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Zinc induced structural changes in the intrinsically disordered BDNF Met prodomain confer synaptic elimination†

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Human brain derived neurotrophic factor (BDNF) encodes a protein product consisting of a C-terminal mature domain (mature BDNF) and an N-terminal prodomain, which is an intrinsically disordered protein. A common single nucleotide polymorphism in humans results in a methionine substitution for valine at position 66 of the prodomain, and is associated with memory deficits, depression and anxiety disorders. The BDNF Met66 prodomain, but not the Val66 prodomain, promotes rapid structural remodeling of hippocampal neurons' growth cones and dendritic spines by interacting directly with the SorCS2 receptor. While it has been reported that the Met66 and Val66 prodomains exhibit only modest differences in structural propensities in the apo state, here we show that Val66 and Met66 prodomains differentially bind zinc (Zn). Zn²⁺ binds with higher affinity and more broadly impacts residues on the Met66 prodomain compared to the Val66 prodomain as shown by NMR and ITC. Zn²⁺ binding to the Met66 and Val66 prodomains results in distinct conformational and macroscopic differences observed by NMR, light scattering and cryoEM. To determine if Zn²⁺ mediated conformational change in the Met66 prodomain is required for biological effect, we mutated His40, a Zn²⁺ binding site, and observed a loss of Met66 prodomain bioactivity. As the His40 site is distant from the known region of the prodomain involved in receptor binding, we suggest that Met66 prodomain bioactivity involves His40 mediated stabilization of the multimeric structure. Our results point to the necessity of a Zn²⁺-mediated higher order molecular assembly of the Met66 prodomain to mediate neuronal remodeling.

Significance to metallomics

The brain derived neurotrophic factor (BDNF) Val66Met variant is a significant risk factor in cognitive decline and psychiatric disorders. The BDNF prodomain region and mature BDNF are expressed in the hippocampus and amygdala, brain regions that accumulate zinc. Zinc, but not other cations present in the synapse, induce structural differences between the Val66 and Met66 prodomains, at physiologically relevant concentrations. Our results suggest that zinc binding confers synaptic remodeling activity to the Met66 prodomain isoform.

Introduction

Neurotrophins, a family of conserved growth factors, were initially identified by their ability to confer neuronal survival and differentiation during development. Of the four neurotrophins found in humans, brain derived neurotrophic factor (BDNF) is the most extensively studied and plays critical roles modulating activity-dependent synaptic plasticity, cell migration, dendritic arborization and even cell death.^{1–4} These diverse responses are regulated by utilization of different protein products of the *bdnf* gene.⁵ BDNF is translated as a precursor, proBDNF (~25.7 kD, aa 1–247), encoding an intrinsically disordered N-terminal prodomain (aa 19–128) and a structured C-terminal mature domain which dimerizes (aa 129–247) (mature BDNF or mBDNF) (Fig. 1A). Proteolytic processing of proBDNF results in the release of dimeric mBDNF and monomeric prodomain. In the adult brain, the prodomain and mBDNF are the predominant

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Fig. 1 (A) Schematic of the proBDNF protein (~25.7 kD, aa 19–247), encoding an intrinsically disordered N-terminal prodomain (~12.5 kD, aa 19–128) and a structured C-terminal mature domain, which forms non-covalent dimers (~13.5 kD aa 129–247) (mature BDNF or mBDNF). (B) Protein purity analysis by using 15% SDS-Page gels of full length Val66-prodomain and Met66-prodomain at 17 μ g and 28 μ g protein loads respectively. (C) Mass spectrometry analysis using an Bruker Autoflex MALDI-TOF, (Bruker Daltonics) mass spectrometer for intact mass detection of unlabeled Val66 and Met66 prodomains clearly distinguishes the 32 atomic mass unit (amu) difference between Val and Met isoforms. The observed monomer peaks are within instrumental error of the expected molecular weights for Val and Met prodomains of 12 492 MW and 12 524 MW respectively.

isoforms found.⁶ The mBDNF protein activates TrkB to promote neuronal survival, growth and development. In contrast, proBDNF binds to a complex of p75^{NTR} and SorCS2 (or Sortilin), which mediates cell death, dendritic remodeling and long term depression (LTD).^{4,7,8} The prodomain together with cleaved mBDNF are co-secreted at the synapse following repeated neuronal stimulation and exposed to an environment that contains cations, including zinc (Zn).⁶ The prodomain region contains a single nucleotide polymorphism, that results in a valine to methionine amino acid change at position 66 (Val66–Met). The Met66 substitution is present in approximately 20% of the human population.⁹ Met66 carriers have increased susceptibility to develop anxiety disorders, depression, memory deficits, reduced hippocampal volume and impaired recovery after traumatic brain injury.^{9–14} Despite the biological significance of the Val66Met polymorphism, the potential impact of metal-induced structural change upon the secreted prodomain of BDNF has not been examined.

The bioactivity of the Met66 prodomain results from its interactions with SorCS2, a Vsp10 domain protein which is expressed on the plasma membrane. The activity of this ligand also requires that SorCS2 co-associates with the p75^{NTR} receptor, a member of the tumor necrosis factor superfamily.⁶ Both proteins are expressed on the postsynaptic region of dendritic spines. Only the Met66 prodomain, but not the Val66 isoform induces acute collapse of mature dendritic spines.¹⁵ Both the Met66 and Val66 isoforms are intrinsically disordered and exhibit limited structural differences in the region of the Val66Met substitution, and the differences in

the interaction with SorCS2 are modest⁶ in the absence of cations and unlikely to account for the dramatic differences in biological effects. This suggests that other co-factors may be required to induce structural differences. mBDNF and the prodomain are co-secreted from dense core vesicles (DCV) into the synaptic cleft,¹⁶ coordinately with synaptic vesicle exocytosis in response to sustained neuronal excitation. Thus the prodomain is exposed to micromolar to millimolar concentrations of divalent metals Ca²⁺, Mg²⁺ and Zn²⁺ present in the synaptic cleft^{17–20} prior to engaging SorCS2.

Here we examine the potential role of physiologically relevant concentrations of divalent cations to induce structural changes in the Met66 vs. Val66 variants of the prodomain which could lead to selective bioactivity of the Met66 prodomain. We demonstrate that both Val66 and Met66 prodomains interact with Zn²⁺ and induce structural differences as determined using NMR. Furthermore, Zn²⁺ binding results in differences in macroscopic oligomerization of the Val66 as compared to the Met66 prodomain, as determined by cryo-electron microscopy (cryoEM). We show that Zn²⁺ interacts with the prodomain His40 to induce intermolecular association. Moreover, we demonstrate that mutation of His40 blocks the prodomain macromolecular structural assembly and abrogates the Met66 prodomain's degenerative bioactivity. Our results identify the unique actions of Zn²⁺ to induce structural changes in this intrinsically disordered protein, and suggest differences that contribute to the acquisition of bioactivity in the Met prodomain.

Methods

Protein expression and purification

The prodomain of BDNF was expressed in *E. coli* using a plasmid pET28 (Novagen, Madison, WI, USA) containing the gene of human BDNF prodomain Val66 or Met66 (amino acids 19–128 preceded by an N-terminal His-6-SUMO tag).²¹ Mutations were introduced into plasmids using Quick-Change mutagenesis (Stratagene) using forward (caggtgtgcggaccaaagg gactctggagag) and reverse (ctctccagagtccttgggtccgcacactg) primers for introduction of His40Lys mutations, then confirmed by sequencing. Unlabeled and isotopically ¹³C–¹⁵N or ¹⁵N-labeled protein samples were expressed in *E. coli* using the Marley isotope exchange protocol.²² Cells were lysed by sonication in 4 M guanidine hydrochloride in the presence of 1 mM PMSF and Sigma Protease inhibitor cocktail. Proteins were washed and initially purified on a Ni-NTA column (Invitrogen) followed by elution in 6 M Urea, 300 mM imidazole pH 6. Protein was dialyzed against at 50 mM Tris 100 mM NaCl, pH 7.4 overnight. To remove the Sumo tag, the protein was incubated with the Ulp-1 protease for 1 h at room temperature, the untagged prodomain was separated from the His-tagged Sumo and His-tagged Ulp-1 by passage through a second Ni-NTA affinity column. All constructs were further purified by HPLC on a Vydac C18 column using a 0.1% formic acid, to 95% acetonitrile gradient. Purified fractions were lyophilized and stored at –80 °C. Sample purity was assessed, by SDS-PAGE

and MALDI-TOF-MS (Fig. 1B and C). The prodomain concentrations were measured by UV absorbance using a calculated OD_{280} of 0.48 (ProtParam) or Bradford protein assay (BioRad).

Mass spectrometry analysis

The integrity of all samples was established by mass spectrometry (Fig. 1C). Prodomain isoforms were tested for high affinity Zn^{2+} binding using MALDI-TOF and direct infusion Electrospray Ionization (ESI) MS. Samples were dissolved in 10 mM Tris buffer with 10 mM piperazine pH 7.0 at a protein concentration of 2.5 μ M in the presence of different concentrations of $ZnCl_2$: 0, 10 μ M, 100 μ M, 1 mM. For MALDI-TOF 1 μ l samples were mixed with 1 μ l matrix (20 mg ml^{-1} sinapinic acid in 50% acetonitrile, 50% water with 0.1% trifluoroacetic acid). 1 μ l aliquots of the mixtures were deposited on a 384-well sample plate, allowed to air-dry, then analyzed by positive ion MALDI-TOF MS (Bruker Autoflex MALDI-TOF, Bruker Daltonics) in linear mode, or directly infused to LTQ-Orbitrap (Thermo Electron) mass spectrometer for intact mass detection.

NMR spectroscopy

Samples for NMR measurement were collected in 10 mM Tris, 100 mM NaCl, 0.1 mM DSS in 90% H_2O /10% D_2O at pH 7.2. NMR data was collected 5 $^{\circ}C$ on Bruker AvanceHD spectrometers at 500 MHz and 600 MHz field strengths at Weill Cornell Medical College and confirmatory experiments at 800 MHz and 900 MHz field strengths at the NYSBC. NMR assignments were obtained by titration to the experimental conditions, starting with our previous prodomain NMR using well-resolved 2D ^{15}N -HSQC assigned resonance changes (Fig. S2A and B, ESI †), 23 (BMRB Entry 19357). Spectra were processed using TopSpin 3.2 (Bruker Corporation) or NMRPipe 24 and analyzed using NMRFAM-Sparky. 25 Zn^{2+} binding was measured using 2D ^{15}N -HSQC NMR spectra collected in the presence or absence of sub-stoichiometric concentrations of Zn^{2+} buffered in 10 mM Tris, 100 mM NaCl, pH 7.2 in 10% D_2O /90% H_2O . NMR ^{15}N longitudinal T_1 with interleaved relaxation delays: 56, 505 (2 $\times$), 838, 1171, 1839, 2170 (2 $\times$) and 2835 ms and ^{15}N transverse T_2 relaxation measurements with interleaved delays: 29.4, 58.8 (2 $\times$), 88.2, 147.0, 117.6 (2 $\times$), 176.4, 205.8 ms, in randomized orders with duplicate points for statistical analysis as described in prior publications. $^{26-28}$ NMR relaxation decay curves were fitted to single exponential decay models to extract longitudinal (R_1) and transverse (R_2) relaxation rates using python scripts incorporated in NMRFAM-Sparky.

NMR translational diffusion measurements were collected on Bruker Avance IIIHD 600 MHz using bionomial gradients as previously described. 6,29 Diffusion measurements were carried out at 5 $^{\circ}C$ using prodomain concentrations ranging from 8.8 μ M to 178 μ M. Diffusion rates were referenced to 1,4-dioxin using the hydrodynamic radius of 2.2 $\text{\AA}$ 30 or DSS with a hydrodynamic radius of 3.5 $\text{\AA}$. The apparent hydrodynamic radius of the protein (R_h^{prot}) was calculated as $R_h^{prot} = (R_h^{ref})(D_{ref})/D_{prot}$, using the measured diffusion rates of the protein (D_{prot}) measured with an internal reference molecule (D_{ref}) of radius (R_h^{ref}). 30 Diffusion rates for Dioxin and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)

reference standard signals were found to be invariant under all prodomain concentrations (all ≤ 1 mg ml^{-1}) tested.

Turbidity measurements

Changes in turbidity upon addition of $ZnCl_2$ were measured using a Varian Cary 50-Bio UV-Vis spectrophotometer employing a 1 cm path length masked quartz cell. Metal cation mediated aggregation kinetics were initiated upon rapid mixing of equal volumes prodomain and Zn^{2+} solutions to give final concentrations ranging from 2 to 10 μ M prodomain and 10 to 100 μ M $ZnCl_2$ chloride in 10 mM Tris, 100 mM NaCl at pH 7.2 in a volume of 250 μ l, turbidity curves were repeated a minimum of 3 times. Turbidity kinetics were followed at 600 nm with data collected at 2 s intervals for up to 20 min immediately following mixing. Aggregate dissociation kinetics were initiated upon five-fold dilution, dissociation changes were followed at 600 nm for up to 20 min. The dead-time of mixing for both formation and dissociation kinetics ranged from approximately 5 to 10 s. Dissociation curves were fitted to single or multiexponential kinetic models using Igor plot (WaveMetrics), with model selection was based on F -test statistical criterion.

Isothermal titration calorimetry (ITC)

For all ITC experiments, prodomain samples were loaded into separate dialysis chambers and exhaustively co-dialyzed into ITC buffer (100 mM NaCl, 10 mM Tris pH 7.2), then centrifuged at 15 000 $\times g$ for 10 min then degassed for 10 min immediately prior to ITC data collection. ITC data was collected on a Nano ITC (TA instruments) at 10 $^{\circ}C$. The sample cell (1 ml) containing 50 μ M prodomain was titrated with 1.5 mM $ZnCl_2$ solutions using 5 μ l injections collected every 500 s with a stirring speed of 300 rpm. The measured protein data were baseline corrected using thermal heats obtained by Zn^{2+} titration into buffer, then fitted, using the Nano Analyze Software (TA Instruments). The values of dissociation constant (K_d) and enthalpy (ΔH) reported constitute averages of 3 experiments performed under the identical conditions, data were fitted using single and two site binding models.

Electron microscopy

Cryo-EM samples were prepared on carbon coated Quantifoil 1.2 μ m holey copper grids, which were glow-charged immediately prior to application of 3 μ l of 20 μ M protein in the presence of 200 μ M $ZnCl_2$ in 10 mM Tris, 10 mM Piperazine 100 mM NaCl at pH 7.0. Following application, the grids were blotted for 2 s then plunge frozen in liquid ethane using a Gatan cryoplunge-3. Images (4k $\times$ 4k) were collected on a Tecnai-F20 at the NY Structural Biology Center operating at 200 kV at 62k $\times$ magnification at final pixel size 1.4 $\text{\AA}$.

Hippocampal culture

Animal care was provided by the Research Animal Resource Center (RARC) at Weill Cornell Medicine following instructions of the "Guide for the care and use of laboratory animals" (National Research Council, USA). The animal research was approved by the Institutional Animal Care and Use Committee

(IACUC) of Weill Cornell. Dissociated cultures of primary hippocampal neurons were prepared from E18 embryos of pregnant C57BL/6 mice. Excised hippocampi were dissociated into cell suspension using pre-warmed papain (Worthington Biochemical Corporation, Lakewood, NJ, USA) containing Hank's Balanced Sodium Solution (HBSS) (GIBCO) supplemented with DNase I, 1.5 mM CaCl_2 , and 0.75 mM EDTA at 37 °C for 20–25 min. After trituration, the neurons were plated on 12 mm poly-L-lysine (1 mg ml^{-1} in dH_2O)-coated coverslips. Prior to plating, coverslips (Electron Microscopy Sciences, Hatfield, PA, USA) were pre-incubated in Neurobasal medium (Gibco, Grand Island, NY, USA), containing 1 mM sodium pyruvate (Gibco), 6 mM Glutamax (Gibco), 10% fetal bovine serum, 0.5% glucose, and $50 \text{ } \mu\text{g ml}^{-1}$ ampicillin. Following 5–6 hours of recovery in serum and glucose, the media was replaced with Neurobasal medium with added B27 (Gibco), 1 mM pyruvate (GIBCO), 2 mM glutamine (Gibco), $50 \text{ } \mu\text{g ml}^{-1}$ ampicillin, and $4 \text{ } \mu\text{M}$ cytosine-1- β -D-arabinofuranoside.^{15,31} The cells were left in the incubator for 18–21 days with occasional addition of $50 \text{ } \mu\text{l}$ per well of media in 24 well plates to prevent evaporation.

Immunostaining

Prior to treatment with prodomain, the 18–21 DIV mature neurons were starved in a pre-warmed Neurobasal media supplemented with 0.5% glucose. Val or Met or His40Lys mutant prod domains were added to the neurons, incubated at a final concentration of 10 ng ml^{-1} for 1 hour at 37 °C, maintained at 5% CO_2 . Following the treatment, neurons were quickly washed in warm $1\times$ HBSS and immediately fixed with 4% paraformaldehyde/4% glucose in $1\times$ PBS for 15 min. For immunostaining, the cells were permeabilized for 15 minutes with 0.1% Triton in $1\times$ PBS containing 5% FBS for blocking and stained for 15 minutes with Alexa Fluor 546 phalloidin (Life Technologies, Thermo-Fisher Scientific) at room temperature to demarcate actin enriched dendritic spines and outline neuronal morphology. Following staining procedure, neurons were rinsed in $1\times$ PBS and coverslips mounted on the slides using ProLong Gold antifade reagent (Invitrogen).

Antibodies

The following primary antibodies were used: chicken polyclonal *anti*-MAP2 (Abcam) to mark dendritic segments with *anti*-chicken 405 nm fluorescent DyLight (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Alexa Fluor 546 phalloidin (Life Technologies, Thermo Fisher Scientific) was used to demarcate actin enriched dendritic spines and the contour of neuronal morphology.

Imaging

The neurons were imaged using Structured Illumination Microscope (N-SIM) Nikon Eclipse Ti (Nikon Instruments Inc., Melville, NY, USA) using $100\times$ Apo TIRF lens with 1.49 N.A. (Nikon Instruments Inc.) and Andor iXon3 DU-897E EMCCD camera (Andor Technology Ltd, Belfast, UK) using 10 MHz, 14-bit, conversion gain of $2.4\times$ or $5\times$ and 50% laser. Exposure times were customized to obtain 4000 maximum counts histogram values for red channel.¹⁵ Super resolution images were generated from illumination pattern at three angles

and in five phases generating 15-image Z-stacks. Acquired images were reconstructed using NIS-Elements software 4.20 with N-SIM analysis plug-in (Nikon Elements). Reconstruction parameters (auto structured illumination contrast, 1.00 apodization filter parameter, 0.05 width of 3D-SIM filter) were kept consistent to generate the best Fourier transforms to visualize thin spines.¹⁵ The thin and mushroom spines were counted by the experimenter blinded to the conditions.

Results

BDNF and its prodomain are highly expressed in diverse brain regions, such as the hippocampus, which are known to have high concentrations of Zn^{2+} , stored in vesicles. In hippocampal neurons, secretion of mature BDNF, prodomain and Zn^{2+} occur upon neuronal depolarization. Prior studies have identified Zn^{2+} binding interactions with the N-terminus of mature BDNF,³¹ however the Zn^{2+} binding to the co-secreted prodomain has never been explored. This is of particular interest, since the Val66 and Met66 prodomain are exposed to high concentrations of this metal and could impact its structure and/or function.

Zinc interacts with prodomain sites at His40, His65 and Glu111

To characterize the interaction of Zn^{2+} with the intrinsically disordered prodomain isoforms we use heteronuclear NMR analysis to identify the individual residues that undergo association and structural change. To identify the specific prodomain residues impacted by Zn binding we have transferring our prior backbone heteronuclear NMR resonance assignments to conditions suitable for Zn titration analysis.²³ The specific backbone $\text{H}^{\text{N}}_{\text{i}}$ and N_{i} resonance assignments for the full length prodomain provide sensitive indicators that are affected by changes in chemical environment and dynamic motions. Addition of sub-stoichiometric concentrations of Zn^{2+} to the prod domains resulted in NMR spectral shifts and peak intensity changes in both Val66 and Met66 isoforms, indicating rapid prodomain- Zn^{2+} equilibrium kinetics, (Fig. 2 and Fig. S1A, B, ESI[†]). Both isoforms display signal changes centered on residues His40, His65 and Glu111 (Fig. 2). Both His and to a lesser degree Glu residues are recognized for a propensity to associate with Zn^{2+} . The residues impacted upon addition of Zn^{2+} are more extensive in the Met66 prodomain impacting 49 residues, compared to 24 residues in the Val66 prodomain under the same conditions (Fig. 2E). The Met66 prodomain displays greater changes in neighboring His40 (residues 37–50), His65 (residues 57–79), and Glu111 (residues 104–119), as compared to the more limited differences observed in the Val66 prodomain (residues 40–49, residues 60–77 and residues 111–112 of the Val66 prodomain) (Fig. 2E). Our NMR results indicate that the Met66 prodomain is significantly more responsive to Zn^{2+} binding than the Val66 isoform.

BDNF Val66 and Met66 prod domains preferentially bind Zn^{2+}

Divalent cations Ca^{2+} , Mg^{2+} and Zn^{2+} are all dynamically regulated within the synaptic cleft, available to interact with

Fig. 2 Superimposed ¹H-¹⁵N HSQC spectra of 8 μ M prodomeins in the absence and presence of 4 μ M Zn²⁺ are shown for (A). Val66 (blue) and Val66 + Zn²⁺ (purple) and (B). Met66 (red) with Met66 + Zn²⁺ (purple). (C and D) A schematic centered at His40, His65 and Leu110 is shown above to provided reference. Addition of sub-stoichiometric Zn²⁺ results in significant attenuation of peak intensity at specific residues. The peak intensities for Val66 and Met66 were normalized to adjacent N- and C-terminal residues which remain unperturbed by isoform variation and Zn²⁺ addition. The peak intensities significantly vary under native conditions for both (C) Val66 (light-blue) and (D) Met66 (light-red) in these three regions, and undergo significant reductions intensity upon Zn²⁺ binding shown for Val66 (blue) and Met66 (red). (E) A summary diagram of the prodomein sequence map regions of significant Zn²⁺ induced peak intensity changes based on moderate or complete intensity loss for Val (top) and Met (bottom), indicate three predominant regions centered neighboring histidine residues His40, His65 and hydrophobic region L110.

prodomein.³² Using simple turbidity analysis to assess cation mediated particle formation, we observed significant UV turbidity changes upon addition of physiologically relevant amount of Zn²⁺ to prodomein (Fig. 3). Higher levels of turbidity were consistently detected for Met66 as compared to the Val66 prodomein, indicating the presence of more abundant and/or larger particles on average. In contrast, turbidity changes were not detected upon addition of significantly high concentrations of Ca²⁺ or Mg²⁺ to the Val66 or Met66 prodomein, applied either individually (Fig. S3C and D) or together at physiologically relevant concentrations (Fig. S3A and B, ESI[†]). These results strongly suggest that prodomein binding, and particulate assembly is specific for Zn²⁺. Under limiting prodomein concentrations, Zn²⁺ association displays sigmoidal kinetics, suggesting an initiation-propagation mechanism that favors more rapid assembly of the Met66 isoform, as compared to the Val66 prodomein (Fig. S3C and D, ESI[†]). Furthermore, we observe Zn²⁺ association with the Met66 and Val66 prodomeins in the setting of millimolar concentrations of Ca²⁺ and Mg²⁺, reflecting conditions present *in vivo*, indicating that divalent ions abundant within the synapse do not compete with Zn²⁺

(Fig. S3A and B, ESI[†]). Zn²⁺ mediated particulates formed were reversible upon dilution (Fig. 3B), with slower kinetics of disassembly consistently observed for Met66 prodomein ($0.83 \pm 0.02 \text{ min}^{-1}$) as compared to the Val66 prodomein which exhibits bi-exponential dissociation rates with fast ($9.6 \pm 0.4 \text{ min}^{-1}$) and slow ($0.88 \pm 0.07 \text{ min}^{-1}$) components (Fig. 3B). Complete return to baseline is not achieved without addition of EDTA, suggesting that a fraction of the macroscopic population is resistant to dissociation. Attempts to identify high affinity (nanomolar) prodomein-Zn interaction by mass spectrometry were unsuccessful, suggesting dissociation during ionization and gas phase analysis, due to weaker, reversible, micromolar Zn²⁺ binding affinity. Furthermore, diagnostic tests for amyloid binding *via* Congo red display no characteristic red shifts, indicating an absence of amyloid structure³³ (Fig. S3E and F, ESI[†]). Collectively this data demonstrates that Zn²⁺ interactions with the Met66 or Val66 prodomein to induce isoform specific differences in macromolecular structure that are largely reversible yet kinetically distinct, suggesting that Met66-Zn aggregates are structurally different from Val66-Zn aggregates in both assembly and dissociation.

Fig. 3 Turbidity changes monitored at 600 nm identifies the formation of aggregates in the presence of 10 μM Val66 and Met66 prod domains in presence of excess (A). 100 μM Zn^{2+} (B). Dissociation of Zn^{2+} aggregates upon dilution to a final concentration of 2 μM prod domain and 40 μM Zn^{2+} , prod domain and Zn^{2+} concentrations that fail to develop turbidity.

Biophysical differences in the interaction of Zn^{2+} with Val66 and Met66 prod domains

To investigate the thermodynamic differences upon the interaction of Zn^{2+} with the Met66 and Val66 prod domains, we used isothermal titration calorimetry (ITC) (Fig. 4). The ITC titration curves can be fitted to a single broad transition with apparent micromolar affinity ($K_D = 12.5 \mu\text{M}$ and $23.8 \mu\text{M}$) for Val66 and Met66 isoforms respectively. These Zn^{2+} titrations are dominated by large endothermic changes that are qualitatively similar in magnitude for both isoforms ($-T\Delta S = -44 \text{ kJ mol}^{-1}$), consistent with a hydrophobically driven process (Fig. 4A), with unfavorable enthalpic contributions data provided in Table S1 (ESI[†]). However, the specific residue changes observed by NMR (Fig. 2) identify multiple regions of the prod domain that are altered upon Zn^{2+} binding, strongly suggesting that the observed curve reflects an average of disparate sites involving inter- and/or intramolecular prod domain–Zn contacts.

His40 is a critical residue for prod domain multimerization

To better understand how Zn^{2+} binding affects the structure of the Met66 and Val66 prod domains, we mutated His40 to Lys to evaluate impact of Zn^{2+} binding (Fig. 4). We found that substitution of His40Lys entirely prevents Zn^{2+} driven turbidity changes at millimolar Zn^{2+} concentrations (Fig. 4C), indicating that His40 is required for Zn^{2+} mediated intermolecular contacts needed for macroscopic particle formation. The effects of His40Lys substitution were then evaluated by ITC, and reveal a multistate binding process upon addition of Zn^{2+} , not discernable with the native prod domains, due to Zn^{2+} driven aggregate association that obscures interpretation. Both His40Lys mutants Val66 and Met66 prod domains display distinctive biphasic ITC transitions (Fig. 4B).

Fig. 4 BDNF prod domain– Zn^{2+} binding measurement. (A) ITC ZnCl_2 titration profile of Val66 (left panel), Met66 (right panel) monitoring Zn^{2+} binding upon injection of Zn^{2+} aliquots to 30 mM prod domain. The ITC experiments indicate that Zn^{2+} binding affinity to full length Val and Met prod domains yield similar affinities (Table S1, ESI[†]). (B) Whereas the Val–H40K (left panel) and Met–H40K (right panel) mutants reveal underlying differences in Zn^{2+} binding affinity in the absence of His40. Note that Met H40K mutations display no apparent aggregate formation at elevated zinc concentrations, whereas the Met H65K mutation retains aggregation characteristics complicating ITC analysis.

The Met66–His40Lys prod domain displays a strong initial enthalpic transition with 10-fold higher affinity ($K_{d1} = 0.4 \pm 0.2 \mu\text{M}$) at 2 : 1 prod domain : Zn^{2+} stoichiometry ($n_1 = 0.5 \pm 0.08$). This initial transition is followed by a weaker secondary transition ($K_{d2} = 41 \pm 36 \mu\text{M}$). The Val66–His40Lys prod domain displays an initial transition with equimolar prod domain–Zn ($n_1 = 1.1 \pm 0.4$), with lower affinity ($K_{d1} = 4 \pm 6 \mu\text{M}$), and a very weak secondary interaction ($K_{d2} = 347 \pm 565 \mu\text{M}$) (Table S1, ESI[†]). Collectively, these results suggest that the Met66 substitution may alter the conformation impacting prod domain– Zn^{2+} affinity, resulting in a higher affinity Zn^{2+} association with Met66 prod domain compared to the Val66 prod domain.

Zinc binding results in different macroscopic structures

The differences in prod domain Zn^{2+} induced spectral changes and binding affinity prompted us to ask whether Met66 and

Fig. 5 Cryo-EM reveals isoform dependent differences upon Zn^{2+} binding. (A) Cryo-EM image of $20\ \mu\text{M}$ Val in the presence of $200\ \mu\text{M}$ Zn^{2+} , the Zn^{2+} induced precipitate results in formation of uniform particles with a rounded appearance. (B) Identically prepared Met prodomain- Zn^{2+} results in aggregates that appear disordered and of varying size. Scale bar shown in (A and B), represent a $100\ \text{nm}$ length. Representative particles are outlined in (A and B), for calculation of (C) area, (D) perimeter and (E) circularity. (C–E) The Val- Zn^{2+} structures display a greater degree of uniformity in comparison to the Met- Zn^{2+} aggregates, suggesting underlying isoform dependent differences in prodomain- Zn^{2+} interactions.

Val66 prodomain- Zn^{2+} complexes adopt different macroscopic forms. To analyze these structures, we employed cryo-EM imaging of the prodomain- Zn^{2+} complexes. Prodomain assemblies display strikingly different morphological appearances by cryo-EM (Fig. 5A and B). The Val66 multimers appear as uniformly shaped clusters of smaller spherical units. Analysis of Zn^{2+} -Val66 prodomain clusters reveal a similarity in particle size with a high degree of circularity and small perimeter to area ratios indicating a reduced complexity of shape (Fig. 5C–E). The smaller spherical units appear to be less than $10\ \text{nm}$ in diameter, which form larger clusters range from 50 – $70\ \text{nm}$ in size. In contrast, the Zn^{2+} -Met66 prodomain multimers appear as irregularly shaped particulates of widely varying size, with reduced circularity and a larger perimeter/area ratio indicating a greater degree of rugosity. The gross difference in macromolecular morphology between Zn^{2+} -associated Val66 and Met66 prodomain likely underscores differences in the residues of the prodomain exposed upon Zn^{2+} binding.

The His40Lys mutant blocks Met66 prodomain gain of function

Given the dramatic Zn^{2+} -mediated changes in multimerization observed in the Met66 vs. Val66 prodomain and the impact of the His40Lys mutation, we investigated the effect of these proteins on cultured hippocampal neurons. Previous findings indicated that application of the Met66 prodomain but not the Val66 prodomain to mature hippocampal neuron cultures

results in the rapid disassembly of synaptic spines and actively eliminates synapses.^{6,15,34–36} In the prior study, the prodomain were exposed to Zn^{2+} which is present in the cell culture media. To directly test whether Zn^{2+} macromolecular association is required for acute negative remodeling of synaptic spines, we compared the bioactivity of Met66 or Val66 prodomain, to Met66 His40Lys or Val66 His40Lys prodomain that fail to associate into macroscopic structure.

To this end, we added recombinant Met66 His40Lys prodomain ($10\ \text{ng}\ \text{ml}^{-1}$), or Met66 prodomain ($10\ \text{ng}\ \text{ml}^{-1}$) or vehicle control to mature hippocampal neurons cultured in Zn^{2+} containing Neurobasal media (Fig. 6A). We conducted a detailed analysis of the effects of the Met66 prodomain on neuronal morphology, specifically examining mature mushroom spines, thin spines and total spines on dendrites using structured illumination microscopy (SIM). Upon addition of the Met prodomain, we observe a transition of the mature mushroom spines into thin/filopodia like protrusions, as demonstrated by a $\sim 60\%$ decrease in mushroom spines and an equal increase in thin spines within 1 hour, without an overall change in spine density (Fig. 6B–D) and consistent with prior studies.¹⁵ In contrast, no acute morphological or density changes were observed in neurons treated with the His40Lys Met66 prodomain mutants (Fig. 6B). No effects of synaptic remodeling were observed following treatment with the Val66 prodomain, consistent with prior results,^{6,15} or with His40Lys Val66 prodomain. These results suggest that Met66 prodomain bioactivity is dependent on interactions of His40 with Zn^{2+} .

Discussion

Both the BDNF prodomain and Zn^{2+} are secreted into the synaptic cleft in response to repeated stimulation of hippocampal neurons.^{16,37} Both BDNF isoforms (including the prodomain) and Zn^{2+} are regionally restricted within the brain, with chelatable Zn^{2+} levels regulated by the expression of zinc transporters.³⁸ Indeed, BDNF is highly expressed in the hippocampus and amygdala,³⁹ regions that exhibit the highest concentrations of chelatable Zn^{2+} in the central nervous system (CNS).⁴⁰ Prolonged neuronal depolarization induces the release of Zn^{2+} into the synaptic cleft, as well as the release of mature BDNF and the prodomain from dense core vesicles.¹⁶ Stimulated secretion of Zn^{2+} in hippocampal tissues has been calculated to approach concentrations of $30\ \mu\text{M}$ or more in the region of mossy fiber input to the CA1 region of the hippocampus.^{41,42} This concentration of Zn^{2+} , in the presence of secreted prodomain, could be utilized to promote the formation of distinct prodomain oligomeric species.

Our prior work demonstrates that the Met66 prodomain isoform elicits morphological changes in neuronal growth cones and dendritic spines through interactions with the SorCS2 receptor, whereas the Val66 isoform was shown to be inert. Furthermore, the Met66 isoform negatively impacts the circuits associated with memory and anxiety disorders^{43–46} and has recently been implicated as an relevant target in Alzheimer's disease.^{47,48}

Fig. 6 BDNF Met prodomain, but not Met-His40Lys, leads to acute dendritic spine changes. (A) DIV20 neurons, visualized by structured illumination microscopy, using actin binding phalloidin-alexa546 and MAP2. (B) Quantitation of spine number; (C) thin spine phenotype; (D) mushroom phenotype; analyzing the effects of the Met (Met) prodomain vs. the Met-His40Lys (Met 40). Error bars are given for $n = 10$ neurons/condition. $**p < 0.001$ (t test).

Our observation that the Val66 and Met66 prodomains differentially interact with Zn^{2+} at physiological concentrations, together with our findings that the Met66 bioactivity is blocked by selective mutation of His40 points to a potential role for Zn^{2+} binding in Met66 *in vivo* bioactivity.

Recent computational studies of the BDNF prodomain by Lohia *et al.*⁴⁹ evaluated differential intramolecular interactions of the Met66 or Val66 prodomains in the apo state. In their computational modeling study, the Met66 prodomain isoform exhibited an enhanced intramolecular association between Met66 with nonlocal C-terminal residues including Met95 as well as local (Phe63) and nonlocal aromatic residues (Phe108, Tyr113), as compared to the Val66 isoform which displays significantly weaker long-range intramolecular contacts between Val66 and C-terminal residues. Their modeling results are consistent with our observations of the Met66 and Val66 prodomains in the absence of Zn^{2+} . Specifically, NMR analysis of the 66-region and C-terminal regions show evidence of chemical exchange dynamics, as monitored by NMR relaxation measurements. The impact of chemical exchange is significantly more prevalent in the Met66 prodomain as compared to the Val66 prodomain (Fig. S4, ESI†). Furthermore, both prodomain isoforms in the apo state display concentration dependent loss in NMR spectral intensities due to chemical exchange localized in the 66-region and the C-terminal, 111-region. In addition, we observe concentration dependent changes of the apo prodomains by NMR diffusion

indicate a greater degree compaction and concentration dependent intermolecular association of the Met66 prodomain, as compared to the Val66 isoform (Fig. S5, ESI†). Our experimental studies are consistent with Lohia's model of Met66 prodomain interactions where the Met66-region and C-terminal regions enhance intramolecular regional self-association.

The differences in the Met66 and Val66 prodomain Zn^{2+} binding and multimerization are critically dependent upon residue His40. In the apo-state both isoforms display dynamic and concentration dependent changes that primarily effect the 66- and 111-regions of the prodomain, whereas the His40 residues are largely unperturbed. Our prior examination of prodomain-SorCS2 interactions in the apo state indicates that the 66-region and the C-terminal 111-region are the regions involved in SorCS2 binding. The His40 region has shown no prior involvement in binding to SorCS2 or its homologous receptor, Sortilin.^{5,50} The importance of Zn^{2+} mediated oligomerization is highlighted by our mutational studies which indicate that residue His40 is essential for intermolecular macroscopic association in both Val66 and Met66 isoforms. ITC analysis indicates similar Zn^{2+} titration energetics for native Val66 and Met66 prodomains, dominated by entropic contributions (Fig. 4A). However, upon His40Lys mutation, two distinct underlying transitions are revealed. The mutant, His40Lys-Met66 displays an initial enthalpically driven interaction upon Zn^{2+} binding, that is of higher affinity as compared

to His40Lys–Val66. This initial transition is followed by a secondary weaker endothermic transition seen in both isoforms (Fig. 4B). NMR changes upon Zn^{2+} addition that simultaneously occur in both Met66 and C-terminal regions of prodomain. The structure conferred by Met66, enhances Zn^{2+} binding and association with the C-terminal portion of the prodomain in comparison to Val66. Collectively these interactions directly impact prodomain– Zn^{2+} macroscopic structure.

Prodomain– Zn^{2+} binding leads to distinct macroscopic differences between Val66 and Met66 prodomain– Zn^{2+} assemblies (Fig. 5A and B). The Val66 prodomain– Zn^{2+} assemblies adopt more uniformly sized particles in the presence of Zn^{2+} , and dissociate rapidly compared to the Met66 prodomain (Fig. 5A). In contrast, Zn^{2+} binding to Met66 prodomain results in irregular assemblies as shown by cryo-EM (Fig. 5B). Differences upon addition of Zn^{2+} are seen in the greater loss in Met66 NMR signal intensities, surrounding residue 66, as well as a modest increase Met66 helical structure shown by circular dichroism (CD) (Fig. S2, ESI[†]) and a greater Met66– Zn^{2+} dissociation timeframe as assessed by turbidity analysis. We hypothesize that Met66 isoform aggregates can provide multivalent interactions that enable effective SorCS2 clustering to initiate negative synaptic remodeling. It is well established that Zn^{2+} can induce conformational changes in other proteins related to neurodegenerative diseases. Binding to Zn^{2+} structurally impacts a number of neurodegeneration-related proteins leading to oligomerization or protein aggregation *in vitro*; this includes the intrinsically disordered proteins TDP-43,⁵¹ tau,⁵² α -synuclein,⁵³ and A β .^{54–56} Ordered aggregation and assembly events, such as fibrils or repeating high-order assemblies are known to initiate signaling complexes utilized in normal physiological responses.^{28,57} In contrast, misfolded, oligomeric pathogenic and nonpathogenic proteins or even small organic molecules forming colloidal aggregates can provoke interactions that often prove cytotoxic.^{58–61}

Promiscuous interactions in the extracellular spaces could potentially disrupt synaptic signaling and channel proteins. The most likely prodomain interaction in this context is SorCS2, that exists as a dimer, as assessed by X-ray crystallography and negatively stained electron microscopy.⁶² The regions of the prodomain surrounding the Val66Met polymorphism directly interact with SorCS2 in the absence of Zn^{2+} , as previously assessed by NMR using a truncated form of the Met66 or Val66 prodomain.⁶ However, the regions in SorCS2 that interact with the prodomain have not been defined, nor has the mechanism by which SorCS2 interacts with the p75^{NTR} co-receptor, as both receptors are required for growth cone retraction and synaptic remodeling. Analysis of the direct interactions of Zn^{2+} –prodomain oligomers with dimeric SorCS2, and with p75^{NTR}, will require additional experimentation.

Author contributions

J. W., H. B. and A. A. prepared protein for biological and biophysical analysis. J. W. and C. B. performed NMR data collection and analysis. A. A., W. J. R. and C. B. performed EM

data collection and analysis. J. W. and C. B. performed ITC analysis. A. A., J. W., H. B., and D. W. performed CD. measurements. J. W., A. A., D. W. and H. B. performed light scattering analysis. T. N. and J. D., performed mass spectrometry and binding analysis. J. G. performed neuronal SIM analysis. C. B., B. L. H., and F. S. L. supervised analysis and designed experiments. C. B., *et al.* wrote the paper, all co-authors contributed to editing.

Conflicts of interest

The authors declare no competing interests.

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