GRP78 INTERACTION MEDIATES THE NEUROPROTECTIVE EFFECTS OF CDNF AND HER-096

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SUMMARY

Unconventional cerebral dopamine neurotrophic factor (CDNF) and its C-terminal domain (C-CDNF) have been reported to display neuroprotective activity *in vitro* and *in vivo*. HER-096 is a brain-penetrating peptidomimetic derived from the active site of human CDNF protein (located at its C-terminal domain) and is developed as a disease-modifying treatment for Parkinson's disease. HER-096 shows similar potency as CDNF and promotes neuroregeneration by restoring proteostasis and by reducing neuroinflammation in an animal model of synucleinopathy (Kulesskaya et al, 2024). Currently, HER-096 is tested in Parkinson's patients in an ongoing Phase 1b clinical trial. Here we show that CDNF and C-CDNF bind to the nucleotide binding domain (NBD) of glucose regulated protein 78 (GRP78), a key ER chaperone and the master regulator of unfolded protein response (UPR) pathway. Mutagenesis analysis revealed that the neuroprotective activity of C-CDNF and HER-096 depends on the interaction with GRP78 and is mainly mediated via UPR, in particular IRE1α and PERK pathways.

METHODS

Small-angle X-ray scattering (SAXS) and data modelling. CDNF WT (aa 25-187) and GRP78-NBD (aa 26-382) were expressed in *E.coli* and purified by affinity chromatography using HisTag. C-CDNF peptides were synthesized using solid state peptide synthesis. Synchrotron SAXS data were collected on the EMBL P12 beamline at PETRA III (DESY, Hamburg, Germany). The ATSAS program DAMMIF was employed for *ab initio* modeling. The hybrid atomic models were generated based on the GRP78-NBD complex with MANF (6ha7.pdb) and CDNF (4bit.pdb) (Graewert et al 2024).

The analysis of the scattering profile from the main peak of the GRP78-NBD complex with CDNF revealed systematic differences compared to the scattering data of pure GRP78-NBD protein (Figure 1A, pink vs orange). As the log I(s) vs s plots reflect reciprocal space, these small differences translate into differences in the overall size of the sample (Figure 1B). In case of GRP78-NBD with C-CDNF, the *ab initio* model showed a slightly larger envelope in the presence of C-CDNF (Figure 1B, green) (Graewert et al 2024).

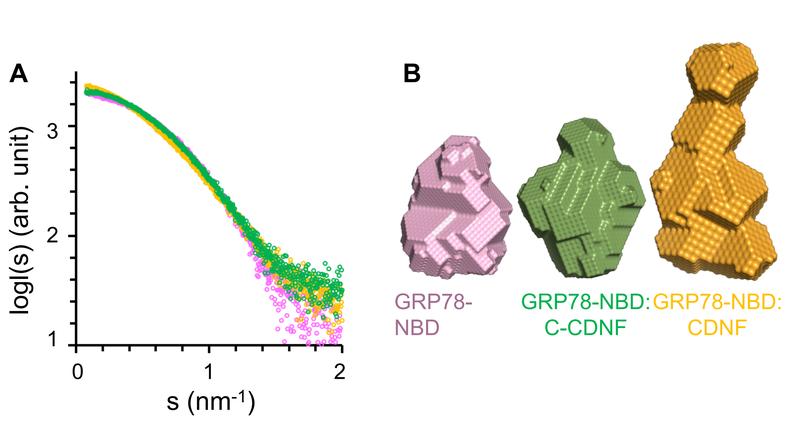

Figure 1. *Solution SAXS results for GRP78-NBD alone and in complex with CDNF or C-CDNF. (**A**) Final SAXS scattering profiles (plotted as log I(s) vs s where s = 4 πsinθ/λ) of the 3 samples. (**B**) Ab initio models generated with Dammif are presented as spheres.*

Binding affinity. Isothermal titration calorimetry (ITC) was used to determine binding affinity of CDNF to GRP78 NBD. Data was collected with MicroCal PEAQ-ITC (Malvern Panalytical, UK). Spectral shift assay was used to determine the binding affinities of C-CDNF and its mutants to GRP78-NBD labelled with fluorescent NT-650-NHS. Samples were analyzed on a Monolith X at 25 °C, with 100% LED power (Table 1) (Graewert et al 2024.

Table 1. The binding data for GRP78-NBD and CDNF or its variants

CDNF variants	Sequence	KD, μM
CDNF (WT)	EECRACAEK[164]	3.3 ±3.72
C-CDNF (WT)	EECRACAEK[164]	0.38 ±0.03
C-CDNF-MT1	EECRACAAA[164]	no interaction
C-CDNF-MT2	AACRACAAK[164]	no interaction
C-CDNF-MT3	EECAACAEA[164]	2.33±0.51

Altered residues are shown in red. Spectral shift assay was used for C-CDNF and its mutants while ITC was used for CDNF.

Immunostaining. Number of TH-positive cells and UPR markers were assessed in rat primary midbrain neuron culture injured with MPP+. Test compounds were applied 4 h before cells were exposed to MPP+ (4 μM) for 48 h. Fixed cells were stained with anti-TH and anti-phospo-IRE1, anti-ATF4 or anti-ATF6 antibodies and secondary antibodies followed by automatic image analysis. Cell surface (cs)GRP78 assessment was done by immunostaining without plasma membrane permeabilization. MPP+ or thapsigargin (Tg) were applied for 15 h to rat mesencephalic culture. Staining with PhenoVue Fluo 555 for plasma membrane was performed before cell fixation with PFA. Then cells were stained with anti-GRP78 and anti-TH antibodies followed by secondary antibodies.

REFERENCES

Graewert et al 2024 *Nat Commun*, 15(1), 8175.

Kulesskaya et al 2024 *Cell Chem Biol*, 31(3), 593-606 e599.

RESULTS

GRP78-CDNF binding interface

The hybrid models for GRP78-NBD with CDNF and C-CDNF were overlayed with SAXS-derived *ab initio* envelopes showing good alignment (Figure 2A and B). According to this model the following CDNF amino acid residues are involved in the salt-bridge formation with GRP78: E156, E163, E173 (Figure 2C). The binding affinities between GRP78-NBD and (C-)CDNF were measured using ITC or spectral shift assays. CDNF and C-CDNF bind to GRP78 with low micromolar or sub-micromolar K_d, respectively (Table 1). C-CDNF mutants with altered residues at the GRP78 interaction interface do not bind or show reduced binding to GRP78 (Table 1) (Graewert et al 2024).

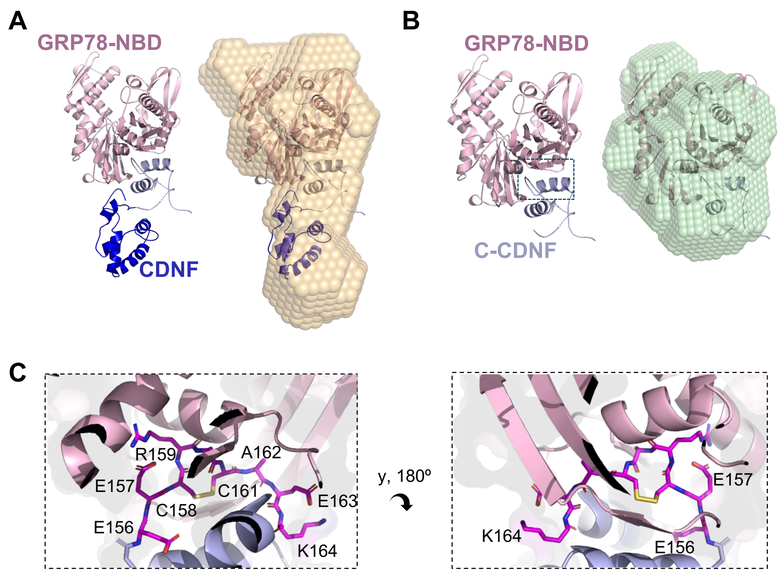

Figure 2. *Hybrid models of GRP78-NBD in complex with CDNF and C-CDNF. (**A**) Hybrid model of CDNF in complex with GRP78-NBD in cartoon representation and its overlay with ab initio reconstruction. (**B**) Hybrid model of C-CDNF in complex with GRP78-NBD in cartoon representation and its overlay with ab initio reconstruction. (**C**) Enlarged view of GRP78-CDNF interaction interface showing the residues that mediate CDNF binding to GRP78.*

Neuroprotection of C-CDNF is mediated via UPR pathway modulation

Treatment with MPP+ leads to activation of UPR pathway signaling in tyrosine hydroxylase (TH)-positive dopamine neurons as assessed by the levels of S724-phosphorylated IRE1 and nuclear ATF4 and ATF6 (Figure 3, red). C-CDNF significantly reduces UPR markers phosphorylated IRE1α and nuclear ATF6 (Figure 3, blue) but C-CDNF mutants MT1 and MT2 which are not able to bind GRP78 show significant loss of this effect (Figure 3, grey and green) (Graewert et al 2024).

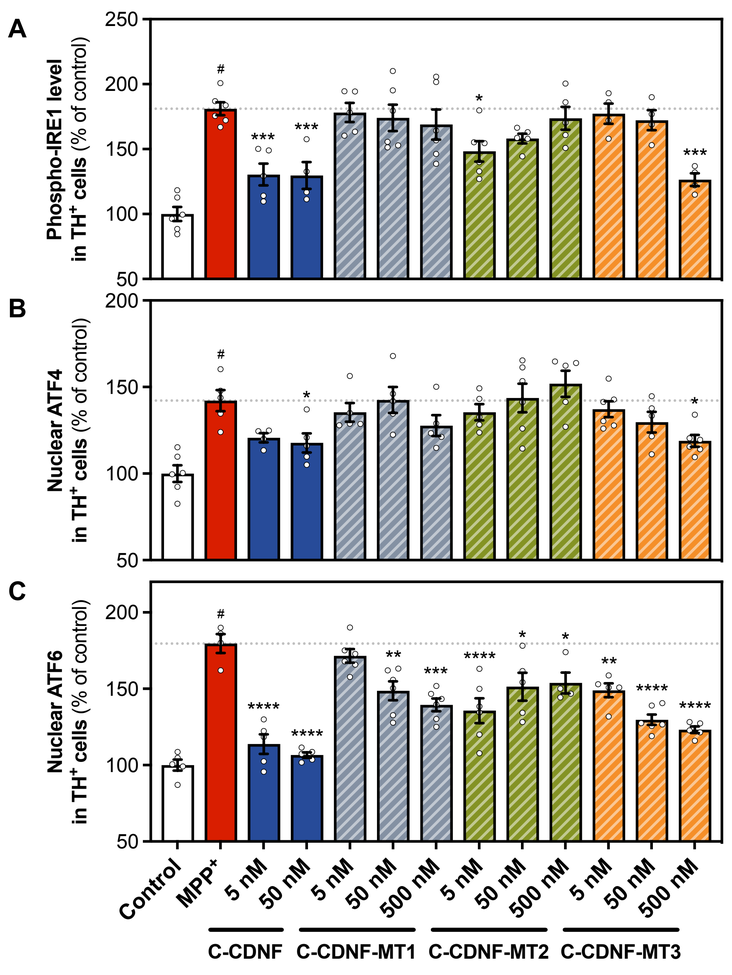

Figure 3. *Effects of WT C-CDNF and its mutants MT1-MT3 on UPR pathway signaling in TH-positive dopamine neurons. (**A**) Effects of WT C-CDNF and its mutants MT1-MT3 on phosphorylated IRE1α. (**B**) Effects of WT C-CDNF and its mutants MT1-MT3 on nucleus-localized ATF4 (PERK signaling). (**C**) Effects of WT C-CDNF and its mutants MT1-MT3 on nucleus-localized ATF6. Different C-CDNF and its mutant concentrations were tested in the background of MPP+. One-way ANOVA with Dunett's test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs MPP+ treated cells; #p<0.0001 MPP+ vs healthy treated cells.*

Neuroprotective activity of C-CDNF depends on GRP78 binding

Treatment with MPP+ results in reduction of TH-positive dopamine neuron survival (Figure 4, red). C-CDNF rescues TH-positive cell survival (Figure 4, blue) but C-CDNF mutants MT1 and MT2 which are not able to bind GRP78 have lost this activity (Figure 3, grey and green). The C-CDNF mutant MT3 showing reduced binding to GRP78 also shows reduced neuroprotective activity (Figure 4, orange) (Graewert et al 2024).

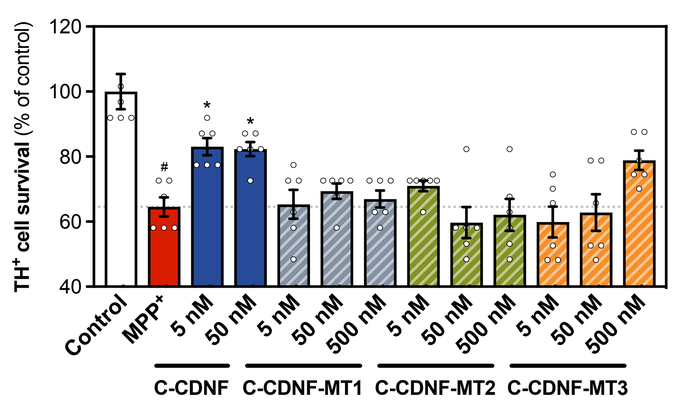

Figure 4. *Effects of WT C-CDNF and its mutants MT1-MT3 on TH-positive dopamine neuron survival in primary rat mesencephalic cells after MPP+ injury. Different C-CDNF and its mutant concentrations were tested in the background of MPP+. Statistics as in Figure 3.*

HER-096 mutants have reduced neuroprotective activity

HER-096 mutants CP-C-187 and CP-C-188 the sequences corresponding to C-CDNF mutants MT1 and MT2, respectively, show reduced neuroprotective activity in primary rat mesencephalic cell culture (Figure 5).

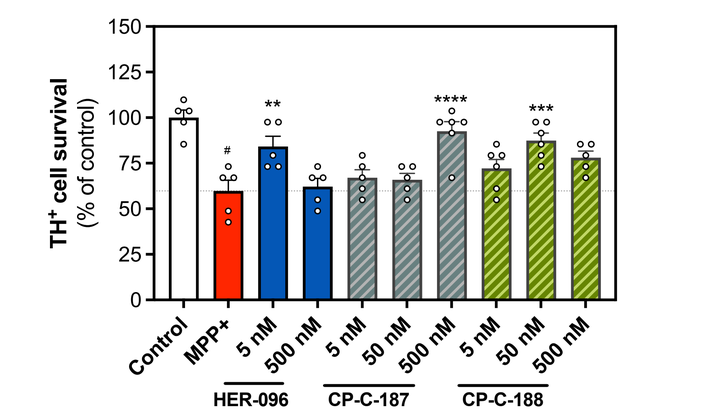

Figure 5. *Effect of HER-096, CP-C-187 and CP-C-188 on TH-positive dopamine neuron survival in primary rat mesencephalic cells after MPP+ injury. Different HER-096 and its mutant concentrations were tested in the background of MPP+. Statistics as in Figure 3.*

csGRP78 expression is enhanced in mesencephalic culture after stress

We tested GRP78 translocation to the cell surface in the primary culture of rat mesencephalic neurons with and without stressors showing that GRP78 is expressed on the surface of TH-positive dopamine neurons in unstressed condition. The csGRP78 expression is enhanced after MPP+ or thapsigargin application for 15 h (Figure 6). This opens the possibility of CDNF or HER-096 binding to GRP78 at the cell surface leading to the downstream signaling events, and/or internalization.

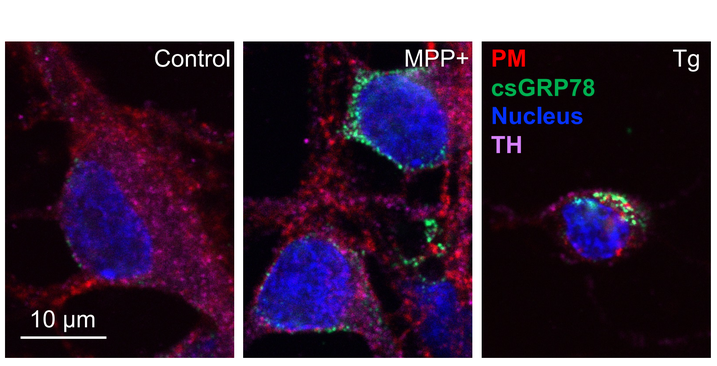

Figure 6. *Confocal images of **unpermeabilized** primary rat mesencephalic cells to assess cell surface GRP78 (csGRP78) on TH-positive cells. Plasma membrane, red; csGRP78, green; TH, magenta. Scale bar, 10 μm.*

CONCLUSIONS

UPR pathway is a key mediator of the neuroprotective activity of C-CDNF where interaction with GRP78 plays a central role. HER-096 is designed based on the GRP78-binding interface and mimics the cytoprotective effects of C-CDNF.

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