

A FUSION PROTEIN

5 RELATED APPLICATION DATA

The present application claims priority from Australian Patent Application No. 2023902574 filed on 14 August 2023 entitled "A Fusion Protein". The entire contents of which is hereby incorporated by reference.

10 SEQUENCE LISTING

The present application is filed with a Sequence Listing in electronic form. The entire contents of the Sequence Listing are hereby incorporated by reference.

FIELD

15 The present disclosure relates to a fusion protein comprising a neurotrophin and a monomeric Ig Fc domain or fragment thereof, and methods of manufacturing the same.

BACKGROUND

20 Neurotrophins are a family of four structurally and functionally related proteins that regulate the growth, maintenance and apoptosis of neurons in the developing nervous system as well as injured neurons. In mammals, there are four neurotrophins, namely, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) with the latter also known as neurotrophin 5 (NT-4/5).

25 Many neurotrophins are currently in clinical trials and have potential for the treatment of various diseases and disorders. However, due to their low concentrations in mammalian tissues, isolation of neurotrophins from natural sources is technically difficult, expensive, and impractical. Recently, efforts have centred on the development of efficient methods for the production of neurotrophins.

30 Mammalian expression systems produce low yields of neurotrophins, are labour and reagent intensive, and as a result are not realistic for scale-up. The *Escherichia coli* expression system does not possess the ability to produce disulfide bonds present in neurotrophins. Thus, the neurotrophins are often incorrectly folded and have a tendency to aggregate in inactive complexes, requiring extensive downstream processing to realise a biologically active dimer. Yeast can offer a high degree of initial purity; however, the yields are low and the expressed
35 neurotrophins may be partially inactive.

It will therefore be apparent to the skilled person that there is a need in the art for improved methods of manufacturing neurotrophins.

SUMMARY

In work leading up to the invention, the inventors sought to provide an improved method of manufacturing a neurotrophin, such that the neurotrophin would: (i) have improved expression levels (e.g., high yield), and/or (ii) be capable of forming a homodimer, and/or (iii) have improved biological activity, and/or (iv) have increased receptor binding affinity. To this end, the inventors surprisingly identified a fusion protein that enables the manufacture of a neurotrophin with improved expression levels and high yields in mammalian cells. Advantageously, the fusion protein is capable of forming a homodimer with improved biological activity and/or improved binding affinity.

Based on the foregoing, the present disclosure provides a fusion protein comprising (i) a neurotrophin; and (ii) a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.

The present disclosure provides a fusion protein comprising:

(i) a neurotrophin; and

(ii) a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.

In one example, the neurotrophin forms a homodimer.

In one example, the fusion protein has improved expression levels, relative to a wild-type or unmodified neurotrophin.

In one example, the fusion protein is characterised by increased activation of a tyrosine receptor kinase (Trk) receptor compared to a wild-type neurotrophin.

In one example, the Trk receptor is a TrkA receptor, a TrkB receptor, a TrkC receptor or a combination thereof. For example, the Trk receptor is a TrkA receptor. In another example, the Trk receptor is a TrkB receptor. In a further example, the Trk receptor is a TrkC receptor.

In one example, the fusion protein is characterised by increased activation of a TrkA receptor compared to a wild-type neurotrophin. In one example, fusion protein is characterised by increased activation of a TrkB receptor compared to a wild-type neurotrophin. In one example, fusion protein is characterised by increased activation of a TrkC receptor compared to a wild-type neurotrophin.

In one example, the fusion protein binds to the TrkA receptor at neutral pH with an affinity constant (K_D) of between 400 to 800 nM.

In one example, the fusion protein binds to the TrkB receptor at neutral pH with an affinity constant (K_D) of between 20 to 60 nM.

In one example, the fusion protein binds to the TrkC receptor at neutral pH with an affinity constant (K_D) of between 0 to 5 nM.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions that reduces dimerization with another monomeric Ig Fc domain or fragment thereof. In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions that inhibits dimerization with another monomeric Ig Fc domain or fragment thereof.

In some examples, the monomeric Ig Fc domain or fragment thereof comprises a substitution at one or more positions selected from the group consisting of 234, 235, 237 and 329 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system. In one example, the monomeric Ig Fc domain or fragment thereof comprises a substitution at 234 and a substitution at position 235 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system. In one example, the monomeric Ig Fc domain or fragment thereof comprises a substitution at position 237 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system. In one example, the monomeric Ig Fc domain or fragment thereof comprises a substitution at position 329 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system. In some examples, the monomeric Ig Fc domain or fragment thereof comprises a substitution at position 234, a substitution at position 235, a substitution at position 237 and a substitution at position 329 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system.

In some examples, the monomeric Ig Fc domain or fragment thereof comprises at least one substitution selected from the group consisting of L234A, L235A, G237A and P329A relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions selected from the group consisting of:

- (i) leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system;
- (ii) leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system;
- (iii) glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system; and
- (iv) proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 and leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system. In one example, the monomeric Ig Fc domain or fragment thereof comprises a glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system. In one example, the monomeric Ig Fc domain or fragment thereof comprises a proline substituted with glycine

at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises:

- (i) leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system;
- (ii) leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system;
- (iii) glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system; and
- (iv) proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site that reduces dimerization with another monomeric Ig Fc domain or fragment thereof. In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site that inhibits dimerization with another monomeric Ig Fc domain or fragment thereof.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions and at least one N-glycosylation site that reduces dimerization with another monomeric Ig Fc domain or fragment thereof. In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions and at least one N-glycosylation site that inhibits dimerization with another monomeric Ig Fc domain or fragment thereof.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site in a CH3 domain.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site:

- (i) at a position corresponding to amino acid 364 of SEQ ID NO: 10 according to the EU numbering system; or
- (ii) at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least two N-glycosylation sites:

- (i) at a position corresponding to amino acid 364 of SEQ ID NO: 10 according to the EU numbering system; and
- (ii) at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the N-terminus of the monomeric Ig Fc domain or fragment thereof is linked to the C-terminus of the neurotrophin. In one example, the C-terminus of the monomeric

Ig Fc domain or fragment thereof is linked to the N-terminus of the neurotrophin. In one example, the N-terminus of the monomeric Ig Fc domain or fragment thereof is linked by a linker to the C-terminus of the neurotrophin. In one example, the C-terminus of the monomeric Ig Fc domain or fragment thereof is linked by a linker to the N-terminus of the neurotrophin.

5 In one example, the linker comprises a serine.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a deletion of positions corresponding to amino acids 1 to 233 of SEQ ID NO: 10 according to the EU numbering system; a leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system; a leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system; and a serine inserted between positions corresponding to amino acid 235 and 236 of SEQ ID NO: 10.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a deletion of positions corresponding to amino acids 1 to 233 of SEQ ID NO: 10 according to the EU numbering system; a leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system; a leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system; a serine inserted between positions corresponding to amino acid 235 and 236 of SEQ ID NO: 10; and a proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a deletion of positions corresponding to amino acids 1 to 235 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a deletion of positions corresponding to amino acids 1 to 235 of SEQ ID NO: 10 according to the EU numbering system; and glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a deletion of positions corresponding to amino acids 1 to 235 of SEQ ID NO: 10 according to the EU numbering system; and proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the neurotrophin is selected from the group consisting of brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4).

35 In one example, the neurotrophin is NT-3. For example, the NT-3 is a human NT-3. In one example, the human NT-3 comprises a sequence set forth in SEQ ID NO: 1.

In one example, the neurotrophin is NT-4. For example, the neurotrophin-4 is a human NT-4. In one example, the human NT-4 comprises a sequence set forth in SEQ ID NO: 4.

In one example, the neurotrophin is BDNF. For example, the BDNF is a human BDNF. In one example, the human BDNF comprises a sequence set forth in SEQ ID NO: 6.

In one example, the neurotrophin is NGF. For example, the NGF is a human NGF. In one example, the human NGF comprises a sequence set forth in SEQ ID NO: 8.

5 In one example, the monomeric Ig Fc domain or fragment thereof comprises a sequence set forth in SEQ ID NO: 2.

In one example, the monomeric Ig Fc domain or fragment thereof comprises a sequence set forth in SEQ ID NO: 35. In one example, the monomeric Ig Fc domain or fragment thereof comprises a sequence set forth in SEQ ID NO: 36. In one example, the monomeric Ig Fc domain or fragment thereof comprises a sequence set forth in SEQ ID NO: 37. In one example, the monomeric Ig Fc domain or fragment thereof comprises a sequence set forth in SEQ ID NO: 38. In one example, the monomeric Ig Fc domain or fragment thereof comprises a consensus sequence comprising a sequence set forth in SEQ ID NO: 39.

10 In one example, the fusion protein comprises a neurotrophin and a sequence set forth in SEQ ID NO: 35. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 36. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 37. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 38. In one example, the fusion protein comprises a consensus sequence comprising a sequence set forth in SEQ ID NO: 39.

20 In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 2. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 6 and a sequence set forth in SED ID NO: 2. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 8 and a sequence set forth in SED ID NO: 2. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 4 and a sequence set forth in SED ID NO: 2.

25 In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 35. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 6 and a sequence set forth in SED ID NO: 35. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 8 and a sequence set forth in SED ID NO: 35. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 4 and a sequence set forth in SED ID NO: 35.

30 In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 36. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 6 and a sequence set forth in SED ID NO: 36. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 8 and a sequence set forth in SED ID NO: 36. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 4 and a sequence set forth in SED ID NO: 36.

In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 37. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 6 and a sequence set forth in SED ID NO: 37. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 8 and a sequence set forth in SED ID NO: 37. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 4 and a sequence set forth in SED ID NO: 37.

In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 38. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 6 and a sequence set forth in SED ID NO: 38. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 8 and a sequence set forth in SED ID NO: 38. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 4 and a sequence set forth in SED ID NO: 38.

In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 1 and a consensus sequence comprising a sequence set forth in SEQ ID NO: 39. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 6 and a consensus sequence comprising a sequence set forth in SEQ ID NO: 39. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 8 and a consensus sequence comprising a sequence set forth in SEQ ID NO: 39. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 4 and a consensus sequence comprising a sequence set forth in SEQ ID NO: 39.

In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 21. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 23. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 24. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 25. In one example, the fusion protein comprises a sequence set forth in SEQ ID NO: 26. In one example, the fusion protein has a consensus sequence comprising a sequence set forth in SEQ ID NO: 28.

The present disclosure also provides a nucleic acid encoding or expressing a fusion protein disclosed herein.

In one example, the nucleic acid encoding the fusion protein is a DNA sequence comprising a sequence set forth in SEQ ID NO: 15. In one example, the nucleic acid encoding the fusion protein is a DNA sequence comprising a sequence set forth in SEQ ID NO: 17.

The present disclosure also provides nucleic acid encoding or expressing a proneurotrophin and a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.

The present disclosure also provides an expression construct comprising a nucleic acid disclosed herein.

In one example, the expression construct comprises a sequence forth in SEQ ID NO: 18. In one example, the expression construct comprises a sequence forth in SEQ ID NO: 20.

In one example, the expression construct comprises a sequence forth in SEQ ID NO: 12. In one example, the expression construct comprises a sequence forth in SEQ ID NO: 14.

The present disclosure also provides a host cell comprising a fusion protein disclosed herein, or expressing a nucleic acid disclosed herein or an expression construct disclosed
5 herein.

In one example, the host cell is a mammalian cell. For example, the mammalian cell is selected from the group consisting of a HEK cell, a CHO cell, a BHK cell, a MDCK cell, a C3H 10T1/2 cell, a FLY I, a Psi-2 cell, a BOSC 23 cell, a PA317 cell, a WEHI cell, a COS cell, a BSC 1 cell, a BSC 40 cell, a BMT 10 cell, a VERO cell, a W138 cell, a MRC5 cell, a
10 A549 cell, a HT1080 cell, a B-50 cell, a 3T3 cell, a NIH3T3 cell, a HepG2 cell, a Saos-2 cell, a Huh7 cell, a HeLa cell, a W163 cell, a 211 cell, a 211 A cell, and derivatives thereof.

In one example, the mammalian cell is a HEK cell. For example, the mammalian cell is a HEK293 cell.

In another example, the mammalian cell is a CHO cell. For example, the mammalian
15 cell is a ExpiCHO cell. In another example, the mammalian cell is a CHOK1 cell.

The present disclosure also provides a method of manufacturing a fusion protein disclosed herein, the method comprising the steps of:

- (a) culturing a cell line comprising a nucleic acid encoding the fusion protein; and
- (b) isolating the fusion protein from the cell line.

20 The present disclosure also provides a composition comprising a supraparticle, wherein the supraparticle comprises a fusion protein disclosed herein.

The present disclosure provides a method for promoting survival of spiral ganglion neurons in an ear of a subject, the method comprising administering the fusion protein, the composition, the nucleic acid or the expression vector described herein.

25 The present disclosure also provides a method of treating an auditory disorder in a subject, the method comprising administering the fusion protein, the composition, the nucleic acid or the expression vector described herein.

The present disclosure also provides use of the fusion protein, the composition, the nucleic acid or the expression vector described herein in the manufacture of a medicament for
30 promoting survival of spiral ganglion neurons in an ear of a subject.

The present disclosure further provides use of the fusion protein, the composition, the nucleic acid or the expression vector described herein in the manufacture of a medicament for the treatment of an auditory disorder.

35 The present disclosure also provides the fusion protein, the composition, the nucleic acid or the expression vector described herein for use in promoting survival of spiral ganglion neurons in an ear of a subject.

The present disclosure also provides the fusion protein, the composition, the nucleic acid or the expression vector described herein for use in the treatment of an auditory disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of a protein purification chromatogram displaying the elution profile of NT3-Fc from a 5 mL HiTrap PrismaA column. (A) Complete affinity purification run. (B) Zoomed-in view of the eluted NT3-Fc peak. Chromatogram traces represent protein absorbance at 280 nM (mAU, solid line), conductivity of the purification solutions (mS/cm; dashed line), and concentration of elution buffer (%; dotted line). The protein fractions marked in grey were pooled.

Figure 2 is a graphical representation of a size exclusion chromatography (SEC) elution profile for NT3-Fc in 1 x PBS. (A) Complete chromatogram. (B) Zoomed-in view of the elution.

Figure 3 is a graphical representation of a buffer exchange elution profile for NT3-Fc into ¼ x PBS on a HiPrep 26/10 Desalting column (Cytiva #17508701). The protein displayed a broad elution profile resulting from protein overloading. The 1x PBS was suitably removed as noted by the elution peak between 80-130 mL (small peak = conductivity trace). The protein fractions marked in grey were pooled.

Figure 4 is a graphical representation of a Coomassie-stained SDS-PAGE of affinity-purified NT3-Fc from (A) HEK293F cells and (B) ExpiCHO-S cells. Samples were incubated with or without a reducing agent (DTT, dithiothreitol) as indicated. NuPAGE BIS-TRIS 4-12%, 200 V, 40 min.

Figure 5 is a graphical representation of (A) a total ion chromatogram of the NT3-Fc sample; and (B) a deconvoluted spectrum of the labelled peaks. Sample diluted 20-fold produced a clean chromatographic peak at 21.2 min containing masses around 42,288Da separated by masses consistent with multiple Hexose and HexNAc variants.

Figure 6 is a graphical representation of (A) total ion chromatograms of the deglycosylated NT3-Fc sample; (B) the deconvoluted MS spectrum of the labelled peak, and (C) the zoomed-in section.

Figure 7 is a pictorial representation of a chromatogram displaying the elution profile of NT3-Fc from a 5 mL HiTrap PrismaA column. The area marked in grey was pooled.

Figure 8 is a graphical representation of a size exclusion chromatography elution profile for NT3-Fc in 1x PBS on a HiLoad 26/600 Superdex 200 pg column. The elution of NT3-Fc displays a uniform profile, shown in a single symmetrical peak. The area marked in grey was pooled. To evaluate the molecular weight of the eluted NT3-Fc homodimer, Bio-Rad protein standards (bovine thyroglobulin [670 kDa], bovine γ -globulin [158 kDa], chicken ovalbumin [44 kDa], equine myoglobin [17 kDa] and vitamin B12 [1.35 kDa] were analysed on the same column. The elution volumes of these standards are indicated with arrows.

Figure 9 is a graphical representation of fully processed SPR binding data showing interactions between injected NT3-Fc with immobilized Trk receptors (TrkA: Top row; TrkB:

middle row and TrkC: bottom row). Triplicate experiments data sets are shown in columns A to C (n=1 (A), n=2 (B), and n=3 (C)). (D) shows the fit of the binding responses at equilibrium ($t = 80-85$ sec, plotted against analyte concentration) to a simple (1:1) binding isotherm.

Figure 10 is a graphical representation of a fully processed SPR binding sensorgrams (solid lines) demonstrating that the NT3-Fc protein produced in (A) CHO cell host (ExpiCHO-S) following transient transfection has the same binding kinetics for TrkC receptor as the NT3-Fc purified following transfection of (B) HEK293Freestyle cells. In this case, the NT3-Fc was immobilised on a solid support. The binding affinity was determined in experiments that consisted of 5 sequential injections of serially diluted TrkC (3-fold, from 243 nM to 3 nM). Overlaid dotted lines represent a global fit of the data to a 1:1 interaction model. (C) Binding interaction parameters extracted from sensorgrams shown in A and B.

Figure 11 is a graphical representation of CellSensor TrkC assay (A) representative dose response curves for fresh NT3 and NT3-Fc. (B) EC_{50} value of NT3 and NT3-Fc according to the dose response curve. Data presented as mean $\pm$ standard error of the mean.

Figure 12 is a graphical representation of (A) NT3-Fc loading capacity of Supraparticles over 84 days. Each data point represented the loading capacity of the Supraparticle at x day after manufacturing. $\sim 4 \mu\text{g}$ NT3-Fc can be loaded in one Supraparticle. (B) Elution of NT3-Fc from the loaded Supraparticle. Data presented as mean $\pm$ standard error of the mean.

Figure 13 is a graphical representation of cat cochlea sections after treated with NT3-Fc loaded supraparticles (SPs) on the round window membrane (RWM), stained with anti-human IgG-HRP and DAB. (A) and (B) a normal hearing cat was implanted bilaterally with NT3-Fc loaded Supraparticles on the RWM for 48 h. (C) A normal hearing cat with no implants, as negative control. (D) A normal hearing cat with NT3-Fc loaded Supraparticles implanted inside the cochlear, as positive control.

Figure 14 is a graphical representation of (A) Scanning electron image of a supraparticle. (B) NT3-SPs implanted onto the cat RWM. (C) Image of the cat cochlea and hair cells with the schematic representation of the supraparticles on the RWM. The arrow depicts the localised region (32kHz) where significant synaptic repair was observed. (D) Cochlear synapses in NT3-SP treated and control ears for the 32kHz cochlear region (closest region measured to the round window membrane). Y axis is synapses/inner hair cell, x axis is the treatment condition, NT is NT3.Fc and Norm is normal (undamaged). Significantly higher synaptic density in surviving inner hair cells was observed in NT3-Fc treated cochleae compared to control (n=8 cats, ANCOVA, $p=0.002$).

Figure 15 is a schematic representation of a modified pCAGGS mammalian expression construct.

Figure 16 is a graphical representation of a chromatogram displaying the elution profile of BDNF-Fc from 5 mL HiTrap PrismaA column. The area marked in grey was pooled.

Figure 17 is a graphical representation of a preparative size exclusion chromatography elution profile for BDNF-Fc (solid line). Bio-Rad protein standards (dotted line) were analysed on the same column to evaluate molecular weights of eluted protein peaks. The molecular weights for these standards are indicated with arrows.

5 **Figure 18** is a pictorial representation of Coomassie-stained SDS-PAGE analysis of purified BDNF-Fc samples. Concentrated samples were incubated at 95°C for five minutes before gel loading. The gels used were NuPAGE Bis-Tris 4-12% in MES-SDS buffer, ran at 180 V for 50 minutes. Samples were analysed under reducing and non-reducing conditions.

10 **Figure 19** is a graphical representation of an analytical size exclusion chromatography of the purified BDNF-Fc dimer sample (~50 µg, solid line). Bio-Rad protein standards (dotted line) were analysed on the same column to evaluate molecular weights of the eluted protein.. The molecular weights for the standards are indicated with arrows.

15 **Figure 20** is a graphical representation of processed SPR binding data (solid line sensorgrams) for recombinant TrkB binding to immobilised BDNF-Fc. The experiment consisted of 8 sequential injections of serially diluted TrkB (2-fold from 320 nM to 2.5 nM). Overlaid dashed lines represent a global fit of the data to a 1:1 interaction model.

Figure 21 is a graphical representation of CellSensor TrkB assay (A) representative dose curves for BDNF and BDNF-Fc. (B) EC₅₀ value of BDNF and BDNF-Fc according to the dose curve. Data presented as mean ± standard error of the mean.

20 **Figure 22** is a graphical representation of the purification profile of NT3.diFc (Prisma, solid line). (A) Two protein peaks representing recombinant NT3.diFc were sequentially eluted with 0.1 M Sodium acetate at pH 4.0 (999.8 min) and at pH 3.5 (1024 min). (B) Zoomed-in view of peaks eluting at pH 4.0 and pH 3.5.

25 **Figure 23** is a graphical representation of analytical size exclusion chromatography of NT3-diFc eluting at pH 4.0 (solid line) and at pH 3.5 (dashed line). A previously purified NT3-Fc sample (dotted line) was included in this figure for comparison purposes. Elution volumes and MW of the Bio-Rad protein standards analysed on the same column are highlighted with black arrows.

30 **Figure 24** is a graphical representation of a Coomassie-stained SDS-PAGE of NT3-diFc samples collected during purification. The concentrated samples were incubated at 95 °C for five minutes before gel loading. The gels used were NuPAGE Bis-Tris 4-12% in MES-SDS buffer, ran at 180 V for 50 minutes. Samples were analysed under reducing (Lanes 2 to 5) and non-reducing (Lanes 6-10) conditions.

35 **Figure 25** is a graphical representation of processed SPR binding data (coloured lines sensorgrams) for graphical representation of processed SPR binding kinetics (solid lines) for recombinant TrkB binding to immobilised NT3-Fc samples (captured onto a Protein A SPR chip). (A) NT3-Fc, (B) NT3-diFc sample eluted at pH 4.0. Both binding experiments consisted

of 5 sequential injections (coloured sensorgrams) of serially diluted TrkB (3-fold from 243 nM to 3 nM). Overlayed dashed lines represent a global fit of the data to a 1:1 interaction model.

Figure 26 is a pictorial representation of the sequence alignment for mature, processed amino acid sequence of **(A)** five mono Fc constructs and **(B)** dimeric Fc construct; hinge region, extra serine insertion, mono Fc mutations responsible for preventing dimerization and LALA mutations are all shaded.

Figure 27 is a graphical representation **(A)** showing the number of cochlear synapses per inner hair cell in mouse explants. Three experimental conditions were examined; Normal, KA Control and KA = NT3-Fc treatment. Explants were treated with kainic acid (KA) to damage the synapses, leading to a reduction in their numbers per inner hair cell. Treatment of the explants with NT3-Fc (at a concentration of 1nM) after KA damaged lead to a recovery of synapses with more synapses observed in the cochlear hair cells. The Normal explants were untreated. Representative images of the cochlear explants are shown **(B)** for each condition showing inner hair cells (grey) containing cochlear synapses (white puncta).

KEY TO SEQUENCE LISTING

SEQ ID NO: 1	Amino acid sequence of a human mature neurotrophin-3
SEQ ID NO: 2	Amino acid sequence of a human monomeric Ig Fc domain (Fc001)
SEQ ID NO: 3	Amino acid sequence of human proneurotrophin-3
SEQ ID NO: 4	Amino acid sequence of a human mature neurotrophin-4
SEQ ID NO: 5	Amino acid sequence of a human proneurotrophin-4
SEQ ID NO: 6	Amino acid sequence of a human mature BDNF
SEQ ID NO: 7	Amino acid sequence of a human proBDNF
SEQ ID NO: 8	Amino acid sequence of a human mature NGF
SEQ ID NO: 9	Amino acid sequence of a human proNGF
SEQ ID NO: 10	Amino acid sequence of a human IgG1 heavy chain
SEQ ID NO: 11	Amino acid sequence of a native human Ig Fc domain with LALA mutations
SEQ ID NO: 12	Amino acid sequence of a NT3-Fc fusion protein construct as cloned in pCAGGS vector (NT3-Fc_pCAGGS MCS)
SEQ ID NO: 13	Amino acid sequence of a dimeric NT3-Fc fusion protein construct as cloned in pCAGGS vector (NT3-Fc007pCAGGS MCS)
SEQ ID NO: 14	Amino acid sequence of a BDNF-Fc fusion protein construct
SEQ ID NO: 15	DNA sequence of a NT3-Fc fusion protein
SEQ ID NO: 16	DNA sequence of a dimeric NT3-Fc fusion protein
SEQ ID NO: 17	DNA sequence of a BDNF-Fc fusion protein
SEQ ID NO: 18	DNA sequence of a NT3-Fc fusion protein construct (NT3-Fc001_pCAGGS MCS)

SEQ ID NO: 19	DNA sequence of a dimeric NT3-Fc fusion protein construct (NT3-Fc007pCAGGS MCS)
SEQ ID NO: 20	DNA sequence of a BDNF-Fc fusion protein construct
SEQ ID NO: 21	Amino acid sequence of NT3Fc001
SEQ ID NO: 22	Amino acid sequence of NT3Fc002
SEQ ID NO: 23	Amino acid sequence of NT3Fc003
SEQ ID NO: 24	Amino acid sequence of NT3Fc004
SEQ ID NO: 25	Amino acid sequence of NT3Fc005
SEQ ID NO: 26	Amino acid sequence of NT3Fc006
SEQ ID NO: 27	Amino acid sequence of NT3Fc007
SEQ ID NO: 28	Amino acid sequence of NT3Fc Consensus sequence
SEQ ID NO: 29	DNA sequence of NT3-Fc001_pCAGGS MCS in pCAGGS vector
SEQ ID NO: 30	DNA sequence of NT3-Fc003_pCAGGS MCS in pCAGGS vector
SEQ ID NO: 31	DNA sequence of NT3-Fc004_pCAGGS MCS in pCAGGS vector
SEQ ID NO: 32	DNA sequence of NT3-Fc005_pCAGGS MCS in pCAGGS vector
SEQ ID NO: 33	DNA sequence of NT3-Fc006_pCAGGS MCS in pCAGGS vector
SEQ ID NO: 34	DNA sequence of NT3-Fc007_pCAGGS MCS in pCAGGS vector
SEQ ID NO: 35	Amino acid sequence of human monomeric Ig Fc domain (Fc003)
SEQ ID NO: 36	Amino acid sequence of human monomeric Ig Fc domain (Fc004)
SEQ ID NO: 37	Amino acid sequence of human monomeric Ig Fc domain (Fc005)
SEQ ID NO: 38	Amino acid sequence of human monomeric Ig Fc domain (Fc006)
SEQ ID NO: 39	Amino acid sequence of human monomeric Ig Fc consensus sequence

DETAILED DESCRIPTION

General

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter.

Those skilled in the art will appreciate that the present disclosure is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present disclosure is not to be limited in scope by the specific examples described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the present disclosure.

Any example of the present disclosure herein shall be taken to apply *mutatis mutandis* to any other example of the disclosure unless specifically stated otherwise. Stated another way, any specific example of the present disclosure may be combined with any other specific example of the disclosure (except where mutually exclusive).

5 Any example of the present disclosure disclosing a specific feature or group of features or method or method steps will be taken to provide explicit support for disclaiming the specific feature or group of features or method or method steps.

10 Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (for example, in cell culture, recombinant proteins, immunology, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present disclosure are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in
15 sources such as, J. Perbal, *A Practical Guide to Molecular Cloning*, John Wiley and Sons (1984), J. Sambrook *et al.* *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), *Essential Molecular Biology: A Practical Approach*, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), *DNA Cloning: A Practical Approach*, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel
20 *et al.* (editors), *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) *Antibodies: A Laboratory Manual*, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan *et al.* (editors) *Current Protocols in Immunology*, John Wiley & Sons (including all updates until present).

25 The description and definitions of variable regions and parts thereof, immunoglobulins, antibodies and fragments thereof herein may be further clarified by the discussion in Kabat *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., 1987 and 1991, Bork *et al.*, *J Mol. Biol.* 242, 309-320, 1994, Chothia and Lesk *J. Mol Biol.* 196:901 -917, 1987, Chothia *et al.* *Nature* 342, 877-883, 1989 and/or or Al-Lazikani *et al.*, *J Mol Biol* 273, 927-948, 1997.
30

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout this specification the word “comprise”, or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element, integer or step,
35 or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Selection Definitions

As used herein, the terms “polynucleotide”, “nucleotide sequence” or “nucleic acid” will be understood to mean a series of contiguous nucleotides (or bases) covalently linked to a phosphodiester backbone. By convention, sequences are presented from the 5’ end to the 3’ end, unless otherwise specified. For example, the nucleic acid is a DNA sequence. In one example, the nucleic acid is an RNA sequence. In another example, the nucleic acid is an mRNA sequence. In one example, the mRNA may be a conventional mRNA (cRNA) or a self-amplifying RNA (sa-mRNA).

The term “sequence identity” is used herein in its broadest sense to include the number of exact nucleotide or amino acid matches having regard to an appropriate alignment using a standard algorithm, having regard to the extent that sequences are identical over a window of comparison. Thus, a “percentage of sequence identity” is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U) or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For example, “sequence identity” may be understood to mean the “match percentage” calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA).

As used herein, the term “encode”, “encodes” or “encoding” refers to a region of a nucleic acid capable of undergoing transcription or translation to produce an RNA, polypeptide or protein. For example, the term “encode”, “encodes” or “encoding” refers to a region of an RNA (e.g., mRNA) capable of undergoing translation into a polypeptide or protein, or, when used in the context of a DNA, a region of DNA capable of undergoing transcription to produce an mRNA which is capable of being translated into a polypeptide or protein.

The term “polypeptide” or “polypeptide chain” will be understood to mean a series of contiguous amino acids linked by peptide bonds. For example, a protein shall be taken to include a single polypeptide chain i.e., a series of contiguous amino acids linked by peptide bonds or a series of polypeptide chains covalently or non-covalently linked to one another (i.e., a polypeptide complex). The series of polypeptide chains can be covalently linked using a suitable chemical or a disulfide bond. Examples of non-covalent bonds include hydrogen bonds, ionic bonds, Van der Waals forces, and hydrophobic interactions.

The term “protein” shall be taken to include a single polypeptide chain, i.e., a series of contiguous amino acids linked by peptide bonds or a series of polypeptide chains covalently or non-covalently linked to one another (i.e., a polypeptide complex). For example, the series of polypeptide chains can be covalently linked using a suitable chemical or a disulphide bond. Examples of non-covalent bonds include hydrogen bonds, ionic bonds, Van der Waals forces, and hydrophobic interactions. In some examples, the protein is a fusion protein. As used herein,

a “fusion protein” is a protein comprising at least two domains that have been joined so that they are translated as a single unit, producing a single protein.

The term “isolated protein” or “isolated polypeptide” is a protein or polypeptide that by virtue of its origin or source of derivation is not associated with naturally associated components that accompany it in its native state; is substantially free of other proteins from the same source. A protein may be rendered substantially free of naturally associated components or substantially purified by isolation, using protein purification techniques known in the art. By “substantially purified” is meant the protein is substantially free of contaminating agents, e.g., at least about 70% or 75% or 80% or 85% or 90% or 95% or 96% or 97% or 98% or 99% free of contaminating agents.

As used herein, the terms “variant” refers to a protein which has undergone substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method.

As used herein “amino acid substitution(s)” refers to the replacement of an amino acid at a particular position in a polypeptide sequence with another amino acid.

For example, the substitution is a conservative substitution. By a conservative substitution it is meant the substitutions of one or more amino acids with alternative amino acids sharing similar properties. The skilled person is aware that various amino acids have similar properties and thus are ‘conservative’. One or more such amino acids of a protein, polypeptide or peptide can often be substituted by one or more other such amino acids without eliminating a desired activity of that protein, polypeptide or peptide. Thus, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted with one another (e.g., amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted with one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains). It will be appreciated that amino acid substitutions within the scope of the present disclosure can be made using naturally occurring or non-naturally occurring amino acids. For example, it is contemplated herein that the methyl group on an alanine may be replaced with an ethyl group, and/or that minor changes may be made to the peptide backbone. Whether or not natural or synthetic amino acids are used, it is preferred that only L- amino acids are present.

The skilled artisan will be aware that an “antibody” is generally considered to be a protein that comprises a variable region made up of a plurality of polypeptide chains, e.g., a

polypeptide comprising a V_L and a polypeptide comprising a V_H . An antibody also generally comprises constant domains, some of which can be arranged into a constant region, which includes a constant fragment or fragment crystallizable (Fc), in the case of a heavy chain. A V_H and a V_L interact to form a Fv comprising an antigen binding region that is capable of specifically binding to one or a few closely related antigens. Generally, a light chain from mammals is either a κ light chain or a λ light chain and a heavy chain from mammals is α , δ , ϵ , γ , or μ . Antibodies can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG₁, IgG₂, IgG₃, IgG₄, IgA₁ and IgA₂) or subclass. The term “antibody” also encompasses humanized antibodies, primatized antibodies, human antibodies and chimeric antibodies.

The terms “full-length antibody”, “intact antibody” or “whole antibody” are used interchangeably to refer to an antibody in its substantially intact form, as opposed to an antigen binding fragment of an antibody. Specifically, whole antibodies include those with heavy and light chains.

As used herein, the term “binds” in reference to the interaction of a tyrosine receptor kinase (Trk) receptor with a fusion protein means that the interaction is dependent upon the presence of a particular structure on the Trk receptor and the fusion protein. For example, fusion protein recognises and binds to a specific structure on the Trk receptor rather than to receptors generally.

As used herein, the term “activate” in reference to the interaction of a tyrosine receptor kinase (Trk) receptor with a fusion protein means that the interaction stimulates the Trk receptor, resulting in a flow on effect.

For the purposes of clarification and as will be apparent to the skilled artisan based on the exemplified subject matter herein, reference to “affinity” in this specification is a reference to the interaction, binding or association of a fusion protein with a Trk receptor.

For the purposes of clarification and as will be apparent to the skilled artisan based on the description herein, reference to an “affinity of at least about” will be understood to mean that the affinity is equal to the recited value or higher (i.e., the value recited as the affinity is lower), i.e., an affinity of 2 nM is greater than an affinity of 3 nM. Stated another way, this term could be “an affinity of X or less”, wherein X is a value recited herein.

The term “soluble” is used in the context of the present disclosure to refer fusion proteins which are expressed in a soluble form. For example, aggregation of foreign proteins into insoluble inclusion bodies is a limiting factor of various recombinant expression systems. In an example, soluble fusion proteins of the disclosure do not aggregate into insoluble inclusion bodies following expression in an appropriate expression system disclosed herein. In an example, fusion proteins expressed in a “soluble form” can be purified, extracted or obtained from a solution as a complete tertiary structure. In certain examples, fusion proteins of the disclosure may have improved solubility. The term “solubility” is used herein to refer to the extent of soluble versus insoluble expression of a fusion protein disclosed herein. In an

example, fusion proteins of the disclosure may have improved solubility relative to wild-type neurotrophin.

In some examples, fusion proteins of the disclosure may have improved translation relative to wild-type neurotrophin. In other examples, fusion proteins of the disclosure may have improved stability relative to wild-type neurotrophin.

As used herein, the terms “treating”, “treat” or “treatment” include administering a fusion protein or composition described herein to thereby reduce or eliminate at least one symptom of a specified disease or condition or to slow progression of the disease or condition.

As used herein, the term “preventing”, “prevent” or “prevention” includes providing prophylaxis with respect to occurrence or recurrence of a hearing disorder or a symptom of a hearing disorder in a subject. An individual may be predisposed to or at risk of developing the disease or disease relapse but has not yet been diagnosed with the disease or the relapse.

An “effective amount” refers to at least an amount effective, at dosages and for periods of time necessary, to achieve the desired result. For example, the desired result may be a therapeutic or prophylactic result. An effective amount can be provided in one or more administrations. In some examples of the present disclosure, the term “effective amount” is meant an amount necessary to effect treatment of a disease or condition as hereinbefore described. In some examples of the present disclosure, the term “effective amount” is meant an amount necessary to effect a change in a factor associated with a disease or condition as hereinbefore described. The effective amount may vary according to the disease or condition to be treated or factor to be altered and also according to the weight, age, racial background, sex, health and/or physical condition and other factors relevant to the mammal being treated. Typically, the effective amount will fall within a relatively broad range (e.g., a “dosage” range) that can be determined through routine trial and experimentation by a medical practitioner. Accordingly, this term is not to be construed to limit the disclosure to a specific quantity. The effective amount can be administered in a single dose or in a dose repeated once or several times over a treatment period.

A “therapeutically effective amount” is at least the minimum concentration required to effect a measurable improvement of a particular disease or condition. A therapeutically effective amount herein may vary according to factors such as the disease state, age, sex, and weight of the patient, and the ability of the fusion protein or composition to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the fusion protein or composition are outweighed by the therapeutically beneficial effects. In one example, a therapeutically effective amount shall be taken to mean a sufficient quantity of fusion protein or composition to reduce or inhibit one or more symptoms of a hearing disorder or a complication thereof.

As used herein, the term “prophylactically effective amount” shall be taken to mean a sufficient quantity of the fusion protein or composition to prevent or inhibit or delay the onset of one or more detectable symptoms of a condition.

As used herein, the term “subject” shall be taken to mean any animal including humans, for example a mammal. Exemplary subjects include but are not limited to humans and non-human primates. For example, the subject is a human.

Fusion Protein

The present disclosure provides a fusion protein comprising (i) a neurotrophin; and (ii) a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.

The term “fusion protein” is used in the context of the present disclosure to refer to a protein created through the joining of two or more originally separate polypeptides or expressing nucleic acid(s) encoding the same in a suitable format to provide a fusion protein.

The fusion protein can be formed via various methods known in the art. For example, a fusion protein can be formed by the joining of two or more polypeptides through a peptide bond formed between the amino terminus of one polypeptide and the carboxyl terminus of another polypeptide. In another example, the fusion protein can be formed through linking of one polypeptide to another through reactions between amino acid side chains (for example disulfide bonds between cysteine residues on each polypeptide). In yet another example, the fusion protein can be formed by the chemical coupling of the two or more polypeptides or it can be expressed as a single polypeptide from a nucleic acid sequence encoding a single contiguous fusion protein. In yet another example, fusion proteins can be made using conventional recombinant techniques in molecular biology to join the two genes in frame into a single nucleic acid sequence, and then expressing the nucleic acid in an appropriate host cell under conditions in which the fusion protein is produced. In other words, methods of manufacturing fusion proteins encompassed by the present disclosure are not particularly limited so long as the resulting fusion protein comprises (i) a neurotrophin; and (ii) a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.

A fusion protein of the disclosure can be provided in a host cell or tissue. Alternatively, in another example, fusion proteins may be provided in a purified or partially purified composition. Accordingly, in certain examples, fusion proteins of the disclosure can be “purified” or “isolated”. These terms are used in the context of the present disclosure to refer to a protein that has generally been separated from the lipids, nucleic acids, other peptides and proteins, and other contaminating molecules with which it is associated in its expression system. Preferably, the purified protein is at least 60% free, more preferably at least 75% free, and more preferably at least 90% free from other components in the expression system. Methods of manufacturing fusion proteins of the disclosure and nucleic acids encoding the same are discussed further below.

Fusion proteins of the disclosure may have improved expression levels. In one example, the fusion protein improves the expression levels of the conjugated neurotrophin, relative to a wild-type or unmodified neurotrophin. In some examples, the fusion protein improves the expression levels of the conjugated neurotrophin in a mammalian cell system, relative to a wild-type or unmodified neurotrophin in a mammalian cell system.

In one example, the fusion protein is capable of forming a homodimer. Accordingly, in an example, fusion proteins of the disclosure can comprise a neurotrophin dimer. In an example, the dimer is provided via dimerization with another neurotrophin of the same type. For example, fusion proteins of the disclosure may comprise a neurotrophin-3 dimer. In another fusion proteins of the disclosure may comprise a BDNF dimer.

Neurotrophins

The present disclosure provides a fusion protein comprising a neurotrophin of the disclosure.

“Neurotrophins” are growth factors expressed in the brain and peripheral tissues, which regulate many aspects of neuronal function including proliferation of neural progenitors, neuronal morphology, synaptic plasticity, and even cell death following injury. Indeed, one cause of spiral ganglion neuron degeneration is the loss of the endogenous supply of neurotrophins. Neurotrophins mediate these actions by activating two different classes of receptors, the tyrosine receptor kinase (Trk) family of receptor tyrosine kinases and p75NTR, a member of the TNF receptor superfamily. Specifically, nerve growth factor (NGF) binds with high affinity to TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4) bind with high affinity to TrkB, and neurotrophin 3 (NT-3) binds with high affinity to TrkC. Apart from activation of TrkC, NT-3 also activates TrkA and TrkB, albeit with lower affinities. Accordingly, in certain examples, neurotrophins encompassed by the present disclosure may be characterised based on their ability to activate a Trk receptor. For example, neurotrophin variants related to a SEQ ID NO described herein may be characterised based on their ability to activate and/or bind relevant Trk receptor at a level corresponding with a counterpart neurotrophin having its amino acid sequence disclosed herein.

Neurotrophins share a number of structural and chemical properties, including more than 50% sequence homology in the primary structure, approximately similar molecular weight, three disulfide bonds that form a cysteine knot and isoelectric points ranging from 9-10. A “wild-type” or “unmodified” neurotrophin is derived from a mammal in natural conditions with no additional modifications or mutations.

It will be understood by the skilled person that neurotrophins are initially synthesised as larger precursor molecules comprising a signal peptide, pro-peptide and peptide. For example, the precursor may be a “proneurotrophin-3”, a “proneurotrophin-4”, a “proBDNF” or a “proNGF”, which undergo proteolytic cleavage to yield the mature neurotrophin.

Proneurotrophins are the precursor forms of neurotrophins and can function as high-affinity apoptotic ligands for selected neural populations.

In one example, the present disclosure also encompasses the precursor form of a fusion protein described herein. For example, a precursor fusion protein may comprise a
5 proneurotrophin and a monomeric Ig Fc domain or fragment thereof as described herein. In one example, the precursor fusion protein comprises a proneurotrophin-3 and a monomeric Ig Fc domain or fragment thereof as described herein. In one example, the precursor fusion protein comprises a proneurotrophin-4 and a monomeric Ig Fc domain or fragment thereof as described herein. In one example, the precursor fusion protein comprises a proBDNF and a monomeric
10 Ig Fc domain or fragment thereof as described herein. In one example, the precursor fusion protein comprises a proNGF and a monomeric Ig Fc domain or fragment thereof as described herein.

Various neurotrophins are known in the art and are, in certain examples, described based on their respective amino acid sequences. Examples of neurotrophins include nerve growth
15 factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4).

Accordingly, in one example, the neurotrophin is NGF, BDNF and NT-3. In another example, the neurotrophin is NGF, BDNF and NT-4. In another example, the neurotrophin is BDNF, NT-3 and NT-4. In another example, the neurotrophin is NGF and BDNF. In another
20 example, the neurotrophin is NGF, NT-3 and NT-4. In another example, the neurotrophin is NGF and BDNF. In another example, the neurotrophin is NGF and NT-3. In another example, the neurotrophin is NGF and NT-4. In another example, the neurotrophin is BDNF and NT-3. In another example, the neurotrophin is BDNF and NT-4. In another example, the neurotrophin is NT-3 and NT-4.

In an example, the neurotrophin is a neurotrophin 3 (NT-3). For example, the neurotrophin can be a mammalian NT-3. In one example, the neurotrophin is a human NT-3. In one example, the NT-3 comprises a sequence set forth in SEQ ID NO: 1. In one example, an NT-3 encompassed by the present disclosure is a variant of SEQ ID NO: 1. For example, the NT-3 can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical
25 to SEQ ID NO: 1. In an example, the variant is identified based on its sequence identity to SEQ ID NO: 1 and its ability to bind/activate Trk at a level corresponding to an neurotrophin comprising an amino acid sequence as shown in SEQ ID NO: 1.

In an example, the neurotrophin is a neurotrophin 4 (NT-4). For example, the neurotrophin can be a mammalian NT-4. In one example, the neurotrophin is a human NT-4.
35 In one example, the NT-4 comprises a sequence set forth in SEQ ID NO: 4. In one example, an NT-4 encompassed by present disclosure is a variant of SEQ ID NO: 4. For example, the NT-4 can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 4. In an example, the variant is identified based on its sequence identity to SEQ

ID NO: 4 and its ability to bind/activate Trk at a level corresponding to an neurotrophin comprising an amino acid sequence as shown in SEQ ID NO: 4.

In an example, the neurotrophin is a nerve growth factor (NGF). For example, the neurotrophin can be a mammalian NGF. In another example, the neurotrophin is a human NGF.

5 In one example, the NGF comprises a sequence set forth in SEQ ID NO: 8. In one example, an NGF encompassed by the present disclosure is a variant of SEQ ID NO: 8. For example, the NGF can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 8. In an example, the variant is identified based on its sequence identity to SEQ ID NO: 8 and its ability to bind/activate Trk at a level corresponding to an neurotrophin
10 comprising an amino acid sequence as shown in SEQ ID NO: 8.

In an example, the neurotrophin is a brain-derived neurotrophic factor (BDNF). For example, the neurotrophin can be a mammalian BDNF. In another example, the neurotrophin is a human BDNF. In one example, the BDNF comprises a sequence set forth in SEQ ID NO: 6. In one example, a BDNF encompassed by the present disclosure is a variant of SEQ ID NO:
15 6. For example, the BDNF can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 6. In an example, the variant is identified based on its sequence identity to SEQ ID NO: 6 and its ability to bind/activate Trk at a level corresponding to an neurotrophin comprising an amino acid sequence as shown in SEQ ID NO: 6.

In one example, the fusion protein is capable of dimerizing with another neurotrophin
20 or fusion protein. In an example, the fusion protein forms a homodimer. In an example, the neurotrophin forms a homodimer when provided as a fusion protein disclosure herein. In such examples, the neurotrophin component of the fusion protein disclosed herein mediates dimerization.

As used herein, the term “dimer” refers to a protein complex including at least two
25 polypeptides. At least two polypeptides may be associated with each other via one or both of covalent and non-covalent (for example, electrostatic, π -effects, van der Waals forces, and hydrophobic effects) interactions. The two polypeptides may have the same amino acid sequence or may be different from each other. In a case where the two polypeptides are identical to each other, the dimer is referred to as a homodimer. In a case where the two
30 polypeptides are different from each other, the dimer is referred to as a heterodimer.

In one example, the neurotrophin component of the fusion protein disclosed herein is provided as a homodimer. For example, a homodimer of the disclosure comprises a NT-3 molecule dimerized with another NT-3 molecule. In another example, the homo dimer is a NT-4 molecule dimerized with another NT-4 molecule. In another example, the homodimer is a
35 BDNF molecule dimerized with another BDNF molecule. In one example, the homodimer is a NGF molecule dimerized with another NGF molecule.

In another example, a neurotrophin of the disclosure is a heterodimer. For example, a NT-3 molecule dimerizes with a NT-4, BDNF or NGF molecule. In another example, a NT-4

molecule dimerizes with a NT-3, BDNF, or NGF molecule. In one example, a BDNF molecule dimerizes with a NT-3, NT-4 or NGF molecule. In one example, a NGF molecule dimerizes with NT-3, NT-4 or BDNF molecule

5 Fragment crystallisable (Fc)

The term “fragment crystallisable” or “Fc” are used herein to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. This term includes Fc domain variants and Fc domain fragments.

Those of skill in the art will be familiar with the human Fc domain region.

10 In one example, a human IgG heavy chain Fc domain region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, Fc domains produced by host cells may undergo post-translational cleavage of one or more, particularly one or two, amino acids from the C-terminus of the heavy chain. Thus, an Fc domain may include a cleaved variant of the full-length heavy chain. This may be the case where the final two C-terminal
15 amino acids of the heavy chain are glycine (G446) and lysine (K447, numbering according to EU index). Therefore, the C-terminal lysine (K447), or the C-terminal glycine (G446) and lysine (K447), of the Fc domain region may or may not be present.

When specified herein, numbering of amino acid residues in the Fc domain region or constant region is according to the EU numbering system, also called the EU index, as
20 described in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

The term “EU numbering system of Kabat” or “EU numbering system” will be understood to mean the numbering of an immunoglobulin heavy chain is according to the EU index as taught in Kabat *et al.*, 1991, Sequences of Proteins of Immunological Interest, 5th Ed.,
25 United States Public Health Service, National Institutes of Health, Bethesda. The EU index is based on the residue numbering of the human IgG1 EU.

The wild-type Fc is homodimeric in nature and this feature is driven by the strong, high-affinity interaction that exists between the two CH3 domains and two disulphide bonds in the hinge region.

30 Fusion proteins of the disclosure comprise a “monomeric Ig Fc domain” or a fragment thereof. Accordingly, the Fc component of disclosed fusion proteins are distinguished from their wild-type counterparts at least in view of their monomeric structure. The term “monomeric Ig Fc domain” is used in the context of the present disclosure to refer to monomeric polypeptides which comprise a sequence of amino acids corresponding to the Fc
35 portion of the heavy chain, e.g., containing a single CH2 domain and a single CH3 domain. Thus, the monomeric Ig Fc domain does not comprise a hinge region. Such monomeric polypeptides will generally comprise amino acid substitution(s), relative to a wild-type Fc, which direct a monomeric structure. For example, the monomeric Ig Fc domain will comprise

N-glycosylation site(s) in the CH3 domain which inhibits dimerization with another CH3 domain.

Various examples of monomeric Fc are known in the art and are discussed further below.

5 Any Ig Fc domain region can be modified to produce a monomeric Ig Fc domain or fragment thereof of the disclosure. As discussed herein, generally an Ig Fc domain is from a human Ig. However, the Ig Fc domain may be derived from an Ig of any other mammalian species, including for example, a Camelid species, a rodent (e.g. a mouse, rat, rabbit, guinea pig) or non-human primate (e.g. chimpanzee, macaque) species. Moreover, the Ig Fc domain
10 may be derived from any immunoglobulin class, including IgM, IgG, IgD, IgA and IgE, and any immunoglobulin isotype, including IgG1, IgG2, IgG3 and IgG4. In certain examples, the Fc domain is an IgG Fc domain (e.g., a human IgG region). In certain examples, the Ig Fc domain is an IgG1 Fc domain (e.g., a human IgG1). In certain examples, the Ig Fc domain is a chimeric Ig Fc domain comprising portions of several different Ig Fc domains. Accordingly, it
15 will be appreciated that the scope of the present disclosure encompasses alleles, variants and mutations of Ig Fc domains which provide a monomeric Ig Fc domain.

In one example, the monomeric Ig Fc domain or fragment thereof is from IgG. For example, the monomeric Ig Fc domain or fragment thereof is from human IgG. In another example, the monomeric Ig Fc domain or fragment thereof is from IgG1. In another example,
20 the monomeric Ig Fc domain or fragment thereof is from human IgG1. In another example, the monomeric Ig Fc domain or fragment thereof is from IgG4. In another example, the monomeric Ig Fc domain or fragment thereof is from human IgG4.

In one example, the monomeric Ig Fc domain or fragment thereof allows for dimerization of the fusion protein. In such examples, the fusion protein described herein forms
25 a homodimer. In such examples, the fusion protein described herein maintains biological activity of the neurotrophin.

In one example, the monomeric Ig Fc domain or fragment thereof does not trigger immune or cellular events. For example, the monomeric Ig Fc domain or fragment thereof does not trigger antibody-dependent cellular cytotoxicity (ADCC). In another example, the
30 monomeric Ig Fc domain or fragment thereof does not trigger complement-dependent cytotoxicity (CDC). In one example, the monomeric Ig Fc domain or fragment thereof does not trigger phagocytosis. In one example, the monomeric Ig Fc domain or fragment thereof does not trigger opsonisation.

In one example, the monomeric Ig Fc domain or fragment thereof facilitates improved
35 expression levels of the fusion protein.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions relative to a sequence set forth in SEQ ID NO: 10. For example,

the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions in the CH3 domain relative to a sequence set forth in SEQ ID NO: 10.

When discussing positioning of a substitution within, e.g., an Ig Fc domain within a fusion protein, the numbering of the position is relative to a sequence set forth in SEQ ID NO: 10 and not relative to the entire fusion protein. Thus, if a neurotrophin is fused to an N-terminal of an Ig domain, when discussing a mutation in the Fc domain, the first residue is the first residue of a sequence set forth in SEQ ID NO: 10.

An Ig Fc domain can be further truncated or include substitution(s) to prevent dimerization and/or reduce ADCC and/or CDC. The ability of an Ig Fc domain fragment to dimerize can be determined using any art recognized assay e.g., gel filtration or size-exclusion chromatography.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site that reduces dimerization with another monomeric Ig Fc domain or fragment thereof. In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site that inhibits dimerization with another monomeric Ig Fc domain or fragment thereof. In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site that prevents dimerization with another monomeric Ig Fc domain or fragment thereof. Some exemplary N-glycosylation sets are described in EP2494061, incorporated herein by reference.

In some examples, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site. For example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site in a CH3 domain.

In some examples, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site at one or more positions selected from the group consisting of 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site:

- (i) at a position corresponding to amino acid 364 of SEQ ID NO: 10 according to the EU numbering system; or
- (ii) at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises at least two N-glycosylation sites:

- (i) at a position corresponding to amino acid 364 of SEQ ID NO: 10 according to the EU numbering system; and
- (ii) at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system.

5 In some examples, the monomeric Ig Fc domain or fragment thereof comprises a substitution at one or more positions selected from the group consisting of 237, 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 10 419, 430, 434, 435, 437, 438 or 439 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system. Some exemplary substitutions are described in US7335742; Ishino et al. 2013, J of Biological Chemistry 288:23:16529-16537; Ying et al. 2012, J of Biological Chemistry 287:23:19399-19408; Wang et al. 2017, Frontiers in Immunology 8:1545; and Wikinson et al. 2013, mAbs 5:3, each of which are incorporated herein by 15 reference.

In some examples, the monomeric Ig Fc domain or fragment thereof comprises a substitution at one or more positions selected from the group consisting of 234, 235, 237 and 329 relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system.

20 In some examples, the monomeric Ig Fc domain or fragment thereof comprises at least one substitution selected from the group consisting of G237A, S298A, E333A, K334A, P329G, L351S, T366R, L368H, P395K, F405E, Y407N, and K409T relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system.

25 In some examples, the monomeric Ig Fc domain or fragment thereof comprises at least one substitution selected from the group consisting of L234A, L235A, G237A and P329A relative to a sequence set forth in SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions selected from the group consisting of:

- (i) serine substituted with alanine at a position corresponding to amino acid 298 of SEQ ID NO: 10 according to the EU numbering system;
- 30 (ii) glutamic acid substituted with alanine at a position corresponding to amino acid 333 of SEQ ID NO: 10 according to the EU numbering system;
- (iii) lysine substituted with alanine at a position corresponding to amino acid 334 of SEQ ID NO: 10 according to the EU numbering system;
- (iv) leucine substituted with serine at a position corresponding to amino acid 351 of SEQ 35 ID NO: 10 according to the EU numbering system;
- (v) threonine substituted with arginine at a position corresponding to amino acid 366 of SEQ ID NO: 10 according to the EU numbering system;

(vi) leucine substituted with histidine at a position corresponding to 368 amino acid of SEQ ID NO: 10 according to the EU numbering system;

(vii) proline substituted with lysine at a position corresponding to amino acid 395 of SEQ ID NO: 10 according to the EU numbering system;

5 (viii) phenylalanine substituted with glutamic acid at a position corresponding to amino acid 405 of SEQ ID NO: 10 according to the EU numbering system;

(ix) tyrosine substituted with asparagine at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system;

10 (x) lysine substituted with threonine at a position corresponding to amino acid 409 of SEQ ID NO: 10 according to the EU numbering system; and

In one example, monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions selected from the group consisting of:

(i) serine substituted with alanine at a position corresponding to amino acid 298 of SEQ ID NO: 10 according to the EU numbering system;

15 (ii) glutamic acid substituted with alanine at a position corresponding to amino acid 333 of SEQ ID NO: 10 according to the EU numbering system;

(iii) lysine substituted with alanine at a position corresponding to amino acid 334 of SEQ ID NO: 10 according to the EU numbering system;

20 (iv) leucine substituted with serine at a position corresponding to amino acid 351 of SEQ ID NO: 10 according to the EU numbering system;

(v) threonine substituted with arginine at a position corresponding to amino acid 366 of SEQ ID NO: 10 according to the EU numbering system;

(vi) leucine substituted with histidine at a position corresponding to 368 amino acid of SEQ ID NO: 10 according to the EU numbering system;

25 (vii) proline substituted with lysine at a position corresponding to amino acid 395 of SEQ ID NO: 10 according to the EU numbering system;

(viii) phenylalanine substituted with glutamic acid at a position corresponding to amino acid 405 of SEQ ID NO: 10 according to the EU numbering system;

30 (ix) tyrosine substituted with asparagine at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system; and

(x) lysine substituted with threonine at a position corresponding to amino acid 409 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises the following amino acid substitutions:

35 (xi) tyrosine substituted with asparagine at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system; and

(xii) lysine substituted with threonine at a position corresponding to amino acid 409 of SEQ ID NO: 10 according to the EU numbering system.

In one example, the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions selected from the group consisting of:

- (i) leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system;
- 5 (ii) leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system;
- (iii) glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system; and
- 10 (iv) proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.

In some examples, the constituent Fc domain regions of a monomeric Ig Fc domain or fragment thereof are linked together by a linker.

Methods of producing single chain Fc domain regions are known in the art (see e.g., US20090252729 and US20110081345).

- 15 To enhance the manufacturability of the fusion proteins disclosed herein, it may be desirable that the monomeric Ig Fc domain does not comprise any non-disulphide bonded cysteine residues. Accordingly, in certain examples the monomeric Ig Fc domain does not comprise free cysteine residues.

20 Linkers

In some examples, components of the fusion protein of the disclosure are linked by a linker.

In some examples, the linker is a polypeptide linker. For example, the linker is a serine residue. In other examples, the linker comprises alanine, alanine and serine.

- 25 Polypeptide linkers of the disclosure are at least one amino acid in length and can be of varying lengths. In some examples, a polypeptide linker of the disclosure is from about 1 to about 50 amino acids in length. In another example, a polypeptide linker of the disclosure is from about 5-10 amino acids in length. In another example, a polypeptide linker of the disclosure is from about 10-20 amino acids in length. In another example, a polypeptide linker of the disclosure is from about 15 to about 50 amino acids in length.

- 30 In some examples, the linker comprises or is a chemical linker. In some examples, the linker is one or more ethylene glycol (EG) units, e.g., 2 or more EG units (i.e., polyethylene glycol (PEG)). In some examples, a linker comprises or consists of a polyethylene glycol (PEG) linker. Polyethylene glycol or PEG refers to a chemical compound composed of repeating ethylene glycol units. An exemplary "PEG linker" comprises a compound of the formula: H-(O-CH₂-CH₂)_n-OH, wherein n is a positive integer (e.g., 1, 10, 20, 50, 100, 200, 300, 400, 500, 600). In some examples, the PEG linker is PEG1000. In some examples, the PEG linker is PEG2000. In some examples, the PEG linker is PEG3000.

In some examples, a fusion protein comprises a monomeric Fc domain or fragment thereof linked to a serine which is in turn linked by a linker to a neurotrophin. In some examples, a fusion protein comprises a monomeric Fc domain or fragment thereof linked by ala-ala-ser which is in turn linked by a linker to a neurotrophin.

5 In other examples, a polypeptide linker comprises or consists of a gly-ser linker. As used herein, the term “gly-ser linker” refers to a peptide that consists of glycine and serine residues. An exemplary gly/ser linker comprises an amino acid sequence of the formula $(\text{Gly}_4\text{Ser})_n$, wherein n is a positive integer (e.g., 1, 2, 3, 4, or 5). In certain examples, the gly/ser linker is $(\text{Gly}_4\text{Ser})_1$. In some examples, the gly/ser linker is $(\text{Gly}_4\text{Ser})_2$. In some examples the
10 gly/ser linker is $(\text{Gly}_4\text{Ser})_3$ or $(\text{Gly}_4\text{Ser})_4$.

Other linkers that are suitable for use in the fusion protein of the disclosure are known in the art, for example, the serine-rich linkers disclosed in US 5525491, the helix forming peptide linkers (e.g., $\text{A(EAAAK)}_n\text{A}$ ($n=2-5$)) disclosed in Arai *et al*, *Protein Eng* 2001;14:529-32, or the stable linkers disclosed in Chen *et al*, *Mol Pharm* 2011;8:457-65.

15 Other linkers include GS linkers (i.e., $(\text{GS})_n$), GGSG linkers (i.e., $(\text{GGSG})_n$), GSAT linkers, SEG linkers, and GGS linkers (i.e., $(\text{GGSGGS})_n$), wherein n is a positive integer (e.g., 1, 2, 3, 4, or 5).

In one example, the N-terminus of the monomeric Ig Fc domain or fragment thereof is linked by a linker to the C-terminus of the neurotrophin. For example, the N-terminus of the
20 monomeric Ig Fc domain or fragment thereof is linked by a serine to the C-terminus of the neurotrophin.

In another example, the C-terminus of the monomeric Ig Fc domain or fragment thereof is linked by a linker to the N-terminus of the neurotrophin.

25 **Fusion Protein Activity**

In certain examples, the present disclosure provides fusion proteins with improved biological activity and/or increased receptor binding affinity.

As used herein, “biologically active” or “biological activity” refers the ability of a fusion protein to affect the normal biological processes of the conjugated neurotrophin, such as e.g.,
30 tyrosine receptor kinase (Trk) receptor activation. For example, improved biological activity refers to an increase in the ability of the fusion protein to affect the normal biological processes, relative to a wild-type or unmodified neurotrophin.

One of skill in the art would appreciate that neurotrophins are biologically active as non-covalently linked homodimers.

35 Fusion proteins of the present disclosure can be readily screened for biological activity and/or binding affinity, e.g., as described below.

In one example, the biological activity of the fusion protein can be assessed *in vivo*, for example, in an animal model. In such examples, the fusion protein can be placed in the cochlea of a subject to determine if the fusion protein directs synaptic repair.

Affinity measurements can be determined by standard methodology, for example, immunoassays, surface plasmon resonance (SPR; e.g., using BiacoreTM technology [Cytiva], Rich and Myszka *Curr. Opin. Biotechnol* 11: 54, 2000; Englebienne *Analyst* 123: 1599, 1998), isothermal titration calorimetry (ITC) or other kinetic interaction assays known in the art. For example, the dissociation rate constant (k_d) or association rate constant (k_a) or affinity constant (K_D) of a fusion protein or a component thereof (e.g., monomeric Ig Fc domain or fragment thereof or neurotrophin) can be determined for fusion proteins described herein and an appropriate control (e.g. wild-type).

In some examples, the fusion protein has a similar K_D or an improved K_D (i.e., a K_D value lower than) for a tyrosine receptor kinase (Trk) receptor compared to an unmodified neurotrophin (e.g., a neurotrophin without a monomeric Ig Fc domain or fragment thereof).

In one example, the fusion protein binds with increased affinity to a Trk receptor compared to an unmodified neurotrophin.

In one example, the fusion protein is characterised by increased activation of a Trk receptor compared to an unmodified neurotrophin.

Binding affinity of the fusion protein can also be determined non-quantitatively using flow cytometry. For example, the fusion protein is added to CHO cells stably expressing a Trk receptor which are then stained with a marker (to detect target binding) and another marker (to detect binding) at acidic (pH 5.5) and neutral (pH 7.4) pH and analysed by flow cytometry. Relative binding to Trk receptor is determined, for example, by calculating mean fluorescence intensity relative to an unmodified neurotrophin.

In one example, the Trk receptor is a TrkA receptor. In another example, the Trk receptor is a TrkB receptor. In a further example, the Trk receptor is a TrkC receptor.

In one example, the fusion protein binds to a TrkA receptor at neutral pH with an affinity constant (K_D) of less than 800 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein binds to a TrkA receptor at neutral pH with an K_D of less than 700 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR. In one example, the fusion protein binds to a TrkA receptor at neutral pH with a K_D of between 400 nM to 800 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR. In one example, the fusion protein binds to a TrkA receptor at neutral pH with a K_D of between 500 nM to 700 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR. In one example, the fusion protein binds to a TrkA

receptor at neutral pH with a K_D of between 600 nM to 700 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR.

5 In one example, the fusion protein comprising an NT-3 binds to a TrkA receptor at neutral pH with an affinity constant (K_D) of less than 800 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein comprising an NT-3 binds to a TrkA receptor at neutral pH with a K_D of less than 700 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkA receptor at neutral pH with a K_D of between 400 nM to 800 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkA receptor at neutral pH with a K_D of between 500 nM to 700 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkA receptor at neutral pH with a K_D of between 600 nM to 700 nM, wherein the TrkA receptor is immobilised on a solid support and the binding of the fusion protein to the TrkA receptor is determined using SPR.

20 In one example, the fusion protein binds to a TrkB receptor at neutral pH with an affinity constant (K_D) of less than 60 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein binds to a TrkB receptor at neutral pH with a K_D of less than 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein binds to a TrkB receptor at neutral/ pH with a K_D of between 20 nM to 60 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein binds to a TrkB receptor at neutral pH with a K_D of between 20 nM to 60 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR.

35 In one example, the fusion protein comprising an NT-3 binds to a TrkB receptor at neutral pH with an affinity constant (K_D) of less than 60 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein comprising an NT-3 binds to a TrkB receptor at neutral pH with an K_D of at least 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein

comprising an NT-3 binds to a TrkB receptor at neutral/ pH with a K_D of between 20 nM to 60 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkB receptor at neutral pH with a K_D of between 30 nM to 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkB receptor at neutral pH with a K_D of between 40 nM to 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR.

10 In one example, the fusion protein comprising a BDNF binds to a TrkB receptor at neutral pH with an affinity constant (K_D) of less than 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein comprising a BDNF binds to a TrkB receptor at neutral pH with an K_D of less than 40 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein comprising a BDNF binds to a TrkB receptor at neutral pH with a K_D of between 20 nM to 60 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein comprising a BDNF binds to a TrkB receptor at neutral pH with a K_D of between 30 nM to 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR. In one example, the fusion protein comprising a BDNF binds to a TrkB receptor at neutral pH with a K_D of between 40 nM to 50 nM, wherein the TrkB receptor is immobilised on a solid support and the binding of the fusion protein to the TrkB receptor is determined using SPR.

25 In one example, the fusion protein binds to a TrkC receptor at neutral pH with an affinity constant (K_D) of less than 10 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein binds to a TrkC receptor at neutral pH with a K_D of less than 5 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR.

30 In one example, the fusion protein binds to a TrkC receptor at neutral pH with a K_D of between 0.1 nM to 5 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR. In one example, the fusion protein binds to a TrkC receptor at neutral pH with a K_D of between 0.5 nM to 2 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR. In one example, the fusion protein binds to a TrkC receptor at neutral pH with a K_D of between 1 nM to 2 nM, wherein the TrkC receptor

is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR.

5 In one example, the fusion protein comprising an NT-3 binds to a TrkC receptor at neutral pH with an affinity constant (K_D) of less than 10nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using surface plasmon resonance (SPR). In one example, the fusion protein comprising an NT-3 binds to a TrkC receptor at neutral pH with a K_D of less than 5 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR.

10 In one example, the fusion protein comprising an NT-3 binds to a TrkC receptor at neutral pH with a K_D of between 0.1 nM to 5 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkC receptor at neutral pH with a K_D of between 0.5 nM to 2 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR. In one example, the fusion protein comprising an NT-3 binds to a TrkC receptor at neutral pH with a K_D of between 1 nM to 2 nM, wherein the TrkC receptor is immobilised on a solid support and the binding of the fusion protein to the TrkC receptor is determined using SPR.

20 In one example, the bioactivity of the fusion protein is assessed. Methods of assessing bioactivity are known in the art and/or are exemplified herein. For example, fusion protein is assessed *in vitro* and/or *ex vivo*. In one example, cell culture assays such as CellSensor™ (<https://www.thermofisher.com/order/catalog/product/K1491>; Gunewardene et al. *J. Control Release* 342: 295-307, 2022.)

25 In one example, the fusion protein has improved biological activity (e.g., improved bioactivity) relative to an unmodified neurotrophin.

For example, the fusion protein has increased bioactivity relative to an unmodified neurotrophin. In an example, bioactivity is determined based on (EC_{50}). For example, a fusion protein with increased bioactivity has an EC_{50} of less than 100 pM. In one example, a fusion protein with increased bioactivity has an EC_{50} of less than 70 pM. In one example, a fusion protein with increased bioactivity has an EC_{50} of less than 60 pM. In one example, the fusion protein has an EC_{50} between 20 pM to 100 pM. In one example, the fusion protein has an EC_{50} between 30 pM to 60 pM. In one example, the fusion protein has an EC_{50} between 50 pM to 60 pM.

35 In one example, the fusion protein has improved solubility relative to a wild-type neurotrophin. Methods for determining the solubility of a fusion protein of the disclosure are known in the art and/or described herein. In an example, solubility is determined using gel filtration or size-exclusion chromatography. As would be understood by the skilled person if a

compound aggregates the mass of the aggregation would be higher than the mass of the compound itself. Thus, the fusion protein can be determined as being predominantly expressed in a soluble form if the mass of the fusion protein is similar to the mass of a wild-type neurotrophin.

5 In another example, the fusion protein has improved expression levels relative to a wild-type neurotrophin. Methods for determining expression levels of a fusion protein of the disclosure are known in the art and/or described herein. In an example, the expression level of the fusion protein is determined using SDS-PAGE.

10 **Methods of Manufacturing Fusion Proteins**

The present disclosure provides nucleic acids, expression constructs and host cells encoding the fusion proteins disclosed herein. Methods of manufacturing fusion proteins comprising expressing these polynucleotides are also provided.

15 As would be understood by the skilled person, manufacturing neurotrophins is difficult and time-consuming in part due to the low yields and the need to refold the neurotrophin isolated from *E. coli* to achieve correct disulphide bond formation. Advantageously, the fusion proteins described herein are synthesised as monomers which subsequently dimerize. Thus, in certain examples, fusion proteins of the disclosure facilitate high yields and the prospect of large-scale manufacturing.

20 In one example, the present disclosure encompasses a nucleic acid encoding a fusion protein disclosed herein. In an example, the nucleic acid encodes a proneurotrophin and a monomeric Ig Fc domain or fragment thereof. In certain examples, the nucleic acid encodes a proneurotrophin and a monomeric Ig Fc domain or fragment thereof comprising a linker or spacer between the proneurotrophin and the monomeric Ig Fc domain or fragment thereof. In
25 an example, the nucleic acid is provided in a host cell suitable for expression of the same.

For example, the proneurotrophin is a proneurotrophin 3 (proNT-3). For example, the proneurotrophin can be a mammalian proNT-3. In one example, the proneurotrophin is a human proNT-3. In one example, the proNT-3 comprises a sequence set forth in SEQ ID NO: 3. In one example, a proNT-3 encompassed by the present disclosure is a variant of SEQ ID
30 NO: 3. For example, the proNT-3 can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 3.

In an example, the proneurotrophin is a proneurotrophin 4 (proNT-4). For example, the proneurotrophin can be a mammalian proNT-4. In one example, the proneurotrophin is a human proNT-4. In one example, the proNT-4 comprises a sequence set forth in SEQ ID NO:
35 5. In one example, a proNT-4 encompassed by present disclosure is a variant of SEQ ID NO: 5. For example, the proNT-4 can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 5.

In an example, the proneurotrophin is a nerve growth factor (proNGF). For example, the proneurotrophin can be a mammalian proNGF. In another example, the proneurotrophin is a human proNGF. In one example, the proNGF comprises a sequence set forth in SEQ ID NO: 9. In one example, a proNGF encompassed by the present disclosure is a variant of SEQ ID NO: 9. For example, the proNGF can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 9.

In an example, the proneurotrophin is a brain-derived neurotrophic factor (proBDNF). For example, the proneurotrophin can be a mammalian proBDNF. In another example, the proneurotrophin is a human proBDNF. In one example, the proBDNF comprises a sequence set forth in SEQ ID NO: 7. In one example, a proBDNF encompassed by the present disclosure is a variant of SEQ ID NO: 7. For example, the proBDNF can comprise a sequence at least 85% or 90% or 95% or 97% or 98% or 99% identical to SEQ ID NO: 7.

In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 1 and a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 2.

In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 6 and a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 2.

In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 21. In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 23. In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 24. In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 25. In one example, the fusion protein comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 26.

In some examples, the fusion protein is expressed by a host cell in a soluble form.

In one example, the nucleic acid encoding the fusion protein is a DNA sequence comprising a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 15. In one example, the nucleic acid encoding the fusion protein is a DNA sequence comprising a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO: 17.

In one example, the expression construct comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence set forth in SEQ ID NO:

18. In one example, the expression construct comprises a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence forth in SEQ ID NO: 20.

In one example, the expression construct encodes a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence forth in SEQ ID NO:

5 12. In one example, the expression construct encodes a sequence having at least 85% or 90% or 95% or 97% or 98% or 99% sequence identity to the sequence forth in SEQ ID NO: 14.

Nucleic acids encoding the fusion protein disclosed herein are typically inserted in an expression construct or vector for introduction into host cells that may be used to produce the desired quantity of the claimed fusion proteins. Accordingly, in some examples, the present
10 disclosure provides expression construct or vectors comprising nucleic acids disclosed herein and host cells comprising these construct or vectors and nucleic acids.

One of the advantages of this approach is that the signal peptide and prodomain are cleaved during synthesis and secretion which provides a neurotrophin which more closely aligns with the naturally occurring counterpart.

15 In one example, the present disclosure provides a nucleic acid encoding or expressing the fusion protein described herein.

In another example, the present disclosure provides a nucleic acid encoding or expressing a proneurotrophin and a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof. For example, the proneurotrophin is a proneurotrophin-3. In
20 one example, the proneurotrophin is a proneurotrophin-4. In one example, the proneurotrophin is a proBDNF. In one example, the proneurotrophin is a proNGF.

In some examples, the constituent Fc domain regions of a monomeric Ig Fc domain or fragment thereof are linked together by a linker.

Numerous expression construct or vector systems may be employed for the purposes of
25 this disclosure and can be prepared by any suitable method including, for example, cloning of appropriate sequences or by direct chemical synthesis by methods such as the phosphotriester method of Narang et al., *Meth. Enzymol.* 68:90-99, 1979; the phosphodiester method of Brown et al., *Meth. Enzymol.* 68:109-151, 1979; the diethylphosphoramidite method of Beaucage et al., *Tetra. Lett.* 22:1859-1862, 1981; the solid phase phosphoramidite triester method described
30 by Beaucage & Caruthers, *Tetra. Letts.* 22(20):1859-1862, 1981, using an automated synthesizer as described in, for example, Needham-VanDevanter et al., *Nucl. Acids Res.* 12:6159-6168, 1984; and, the solid support method of U.S. Pat. No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This can be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a
35 DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is generally limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Exemplary nucleic acids sequences encoding a monomeric Ig Fc domains can be prepared by cloning techniques. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook et al., supra, Berger and Kimmel (eds.), supra, and Ausubel, supra. Product information from manufacturers of biological reagents and experimental equipment also provide useful information. Such manufacturers include the SIGMA Chemical Company (Saint Louis, Mo.), R&D Systems (Minneapolis, Minn.), Pharmacia Amersham (Piscataway, N.J.), CLONTECH Laboratories, Inc. (Palo Alto, Calif.), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, Wis.), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, Md.), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen (San Diego, Calif.), and Applied Biosystems (Foster City, Calif.), Thermo Fisher Scientific as well as many other commercial sources known to one of skill.

Nucleic acids can also be prepared by amplification methods. Amplification methods include polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR). A wide variety of cloning methods, host cells, and in vitro amplification methodologies are well known to persons of skill.

The term "vector" or "expression vector" or "expression construct" is used herein interchangeably for the purposes of the specification and claims, to mean vectors used in accordance with the present disclosure as a vehicle for introducing into and expressing a desired gene in a cell. As known to those skilled in the art, such vectors may easily be selected from the group consisting of plasmids, phages, viruses and retroviruses.

More generally, once a vector or nucleic acid sequence encoding the fusion protein has been prepared, the expression vector may be introduced into an appropriate host cell. That is, the host cells may be transfected. Introduction of the expression vector into the host cell can be accomplished by various techniques well known to those of skill in the art. These include, but are not limited to, transfection, electroporation, protoplast fusion, cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A. A. G. "Mammalian Expression Vectors" Chapter 24.2, pp. 470-472 Vectors, Rodriguez and Denhardt, Eds. (Butterworths, Boston, Mass. 1988). Most preferably, vector introduction into the host cell is via transfection (e.g. calcium phosphate precipitation, FuGENE, Lipofectamine (trade names) and polyethylenimine (PEI)). The transformed host cells are grown under conditions appropriate to the production of the fusion protein, and assayed for fusion protein expression. The nucleic acid sequence encoding a fusion protein of the disclosure can be transiently transfected into a host cell. The cell line may also be created with stable integration of the nucleic acid sequence encoding the fusion protein together with a sequence encoding a selectable genetic marker e.g. glutamine synthetase (GS) or dihydrofolate reductase (DHFR), to allow the use of drug selection to derive cell lines producing the protein of interest.

Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or fluorescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

5 In certain examples, *in vitro* production can be “scaled-up” to give large amounts of a desired fusion protein. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography
10 methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-) affinity chromatography.

Isolation and purification of expressed fusion protein can be carried out by conventional means using, for example, preparative chromatography and immunological separations. Once expressed, the fusion protein can be purified according to standard procedures of the art,
15 including ammonium sulfate precipitation, affinity columns, column chromatography, and the like (see, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y., 1982 and Janson, Jan-Christer, *Protein Purification: Principles, High Resolution Methods, and Applications*, 3rd Edition, Wiley Series in Methods of Biochemical Analysis). Substantially pure compositions of at least about 90 to 95% homogeneity are disclosed herein, and 98 to 99% or more
20 homogeneity can be used for pharmaceutical purposes. Once purified, partially or to homogeneity as desired, if to be used therapeutically, the fusion protein should be substantially free of endotoxin.

Non-limiting examples of suitable mammalian cells include a HEK cell, a CHO cell, a BHK cell, a MDCK cell, a C3H 10T1/2 cell, a FLY I, a Psi-2 cell, a BOSC 23 cell, a PA317
25 cell, a WEHI cell, a COS cell, a BSC 1 cell, a BSC 40 cell, a BMT 10 cell, a VERO cell, a W138 cell, a MRC5 cell, a A549 cell, a HT1080 cell, a B-50 cell, a 3T3 cell, a NIH3T3 cell, a HepG2 cell, a Saos-2 cell, a Huh7 cell, a HeLa cell, a W163 cell, a 211 cell, a 211 A cell, and derivatives thereof.

In one example, the host cell is a HEK cell. For example, the HEK cell is HEK 293 cell.
30 In one example, the HEK cell is a Freestyle HEK 293-F cell. In another example, the HEK cell is a HEK 293T cell. In one example, the host cell is a CHO cell. In one example, the CHO cell is a ExpiCHO cell. In one example, the CHO cell is a CHOK1 cell. In one example, the host cell is a BHK cell. In one example, the host cell is a MDCK cell.

35 **Supraparticles**

The present disclosure provides a composition comprising a supraparticle, wherein the supraparticle comprises the fusion protein described herein. The present disclosure also

provides a composition comprising a supraparticle, wherein the supraparticle comprises the nucleic acid or expression vector described herein.

The term “supraparticle” is used in the context of the present disclosure to refer to agglomerated particles comprising a network of pores. The network of pores provides
5 supraparticles with a large pore volume and surface area for carrying a payload e.g., a fusion protein. A large pore volume and surface area is advantageous as it can enhance the amount of payload that can be carried by supraparticles. In an example, a supraparticle is agglomerated nanoparticles.

In an example, the supraparticle comprises at least 1.5 µg of a fusion protein. In another
10 example, the supraparticle comprises at least 2.0 µg of a fusion protein. In another example, the supraparticle comprises at least 3.0 µg of a fusion protein. In another example, the supraparticle comprises at least 4.0 µg of a fusion protein. In another example, the supraparticle comprises at least 5.0 µg of a fusion protein. In another example, the supraparticle comprises at least 6.0 µg of a fusion protein. In another example, the supraparticle comprises at least 7.0
15 µg of a fusion protein. In another example, the supraparticle comprises at least 8.0 µg of a fusion protein. In another example, the supraparticle comprises at least 9.0 µg of a fusion protein. In another example, the supraparticle comprises at least 10 µg of a fusion protein. In another example, the supraparticle comprises at least 11 µg of a fusion protein. In another example, the supraparticle comprises at least 12 µg of a fusion protein. In another example, the
20 supraparticle comprises at least 15 µg of a fusion protein. In another example, the supraparticle comprises at least 20 µg of a fusion protein.

In an example, the supraparticle comprises at least 1.5 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 2.0 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 3.0 µg of a nucleic
25 acid or expression vector. In another example, the supraparticle comprises at least 4.0 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 5.0 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 6.0 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 7.0 µg of a nucleic acid or expression vector. In another example, the
30 supraparticle comprises at least 8.0 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 9.0 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 10 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 11 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 12 µg of a nucleic
35 acid or expression vector. In another example, the supraparticle comprises at least 15 µg of a nucleic acid or expression vector. In another example, the supraparticle comprises at least 20 µg of a nucleic acid or expression vector.

Therapeutic efficacy may be improved by administering supraparticles comprising multiple different fusion proteins. Thus, in an example, supraparticles can comprise at least two, at least three, at least four, at least five different fusion proteins. For example, the supraparticle comprises a fusion protein comprising an NGF and a fusion protein comprising an NT-3. In another example, the supraparticle comprises a fusion protein comprising an NT-4 and a fusion protein comprising an NT-3. In a further example, the supraparticle comprises a fusion protein comprising a BDNF and a fusion protein comprising an NT-3.

Therapeutic efficacy may be improved by administering supraparticles comprising multiple different nucleic acids or expression vectors. Thus, in an example, supraparticles can comprise at least two, at least three, at least four, at least five different nucleic acids or expression vectors. For example, the supraparticle comprises a fusion protein comprising an NGF and a fusion protein comprising an NT-3. In another example, the supraparticle comprises a fusion protein comprising an NT-4 and a fusion protein comprising an NT-3. In a further example, the supraparticle comprises a fusion protein comprising a BDNF and a fusion protein comprising an NT-3.

In one example, the supraparticle comprises at least two payloads, one payload being the fusion protein, the other payload being any agent useful for treating a disorder. In one example, the supraparticle comprises at least two payloads, one payload being the nucleic acid or expression vector, the other payload being any agent useful for treating a disorder. Examples of agents include biological products such as polynucleotides, antibodies, monoclonal antibodies, antibody fragments, antibody-drug conjugates, proteins, biologically active proteins, fusion proteins, recombinant proteins, peptides, polypeptides, synthesized polypeptides, vaccines, therapeutic serums, viruses, polynucleotides, cells such as stem cells or parts thereof as well as small molecules. Exemplary agents include neurotrophic factors, steroids or antioxidants. Exemplary neurotrophic factors can include agents discussed above with known therapeutic efficacy for directly or indirectly enhancing survival of cells from the auditory system and/or their synaptic connections. For example, some exemplary neurotrophic factors include members of the ciliary neurotrophic factor (CNTF) family such as CNTF, Leukemia inhibitory factor (LIF), Interleukin-6 (IL-6), Glia maturation factor (GMF), insulin-like growth factor-1 (IGF-1), Neuregulin 1, Neuregulin 2, Neuregulin 3 and Neuregulin 4, vascular endothelial growth factor (VEGF), members of the Glial Cell Derived Neurotrophic Factor (GDNF) family such as GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN), ephrins such as A1, A2, A3, A4, A5, B1, B2 and B3, interleukins such as IL-11, antibodies or other binding proteins such as anti-Tropomyosin receptor kinase (Trk)B, anti-TrkC or binding proteins that interact with p75 neurotrophin receptor. For example, p75 neurotrophin receptor antagonists. In another example, neurotrophic factors include nucleic acids. For example, the neurotrophic factor can comprise a gene therapy, silencing RNA such as a siRNA or miRNA, expression constructs such as DNA plasmids comprising a nucleic acid

of interest. In an example, the neurotrophic factor is an expression construct comprising a nucleic acid encoding an opsin(s). In an example, supraparticles can comprise antineoplastic agents including cisplatin or related compounds, antibiotics including aminoglycosides such as tobramycin or related compounds, loop diuretics such as furosemide, antimetabolites such as methotrexate, salicylates such as aspirin or a radioactive moiety and a neurotrophin.

Various otic interventions such as surgical procedures and implantation of hearing devices can result in side effects such as tissue damage, inflammation and/or infection in the middle and inner ear. Biological response(s) mounted against such side effects can indirectly affect the growth or survival potential of cells from the auditory system and/or their synaptic connections. Thus, in an example, the fusion proteins of the disclosure assist in tissue repair, reducing inflammation and/or reducing infection.

In an example, supraparticles of the present disclosure are produced from nanoparticles having a diameter of between about 1 nm and 100 nm. In another example, supraparticles are produced from microparticles having a diameter of between about 0.1 μm and 100 μm . In another example, supraparticles are produced from nanoparticles and microparticles.

Exemplary particles forming the supraparticles of the present disclosure include organic particles, inorganic particles, metal particles or a combination thereof. Exemplary organic particles include polymeric particles such as polyglycolic acid (PGA), polylactic acid (PLA), poly(methacrylic acid), poly(ethacrylic acid), polyacrylic acid (PAA), poly(N-isopropylacrylamide), poly(N,N-dimethylacrylamide), polyamides, poly-2-hydroxy butyrate (PHB), gelatines, polycaprolactone (PCL), and poly (lactic-co-glycolic acid) (PLGA). Exemplary inorganic particles include mineral fillers such as heavy fillers or high density fillers, pigments, clays and other synthetic particles. Other exemplary inorganic particles include dense minerals such as barite, hematite, magnesium oxide, inorganic oxides including titanium dioxide, calcium oxide, zinc oxide, magnesium oxide, cerium oxide, zirconium dioxide, and silicon dioxide. In an example, the material is silicon dioxide (i.e. silica). Thus, in an example, the supraparticles can be referred to as silica supraparticles. Exemplary metal particles include gold, silver, and copper. In one example, supraparticles may comprise the same particles. For example, supraparticles can substantially consist of silica particles. In another example, supraparticles may comprise different particles; for example silica and clay particles. In other examples, supraparticles can comprise at least three, at least four, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 different particles.

In an example, supraparticles comprise polyelectrolytes or polyelectrolyte material. Examples of such supraparticles are disclosed in WO 2006/037160. In this example, the polyelectrolyte may be a positively charged polyelectrolyte (or have the ability to be positively charged) or a negatively charged polyelectrolyte (or have the ability to be negatively charged) or have a zero net charge.

Supraparticles of the present disclosure can have various shapes. For example, supraparticles can have a spherical shape. Exemplary spherical shapes include spheres and ovoids. In another example, supraparticles have a non-spherical shape. Exemplary non-spherical shapes include dumbbell, hemisphere, disc, tetrahedron, fibre, spherocylinder and irregular shapes. In an example, supraparticles can have an ordered structure. For example, supraparticles may comprise an ordered array.

Spherical supraparticles of the present disclosure may be characterised by their diameter. For example, supraparticles of the present disclosure have a diameter greater than 100 μm . For example, supraparticles of the present disclosure can have a diameter of at least about 150 μm , about 200 μm , about 250 μm , about 300 μm , about 350 μm , about 400 μm , about 450 μm , about 500 μm , about 550 μm , about 600 μm , about 650 μm , about 700 μm , about 750 μm , about 800 μm , about 850 μm , about 900 μm , about 950 μm , about 1000 μm . For example, supraparticles can have a diameter of about 550 μm . This diameter of supraparticle is advantageous as it allows for high drug loading while facilitating inner ear delivery via cannula. In other examples, supraparticles can have a diameter of between about 150 μm and about 1000 μm , about 200 μm and about 900 μm , about 300 μm and about 800 μm . In another example, supraparticles can have a diameter of between about 400 μm and about 600 μm . In another example, supraparticles can have a diameter of between about 450 μm and about 550 μm . In another example, supraparticles can have a diameter of between about 520 μm and about 580 μm . In another example, supraparticles can have a diameter of between about 460 μm and about 540 μm . In another example, supraparticles can have a diameter of between about 470 μm and about 530 μm . In another example, supraparticles can have a diameter of between about 480 μm and about 520 μm . In another example, supraparticles can have a diameter of between about 490 μm and about 510 μm . In other examples, supraparticles can be characterised by the width across the widest point of their three dimensional structure. For example, supraparticles can have a width consistent with the above exemplified diameters.

In an example, the supraparticles can be manufactured from a composition comprising nanoparticles and alginic acid or a polysaccharide derivative thereof. Various examples of nanoparticles are provided above. In an example, the nanoparticles have a bimodal pore structure. Nanoparticles can be produced using various methods. One such method is described in Cui et al. 2015, ACS Nano, 9, 1571-1580.

In another example, supraparticles according to the present disclosure are produced by electrospraying. Examples of electrospraying are reviewed in Jaworek A., 2007 Powder Technology 1, 18 – 35. An example of electrospraying is also exemplified below. In an example, the present disclosure encompasses a method of manufacturing supraparticles, the method comprising electrospraying a composition comprising nanoparticles and alginic acid or a polysaccharide derivative thereof into a di-cationic aqueous solution.

One of skill in the art will appreciate that electrospray parameters can be optimized based on the type of the solution used for electro spraying. For example, voltage and flow rates can be optimised to provide supraparticles of a desired size. In an example, the flow rate is about 6 - 10 mL h⁻¹. In another example, the flow rate is about 7 - 9 mL h⁻¹. In another example, the flow rate is about 8 mL h⁻¹. In an example, the voltage is between about 10 and 25 KV. In another example, the voltage is between about 11 and 20 KV. In another example, the voltage is between about 12 and 14 KV. In another example, the voltage is about 13 KV.

In an example, supraparticles are produced by electrospraying a nanoparticle solution. In an example, the concentration of nanoparticles in solution is about 20 mg/mL. In an example, the concentration of nanoparticles in solution is about 30 mg/mL. In an example, the concentration of nanoparticles in solution is about 40 mg/mL. In an example, the concentration of nanoparticles in solution is about 50 mg/mL. In an example, the concentration of nanoparticles in solution is about 60 mg/mL.

In another example, supraparticles are produced by electrospraying a nanoparticle solution comprising alginic acid or a derivative thereof. In an example, the nanoparticle solution is prepared from 5 mg mL⁻¹ alginic acid solution. In an example, the nanoparticle solution is prepared from 10 mg mL⁻¹ alginic acid solution. In an example, the nanoparticle solution is prepared from 20 mg mL⁻¹ alginic acid solution. In an example, the nanoparticle solution is prepared from 30 mg mL⁻¹ alginic acid solution. In another example, the nanoparticle solution is prepared from 5 mg mL⁻¹ to 30 mg mL⁻¹ alginic acid solution. In another example, the nanoparticle solution is prepared from 10 mg mL⁻¹ to 30 mg mL⁻¹ alginic acid solution. In another example, the nanoparticle solution is prepared from 20 mg mL⁻¹ to 30 mg mL⁻¹ alginic acid solution.

In an example, the nanoparticle solution is prepared from 5 mg mL⁻¹ alginic acid in water. In an example, the nanoparticle solution is prepared from 10 mg mL⁻¹ alginic acid in water. In an example, the nanoparticle solution is prepared from 20 mg mL⁻¹ alginic acid in water. In an example, the nanoparticle solution is prepared from 30 mg mL⁻¹ alginic acid in water. In another example, the nanoparticle solution is prepared from 5 mg mL⁻¹ to 30 mg mL⁻¹ alginic acid in water. In another example, the nanoparticle solution is prepared from 10 mg mL⁻¹ to 30 mg mL⁻¹ alginic acid in water. In another example, the nanoparticle solution is prepared from 20 mg mL⁻¹ to 30 mg mL⁻¹ alginic acid in water.

In another example, supraparticles are produced by electrospraying a nanoparticle solution comprising alginic acid.

Alginic acid derivatives are not particularly limited so long as they form a gel at a defined temperature. In an example, the alginic acid derivative is a polysaccharide derivative. Alginic acid derivatives include various alginic acid salt forms. Examples include sodium alginate, potassium alginate and calcium alginate. Other examples include barium alginate and

strontium alginate. In an example, the alginic acid is sodium alginate. In another example, supraparticles are produced by electrospraying a nanoparticle solution comprising alginic acid.

Alginates of various viscosities may be used to produce supraparticles according to the present disclosure depending on the desired size and shape of supraparticle. For example, 5 alginate having a viscosity of about 20 to 300 mPa*s can be used. In another example, the alginate has a viscosity of about 20 to 200 mPa*s. In an example, the alginate has a viscosity of 20 mPa*s. In another example, the alginate has a viscosity of 100 mPa*s. In another example, the alginate has a viscosity of 200 mPa*s.

10 In an example, supraparticles are produced by electrospraying a composition comprising nanoparticles into an aqueous solution. In an example, this is a di-cationic aqueous solution. Exemplary di-cationic components include Ca^{2+} and Ba^{2+} . For example, the aqueous solution can comprise calcium chloride. In another example, the aqueous solution comprises barium chloride.

15 In an example, supraparticles are produced by electrospraying a composition wherein the concentration of alginate in the composition is 5 mg mL^{-1} to 30 mg mL^{-1} , the concentration of nanoparticles in the composition is 20 mg mL^{-1} to 50 mg mL^{-1} and the voltage is 10 kV to 25 kV. In another example, supraparticles are produced by electrospraying a composition wherein the concentration of alginate in the composition is 10 mg mL^{-1} to 30 mg mL^{-1} , the concentration of nanoparticles in the composition is 30 mg mL^{-1} to 50 mg mL^{-1} and the voltage 20 is 11 kV to 21 kV. In another example, supraparticles are produced by electrospraying a composition wherein the concentration of alginate in the composition is 20 mg mL^{-1} to 30 mg mL^{-1} , the concentration of nanoparticles in the composition is 35 mg mL^{-1} to 45 mg mL^{-1} and the voltage is 12 kV to 14 kV. In these examples, the flow rate can be 8 mL h^{-1} .

25 In another example, supraparticles are produced by electrospraying a composition wherein the concentration of alginate in the composition is 30 mg mL^{-1} , the concentration of nanoparticles in the composition is 40 mg mL^{-1} , the voltage is 13kV, and the flow rate is 8 mL h^{-1} .

30 In an example, supraparticles manufactured using methods defined herein are subjected to calcination to remove alginic acid or a derivative thereof. In an example, calcination is performed at around 500°C . In another example, calcination is performed at around 550°C . . In another example, calcination is performed at around 600°C . In another example, calcination is performed at around 650°C . In another example, calcination is performed at around 700°C . In an example, calcination is performed for about 6 to about 30 hours. In another example, calcination is performed for about 10 hours. In another example, calcination is performed for 35 about 20 hours. In another example, calcination is performed for about 30 hours.

Methods of producing supraparticles loaded with fusion proteins are not particularly limited so long as the resulting supraparticle can be loaded with at least $1.5 \mu\text{g}$ of a fusion protein. Preferably, the resulting supraparticle can deliver the payload to an ear of a subject.

Exemplary methods of loading are reviewed in Wang et al. (2009) J. Mater. Chem. 19, 6451 and include fusion protein encapsulation and entrapment. In one non-limiting example, supraparticles may be loaded by contacting the supraparticle with an aqueous solution of the fusion protein followed by a period of incubation. The fusion protein solution can contain an excess of the amount of fusion protein to be loaded onto the supraparticle and incubation can occur at room temperature. Agitation of the solution containing the supraparticle and the fusion protein may be used to enhance loading of the fusion protein.

One of skill in the art will appreciate that the required level of fusion protein will likely be influenced by the fusion protein itself and the indication being treated according to the present disclosure.

Supraparticles can have a pore size selected from the examples discussed below. In an example, a supraparticle is microporous. The term “microporous” is used in the context of the present disclosure to refer to particles having a pore size of less than about 2 nm. For example, microporous supraparticles can have a pore size of about 0.5 nm to about 2 nm. In other examples, a microporous supraparticle has a pore size of about 1 nm to about 2 nm, about 1.5 nm to about 2 nm. In another example, a supraparticle is mesoporous. The term “mesoporous” is used in the context of the present disclosure to refer to particles having pores with diameters between about 2 nm and about 50 nm. For example, a mesoporous supraparticle has a pore size of about 2 nm to about 50 nm. In other examples, a mesoporous supraparticle has a pore size of about 2 nm to about 40 nm, about 2 nm to about 30 nm. In another example, the supraparticle is macroporous. The term “macroporous” is used in the context of the present disclosure to refer to particles having a pore size of greater than about 50 nm. For example, a macroporous supraparticle has a pore size of about 50 nm to about 500 nm. In other examples, a macroporous supraparticle has a pore size of about 50 nm to about 250 nm, about 50 nm to about 150 nm, about 50 nm to about 100 nm.

In an example, the supraparticle is comprised of microporous nanoparticles. In an example, the supraparticle is comprised of nanoparticles having a pore size of about 0.5 nm to about 2 nm. In other examples, the supraparticle is comprised of nanoparticles having a pore size of about 1 nm to about 2 nm, about 1.5 nm to about 2 nm. In another example, the supraparticle is comprised of mesoporous nanoparticles. In an example, the supraparticle is comprised of nanoparticles having a pore size of about 2 nm to about 50 nm. In other examples, the supraparticle is comprised of nanoparticles having a pore size of about 2 nm to about 40 nm, about 2 nm to about 30 nm. In another example, the supraparticle is comprised of macroporous nanoparticles. In an example, the supraparticle is comprised of nanoparticles having a pore size of about 50 nm to about 95 nm. In other examples, the supraparticle is comprised of nanoparticles having a pore size of about 50 nm to about 85 nm, about 50 nm to about 75 nm, about 50 nm to about 65 nm.

In other examples, the supraparticle comprises pore sizes ranging from 1 nm to 200 nm. It will be understood by the person skilled in the art that supraparticles can comprise a range of pore sizes. For example, a single supraparticle can be microporous, mesoporous and macroporous.

5 It will be appreciated by the person skilled in the art that supraparticle pore size can be measured by, for example, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and X-Ray computed tomography. One of skill in the art can identify supraparticles having the above exemplified pore sizes by measuring the width across the widest point of their three dimensional structure. In an example, the widest point or a pore may
10 be at the surface of the supraparticle.

Uses of Fusion Proteins

The present disclosure provides a method for promoting survival of spiral ganglion neurons in an ear of a subject, the method comprising administering the fusion protein or the
15 composition described herein. The present disclosure also provides a method of treating an auditory disorder in a subject, the method comprising administering the fusion protein or the composition described herein. The present disclosure provides a method for promoting survival of spiral ganglion neurons in an ear of a subject, the method comprising administering the nucleic acid or expression vector described herein. The present disclosure also provides a
20 method of treating an auditory disorder in a subject, the method comprising administering the nucleic acid or expression vector described herein.

The present disclosure also provides use of the fusion protein or the composition described herein in the manufacture of a medicament for promoting survival of spiral ganglion neurons in an ear of a subject. The present disclosure further provides use of the fusion protein
25 or the composition described herein in the manufacture of a medicament for the treatment of an auditory disorder. The present disclosure also provides use of the nucleic acid or expression vector described herein in the manufacture of a medicament for promoting survival of spiral ganglion neurons in an ear of a subject. The present disclosure further provides use of the nucleic acid or expression vector described herein in the manufacture of a medicament for the
30 treatment of an auditory disorder.

The present disclosure also provides the fusion protein or the composition described herein for use in promoting survival of spiral ganglion neurons in an ear of a subject. The present disclosure also provides the fusion protein or the composition described herein for use in the treatment of an auditory disorder. The present disclosure also provides the nucleic acid
35 or expression vector described herein for use in promoting survival of spiral ganglion neurons in an ear of a subject. The present disclosure also provides the nucleic acid or expression vector described herein for use in the treatment of an auditory disorder.

Accordingly, in an example, fusion proteins or compositions according to the present disclosure are administered to a subject in an amount effective to treat a disease or disorder in the subject. In another example, nucleic acids or expression vectors according to the present disclosure are administered to a subject in an amount effective to treat a disease or disorder in the subject. In an example, the disease or disorder is hearing loss. The term “hearing loss” is used in the context of the present disclosure to refer to any reduction in a subject's ability to detect or process sound. Thus, reference to hearing loss encompasses a partial hearing deficit or total inability to hear.

In some examples, the fusion protein treats hearing loss. In some examples, the composition, nucleic acids and expression vectors described herein treat hearing loss. In such examples, treatment repairs synaptic connections in the cochlea of a subject.

In an example, the hearing loss is characterised as sensorineural hearing loss (SNHL). SNHL is used in the context of the present disclosure to refer to hearing loss resulting from damage to the delicate sensory hair cells within the cochlea, or loss of their synaptic connections with spiral ganglion neurons (SGNs) or dysfunction of the cochlear Schwann cells or glial cells. In an example, the hearing loss is characterised as presbycusis. In another example, the hearing loss is noise induced. In another example, the hearing loss is disease induced or genetic. In another example, the hearing loss is induced by exposure to ototoxins, for example aminoglycosides.

In an example, the subject is a mammal. In one example, the subject is a human. For example, the human subject can be an adult. In an example, the human subject is a child. Other exemplary mammalian subjects include companion animals such as dogs or cats, or livestock animals such as horses or cows. Terms such as “subject”, “patient” or “individual” are terms that can, in context, be used interchangeably in the present disclosure.

In an example, a fusion protein or composition according to the present disclosure are administered intraperitoneally. The present disclosure also encompasses methods of delivering a fusion protein or composition to a cell, tissue or organ in a subject via the ear, the method comprising administering to the ear of a subject a fusion protein or composition according to the present disclosure. In this example, the method may deliver a fusion protein or composition to a cell from a subject's inner ear, middle ear and/or vestibular system. In another example, the method delivers a fusion protein or composition to a neural cell, neural tissue or the brain of a subject. In an example, a nucleic acid or expression vector according to the present disclosure are administered intraperitoneally. The present disclosure also encompasses methods of delivering a nucleic acid or expression vector to a cell, tissue or organ in a subject via the ear, the method comprising administering to the ear of a subject a nucleic acid or expression vector according to the present disclosure. In this example, the method may deliver a nucleic acid or expression vector to a cell from a subject's inner ear, middle ear and/or vestibular system. In another example, the method delivers a nucleic acid or expression vector

to a neural cell, neural tissue or the brain of a subject. In an example, a fusion protein or composition is administered onto the tympanic membrane. In this example, a fusion protein or composition may be formulated for topical administration (e.g. drops, gels, foams, sprays). In an example, a nucleic acid or expression vector is administered onto the tympanic membrane. In this example, a nucleic acid or expression vector may be formulated for topical administration (e.g. drops, gels, foams, sprays).

In another example, compositions of the disclosure are administered to the “middle ear” cavity. In another example, fusion proteins, nucleic acids or expression vectors of the disclosure are administered to the “middle ear” cavity. The term “middle ear” in the context of the present disclosure is used to refer to the space between the tympanic membrane and the inner ear. Thus, the middle ear is external to all inner ear tissue. For example, a fusion protein or composition can be administered to the middle ear via injection through the tympanic membrane. In this example, a fusion protein or composition may be administered as a depot injection. In another example, an opening in the tympanic membrane can be produced by a treating clinician to facilitate access of a fusion protein or composition to the middle ear. When administering a fusion protein or composition to the middle ear, the fusion protein or composition can be administered onto the round and/or oval window(s). For example, the nucleic acid or expression vector can be administered to the middle ear via injection through the tympanic membrane. In this example, the nucleic acid or expression vector may be administered as a depot injection. In another example, an opening in the tympanic membrane can be produced by a treating clinician to facilitate access of the nucleic acid or expression vector to the middle ear. When administering the nucleic acid or expression vector to the middle ear, the nucleic acid or expression vector can be administered onto the round and/or oval window(s).

In another example, the fusion protein or composition is administered into the inner ear. For example, the fusion protein or composition can be administered to the cochlea. In an example, the fusion protein or composition can be administered to the basal turn of the cochlea. In another example, the nucleic acid or expression vector is administered into the inner ear. For example, the nucleic acid or expression vector can be administered to the cochlea. In an example, the nucleic acid or expression vector can be administered to the basal turn of the cochlea. Surgical techniques to gain access to the cochlea or other structures of the inner ear are known in the art. Exemplary techniques for surgically accessing the human cochlea are described in, for example, Clark GM, et al., "Surgery for an improved multiple-channel cochlear implant", Ann Otol Rhinol Laryngol 93:204-7, 1984, and in Clark GM, et al., "Surgical and safety considerations of multichannel cochlear implants in children", Ear and Hearing Suppl. 12:15S-24S, 1991.

Various otic interventions such as surgical procedures and implantation of hearing devices can result in side effects such as tissue damage, inflammation and/or infection in the

middle and inner ear. Biological response(s) mounted against such side effects can indirectly affect the growth or survival potential of cells from the auditory system and/or their synaptic connections. Thus, in an example, neurotrophins assist in tissue repair, reducing inflammation and/or reducing infection.

5 Combination of the fusion protein or composition with otic intervention is discussed above. In these examples, otic intervention may occur simultaneously with administration of the fusion protein. For example, a cochlear device can be implanted simultaneously with the fusion protein. However, increased survival of spiral ganglion neurons can improve the utility of a cochlear implant. Thus, it may be desirable to implant a cochlear device after the fusion
10 protein has been administered. For example, a cochlear device can be implanted about one month, about two months, about three months, about six months after the fusion protein has been administered. In these examples, additional fusion proteins can be administered with the cochlear device.

 In an example, a first dose of the fusion protein or composition is administered to the
15 subject's cochlea and at least a second dose of the fusion protein or composition is administered onto the subject's round and/or oval window(s).

 Combination of the nucleic acid or expression vector with otic intervention is discussed above. In these examples, otic intervention may occur simultaneously with administration of the nucleic acid or expression vector. For example, a cochlear device can be implanted
20 simultaneously with the nucleic acid or expression vector. However, increased survival of spiral ganglion neurons can improve the utility of a cochlear implant. Thus, it may be desirable to implant a cochlear device after the nucleic acid or expression vector has been administered. For example, a cochlear device can be implanted about one month, about two months, about three months, about six months after the nucleic acid or expression vector has been
25 administered. In these examples, additional fusion proteins can be administered with the cochlear device.

 In an example, a first dose of the nucleic acid or expression vector is administered to the subject's cochlea and at least a second dose of the nucleic acid or expression vector is administered onto the subject's round and/or oval window(s).

30 In an example, a therapeutically effective amount of the fusion protein or composition is administered to an ear of the subject. In an example, multiple fusion proteins or compositions are administered to an ear of the subject. For example, at least two, at least three, at least four, at least five, at least ten, at least 20 fusion proteins or compositions can be administered to an ear of the subject. In another example, about one to 10 fusion proteins or compositions are
35 administered. In other examples about two to 9, about three to 8, about four to 7, about 5 to 6 fusion proteins or compositions are administered to an ear of the subject. In an example, a therapeutically effective amount of the nucleic acid or expression vector is administered to an ear of the subject. In an example, multiple nucleic acids or expression vectors are administered

to an ear of the subject. For example, at least two, at least three, at least four, at least five, at least ten, at least 20 nucleic acids or expression vectors can be administered to an ear of the subject. In another example, about one to 10 nucleic acids or expression vectors are administered. In other examples about two to 9, about three to 8, about four to 7, about 5 to 6
5 nucleic acids or expression vectors are administered to an ear of the subject.

One skilled in the art would be able, by routine experimentation, to determine what an effective, non-toxic amount of a fusion protein, composition, nucleic acid or expression vector would be for the purpose of treating an auditory disorder. For example, a therapeutically active amount of a fusion protein, composition, nucleic acid or expression vector may vary according
10 to factors such as the disease stage and weight of the subject, and the ability of the fusion protein to elicit a desired response in the subject. The dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. Generally, however, an effective dosage is expected to be in the
15 range of about 1 to 200 mg/kg body weight.

Kits and Other Compositions of Matter

Another example of the disclosure provides kits containing a fusion protein, composition, nucleic acid or expression vector of the present disclosure useful promoting
20 survival of spiral ganglion neurons in an ear of a subject in need thereof, the kit comprising:

- (i) at least one fusion protein, composition, nucleic acid or expression vector of the disclosure;
- (ii) instructions for using the kit for promoting survival of spiral ganglion neurons in the ear of the subject; and
- 25 (iii) optionally, at least one additional therapy.

The disclosure also provides a kit for treating an auditory disorder in a subject in need thereof, the kit comprising:

- (i) at least one fusion protein, composition, nucleic acid or expression vector of the disclosure;
- 30 (ii) instructions for using the kit for promoting survival of spiral ganglion neurons in the ear of the subject; and
- (iii) optionally, at least one additional therapy.

In accordance with this example of the disclosure, the instructions (or package insert) is on or associated with the container. Suitable containers include, for example, bottles, vials,
35 syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The label or package insert indicates that the fusion protein, composition, nucleic acid or expression vector is used for treating a subject eligible for treatment, e.g., one having or predisposed to developing a condition described herein, with specific guidance regarding

dosing amounts and intervals of the fusion protein, composition, nucleic acid or expression vector and any other medicament being provided. The kit may further comprise an additional container comprising a pharmaceutically acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution, and/or dextrose solution.

- 5 The kit may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The kit optionally further comprises a container comprising a second medicament, wherein the fusion protein, composition, nucleic acid or expression vector is a first medicament, and which article further comprises instructions on the package insert for treating
10 the subject with the second medicament, in an effective amount. The second medicament may be a therapeutic protein set forth above.

The present disclosure includes the following non-limiting Examples.

15 **Example 1: Production and purification of neurotrophin-3 fusion protein in HEK293Freestyle Cells**

Cell culture and transfections

A codon-optimised synthetic DNA fragment was synthesised by GenScript and sub-cloned into a modified pCAGGS mammalian expression vector (Miyazaki et.al., Gene 79:269-
20 277, 1989) via the SacI-XhoI restriction enzyme sites (Figure 15). HEK293T cells were grown as adherent cultures in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) with 10% v/v foetal bovine serum (Serana) and 1x GlutaMAX™ Supplement (Thermo Fisher Scientific) in a humidified incubator at 37°C and 5% CO₂. For both transient transfection, 4 x 10⁵ cells in 2 mL were seeded into a 6-well tissue culture plate the day before transfection.
25 Prior to transfection, the culture volume was reduced to 1 mL and transfection performed using 1 µg plasmid DNA and 4 µL of FuGENE® Transfection Reagent (Promega) according to the recommended protocol. After 24 hours, the culture medium was replaced with 1mL of serum-free Freestyle 293 Expression Medium (Thermo Fisher Scientific) and harvested after a further 48 hours of culture, clarified by a brief centrifugation and stored at -20°C.

30 Freestyle HEK 293-F cells (Thermo Fisher Scientific) were grown as suspension cultures in Freestyle 293 Expression Medium using a humidified shaking incubator (37°C, 5% CO₂, 130 rpm). The cells were maintained and scaled-up to 1,800 mL with a viable cell density (ViCD) of 2.1x10⁶ cells/mL in Erlenmeyer shaker flasks (Corning) for setting up transfection experiment using polyethylenimine (PEI, Polysciences) at a ratio of 1:3 [DNA:PEI] with 1 µg
35 plasmid per mL of culture. The transfection mix was prepared by adding the plasmid DNA solution to warm (37°C) PBS and then the PEI solution was added followed by gentle mixing and incubation for 15 minutes at room temperature.

Protein production was carried out according to the standard protocol developed by CSIRO. The culture was maintained in a humidified incubator (37°C, 5% CO₂, 130 rpm) for the duration of the production period (9 days). At the end of production, cells were removed by centrifugation 4000rpm for 20 min at 4°C and 0.2 µm filtered (Nalgene Rapid Flow Filter) to collect protein supernatant prior to freezing at -80°C.

Affinity Chromatography

Prior to affinity chromatography, systems were cleaned/sanitised with 1 M NaOH + 2 M NaCl including a 30-minute incubation. Alkali-resistant columns were also treated with 1 M NaOH and allowed to be sanitised over a 30-minute period. Chromatogram displaying the elution profile of NT3-Fc is shown in Figure 1. The elution occurred as a single elution peak, with a small shoulder. The UV detector was at saturation. The area shaded in was pooled, ready for loading onto the Superdex 200 pg 26/600 column.

Table 1. Column and run information

Column	MabSelect PrismA (Cytiva)
Column volume (CV)	2x 5 mL
Column sanitisation	1 M NaOH + 2 M NaCl
Load (volume)	Clarified HEK293F supernatant (~2 L)
Equilibration solution(s)	1x PBS pH 7.4
Elution	0.1 M Citrate pH 3.0, 0.15 M NaCl (neutralised with 1/6th vol 3 M Tris pH 8)

Table 2. Spectrophotometric analysis A_{280nm} (E0.1% = 1.696)

Run type	Pooled fractions	Volume (mL) + 3 M Tris	mg/mL	mg total
Affinity	5.A.4-5.B.5	18	4.93	88.74

Preparative size exclusion chromatography (SEC)

Calibration of the column conformed to expected specifications. The total protein volume of 32 mL was divided into three portions, each portion of ~10 mL for loading onto the Superdex 200 pg 26/600 column. There were a total of three SEC runs. The elution profile of each SEC run was identical, in 1x PBS. There was a major peak eluting at the expected volume for NT3-Fc, with minor aggregate peaks eluting in the void volume and as a shoulder before the main peak (Figure 2).

Table 3. Spectrophotometric analysis A_{280nm} (E0.1% = 1.696) in 1xPBS

Run	Pool fracs	mL	mg/mL	mg
SEC #1	5.H.2 – 6.A.6	32	0.8	64
SEC #2	5.H.1 – 6.A.5	20		
SEC #3	5.G.4 – 5.H.3	28		
SEC not concentrated		80	0.8	64
SEC concentrated		25	2.19	54.75

Fractions from the main peak were pooled and combined across the 3 SEC runs. From the pooled fractions (~80 mL), the concentration was measured to be ~0.8 mg/mL. The sample was concentrated on a 10 kDa MWCO spin filter to a volume of ~25 mL, then filtered aseptically through 0.2 µm. The concentration of the final concentrated sample was 2.19 mg/mL. An estimated ~10mg was lost on concentrating which is an expected loss for this step.

Desalt exchange into 1/4 x PBS

The purpose of running a final desalting column was to buffer exchange from 1xPBS to 1/4x PBS (~2.5 mM phosphate and 35 mM NaCl). The broad elution and indistinct peak shape were due to protein overloading (Figure 3). Protein was suitably buffer exchanged as noted by the lower conductivity trace of the 1/4x PBS for the NT3-Fc elution peak vs 1x PBS which elutes between 80-130 mL (small peak = conductivity trace).

Table 4. Spectrophotometric analysis A_{280nm} (E0.1% = 1.696) in 1/4xPBS

Run	Pool frags	mL	mg/mL	mg
Desalting	5.C.4 – 5.F.5	32	1.59	50.88

15

SDS-PAGE Analysis

All samples were incubated at 95°C for five minutes prior to gel loading. Gels were NuPAGE BIS-TRIS 4-12%, MES-SDS buffer, run at 200 V for 40 minutes. Six samples equivalent to 5 µg (Affinity load, flow through and eluate in both 1x PBS and 1/4x PBS) were collected and either treated with (R) or without (NR) the reducing agent (DTT).

A faint band corresponding to ~40 kDa may indicate NT3-Fc was found in the culture supernatant (Affinity load, Figure 4A). There was a difference in protein band intensity of the indicated NT3-Fc (NR) when comparing Affinity load and Affinity flow through (see ~40 kDa, lanes 1 vs 2) indicating complete affinity capture. The elution profile was as anticipated. The affinity eluate in the non-reduced form displayed a small degree of band laddering at higher molecular weights (lane 4), however, it was not visible in the reduced form (lanes 5 & 6). A faint band corresponding to ~75 kDa in the affinity elute possibly indicated the dimer form of NT3-Fc. Despite band laddering, the NT3-Fc sample was pure with no other contaminating protein.

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Mass Spectrometric Analysis

MS analysis of the untreated sample yielded multiple masses around 42289 Da, separated by hexose and N-acetyl hexose masses (Figure 5). The sample was subsequently deglycosylated with PNGaseF and analysed by rpHPLC-MS (reverse-phase liquid chromatography coupled to electrospray mass spectrometry). The sample was analysed both reduced and non-reduced.

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The deglycosylated sample yielded a mass of 37604 Da (Figure 6), ~128 Da less than the anticipated mass of 37731.55 Da. This apparent difference was explained by the loss of C-terminal Lysine, a common modification that occurs for antibodies or Fc-fusions.

5 Endotoxin Testing

 The pooled and filtered protein sample was diluted 1:10 with fresh sterile MilliQ (MQ) water, previously tested and passed for endotoxin levels. The Endotoxin level was measured using an Endosafe® PTS assay. Cartridge Sensitivity 5 - 0.05 EU/mL

10 **Table 5. Endotoxin Level**

MQ water H ₂ O	<0.050 EU/mL
Concentrated NT3-Fc	<0.516 EU/mL

Final product detail and dispensing

 Approximately 54 mg NT3-Fc protein was captured by a three-step purification regime: Protein-A (PrismaA) followed by preparative SEC (Superdex 200 pg 26/600), and final desalt
15 exchange in ¼x PBS buffer. Due to the high concentration of the sample, multiple preparative SEC steps were performed. All preparative SEC runs were pooled. The pooled sample displayed a uniform profile when analysed by analytical SEC. The final PBS buffer was diluted into ¼ x PBS (2.5 mM phosphate, 35 mM NaCl). Intact-MS shows NT3-Fc is of expected size when accounting for loss of terminal lysine. Final samples were filtered through a 0.2 µm filter
20 into 50, 100 and 500 µL aliquots and stored at 4 °C. The final samples passed endotoxin testing.

Example 2: Production and purification of neurotrophin-3 fusion protein in ExpiCHO Cells

Cell culture and transfections

25 A codon-optimised synthetic DNA fragment was synthesised by GenScript and sub-cloned into a modified pCAGGS mammalian expression vector (Miyazaki et.al., Gene 79:269-277, 1989) via the SacI-XhoI restriction enzyme sites (Figure 15).

ExpiCHO Cells

30 Transient transfection of suspension-adapted cultures of ExpiCHO cells (ThermoFisher Scientific) was performed using Thermo Fisher Scientific ExpiCHO-S™ Expression System (Cat # A29129). Cell culture, growth parameters, as well as transient expression and feeding, were as per the manufacturer’s recommendations. Briefly, a vial of ExpiCHO-S™ cells was thawed into ExpiCHO Expression Medium (Cat # A2910002) in a 125 mL shake and cultured
35 in a humidified and shaking CO₂ incubator (37°C, 5% CO₂, 130 rpm). The culture was routinely passaged and expanded every 3 – 4 days to reach the required transfection volume.

- On the day of transfection, the cell density was adjusted to 6.0×10^6 cells/mL and transferred into a 500 mL shake flask, with a total cell volume of 100 mL, ready for transfection. The transfection was performed as per the manufacturer's recommendation with plasmid DNA (a final concentration of 1 mg DNA per litre of culture). Protein production was carried out using the standard feeding regime as per the manufacturer's protocol. The culture supernatant was harvested nine days post-transfection by centrifugation at 4000 rpm for 30 mins at 4°C under low endotoxin conditions, followed by 0.2 µm sterile filtration and storage at 4°C.

Affinity Chromatography

- NT3-Fc purification from ExpiCHO_S culture supernatant was performed using a similar methodology to that described in Example 1.

Protein elution from PrismA occurred as a single elution peak, with a jagged peak due to the saturation of the UV detector (Figure 7).

Table 6. Column and run information

Column	HiTrap MabSelect PrismA
Column volume (CV)	5 mL
Column sanitisation	1 M NaOH + 2 M NaCl (30 min incubation)
Load (volume)	~120 mL
Equilibration solution(s)	1xPBS pH 7.4
Elution	0.1 M Citrate pH 3.5, 0.15 M NaCl (neutralised with 1/6th vol 3 M Tris pH 8.0)

Table 7. Spectrophotometric analysis A_{280nm} ($E_{0.1\%} = 1.696$)

Run type	Pooled fractions	Volume (mL) + 3 M Tris	mg/mL	mg total
Affinity	G.10 – H.10	7.20	3.23	23.25

Preparative size exclusion chromatography (SEC)

- The pooled eluate from affinity chromatography eluted on a preparative SEC at an expected molecular weight (Figure 8).

Table 8. Column and run information

Column	HiLoad 26/600 Superdex 200 pg (Cytiva)
Column volume (CV)	318 mL
Column sanitisation	1 M NaOH + 2 M NaCl (30 min incubation)
Load (volume)	~7.2 mL
Equilibration and elution	¼ xPBS pH 7.4

Table 9. Spectrophotometric analysis A_{280nm} ($E_{0.1\%} = 1.696$)

Run type	Pooled fractions	Volume (mL)	mg/mL	Total (mg)
GF run	D.3 – F.12	32.00	0.55	17.6

Concentrating the protein

From the pooled fraction (~32 mL), the concentration was measured to be 0.55 mg/mL. The sample was concentrated on a 10 kDa MWCO spin filter to a volume of ~8.3 mL, then filtered through 0.2 µm. The concentration of the final concentrated sample was 1.90 mg/mL.

5 An estimated ~2 mg was lost on concentrating.

SDS-PAGE Analysis

All samples were treated with iodoacetamide and then incubated at 95°C in the sample loading buffer for five minutes prior to gel loading. Gels were NuPAGE BIS-TRIS 4-12%, MES-SDS buffer, run at 180 V for 45 minutes. There was a major band at ~ 40 kDa, corresponding to NT3-Fc. (lanes 3 & 4, Figure 4B).

10

Endotoxin Testing

The pooled and filtered protein sample was diluted 1:10 with fresh sterile MilliQ (MQ) water, previously tested and passed for endotoxin levels. The Endotoxin level was measured using an Endosafe® PTS assay. Cartridge Sensitivity 5 - 0.05 EU/mL

15

Table 10. Endotoxin Level

MQ water H ₂ O	<0.050 EU/mL
Concentrated NT3-Fc	<0.50 EU/mL

Product detail and dispensing

Approximately 15.77 mg NT3-Fc protein was captured by a two-step purification regime: Affinity HiTrap MabSelect Prisma column followed by preparative SEC (S200pg 26/60). Final samples were filtered through 0.2 µm filter into 5 x 1 mL, 5 x 0.5 mL, 8 x 0.1 mL aliquots and stored at 4 °C.

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Example 3: Neurotrophin-3 fusion protein characteristics

Surface Plasmon Resonance (SPR) for NT3-Fc binding to immobilized Trk receptors

SPR measurements were performed using a Biacore T200 instrument (Cytiva). Initially, a multicycle kinetics approach was utilized to demonstrate binding interactions for injected NT3-Fc protein with Trk receptors immobilized on the chip surface. Pilot capture experiments were performed with Trk proteins at different concentrations so that roughly equal amounts of each Trk could be captured when the subsequent binding experiments were performed. All SPR binding experiments were performed at 25°C with 1x HBS-EP+ as the instrument running buffer. Trk receptors were diluted in the SPR binding buffer to 1 µg/mL (TrkC, TrkB) and 5 µg/mL (TrkA) and captured onto the anti-histidine mAb chip surface at the start of each binding cycle. Following capture, NT3-Fc was injected over the Trk receptor surfaces for 90-sec at 30

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μL/min and then allowed to dissociate in the running buffer for 300 - sec. The anti-histidine mAb surface was regenerated with a single 60-sec injection of 10 mM Glycine pH 1.5. This binding cycle (1. Capture, 2. Analyte injection, and 3. Regeneration) was repeated several times with different analyte (NT3-Fc) concentrations (diluted 3-fold) being injected. The experimental design for this assay is outlined in Table 11.

Table 11. Binding cycle Conditions

Ligand ID	Flow Cell	Concentration (μg/mL)	Flow Rate (μL/mL)	Contact Time (s)	
TrkC	2	1	10	60	
TrkB	3	1	10	60	
TrkA	4	5	10	60	
Analyte	Flow Cell	Concentration (nM)	Flow Rate (μL/mL)	Contact Time (s)	Dissociation (sec)
NT3-Fc	1, 2, 3 & 4	0.25, 0.5 1 2 3 8 16 32 64 128 & 256 nM	30	90	300
Regeneration	Flow Cell	Concentration (nM)	Flow Rate (μL/mL)	Contact Time (s)	
Glycine pH 1.5	1, 2, 3 & 4	10	30	60	

SPR binding assays for TrkC binding to immobilized NT3-Fc

SPR experiments were performed at 25°C with 1x HBS-EP+/N+ (10 mM HEPES, 500 mM NaCl, 3 mM EDTA, 0.05%(v/v) Tween 20) as the instrument (Biacore T200) running buffer. A multi-cycle kinetics approach was utilised to determine the binding affinity for recombinant human TrkC interacting with immobilised NT3-Fc sample.

Briefly, the purified protein sample was diluted in the instrument running buffer and captured onto the SPR chip surfaces containing an amine-coupled Protein A (Cytiva, Cat#:29127556) at the start of each binding cycle. Typically, these captures were carried for 60 seconds at a constant flow rate of 10 μL/min. Pilot experiments were performed to determine the appropriate dilution of NT3-Fc to achieve consistent capture levels of 300 RU (1 RU = 1 pg of protein/mm²). Following capture, TrkC protein (Sino Biological, Cat#10048-H08H) was injected for 90 sec at 30 μL/min and then dissociated in the running buffer for 180 sec. Protein A chip surface was regenerated between each binding with a 30-sec injection of 10 mM Glycine at 30 μL/min.

Final SPR binding data and the binding interaction summaries derived from these experiments are shown in Figure 10, Figure 25 and Table 22. The estimated affinity (K_D value) for TrkC binding to immobilised NT3-Fc samples was estimated to be around 50 to 150 nM.

SPR data processing and analyses

SPR experimental data were processed and analysed using Scrubber Software (www.biologic.com.au). Flow cell 1 and “zero-buffer-blank” injection were utilized to reference binding signals. Affinity Parameter (K_D) was derived by fitting each set of experimental data at equilibrium to a Langmuir 1:1 binding model.

Affinity Determinations

Binding data showing interactions between injected NT3-Fc produced from HEK cells with immobilized Trk receptors is illustrated in Figure 9. The assay configuration for these initial SPR experiments was with the dimeric NT3-Fc in the solution injected over immobilized Trk receptors. The Langmuir 1:1 binding model was used to extract the affinity parameters.

Table 12. Equilibrium dissociation constant (K_D) estimates obtained for NT3-Fc interacting with immobilized Trk receptors. Reported numbers represent average values $\pm$ standard deviation obtained from three independent experiments (n=3)

	K_D (nM)		
	TrkC	TrkB	TrkA
NT3-Fc	1.35 $\pm$ 0.08	45.2 $\pm$ 1.7	626 $\pm$ 16

In SPR experiments, the multimeric status of the injected reagent matters. If the injected species (in solution) is a monomer (TrkC), estimated binding parameters represent true affinity. If, on the other hand, the injected species is a dimer, the avidity (sum of affinities) is measured. The model used to extract K_D values in Table 12 was a 1:1 model therefore these estimates only represent apparent affinities. Nevertheless, this assay approach is useful because it permits a direct comparison of NT3-Fc interacting with different Trk receptors in the same assay. Furthermore, it is also relevant in the biological context where an NT3 homodimer interacts with immobilised Trk receptors

SPR binding data and the binding interaction summaries for a monomeric TrkC interacting immobilized NT3-Fc are shown in Figure 10. In this case, true affinity estimates are generated. Importantly, these results demonstrate that purified NT3-Fc produced in a CHO cell host (ExpiCHO) following transient transfection has the same binding kinetics for TrkC receptor as NT3-Fc purified following transfection of HEK293Freestyle cells.

In vitro bioactivity

The CHO K1 cell line with a CellSensor construct (TrkC-NFAT-bla) was cultured in growth medium (DMEM with GlutaMAX) supplemented with 10% dialyzed FBS, 1 $\times$ MEM NEAA (minimum essential medium nonessential amino acid) solution, 25 mM Hepes,

blasticidin (5 µg/mL), and Zeocin (200 µg/mL). Cells were seeded in 96-well plate at 1.0×10^4 cells per well in a medium without blasticidin and Zeocin overnight. The next day, the cells were treated with the fusion protein for 5 hours. Subsequently, the wells were loaded with β -lactamase LiveBLAzer-FRET B/G substrate for 2 hours. Fluorescence at 450 and 510 nm was recorded on a plate reader. Figure 11 illustrates the bioactivity of NT3 and NT3-Fc.

Example 4: Neurotrophin-3 fusion protein loaded onto supraparticles characteristics

Loading

Each supraparticle (SP) was loaded in a solution containing 5.5 µg of the fusion protein per particle and loaded over 3 days at room temperature. First, the SPs were sterilized with 100 µL of ethanol (80% v/v) at room temperature (~22 °C) for 4 h. The SPs were then washed with sterile Milli-Q water six times. Next, a phosphate buffer solution containing the fusion protein was added to the SPs at a ratio of 5.5 µg of the fusion protein per particle. After 3 days, the supernatant was withdrawn for analysis. The concentration of the fusion protein in the supernatant was measured using Nanodrop and MicroBCA assays. The amount of fusion protein loaded into the SPs was calculated by subtracting the amount of protein in the supernatant from the amount of protein in the original stock solution (i.e., the preloaded solution minus the postloaded solution). Figure 12A illustrates that the NT3-Fc loading capacity remains consistent over 84 days.

Elution

The loaded particles were individually separated and transferred to 100 µL of phosphate-buffered solution (PBS) containing 0.2 % BSA and 0.05% Sodium Azide. The elution profile of the particles was analysed for 63 days. At specific timepoints (day 3, 7, 14, 21, 28, 35, 42, 49, 56, 63 days), the PBS solution was collected as elution samples. The protein concentration in each elution sample was determined by ELISA. Fresh PBS was replenished after each collection. Figure 12B illustrates the elution profile of NT3-Fc from the loaded Supraparticle.

Bioactivity of eluted fusion protein

The CHO K1 cell line with a CellSensor construct (TrkC-NFAT-bla) was cultured in growth medium (DMEM with GlutaMAX) supplemented with 10% dialyzed FBS, 1× MEM NEAA (minimum essential medium nonessential amino acid) solution, 25 mM Hepes, blasticidin (5 µg/mL), and Zeocin (200 µg/mL). Cells were seeded in 96-well plate at 1.0×10^4 cells per well in a medium without blasticidin and Zeocin overnight. The next day, the cells were treated with the eluted fusion protein at the concentration of 50 pM for 5 h. Subsequently, the wells were loaded with β -lactamase LiveBLAzer-FRET B/G substrate for 2 h. Fluorescence at 450 and 510 nm was recorded on a plate reader.

In vivo pharmacokinetic study

A normal hearing cat was implanted bilaterally with NT3-Fc loaded supraparticles (SPs) via a round window approach. Surgery was performed using aseptic surgical techniques to expose the round window membrane. SPs (n = 30 SPs) were placed on the round window membrane using a 21-gauge polyurethane catheter, fibrin sealant was placed on top of the particles, and Kwik seal was then applied over the hardened fibrin sealant to maintain the particle placement. The surrounding muscle layers were sutured, and the skin incision closed with staples. The treatment period lasted for 48 h.

At the completion of the treatment period, the animal was terminated and intracardially perfused with 0.9% NaCl (37 °C) followed by 10% Neutral Buffered Formalin (10% NBF; 4 °C). The bulla was removed from the temporal bones and the cochleae dissected. The cochleae were then post-fixed in 10% NBF and then transferred to 10% ethylenediamine tetraacetic acid (EDTA) in PBS at room temperature for decalcification. The cochleae were further sectioned using a cryostat. The sectioned cochlea tissue was blocked for endogenous peroxidase with EPB solution (3% H₂O₂) for 20 min, followed by 20 min treatment in 0.1% Tween-20 PBS. Then the tissue was treated with Goat anti-human IgG (Fc specific) antibody (1:500 in 0.1% Tween-20 PBS) for 4 hours, washed with PBS and then treated with DAB solution for 1 min. The tissue was imaged after rinsed in water and dehydrated with ethanol.

A normal hearing cat with no implants was used as negative control and a normal hearing cat with NT3-Fc loaded supraparticles implanted via an intracochlear approach was used as positive control. Figure 13 shows cat cochlea sections after treatment with NT3-Fc loaded SPs on the round window membrane (RWM) for 48 h, stained with anti-human IgG-HRP and DAB.

In vivo efficacy study

This study investigated the efficacy study of NT3-Fc SPs delivered to the round window membrane of noise exposed cats. Cats (n=8) were bilaterally deafened (124dB 16kHz pure tone for 100 min under anaesthesia) 3 days prior to implant surgery. The NT3-SPs (n=25) were unilaterally implanted on the round window membrane using the same surgical approach as above, achieving a total dosage of approximately 105ug NT3 per ear over a two-month treatment duration. The contralateral ear was not implanted. The cochleae were processed for surface preparations to quantify cochlear hair cells and synapses in the NT3-SP treated and unimplanted control ears. Analysis of the cochlear synapses at the highest frequency measured (32kHz cochlear region - in closest proximity to the round window membrane), showed a significant effect of NT3-SP treatment on cochlear synapse repair (ANCOVA p=0.002). Figure 14 illustrates the NT3-Fc SPs implanted on the cat RWM and the Significantly higher synaptic density for 32kHz cochlear region in the NT3-Fc treated cochleae compared to control (n=8 cats).

Example 5: Production and purification of BDNF fusion protein in ExpiCHO Cells

Cell culture and transfections

A gene construct, encoding the human BDNF gene fused to a non-dimerizing version from a human antibody Fc gene fragment, was synthesised by GenScript and sub-cloned into a modified pCAGGS mammalian expression via the SacI-XhoI restriction enzyme sites (Figure 15).

ExpiCHO Cells

Transient transfection of suspension-adapted cultures of ExpiCHO cells (ThermoFisher Scientific) was performed using Thermo Fisher Scientific ExpiCHO-S™ Expression System (Cat # A29129). Cell culture, growth parameters, as well as transient expression and feeding, were as per the manufacturer’s recommendations. Briefly, a vial of ExpiCHO-S™ cells was thawed into ExpiCHO Expression Medium (Cat # A2910002) in a 125 mL shake and cultured in a humidified and shaking CO₂ incubator (37°C, 5% CO₂, 130 rpm). The culture was routinely passaged and expanded every 3 – 4 days to reach the required transfection volume. On the day of transfection, the cell density was adjusted to 6.0 x 10⁶ cells/mL and transferred into a 500 mL shake flask, with a total cell volume of 100 mL or 150 mL, ready for transfection. The transfection was performed as per the manufacturer’s recommendation with plasmid DNA (a final concentration of 1 µg DNA per litre of culture).

Protein yield monitoring

BDNF-Fc production was carried out using the standard feeding regime as per the manufacturer’s protocol. The cultures were harvested on day 7 (PF23-11-12, Viability: 94.9% ViCD: 8.52 x 10⁶ cells/mL) days post-transfection by centrifugation at 4000 rpm/30 mins/4°C under low endotoxin conditioning, followed by 0.2 µm sterile filtration. Cell samples were measured for cell counting using trypan blue staining.

Production yields were monitored using SPR. The Fc moiety enables the binding of the fusion protein to a Protein A SPR chip docked inside an SPR instrument (Biacore 8K, 8-channel instrument, Cytiva). Specifically, a calibration-dependent concentration assay (CDCA) was used to monitor the amount of secreted BDNF-Fc protein. This approach uses a reference sample to establish a standard curve (SPR binding response vs injected concentration). The outcomes of these SPR experiments are shown in Table 13.

Table 13. Relative active concentration of BDNF-Fc in ExpiCHO culture supernatant.

Day	µg protein/mL culture
3	19.6
6	48.8
7	55.4

Purification Process Summary:

BDNF-Fc was purified using a two-step chromatography method: 1) PrismA affinity chromatography column, and 2) Size exclusion chromatography column. The final protein sample was concentrated to 0.8 mg/mL and analysed by SDS-PAGE, analytical SEC, SPR and Mass Spectroscopy.

Affinity Chromatography on PrismA column

The elution occurred as a single elution peak (Figure 16).

Table 14. PrismA Column and run information

Column	HiTrap MabSelect PrismA
Column volume (CV)	5 mL
Column sanitisation	1 M NaOH + 2 M NaCl (20 min incubation)
Load (volume)	190 mL
Equilibration solution(s)	1 x PBS pH 7.4
Elution	0.1 M Citrate pH 3.5, 0.15 M NaCl (neutralised with 1/6th vol 3 M Tris pH 8.0)

Table 15. Spectrophotometric analysis

Run type	Pooled fractions	Volume (mL) + 3 M Tris	mg/mL	mg total
Affinity	A6-B12	7.0	0.8	5.6

Preparative SEC

Calibration of the column conformed to expected specifications. A major protein peak eluted at molecular weight is expected for the BDNF-Fc dimer (~85 kDa).

Table 16. Preparative SEC Column and run information

Column	HiLoad 26/600 Superdex 200 pg (Cytiva)
Column volume (CV)	318 mL
Column sanitisation	1 M NaOH + 2 M NaCl (30 min incubation)
Load (volume)	~7.2 mL
Equilibration and elution	¼x PBS pH 7.4

Collected fractions from the preparative SEC (Figure 17) spanning the major 85 kDa peak were combined and buffer exchange by two rounds of concentration and dilution into 1x PBS using an Amicon® Ultra Centrifugal Filter (10 kDa MWCO; Merck). The summary of this process is outlined in Table 17.

Table 17. Preparative SEC sample processing

BDNF-Fc sample from SEC				Final BDNF-Fc sample after concentration & buffer exchange				Protein recovery
Sample Buffer	Con. (mg/mL)	Volume (mL)	Amount (mg)	Sample Buffer	Con. (mg/mL)	Volume (mL)	Amount (mg)	%
1x PBS + 350 mM NaCl	0.08	30	2.4	1 x PBS	0.8	2.0	1.6	67

SDS-PAGE

All three samples were analysed by SDS-PAGE under reducing and non-reducing conditions (Table 18). The protein migrated on SDS-PAGE (Figure 18) at ~45 kDa. No significant migration differences for these proteins were observed under reducing and non-reducing conditions. This observation was consistent with BDNF-Fc being a correctly processed mature non-covalent dimer that dissociates.

Table 18. SDS-PAGE Lanes

Lane	Sample Description
SB	SeeBlue Plus 2 Marker
R	Reduced BDNF-Fc
NR	Non-reduced BDNF-Fc

Size exclusion chromatography

Analytical size exclusion chromatography was first performed in a high salt buffer (1x PBS +350 mM NaCl). The protein sample eluted at an expected molecular weight of ~85 kDa, consistent with the molecular weight of the BDNF-Fc dimer (Figure 19).

Table 19. SEC Column and run information

Column	Superdex 200 10/300 GL (Cytiva)
Column volume (CV)	~2.4 mL
Sample load (volume)	~50 µg
Equilibration and elution	1 x PBS +350 mM NaCl (Final [NaCl] = 500 mM)

Final processing

The BDNF-Fc sample was filtered through a 0.2 µm Nanosep filter (Cytiva) and stored at 4°C.

Example 6: BDNF fusion protein characteristics

SPR binding assays

All SPR experiments were performed at 25°C with 1x HBS-EP+/N+ (10 mM HEPES, 500 mM NaCl, 3 mM EDTA, 0.05%(v/v) Tween 20) as the instrument (Biacore 8K) running buffer. A multi-cycle kinetics approach was utilised to determine the binding affinity for recombinant human TrkB interacting with immobilised BDNF-Fc sample.

Briefly, the purified protein sample was diluted in the instrument running buffer and captured onto the SPR chip surfaces containing an amine-coupled Protein A (Cytiva, Cat#:29127556) at the start of each binding cycle. Typically, these captures were carried for 60 seconds at a constant flow rate of 10 µL/min. Pilot experiments were performed to determine the appropriate dilution of BDNF-Fc to achieve consistent capture levels of 300 RU (1 RU = 1 pg of protein/mm²). Following capture, TrkB protein (Sino Biological, Cat#10047-H08H) was injected for 90 sec at 30 µL/min and then dissociated in the running buffer for 180 sec. Protein A chip surface was regenerated between each binding with a 30-sec injection of 10 mM Glycine at 30 µL/min.

Final SPR binding data and the binding interaction summaries derived from these experiments are shown in Figure 20 and Table 20. The estimated affinity (K_D value) for TrkB binding to immobilised BDNF-Fc samples was estimated to be 32 nM.

Table 20. Binding interaction parameters from SPR experiments shown in Figure 20.

Capture Solution	Analyte 1	k _a (M ⁻¹ s ⁻¹)	k _d (s ⁻¹)	K _D (nM)
BDNF-Fc	TrkB	6.02 x 10 ⁰⁵	1.91 x 10 ⁻⁰²	32

Endotoxin test

The Endotoxin level was measured using an Endosafe® PTS assay (Cartridge Sensitivity 5 - 0.05 EU/mL) BDNF-Fc Endotoxin Level: <0.500 EU/mL.

In vitro bioactivity of BDNF-Fc

The CHO K1 cell line with a CellSensor construct (TrkB-NFAT-bla) was cultured in growth medium (DMEM with GlutaMAX) supplemented with 10% dialyzed FBS, 0.1 mM NEAA (nonessential amino acid) solution, 25 mM HEPES, Penicillin (100 U/mL) / Streptomycin (100 µg/mL), blasticidin (5µg/mL), and Zeocin (200 µg/mL). Cells were seeded in 96-well plate at 1.0 × 10⁴ cells per well in a medium without blasticidin and Zeocin overnight. The next day, the cells were treated with BDNF and BDNF-Fc for 5 hours. Subsequently, the wells were loaded with LiveBLAzer-FRET B/G substrate for 2 hours. Fluorescence at 450 and 510 nm was recorded on a plate reader. Figure 21 illustrates the bioactivity of BDNF and BDNF-Fc.

Example 7: Purification and analysis of a dimeric neurotrophic fusion protein

To demonstrate the importance of the fusion protein comprising monomeric Ig Fc domain in improving expression levels (e.g., high yield) and/or improving biological activity and/or increasing receptor binding, a human NT3 was fused to a dimerizing human Fc protein fragment (hereafter “NT3-diFc”) in a transient ExpiCHO cell culture.

When compared with the original NT3-Fc, the produced amount of active NT3-diFc was significantly lower, likely owing to the complexity of forming an appropriately assembled molecule that dimerises through both the Fc and NT3 moieties.

NT3-diFc material was purified by Affinity HiTrap MabSelect PrismaA chromatography (Figure 22).

Size exclusion chromatography

NT3-diFc and NT3-Fc samples were analysed using analytical SEC. NT3-diFc (PrismaA pH 4.0) sample eluted from Superdex 200 10/300 SEC column in three major peaks with estimated molecular weights of 1) >670 kDa (9.5 mL), ~80 kDa (14.9 mL) and ~50 kDa (15.7 mL). NT3-diFc (PrismaA pH 3.5 sample) predominantly eluted as a high molecular weight aggregate (> 670 kDa). In comparison, NT3-Fc eluted at 14.0 mL on the same column under identical conditions (Figure 23).

SDS-PAGE

NT3-diFc migrated on SDS-PAGE as a major band (Figure 24, shown with arrows and Table 21) at approximately 40 kDa under reducing and 80 kDa under non-reducing conditions, which is consistent with a correctly processed mature NT3-diFc (expected Mw ~39 kDa for reduced monomer and ~78 kDa for non-reduced dimer). Non-reduced samples contained many protein bands migrating at higher molecular weight indicating a high aggregation propensity for this construct.

Table 21. SDS-PAGE Lanes

Lane	Sample + details	Reducing agent (βME)
1	MW marker	N/A
2	Starting Material (ExpiCHO supernatant)	Yes
3	PrismaA column Flow-through	
4	Protein Peak eluting at pH 4.0	
5	Protein Peak eluting at pH 3.5	
6	Starting Material (ExpiCHO supernatant)	No
7	PrismaA column Flow-through	
8	Protein Peak eluting at pH 4.0	
9	Protein Peak eluting at pH 3.5	

SPR assay

SPR binding experiments with recombinant TrkC (receptor) confirmed that NT3-diFc was active as it bound to TrkC with an equivalent affinity as NT3-Fc (Figure 25 and Table 22).

5 SPR binding activity of NT3-diFc localized to the 14.9 mL (80 kDa) peak.

Table 22. Binding interaction parameters

Sample	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
NT3-Fc	1.22×10^5	6.89×10^{-3}	56
NT3-Fc (pH 4.0)	8.56×10^4	6.26×10^{-3}	73

Example 8: Purification and analysis of alternative neurotrophic fusion protein

10 The NT3-Fc constructs in Table 23 were designed to investigate the production and purification of NT3-Fc further. Figure 26 shows sequence alignments of these constructs. Table 24 provides a summary of expression and purification outcomes

Table 23. Construct Information

Construct Id (in pCAGGS)	Construct Description
NT3-Fc001 (NT3-Fc)	3-point mutations from native Fc, missing 1/2 hinge region, contains LALA mutations, extra Ser after LALA (AAS)
NT3-Fc003	3-point mutations from native Fc, missing 1/2 hinge region, contains LALA mutations, extra Ser after LALA (AAS) and P329G
NT3-Fc004	3-point mutations from native Fc, missing 1/2 hinge region, contains LALA mutations, no AAS linker and P329G
NT3-Fc005	3-point mutations from native Fc, missing 1/2 hinge region, contains LALA mutations, no AAS linker and G237A
NT3-Fc006	3-point mutations from native Fc, missing 1/2 hinge region, contains LALA mutations, no AAS linker and P329G
NT3-Fc007 (NT3-diFc)	Contains Native Fc from human IgG1, except for LALA mutations. This construct is expected to dimerise through NT3 and Fc.

15 Table 24. Expression and purification result summary

	Expression culture	Protein Produced	Protein Purified	Predicted Yield	Binding Test	Binding outcome
NT3-Fc001	1 mL HEK293T cells	Yes	No	~70 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
NT3-diFc	1 mL HEK293T cells	Yes	No	~1 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
Construct	Expression culture	Protein Produced	Protein Purified	Predicted Yield	Binding Test	Binding outcome
NT3-Fc001	1 L Expi CHO cells	Yes	Yes	~482 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
NT3-Fc003	1 L Expi CHO cells	Yes	Yes	~473 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
NT3-Fc004	1 L Expi CHO cells	Yes	Yes	~468 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
NT3-Fc005	1 L Expi CHO cells	Yes	Yes	~281 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
NT3-Fc006	1 L Expi CHO cells	Yes	Yes	~352 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC
NT3-Fc007	1 L Expi CHO cells	Yes	Yes	~25 µg/mL	SPR, Capture on Protein A Chip	active/binds TrkC

Example 9: Mouse Cochlear Explant Synaptopathy Assay

20 Materials and Methods

Postnatal day 3-5 mouse pups were culled, and cochleae were isolated from the temporal bones. Explants were cultured in culture medium (Dulbecco's Modified Eagle Medium supplemented with 1% FBS, 1% N-2, ampicillin and amphotericin B) overnight (37°C 5% CO₂). Cochlear synapse damage (synaptopathy) was induced using kainic acid (KA) (0.5 mM) then explants were cultured for three days in NT3-Fc diluted in culture medium. After the three-day culture period, explants were fixed in PFA and stored at 4°C in PBS.

To detect and image cochlear hair cells and their synapses (the pre and post synaptic puncta) explants were incubated in the following primary antibodies overnight at 4°C: PSD-95 (postsynaptic puncta), CtBP2 (presynaptic puncta), and Myo7A (inner hair cells, IHCs). Secondary antibodies, which fluoresce under the confocal microscope at given wavelengths, include: 405 Anti-Rabbit IgG (Blue), 488 Anti-Mouse IgG2a (green) and 568 Anti-Mouse IgG1 (Red). Slides containing the explants were cover slipped prior to imaging.

Explants were first imaged at 10x magnification with a fluorescence microscope. The cochlear length was measured in ImageJ, and the mid-cochlear region (50% along the length of the cochlea) was identified. This region was then imaged at 63x using the Stellaris 5 Leica confocal microscope. Same region of the cochlea was analysed across all samples.

High power images were analysed using specialised software (Imaris). IHCs and synapses/IHC were counted. Cochlear synapses were defined as counts of presynaptic and postsynaptic puncta within one micron of one another and quantified as the number of synapses per inner hair cell.

Results

Treatment of the explants with NT3-Fc (at a concentration of 1 nM) after KA damage lead to a recovery of synapses with more synapses observed in the cochlear hair cells (Figure 27).

CLAIMS:

1. A fusion protein comprising:
 - (i) a neurotrophin; and
 - (ii) a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.
2. The fusion protein of claim 1, wherein the neurotrophin forms a homodimer.
3. The fusion protein of claim 1 or 2, wherein the fusion protein has improved expression levels in a mammalian cell, relative to a wild-type or unmodified neurotrophin.
4. The fusion protein of any one of claims 1 to 3, wherein the fusion protein is characterised by increased activation of a tyrosine receptor kinase (Trk) receptor compared to a wild-type neurotrophin.
5. The fusion protein of claim 4, wherein the Trk receptor is a TrkA receptor, a TrkB receptor, a TrkC receptor or any combination thereof.
6. The fusion protein of claim 5, wherein the fusion protein binds to the TrkA receptor at neutral pH with an affinity constant (K_D) of between 400 to 800 nM.
7. The fusion protein of claim 5 or 6, wherein the fusion protein binds to the TrkB receptor at neutral pH with an affinity constant (K_D) of between 20 to 60 nM.
8. The fusion protein of any one of claims 5 to 7, wherein the fusion protein binds to the TrkC receptor at neutral pH with an affinity constant (K_D) of between 0 to 5 nM.
9. The fusion protein of any one of claims 1 to 8, wherein the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions and/or at least one N-glycosylation site that reduces and/or inhibits dimerization with another monomeric Ig Fc domain or fragment thereof.
10. The fusion protein of any one of claims 1 to 9, wherein the monomeric Ig Fc domain or fragment thereof comprises one or more amino acid substitutions selected from the group consisting of:
 - (i) leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system;

- (ii) leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system;
- (iii) glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system; and/or
- 5 (iv) proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.
11. The fusion protein of any one of claims 1 to 9, wherein the monomeric Ig Fc domain or fragment thereof comprises:
- 10 (i) leucine substituted with alanine at a position corresponding to amino acid 234 of SEQ ID NO: 10 according to the EU numbering system;
- (ii) leucine substituted with alanine at a position corresponding to amino acid 235 of SEQ ID NO: 10 according to the EU numbering system;
- (iii) glycine substituted with alanine at a position corresponding to amino acid 237 of SEQ ID NO: 10 according to the EU numbering system; and
- 15 (iv) proline substituted with glycine at a position corresponding to amino acid 329 of SEQ ID NO: 10 according to the EU numbering system.
12. The fusion protein of any one of claims 1 to 11, wherein the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site in a CH3 domain.
- 20 13. The fusion protein of any one of claims 1 to 12, wherein the monomeric Ig Fc domain or fragment thereof comprises at least one N-glycosylation site:
- (i) at a position corresponding to amino acid 364 of SEQ ID NO: 10 according to the EU numbering system; or
- 25 (ii) at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system.
14. The fusion protein of any one of claims 1 to 13, wherein the monomeric Ig Fc domain or fragment thereof comprises at least two N-glycosylation sites:
- 30 (i) at a position corresponding to amino acid 364 of SEQ ID NO: 10 according to the EU numbering system; and
- (ii) at a position corresponding to amino acid 407 of SEQ ID NO: 10 according to the EU numbering system.
- 35 15. The fusion protein of any one of claims 1 to 14, wherein the N-terminus of the monomeric Ig Fc domain or fragment thereof is linked by a linker to the C-terminus of the neurotrophin.

16. The fusion protein of any one of claims 1 to 14, wherein the C-terminus of the monomeric Ig Fc domain or fragment thereof is linked by a linker to the N-terminus of the neurotrophin.
- 5 17. The fusion protein of claim 15 or 16, wherein the linker comprises serine.
18. The fusion protein of any one of claims 1 to 17, wherein the neurotrophin is selected from the group consisting of brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4).
- 10 19. The fusion protein of claim 18, wherein the neurotrophin is NT-3.
20. The fusion protein of claim 19, wherein the NT-3 is a human NT-3.
- 15 21. The fusion protein of claim 20, wherein the human NT-3 is set forth in SEQ ID NO: 1.
22. The fusion protein of claim 18, wherein the neurotrophin is BDNF.
- 20 23. The fusion protein of claim 22, wherein the BDNF is a human BDNF.
24. The fusion protein of claim 23, wherein the human BDNF is set forth in SEQ ID NO: 6.
- 25 25. The fusion protein of any one of claims 1 to 24, wherein the monomeric Ig Fc domain or fragment thereof is set forth in SEQ ID NO: 2.
26. The fusion protein of any one of claims 1 to 14, comprising a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 2.
- 30 27. The fusion protein of any one of claims 1 to 14, comprising a sequence set forth in SEQ ID NO: 6 and a sequence set forth in SED ID NO: 2.
28. The fusion protein of any one of claims 1 to 14, comprising a sequence set forth in SEQ ID NO: 1 and a sequence set forth in SED ID NO: 2.
- 35 29. A nucleic acid encoding or expressing the fusion protein of any one of claims 1 to 28.

30. A nucleic acid encoding or expressing a proneurotrophin and a monomeric immunoglobulin (Ig) fragment crystallizable (Fc) domain or fragment thereof.
31. The nucleic acid of claim 30, wherein the nucleic acid sequence is a DNA sequence
5 comprising a sequence set forth in SEQ ID NO: 15.
32. The nucleic acid of claim 30, wherein the nucleic acid sequence is a DNA sequence comprising a sequence set forth in SEQ ID NO: 17.
- 10 33. An expression construct comprising the nucleic acid of any one of claims 29 to 32.
34. The expression construct of claim 33, comprising a sequence forth in SEQ ID NO: 18.
35. The expression construct of claim 33, comprising a sequence forth in SEQ ID NO: 20.
15
36. The expression construct of claim 33, comprising a sequence forth in SEQ ID NO: 12.
37. The expression construct of claim 33, comprising a sequence forth in SEQ ID NO: 14.
- 20 38. A host cell comprising the fusion protein of any one of claims 1 to 28, or expressing the nucleic acid of any one of claims 29 to 32 or the expression construct of any one of claims 33 to 37.
39. The host cell of claim 38, wherein the host cell is a mammalian cell.
25
40. The host cell of claim 39, wherein the mammalian cell is selected from the group consisting of a HEK cell, a CHO cell, a BHK cell, a MDCK cell, a C3H 10T1/2 cell, a FLY I, a Psi-2 cell, a BOSC 23 cell, a PA317 cell, a WEHI cell, a COS cell, a BSC 1 cell, a BSC 40 cell, a BMT 10 cell, a VERO cell, a W138 cell, a MRC5 cell, a A549 cell, a HT1080 cell, a B-
30 50 cell, a 3T3 cell, a NIH3T3 cell, a HepG2 cell, a Saos-2 cell, a Huh7 cell, a HeLa cell, a W163 cell, a 211 cell, a 211 A cell, and derivatives thereof.
41. The host cell of claim 39 or 40, wherein the mammalian cell is a HEK cell, preferably a HEK293 cell.
35
42. The host cell of claim 39 or 40, wherein the mammalian cell is a CHO cell, preferably a CHOK1 cell.

43. A method of manufacturing the fusion protein of any of claims 1 to 28, the method comprising the steps of:

- (a) culturing a cell line comprising a nucleic acid encoding the fusion protein; and
- (b) isolating the fusion protein from the cell line.

5

44. A composition comprising a supraparticle, wherein the supraparticle comprises the fusion protein of any one of claims 1 to 28.