted the links between inositol depletion and the downstream effects on inositol phosphates and phosphoinositides in disease states. For this research, many advances have employed simple model systems that include the social amoeba *D. discoideum* and the yeast *S. cerevisiae*, since these models enable a range of experimental approaches that are not possible in mammalian models. In this review, we discuss recent findings initiated in simple model systems and translated to higher model organisms where the effect of altered inositol, inositol phosphate and phosphoinositide levels impact on bipolar disorder, Alzheimer disease, epilepsy and autophagy.

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Sources of inositol

Myo-inositol is a stereoisomer of inositol that has critical roles in eukaryotes as a structural constituent of both inositol phosphates that act as second messengers in intracellular signalling pathways and phosphoinositides that have important roles in lipid signalling, cell signalling and membrane trafficking (**Figure 1**). *Myo*-inositol is naturally most abundant in fruits, vegetables, grains and nuts (Clements and Darnell, 1980). Dietary inositol intake provides one of three means to maintain cellular inositol levels through the Na⁺-*myo*-inositol transporters (SMITs) and the H⁺-*myo*-inositol transporter (HMIT) (Calker and Belmaker, 2000). In addition, inositol can be recycled in the phosphatidylinositol (PI) cycle from inositol phosphates, which include inositol 1,4,5-trisphosphate (InsP₃) and inositol 1,4-bisphosphate (InsP₂) and higher order inositol phosphates such as InsP₄, InsP₅ and InsP₆ (Irvine and Schell, 2001) (**Figure 1**). Inositol can also be obtained by *de novo* synthesis, in which glucose 6-phosphate is converted to *myo*-inositol 3-phosphate by *myo*-inositol-3-phosphate synthase (INO1), followed by dephosphorylation to inositol by inositol monophosphatase (IMPase) (Loewus et al., 1980). The relative contribution of these mechanisms to inositol homeostasis is likely to vary between different cell types and tissues. In the brain, the expression of INO1 is confined to the vasculature (Wong et al., 1987) and thus cortical neurons are likely to rely on extracellular uptake and recycling of inositol (Di Daniel et al., 2006). Indeed, HMIT is expressed in several brain regions, including neurons of the frontal cortex, and SMIT2 is widely expressed in brain (Di Daniel et al., 2006). In testis, on the contrary, INO1 is expressed in the epithelial cells of the seminiferous tubules rather than in the blood vessels (Wong et al., 1987) and thus these cells are likely to rely on synthesis or recycling of inositol. In mammals, plasma concentrations of inositol range from 25 to 100 μM while intracellular concentrations are several-fold higher than in the circulation and the highest concentration is found in the brain (Kollros et al., 1990).

Regulation of inositol synthesis

The inositol biosynthetic pathway has been highly conserved throughout evolution (Majumder et al., 2003) and is essential for viability in a variety of model organisms, including *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, *Arabidopsis thaliana* and mice (Culbertson and Henry, 1975; Lester and Gross, 1959; Martin and Smith, 2005; Meng et al., 2009; Ohnishi et al., 2014). Owing to the essential role of inositol in cell function, studying the regulation of inositol biosynthesis is difficult in humans and other complex organisms (Keith et al., 1977), thus much of our understanding of inositol metabolism is based on extensive research in the yeast *S. cerevisiae* (Bachhawat et al., 1995; Dean-Johnson and Henry, 1989; Ju et al., 2004; Lopes et al., 1993) and the amoeba *D. discoideum* (Adley et al., 2006; Chang et al., 2012; Fischbach et al., 2006; Frej et al., 2016; Williams et al., 1999, 2002; Xu et al., 2007).

Inositol metabolism is tightly regulated. At the transcriptional level, *INO1* expression in *S. cerevisiae* is repressed by inositol and this occurs by the binding of the overproducer of inositol protein 1 (Opi1) to the Ino2/Ino4 transcription regulator complex at the upstream activation sequence in the *INO1* promoter (UAS_{INO1}) (Greenberg and Lopes, 1996). When cells experience a reduction in inositol levels, the suppressor of choline sensitivity protein 2 (Scs2) sequesters Opi1 in the ER and this relieves the repression of the Ino2/Ino4 complex, which activates *INO1* transcription (Felberbaum et al., 2012). *INO1* transcription is also regulated by

sumoylation, which is a post-translational protein modification by highly conserved small ubiquitin-related modifier (SUMO) proteins (Wang and Dasso, 2009). Mutations in the two yeast SUMO proteases, ubiquitin-like specific protease 1 (*ULP1*) and *ULP2*, decrease *INO1* gene transcription, which causes impaired cell growth in the absence of inositol and leads to an accumulation of sumoylated *Scs2* (Felberbaum et al., 2012). *INO1* transcription is also tightly regulated by the presence of choline, *INO1* protein levels and cellular stresses like the unfolded protein response (UPR) (Brickner and Walter, 2004; Konarzewska et al., 2012; Shetty and Lopes, 2010).

At the translational level, the *de novo* biosynthesis of inositol is primarily regulated by *INO1* (**Figure 1**), which is encoded by the *INO1* gene in yeast and the *ISYNA1* gene in humans (Loewus et al., 1980). The activity of yeast *INO1* is regulated by phosphorylation at five sites in the protein (Deranieh et al., 2013). *INO1* protein extracted from rat brain is also phosphorylated (Parthasarathy et al., 2013), where three of the phosphosites identified in yeast *INO1* (S184, S296, S374) are conserved in human *INO1* (S177, S279, S357) and phosphorylation at these sites was demonstrated to regulate *INO1* activity in both organisms (Deranieh et al., 2013). In *D. discoideum*, the enzyme has also been shown to contribute to a macromolecular complex (Frej et al., 2016), which provides a potential mechanism for regulation of its activity by sequestration (Randise-Hinchliff and Brickner, 2016). In this model, in addition to the established role for *INO1* in *de novo* inositol production, the enzyme is also involved in cell growth, cell shape and metabolic regulation (Frej et al., 2016).

The IMPase enzyme, which is encoded by the *INM1* and *INM2* genes in yeast and the *IMPA1* and *IMPA2* genes in humans, also has an important role in maintaining intracellular levels of *myo*-inositol. These protein control the dephosphorylation of inositol monophosphate isomers to produce *myo*-inositol as the final step of recycling inositol 1,4,5-trisphosphate (*InsP₃*) to produce inositol (Ferruz et al., 2016). By this mechanism, IMPase regulates inositol homeostasis via phosphoinositide recycling and *de novo* inositol biosynthesis from glucose 6-phosphate (**Figure 1**). In yeast, IMPase expression increases in the presence of inositol, although inositol does not affect enzyme activity *in vitro* (Murray and Greenberg, 1997). The enzyme requires magnesium ions for activity and is inhibited by lithium, which is a drug that is used as a mood-stabilizing treatment (Ferruz et al., 2016). It is not known whether IMPase is post-translationally modified, however it is clear that the amino (N)-terminal region of the protein is important for its activity (Whiting et al., 1990).

The metabolic effects of inositol depletion

Inositol has a critical role as a precursor in the synthesis of phosphoinositides, which include phosphatidylinositol (PI) and its phosphorylated derivatives such as PIP, PIP₂ and PIP₃ (**Figure 1**). Phosphoinositides comprise a phosphatidic acid (PA) backbone (glycerol linked to two fatty acid tails) that is linked to inositol via a phosphate group, with further phosphorylations occurring at positions three, four and five on the inositol ring. Phosphoinositides reside in the inner leaflet of the plasma membrane and their levels are maintained at steady state by phosphorylation and dephosphorylation reactions that are performed by specific kinases and phosphatases and by phospholipase C dependent breakdown (Kurnasov et al., 2013). Controlled synthesis of the different phosphoinositides can occur in diverse intracellular compartments for distinct and independently regulated functions

with differing target enzymes. Phosphoinositides take part in a wide variety of cellular functions including motility and intracellular membrane trafficking (Cremona and De Camilli, 2001).

Inositol phosphates are the second major class of molecules derived from inositol (**Figure 1**) and originate from the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C (PLC) to produce inositol 1,4,5-trisphosphate (InsP₃) (Ferruz et al., 2016). InsP₃ has a central role in triggering the release of intracellular calcium, but it can also be phosphorylated to form higher order phospholipids like inositol phosphates (InsP₄, InsP₅, InsP₆) or inositol pyrophosphates (InsP₇ and InsP₈) (Pattni and Banting, 2004) that are involved in the regulation of gene expression and in protein post-translational modifications (Steger et al., 2003). These molecules are critically important for a range of cellular functions, including phospholipid synthesis, the UPR and protein secretion (Deranieh and Greenberg, 2009).

Several recent studies have demonstrated a key role of inositol supply in regulating phosphoinositide levels. In *D. discoideum*, genetic ablation of the *INO1* gene permitted an analysis of the effects of inositol depletion on phosphoinositide levels, since all inositol is then provided through media supplementation (Frej et al., 2016) (**Figure 2**). Starving these cells of inositol for 24 hours reduced the intracellular level of *myo*-inositol by 40% (Frej et al., 2016). Analysis of phosphoinositides in this model included the differentiation of the two distinct types of phosphoinositide, where the predominant type contains an ether linkage of the fatty acids to glycerol in *D. discoideum* and other lower organisms, whereas the predominant species in animals contains a diacyl linkage (Clark et al., 2014). Inositol depletion in *D. discoideum ino1* cells led to a rapid reduction of PI and PIP levels and this was true for both diacyl and ether species. Interestingly, diacyl-PIP₂ also decreased, but the ether-PIP₂ remained unchanged, which suggests that the diacyl-PIP₂ found in animals is a more labile form of PIP₂ than the evolutionarily older ether-PIP₂ (Frej et al., 2016). In addition, loss of the *INO1* protein reduced ether-PIP₃ levels for all inositol supplementation regimes. Finally, inositol depletion caused a rapid (12h) reduction in phosphatidylserine, but did not significantly change other glycerophospholipids like phosphatidylethanolamine or phosphatidylcholine. In yeast, inositol depletion also modifies total lipid content, including causing a decrease in PI and an increase in phosphatidylserine (Yamagami et al., 2015). These data suggest that decreasing inositol levels may cause a profound change in cellular phosphatidylinositol signalling.

Alteration of the levels of phosphoinositides through inositol depletion may have major effects on cellular function. For example, PIP₂ is an important phospholipid component of cell membranes and is a substrate for a number of signalling proteins that regulate the function of many membrane proteins and ion channels (Ho et al., 2012). PIP₂ has a critical role in cytokinesis (Abe et al., 2012), vesicle formation and transport (Klopfenstein et al., 2002), and membrane traffic at synapses in the brain (Cremona and De Camilli, 2001). PIP₂ is also involved in neurotransmitter secretion, nucleation of clathrin coats and the formation of a cytoskeletal scaffold to promote endocytosis at the synapse, where PIP₂ dephosphorylation accompanies the release of newly formed vesicles (Cremona and De Camilli, 2001). Studies in yeast have provided further support for the importance of inositol for PIP₂ homeostasis: removal of *INO1* activity that causes inositol depletion triggers altered vacuolar structure and decreased vacuolar ATPase activity and proton pumping in isolated vacuolar vesicles (Deranieh et al., 2015). The V-ATPase complex couples ATP hydrolysis to proton transport into intracellular compartments and across the plasma membrane (Stransky et al., 2016) and its functions include membrane trafficking, protein processing and degradation, and the

coupled transport of small molecules. PIP₃ is another example of a phosphoinositide that is important for the regulation of the survival, connectivity and synaptic function of the neurons in the central nervous system (Waugh, 2015). Thus, inositol depletion, through reducing the levels of phosphoinositides such as PIP₂ and PIP₃, may have wide-ranging effects on cell signalling in health and disease.

Inositol perturbation and disease

Altered inositol metabolism has been implicated in various human diseases that include neuropsychiatric, neurodegenerative and neurological diseases (Croze et al., 2015; Das et al., 1994; Prieto et al., 2011; Shimon et al., 1998; Teo et al., 2009; Toker et al., 2014; Trushina et al., 2012). The role of phosphoinositide metabolic pathways and the enzymes that have been implicated in neurological diseases have been comprehensively reviewed elsewhere (Waugh, 2015). Here, we will focus on bipolar disorder, Alzheimer disease and epilepsy.

Inositol imbalance and bipolar disorder

Bipolar disorder is a devastating neuropsychiatric disorder that leads to severe mood swings and causes a dramatic reduction in quality of life and a significant increase in the likelihood of suicide (Fajutrao et al., 2009). Perhaps the most clearly demonstrated link between alterations in inositol levels and disease is in the treatment of bipolar disorder. Clinical studies have also shown that 10 out of 15 patients experienced a reduced severity of bipolar disorder when their inositol intake was reduced (Shaldubina et al., 2006). Treatment of patients with either of two drugs most commonly prescribed for the disease, lithium and valproic acid (VPA), caused changes in inositol levels in the frontal and temporal lobes, the cingulate gyrus, and basal ganglia (Silverstone et al., 2005). Additionally, a decrease in inositol levels was reported in patients with bipolar disorder that were undergoing drug therapy (Davanzo et al., 2001).

Clinical studies demonstrating a link between inositol depletion and successful treatment of bipolar disorder have received support from experiments in multiple model systems, which gave rise to the 'inositol depletion hypothesis' (Berridge et al., 1989). Initially, lithium was shown to reduce InsP₃ levels in rat cerebral cortex slices (Kennedy et al., 1989). Lithium and VPA were also found to decrease *myo*-inositol and increase the inositol monophosphate concentration in rat brain extract (O'Donnell et al., 2000). Lithium was then shown to act by directly inhibiting purified bovine IMPase (Saudek et al., 1996), which suggests that lithium treatment reduces both inositol recycling and biosynthesis, leading to a reduction of an overactive inositol-dependent signalling pathway in the brain. This effect was then proposed as the therapeutic mechanism of action in bipolar disorder treatment (Berridge et al., 1989). Subsequent studies using the structurally distinct compound VPA (Terbach and Williams, 2009) also showed effects similar to that of lithium treatment. In both yeast and *D. discoideum*, lithium and VPA lower intracellular InsP₃ levels and elevate *INO1* transcription (Eickholt et al., 2005; Ju and Greenberg, 2003; Vaden et al., 2001; Williams et al., 2002). In cultured primary rat sensory neurons, this common inositol-depleting effect was also observed with a third bipolar disorder treatment, carbamazepine, and cellular changes were reversed by the addition of exogenous inositol, which confirmed inositol depletion as a mechanism of action for structurally disparate bipolar disorder treatments (Williams et al., 2002). A mechanism for

the effect of VPA on inositol-linked effects was then suggested by studies in yeast, in which VPA inhibited INO1 activity *in vivo* (Ju et al., 2004), and this mechanism has been supported in experiments using mouse brain extracts and human frontal cortex (Shaltiel et al., 2004). However, the inhibitory effect of VPA was absent against the purified enzyme (Ju et al., 2004), which suggests an indirect mechanism of action of VPA-dependent INO1 inhibition.

Although inositol phosphates have been the main focus for the effects of bipolar disorder treatments (relating to the inositol depletion effect) these changes are likely to impact on phosphatidylinositol signalling. Studies in *D. discoideum* have shown that VPA causes an acute reduction in phosphoinositides including PIP₂ (Xu et al., 2007)(Chang et al., 2014) although again the exact mechanism of this effect remains to be established. Lithium also suppresses PIP₃-mediated signalling in this model and in a human neutrophil cell line during chemotaxis (King et al., 2009). In yeast, treatment with VPA also compromised the homeostasis of PIP₂ and reduced V-ATPase proton transport in inositol-depleted wild-type yeast cells (Deranieh et al., 2015). The perturbation of the V-ATPase was proposed to be a consequence of the change in the PIP₂ dynamics during low inositol conditions (Deranieh et al., 2015). Thus these studies, initiated in simple models and translated to mammalian models, suggest a link between inositol deregulation and downstream effects on phosphoinositides.

Regulation of inositol signalling in bipolar disorder further links to calcium homeostasis, through InsP₃-dependent calcium release (Deranieh and Greenberg, 2009). The free cytosolic concentration of calcium is an important regulator of numerous cellular functions, which includes neurotransmitter release, activation of ion channels and cell death, and calcium is elevated in patients during mania and depression along with a reduction in the activity of the Na⁺/K⁺-ATPase (NKA) pump (Looney and El-Mallakh, 1997; Warsh et al., 2004). NKA is a membrane-bound enzyme that is abundant in brain tissue and has been linked to bipolar disorder pathophysiology and NKA-dependent signal transduction pathways are dependent on calcium. Interestingly, lithium and VPA treatment reduce behavioural abnormalities associated with mania in the heterozygous *Myshkin* mouse (*Myk*^{+/+}) that has an inactive neuron-specific α 3 isoform of NKA (ATP1A3) (Kirshenbaum et al., 2011). Despite its efficacy, lithium has a narrow therapeutic window and a number of unwanted side effects (McKnight et al., 2012) and this has triggered a search for alternative treatments that act through a common mechanism. Ebselen (2-phenyl-1,2-benzisoxazol-3(2H)-one) provides one such compound, a seleno-organic drug with anti-inflammatory, anti-atherosclerotic and cytoprotective properties (Schewe, 1995) that functions as a lithium mimetic and has been tested in phase 1 clinical trials (Singh et al., 2016). Ebselen inhibits IMPase in the human brain, therefore reducing inositol levels and thus affecting the phosphoinositide cycle (Masaki et al., 2016), which makes it a promising treatment for bipolar disorder and other neurodegenerative diseases.

Inositol imbalance and Alzheimer disease

Inositol imbalance has also been linked to a range of neurodegenerative diseases including Alzheimer disease (AD), which is the most common form of dementia that presently affects an estimated 46.8 million people worldwide (Alzheimer's Disease International, 2015). Indeed, the activity and levels of the INO1 enzyme were found to be higher in the post-mortem brains of patients with Alzheimer disease (Shimon et al., 1998) and who also show an increased level of brain *myo*-inositol compared to healthy individuals (Griffith et al., 2008). Lithium has also

been shown to improve behavioural and cognitive deficits in Alzheimer disease and other neurodegenerative disease models that include lateral sclerosis, fragile X syndrome, Huntington, and Parkinson diseases (Chiu and Chuang, 2011) and VPA has shown efficacy in animal models of Alzheimer disease (Qing et al., 2008). The pathology of AD involves an accumulation of amyloid- β (A β) peptides into plaques, and the formation of neurofibrillary tangles, leading to neuronal cell death in brain regions associated with learning and memory, especially in the cortex and the hippocampus (Pereira et al., 2004). *Scyllo*-inositol and *myo*-inositol, two naturally occurring stereoisomers of inositol, inhibit A β production and plaque accumulation in the brain of a transgenic mouse model of Alzheimer disease that demonstrates age-associated cognitive deficits and AD-like pathology (Fenili et al., 2007). *Scyllo*-inositol has also been shown to reverse memory deficits in these mice (McLaurin et al., 2006).

Alzheimer disease pathology has been linked to dysregulation of energy metabolism and this is critically linked to inositol regulation. The brain has high energy requirements and relies on a supply of glucose and oxygen to allow for neurotransmitter production and recycling, vesicular trafficking and maintenance of ion gradients for the propagation of action potentials and memory. For this purpose, the brain uses about 20% of the oxygen and 25% of the glucose consumed by the human body (Be' langer et al., 2011). Energy metabolism has been previously associated with inositol regulation in which a generation of *S. cerevisiae* glycolytic mutants (lacking triose phosphate isomerase 1 (Tpi1) and phosphoglycerate kinase 1 (Pgk1)) caused an accumulation of dihydroxyacetone phosphate (DHAP) that was inhibitory to INO1 and led to inositol auxotrophy (Shi et al., 2005). Additionally, the carbohydrate metabolites glyceraldehyde 3-phosphate (G3P) and oxaloacetate (OAA) inhibit the activity of human and yeast INO1 proteins *in vitro* (Shi et al., 2005). Inositol and glucose imbalance therefore has a major impact on brain energy consumption.

Metabolomic studies have also shown that a range of fundamental changes occur during Alzheimer disease progression. These changes include elevated protein degradation, glucose breakdown and pyruvate oxidation (Hoyer, 1990), increased levels of some amino acids, serotonin and catecholamine metabolites and alterations in purine metabolic pathways (Hoyer, 1990; Kaddurah-Daouk et al., 2013). A link between these changes and inositol has recently been suggested through studies in *D. discoideum*, in which the loss of INO1 (rather than inositol depletion *per se*) caused a shift to catabolic metabolism that was marked by an increase in some amino acids, products of nucleoside breakdown and an accumulation of Krebs cycle metabolites (Frej et al., 2016). Thus, further studies are necessary to investigate if the levels of both inositol and INO1 may modulate the regulation of metabolism in brains of Alzheimer disease patients.

Inositol, phosphoinositides and epilepsy

Epilepsy is a severe neurological disorder in which patients exhibit repeated seizures and this can have a devastating effect on quality of life. Epilepsy is estimated to affect around 50 million people worldwide (WHO, 2012) and current treatments do not control seizures in 30% of patients (Kwan, 2004). A series of recent papers have demonstrated a role for altered inositol and phosphoinositides in seizure activity and as a target for epilepsy treatments.

Inositol levels and seizure have been recently linked in a range of studies. Inositol pre-treatment has been shown to reduce the induction of seizures in multiple models (Belmaker and Bersudsky, 2007; Kotaria et al., 2013; Nozadze et al., 2011; Solomon et al., 2007), and seizure activity increases Na⁺/myo-inositol co-transporter levels (Nonaka et al., 1999; Solomon et al., 2013, 2010). Elevated inositol levels are also found in brain regions that are involved in seizure occurrence (Wellard et al., 2003). In a recent study, induction of spontaneous seizures showed an initial 20% increase in hippocampal inositol levels (Pascente et al., 2016), suggesting a seizure-induced change in inositol signalling in the brain (Y. Wu et al., 2015). This elevated level was then shown to persist in animals that went on to develop epilepsy (i.e. repeated seizures), but did not persist in animals that did not develop epilepsy. These data suggest an association between long-term elevated inositol levels and the development of epilepsy, strongly supporting a role for inositol levels in controlling seizures and with seizure activity altering these levels.

Recent studies have also suggested a role for phosphoinositides in seizure activity (**Figure 3**). Studies in *D. discoideum* have shown that VPA and components of the medium chain triglyceride (MCT) ketogenic diet act through regulation of phosphoinositides (Chang et al., 2012; Xu et al., 2007). These studies have then been translated into *in vitro* and *in vivo* animal models of epilepsy to show that seizure activity decreases brain PIP₃ levels and that VPA acts to protect against this reduction (Chang et al., 2014). The studies also show that novel compounds identified in *D. discoideum* provide potent seizure control (Chang et al., 2015a, 2015b, 2013, 2012). The breadth of functions regulated by phosphoinositides that relate to seizures and epilepsy make it difficult to identify a single mechanism of action for these compounds in seizures. PIP₃ provides a key phosphoinositide in a wide variety of cellular functions (Vanhaesebroeck et al., 2012) and has many activities that relate to potential seizure roles such as voltage-gated channel regulation (Viard et al., 2004), regulation of neuronal excitability (MacGregor et al., 2002) and the insertion of ion channels into synaptic plasma membranes (Lhuillier and Dryer, 2002). In addition, PIP₂ also regulates epilepsy-related ion channel activity (Alberdi et al., 2015) and a recent study of a severe form of epilepsy (DOORS syndrome) has identified a causative mutation in the Rab GTPase-activating protein TBC1D24 that alters a PIP₂/PIP₃ binding pocket; the synaptic defects that result are reversed by elevating PIP₂ levels (Fischer et al., 2016). These studies therefore highlight a major role of phosphoinositides in seizure activity and protection against changes in phosphoinositide levels as a potential therapeutic drug action.

Autophagy in neurodegenerative diseases

Autophagy is a process by which the degradation and recycling of cellular material provides essential molecules and energy for continued cell function, or to fulfil a protective role by clearing damaged and dysfunctional cellular components (Otto et al., 2003). It is triggered by cell stress, cellular differentiation and under some developmental conditions. Under stress conditions like inflammation, autophagy degrades unwanted proteins however the aberrant activation of autophagy may also cause cell death (Kosta et al., 2004). The most common form of autophagy, macroautophagy, involves the transport of cytosolic components and entire organelles to the lysosomal compartment via specific double-membrane vesicles, called autophagosomes. It is a process tightly regulated by the activity of the nutrient-sensing target of rapamycin (mTOR) protein kinase, the energy-sensing AMP-activated protein kinase (AMPK) and over 30 autophagy-related (ATG) proteins (Akers et al., 2012). AMPK is activated under low-energy conditions, leading to autophagy induction, whereas under normal

physiological conditions (high energy levels, amino acids, or growth factors), mTOR inhibits the canonical autophagy pathway by hyperphosphorylation of Atg13 in yeast or ATG13/ULK1/ULK2 in mammals (Akers et al., 2012). Nutrient depletion, reactive oxygen species, and rapamycin treatment result in a rapid dephosphorylation of ATG13, an increase in Atg1 kinase activity and an induction of autophagy (Kamada et al., 2000). Autophagosome formation starts with ATG1 membrane localisation followed by the nucleation of the forming autophagosome. The phagophore is then elongated and ubiquitin-like proteins modulate conjugation of ATG8 to phosphatidylethanolamine (PE) and assembly of an ATG12/ATG5 in the autophagosome membrane (Mizushima et al., 2002). ATG8-PE remains at the membrane, and autophagosome maturation continues with elongation, completion, fusion with lysosomes, and the degradation and recycling of the sequestered cellular material. These processes are controlled by the dynamic regulation of inositol phosphates and phosphoinositides and their binding partners (Lystad and Simonsen, 2016) (Vicencio et al., 2009). Mutations in the autophagy-related genes have been associated with various human diseases such as neurodegenerative diseases, infectious diseases and cancers (Jiang and Mizushima, 2014).

Autophagy and bipolar disorder

Autophagy has been suggested to be involved in bipolar disorder since the three structurally distinct bipolar disorder drugs carbamazepine, VPA and lithium all induce autophagy via inositol depletion in several mammalian cell lines (Sarkar et al., 2005). Lithium reduces InsP_3 levels and induces autophagy, resulting in the clearance of aggregates of proteins (Sarkar et al., 2005). This lithium effect is not mediated by a second lithium target, glycogen synthase kinase 3 β (GSK3 β), but by IMPase inhibition since a specific inhibitor (L-690,330) causes similar effects. Furthermore, elevating InsP_3 reduces lithium-induced clearance of protein aggregates and inositol inhibits the degradation of autophagy substrates (Sarkar et al., 2005). An inositol depletion-triggered effect of carbamazepine and VPA also induces autophagy leading to the killing of mycobacteria within primary human monocyte-derived and alveolar macrophages and this process is independent of the mTOR pathway known to regulate autophagy (Ghavami et al., 2014), which suggests that inositol depletion may control autophagy via a novel *myo*-inositol-dependent activation pathway (Schiebler et al., 2015).

Autophagy and Alzheimer disease

Autophagy has been widely implicated in the progression of Alzheimer disease. In a mouse model of Alzheimer disease, the accumulation of autophagosomes in neurons promotes the production of A β peptides and is accelerated by the overproduction of SUMO1 (Cho et al., 2015). Depleting several autophagy proteins (ATG5, ATG7 and ATG12) halts the SUMO1-mediated A β production, supporting a role for autophagosome contribution to A β production and disease pathogenesis (Cho et al., 2015). The accumulation of waste proteins and toxic aggregates in the brains of patients with Alzheimer disease could also be a result of the dysregulation of autophagy. One possible cause for the accumulation of autophagosomes is an inhibition of the fusion of autophagosome with lysosomes (to give autolysosomes). It is likely that although SUMO1 increases the autophagic flux, autolysosome formation process is defective, thus promoting autophagosome accumulation instead of the degradation of waste proteins. This is supported by the finding that SUMO1-overexpressing cells have a higher autophagosome to autolysosome ratio than cells treated with trehalose, which induces autophagy (Cho et al., 2015). Triggering autophagy by depleting inositol may allow for

autolysosome formation to complete and result in the clearance of accumulated dysfunctional proteins. Furthermore, inositol polyphosphate-5-phosphatase E (INPP5E), which hydrolyses InsP_3 and PIP_2 , is a positive regulator of autophagy (Hasegawa et al., 2016). PIP_2 has been shown to counteract cortactin-mediated actin filament stabilization on lysosomes to allow for their fusion with autophagosomes. Both an excess or depletion of PIP_2 on lysosomes have been reported to inhibit autophagy during autolysosome formation and lysosomal degradation, which suggests that an optimal level of lysosomal PIP_2 is essential for autophagy (Hasegawa et al., 2016). Since inositol is a precursor of PIP_2 , changes in inositol levels can have an impact on the later stages of autophagy. Inositol depletion can thus have a therapeutic value as a regulator of autophagy and sumoylation in the pathogenesis of Alzheimer's disease.

Autophagy and epilepsy

Impaired autophagy has been proposed to contribute to epileptogenesis, through the mTOR pathway, that plays a role in neuronal development and the proper functioning of mature neurons. Dysregulation of mTOR activity is often observed in a number of neurological diseases (Ghavami et al., 2014). Hyperactivation of mTOR has been associated with the development of temporal lobe epilepsy (TLE) in humans and in animal models (Buckmaster et al., 2009; Hartman et al., 2012; Macias et al., 2013; Sha et al., 2012; Zeng et al., 2009), though it is not known which specific processes involved in the generation of epileptic seizures require mTOR activity. The mTOR inhibitor rapamycin moderates spontaneous seizure development in animal TLE models (Drion et al., 2016; van Vliet et al., 2012; Zeng et al., 2009), corroborating the epileptogenic effects of mTOR dysfunction. Furthermore, mice lacking Atg7, a key protein for autophagy initiation, develop spontaneous seizures (McMahon et al., 2012). Autophagy thus may be of interest as a potential therapeutic target for epilepsy treatment and/or prevention. A number of anti-epileptic drugs VPA, carbamazepine, vigabatrin, acetazolamide, clonazepam, lamotrigine, and levetiracetam have been indeed demonstrated to affect autophagy (Cataldi et al., 2005; Fu et al., 2010; Mohammadpour et al., 2014; Puls et al., 2013; Schiebler et al., 2015; Vogel et al., 2016; Watanabe et al., 2010; H. Wu et al., 2015).

Inositol depletion and decrease in the InsP_3 levels has been shown to stimulate autophagy (by an mTOR-independent pathway) (Vicencio et al., 2009) while increasing InsP_3 levels has been demonstrated to inhibit autophagy induced by nutrient depletion (Criollo et al., 2007). This is likely related to the role of the InsP_3 receptor (InsP_3R), as either promoting or suppressing autophagy, depending on cellular conditions (Vicencio et al., 2009). The InsP_3R is the main intracellular calcium release channel governing calcium efflux from the endoplasmic reticulum into the cytosol (Vicencio et al., 2009). Under stress conditions Beclin 1 can interact with InsP_3R leading to an increased Ca^{2+} release, which can diffuse into cytosol to stimulate the induction of autophagy (Vicencio et al., 2009). Also, Levetiracetam has been reported to reduce the InsP_3 -dependent calcium release initiated by a G_q -coupled receptor activation (a process that plays a role in triggering and maintaining seizures) in rat PC12 cells at concentrations that are in the range observed in the plasma of patients treated with the drug (Cataldi et al., 2005).

Conclusions

In conclusion, inositol homeostasis is regulated by uptake, biosynthesis and recycling mechanisms. Inositol is vital for maintaining proper cell function and inositol depletion triggers molecular changes that can eventually lead to a disease state. A series of recent studies in simple model systems has shown that a reduction of inositol levels is likely to cause changes in both inositol phosphate and phosphatidylinositol signalling that widely impacts on numerous cellular functions including transcription, metabolism, ion channel and synaptic function, calcium homeostasis, and energy metabolism, and to directly impact on autophagy. Through these numerous mechanisms, dysregulated inositol levels are likely to play an important part in a range of neurological diseases that include bipolar disorder, Alzheimer disease and epilepsy.

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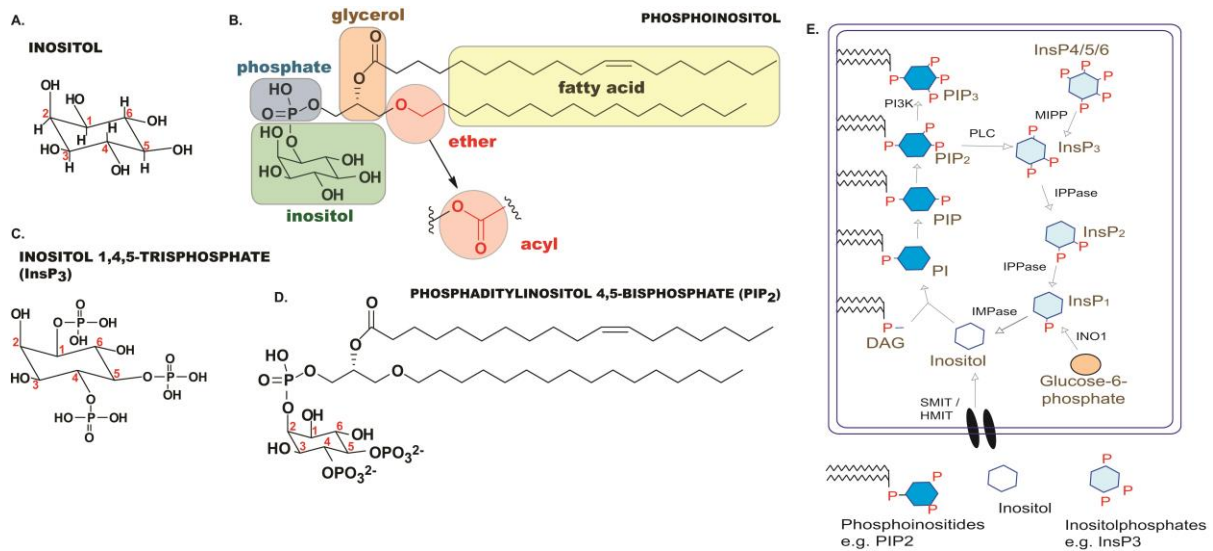


Figure 1. The structure of inositol and its derivatives, and inositol-dependent signalling.

A. Inositol is a polyalcohol that comprises a six ring carbon with hydroxyl groups on each carbon and provides a key part of two families of chemicals. **B.** The first family is phosphoinositides, which includes phosphoinositol, and comprises an inositol head group (green) that is linked via a phosphate group to a glycerol backbone with two attached fatty acids. Two species of phosphoinositide are found, where the linkage to one fatty acid is through either an ether or an acyl group. **C.** The second family is inositol phosphates such as inositol trisphosphate, in which the inositol group is phosphorylated at defined carbons (e.g. positions 1, 4 and 5). **D.** Key phosphoinositides include PIP₂ (shown) or PIP₃, in which the inositol head group of the phosphoinositide is phosphorylated on defined carbons (e.g. position 4 and 5). **E.** Inositol can enter cells through Na⁺-myo-inositol transporters (SMITs) and the H⁺-myo-inositol transporter (HMIT). Inositol and diacylglycerol (DAG) then provide a variety of phosphoinositides including phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP₂), which can then be further phosphorylated by the phosphatidylinositol bisphosphate 3-kinase (PI3K) protein family to form phosphatidylinositol trisphosphate (PIP₃), or degraded by phospholipase C (PLC) activity to form inositol phosphates that include inositol trisphosphate (InsP₃). InsP₃ can also be formed by the dephosphorylation of higher order inositol phosphates (InsP₄, InsP₅, InsP₆), which is catalysed by multiple inositol-polyphosphate phosphatase (MIPP). InsP₃ is broken down by sequential removal of the phosphate groups by inositol polyphosphate phosphatase (IPP) to form inositol bisphosphate (InsP₂) and inositol monophosphate (InsP₁) and by inositol monophosphatase (IMPase) to form inositol. Inositol may also be produced through *de novo* biosynthesis from glucose-6-phosphate by myo-inositol synthase (INO1).

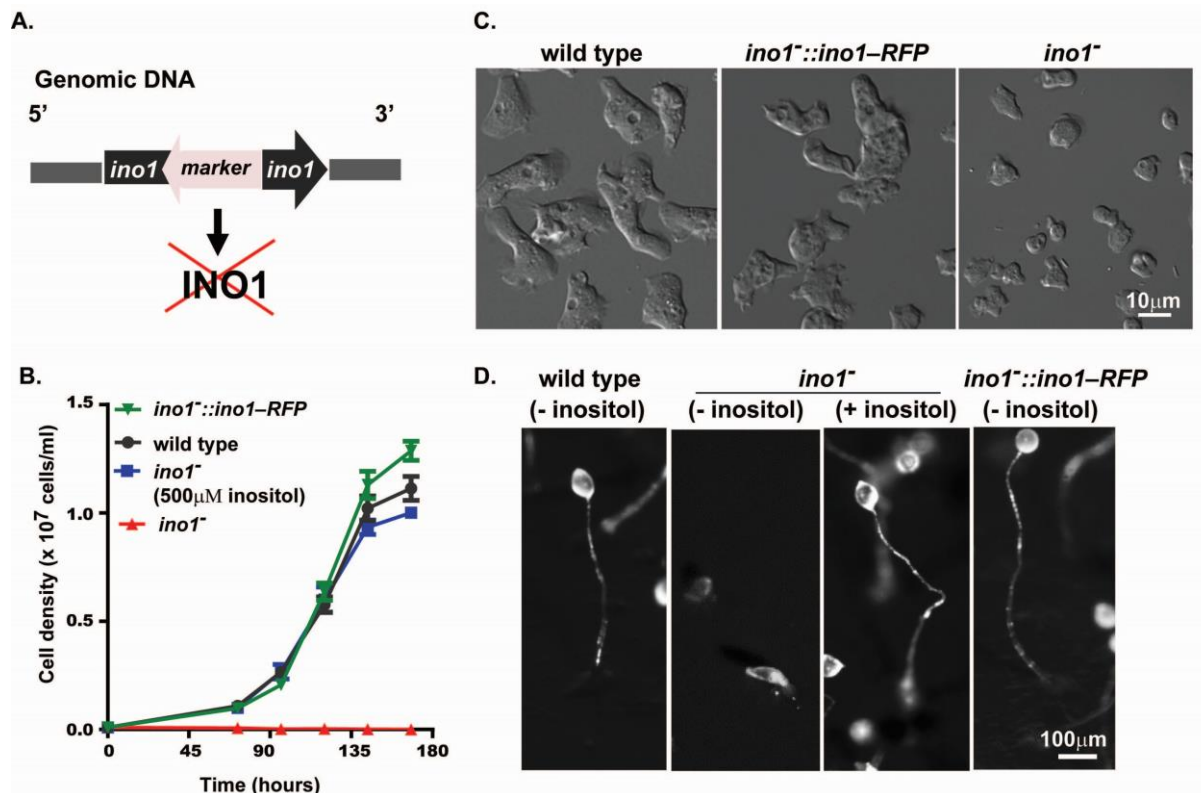


Figure 3. The social amoeba *Dictyostelium discoideum* has been used to investigate the role of inositol, inositol phosphates and phosphoinositides. **A.** The gene encoding for the INO1 protein in *Dictyostelium* has been removed and INO1 is not produced in *ino1* cells. **B.** Compared to wild-type vegetative cells (left), the *ino1* cells starved of inositol for 30 hours (right) are visibly more rounded. **C.** *Dictyostelium* cells that cannot produce INO1 require inositol supplementation in order to proliferate. Restoring INO1 production in these cells (*ino1*::*ino1*-RFP) rescues the growth of the *ino1* mutant cells in the absence of inositol. **D.** Inositol is required for development in *Dictyostelium*. *Ino1* mutant cells are not able to develop without inositol treatment. Restoring INO1 production in these cells (*ino1*::*ino1*-RFP) rescues the development of the *ino1* mutant cells in the absence of inositol. Since the inositol auxotrophic *ino1* mutant is able to grow and develop when supplied with exogenous inositol, these mutant cells can be used to study the effects of inositol depletion.

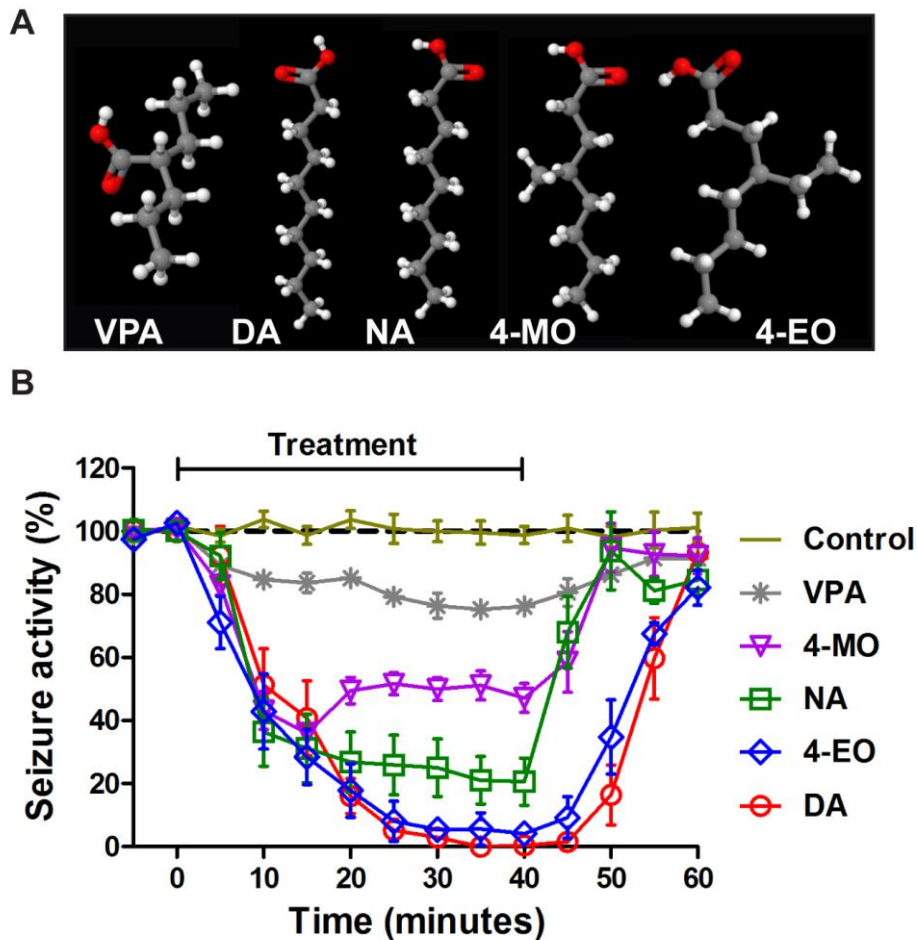


Figure 4. New treatments for epilepsy that were identified in *Dictyostelium discoideum*, which regulate phosphoinositide signalling and show efficacy in seizure models. A. Through the analysis of the mechanism of action of valproic acid (VPA), a range of related chemicals that include decanoic acid (DA), nonanoic acid (NA), 4-methyloctanoic acid (4-MO) and 4-ethyloctanoic acid (4-EO) were identified. **B.** These compounds show strong seizure control in a generalised seizure model in which seizure-like activity is induced in a rat hippocampal slice and is controlled by the addition of compounds (from Chang et al, 2012, 2016).