Increased proNGF and decreased BDNF in non-Alzheimer's disease tauopathies

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Abstract

Alterations in the expression and signaling of brain derived neurotrophic factor (BDNF) and the precursor to nerve growth factor (NGF), proNGF, play a role in the neuronal and cognitive dysfunction of Alzheimer's disease. Aggregated amyloid-β has been shown to down-regulate specific BDNF transcripts in Alzheimer's disease, but the role of tau pathology in neurotrophin dysregulation has not been investigated. We measured levels of BDNF mRNA and protein using real time quantitative RT-PCR and ELISA and proNGF protein using Western blotting in parietal cortex of subjects with tauopathies, neurodegenerative diseases exhibiting tau pathology without amyloid-β accumulation. We observed a significant increase in the level of proNGF protein in Pick's disease and a significant decrease in BDNF mRNA and protein levels in Pick's disease and corticobasal degeneration, but no neurotrophin alterations in progressive supranuclear palsy. The decrease in total BDNF mRNA levels in these tauopathies was predominantly due to down-regulation of transcript IV. These findings implicate tau pathology in neurotrophin dysregulation, which may represent a mechanism through which tau confers toxicity in Alzheimer's disease and related non-Alzheimer's dementias.

Keywords: Alzheimer's disease, tauopathy, tau, neurotrophin, nerve growth factor, brain-derived neurotrophic factor, Pick's disease, corticobasal degeneration, progressive supranuclear palsy, post mortem brain

1. Introduction:

In Alzheimer's disease (AD), the degree of synaptic loss in basal forebrain cholinergic neurons, entorhinal cortex, hippocampus and cortical regions of the brain correlate strongly with the severity of dementia (Scheff and Price, 2003, Terry et al., 1991). AD brain exhibits intraneuronal neurofibrillary tangles caused by post-translational modifications to tau, a microtubule binding protein, as well as extracellular senile plaques caused by aggregation of the amyloid-β (Aβ) peptide. The amyloid cascade hypothesis of Alzheimer's disease proposes that amyloid-β accumulation is the initial insult leading to degeneration of central nervous system neurons (Hardy and Selkoe, 2002). Importantly, recent evidence argues that Aβ toxicity is mediated through tau (Ittner et al., 2010, LaFerla, 2010, Roberson et al., 2007).

Neurotrophins regulate neuronal survival, differentiation, and function in the peripheral and central nervous systems (Huang and Reichardt, 2001). Dysregulation of the levels of neurotrophins or their receptors, or alterations in neurotrophin function or trafficking can damage neurons, leading to gradual neuronal degeneration (Mufson et al., 2007).

Brain-derived neurotrophic factor (BDNF) promotes neuronal survival, synaptic plasticity, and memory consolidation (Fahnestock, 2011, Lu, 2003, Yamada et al., 2002). BDNF mRNA and protein are decreased in post-mortem brain tissue from subjects with mild cognitive impairment (MCI) and AD (Connor et al., 1997, Ferrer et al., 1999, Garzon et al., 2002, Hock et al., 2000, Holsinger et al., 2000, Michalski and Fahnestock, 2003, Peng et al., 2005, Phillips et al., 1991), and the reduction in BDNF correlates with the degree of cognitive impairment (Peng et al., 2005). BDNF administration can rescue synaptic loss and cognitive dysfunction in animal models, implicating loss of BDNF as a contributing factor to AD (Arancibia et al., 2008, Blurton-Jones et al., 2009, Nagahara et al., 2009). Down-regulation of BDNF does not exacerbate Aβ deposition

or tau pathology in a 3X transgenic mouse model of Alzheimer's disease, further confirming that the decrease in BDNF is not a cause of, but is rather a consequence of, Aβ and tau pathology (Castello et al., 2012). Soluble, oligomeric $A\beta_{42}$ has been shown to be at least partially responsible for down-regulation of BDNF (Garzon and Fahnestock, 2007, Peng et al., 2009, Tong et al., 2001, Tong et al., 2004). Although BDNF is not down-regulated in tau transgenic mice (Burnouf et al., 2012), whether tau contributes to BDNF down-regulation in human tissue has not been investigated.

Basal forebrain cholinergic neurons (BFCNs) are crucial for learning and memory and require nerve growth factor (NGF) for survival and function (Gutierrez et al., 1997, Woolf et al., 2001). In AD, BFCNs undergo degeneration which is associated with decreased NGF-immunoreactive protein in BFCN cell bodies and increased NGF precursor (proNGF) in BFCN target tissues such as hippocampus and cortex (Fahnestock et al., 1996, Fahnestock et al., 2001, Mufson et al., 1995, Peng et al., 2004, Salehi et al., 2006, Scott et al., 1995). The high-affinity receptor for NGF, tropomyosin receptor kinase A (TrkA), is reduced in BFCNs in AD (Counts et al., 2004, Ginsberg et al., 2006), which could account for proNGF accumulation in BFCN target tissues due to impaired retrograde transport of this protein (Bradbury, 2005, Reichardt and Mobley, 2004, Salehi et al., 2004, Salehi et al., 2006). Tau dysfunction can also contribute to impairment of axonal transport (Cowan et al., 2010, Ittner et al., 2010, Niewiadomska and Baksalerska-Pazera, 2003, Niewiadomska et al., 2005, Vossel et al., 2010) which may lead to proNGF accumulation in BFCN target tissue. To determine whether tau contributes to neurotrophin dysregulation in neurodegenerative diseases, we analyzed proNGF protein and BDNF mRNA and protein levels in post mortem parietal cortex from subjects with tauopathies, a class of neurodegenerative diseases that exhibit tau pathology in the absence of Aβ accumulation.

2. Methods:

Human brain tissue samples

Postmortem parietal cortex from subjects with tauopathies including Pick's disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and from age-matched controls were provided by Dr. Virginia M.Y. Lee (University of Pennsylvania, USA). There were no differences in age, gender, post-mortem interval, or yield of protein or RNA between groups. Subject characteristics are shown in Table 1.

ProNGF Western blotting

Human normal and tauopathy parietal cortex tissue, n=6 per group, were used for proNGF quantification. Frozen tissue samples (40-90mg) were sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in a 1:10 w/v ratio in homogenization buffer [0.05 M Tris-Cl pH 7.5, 0.5% Tween-20, 10 mM ethylenediaminetetraacetic acid (EDTA), 2μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF)]. Homogenates were kept on ice for 15 min and then centrifuged for 15 min at 9500 × g at 4°C. Equal volumes of supernatants were assayed for total protein using the D_C protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Total protein (30μg from each sample) was loaded onto 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Oakville, Canada). Membranes were blocked for 1 hour in TBS-T [50 mM Tris-Cl, pH 8.0, 133 mM NaCl, 0.2% (v/v) Tween-20] with 5% (w/v) Carnation nonfat milk powder (Nestle, North York, Canada) and probed with a 1:500 dilution of affinity-purified polyclonal rabbit anti-NGF (H-20,

Santa Cruz Biotechnology, CA, USA) overnight at 4°C. The membrane was rinsed in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (1:5000, Amersham) in TBS-T + 5% nonfat milk powder for 1 hour at room temperature. Bands were visualized by ECL^{TM} chemiluminescence (Amersham) on KODAK film (Kodak X-OMAT LS, Kodak, Vancouver, BC, Canada). The same membrane was then washed in TBS-T and re-probed with monoclonal mouse anti-β-actin (1:10000 dilution, Sigma, St Louis, MO, USA), a validated housekeeping gene, followed by HRP-conjugated sheep anti-mouse IgG secondary antibody (1:5000 dilution, Amersham).

Each Western blot contained a standard curve consisting of 5, 7.5, 10, 15, 20, 30, and 60μg of total protein from a single human cortex sample, common to all blots. Standard curves for proNGF and beta-actin were used to normalize pixel values between blots and to ensure that blots were not oversaturated and spanned the linear range of detection for both targets. Recombinant proNGF and 2.5S (mature) NGF were used as positive controls in all blots to confirm efficacy of the antibodies. Furthermore, we assessed specificity of the bands by blocking with 5-fold molar excess of NGF peptide. The pixel value of the immunoreactive bands was determined by densitometry of films using an HP Scanjet scanner and Scion Image beta 4.01 software, with local background subtracted. Samples were analyzed 3 times in independent experiments, and the mean pixel values from the three experiments were used for further statistical analysis. One outlier in the control group (outlier defined as more than 1.5 quartiles below the first quartile or above the third quartile) was excluded from subsequent analysis. Between-group differences were analyzed by one-way analysis of variance, followed by a Dunnett's post-hoc test (SPSS version 17, SPSS Inc., Chicago, IL).

BDNF qRT-PCR

For BDNF mRNA analysis, Pick's disease (PiD, n=8), corticobasal degeneration (CBD, n=12), progressive supranuclear palsy (PSP, n=13), and control (n=12) samples were used. Two independent RNA extractions were performed for each sample by independent investigators. RNA was extracted using the RNeasy® minikit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol, with minor modifications. Briefly, the sample was homogenized in Trizol™ (1mL per 100mg; Invitrogen, Burlington, Canada) and centrifuged for 3 minutes at 5500 x g at 4ºC. The supernatant was removed and mixed with chloroform (5:1 ratio) followed by a 3 minute incubation and centrifuged at 9500 x g for 15 minutes. In a fresh microtube, an equal volume of 70% EtOH (-20°C) was added to the upper, aqueous phase. The RNA was bound to an RNeasy® spin column and treated as per the manufacturer's protocol for the remaining extraction, including a treatment with 30 units of RNase-free DNase I (Qiagen) for 15 minutes prior to elution. The concentration and purity of RNA was measured by spectrophotometry at 260 and 280 nm in a Beckman DU™ Series 60 Spectrophotometer (Beckman Instruments Inc, Fullerton, CA, USA).

RNA (1µg) was reverse transcribed in 20µL total volume containing 200 units of Superscript™ II reverse transcriptase (RT), 250ng of random primers, 0.5mM each deoxynucleotide triphosphates, 1X first strand buffer, 0.05mM dithiothreitol, and 2 units of RNaseOUT™ (Invitrogen, Burlington, Canada). As a negative control, a separate reaction was prepared without RT. For analysis of individual BDNF transcripts III, V, Vh, VII, and IX, 2µg of RNA was reverse transcribed instead of 1µg. For analysis of transcript IV, 4µg of RNA was reverse transcribed in 20µL total volume using Superscript™ III following the manufacturer's instructions (Invitrogen). The reaction was carried out in a GeneAmp® 2400 PCR thermocycler

(Applied Biosystems, Carlsbad, California) for 10 minutes at 25ºC, 50 minutes at 42ºC, and 15 minutes at 70ºC, followed by cooling to 4ºC. Absolute quantitative real-time PCR (qPCR) was carried out on cDNA from 50ng of RNA (up to 200ng for transcripts). The 20μL reaction mixture included Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), ROX reference dye and primers (listed in Table 2) and was amplified using an MX3000P machine (Stratagene, La Jolla, CA, USA) for 2 min. at 50ºC and 2 min. at 95ºC followed by 40 cycles of 95ºC for 15 s (30 s for BDNF transcripts), 58ºC for 30 s and 72ºC for 30 s (45 s for BDNF transcripts). A standard curve was constructed for total BDNF and for each BDNF transcript using cDNA pooled from SH-SY5Y cell and post-mortem human hippocampal tissue RNA. The housekeeping gene β-actin was quantified against a qPCR plasmid standard (Invitrogen). A negative control containing no template was included. All samples were assayed in triplicate (duplicate for transcript IV). Copy numbers were determined with MXPro MX3000P software. R^2 values were >0.995 and efficiencies were > 90%. A dissociation curve indicated that a single product was obtained for each transcript.

One outlier (assessed with a Grubbs' outlier test, GraphPad), was identified in each of the control and CBD groups and was excluded from analysis. Analysis of BDNF or BDNF transcript to β-actin ratios revealed a skewed distribution, so the sample ratios were log transformed prior to statistical analysis. Total BDNF results were subsequently analyzed using a 6-level repeated measures ANOVA followed by a Dunnett's *post-hoc* test compared to age-matched controls. This allowed us to account for measurements from six independent reverse transcriptions. Expression of BDNF transcript IV was analyzed by a one-way ANOVA followed by a *post hoc* Dunnett's test. For each of the other BDNF transcripts, the mean value from three independent reverse transcriptions was used to perform a one-way ANOVA. All graphs were created using GraphPad

Prism (Version 3.03, GraphPad Software, San Diego, CA). Data was considered significant when $p<0.05$.

BDNF ELISA

A BDNF ELISA was carried out in duplicate using the Human BDNF DuoSet® ELISA kit (R & D Systems, Minneapolis, MN, USA). Protein isolation was carried out as described above for Western blots except that for some samples homogenization buffer contained protease inhibitor tablets (Roche, Mississauga, Ontario) instead of individually added protease inhibitors, and the ratio of tissue to homogenization buffer was either 1:10 or 1:15. Each sample was adjusted to 1 mg/ml total protein and analyzed according to the kit protocol. A standard curve using recombinant BDNF protein was run on each ELISA plate.

Results:

Increased proNGF protein in Pick's disease:

ProNGF levels in post mortem parietal cortex samples from non-AD tauopathies were compared to age matched controls. A representative Western blot is shown in Figure 1A. β-actin did not differ between groups (p=0.6, one-way ANOVA) and was used to normalize proNGF pixel values between samples. Mature NGF was not detectable in human cortical tissue, as previously reported (Fahnestock et al., 2001). A one-way ANOVA showed an overall significant effect of group (p=0.007) in the level of 34kDa proNGF (normalized to β-actin). This was followed by a *post hoc* Dunnett's test that revealed a statistically significant increase in proNGF in Pick's disease (PiD) compared to age-matched controls (p=0.02). The increase represents a 50% elevation in proNGF protein in PiD relative to controls and an effect size of 1.60. This increase was not observed in corticobasal degeneration (CBD, p=0.68) or progressive supranuclear palsy (PSP, p= 0.83) (Figure 1B).

Decreased BDNF mRNA in Pick's disease and corticobasal degeneration:

BDNF mRNA was measured in post mortem parietal cortex of subjects with PiD (n=8), CBD (n=11), PSP (n=13) and age-matched controls (n=11) by quantitative real-time RT-PCR (qRT-PCR). A total of 6 independent RT products, with three PCR reactions per RT, were analyzed for total BDNF and β-actin copy numbers. β-actin mRNA did not differ between groups (p=0.35) and was used to normalize BDNF and transcript levels. A 6-level repeated measures ANOVA (p=0.02) followed by a Dunnett's *post-hoc* test revealed a statistically significant down-regulation of total BDNF mRNA in PiD (p=0.03) and CBD (p=0.04) compared

to the age-matched control group (Figure 2). In both groups, this represents approximately a 55% reduction in total BDNF/β-actin copy number ratio when compared with control with an effect size for PiD versus control of 1.19 and for CBD versus control of 1.05. There was no reduction in total BDNF mRNA in PSP subjects compared to controls (p=0.86).

BDNF transcript down-regulation:

In AD, BDNF transcripts I, II, IV and VI are specifically down-regulated (Garzon et al., 2002). As in AD, expression of BDNF transcript IV was down-regulated in the two tauopathy groups exhibiting total BDNF down-regulation, PiD (n=8) and CBD (n=11) compared to control (n=11) (Figure 3, one-way ANOVA, p=0.006 followed by Dunnett's *post-hoc* test, p=0.01 and p=0.03, respectively). These results represent a BDNF transcript IV/β-actin copy number ratio of approximately 75% below control levels in both the PiD and CBD groups. Effect sizes are 1.33 for PiD versus control and 1.14 for CBD versus control. No significant change was observed in the level of BDNF transcript IV in PSP compared to control (p=0.88) (Figure 3). In contrast to AD, there were no transcript-specific differences in PiD and PSP when compared to age-matched controls for transcripts I, II or VI (Figure 4A,B,C, one-way ANOVA, p>0.05). Similar to AD, transcript VII was not down-regulated in PiD or CBD (Figure 4D, one way ANOVA, p>0.05). Combining PiD and CBD groups did not alter the results. Transcripts III, V, Vh, and IX fell below the limit of detection, defined as the amount of product obtained from 5ag of standard transcript cDNA. Levels of BDNF transcripts other than transcript IV were not analyzed in PSP. Although specific BDNF transcripts other than transcript IV may change in PSP, because transcript IV comprises more than 50% of the total BDNF mRNA in cortical tissue (Garzon and

Fahnestock, 2007), the physiological relevance of the contributions of other transcripts with no change in total BDNF mRNA or protein is considered minor.

Decreased BDNF protein in Pick's disease and corticobasal degeneration:

BDNF protein was measured by ELISA in post mortem parietal cortex of subjects with PiD (n=9), CBD (n=12), PSP (n=13) and age-matched controls (n=12). A one-way ANOVA ($p=0.01$) followed by a Dunnett's *post-hoc* test revealed a statistically significant down-regulation of BDNF protein in PiD ($p=0.04$) and CBD ($p=0.04$) compared to the age-matched control group (Figure 5). This represents a 44% reduction in BDNF protein in PiD and a 41% reduction in BDNF protein in CBD when compared with controls, confirming the mRNA data. There was no reduction in BDNF protein in PSP subjects compared to controls ($p=1.0$). There was a highly significant correlation between levels of BDNF protein measured by ELISA and levels of BDNF Transcript IV mRNA (Pearson correlation, $r^2=0.501$, $p=0.001$), further confirming that mRNA levels are representative of BDNF protein levels.

3. Discussion:

This is the first study to implicate tau as a potential mediator of neurotrophin dysregulation in human subjects with tauopathies. We show that proNGF protein is elevated in the parietal cortex of individuals with PiD, but not in those with CBD or PSP compared to age-matched controls. We also show that total BDNF mRNA and protein are decreased in PiD and CBD, but not in PSP, and that this decrease is predominantly attributable to reduced BDNF transcript IV.

A decrease in BDNF mRNA as well as mature and proBDNF protein in AD and MCI brain tissue has been identified previously in several studies (Connor et al., 1997, Ferrer et al., 1999, Garzon et al., 2002, Hock et al., 2000, Holsinger et al., 2000, Michalski and Fahnestock, 2003, Peng et al., 2005, Phillips et al., 1991). Soluble, oligomeric Aβ is sufficient to induce BDNF down-regulation *in vitro* (Garzon and Fahnestock, 2007, Tong et al., 2004). Importantly, a growing body of evidence suggests that tau is required for many of the toxic effects initiated by Aβ (Ittner et al., 2010, LaFerla, 2010, Morris et al., 2011, Roberson et al., 2007, Vossel et al., 2010). BDNF down-regulation in the parietal cortex of subjects with PiD and CBD provides evidence that tau may contribute to alterations in BDNF levels in AD and non-AD tauopathies. AD brain exhibits a reduction in BDNF transcripts I, II, and IV, and a trend toward down-regulation of transcript VI (Garzon et al., 2002). In PiD and CBD, we observed statistically significant down-regulation of transcript IV, which accounts for 50% of the total BDNF mRNA in the human cortex (Garzon & Fahnestock, 2007). This suggests that tau may be involved in Aβ-induced BDNF transcript IV down-regulation in Alzheimer's disease. Here, we have also shown a significant reduction in the levels of BDNF protein in PiD and PSP which further confirms BDNF down-regulation in those tauopathies.

It should be noted that the β-actin housekeeping gene used in this study corrects for loading artifacts and large variations in cell loss between samples but may not account for smaller differences in cell loss. However, considering the dramatic increase in proNGF, the profound effect size in total BDNF observed in our study, and the specific changes to transcript IV and not to other BDNF transcripts, it is unlikely that the results can be accounted for by variations in cell loss between samples. One potential mechanism underlying BDNF down-regulation may be a reduction in CREB signaling, which has been reported in mice over-expressing tau_{P301L} (Ljungberg et al., 2012). This may have significant impact on total BDNF levels since it is well accepted that CREB signaling participates in Ca^{2+}-dependent BDNF transcription (Tao et al., 1998). It is noteworthy that treatment with lithium increases BDNF expression in the brain. The neuroprotective effect of lithium, in part, could be due to inhibition of glycogen synthase kinase 3 (involved in tau phosphorylation) leading to CREB activation (Wada et al., 2005).

In contrast to the hypothesis that tau is associated with BDNF down-regulation, it is important to note that one study which employed the THY-Tau22 mouse model of tauopathy did not find a reduction in BDNF (Burnouf et al., 2012). Considering the limitations inherent in mouse models, it is quite possible that the pathological modifications to tau in human disease are not adequately represented in this particular transgenic model. Future functional studies will be required to firmly establish that tau contributes to BDNF down-regulation and to further explore possible mechanisms.

Parietal cortex was used here to allow for comparisons between this study and our previous studies showing neurotrophin dysregulation in parietal cortex from Alzheimer's disease affected individuals (Garzon et al., 2002, Peng et al., 2004, Peng et al., 2005). In this study, we

found no total BDNF down-regulation in the parietal cortex of subjects with PSP. The different brain areas affected in the three tauopathies may account for why total BDNF mRNA is down-regulated in PiD and CBD, but not in PSP. In PSP, degeneration is most pronounced in the midbrain and subcortical frontal lobe (Murray et al., 2005). In contrast, CBD affects the parietofrontal cortex and PiD exhibits degeneration in the frontal and temporal cortex (Murray et al., 2005, Rossor, 2001). Compared to PiD and CBD, areas affected by PSP are more spatially distant from parietal cortex used in this study.

ProNGF is elevated in the cortex and hippocampus of AD and MCI brain tissue (Fahnestock et al., 2001, Peng et al., 2004). Pathologically modified tau leads to axonal trafficking impairment (Cooper et al., 1994, Niewiadomska and Baksalerska-Pazera, 2003, Niewiadomska et al., 2005, Salehi et al, 2004, 2006; Schindowski et al., 2008, Vossel et al., 2010, Belarbi et al., 2011). Similar to AD, we show here that levels of proNGF protein are increased in PiD compared to controls. Consistent with the recent finding that tau is required for Aβ-induced impairment of protein transport (Vossel et al., 2010), our data suggest that tau may be a critical player in the accumulation of proNGF protein in AD as well as in non-AD tauopathies.

Differences in NGF protein levels have also been assessed in tau transgenic mice (Belarbi et al., 2011). In the THY-Tau22 mouse model of tauopathy, the authors used an ELISA which does not distinguish between proNGF and mature NGF to demonstrate that NGF-immunoreactive material accumulates in these mice relative to controls, in apparent agreement with our human results. Although western blot analysis implicated mature NGF and did not detect a change in proNGF protein, it should be noted that differences in mature NGF levels between rodents and human (Fahnestock et al., 2001), differences in sensitivity between Western

blots and ELISAs and the possibility that the model does not fully recapitulate human tauopathy pathology may explain the lack of observed difference in proNGF in THY-Tau22 mice.

Increased proNGF protein was not observed in CBD or PSP. In addition to differences in affected brain areas in the various tauopathies as mentioned above, the lack of proNGF accumulation in CBD and PSP may arise because AD and PiD show several consistent biochemical changes to tau which differ from those reported in CBD and PSP (Lee et al., 2001). Pick's disease (PiD) and AD are the most similar, as they share common behavioral impairments (such as primary cortical dementias compared to PSP and CBD with predominant motor dysfunction) and they show similar pathology (Marcinkowski, 1996). 3R-tau is the predominant isoform of tau in the PiD brain, whereas 4R-tau is predominant in CBD and PSP (Janus 2008). In addition to altered isoform ratio and differences in phosphorylation profiles (Yoshida, 2006), AD and PiD also demonstrate a truncated form of the tau protein (Arai et al., 2003). Therefore, the finding that proNGF is altered in AD and PiD, but not in CBD or PSP, may be attributed to the specific changes in tau pathology altering axonal transport and accumulation of proNGF in target tissue. Whether specific alterations in tau are responsible for altered protein trafficking requires further investigation.

In summary, we show that changes in both proNGF and BDNF in tauopathies closely resemble what has been reported previously in AD. This suggests that Aβ pathology is not a prerequisite to neurotrophin dysregulation and that tau pathology may be an important contributor to changes in neurotrophin expression in AD and non-AD tauopathies. In tauopathies, down-regulation of BDNF transcript IV, a transcript which is also regulated by amyloid-β, is consistent with the hypothesis that amyloid-β down-regulates BDNF transcript IV via tau. Our findings indicate that pathologically modified tau may confer toxicity through down-

regulation of BDNF and accumulation of proNGF protein, suggesting a novel role for tau-mediated neurotrophin dysregulation in human neurodegenerative disorders.

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5. Disclosure statement

The authors declare that they have no actual or potential conflicts of interest.

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Figure legends

Figure 1: ProNGF is increased in Pick's disease parietal cortex. Control, n=5; Pick's disease (PiD), n=6; corticobasal degeneration (CBD), n=6; progressive supranuclear palsy (PSP), n=6. **(**A) Representative Western blot of tauopathy parietal cortex samples. 30 μg total protein was loaded per lane. Blots were probed with NGF antibody (H-20) and reprobed with β-actin antibody. (B) A significant increase in the ratio of proNGF (34kDa) to β-actin was observed in PiD compared to control brain. This increase was not observed in CBD or PSP. All samples were measured in 3 separate experiments, and the mean of pixel values was used for statistical analysis. Error bars represent standard error of the mean (S.E.M). *****p=0.02, one-way ANOVA and *post hoc* Dunnett's test.

Figure 2. BDNF mRNA is down-regulated in corticobasal degeneration and Pick's disease compared to controls. Quantitative real-time RT-PCR (qRT-PCR) for total BDNF mRNA normalized to β-actin mRNA. Control, n=11; Pick's disease (PiD), n=8; corticobasal degeneration (CBD), n=11; progressive supranuclear palsy (PSP), n=13. Each BDNF/β-actin ratio was log-transformed to obtain a normal distribution. Each point on this graph represents the mean of 6 independent reverse transcriptions (each subjected to three PCR reactions) per sample. The horizontal line represents the mean of each group. A significant decrease in BDNF mRNA was observed in PiD (p=0.03) and CBD (p=0.04), but not in PSP (p=0.86), when compared to controls. **p*<0.05, 6-level repeated measures ANOVA and *post hoc* Dunnett's test.

Figure 3. BDNF transcript IV is down-regulated in Pick's disease and corticobasal degeneration. Control, n=11; Pick's disease (PiD), n=8, corticobasal degeneration (CBD), n=11 progressive supranuclear palsy (PSP), n=13. RNA was extracted from each sample, and cDNA

from 200ng of RNA was analyzed using quantitative real-time PCR (qRT-PCR) for BDNF transcript IV and from 20ng RNA for β-actin. Each ratio of BDNF transcript IV copies to β-actin copies per 1ng of cDNA was converted to its natural logarithm to obtain a normal distribution. One-way ANOVA revealed significant differences between groups (p=0.006), and *post hoc* Dunnett's test displayed down-regulation of transcript IV in PiD and CBD groups (p=0.01 and p=0.03, respectively), but not in PSP (p=0.88).

Figure 4. No change in expression of BDNF transcripts I, II, VI or VII in Pick's disease or corticobasal degeneration. Control, n=11; Pick's disease (PiD), n=8 and corticobasal degeneration (CBD), n=11. RNA was extracted from each sample, and cDNA from 50ng of RNA (150ng RNA for transcript VII) was analyzed using quantitative real-time PCR (qRT-PCR) for each BDNF transcript and normalized to β-actin values. Each ratio was converted to its natural logarithm to obtain a normal distribution. Shown here are results from transcript transcript I (A), transcript II (B), transcript VI (C), and transcript VII (D). In these graphs, each square represents the mean of 3 independent reverse transcriptions per sample. The horizontal line represents the mean of each group. None of the transcripts examined demonstrated significant down-regulation compared to controls (one-way ANOVA, $p>0.05$).

Figure 5. BDNF protein is decreased in corticobasal degeneration and Pick's disease compared to controls. Control, n=12; Pick's disease (PiD), n=9; corticobasal degeneration (CBD), n=12; progressive supranuclear palsy (PSP), n=13. The horizontal line represents the mean of each group. A significant decrease in BDNF protein as measured by ELISA was observed in PiD (p=0.04) and CBD (p=0.04), but not in PSP (p=1.0), when compared to controls. *$p<0.05$, one-way ANOVA (p=0.01) and *post hoc* Dunnett's test.

Table 1: Subject Characteristics

(A) Characteristics of samples analyzed for proNGF; (B) Characteristics of samples analyzed for BDNF mRNA; (C) Characteristics of samples analyzed for BDNF protein. Expressed as mean ± standard deviation (age and protein yield), range (age and yield of mRNA), and percent of males. P-value determined by one-way ANOVA.

1A.

	Clinical Diagnosis				**P-value**
	Normal (n = 6)	**PiD (n = 6)**	**CBD (n=6)**	**PSP (n=6)**	
Age (years) at death: mean ± SD (Range)	72.3 ± 2.4 (69 – 75)	68.3 ± 6.8 (59 – 76)	70.7 ± 4.5 (62 – 74)	72.8 ± 3.2 (70 – 79)	0.34
Number (%) of Males	3 (50%)	4 (67%)	3 (50%)	2 (33%)	0.72
Yield of protein (μg/mg)	44 ± 5	40 ± 5.8	42.5 ± 6.6	42.3 ± 5.3	0.69

1B.

	Clinical Diagnosis				**P-value**
	Normal (n = 11)	**PiD (n = 8)**	**CBD (n=11)**	**PSP (n=13)**	
Age (years) at death: mean ± SD (Range)	68.1 ± 11.6 (36 – 81)	62.3 ± 11.0 (42 - 76)	70.1 ± 6.3 (56 – 80)	68.8 ± 8.0 (48 – 79)	0.73
Number (%) of Males	5 (45%)	4 (50%)	5 (45%)	5 (38%)	0.78
Yield of mRNA (μg/mg)	0.05 – 0.49	0.3 – 0.54	0.15 – 0.52	0.17 – 0.45	0.73

1C.

	Clinical Diagnosis				**P-value**
	Normal (n = 12)	**PiD (n = 9)**	**CBD (n=12)**	**PSP (n=13)**	
Age (years) at death: mean ± SD (Range)	68.6± 11.2 (36 – 81)	65.4±10.3 (42–76)	69.4 ± 6.4 (56 – 80)	68.8 ± 8.0 (48–79)	0.77
Number (%) of Males	5 (42%)	5 (56%)	5 (42%)	5 (38%)	0.89
Yield of protein (μg/mg)	44.4 ± 6.7	44.1 ±11.5	49.9 ± 6.9	45.2 ± 13.9	0.49

Table 2: Primers used for quantitative real-time PCR.

Forward primer sequences for BDNF exons Vh and IX, and reverse sequences for exons III, Vh, and IX were taken from Pruunsild et al. (2007) (Pruunsild et al., 2007). All other primers were designed using Primer3 version 4.0 (online version, Massachusetts Institute of Technology) using the NCBI reference number listed. Location of primers in specific exons was confirmed by referring to the intron-exon boundaries identified previously (Pruunsild et al., 2007). For transcripts that were detected prior to a cycle threshold of 32, a dissociation curve confirmed that only one major product was amplified.

Target	Product Size	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
β-actin (NM_001101.3)	109bp	CTCTTCCAGCCTTCCTTC	TGTTGGCGTACAGGTCTT
Total BDNF (EF689009)	249bp	AAACATCCGAGGACAAGGTG	AGAAGAGGAGGCTCCAAAG
BDNF Exon I (EF689021)	286bp	GCGGATATTGCAAAGGGTTA	ACCTTGTCCTCGGATGTTTG
BDNF Exon II (NM_001143806.1)	148bp	GCGGTGTAGGCTGGAATAGA	ACCTTGTCCTCGGATGTTTG
BDNF Exon III (NM_001143807.1)	487bp	TTAGAGGGTTCCCGCTTTCT	GACCCTCATGGACATGTTTGCAGC
BDNF Exon IV (NM_170733.3)	382bp	GAGTATTACCTCCGCCATGC	ATTCACGCTCTCCAGAGTCC
BDNF Exon V (EF689011)	219bp	CATGCTCTGTGCGATTTCAT	ATTCACGCTCTCCAGACTCC
BDNF Exon Vh (EF689013)	495bp	GGCTGGAACACCCCTCGAA	GACCCTCATGGACATGTTTGCAGC
BDNF Exon VI (EF689014)	198bp	AACCACGATGTGACTCCGC	ATTCACGCTCTCCAGAGTCC
BDNF Exon VII (EF689017)	257bp	CTACCGCTGGGAACTGAAAG	ATTCACGCTCTCCAGAGTCC
BDNF Exon IXabcd (NM_170735.5)	597bp	TTTCTCGTGACAGCATGAG	GTCCTCATCCAACAGCTCTTCTATC

Figure 1

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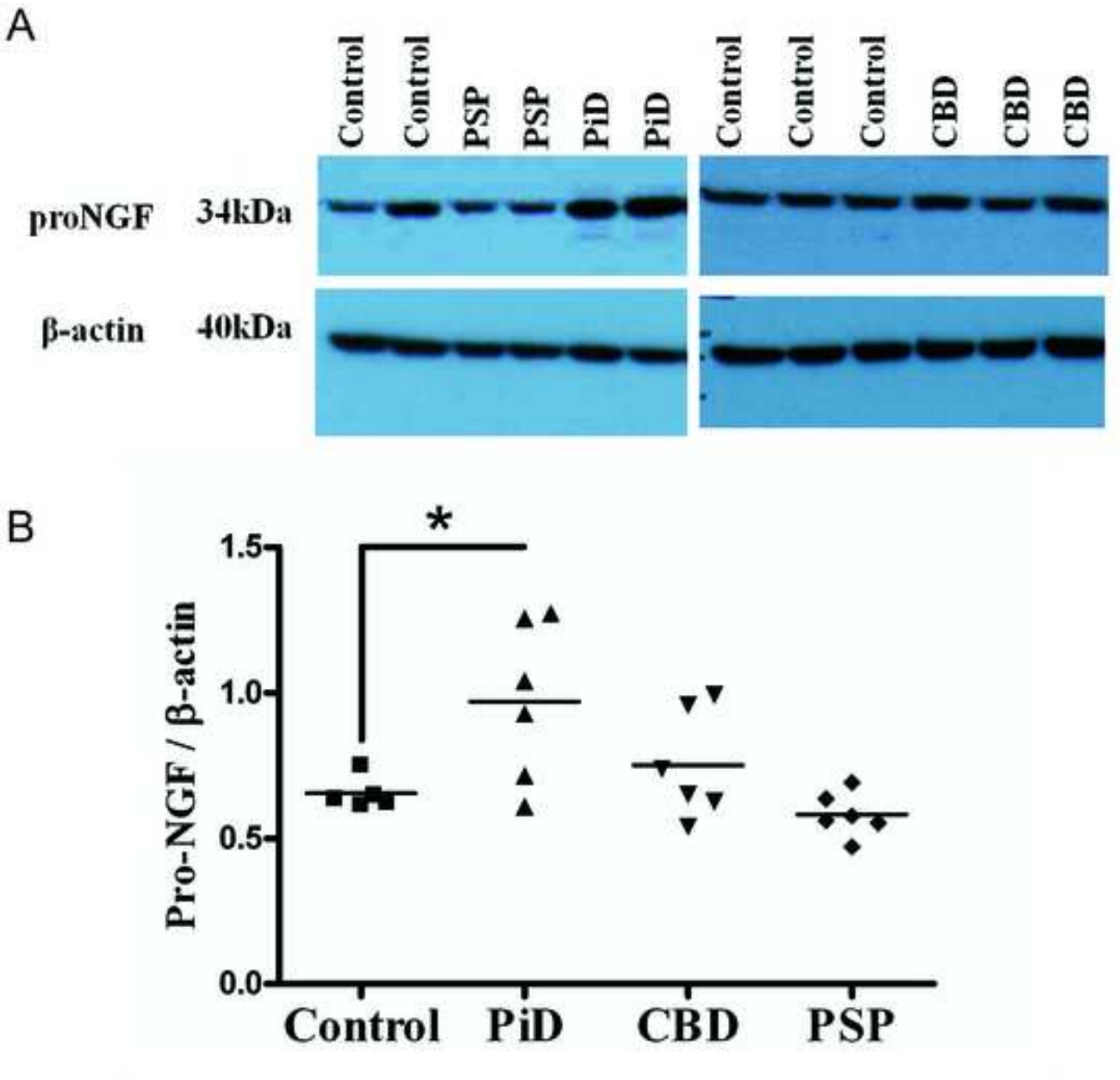

Figure 2

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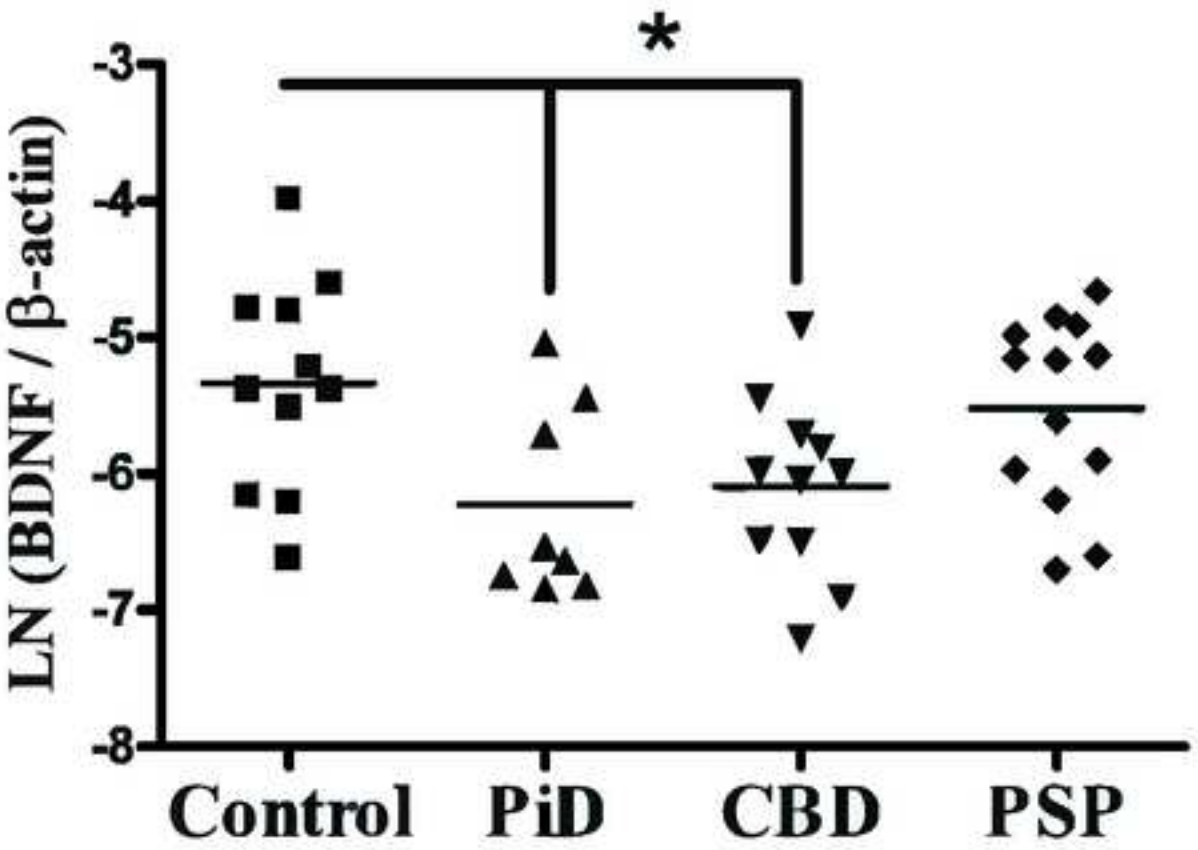

Figure 3

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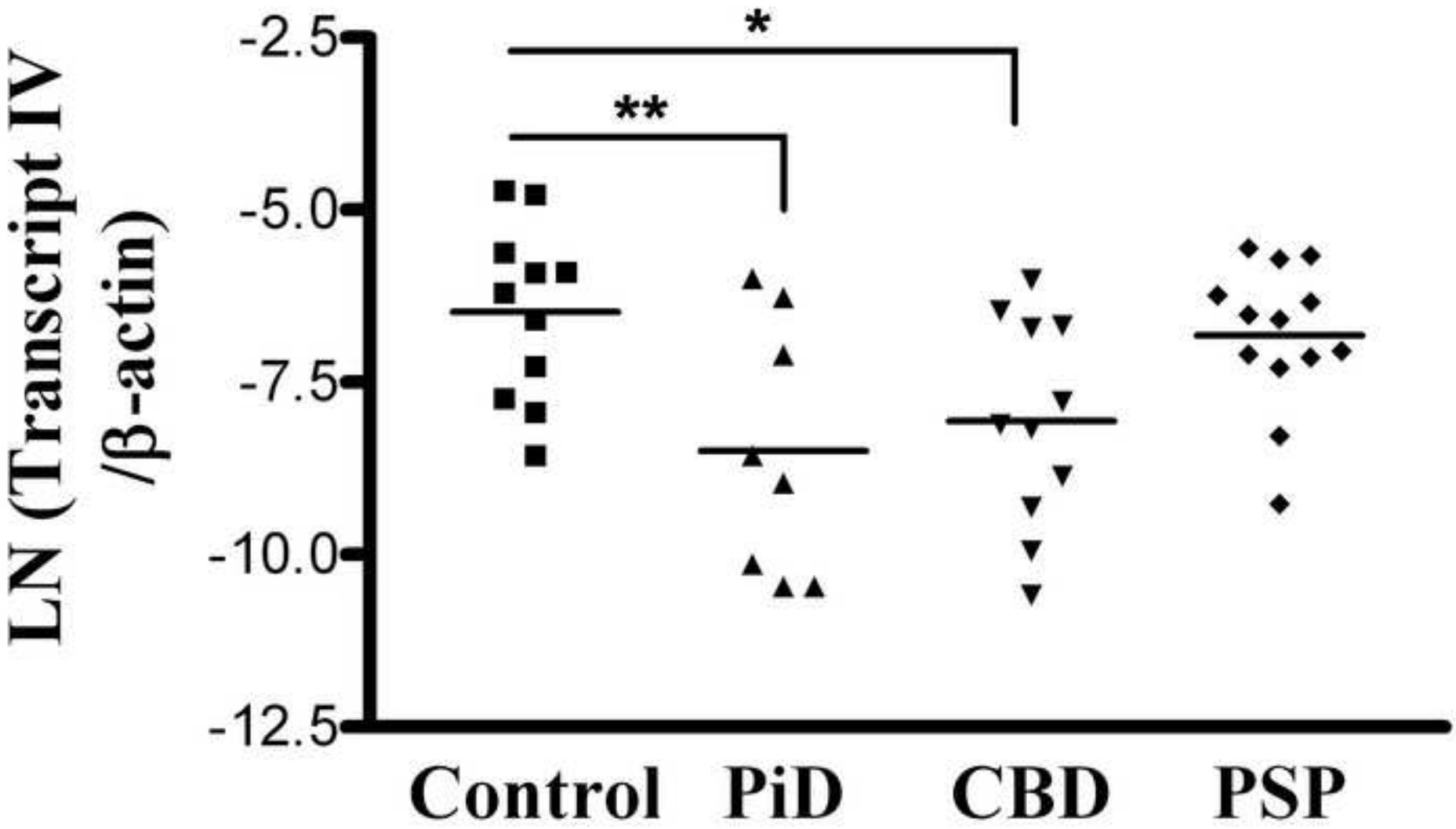

Figure 4

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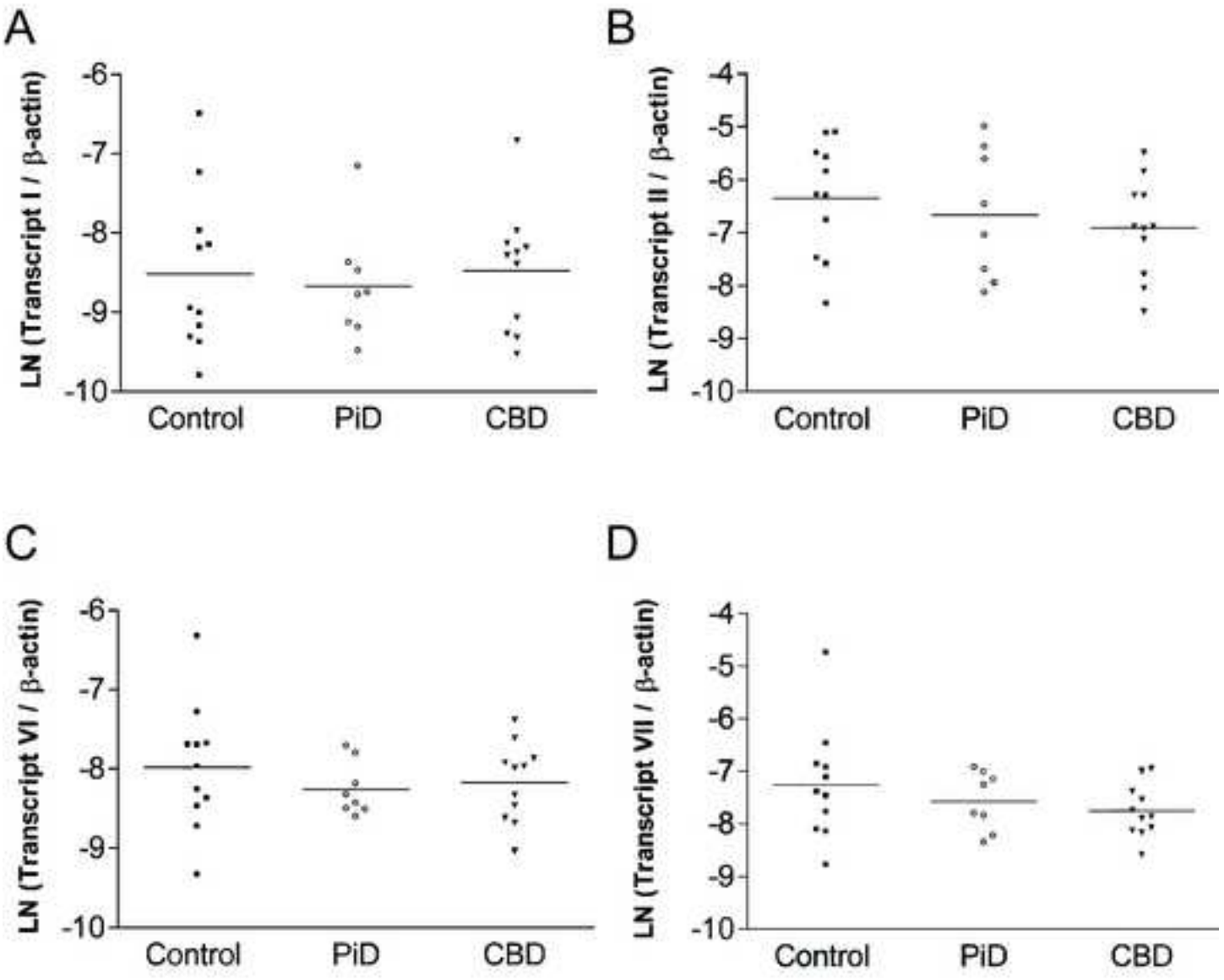

Figure 5

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