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The effects of CNS-active Valproic acid constitutional isomers, cyclopropyl analogues and amide derivatives on neuronal growth cone behavior

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Running title: Second Generation VPA as Potential Mood Stabilizers

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Abbreviations: VPA, valproic acid; DIA, diisopropylacetic acid; PIA, propylisopropylacetic acid; VCA, valnoctic acid; VCD, valnoctamide; VPD, valpromide; DID, diisopropylacetamide; PID, propylisopropylacetamide; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid; TMCD, 2,2,3,3-tetramethylcyclopropanecarboxamide; MTMCD, N-methyl-2,2,3,3,-tetramethylcyclopropane carboxamide; TMCU, 2,2,3,3-tetramethylcyclopropanecarbonylurea; DRG, dorsal root ganglia; GSK-3β, glycogen synthase kinase 3β; $InsP_3$, inositol-1,4,5-trisphosphate; InsP, Inositol phosphates.

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Abstract

Valproic acid (VPA) is an effective antiepileptic drug with an additional activity for the treatment of bipolar disorder. It has been assumed that both activities arise from a common target. At the molecular level VPA targets a number of distinct proteins that are involved in signal transduction. VPA inhibition of inositol synthase reduces the cellular concentration of myo-inositol, an effect common to the mood stabilizers lithium and carbamazepine. VPA inhibition of histone deacetylases (HDACs) activates Wnt signaling via elevated β-catenin expression and causes teratogenicity. Given the VPA chemical structure, it may be possible to design VPA derivatives and analogues that modulate specific protein targets, but leave the others unaffected. Indeed it has been shown that some non-teratogenic VPA derivatives retain antiepileptic and inositol signaling effects. In this study, we describe a further set of VPA analogues and derivatives that separate anticonvulsant activity from effects on neuronal growth cone morphology. Lithium, carbamazepine and VPA induce inositol-dependent spread of neuronal growth cones, providing a cell-based assay that correlates with mood stabilizing activity. We find that two constitutional isomers of VPA, propylisopropylacetic acid (PIA) and diisopropylacetic acid (DIA), but not their corresponding amides, and N-methyl-2,2,3,3-tetramethylcyclopropanecarboaxamide (MTMCD) are more effective than VPA in increasing growth cone spreading. We show that these effects are associated with inositol depletion, and not changes in β-catenin mediated Wnt signaling. These results suggest a route to a new generation of CNS-active VPA analogues that specifically target bipolar disorder.

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Introduction

VPA is an eight-carbon, branched side chain carboxylic acid with many clinical uses. It has both antiepileptic and mood stabilizing activity, but also provides effective prophylaxis for neuropathic pain and migraine (Bourgeois et al., 2002). The relationship between the antiepileptic and mood stabilizing activity is particularly interesting as it may reveal information about the molecular origins of bipolar mood disorder, a chronic and disabling illness, which affects 1% of the population and when untreated carries a 10-20% of patients a lifetime risk of suicide (Muller-Oerlinghausen et al., 2002).

Bipolar disorder and epilepsy have some common features, such as their episodic nature and associated kindling phenomena, and this led to the use of the antiepileptic drugs (AEDs) carbamazepine (CBZ) and subsequently, valproic acid (VPA) in the treatment of bipolar disorder (Macdonald and Young, 2002). Although pharmacological treatment proves effective in many patients, relapse rates of bipolar disorder are 73% during 5 or more years, and about 30% of epileptic patients are resistant to multiple AEDs (Gitlin et al., 1995; Kwan and Brodie, 2005). Furthermore co-morbidity and severe adverse effects can further complicate treatment in both disorders (Macdonald and Young, 2002). Hence, there is a pressing need for developing better and safer mood stabilizing agents.

The clinical use of VPA is limited by two rare, but potentially life-threatening, adverse effects teratogenicity and hepatotoxicity (Tomson and Battino, 2005; Dreifuss et al., 1987). Recently, a second generation of AEDs has been developed based on the structure of VPA that improve antiepileptic activity while avoiding these side-effects. These second generation drugs include specific amide and alkyl amide derivatives of

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VPA, and amide derivatives of a cyclopropyl analogue of VPA 2,2,3,3-tetramethylcyclopropanecarboxylic acid (TMCA) (Sobol et al., 2004; Bialer, 2006).

The situation with bipolar drugs is more complex. Here, a major limitation for the development of drugs with improved mood-stabilizing activity is our poor knowledge of the mechanism of treatment for bipolar disorder. In contrast to epilepsy, no animal models in bipolar disorder are universally accepted and no model is able to exhibit the characteristic mood swings (Machato-Vieira et al., 2004). Although most AEDs have now been investigated for their mood stabilizing effects, only a few demonstrated clinical efficacy in patients (Macdonald and Young, 2002). This suggests the mechanism of drug action in mood disorder and in epilepsy only partially overlaps.

One approach to identify the targets of VPA in the treatment of bipolar disorder is to seek common effects with other mood stabilizers. Williams et al. (2002) have recently found that, in common to VPA, both lithium and CBZ increase spreading of growth cones from rat dorsal root ganglia (DRG). This effect can be prevented by exogenous myo-inositol. Furthermore lithium and VPA lower *in vivo* concentrations of inositol and inositol-1,4,5-triphosphate ($InsP_3$) in a range of cell systems. These findings fit the hypothesis originally proposed by Berridge et al. (1989) for lithium that proposes mood stabilizers function by attenuation of an overactive inositol phosphate (InsP) signal transduction pathway. Use of this neuronal system offers a rapid cell based assay to investigate and screen potential new mood disorder drugs (Eickholt et al, 2005).

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A number of the molecular VPA targets have been identified. VPA inhibits inositol synthase thereby leading to depletion of the cellular concentration of myo-inositol (Shaltiel et al., 2004). Initially, VPA was also proposed to inhibit GSK-3, a protein kinase important for cell signaling. This report has not been substantiated (Phiel et al 2001, Ryves et al, 2005), and it was further shown that a range of VPA derivatives also had no direct effect on GSK-3 activity (Eickholt et al, 2005). It was subsequently shown that in some cell types VPA mimics the loss of GSK-3 in the Wnt signaling pathway by inhibition of histone deacetylases (HDACs). This direct inhibition may explain the effect of VPA on gene expression and animal development.

VPA increases transcription of the β-catenin gene and mimics the effect of Wnt stimulation (Phiel et al 2001). Wnt stimulation blocks GSK-3 activity leading to a build up of β-catenin, which in turn binds to TCF family transcription factors and mediated changes in gene expression. Lithium inhibits GSK-3 elevating β-catenin. Consequently, VPA and lithium may have similar effects via different molecular mechanisms (Harwood and Agam, 2003). Given the existence of an alternative signaling target in addition to InsP signaling, it is important to examine which pathway is targeted by VPA related compounds.

In this report, we address the question of whether the anti-epileptic and mood stabilizing properties of VPA arise from the same molecular targets or represent distinct effects that overlap with the VPA molecule. To do this, we evaluated the effects of a number of CNS-active VPA constitutional isomers, cyclopropyl analogues and amide derivatives on DRG growth cones spreading and on the cellular concentration of $InsP_3$. The compounds were also evaluated for their ability to

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modulate the canonical Wnt signaling pathway. All tested compounds possess eight carbon atoms in their major chemical template and have previously been shown to possess antiepileptic activity (Isoherranen et al., 2003). We find little correlation between those drugs that have antiepileptic activity and those that cause growth cones spreading and inositol depletion. This suggests that the antiepileptic and mood stabilizing activities of VPA, may arise through different mechanisms and these results indicate a potential route to specific mood stabilizing drugs structurally based on VPA analogous and derivatives.

Materials and Methods

Materials. Solvents and drugs were purchased from Sigma–Aldrich (Rehovot, Israel). VPA was a gift from Teva Pharmaceutical Industries, Petach Tikva, Israel. VPA's analogues and derivatives were synthesized according to the synthetic procedures previously described in the following references: propylisopropylacetic acid (PIA), (Bojic et al., 1996); diisopropylacetic acid (DIA), (Haj-Yehia and Bialer, 1990); valnoctic acid (VCA), valnoctamide (VCD), (Radatz et al., 1998); 2,2,3,3,-tetramethylcyclopropanecarboxamide (TMCD), N-methyl-2,2,3,3-tetramethylcyclopropanecarboxamide (MTMCD) (Bialer et al., 1996); 2,2,3,3-tetramethylcyclopropanecarbonylurea (TMCU), (Sobol et al., 2004). All VPA derivatives used in the *in vitro* experiments were dissolved in water or ethanol to result in stock solutions of 0.2M.

Dorsal Root Ganglion Explant Culture. Dorsal root ganglia (DRG) were dissected from the spinal cord area of P0 Sprague Dawley rat pups and cultured individually on poly-ornithine/laminin coated coverslips in serum-free medium at 37°C with 5% CO_2 (Bottenstein et al., 1979). Media was supplemented with mouse NGF-7s (25 ng/ml;

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the optimal concentration for growth stimulation was determined for each batch) in order to promote neuron survival and axon outgrowth (Williams et al, 2002). After allowing attachment for 24 hours, the antimitotic agent cytosine β–arabinofuranoside (Ara-C; 10 μM) was added for 24 hours to kill non-neuronal cells. DRG explants were then changed to fresh serum-free media for a further 24 hours. Following the development of an extensive axonal outgrowth in this three-day period, drugs were added at 1.5 times the therapeutic level. i.e. 1mM sodium chloride (control), 2mM lithium chloride, or 3mM VPA (with or without 2mM myo-inositol). After 48-hour exposure, the explants were fixed using 4% paraformaldehyde in PBS for 20 min at room temperature. Growth cones images were recorded using an inverted microscope and spread areas measured using NIH image analysis software. Growth cones were identified by their distinctive morphology, comprising of membrane extension beyond the boundary of the extending axon. DRG growth cones were not routinely stained, but antibody staining for tubulin and phalloidin staining for F-actin demonstrated that the delineation between axonal microtubule and F-actin exactly coincided with the morphology of the measured growth cone (data not shown). Data was collected and analysed using a double blind protocol. Between 10-50 growth cones were formed per DRG. Drug concentrations that reduced growth cone numbers below this number were considered toxic and the data rejected. Each experiment comprised 1-2 DRG and was repeated in triplicate; giving between 50-150 growth cone measurements per drug. In the experiments described here, the dose of NGF caused a generally higher degree of growth cone spreading, hence sizes are slightly higher than previously reported. All chemicals were supplied by Sigma-Aldrich, UK unless otherwise stated.

***Dictyostelium* Cell Culture and $InsP_3$ assays.** Wild-type *Dictyostelium discoideum* cells (Ax2) were grown at 22ºC for 20 h in AX media at 1 x 10^6 cells/ml in the

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presence of drugs at indicated concentrations or with vehicle-only control. Cells were washed in the presence of the drug and lysed in perchloric acid. $InsP_3$ levels were measured by isotope dilution binding assay (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) as previously described (Williams et al., 1999). Protein was measured by Bradford assay (Bio-Rad Laboratories, Hemel Hempstead, UK).

TCF promoter activity

Activation of TCF promoter activity was monitored using the TOPflash assay (Fahnert et al 2004). Human embryonic kidney epithelial cells transfected with TCF-luciferase reporter plasmid were seeded at $1x10^4$ cells per well of 96 well black-walled dishes in 50µl DMEM medium and incubated at 37°C for 48h. On the third day 30µl DMEM was added to each well and the drugs, dissolved in pure ethanol, were added in triplicates to give a final concentration of 3mM. The luminescence assay was performed by removing the medium and washing the cells twice with PBS. The cells were incubated with the drugs for 48h and the luminescence was read after 5min incubation with a luminescence substrate. For control wells 10µl of 1% ethanol in DMEM was added.

Statistical Analysis. The p values were calculated by analysis of variance with Dunn's multiple comparison test for post hoc pairwise comparison with the control value. All statistical analyses were performed with GraphPad InStat, version 3.01 (GraphPad Software, San Diego, CA, U.S.A.). Data are expressed as mean ± S.D. A p value of ≤ 0.05 was considered significant.

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Results

Effect of VPA constitutional isomers and their amide derivatives on the morphology of DRG neuronal growth cones

We used a modified version of the DRG cell based assay described by Williams and co-workers (2002) to compare the action of VPA analogues. In this assay, DRG excised from PO newborn rats are cultured as explants for 72 hours in nerve growth factor (NGF) supplemented medium. Under these conditions explants project axonal outgrowths that extend through migration of their growth cones. Cultures were treated with drugs for a further 48 hours then fixed and observed under phase contrast microscopy. Growth cone morphologies were scored by measuring the spread area of each growth cone and analyzed to give a numerical value. The advantage of this modified method over that previously reported (Williams et al, 2002) is that we were able to collect data from all growth cone morphologies. This complete range of size measurements made possible a statistical analysis.

Lithium chloride was used as a positive control and all treatments were compared to drug free, solvent controls. The effects of VPA were compared to the VPA constitutional isomers, VCA, DIA and PIA, three constitutional isomers of VPA with modified aliphatic moieties, and their amide derivatives (Figure 1). A substantial change in the distribution in growth cone sizes was observed following treatment with 2mM lithium chloride or 3mM VPA (Figure 2), with cultures showing fewer small growth cones and an increased proportion of growth cones with large spread areas. Very little effect was seen in cultures treated with 1 mM VPA.

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PIA and DIA strongly increased growth cone spreading at 1mM concentrations (Figure 2). VCA gave a strong effect at 3mM, but a weak effect at 1mM. The stronger effect of PIA and DIA than observed for 1mM VPA suggested a higher potency of these isomers. To examine this further we tested both drugs at 0.5mM. PIA again exhibited a strong increase in growth cone spread area, however no effect was observed for DIA. These results suggested an order of potency with PIA > DIA > VCA.

The common effects of mood stabilizers on growth cone size are reversed by addition of myo-inositol to the medium, consistent with the proposed mechanism via inositol depletion (Williams et al., 2002). Inositol reversal has previously been used to identify constitutional isomers of VPA that possess inositol-depleting activity (Eickholt et al., 2005). In all cases the effects of PIA, DIA and VCA can be reversed by addition of myo-inositol to the medium (Figure 2).

Valpromide (VPD), the corresponding amide of VPA, has antiepileptic activity but does not increase growth cone spreading (Shaltiel et al, 2004). We therefore tested the corresponding amides of the constitutional VPA isomers (PIA, DIA, and VCA), which have been shown to have significant antiepileptic activity (Isoherranen et al., 2003, Table 1). Again, these compounds have no affect on growth cone spreading at 1mM or 3mM (Figure 3). Finally, we tested two unsaturated VPA derivatives 4-ene-VPA and 4-yne-VPA. These compounds were toxic to the DRG at 3mM, but increased spreading at 1mM (data not shown).

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Effect of cyclopropyl VPA analogue and its amide derivatives on the morphology of DRG neuronal growth cones

The cyclopropyl VPA analogue TMCA and its amide derivatives, TMCD, MTMCD and TMCU, all possess anticonvulsant activity in rodent models (Isoherranen et al., 2003; Sobol et al, 2004, Winkler et al, 2005a). Neither TMCA, TMCD nor TMCU showed an effect on growth cone morphology at concentrations of 3mM and were toxic at higher concentrations (Figure 3). In contrast, the N-methylamide of TMCA (MTMCD) was more potent than VPA, causing a substantial increase in growth cone area at 1mM, a result consistent with previous observations (Shaltiel et al, 2004). We observed a strong effect on growth cones when the concentration of MTMCD was lowered further to 0.5mM. We find that addition of 2mM myo-inositol to the medium of growth cones treated with 1mM MTMCD (Figure 4) reverses its effect, consistent with the mode of action of VPA and suggests an inositol depletion mechanism.

Effects of VPA isomers and derivatives on $InsP_3$.

In all cases the increase in growth cone area is reversed by addition of myo-inositol, indicative of an inositol depletion mechanism. It is not possible to directly measure inositol or inositol phosphate concentrations in DRG explants, so we examined $InsP_3$ in an alternative system, *Dictyostelium* amoeba. This has previously proven useful to measure inositol depletion due to VPA and other related molecules (Williams et al 2002; Eickolt et al 2005). To test the effects of the VPA derivatives described above on inositol based signalling, we measured their effects on $InsP_3$ concentrations in *Dictyostelium*. For technical reasons cells were treated at 0.5mM for 20 hours. PIA, the most potent branched constitutional isomer, caused a significant decrease in $InsP_3$ (Figure 5). PID, the corresponding amide of PIA, had no effect on $InsP_3$

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concentration, an observation consistent with its lack of effect on growth cone spreading. All of the other compounds tested (DIA, VCA, DID, TMCA, TMCD, TMCU, MTMCD, 4-ene-VPA), except 4-yne-VPA, had no significant effect on $InsP_3$ (data not shown).

The effect of VPA derivatives and analogous on the Wnt signal transduction pathway

Given the potential for VPA to stimulate TCF transcriptional activity via HDAC inhibition (Phiel et al., 2001), we examined whether the compounds identified above stimulate this pathway. We used a TCF-luciferase based reporter assay in HEK293 cells (a TOPflash assay) to examine the effects of each VPA isomer, analogue and their derivatives. As a control we used lithium to stabilize β-catenin protein via inhibition of GSK-3. This caused a significant increase in TCF-mediated gene expression as measured by luminescence at 3 mM; a concentration only slightly higher than its Ki of 2 mM against GSK-3 (Figure 6). VPA treatment (3 mM) induces a significant but smaller induction of luciferase gene expression. At this concentration, none of the three VPA isomers, TMCA nor its amide derivatives, caused a significant increase in luminescence compared to an untreated control.

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Discussion

These experiments map the structural elements of the VPA molecule that affect neuronal growth cone behavior and inositol depletion. We show that structurally different groups of VPA-related compounds cause growth cone spreading. First, there is a class of branched constitutional isomers of VPA, which include PIA, DIA and VCA. The most potent isomer was PIA, which caused growth cone spreading at 0.5mM, a significant lower concentration that seen with VPA. Second, we found that one of the amide derivatives of a cyclopropyl analog of VPA, MTMCD, had activity on DRG growth cones. This was previously noted by Shaltiel et al (2004), but here we show that this property is unique among the cyclopropyl analogs that have been tested

The effects of all compounds that increased growth cone spreading were reversed by addition of myo-inositol, consistent with the inositol depletion mechanism reported for VPA. The inositol reversal of MTMCD reported here is also consistent with the strong inhibition of inositol synthase activity reported by Shaltiel et al (2004). Finally, we show that PIA reduces $InsP_3$ in the *Dictyostelium* based assay again consistent with a mechanism of inositol depletion. It is unclear why the other branched constitutional isomers, DIA, VCA and the VPA amide analogue MTMCD, failed to significantly reduce $InsP_3$ in this assay. This may arise from differences in cellular uptake, pharmacokinetics or inositol synthase structure; furthermore many of the compounds exhibited toxic effects on *Dictyostelium* above 0.5mM.

We therefore have found two independent structural determinants, modification of the branching at C-2 of VPA and the derivatization by methylamine of the carboxylic group of TMCA, that cause growth cone spreading via an inositol depletion mechanism. These can be added to those reported in the earlier study of Eickholt et al

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(2005), who also identified another branched constitutional isomer of VPA, 2-ethyl-4-methylpentanoic acid, that possesses growth cone spreading and inositol depleting activity. This earlier study also suggested a third structural feature of unsaturated C-C bonds, as seen for the VPA analogues 2-methyl-2-pentenoic acid, (S)-2-pentyl-4-pentynoic acid and (S)-2-propyl-4-hexynoic acid which contribute to inositol depletion, however these molecules were also teratogenic (Eickholt et al., 2005). Consistent with this structural feature we found that the two unsaturated VPA derivatives 4-ene-VPA and 4-yne-VPA also caused growth cone spreading, and 4-yne-VPA lowered $InsP_3$ in *Dictyostelium* (data not shown).

Importantly these structural determinants show little overlap with those of VPA derivatives and analogues possessing potent anticonvulsant activity (Table 1). The corresponding amide of VPA, VPD (Shaltiel et al, 2004), and its CNS-active constitutional isomers: VCD, PID and DID (Isoherranen et al, 2003; Winkler et al., 2005b) had no effect on growth cone morphology. In contrast, PIA, DIA and VCA, the constitutional isomers of VPA that increase growth cone spreading have higher MES-ED_{50} value in mice compared to their corresponding amides (Table 1). There is also little correlation between growth cone spreading and MES-ED_{50} of TMCA, the cyclopropyl analogue of VPA and its amide derivatives. All have lower (more potent) MES-ED_{50} values than VPA, and that of VPA is >16 times higher than that measured for TMCU, however, only MTMCD caused growth cone spreading in these and previous experiments (Shaltiel et al, 2004).

Due to similarities between bipolar disorder and epilepsy, it has been proposed that they could have a common origin. Consistent with this hypothesis, the AEDs VPA and CBZ are effective mood stabilizers. New AEDs are now routinely evaluated for

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mood stabilizing properties (Gajwani et al, 2005). The results presented here show no significant correlation between MES-ED_{50} values and growth cone spreading at therapeutically used concentrations (Table 1). One caveat is that amides of several VPA analogues can reach higher concentrations in the brain than their corresponding acids (Blotnik et al., 1996), we cannot exclude the possibility that the amides of several VPA analogues tested by us (Figure 1) achieve higher brain concentrations than could be tested in these cell based assays. Taken together these results demonstrate that anticonvulsant activity and growth cone effects are likely to arise from different molecular targets that interact by distinct pharmacophore molecular conformations of VPA and its isomers. Given the correlation between increased growth cone spreading and other mood stabilizers, we suggest by extrapolation that the anticonvulsant and mood stabilizing properties of VPA may arise through different molecular mechanisms.

As lithium inhibits GSK-3, a protein kinase central component of the Wnt signaling pathway, it has been proposed that this signaling pathway may be a target of mood stabilizer drugs. Indeed the action of lithium shows many similarities to Wnt stimulation on regenerating neurons (Lucas et al, 1998). Furthermore, as VPA has been shown to elevate expression of β-catenin and hence mimic the effect of lithium on the Wnt pathway, it has been proposed that VPA may also act on the Wnt signaling pathway (Phiel et al, 2001). To test the possibility that the VPA analogues could elicit a Wnt signaling response we looked at their ability to activate TCF promoter activity, the nuclear target of β-catenin. We found no stimulation of TCF promoter activity of any of the VPA constitutional isomers and derivatives used in this study. These results further indicate that VPA can cause growth cone spreading independent of Wnt pathway activation, an activity also seen with CBZ (Williams et al, 2002; Ryves et al,

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2005). GSK-3 has also been associated with rapid changes in growth cone structure during axon guidance. Here, inhibition of GSK-3 blocks Sem3A induced growth cone collapse (Eickholt et al, 2002). Although unrelated to the changes of growth cone morphology examined in this study, we can exclude a similar mechanism of VPA action, because a reduction in cellular inositol would reduce cellular concentrations of phosphatidyl (3,4,5) trisphosphate (PIP_3) and lead to elevated GSK-3 activity; the opposite effect to that seen in the Sem3A collapse studies.

In conclusion, we have shown that structural modifications of the alkyl side chain moieties on the VPA core structure, to form its constitutional isomers PIA and DIA, potentiate the activity on the DRG. The structural activity relationships described here indicate the different pharmacological activity seen with the VPA molecule and its constitutional isomers, may relate to different structural requirements or conformations adopted by the side chain moieties of these isomers in their interactions with cellular VPA targets. In addition, MTMCD, an amide derivative of cyclopropyl VPA analogue, was the only amide analog demonstrating growth cone spreading effect, suggesting further investigation in elucidating the exact structural requirements for amides of VPA analogues for the growth cone spreading effect. Our results provide new strategies for future design of second generation to VPA CNS-active drugs with potential mood stabilizing activities, based on structural modifications of the alkyl side chain moieties of the VPA core structure.

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Footnotes

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Legends for Figures

Figure 1. Chemical structures of the tested compounds. VPA, valproic acid; 4-ene-VPA, 4-ene-valproic acid; 4-yne-VPA, 4-yne-valproic acid; DIA, diisopropylacetic acid; PIA, propylisopropylacetic acid; VCA, valnoctic acid; VCD, valnoctamide; VPD, valpromide; DID, diisopropylacetamide; PID, propylisopropylacetamide; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid; TMCD, 2,2,3,3-tetramethylcyclopropanecarboxamide; MTMCD, N-methyl-2,2,3,3,-tetramethylcyclopropanecarboxamide; TMCU, 2,2,3,3-tetramethylcyclopropanecarbonylurea.

Figure 2. The effects of aliphatic VPA constitutional isomers on rat DRGs growth cones spreading. These compounds demonstrate low anticonvulsant potency in animal models of epilepsy (Table 1, Haj-Yehia and Bialer 1990). A histogram shows the percentage of growth cones with spread areas within 50 μm^2 class intervals. The abbreviations for the various compounds are given in Figure 1. (a) Untreated control experiment, comprising 119 growth cones derived from 9 DRG explants, (*b*) denotes where a small growth cone would be seen (*c*) denotes where a spread growth cone would be. (b) DIC image of a small growth cone (x64). (c) DIC image of a spread growth cone (x64). (d) 2mM LiCl treated experiment, comprising 79 growth cones derived from 3 DRG explants. (e) 3mM VPA treated experiment, comprising 146 growth cones derived from 6 DRG explants. (f) 1mM VPA treated experiment, showing a sub-optimal dose for growth cone spreading (110 growth cones from 6 DRG explants). (g) 3mM VCA treated experiment, comprising 89 growth cones derived from 4 DRG explants. (h) 1mM VCA treated experiment, comprising 124

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growth cones derived from 4 DRG explants. (i) 1mM VCA treatment with 2mM myo-inositol. Data comprises 106 growth cones derived from 3 DRG explants. (j) 1mM DIA treatment experiment, comprising 75 growth cones derived from 3 DRG explants. (k) 0.5mM DIA treatment experiment, comprising 61 growth cones derived from 4 DRG explants. (l) 1mM DIA treatment with 2mM myo-inositol. Data comprises 60 growth cones derived from 3 DRG explants. (m) 1mM PIA treated experiment, comprising 99 growth cones derived from 4 DRG explants. (n) 0.5mM PIA treated experiment, comprising 73 growth cones derived from 3 DRG explants (o) 1mM PIA treatment with 2mM myo-inositol. Data comprises 61 growth cones derived from 3 DRG explants. Addition of myo-inositol reverses the effect of DIA and PIA induced growth cone spreading. Addition of myo-inositol also reduces the median growth cone size of VCA treated experiment significantly ($p<0.05$)**.** Total data of (a & d-o) was analyzed by a Kruskal-Wallis test, followed by Dunn's multiple comparison test of each experiment vs. control: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 3. The effects of VPA and aliphatic VPD constitutional isomers on rat DRGs growth cones spreading. The VPD constitutional isomers exhibit potent anticonvulsant activity in animal models of epilepsy (Table 1, Haj-Yehia and Bialer, 1990; Winkler et al, 2005b). A Histogram shows the percentage of growth cones with spread areas within 50 μm^2 class intervals. The abbreviations for the various compounds are given in Figure 1. (a) Untreated control experiment, as shown in figure 2. (b) 3mM VPA treated experiment, as shown in figure 2. (c) 3mM VPD treated experiment, comprising 69 growth cones derived from 3 DRG explants. (d) 3mM DID treated experiment, comprising 110 growth cones derive from 3 DRG explants. (e) 3mM PID treated experiment, comprising 75 growth cones derived from

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4 DRG explants. (f) 3mM VCD treated experiment, comprising 60 growth cones derived from 3 DRG explants. Total data of (a-f), was analyzed by a Kruskal-Wallis test, followed by Dunn's multiple comparison test of each experiment vs. control: ***$p<0.001$.

Figure 4. The effects of VPA analogue (TMCA) and its amide derivatives on rat DRGs growth cones spreading. The amide derivatives of TMCA demonstrate potent anticonvulsant activity in animal models of epilepsy (Table 1, Sobol et al, 2004), where as TMCA shows only weak anti-seizure activity. A Histogram shows the percentage of growth cones with spread areas within 50 μm^2 class intervals. The abbreviations for the various compounds are given in Figure 1. Untreated control experiment, as well as 3mM VPA and 2mM LiCl treated experiments as in figure 2. (a) 3mM TMCA treated experiment, comprising 97 growth cones derived from 4 DRG explants. (b) 3mM TMCD treated experiments, comprising 63 growth cones derived from 3 DRG explants. (c) 1mM TMCU treated experiment, comprising 73 growth cones derived from 4 DRG explants. (d) 0.5mM MTMCD treated experiment**,** comprising 50 growth cones derived from 2 DRG explants. (e) 1mM MTMCD treated experiment, comprising 79 growth cones derived from 4 DRG explants. (f) 1mM MTMCD treatment with 2mM myo-inositol. Data comprises 60 growth cones derived from 3 DRG explants. Addition of myo-inositol reverses the effect of MTMCD induced growth cone spreading. Total data of (a), (b), (c), (d), (e), (f) was analyzed by a Kruskal-Wallis test, followed by Dunn's multiple comparison test of each experiment vs. control: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

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Figure 5. Effects of VPA derivatives, its analogues and their amides on *Dictyostelium* $InsP_3$ levels. *D. discoideum* cells were grown overnight in complete medium in the presence of VPA or one of the tested compounds at 0.5mM or with lithium at 2mM. Changes in $InsP_3$ levels were subsequently measured by isotope dilution assay and compared with vehicle-only. Comparison of relative efficacy of VPA derivatives and their analogues to cause $InsP_3$ depletion expressed as a percentage of that found for control. Results represent three experiments assayed in triplicate (± S.D); *, $p<0.05$.

Figure 6. Screening for Wnt pathway modulators.

Cells were grown for 48h in complete medium in the presence of lithium, VPA or one of its derivatives and analogues at 3mM. Luminescence was read after 5min incubation with luminescence substrate. The histograms show the relative light unites for the corresponding drugs assayed in triplicates (Mean ± S.D). The abbreviations for the various compounds are given in Figure 1.

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Tables

Table 1

Activity of VPA derivatives and analogues as anticonvulsants (MES test in mice, i.p.) and their effects on the median growth cone area from rat dorsal root ganglia.

Drug	MES ED_{50} (mg/kg)	Median Spread Area (μm^2)
Control	NA	70
3mM VPA	263[a]	228***
2mM LiCl	NA	118**
1mM DIA	238[b]	191***
1mM PIA	>300[d]	102*
1mM VCA	269[b]	84
1mM 4-ene-VPA	350[c]	95
1mM 4-yne-VPA	NT	83
3mM DID	87[b]	65
3mM PID	122[d]	73
3mM VCD	58[b]	61
3mM VPD	56[b]	52
1mM MTMCD	98[e]	101*
1mM TMCU	90[e]	51
3mM TMCA	194	78
3mM TMCD	>120[e]	62

The abbreviations for the various compounds are given in Figure 1.

The differences in the median values were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparison test of each drug treatment vs. control: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

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NA- not applicable; NT-not tested

[a] Data from White et al. (2002)

[b] Data from Haj-Yehia and Bialer (1990)

[c] Data from Loescher W. et al. (1985)

[d] Data from Spiegelstein et al. (1999)

[e] Data from Sobol et al. (2004)

[f] TMCU is insoluble at concentrations above 1mM and therefore could only be tested at lower concentrations.

Figure 1

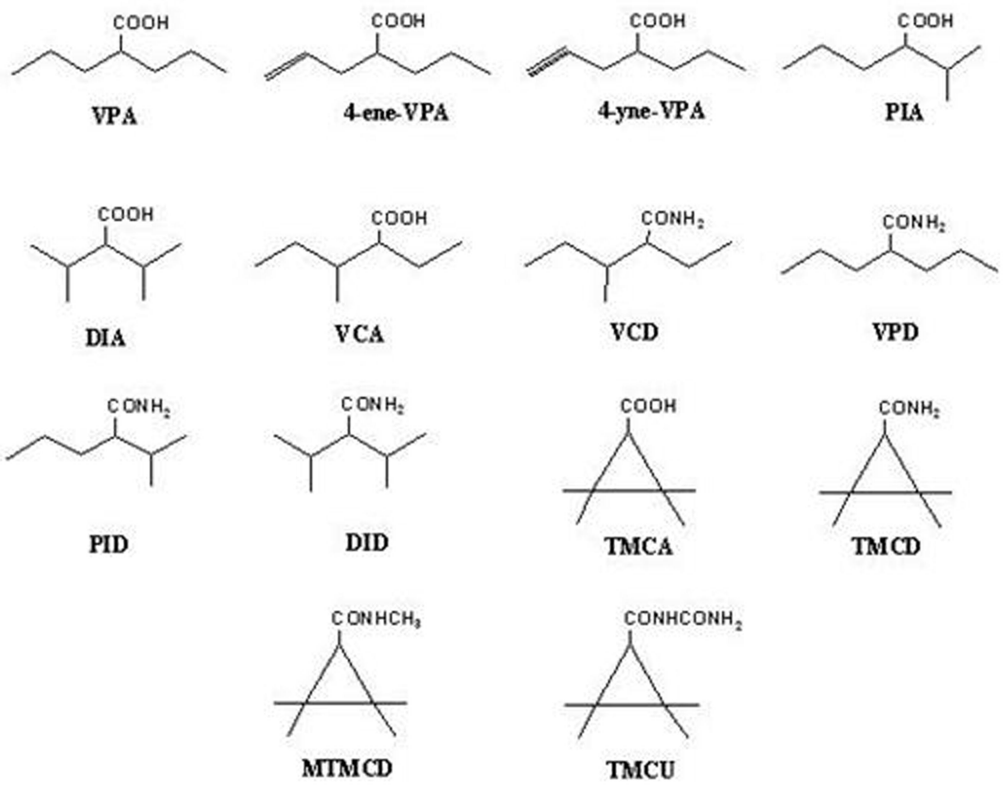

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Figure 2

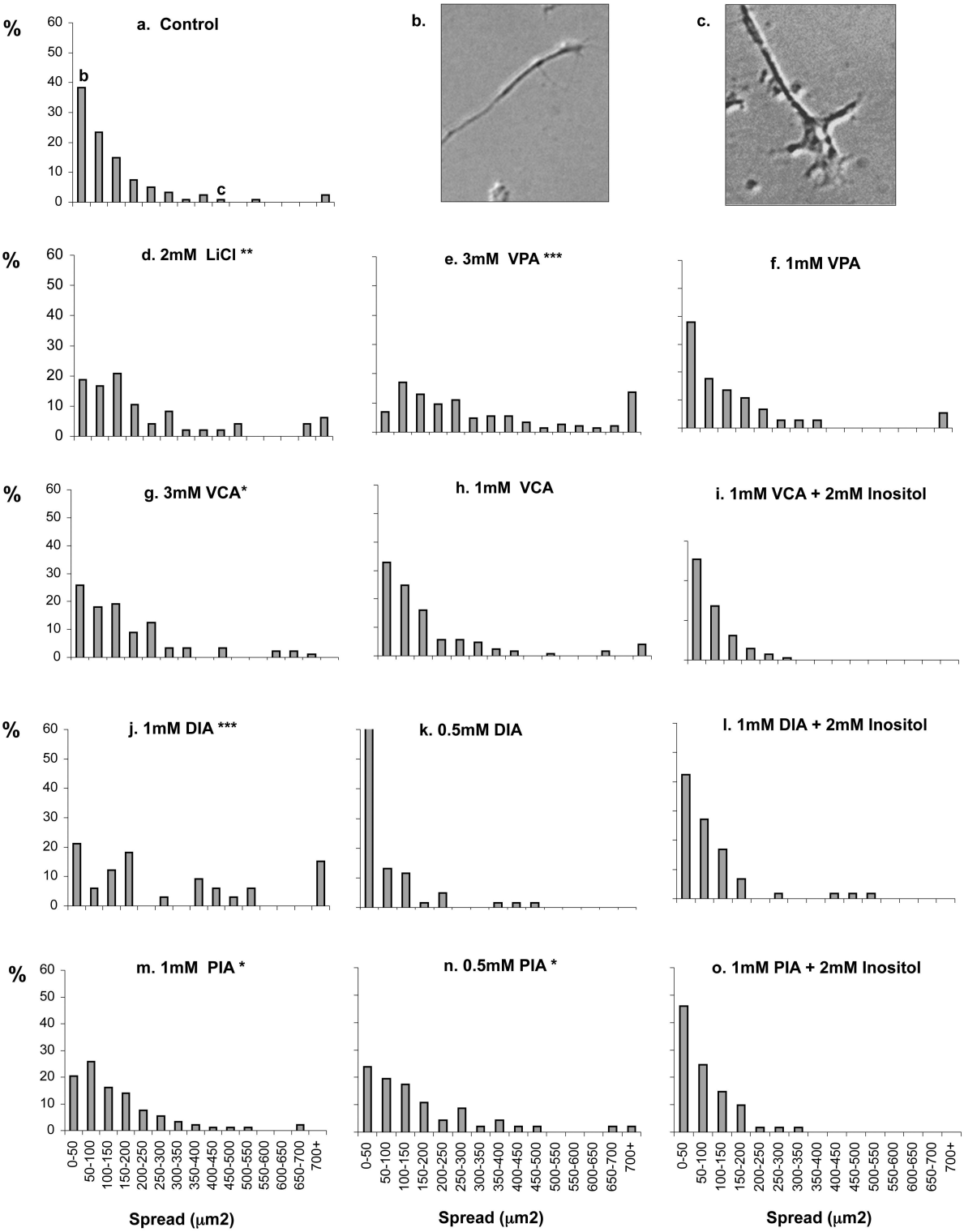

Figure 3

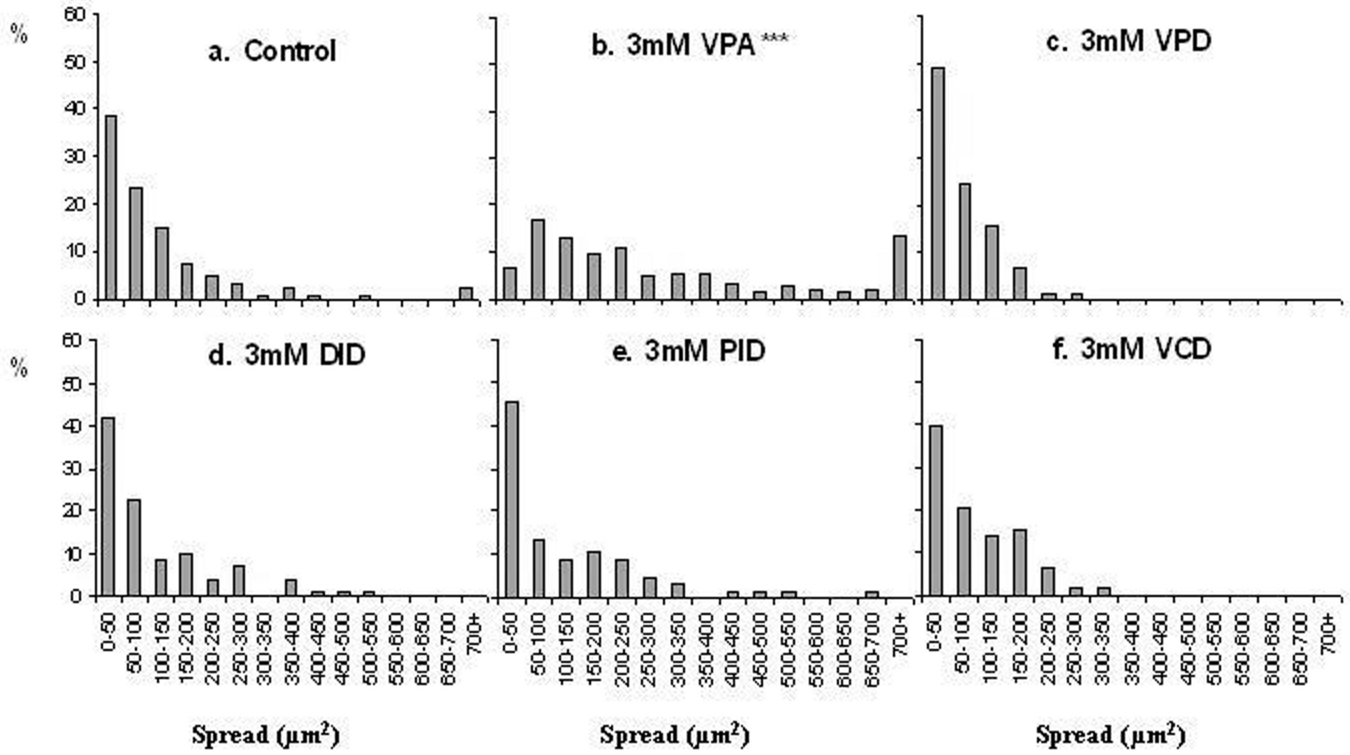

Figure 4

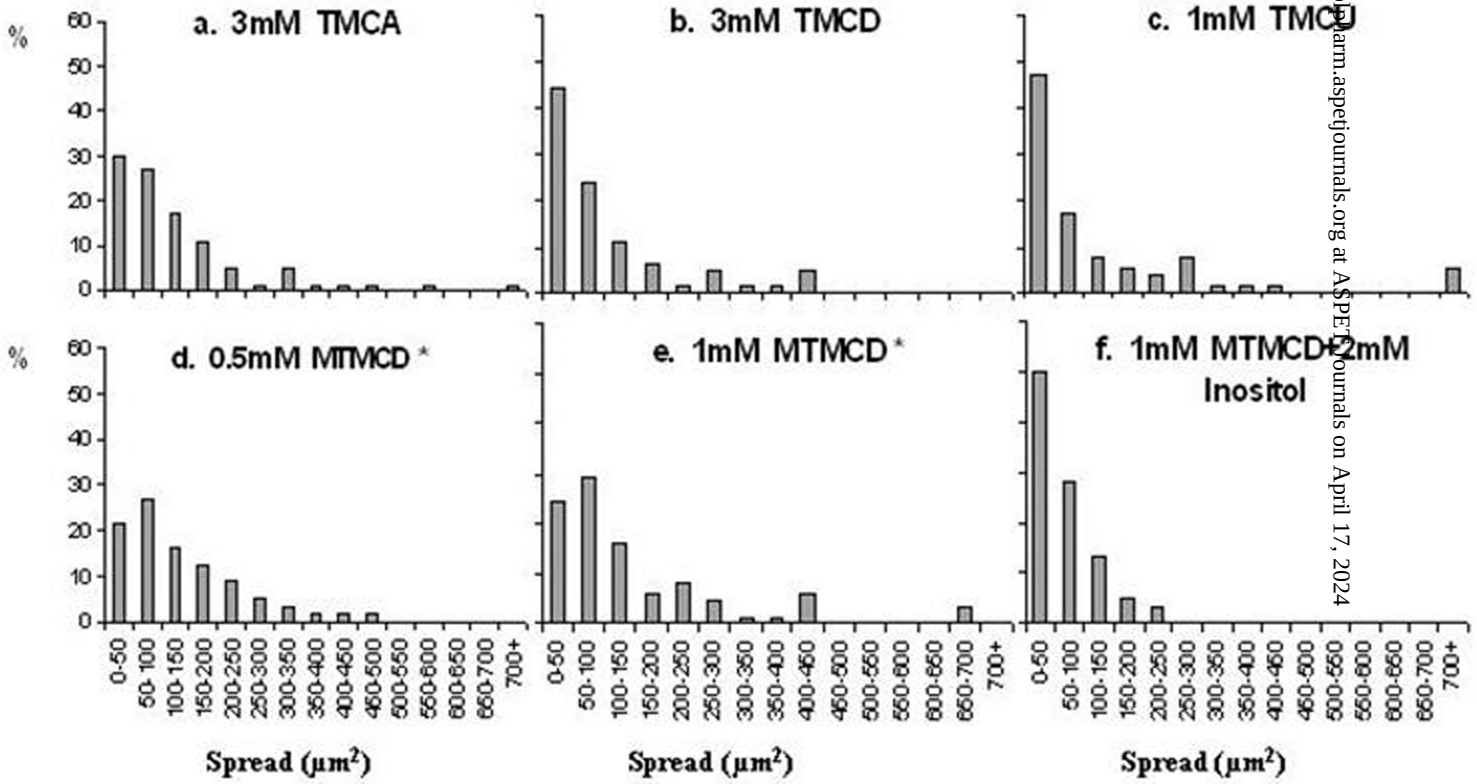

Figure 5

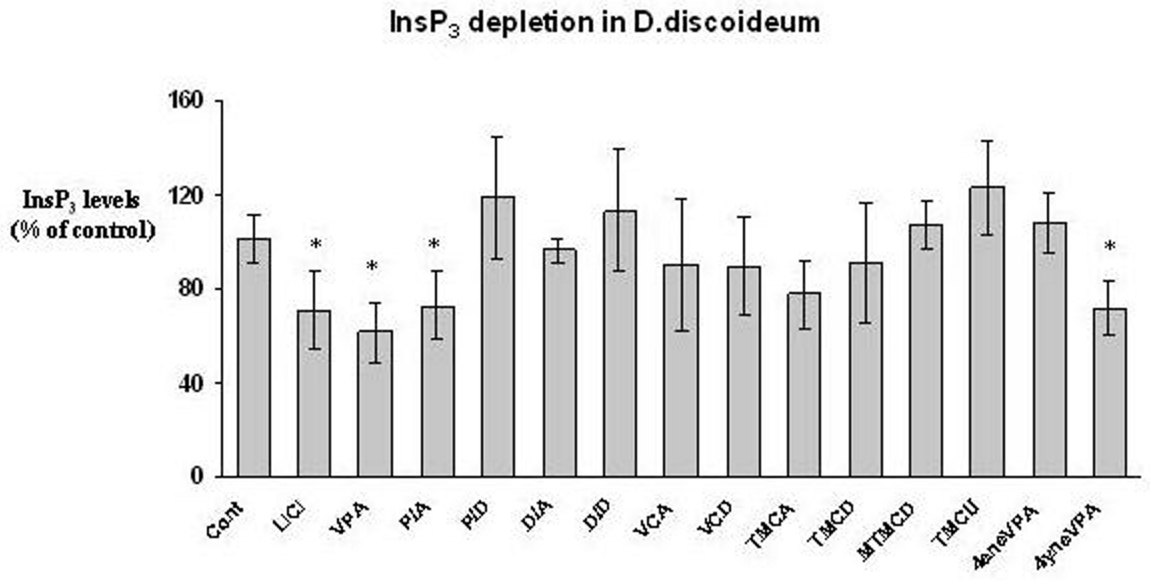

Figure 6