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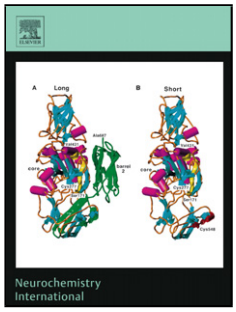

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The age- and amyloid-β-related increases in Nogo B contribute to microglial activation

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ABSTRACT

The family of reticulons include 3 isoforms of the Nogo protein, Nogo A, Nogo B and Nogo C. Nogo A is expressed on neuronal tissue and its primary effect is widely acknowledged to be inhibition of neurite outgrowth. Although both Nogo B and Nogo C are also expressed in neuronal tissue, their roles in the CNS remain to be identified. In this study, we set out to assess whether expression of Nogo A or Nogo B was altered in tissue prepared from aged rats in which increased microglial activation is accompanied by decreased synaptic plasticity. The data indicate that Nogo B, but not Nogo A, was markedly increased in hippocampal tissue prepared from aged rats and that, at least *in vitro*, Nogo B increased several markers of microglial activation. In a striking parallel with the age-related changes, we demonstrate that intracerebroventricular delivery of amyloid-β $(A\beta)_{1\text{-}40}$ + $A\beta_{1\text{-}42}$ for 8 days was associated with a depression of long-term potentiation (LTP) and an increase in markers of microglial activation and Nogo B. In both models, evidence of cell stress was identified by increased activity of caspases 8 and 3 and importantly, incubation of cultured neurons in the presence of Nogo B increased activity of both enzymes. The data identify, for the first time, an effect of Nogo B in the brain and specifically show that its expression is increased in conditions where synaptic plasticity is compromised.

Key words: Nogo; hippocampus; amyloid-β; age; neuroinflammation; microglial activation; synaptic deterioration; caspase; long term potentiation.

INTRODUCTION

Several markers of microglial activation are increased in the brain of aged animals; these include cell surface markers like CD11b, CD40 and major histocompatibility complex Class II (MHCII), while increased production of inflammatory cytokines has also been reported (see Clarke, et al., 2008, Loane, et al., 2009, Lynch, 2009). These changes are coupled with a deficit in synaptic plasticity, characterized by a decrease in the ability of animals to sustain long-term potentiation (LTP), and a convincing correlation between neuroinflammatory changes and inhibition of synaptic function (Clarke, et al., 2008, Downer, et al., 2010), and therefore cognitive function (Hein, et al., 2009, Moore, et al., 2009), has been described by several groups (see Lynch, 2010). Several of these inflammatory changes have also been identified in tissue prepared from animals treated with amyloid-β (Aβ) (Clarke, et al., 2007, Lyons, et al., 2007) and a deficit in synaptic function, both behavioural function and LTP, has been reported. When inflammatory changes are inhibited, for example by the anti-inflammatory cytokine IL-4, by the inhibitor of microglial activation minocycline, or by the polyunsaturated fatty acid eicosapentaenoic acid, the deficits in synaptic function are ameliorated in both aged and Aβ-treated (Lynch, et al., 2007, Lyons, et al., 2007, Minogue, et al., 2007, Nolan, et al., 2005) animals, suggesting a causal relationship between neuroinflammatory changes and loss of plasticity.

Under normal conditions, microglia are in a resting state because appropriate cell-cell interactions are maintained and concentrations of inflammatory cytokines like interferon-γ (IFNγ), which leads to their activation, are low. This homeostatic arrangement is disrupted in the brain of aged and Aβ-treated animals (see Lynch, 2010). However it is undoubtedly the case that other factors also play a role in modulating microglial activation; for example in ischaemic injury, traumatic brain injury and spinal cord injury numerous factors are released from infiltrating cells including matrix metalloproteinases, reactive oxygen and nitrogen species and heat shock proteins which may contribute to activation of both astrocytes and microglia (Beck, et al., 2010, Kim and Suh, 2009) and consequently contribute to poor recovery. Interestingly minocycline improves functional outcome identifying the negative impact of microglial activation on recovery (Stirling, et al., 2004)

although it is widely recognized that endogenous inhibitory factors, including a family of myelin-associated glycoproteins generated from the *nogo/RTN-4* gene, play a major role in preventing axonal regeneration and therefore functional recovery (Kilic, et al., 2010).

The family of reticulons include 3 isoforms of the Nogo protein, Nogo A, Nogo B and Nogo C. Nogo-A is mainly expressed on neuronal tissue, especially oligodendrocytes in the adult CNS (Chen, et al., 2000, GrandPre, et al., 2000, Prinjha, et al., 2000), and both Nogo A and Nogo B are expressed on central and peripheral neurons (Huber, et al., 2002, Josephson, et al., 2001, Oertle, et al., 2003, Wang, et al., 2002). Nogo-B and Nogo C are expressed in several peripheral tissues, with Nogo C particularly expressed on muscle (Josephson, et al., 2001, Morris, et al., 1999, Oertle, et al., 2003). With respect to the brain, protein and mRNA levels of all three isoforms have been detected in the spinal cord, and cerebral cortex, hippocampus and cerebellum brain regions (Chen, et al., 2000, Huber, et al., 2002, Yan, et al., 2006).

A great deal of evidence has been accumulated highlighting the negative influence of Nogo A on functional outcome and repair in a variety of models of neurogenerative change (Freund, et al., 2009, Kilic, et al., 2010, Marklund, et al., 2007). Fewer studies have investigated potential roles for Nogo B in the CNS. Here we report that Nogo B, but not Nogo A, was increased in hippocampal tissue prepared from aged rats and rats which received an intracerebroventricular infusion of Aβ for 8 days. This increase was coupled with evidence of microglial activation and increased caspase 3 activity. Significantly, we observed that Nogo B was capable of triggering microglial activation in a culture of mixed glial cells and that it potently increased caspase 3 activity in neuronal cultures. These findings highlight previously-unidentified effects of Nogo B in the brain.

MATERIALS AND METHODS

Animals

Male Wistar rats (Bantham and Kingman, UK) aged 3 months (250–350g) or 22 months (550–650g) were housed under a 12 h light/dark schedule and at an ambient temperature of 22-23°C. Rats were maintained in the BioResources Unit at Trinity College, Dublin, under veterinary supervision throughout the study and experiments were performed under a license issued by the Department of Health (Ireland) and in accordance with the guidelines laid down by the local ethical committee.

In one study, in which only young rats were used, rats were randomly assigned to a group which were treated with a cocktail of $A\beta_{1\text{-}40}$ + $A\beta_{1\text{-}42}$, and a control group which received the reverse peptide, $A\beta_{40\text{-}1}$ for a period of 8 days (Miller, et al., 2009). 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 stereotaxically 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 $A\beta_{1\text{-}40}$ (26.9μM) and $A\beta_{1\text{-}42}$ (36.9μM; aggregated for 24 h at 25°C and 37°C for 48 h; Biosource, Belgium) or control peptide $A\beta_{40\text{-}1}$ (63.8μM) intracerebroventricularly at the rate of 0.25μl/h (± 0.05μl) for 8 days. Analysis of the Aβ 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.

Analysis of LTP

LTP was assessed in young and aged animals and in animals which received $A\beta_{1-40}$ + $A\beta_{1-42}$ or control peptide. 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). Briefly, a bipolar stimulating electrode was positioned in the perforant path (4.4mm lateral to lambda), 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) and test shocks were delivered to the perforant path at 30 sec intervals for up to an hour allowing the response to stabilize. Recordings were made for 10 min before and 40 min after tetanic stimulation (3 trains of stimuli; 250Hz for 200 msec; 30 sec intertrain interval) and at the end of this time, rats were killed by decapitation, the brains were rapidly removed and the hippocampus and cortex were dissected free. Tissue was divided into one portion which was flash frozen and later used for PCR, and a second portion, which was cross-chopped (350µm x 350µm) and stored at –80°C in Krebs buffer (composition in mM; NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10) with added $CaCl_2$ (1.13mM) and 10% dimethyl sulphoxide (DMSO); the latter portion was used for Western immunoblotting and for analysis of activities of caspases 8 and 3.

Preparation and treatment of primary glial and neuronal cultures

Mixed glial cultures and neuronal cultures were prepared from the cortices of 1 day old Wistar rats (Trinity College, Dublin, Ireland) as previous described (Loane, et al., 2009). Briefly, cortical tissue was dissected free, cross-chopped and triturated in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, UK) supplemented with 10% Fetal Bovine Serum (FBS). The cells were passed through a sterile mesh filter (40µm), centrifuged at 2000 x g for 3 min and plated (2.5×10^5 cells/ml). To prepare neuronal cultures, tissue was cross-chopped and incubated in phosphate-buffered saline (PBS) with trypsin (0.25µg/ml, Sigma, UK) for 25 min at 37°C, triturated in PBS containing soybean trypsin inhibitor (0.2µg/ml, Sigma, UK) and DNase (0.2mg/ml, Sigma, UK) and gently passed through a sterile mesh filter (40µm). The suspension was centrifuged at 2000 x g for 3 min at 20°C, and the pellet was resuspended in warm NeuroBasal Media (NBM, Invitrogen, UK), supplemented with 10% FBS, penicillin (100 units/ml, Invitrogen, UK) and B27 (Gibco). After 48 h the neurons were treated with Ara-C (5µg/ml, Sigma) for 24 h.

Glia were treated after 12 days in culture with Nogo B (10 or 100ng/ml) and, after 24 h, cells were harvested for analysis of MHCII mRNA and CD11b mRNA by PCR. Neurons were similarly treated with Nogo B after 7 days in culture and, in this case, cells were harvested for analysis of activities of caspases 8 and 3. In both cases, cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2:95% air and media was replaced every 3 days.

Neurons were also treated with $A\beta_{1-40}$ (4.2µM) + $A\beta_{1-42}$ (5.6µM) or control peptide (10µM) for 48 h and treated 24 h subsequently with the Nogo receptor (NgR) antagonist NEP1-40 (Alpha Diagnostic Intl. Inc., Texas 78244 USA). The cells were harvested 24 h later and assessed for caspase 3 activation. Supernatants were assessed for LDH release as a measure of cytotoxicity (Promega, Southampton, UK).

Assessment of caspase activity

Activities of caspase 8 and caspase 3 were analysed using a colorimetric method (Biomol, UK), in samples of cortical and hippocampal homogenate prepared from young and aged rats, and rats treated with $A\beta_{1-40}$ + $A\beta_{1-42}$ or control peptide. Enzyme activity was also assessed in harvested cortical neurons. Samples and standards (25µl) were added to

assay buffer (25µl) in a 96 well plate and aliquots of substrates for caspases 8 and 3 (Ac-IETD-p-nitroanilide; pNA and Ac-DEVD-pNA respectively; 50µl; 200µM final concentration) were added to start the reaction. Absorbance was measured continuously from 0-20 min at 405nm, values were calculated with respect to the pNA calibration standard (100µl) and enzyme activity was expressed as pmol/min/mg protein.

Real-time PCR analysis of MHCII and CD11b mRNA expression

Total RNA was extracted from snap-frozen hippocampal tissue and harvested mixed glial cells using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Germany) as per manufacturer's instructions. Denaturing agarose gel electrophoresis was used to assess the RNA integrity. Total RNA concentrations were determined by spectrophotometry, samples were equalised and stored at -80^{o}C until required for cDNA synthesis. cDNA synthesis was performed on 1µg total RNA using a High Capacity cDNA RT kit (Applied Biosystems, Germany). Real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems, Germany) which contain forward and reverse primers, and a FAM-labelled MGB Taqman probe for each gene of interest; the assay IDs for MHCII and CD11b were Rn01768597_m1 and Rn00709342_m1, respectively. Real-time PCR was conducted using an ABI Prism 7300 instrument (Applied Biosystems, Germany). A 20µl volume was added to each well (9µl of diluted cDNA, 1µl of primer and 10µl of Taqman® Universal PCR Master Mix). Samples were assayed in duplicate in one run (40 cycles), which consisted of 3 stages, 95°C for 10 min, 95°C for 15 sec for each cycle (denaturation) and finally the transcription step at 60°C for 1 min. β-actin was used as endogenous control to normalize gene expression data, and β-actin expression was conducted using a gene expression assay containing forward and reverse primers (primer limited) and a VIC-labelled MGB Taqman probe from Applied Biosystems (Germany; Assay ID: 4352341E). Gene expression was calculated relative to the endogenous control samples and to the control sample giving an RQ value (2^{-DDCt}, where CT is the threshold cycle).

Analysis of Nogo, ICAM, CD86 and synaptophysin by Western immunoblotting

Tissue was equalized for protein, aliquots (10µl) were loaded onto 10% NuPAGE® Novex Bis-Tris gels (Invitrogen, UK), and were separated by application of a constant voltage (170V; 70 min) and transferred onto nitrocellulose strips (30V; 65 min). Proteins were immunoblotted for 2 h at room temperature with antibodies (1:100 in milk for Nogo A (Abcam, UK) and B (Chemicon International, USA); 1:200 in Tris-buffered saline (TBS)-Tween containing 2% BSA for CD86 and (R&D Systems, USA) ICAM-1 (Santa Cruz Biotechnology Inc., USA) and 1:400 in 1% BSA for synaptophysin (Sigma-Aldrich, UK) respectively). Membranes were washed three times in TBS-Tween, incubated with the appropriate secondary antibody (1:1000), stripped with 'Reblot' (1:10 dilution; Chemicon International, USA) and stained for actin expression to ensure equal loading of protein. Actin expression was assessed using a mouse monoclonal IgG_1 antibody (1:10000 in TBS-Tween containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA). Immunoreactive bands were detected using peroxidase-conjugated anti-mouse IgG (Sigma) and ECL (GE Healthcare, UK) chemiluminescence and were quantified using densitometry (Labworks, UVP BioImaging Systems, UK).

Statistical analysis

Data were analyzed using either Student's t-test for independent means, or analysis of variance (ANOVA) followed by post hoc Student Newman–Keuls test to determine which conditions were significantly different from each other. Data are expressed as means ± SEM.

RESULTS

LTP in perforant path-granule cell synapses was markedly decreased in aged, compared with young, rats (***$p < 0.001$; ANOVA; Figure 1a) as previously described (Loane, et al., 2009); the mean percentage changes (± SEM) in population epsp slope in the last 10 minutes of the experiment were 130.3 ± 0.57 and 107.6 ± 0.56 in young and aged rats respectively. Nogo A and Nogo B were assessed in hippocampal tissue prepared from young and aged rats and the data indicate that, while there was no change in Nogo A, Nogo B was significantly increased in tissue prepared from aged, compared with young, rats (**$p < 0.01$; student's t-test for independent means; Figures 1b, c). These changes were accompanied by evidence of microglial activation; thus increases in MHCII mRNA and in CD11b mRNA were observed in hippocampal tissue prepared from aged, compared with young, rats (*$p < 0.05$; ***$p < 0.001$; student's t-test for independent means; Figures 2a, b). In addition, both CD86 and ICAM-1 were increased (*$p < 0.05$; student's t-test for independent means; Figures 2c, d) which is a further indication of an age-related increase in microglial activation, and which supports previous evidence (Griffin, et al., 2006).

Activities of caspases 8 and 3 were assessed in hippocampal tissue prepared from young and aged rats as a measure of cell stress and both were found to be significantly increased in tissue prepared from aged, compared with young, rats (***$p < 0.001$; student's t-test for independent means; Figures 3a, b). These changes were paralleled by a significant decrease in synaptophysin (*$p < 0.05$; student's t-test for independent means; Figure 3c).

Having shown that the age-related deficit in LTP was coupled with increased expression of Nogo B and increased microglial activation, we investigated whether a similar coupling was observed in tissue prepared from Aβ-treated rats. Figure 4 shows that chronic infusion of Aβ for 8 days led to a marked decrease in LTP; the mean percentage changes (± SEM) in population epsp slope in the last 10 minutes of the experiment were 114.8 ± 0.78 in rats treated with the control peptide $A\beta_{40\text{-}1}$ and 87.0 ± 0.60 in rats treated with $A\beta_{1\text{-}42}$ + $A\beta_{1\text{-}40}$. Analysis of tissue prepared from these rats revealed that Nogo B was significantly increased (*$p < 0.05$; student's t-test for independent means; Figure 4c), whereas Nogo A was similar in both cohorts of animals (Figure 4b).

We next evaluated MHCII and CD11b mRNA as indicators of microglial activation and observed that both were significantly increased in hippocampal tissue prepared from rats which received $A\beta_{1\text{-}42}$ + $A\beta_{1\text{-}40}$ compared with control (*$p < 0.05$; ***$p < 0.001$; student's t-test for independent means; Figures 4a, b) and these changes were paralleled by an Aβ-induced increases in CD86 and ICAM-1 (*$p < 0.05$; ***$p < 0.001$; student's t-test for independent means; Figure 4c). As in the case of aged animals, the increase in microglial activation was accompanied by increased activity of caspases 8 and 3 (***$p < 0.001$; student's t-test for independent means; Figures 5d, e).

Because the Aβ-induced and age-associated increases in markers of microglial activation were mirrored by an increase in Nogo B, we considered that there might be a causal relationship between them and assessed the effect of Nogo B on MHCII and CD11b mRNA in mixed glial cultures. The data indicate that the higher, but not the lower, concentration of Nogo B significantly increased both measures (**$p < 0.01$; 100ng/ml Nogo B vs control; $^{++}p < 0.01$; 100ng/ml vs 10ng/ml Nogo B; ANOVA; Figures 6a, b), while 100ng/ml Nogo B also significantly increased ICAM-1 (*$p < 0.05$; ANOVA; Figure 6d) but did not significantly increase CD86 (Figure 6c). In addition, we assessed the effect of Nogo B on caspase activity in neurons and report that 100ng/ml significantly increased activity of both caspases 8 and 3 (***$p < 0.001$; 100ng/ml Nogo B vs control; $^{+++}p < 0.001$; 100ng/ml vs 10ng/ml Nogo B; student's t-test for independent means; Figures 7a and b respectively).

Since Aβ infusion into the rat brain induced caspase 3 activation and increased Nogo B expression we investigated whether antagonism of the NgR by NEP1-40 peptide could prevent Aβ-induced caspase 3 activation in a culture system (Figure 8). Caspase-3 activity was significantly increased in Aβ-treated neurons, and NEP1-40 significantly attenuated the effect of the Aβ ($^{*}p < 0.05$; $^{+}p < 0.05$; ANOVA; Figure 8).

DISCUSSION

We set out to investigate the possible impact of Nogo B on the deterioration of synaptic function in aged rats and in rats treated chronically with Aβ, and report that the deficit in LTP in both models was accompanied by increased hippocampal concentration of Nogo B. The evidence suggests that Nogo B negatively impacts on synaptic integrity by increasing activities of caspases 8 and 3 while it also increases microglial activation.

First we showed that Nogo B was markedly increased in hippocampal tissue prepared from aged rats in which LTP in perforant path-granule cell synapses was decreased. The age-related deficit in LTP is well-documented and several authors have reviewed the many changes which contribute to the deficit; these include synaptic loss, changes in expression of specific receptors, alterations in signalling cascades and gene transcription and dysfunction in homeostatic mechanisms especially in terms of calcium handling and oxidative processes (Burke and Barnes, 2006, Lynch, et al., 2006, Lynch, 2004, Serrano and Klann, 2004). In this laboratory, we have coupled the age-related deficit in LTP with evidence of microglial activation (Lynch, 2010), and the findings presented here support this, by showing that MHCII mRNA and CD11b mRNA are upregulated in hippocampus of aged, compared with young rats. We have reported a similar age-related change in MHCII mRNA, though not in CD11b mRNA, previously (Lyons, et al., 2009). CD86 and ICAM-1 are expressed on activated microglia and age-related increases in both markers are demonstrated here and elsewhere (Downer, et al., 2010). Thus the increase in microglial activation is accompanied by increased Nogo B, but not Nogo A and, to our knowledge, there are no previous data which have reported a similar change although, interestingly, a positive correlation between expression of genes associated with inflammation and Nogo B has been suggested (Yu, et al., 2009).

We made a similar link between increased hippocampal Nogo B, increased microglial activation, and the deficit in LTP induced by Aβ. Once again, the change was specific to Nogo B, with no change observed in Nogo A, and similarly no change in Nogo receptor (NgR) was observed (data not shown). Intracerebroventricular delivery of a cocktail of $A\beta_{1\text{-}40+1\text{-}42}$ for 8 days resulted in a marked decrease in the ability of rats to sustain LTP and, although LTP has not been evaluated previously using this delivery regime, delivery of Aβ for 28 days has a negative effect on spatial learning (Frautschy, et al., 2001), inhibits LTP (Miller, et al., 2009) and increases microglial activation (Frautschy, et al., 2001, Piazza and Lynch, 2009). As in the case of aged animals the deficit in LTP is coupled with evidence of increased microglial activation and also increased Nogo B, strengthening the correlation between these 3 factors.

A significant and novel finding in the present study is that Nogo B activates microglia *in vitro*, increasing cell surface markers, MHCII and CD11b. There are no previous reports of such an action. However activation of microglia and macrophages, accompanied by upregulation of NgR, has been reported following traumatic brain injury and in the lesion which characterize multiple sclerosis (David, et al., 2008) and, in an animal model of Alzheimer's disease the plaque-associated activated microglia (Simard, et al., 2006) also seem to be associated with increased expression of NgR (Park, et al., 2006). Interestingly, a role for Nogo B in recruitment of macrophages was implicated by *in vitro* analysis of the effect of Nogo B on chemotaxis and by the finding that infiltration of F4/80 positive

macrophages was markedly reduced in ischaemic tissue in Nogo$^{-/-}$ mice (Yu, et al., 2009). At least in the broadest sense, these findings are consistent with the present observation, as is the finding that there is a decrease in expression of several genes associated with inflammation in Nogo$^{-/-}$ mice (Yu, et al., 2009)

Whereas a vital role for Nogo A in blocking neurite outgrowth is widely recognized (Yan, et al., 2006), the function of Nogo B, particularly in the brain where expression has been reported (Acevedo, et al., 2004, Chen, et al., 2000, Huber, et al., 2002, Yan, et al., 2006), is almost unexplored. However the presence of the 3 Nogo transcripts in the hippocampus during development points to a physiological role (Meier, et al., 2003) but the nature of the role, specifically in the context of Nogo B is currently unclear. Its marked expression in blood vessels, and in cultured endothelial cells and smooth muscle cells, has led to the proposal that it is an important factor in vascular remodelling and there is certainly evidence to support this (Acevedo, et al., 2004). Data from a recent study demonstrated that recovery of blood flow following ischaemic injury to the hindlimb, and the associated angiogenesis and arteriogenesis, was reduced in Nogo$^{-/-}$ mice (Yu, et al., 2009). The evidence linked this poor recovery with a Nogo B-associated decrease in the recruitment of macrophages to the ischaemic tissue (Yu, et al., 2009). In this context therefore, it was somewhat surprising that genetic deletion of Nogo A and B was associated with reduced recovery in 12 month-old rats following traumatic brain injury (Marklund, et al., 2009). Nogo B, but particularly Nogo A, was increased in the hippocampus following deafferentation and, in the case of Nogo B, this upregulation persisted until axonal sprouting peaked at which time its expression returned to control levels (Meier, et al., 2003)

It is known that increased Nogo A expression is associated with conditions characterized by cell stress, for example in amyotrophic lateral sclerosis (ALS) and in an animal model of ALS, while Nogo A autoantibodies have been found in serum and cerebrospinal fluid of patients with multiple sclerosis (Yan, et al., 2006). Here we did not find any age-related or Aβ-associated change in Nogo A but the age-related and Aβ-associated increase in Nogo B was associated with increases in the activities of caspases 8 and 3. These enzymes are indicators of cell stress, although not necessarily cell death (Garnier, et al., 2004) and taken with the findings of Meier and colleagues (Meier, et al., 2003), the data suggest a coupling between tissue or cell stress and Nogo B, at least in hippocampus. One of the significant findings in this study is that Nogo B induced an increase in activities of caspases 8 and 3 in cultured neurons suggesting that it can trigger cell stress. In this context, it is interesting that Nogo B, like the other Nogo family members, is abundant in the endoplasmic reticulum (Teng, et al., 2004) and its overexpression induces endoplasmic reticulum stress, which at least in some cases is linked with apoptosis (Kuang, et al., 2006). It has been proposed that an apoptotic signal arises as a consequence of the interaction between Nogo B and Bcl-2 which prevents its translocation from the endoplasmic reticulum to mitochondria (Tagami, et al., 2000). Consistent with this, it has been shown that expression of Nogo B in cancer cell lines can induce apoptosis (Kuang, et al., 2005) although this has been disputed (Oertle, et al., 2003). Here we show that the NgR antagonist NEP1-40 attenuated the Aβ-induced increase in caspase 3 activation which is broadly consistent with the finding that NEP1-40 reduced caspase-3 activation as a result of focal cerebral ischemic injury (Wang, et al., 2008).

Several factors are capable of inducing microglial activation which is often accompanied by evidence of neuronal stress, presumably as a consequence of the actions of the release neuromodulatory molecules, like inflammatory cytokines, chemokines, and oxidative and nitrogen species (Lynch, 2009). Under resting conditions concentration of these factors is low and, together with appropriate interactions between cells, microglia are

maintained in a quiescent state. The findings of this study identify Nogo B as another factor which contributes to activation of microglia and highlights the need to establish the mechanisms by which its expression is modulated.

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FIGURE LEGENDS

Figure 1: The age-related decrease in LTP is accompanied by increased Nogo B in hippocampus.

(a) LTP was significantly decreased in aged (22 months), compared with young (3 months), rats (***$p < 0.001$; $n = 8$). Nogo A (b) was unchanged but Nogo B (c) was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (***$p < 0.001$; student's t-test for independent means). Data are expressed as means ± SEM (n=6).

Figure 2: Markers of microglial activation are increased in hippocampus of aged rats.

MHCII mRNA (a) and CD11b mRNA (b) were significantly increased in hippocampal tissue prepared from aged, compared with young, rats (***$p < 0.001$; student's t-test for independent means) and these were accompanied by increases in CD86 (c) and ICAM-1 (d; *$p < 0.05$; Student's t-test for independent means). Sample immunoblots for CD86 and ICAM-1 are shown. Data are expressed as means ± SEM (n=8).

Figure 3: Age-related increases in activities of caspases 8 and 3 in hippocampus are accompanied by a decrease in synaptophysin.

Activities of caspase 8 (a) and caspase 3 (b) were significantly increased in tissue prepared from aged, compared with young, rats (***$p < 0.001$; Student's t-test for independent means; $n = 6$) and this was accompanied by an age-related decrease in synaptophysin (c; *$p < 0.05$; Student's t-test for independent means; n=8).

Figure 4: The Aβ-induced decrease in LTP is accompanied by increased Nogo B in hippocampus.

(a) LTP was significantly decreased in rats which were infused intracerebroventricularly for 8 days with $A\beta_{1\text{-}40}$ (26.9μM) and $A\beta_{1\text{-}42}$ (36.9μM), compared with rats which received the control peptide $A\beta_{40\text{-}1}$ (63.8μM; ***$p < 0.001$; ANOVA; n=6). Nogo A (b) was unchanged but Nogo B (c) was significantly increased in hippocampal tissue prepared from Aβ-treated, compared with control-treated, rats (*$p < 0.05$; student's t-test for independent means). Data are expressed as means ± SEM (n=6).

Figure 5: Aβ induces changes in microglial activation and activities of caspases 8 and 3.

MHCII mRNA (a), CD11b mRNA (b), CD86 (c) and ICAM-1 (d) were significantly increased in tissue prepared from Aβ-treated, compared with control-treated, rats (*$p < 0.05$; ***$p < 0.001$; student's t-test for independent means); sample immunoblots for CD86 and ICAM-1 are shown. Activities of caspase 8 (e) and caspase 3 (f) were also significantly increased in tissue prepared from Aβ-treated, compared with control-treated, rats (***$p < 0.001$; Student's t-test for independent means; n=6). Data are expressed as means ± SEM (n=6).

Figure 6: Nogo B increases expression of markers of microglial activation.

Incubation of mixed glia in the presence of Nogo B (100ng/ml, but not 10ng/ml) increased

MHCII mRNA (a), CD11b mRNA (b), CD86 (c) and ICAM-1 (d; *$p < 0.05$; **$p < 0.01$; ANOVA) and the difference induced by 100ng/ml compared with 10ng/ml was significant in the case of MHCII mRNA and CD11b mRNA ($^{++}p < 0.01$; ANOVA). Data are expressed as means ± SEM (n=6).

Figure 7: Nogo B increases activities of caspases 8 and 3 in cultured neurons.
Incubation of cortical neurons in the presence of Nogo B (100ng/ml, but not 10ng/ml) increased activity of caspase 8 (a) and caspase 3 (b; ***$p < 0.001$; ANOVA) and a significant difference between the effects of 100ng/ml and 10ng/ml Nogo B was observed ($^{+++}p < 0.001$; ANOVA). Data are expressed as means ± SEM (n=6).

Figure 8: NgR antagonist NEP1-40 rescued the Aβ-induced activation of caspase 3 in cultured neurons.
Aβ significantly induced caspase 3 activation after 48 h in cultured neurons (*$p < 0.05$; ANOVA) and this was significantly attenuated by treatment with NEP1-40 (1μM; $^{+}p < 0.05$; ANOVA).

REFERENCES

1. Acevedo, L., Yu, J., Erdjument-Bromage, H., Miao, R. Q., Kim, J. E., Fulton, D., Tempst, P., Strittmatter, S. M., and Sessa, W. C., 2004. A new role for Nogo as a regulator of vascular remodeling. Nat Med 10**,** 382-388.

2. Beck, K. D., Nguyen, H. X., Galvan, M. D., Salazar, D. L., Woodruff, T. M., and Anderson, A. J., 2010. Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. Brain.

3. Burke, S. N., and Barnes, C. A., 2006. Neural plasticity in the ageing brain. Nat Rev Neurosci 7**,** 30-40.

4. Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A. A., Christ, F., and Schwab, M. E., 2000. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403**,** 434-439.

5. Clarke, R. M., Lyons, A., O'Connell, F., Deighan, B. F., Barry, C. E., Anyakoha, N. G., Nicolaou, A., and Lynch, M. A., 2008. A pivotal role for interleukin-4 in atorvastatin-associated neuroprotection in rat brain. J Biol Chem 283**,** 1808-1817.

6. Clarke, R. M., O'Connell, F., Lyons, A., and Lynch, M. A., 2007. The HMG-CoA reductase inhibitor, atorvastatin, attenuates the effects of acute administration of amyloid-beta1-42 in the rat hippocampus in vivo. Neuropharmacology 52**,** 136-145.

7. David, S., Fry, E. J., and Lopez-Vales, R., 2008. Novel roles for Nogo receptor in inflammation and disease. Trends Neurosci 31**,** 221-226.

8. Downer, E. J., Cowley, T. R., Lyons, A., Mills, K. H., Berezin, V., Bock, E., and Lynch, M. A., 2010. A novel anti-inflammatory role of NCAM-derived mimetic peptide, FGL. Neurobiol Aging 31**,** 118-128.

9. Frautschy, S. A., Hu, W., Kim, P., Miller, S. A., Chu, T., Harris-White, M. E., and Cole, G. M., 2001. Phenolic anti-inflammatory antioxidant reversal of Abeta-induced cognitive deficits and neuropathology. Neurobiol Aging 22**,** 993-1005.

10. Freund, P., Schmidlin, E., Wannier, T., Bloch, J., Mir, A., Schwab, M. E., and Rouiller, E. M., 2009. Anti-Nogo-A antibody treatment promotes recovery of manual dexterity after unilateral cervical lesion in adult primates--re-examination and extension of behavioral data. Eur J Neurosci 29**,** 983-996.

11. Garnier, P., Prigent-Tessier, A., Van Hoecke, M., Bertrand, N., Demougeot, C., Sordet, O., Swanson, R. A., Marie, C., and Beley, A., 2004. Hypoxia induces caspase-9 and caspase-3 activation without neuronal death in gerbil brains. Eur J Neurosci 20**,** 937-946.

12. GrandPre, T., Nakamura, F., Vartanian, T., and Strittmatter, S. M., 2000. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403**,** 439-444.

13. Griffin, R., Nally, R., Nolan, Y., McCartney, Y., Linden, J., and Lynch, M. A., 2006. The age-related attenuation in long-term potentiation is associated with microglial activation. J Neurochem 99**,** 1263-1272.

14. Hein, A. M., Stasko, M. R., Matousek, S. B., Scott-McKean, J. J., Maier, S. F., Olschowka, J. A., Costa, A. C., and O'Banion, M. K., 2009. Sustained hippocampal IL-1beta overexpression impairs contextual and spatial memory in transgenic mice. Brain Behav Immun.

15. Huber, A. B., Weinmann, O., Brosamle, C., Oertle, T., and Schwab, M. E., 2002. Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. J Neurosci 22**,** 3553-3567.

16. Josephson, A., Widenfalk, J., Widmer, H. W., Olson, L., and Spenger, C., 2001. NOGO mRNA expression in adult and fetal human and rat nervous tissue and in weight drop injury. Exp Neurol 169**,** 319-328.

17. Kilic, E., Elali, A., Kilic, U., Guo, Z., Ugur, M., Uslu, U., Bassetti, C. L., Schwab, M. E., and Hermann, D. M., 2010. Role of Nogo-A in neuronal survival in the reperfused ischemic brain. J Cereb Blood Flow Metab.

18. Kim, H. S., and Suh, Y. H., 2009. Minocycline and neurodegenerative diseases. Behav Brain Res 196**,** 168-179.

19. Kuang, E., Wan, Q., Li, X., Xu, H., Liu, Q., and Qi, Y., 2005. ER Ca2+ depletion triggers apoptotic signals for endoplasmic reticulum (ER) overload

response induced by overexpressed reticulon 3 (RTN3/HAP). J Cell Physiol 204**,** 549-559.

20. Kuang, E., Wan, Q., Li, X., Xu, H., Zou, T., and Qi, Y., 2006. ER stress triggers apoptosis induced by Nogo-B/ASY overexpression. Exp Cell Res 312**,** 1983-1988.

21. Loane, D. J., Deighan, B. F., Clarke, R. M., Griffin, R. J., Lynch, A. M., and Lynch, M. A., 2009. Interleukin-4 mediates the neuroprotective effects of rosiglitazone in the aged brain. Neurobiol Aging 30**,** 920-931.

22. Lynch, A. M., Loane, D. J., Minogue, A. M., Clarke, R. M., Kilroy, D., Nally, R. E., Roche, O. J., O'Connell, F., and Lynch, M. A., 2007. Eicosapentaenoic acid confers neuroprotection in the amyloid-beta challenged aged hippocampus. Neurobiol Aging 28**,** 845-855.

23. Lynch, G., Rex, C. S., and Gall, C. M., 2006. Synaptic plasticity in early aging. Ageing Res Rev 5**,** 255-280.

24. Lynch, M. A., 2004. Long-term potentiation and memory. Physiol Rev 84**,** 87-136.

25. Lynch, M. A., 2009. The multifaceted profile of activated microglia. Mol Neurobiol 40**,** 139-156.

26. Lynch, M. A., 2010. Age-related neuroinflammatory changes negatively impact on neuronal function. Front Aging Neurosci 1**,** 6.

27. Lyons, A., Griffin, R. J., Costelloe, C. E., Clarke, R. M., and Lynch, M. A., 2007. IL-4 attenuates the neuroinflammation induced by amyloid-beta in vivo and in vitro. J Neurochem 101**,** 771-781.

28. Lyons, A., Lynch, A. M., Downer, E. J., Hanley, R., O'Sullivan, J. B., Smith, A., and Lynch, M. A., 2009. Fractalkine-induced activation of the phosphatidylinositol-3 kinase pathway attentuates microglial activation in vivo and in vitro. J Neurochem 110**,** 1547-1556.

29. Marklund, N., Bareyre, F. M., Royo, N. C., Thompson, H. J., Mir, A. K., Grady, M. S., Schwab, M. E., and McIntosh, T. K., 2007. Cognitive outcome following brain injury and treatment with an inhibitor of Nogo-A in association with an attenuated downregulation of hippocampal growth-associated protein-43 expression. J Neurosurg 107**,** 844-853.

30. Marklund, N., Morales, D., Clausen, F., Hanell, A., Kiwanuka, O., Pitkanen, A., Gimbel, D. A., Philipson, O., Lannfelt, L., Hillered, L., Strittmatter, S. M., and McIntosh, T. K., 2009. Functional outcome is impaired following traumatic brain injury in aging Nogo-A/B-deficient mice. Neuroscience 163**,** 540-551.

31. Meier, S., Brauer, A. U., Heimrich, B., Schwab, M. E., Nitsch, R., and Savaskan, N. E., 2003. Molecular analysis of Nogo expression in the hippocampus during development and following lesion and seizure. FASEB J 17**,** 1153-1155.

32. Miller, A. M., Piazza, A., Martin, D. S., Walsh, M., Mandel, A., Bolton, A. E., and Lynch, M. A., 2009. The deficit in long-term potentiation induced by chronic administration of amyloid-beta is attenuated by treatment of rats with a novel phospholipid-based drug formulation, VP025. Exp Gerontol 44**,** 300-304.

33. Minogue, A. M., Lynch, A. M., Loane, D. J., Herron, C. E., and Lynch, M. A., 2007. Modulation of amyloid-beta-induced and age-associated changes in rat hippocampus by eicosapentaenoic acid. J Neurochem 103**,** 914-926.

34. Moore, A. H., Wu, M., Shaftel, S. S., Graham, K. A., and O'Banion, M. K., 2009. Sustained expression of interleukin-1beta in mouse hippocampus impairs spatial memory. Neuroscience.

35. Morris, N. J., Ross, S. A., Neveu, J. M., Lane, W. S., and Lienhard, G. E., 1999. Cloning and characterization of a 22 kDa protein from rat adipocytes: a new member of the reticulon family. Biochim Biophys Acta 1450**,** 68-76.

36. Nolan, Y., Maher, F. O., Martin, D. S., Clarke, R. M., Brady, M. T., Bolton, A. E., Mills, K. H., and Lynch, M. A., 2005. Role of interleukin-4 in regulation of age-related inflammatory changes in the hippocampus. J Biol Chem 280**,** 9354-9362.

37. Oertle, T., Huber, C., van der Putten, H., and Schwab, M. E., 2003. Genomic structure and functional characterisation of the promoters of human and mouse nogo/rtn4. J Mol Biol 325**,** 299-323.

38. Oertle, T., Merkler, D., and Schwab, M. E., 2003. Do cancer cells die because of Nogo-B? Oncogene 22**,** 1390-1399.

39. Park, J. H., Gimbel, D. A., GrandPre, T., Lee, J. K., Kim, J. E., Li, W., Lee, D. H., and Strittmatter, S. M., 2006. Alzheimer precursor protein interaction with the Nogo-66 receptor reduces amyloid-beta plaque deposition. J Neurosci 26**,** 1386-1395.

40. Piazza, A., and Lynch, M. A., 2009. Neuroinflammatory changes increase the impact of stressors on neuronal function. Biochem Soc Trans 37**,** 303-307.

41. Prinjha, R., Moore, S. E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D. L., and Walsh, F. S., 2000. Inhibitor of neurite outgrowth in humans. Nature 403**,** 383-384.

42. Serrano, F., and Klann, E., 2004. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing Res Rev 3**,** 431-443.

43. Simard, A. R., Soulet, D., Gowing, G., Julien, J. P., and Rivest, S., 2006. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. Neuron 49**,** 489-502.

44. Stirling, D. P., Khodarahmi, K., Liu, J., McPhail, L. T., McBride, C. B., Steeves, J. D., Ramer, M. S., and Tetzlaff, W., 2004. Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. J Neurosci 24**,** 2182-2190.

45. Tagami, S., Eguchi, Y., Kinoshita, M., Takeda, M., and Tsujimoto, Y., 2000. A novel protein, RTN-XS, interacts with both Bcl-XL and Bcl-2 on endoplasmic reticulum and reduces their anti-apoptotic activity. Oncogene 19**,** 5736-5746.

46. Teng, F. Y., Ling, B. M., and Tang, B. L., 2004. Inter- and intracellular interactions of Nogo: new findings and hypothesis. J Neurochem 89**,** 801-806.

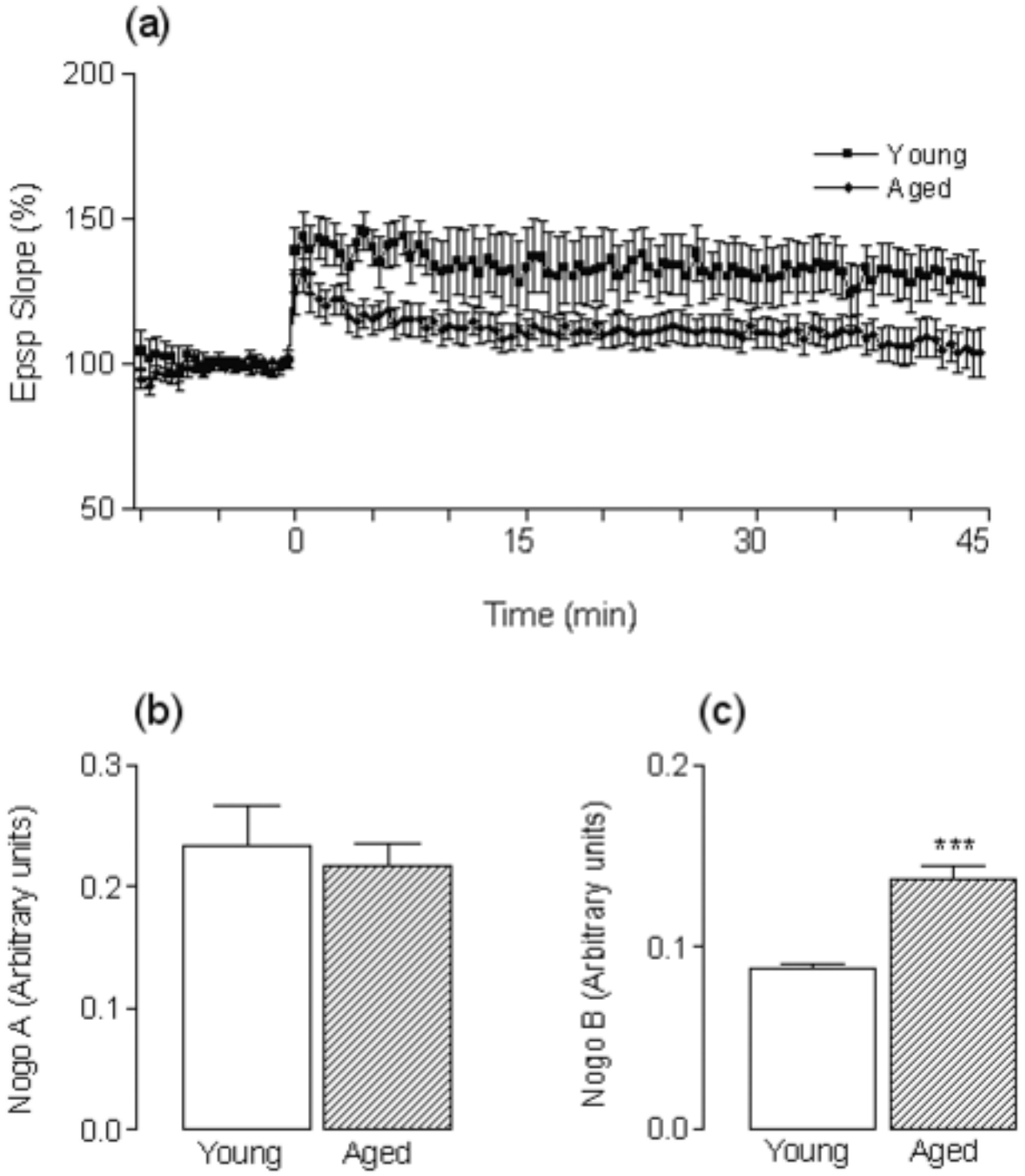
47. Wang, Q., Gou, X., Xiong, L., Jin, W., Chen, S., Hou, L., and Xu, L., 2008. Trans-activator of transcription-mediated delivery of NEP1-40 protein into brain has a neuroprotective effect against focal cerebral ischemic injury via inhibition of neuronal apoptosis. Anesthesiology 108**,** 1071-1080.

48. Wang, X., Chun, S. J., Treloar, H., Vartanian, T., Greer, C. A., and Strittmatter, S. M., 2002. Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact. J Neurosci 22**,** 5505-5515.

49. Yan, R., Shi, Q., Hu, X., and Zhou, X., 2006. Reticulon proteins: emerging players in neurodegenerative diseases. Cell Mol Life Sci 63**,** 877-889.

50. Yu, J., Fernandez-Hernando, C., Suarez, Y., Schleicher, M., Hao, Z., Wright, P. L., DiLorenzo, A., Kyriakides, T. R., and Sessa, W. C., 2009. Reticulon 4B (Nogo-B) is necessary for macrophage infiltration and tissue repair. Proc Natl Acad Sci U S A 106**,** 17511-17516.

- Age- and amyloid-β-related upregulation in Nogo B expression *in vivo*.
- Increase in Nogo B expression parallels evidence of microglial activation and activation of caspases 8 and 3 *in vivo*.
- Nogo B is capable of triggering microglial activation in cultures of mixed glial cells.
- Nogo B negatively impacts on synaptic integrity by increasing activities of caspases 8 and 3 in neuronal cultures.



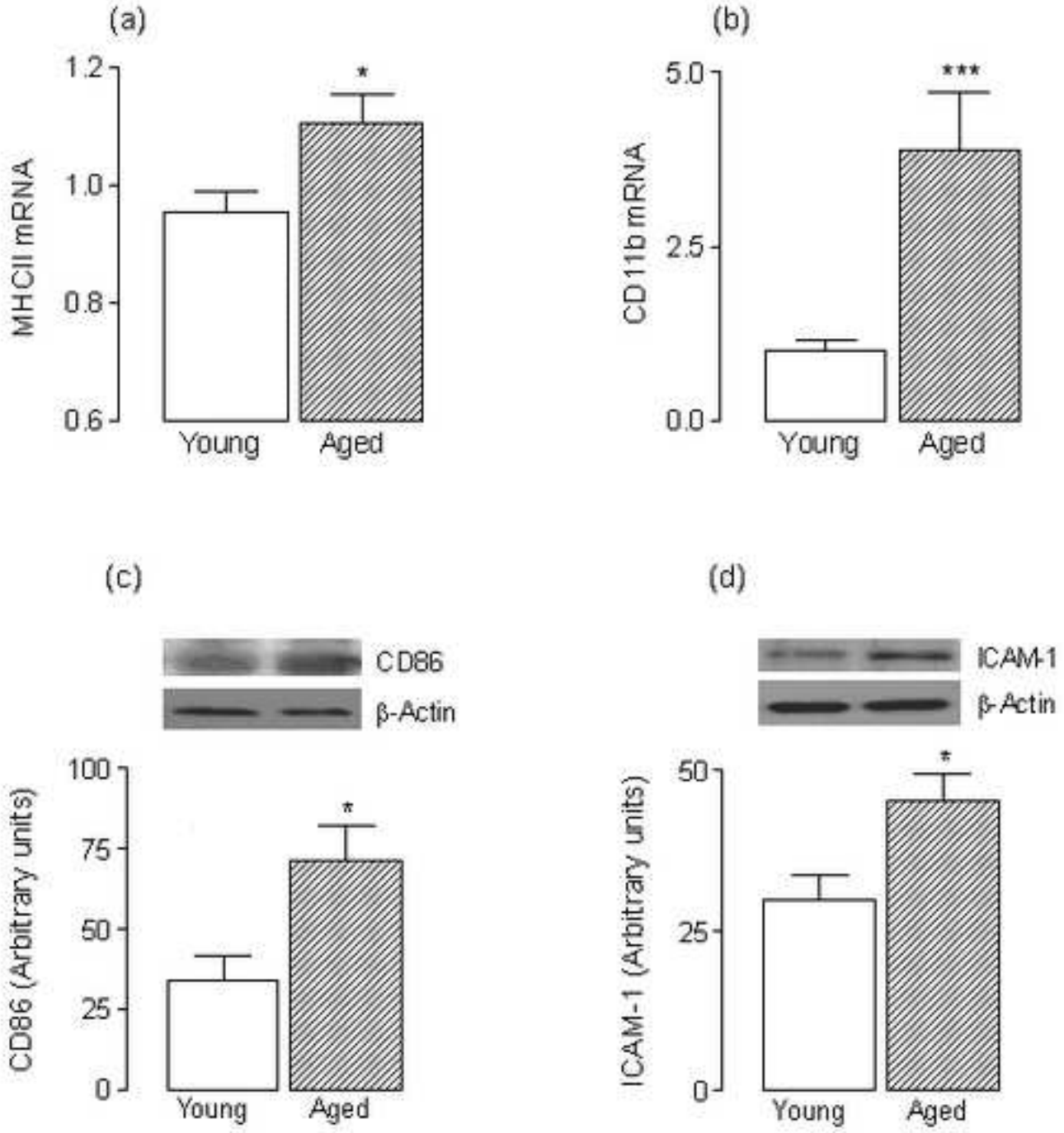
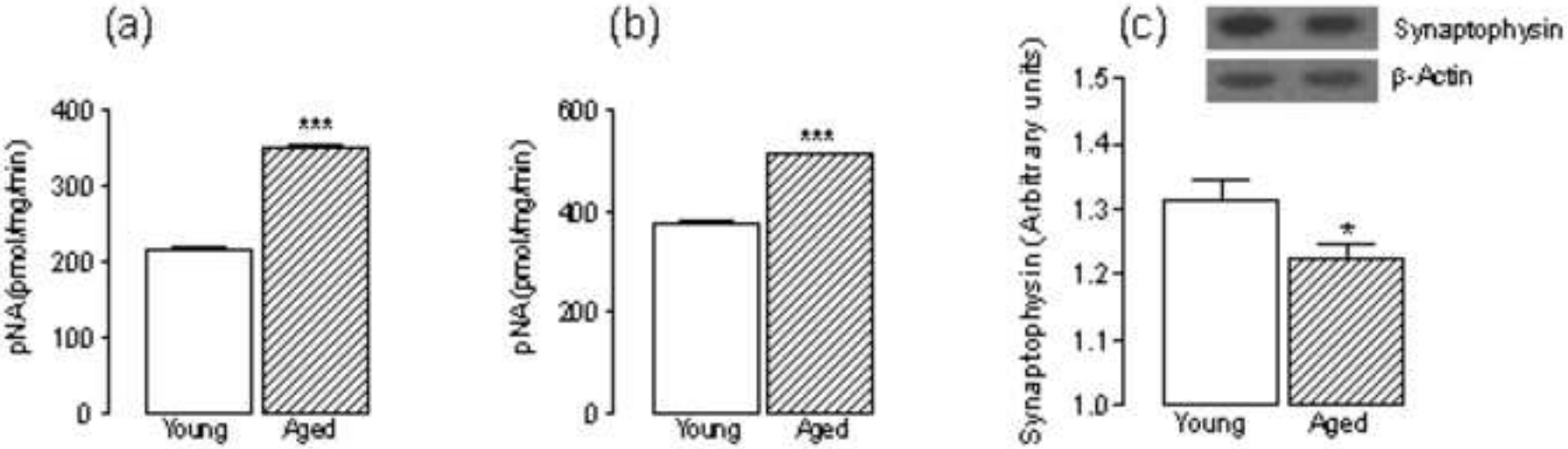


Figure 3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pNA (pmol/mg/min)
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***
Young
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(b)
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Young
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(c)
Synaptophysin
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Young
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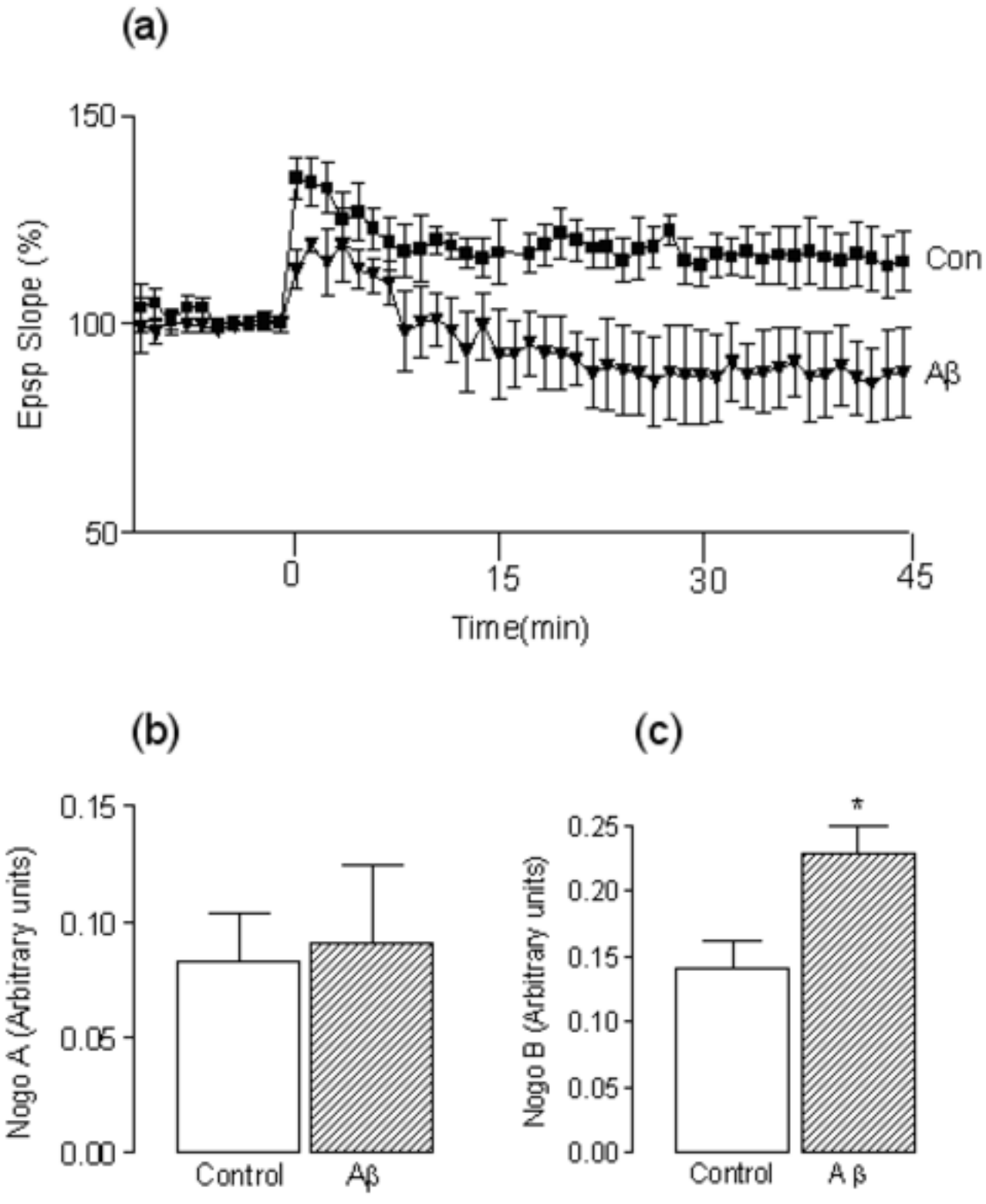


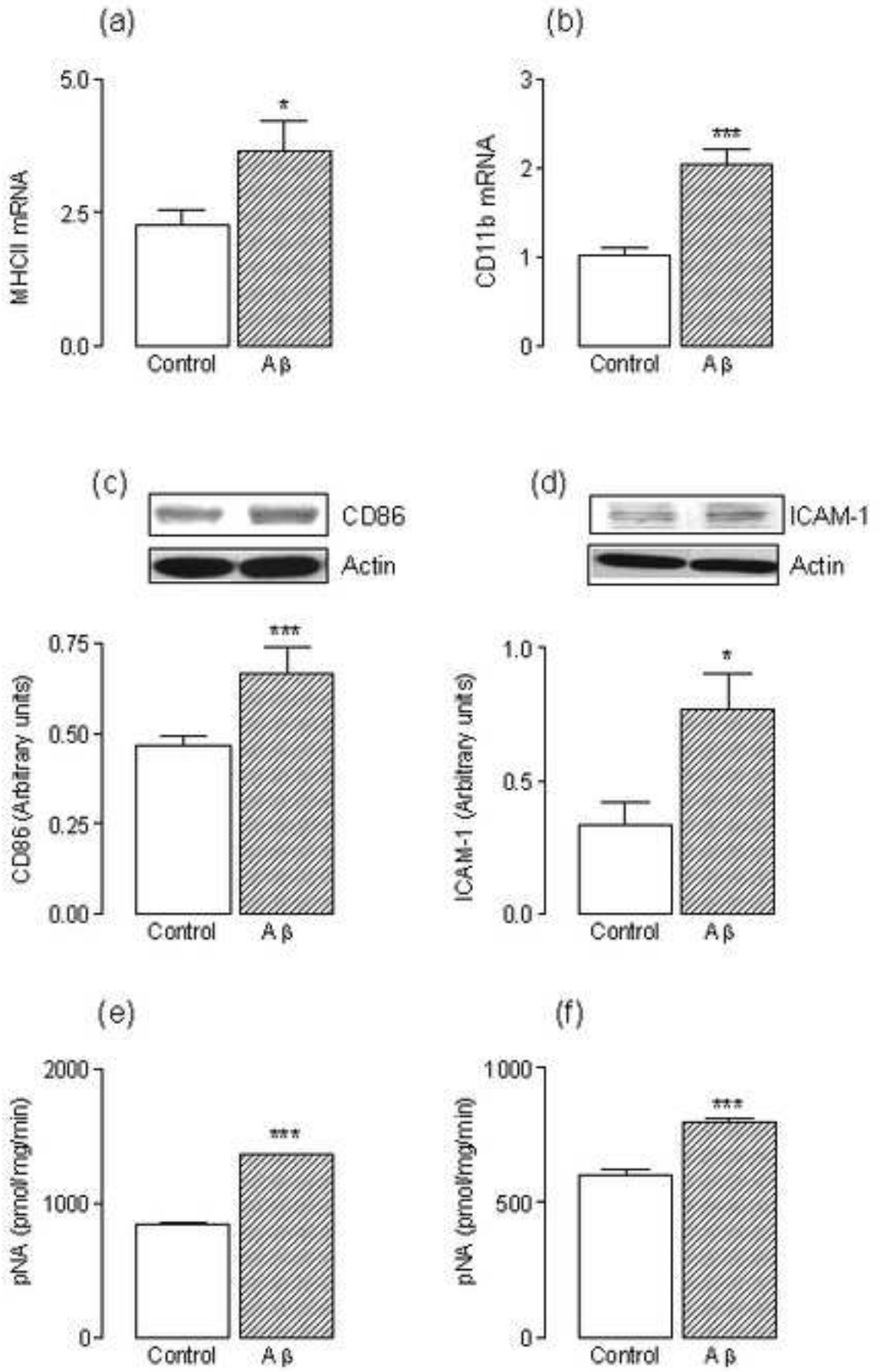
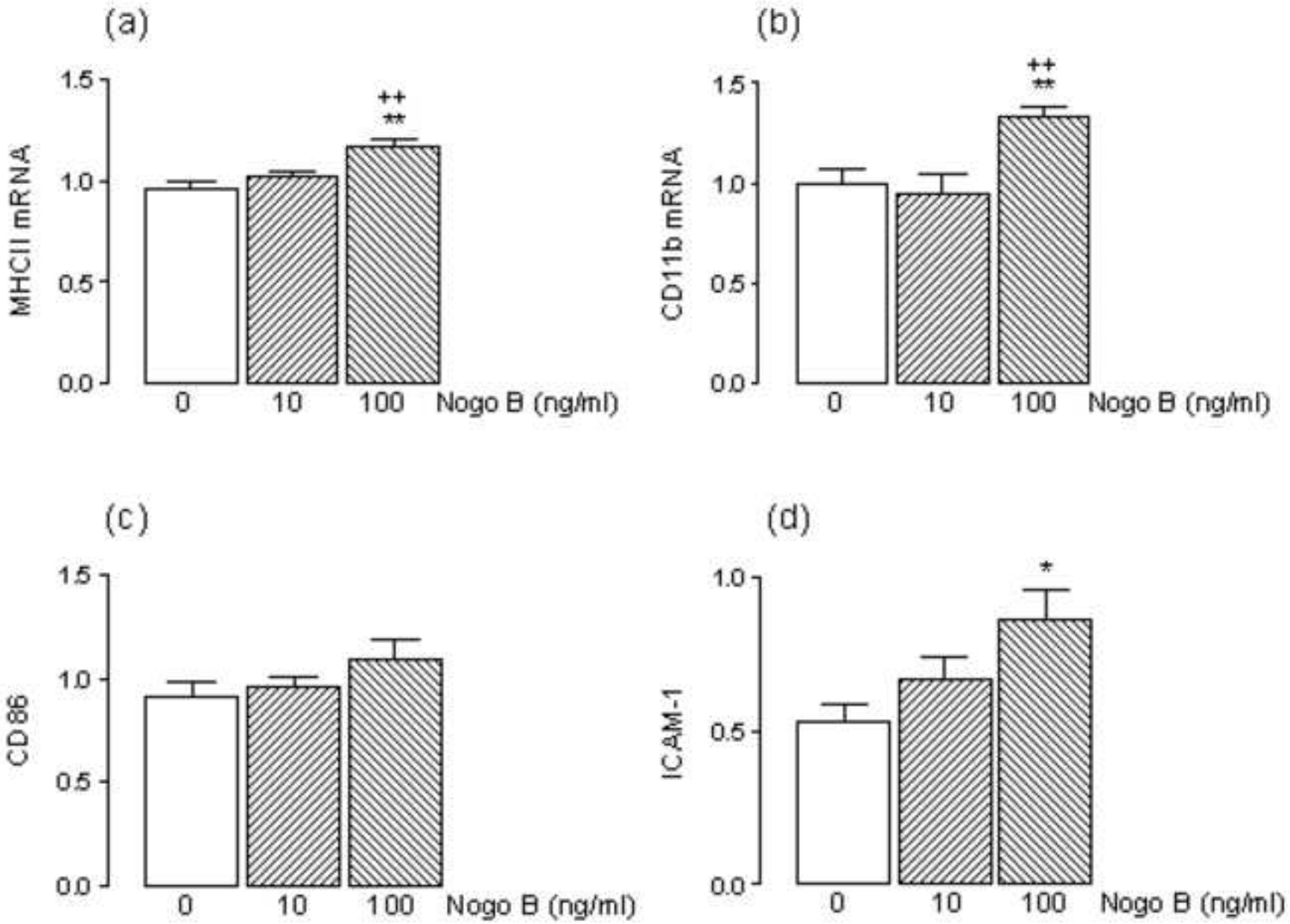


Figure 6


(a)
MHCII mRNA
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Nogo B (ng/ml)
(b)
CD11b mRNA
1.5
1.0
0.5
0.0
++
**
0
10
100
Nogo B (ng/ml)
(c)
CD 86
1.5
1.0
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Nogo B (ng/ml)
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Nogo B (ng/ml)

Figure 7

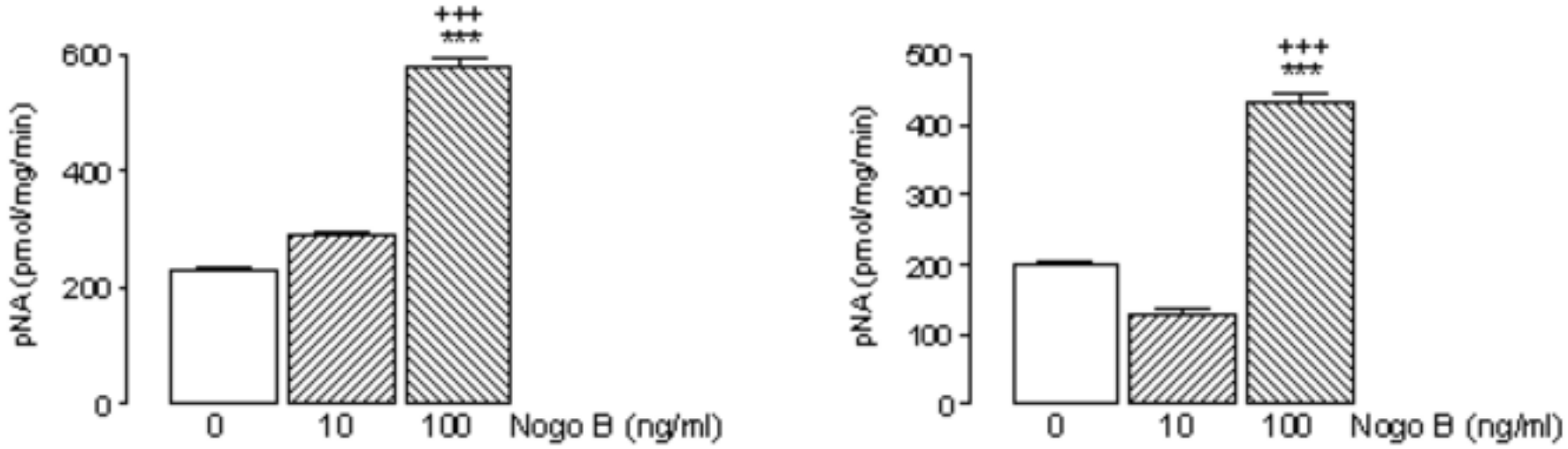
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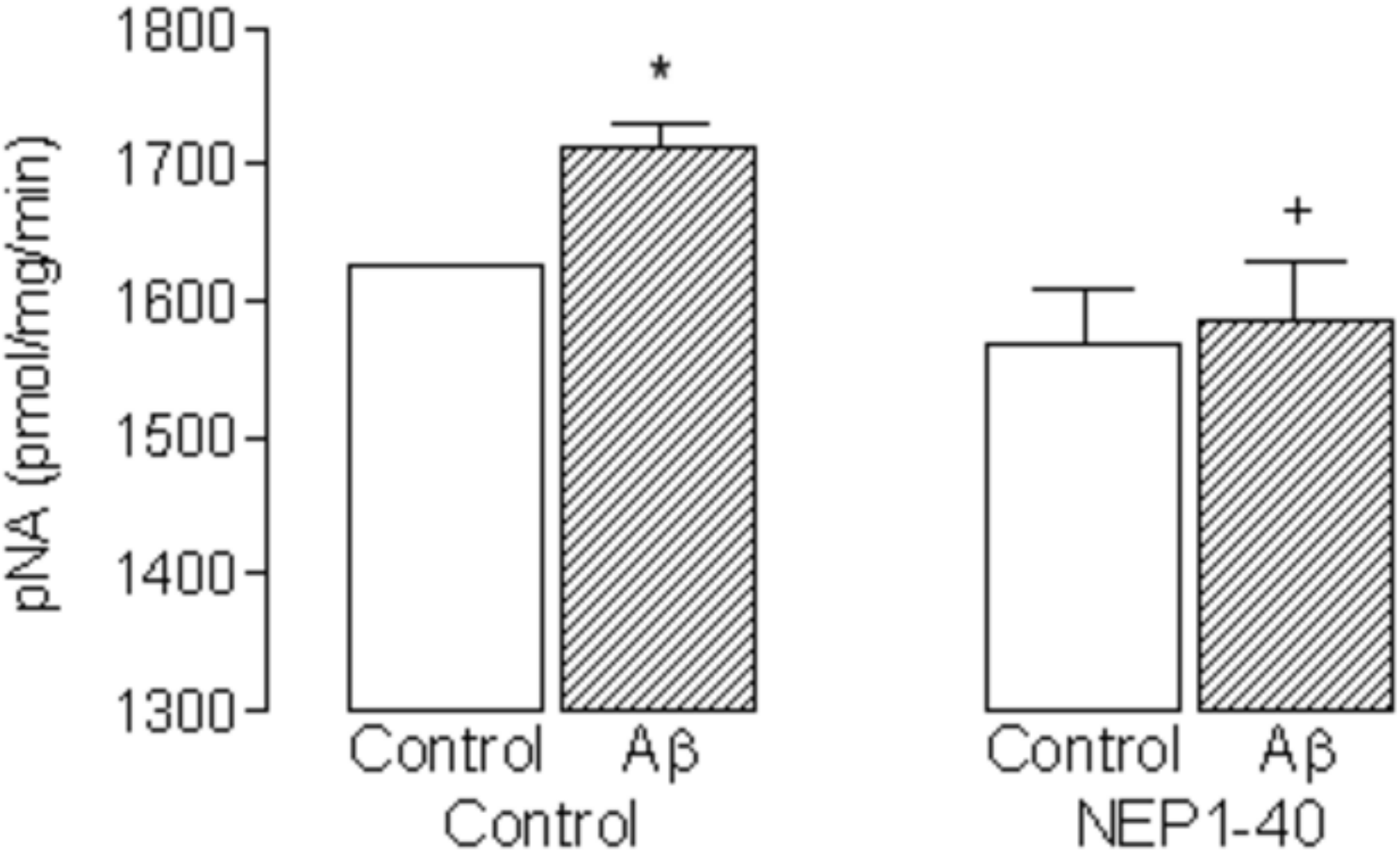
Figure 8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