Supplementary Materials: Characterization, Stability, and in Vivo Efficacy Studies of Recombinant Human CNTF and its Permeation into the Neural Retina in ex Vivo Organotypic Retinal Explant Culture Models

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Protein expression and purification

For rhCNTF protein production, Rosetta 2(DE3)pLysS (Novagen) E. coli cells were transformed with expression plasmid pOPINF-hCNTF and the expression carried out as described earlier (Itkonen et al., 2014). The subsequent purification of soluble rhCNTF from clarified cell lysates was carried out under native conditions by batch-mode immobilized metal-ion affinity chromatography (IMAC) with Protino® nickel iminodiacetic acid (Ni-IDA) resin (Macherey Nagel, Germany). Detected with SDS-PAGE analysis, the imidazole-eluted fractions containing rhCNTF were pooled, buffer exchanged and concentrated before final purification with size-exclusion chromatography (SEC). Pooled protein was loaded onto a Superdex 200 prep grade-packed C16/40 column and the elution, collection and handling of protein fractions carried out as described previously (Itkonen et al., 2014). Purified protein was kept either on ice at 4 °C or snap-frozen with liquid N₂ for storage at -80 °C.
### Table S1. Layout of the ThermoFluor buffer, salt, and pH screen.

|   | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   |
|---|------|------|------|------|------|------|------|------|------|------|------|------|
|   | Na Acetate | Na Acetate | Na Acetate | Na Acetate | HEPES | HEPES | HEPES | HEPES | Na Acetate | Na Acetate | Na Acetate | Na Acetate |
| A | pH 3.5 | 50 mM NaCl | pH 3.5 | 150 mM NaCl | pH 3.5 | 500 mM NaCl | pH 3.5 | 150 mM NaCl | pH 3.5 | 500 mM NaCl | pH 3.5 | 150 mM NaCl | pH 3.5 |
|   | Citric Acid | Citric Acid | Citric Acid | Citric Acid | Imidazole | Imidazole | Imidazole | Imidazole | Ammonium Acetate | Ammonium Acetate | Ammonium Acetate | Ammonium Acetate |
| B | pH 4.0 | 50 mM NaCl | pH 4.0 | 150 mM NaCl | pH 4.0 | 500 mM NaCl | pH 4.0 | 150 mM NaCl | pH 4.0 | 500 mM NaCl | pH 4.0 | 150 mM NaCl | pH 4.0 |
|   | Ammonium Acetate | Ammonium Acetate | Ammonium Acetate | Ammonium Acetate | Na-K Phosphate | Na-K Phosphate | Na-K Phosphate | Na-K Phosphate | Na-K Phosphate | Na-K Phosphate | Na-K Phosphate | Na-K Phosphate |
| C | pH 4.5 | 50 mM NaCl | pH 4.5 | 150 mM NaCl | pH 4.5 | 500 mM NaCl | pH 4.5 | 150 mM NaCl | pH 4.5 | 500 mM NaCl | pH 4.5 | 150 mM NaCl | pH 4.5 |
|   | Na Citrate | Na Citrate | Na Citrate | Na Citrate | Bicine | Bicine | Bicine | Bicine | MES | MES | MES | MES |
| D | pH 5.0 | 50 mM NaCl | pH 5.0 | 150 mM NaCl | pH 5.0 | 500 mM NaCl | pH 5.0 | 150 mM NaCl | pH 5.0 | 500 mM NaCl | pH 5.0 | 150 mM NaCl | pH 5.0 |
|   | Na Citrate | Na Citrate | Na Citrate | Na Citrate | CHES | CHES | CHES | CHES | Na Phosphate | Na Phosphate | Na Phosphate | Na Phosphate |
| E | pH 5.5 | 50 mM NaCl | pH 5.5 | 150 mM NaCl | pH 5.5 | 500 mM NaCl | pH 5.5 | 150 mM NaCl | pH 5.5 | 500 mM NaCl | pH 5.5 | 150 mM NaCl | pH 5.5 |
|   | MES | MES | MES | MES | CAPS | CAPS | CAPS | CAPS | Triethanolamine | Triethanolamine | Na-K Phosphate | Na-K Phosphate |
| F | pH 6.0 | 50 mM NaCl | pH 6.0 | 150 mM NaCl | pH 6.0 | 500 mM NaCl | pH 6.0 | 150 mM NaCl | pH 6.0 | 500 mM NaCl | pH 6.0 | 150 mM NaCl | pH 6.0 |
|   | MES | MES | MES | MES | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate |
| G | pH 6.5 | 50 mM NaCl | pH 6.5 | 150 mM NaCl | pH 6.5 | 500 mM NaCl | pH 6.5 | 150 mM NaCl | pH 6.5 | 500 mM NaCl | pH 6.5 | 150 mM NaCl | pH 6.5 |
| H | HEPES | HEPES | HEPES | HEPES | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate | Na Acetate |
| I | pH 7.0 | 50 mM NaCl | pH 7.0 | 150 mM NaCl | pH 7.0 | 500 mM NaCl | pH 7.0 | 150 mM NaCl | pH 7.0 | 500 mM NaCl | pH 7.0 | 150 mM NaCl | pH 7.0 |

All buffers used at 100 mM concentration.
Table S2. Tissue processing procedure.

| Solvent      | Time     |
|--------------|----------|
| Tap water    | 10 minutes |
| 80% ethanol  | 30 minutes |
| 94% ethanol  | 30 minutes |
| 94% ethanol  | 30 minutes |
| 99% ethanol  | 45 minutes |
| 99% ethanol  | 30 minutes |
| Xylene       | 20 minutes |
| Xylene       | 20 minutes |
| Liquid paraffin | 1 hour    |
| Liquid paraffin | 1 to 24 hours |

Table S3. H&E staining protocol.

| Reagent                      | Time         |
|------------------------------|--------------|
| Xylene                       | 2 x 5 minutes|
| 100 % ethanol                | 2 x 2 minutes|
| 94 % ethanol                 | 2 x 2 minutes|
| Rinsing with distilled water | 20 seconds   |
| Delafield hematoxylin        | 9 minutes    |
| Rinsing with tap water       | 5 minutes    |
| 1 % HCl in 70% ethanol       | 4-5 seconds  |
| Rinsing with tap water       | 10 minutes   |
| 1 % eosin                    | 30 seconds   |
| 94 % ethanol                 | 2 x 2 minutes|
| 100 % ethanol                | 2 x 2 minutes|
| Xylene                       | 2 x 5 minutes|

Xylene (BDH Prolabo, VWR Chemicals, France), and 100% and 94% ethanol (Altia Oyj, Finland) were purchased from manufacturers. Delafield hematoxylin, 1% HCl in 70% ethanol and 1% eosin were prepared in the University of Eastern Finland. Prior staining, the 1% eosin was filtered and 1 % of glacial acetic acid (BDH Prolabo, VWR Chemicals, France) was added.

Immunofluorescence staining of rat retinal explants

Immunofluorescence staining was carried out to assess the localization of labeled rhCNTF. Slides with fixed sections of retinal tissue were washed 3 times with PBS followed by incubation with blocking buffer (10% goat serum, 1% BSA, 0.5% Triton-X) for 1 h at room temperature. Next, samples were incubated with primary polyclonal antibody against Iba-1 (1:200, FUJIFILM Wako Chemicals U.S.A. Corp), a specific microglial marker, diluted in IHC antibody buffer (3% goat serum, 1% BSA, 0.5% Triton-X) overnight at 4 °C. Next day, the slides were washed 3 times for 5 min with PBS and incubated with Alexa Fluor™ 568-conjugated secondary antibody (1:350, Thermo Fisher Scientific) diluted in IHC antibody buffer for 1 h at room temperature. Further, to visualize cell nuclei, counterstaining was carried out by incubating the slides in 4’,6-diamidino-2-pphenylindole (DAPI) diluted in PBS (1:5000) for 5 min. Finally, samples were washed 3 times for 5 min with PBS, dried out, embedded with Fluoromount-G™ antifade reagent (Thermo Fisher Scientific), and closed with cover glasses.
**Table S4.** Heat map of rhCNTF Ts measured in ThermoFluor screen.

Cells with underlined results indicate buffers chosen for further studies.

|    | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   |
|----|------|------|------|------|------|------|------|------|------|------|------|------|
| A  | 37.3 | 27.4 | 26.3 | 26.3 | 54.2 | 54.2 | 56.2 | 57.2 | 53.2 | 54.2 | 55.2 | 57.2 |
| B  | 45.2 | 45.2 | 45.2 | 41.2 | 52.2 | 53.2 | 54.2 | 59.3 | 52.2 | 54.2 | 54.2 | 55.2 |
| C  | 43.2 | 41.2 | 41.2 | 43.2 | 53.2 | 56.2 | 55.2 | 57.2 | 53.2 | 54.2 | 56.2 | 56.2 |
| D  | 53.2 | 54.2 | 52.2 | 51.2 | 51.2 | 52.2 | 54.2 | 56.2 | 52.2 | 53.2 | 55.2 | 59.2 |
| E  | 58.2 | 57.2 | 56.2 | 53.2 | 52.2 | 51.2 | 52.2 | 54.2 | 56.2 | *    | 50.2 | 52.2 |
| F  | 50.2 | 50.2 | 51.2 | 52.2 | 38.3 | 43.2 | 43.2 | 45.2 | 54.2 | 56.2 | 56.2 | 57.2 |
| G  | 51.2 | 52.2 | 53.2 | 55.2 | 42.2 | 42.2 | 43.2 | 43.2 | *    | 50.2 | 56.2 | 58.2 |
| H  | 52.2 | 53.2 | 54.2 | 56.2 | 46.2 | 47.2 | 47.2 | 46.2 | 58.2 | 56.2 | 53.2 | 54.2 |

Blue corresponds to the lowest Ts and red to the highest Ts.

*No discernible peak on derivative plot, no Ts determined.*
|                        | Buffer M |                 | Buffer C |                 |
|------------------------|----------|-----------------|----------|-----------------|
| **Days post-purification** | 2       | 14              | 28       | 48              | 52       |
| **Size**               |          |                 |          |                 |          |
| Monomer/dimer          | Rₘ ± SD (nm) | 3.44 ± 0.21    | 3.45 ± 0.11 | 3.52 ± 0.53    | 3.83 ± 0.25 | 2.86 ± 0.18 |
| Peak PdI              |          | 0.02           | 0.07     | 0.04            | 0.11     | 0.02       |
| HMW oligomers          | Rₘ ± SD (nm) | N/A            | 66 ± 24  | 129 ± 87        | 31 ± 0   | 48 ± 33    |
| Relative % of HMW      |          |                 |          |                 |          |
| By intensity distribution | N/A     | 22.6 ± 2.5  | 11.8 ± 9.5 | 19.9 ± 0.0     | 45.1 ± 28.2 |
| By volume distribution |          | < 0.7         | < 0.6    | 0               | < 0.2    |
| HMW oligomers          |          |                 |          |                 |          |

HMW oligomers Rₘ 30-200 nm.
| Table S6. Rh estimation of rhCNTF stored at -80°C. |
|-------------------------------------------------|

**A) Thawed sample; unmixed and uncentrifuged**

| Days post-thaw | 12 | 25 | 26 | 45 | 46 |
|----------------|----|----|----|----|----|
| **Size**       |    |    |    |    |    |
| Monomer/dimer  | Rs ± SD (nm) | 2.69 ± 0.09 | 3.82 ± 0.22 | 3.43 ± 0.33 | 2.68 ± 0.53 | 4.04 ± 0.02 |
|                | Peak PdI  | 0.05   | 0.06  | 0.04  | 0.11  | 0.06   |
| HMW oligomers  | Rs ± SD (nm) | 91.0 ± 16 | 57.0 ± 42 | 95 ± 21 | 79 ± 48 | 92 ± 49 |

Relative % of HMW

| By intensity distribution | 14.5 ± 4.2 | 12.3 ± 3.4 | 15.4 ± 0.4 | 57.0 ± 27.8 | 11.5 ± 1.0 |
| By volume distribution   | 0           | <0.3       | <0.8       | <0.1        | 0           |

**B) Thawed sample; centrifugally cleared supernatant**

| Days post-thaw | 10 | 12 | 24 | 25 | 26 | 45 | 46 |
|----------------|----|----|----|----|----|----|----|
| **Size**       |    |    |    |    |    |    |    |
| Monomer/dimer  | Rs ± SD (nm) | 2.55 ± 0.25 | 3.56 ± 0.30 | 3.63 ± 0.20 | 2.62 ± 0.39 | 3.87 ± 0.25 |
|                | Peak PdI  | 0.12   | 0.09  | 0.06  | 0.09  | 0.09  |
| HMW oligomers  | Rs ± SD (nm) | N/A   | N/A   | 113 ± 0 | 89 ± 17 | 114 ± 0 |

Relative % of HMW

| By intensity distribution | N/A | 17.2 ± 0.0 | 5.8 ± 0.0 | 34.7 ± 9.4 | 6.2 ± 0.0 |
| By volume distribution   | 0    | 0          | 0        | 0         | 0       |

**A) Thawed sample; unmixed and uncentrifuged**

| Days post-thaw | 10 | 12 | 24 | 25 | 26 | 45 | 46 |
|----------------|----|----|----|----|----|----|----|
| **Size**       |    |    |    |    |    |    |    |
| Monomer/dimer  | Rs ± SD (nm) | 3.02 ± 0.37 | 3.17 ± 0.17 | 2.66 ± 0.25 | 3.33 ± 0.44 | 2.92 ± 0.05 | 3.18 ± 0.16 | 3.19 ± 0.16 |
|                | Peak PdI  | 0.05   | 0.01  | 0.09  | 0.07  | 0.03  | 0.04  | 0.03   |
| HMW oligomers  | Rs ± SD (nm) | 113 ± 78 | 162 ± 1 | 143 ± 45 | N/A   | 95 ± 21 | 118 ± 64 | 109 ± 34 |

Relative % of HMW

| By intensity distribution | 12.7 ± 4.4 | 19.6 ± 12.2 | 16.5 ± 4.3 | N/A | 15.4 ± 0.4 | 20.2 ± 3.2 | 18.3 ± 5.3 |
| By volume distribution   | 0           | 0           | 0         | 0   | 0           | 0           | 0          |

**B) Thawed sample; centrifuged cleared supernatant**

| Days post-thaw | 10 | 12 | 24 | 25 | 26 | 45 | 46 |
|----------------|----|----|----|----|----|----|----|
| **Size**       |    |    |    |    |    |    |    |
| Monomer/dimer  | Rs ± SD (nm) | 2.89 ± 0.4 | 3.02 ± 0.04 | 2.84 ± 0.14 | 2.92 ± 0.11 | 3.27 ± 0.05 | 3.08 ± 0.31 | 3.33 ± 0.33 |
|                | Peak PdI  | 0.08   | 0.03  | 0.04  | 0.09  | 0.02  | 0.04  | 0.07   |
| HMW oligomers  | Rs ± SD (nm) | N/A   | 99 ± 70 | 80 ± 58 | N/A   | 81 ± 4 | 109 ± 54 | N/A   |

Relative % of HMW
By intensity distribution

|        | N/A | 16.0 ± 6.6 | 15.2 ± 3.7 | N/A | 32.3 ± 3.5 | 18.9 ± 9.6 | N/A |
|--------|-----|-------------|-------------|-----|-------------|-------------|-----|

By volume distribution

|        | 0   | < 0.1       | 0           | 0   | 0           | 0           | 0   |

HMW oligomers R = 30-200 nm.

Figure S1. Mean and SD of recorded scotopic α and β wave amplitudes in the 2nd study set 1 week after intravitreal injection (n=6).
Figure S2. Mean and SD of recorded scotopic α and β wave amplitudes in the 2nd study set 2 weeks after intravitreal injection (n=6).
Figure S3. Left eye α wave distribution 1 week post-injection, scotopic 0.05 cd × s/m² ERG. The α wave amplitudes were significantly lower in the MES treated group than in the NControl group (Kruskal-Wallis test, p = 0.016). The NControl group α wave values did not statistically significantly differ from the 1 µg CNTF treated group (Kruskal-Wallis test, p = 0.411).

Figure S4. Left eye β wave distribution 1 week post-injection, scotopic 0.5 cd × s/m² ERG. Left eye β wave amplitudes were significantly higher in the MES treated group than in 1 µg CNTF treated group (Kruskal-Wallis test, p = 0.018) and NControl group (p = 0.025). However, the 1 µg CNTF treated group and the NControl group did not show any statistically significant difference between their mean values (p = 0.831).
**Figure S5.** Left eye β wave distribution 2 weeks post-injection, photopic $1 \text{ cd} \times s/m^2$ ERG. Left eye β wave amplitudes were significantly higher in the NControl group than in MES treated group (Kruskal-Wallis test, $p = 0.007$). The difference between 1 µg CNTF group and MES group was not statistically significant ($p = 0.058$).

**Figure S6.** rhCNTF penetration in bovine retinal explant. NT-647 labeled rhCNTF (red) readily penetrates into the retina after apical administration, with fluorescence observed in the neural retina in layers ranging from GCL to OPL, and even the ONL. ILM, inner limiting membrane; GCL, ganglion cell layer; ILM, inner limiting membrane; IPL, Inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.
Calculations of intravitreal half-life of CNTF based on $R_h$

Based on the comprehensive collection of the intravitreal pharmacokinetic parameters of volume of distribution ($V_{ss,ivt}$) and clearance ($CL_{ivt}$) of intravitreal biologicals in rabbit and human eye (del Amo et al., 2015; del Amo & Urtti 2016) the following calculations were done:

1. A linear correlation between $R_h$ (Shatz et al., 2016) and $CL_{ivt}$ that allowed the calculation of rabbit $CL_{ivt}$ CNTF. Based on its $R_h$ of 2.95 nm and 3.32 nm, rabbit $CL_{ivt}$ CNTF are expected to be 0.025 ml/h and 0.024 ml/h respectively.

2. Human $V_{ss,ivt}$ and $CL_{ivt}$ approximates four and two times the rabbit $V_{ss,ivt}$ and $CL_{ivt}$ respectively. Therefore, human CNTF $CL_{ivt}$ are predicted to be 0.050 ml/h and 0.047 ml/h for each $R_h$ which correspond to half-lives of 4.68 and 4.97 days (based on the average rabbit $V_{ss,ivt}$ of 2.04 ml for macromolecules, the expected human $V_{ss,ivt}$ is 8.16 ml).

References

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