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Anne-Marie Miller, Alessia Piazza, Darren S.D. Martin, Michelle Walsh, Arkady Mandel, et al.. The deficit in long-term potentiation induced by chronic administration of amyloid- β -is attenuated by treatment of rats with a novel phospholipid-based drug formulation, VP025. *Experimental Gerontology*, 2009, 44 (4), pp.300. 10.1016/j.exger.2008.12.001 . hal-00493095

HAL Id: hal-00493095

<https://hal.science/hal-00493095>

Submitted on 18 Jun 2010

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Accepted Manuscript

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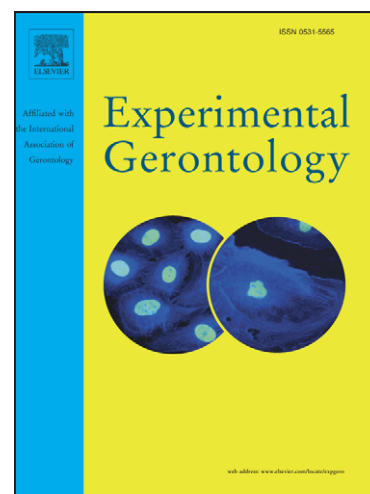
PII: S0531-5565(08)00405-1
DOI: [10.1016/j.exger.2008.12.001](https://doi.org/10.1016/j.exger.2008.12.001)
Reference: EXG 8570

To appear in: *Experimental Gerontology*

Received Date: 28 October 2008
Accepted Date: 1 December 2008

Please cite this article as: Miller, A-M., Piazza, A., Martin, D.S.D., Walsh, M., Mandel, A., Bolton, A.E., Lynch, M.A., The deficit in long-term potentiation induced by chronic administration of amyloid- β -is attenuated by treatment of rats with a novel phospholipid-based drug formulation, VP025, *Experimental Gerontology* (2008), doi: [10.1016/j.exger.2008.12.001](https://doi.org/10.1016/j.exger.2008.12.001)

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The deficit in long-term potentiation induced by chronic administration of amyloid- β is attenuated by treatment of rats with a novel phospholipid-based drug formulation, VP025

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Running Title: VP025 attenuates the A β -induced inhibition of LTP

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Key Words: Long-term potentiation (LTP), hippocampus, caspase 3, amyloid- β , phospholipid particles

ABSTRACT

Amyloid- β (A β) peptides, the primary component of the amyloid plaques in Alzheimer's disease (AD), exert profound effects on neurons in vitro and negatively impact on neuronal function in vivo. One of the consequences of increased A β in the brain, either as a result of overexpression of the precursor amyloid precursor protein in transgenic mice, or injection into the brain is a decrease in one form of synaptic plasticity, long-term potentiation (LTP) in the hippocampus. Here we investigated the effect of infusion of A β for 28 days on LTP in dentate gyrus of rats and demonstrate that it was profoundly decreased compared with control-treated rats. We show that this effect is accompanied by increased activity of caspase 3, which is an indicator of cell stress. Significantly these changes were attenuated in animals which were pretreated with particles incorporating phosphatidylglycerol (VP025) and the evidence indicated that even when treatment was given 2 weeks after the start of the A β infusion, VP025 was capable of attenuating A β -induced changes. The evidence suggests that activation of caspase 3 was mediated by an A β -induced increase in sphingomyelinase, with the subsequent production of ceramide which is known to have a detrimental effect on neuronal function.

1. INTRODUCTION

Deterioration in neuronal function has been consistently described in rats treated with A β and one manifestation of this is a decrease in the ability of these animals to sustain LTP in the hippocampus. Data from this laboratory have established that the A β -induced decrease in LTP is associated with microglial activation and increased concentration of inflammatory cytokines in hippocampus (Minogue et al., 2007). Data from a number of studies have shown that if microglial activation is attenuated, then the depression in LTP is also attenuated suggesting that the inflammatory conditions which prevail following A β administration are detrimental to neuronal function (Lynch et al., 2007; Lyons et al. 2007; Minogue et al., 2007). A similar inverse correlation between maintenance of LTP and inflammatory changes has been identified in rats following treatment with the immune modulator, lipopolysaccharide (LPS) but these changes were prevented by treatment of rats with the polyunsaturated fatty acid, eicosapentaenoic acid (Lonergan et al., 2004) or by treatment of rats with a preparation of phospholipid nanoparticles incorporating phosphatidylserine (Nolan et al., 2004). Phosphatidylglycerol shares some properties with phosphatidylserine and we have recently found that particles incorporating phosphatidylglycerol (VP025) attenuate the age-related and LPS-induced depression of LTP (Martin et al., submitted) and ameliorates the 6-hydroxydopamine (6OHDA)-induced cell loss in the striatum (Crotty et al., 2008).

In addition to the inverse relationship between A β -induced microglial activation and decreased LTP, we have found that decreased LTP is associated with activation of caspase 3 (Minogue et al., 2003). It is known from in vitro analysis that A β induces neuronal stress and increases caspase 3 activation as a consequence of increased permeability of the mitochondrial membrane with the subsequent release of cytochrome c (Green and Kroemer, 2004; Minogue et al., 2003). Among the

factors which contribute to loss of mitochondrial membrane patency and ultimately, activation of caspase 3, is ceramide, which is generated when sphingomyelinase hydrolyses the sphingophospholipid, sphingomyelin. It has been suggested that caspase 8 can act on membrane-associated sphingomyelinase in endosomes or lysosomes, and in this way lead to the generation of ceramide (Smyth et al., 1996) which can increase mitochondrial membrane permeability and trigger cytochrome c release (Roberts, 2005; Siskind et al., 2006).

Here, we investigated the effects of chronic administration of A β ₁₋₄₀ and A β ₁₋₄₂ on LTP in perforant path-granule cell synapses and asked whether treatment of rats with VP025, which has been shown to have neuroprotective effects, might modulate A β -induced changes. The data indicate that A β treatment decreased LTP and that this, as well as the associated increase in caspase 3 activity, was attenuated in VP025-treated animals. The evidence is consistent with the proposal that the A β -induced increase in caspase 3 is dependent on activation of sphingomyelinase.

2. MATERIALS AND METHODS

2.1 Treatment of animals

Male Wistar rats aged 3-4 months (Bioresources Unit, Trinity College, Dublin, Ireland) were used in these studies. Animals were housed in groups of 4-6 at 22-23°C, and were maintained on a 12-hour light schedule, under veterinary supervision in the BioResources Unit. The experiments were performed under a license issued by the Department of Health and Children (Ireland), with the approval of the local ethics committee and in accordance with local guidelines.

Rats were randomly divided into 8 groups, half of which were treated with a cocktail of A β ₁₋₄₀ + A β ₁₋₄₂, while the other half received the reverse peptide, A β ₄₀₋₁. Animals were anaesthetized with ketamine (75mg/kg) and xylazine (10mg/kg) and implanted with osmotic mini-pumps (model 2004, Alzet, USA). The pump was implanted subcutaneously in the mid-scapular region and was attached via polyvinylchloride tubing (Alzet, 0.69mm diameter) to a chronic indwelling cannula (Alzet, Infusion Kit II), which was positioned stereotactically in the ventricle (0.9mm posterior to bregma, 1.3 lateral to the midline and 3.5mm ventral to the dura). The cannula was affixed to the skull using cryanoacetate gel and was secured in place by a smooth covering of dental cement (Stoelten, USA). Post-operative care included a subcutaneous injection of the analgesic Rimadil (5mg/kg). The pumps delivered a cocktail of A β ₁₋₄₀ (26.9 μ M) and A β ₁₋₄₂ (36.9 μ M; aggregated for 24 h at 25°C and 37°C for 48 h; Biosource, Belgium) or control peptide A β ₄₀₋₁ (63.8 μ M) intracerebroventricularly at the rate of 0.25 μ l/h (\pm 0.05 μ l) for 28 days. Analysis of the A β preparation by the thioflavin T fluorescent assay and gel electrophoresis revealed the presence of low oligomeric species; the predominant form (36% of the total) was the 13.5kDa species.

Rats which received reverse peptide (hereafter referred to as control-treated rats) and those which received A β ₁₋₄₀ + A β ₁₋₄₂ (hereafter described as A β -treated rats) were further subdivided. In one experiment, rats were injected intramuscularly with saline or VP025 (150 μ l; 1.2 \times 10⁷ particles/ml) 14, 13 and 1 day prior to implantation of the minipumps and delivery of A β for 28 days (pretreated group); in this experiment there were 4 subgroups of rats, control-treated, A β -treated, VP025-treated and

A β +VP025-treated. In the second experiment, saline or VP025 was injected 15, 16 and 27 days following surgery, i.e. 2 weeks after A β treatment began (intervention group); this was to assess whether intervention with VP025 would ameliorate the effect of A β .

2.2 Analysis of LTP

At the end of the period of A β delivery, rats were anaesthetized with urethane (1.5mg/kg) and assessed for their ability to sustain LTP in perforant path-granule cell synapses as described previously (Nolan et al., 2005). A bipolar stimulating electrode was positioned in the perforant path (4.4mm lateral to lambda) and a unipolar recording electrode was positioned in the dorsal cell body region of the dentate gyrus (2.5mm lateral and 3.9mm posterior to bregma). Test shocks were delivered at 30 sec intervals, and after a period of recording which allowed stabilization, responses were recorded for 10 min before and 40 min after, tetanic stimulation (3 trains of stimuli; 250 Hz for 200 msec; 30 sec intertrain interval). At the end of the period of electrophysiological recording, rats were killed by decapitation, the brains were rapidly removed and the hippocampus and cortex were dissected free. Tissue was cross-chopped (350 μ m x 350 μ m) and stored at -80°C in Krebs buffer (composition in mM; NaCl 136, KCl 2.54, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.18, NaHCO₃ 16, glucose 10) with added CaCl₂ (1.13 mM) and 10% dimethyl sulphoxide (DMSO) until required for analysis.

2.3 Preparation of neuronal cultures

Primary cortical neurons were isolated and prepared from 1-day old Wistar rats as described previously (Lyons et al., 2007). Briefly, cells were maintained in neurobasal medium (NBM; Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO₂:95% air. Media was replaced every 3 days and cells were grown in culture for up to 7 days prior to treatment. Cells were treated with A β ₁₋₄₂ (10 μ M; aggregated according to manufacturer's instructions; Biosource, USA) for 24 h. In some cases, cells were cocultured for 24h with A β in the presence or absence of the neutral sphingomyelinase inhibitor, GW4869 (20 μ M). GW4869 was dissolved in DMSO (0.001%). In other experiments, cells were incubated in the presence of ceramide (100 μ M; Sigma, UK) for 24h; ceramide was dissolved in DMSO (0.005%) and diluted with NBM (1:200 dilution) for cell treatment. In the experiments in which DMSO was used as diluent, the appropriate concentration of DMSO was used in the vehicle controls. At the end of the treatments, supernatants were removed and cells were harvested for analysis of activities of sphingomyelinase and caspase and for assessment of cell viability.

2.4 Assessment of caspase activity

Activities of caspases 3 and 8 were assessed in samples of cortical or hippocampal tissue and in cultured neuronal cells according to the manufacturer's instructions (Biomol, UK). Briefly, samples were equalized for protein concentration in assay buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol; pH 7.4) to give a final concentration of 0.8mg/ml. Aliquots (25 μ l) of supplied enzyme solution were added to 96-well microplates, sample or assay buffer (25 μ l)

was added and incubation continued at room temperature 21°C for 10 min. Substrate (Ac-DEVD-pNA (caspase-3) or Ac-IETD-pNA (caspase-8); 50µl; final concentration 200µM) was added to start the reaction and fluorescence was measured continuously for 6 min at 405nm. Data are presented as means \pm SEM and are expressed as pNA produced (pmol/min/mg).

2.5 Assessment of sphingomyelinase activity

Sphingomyelinase activity was assessed in aliquots of cortical tissue. Samples were equalized for protein and sphingomyelinase activity was assessed using the Amplex Red assay kit (Molecular Probes, UK) according to the manufacturer's instructions. Briefly, a working solution (100µl) containing Amplex Red reagent (100µM), horseradish peroxidase (2U/ml), choline oxidase (0.2U/ml), alkaline phosphatase (8U/ml) and sphingomyelin (0.5mM) was added to samples or controls (100µl) and incubated for 30 min at 37°C. Fluorescence was measured (544nm excitation and 590 emission) at several time points (0, 5, 15, 25 and 60 min), values were corrected for background and mean fluorescent values were calculated.

2.6 Statistical analysis

Data were analysed using either the Student's t-test for independent means, or analysis of variance (ANOVA) or multifactorial ANOVA followed by Student Newman-Keuls or Bonferoni post hoc tests to determine which conditions were significantly different from each other. All data are expressed as means \pm standard error of the means.

3. RESULTS

We report that delivery of a high frequency train of stimuli to the perforant path induced an immediate and sustained increase in population excitatory post synaptic potential (epsp) slope in control-treated rats, but treatment of rats with A β led to a marked time-related decline in epsp slope; statistical analysis revealed a significant difference between control-treated and A β -treated rats ($^{***}p < 0.001$; ANOVA; Figures 1a and 1b). Pretreatment of rats with VP025 significantly attenuated the A β -induced change ($^{+++}p < 0.001$; ANOVA; Figure 1a), while VP025 treatment did not affect the response to tetanic stimulation. Similarly the A β -induced depression in LTP was attenuated in animals which received VP025 2 weeks after A β infusion began ($^{+++}p < 0.001$; ANOVA; Figure 1b).

Hippocampal tissue prepared from these animals was assessed for caspase 3 activity and the data show that there was a significant A β -induced effect in both experiments ($^{***}p < 0.001$; ANOVA; Figures 1c and 1d). Post hoc analysis indicated that the significant A β -induced change was significantly attenuated in tissue prepared from A β -treated rats which received VP025 as a pretreatment or as an intervention treatment ($^{+++}p < 0.001$; ANOVA; Figures 1c and 1d). Analysis of caspase 3 in cortical tissue prepared from the same rats revealed similar findings; thus there was a significant A β effect ($^{***}p < 0.001$; ANOVA; Figure 2c) and this A β -induced change was significantly attenuated in tissue prepared from rats which were pretreated with VP025 ($^{+++}p < 0.001$; ANOVA; Figure 2c). The changes in activities

of caspase 8 ($***p < 0.001$; ANOVA; Figure 2a) and sphingomyelinase ($*p < 0.05$; mixed factorial ANOVA; Figure 2b) mirrored the changes in caspase 3 with a significant A β effect in each case that was attenuated in VP025-treated rats, although this attenuation was statistically significant only in the case of caspase 8 ($***p < 0.001$; ANOVA; Figure 2a).

In an effort to understand the mechanism by which A β induces activation of caspase 3, we undertook a series of experiments in cultured neurons prepared from cortical tissue obtained from neonatal rats. We first showed that A β significantly increased caspase 3 activity in these cells ($***p < 0.001$; ANOVA; Figure 2d) and that this effect was inhibited in cells which were incubated in A β and the sphingomyelinase inhibitor, GW4869 ($**p < 0.01$; ANOVA). This suggests that ceramide, which is the product of sphingomyelinase, mediates the effect of A β and, consistent with this, we show that ceramide, like A β , significantly increased caspase 3 activity ($**p < 0.01$; student t-test for independent means; Figure 2e). We also show that incubation of cells in the presence of A β decreased cell viability ($*p < 0.05$; ANOVA; Figure 2f) and that this was inhibited by including GW4869 in the incubation medium ($*p < 0.05$; ANOVA; Figure 2f; compared with A β alone). Consistent with the view that the effects of A β are mediated by sphingomyelinase is the finding that ceramide also significantly decreased cell viability ($***p < 0.001$; student t-test for independent means; Figure 2g).

4. DISCUSSION

Our objective was to assess whether a chronic infusion of A β for 28 days would exert an inhibitory effect on LTP and to establish whether any A β -induced effect might be modulated by pre-treatment or intervention treatment with VP025. We demonstrate that A β significantly decreased LTP and increased caspase 3 activity and that VP025 inhibited these effects of A β . The evidence suggests that the A β -induced activation of sphingomyelinase mediates the effect of A β .

An important finding of the present study is that chronic infusion of A β_{1-40} and A β_{1-42} inhibited LTP and while several groups have reported that acute exposure to different species of A β inhibits LTP in vivo (Freir et al., 2001; Klyubin et al., 2004; Minogue et al., 2003; Walsh et al., 2002) and in vitro (Freir et al., 2001; Puolivali et al., 2002; Saleshando and O'Connor, 2000; Wang et al., 2004), few studies have examined the effect of chronic infusion of A β on hippocampal function. However one study reported that LTP in CA1 was decreased in hippocampal slices obtained from rats which received A β_{1-40} for 10-11 days (Itoh et al., 1999) while others reported that hippocampal-dependent learning is depressed in rats treated chronically with A β_{1-40} or A β_{1-40} +A β_{1-42} (Frautschy et al., 2001; Nitta et al., 1997) and the findings of the present study are consistent with this. Here we coupled the A β -induced inhibition of LTP with increased activity of caspase 3 in hippocampus; this is indicative of cell stress, although not necessarily cell death (Garnier et al., 2004), and consistent with several reports which show that A β increases cell stress in vivo and in vitro (Boland and Campbell, 2004; Clarke et al., 2007; Lyons et al., 2007).

Importantly we show that treatment of rats with VP025 restores their ability to sustain LTP, even if treatment is not started until 2 weeks after A β infusion begins.

Consistent with the relationship between LTP and caspase 3 activity, the data show that VP025 also reversed the A β -induced increase in enzyme activity. These findings reveal a neuroprotective effect of VP025 and therefore provide support for our recent findings which demonstrated that VP025 overcame the inhibitory effects of age and LPS on LTP (Martin et al., submitted) and an earlier report which indicated that treatment of rats with VP025 attenuated the loss of dopaminergic neurons following 6OHDA injection (Crotty et al., 2008). Interestingly VP015 exerted similar neuroprotective effects and attenuated LPS- and age-associated deficits in LTP (Nolan et al., 2004; Nolan et al., 2005); these effects were coupled with the anti-inflammatory actions of VP015 which are shared by VP025 (Martin et al., submitted).

The A β -induced increase in activity of caspase 3 in hippocampus was mirrored by a similar change in cortical tissue, which was accompanied by increased activity in caspase 8 and sphingomyelinase. The coupled increases in activation of these enzymes is consistent with the fact that the executioner caspase 3 is a major substrate for the initiator caspase 8 (Stennicke et al., 1998), with the evidence which indicated that caspase 8 can activate sphingomyelinase (Heinrich et al., 2004) and with the demonstration that TNF α -induced apoptosis in oligodendrocytes is dependent on sequential activation of caspase 8, neutral sphingomyelinase and caspase 3 (Testai et al., 2004). Numerous studies have reported that A β increases caspase 3 activity (Awasthi et al., 2005; Boland and Campbell, 2004) and caspase 8 activity (Awasthi et al., 2005) in cultured neurons while increases in both enzymes have also been reported in the AD brain (Rohn et al., 2002).

In an effort to obtain additional evidence to support the proposal that sphingomyelinase plays a role in activating caspase 3, we undertook a series of experiments in vitro and demonstrate that, in cortical neurons, the A β -induced activation of caspase 3 was blocked by the sphingomyelinase inhibitor GW4869, suggesting that ceramide may trigger the increase in caspase 3 activity. This was confirmed by the finding that the stimulatory effect of A β on caspase 3 activity was mimicked by exposure of neurons to ceramide. We found that the A β decreased cell viability and that this, like its effect on caspase 3 was attenuated by GW4869 and mimicked by ceramide. The findings are consistent with others which have shown that A β increased ceramide in human primary neurons (Jana and Pahan, 2004) and that ceramide increases activity of caspases 9 and 3 in cortical neurons (Movsesyan et al., 2002).

The present findings identify a causal relationship between activation of sphingomyelinase and caspase 3 and reveal that the A β -associated decrease in LTP is coupled with upregulation of enzyme activity. Importantly, we identify a neuroprotective effect of VP025 and show that it attenuates the A β -induced increase in caspase 3 and deficit in LTP. The data are consistent with the proposal that activation of sphingomyelinase activity, which we have also observed in the aged brain, is a key factor in modulating activation of caspase 3 and controlling neuronal function.

FIGURE LEGENDS

Figure 1: VP025 treatment attenuates A β induced changes in LTP and caspase 3 activity. (a) and (b) LTP in dentate gyrus was significantly decreased in rats which received A β_{1-40} and A β_{1-42} intracerebroventricularly for 28 days ($^{***}p < 0.001$; ANOVA $n = 4-6$). Pretreatment of rats with VP025 14, 13 and 1 day prior to the start of A β infusion inhibited the A β -induced deficit in LTP (a) and similarly, the A β -induced decrease was inhibited when VP025 was given 15, 16 and 27 days after A β infusion started (b; $^{+++}p < 0.001$; ANOVA; $n = 4-6$). (c) and (d) Caspase 3 activity was significantly increased in hippocampal tissue prepared from A β -treated rats ($^{***}p < 0.001$; ANOVA; $n = 4-6$) and both VP025 treatment schedules significantly inhibited this change ($^{+++}p < 0.001$; ANOVA; $n = 4-6$).

Figure 2: The A β -induced increase in caspase 3 activity is accompanied by increased sphingomyelinase activity. (a)-(c) A β significantly increased activities of caspases 8 (a) sphingomyelinase (b) and caspase 3 (c) in cortical tissue ($^{*}p < 0.05$; multifactorial ANOVA; $^{***}p < 0.001$; ANOVA; $n = 5-6$); pretreatment of rats with VP025 14, 13 and 1 day prior to the start of A β infusion inhibited these A β -induced changes and the effects were significant in the case of caspases 8 and 3 ($^{+++}p < 0.001$; ANOVA; $n = 5$). (d) A β significantly increased caspase 3 in cortical neurons ($^{***}p < 0.001$; ANOVA; $n = 6$) and this was inhibited by GW4869 (20 μ M; $^{++}p < 0.01$; ANOVA; $n = 5$), GW4869 alone also decreased activity of caspase 3 ($^{***}p < 0.001$; ANOVA; $n = 5$). (e) and (g) Incubation of cortical neurons in the presence of ceramide (100 μ M) significantly increased caspase 3 activity (e; $^{**}p < 0.01$; student's t-test for independent means; $n = 6$) and decreased cell viability (g; $^{***}p < 0.001$; student's t-test for independent means; $n = 6$). (f) Incubation of cortical neurons in the presence of A β (10 μ M) significantly decreased cell viability ($^{*}p < 0.05$; ANOVA; $n = 6$) and this was significant attenuated by inclusion of GW4869 (20 μ M) in the incubation medium ($^{*}p < 0.05$; ANOVA; $n = 6$).

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Figure 1

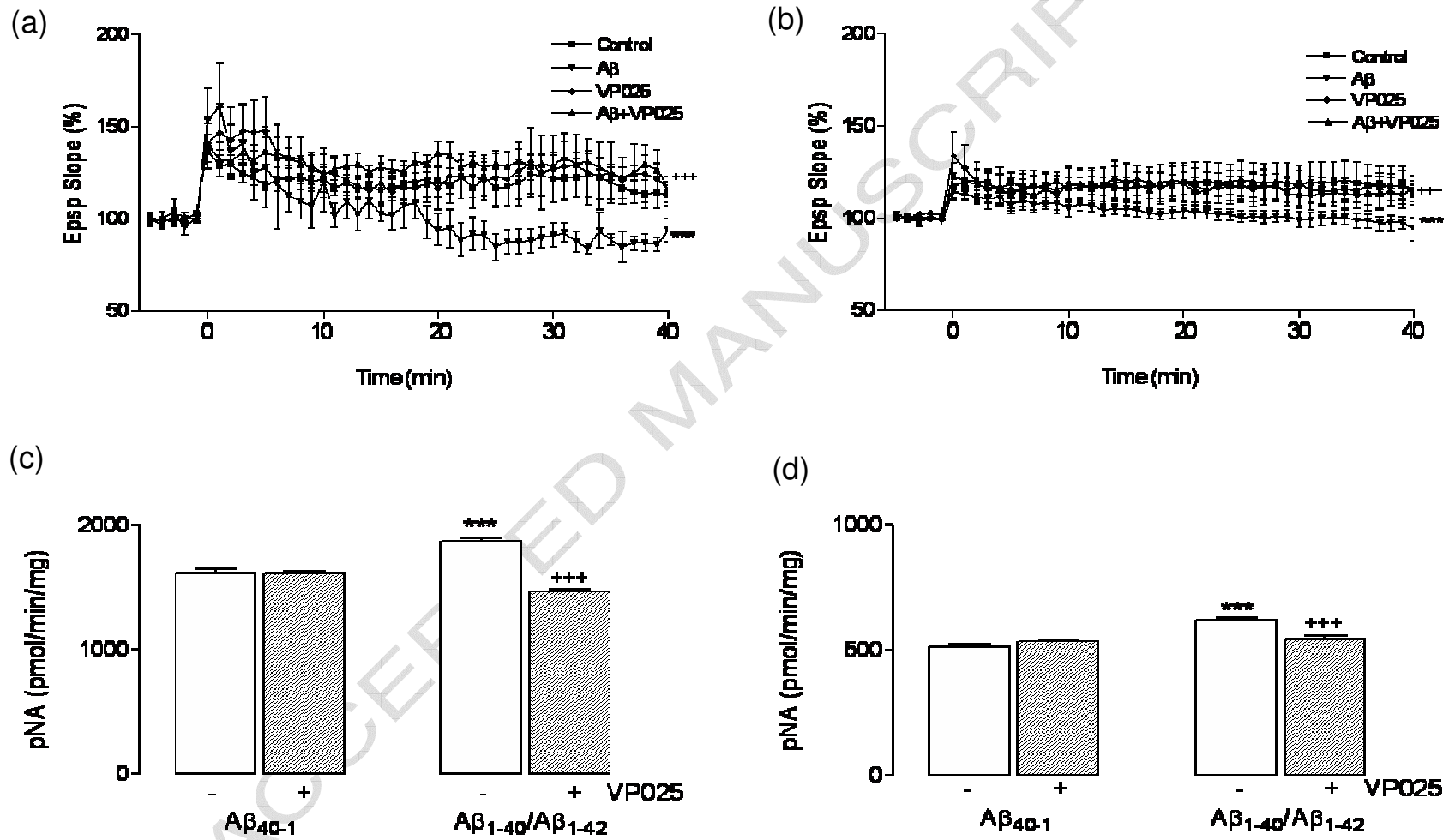


Figure 2

