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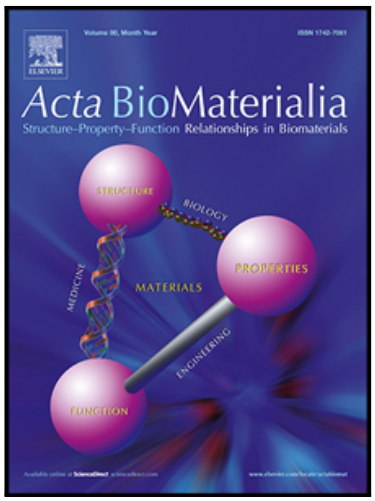


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PII: S1742-7061(21)00556-0
DOI: https://doi.org/10.1016/j.actbio.2021.08.030
Reference: ACTBIO 7568

To appear in: *Acta Biomaterialia*

Received date: 29 April 2021
Revised date: 8 August 2021
Accepted date: 19 August 2021

Please cite this article as: Melissa L.D. Rayner , Adam G.E. Day , Kulraj S. Bhangra , John Sinden , James B. Phillips , Engineered neural tissue made using clinical-grade human neural stem cells supports regeneration in a long gap peripheral nerve injury model, *Acta Biomaterialia* (2021), doi: https://doi.org/10.1016/j.actbio.2021.08.030

# Engineered neural tissue made using clinical-grade human neural stem cells supports regeneration in a long gap peripheral nerve injury model

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**Abstract**

A surgical autograft remains the clinical gold-standard therapy for gap repair following peripheral nerve injury, however, challenges remain with achieving full recovery and reducing donor-site morbidity. Engineered neural tissue (EngNT) manufactured using differentiated CTX0E03 human stem cells (EngNT-CTX) has been developed as a potential 'off the shelf' allogeneic autograft replacement. Ensheathed within a collagen membrane developed to facilitate biomechanical integration, EngNT-CTX was used to bridge a critical-length (15 mm) sciatic nerve gap injury in athymic nude rats. The effectiveness of EngNT-CTX was compared to an autograft using outcome measures that assessed neuronal regeneration and functional recovery at 8 and 16 weeks. At both time points EngNT-CTX restored electrophysiological nerve conduction and functional reinnervation of downstream muscles to the same extent as the autograft. Histological analysis confirmed that more motor neurons had successfully regenerated through the repair in EngNT-CTX in comparison to the autograft at 8

weeks, which was consistent with the electrophysiology, with the number of motor neurons similar in both groups by 16 weeks. The total number of neurons (motor + sensory) was greater in autografts than EngNT-CTX at 8 weeks, indicating that more sensory fibres may have sprouted in those animals at this time point. In conclusion, this study provides evidence to support the effectiveness of EngNT-CTX as a replacement for the nerve autograft, as the functional regeneration assessed through histological and electrophysiological outcome measures demonstrated equivalent performance.

Statement of significance: Following injury a peripheral nerve has the capacity to regenerate naturally, however, in the case of severe damage where there is a gap the current gold-standard microsurgical intervention is an autograft. This is associated with serious limitations including tissue availability and donor-site morbidity.
Tissue engineering aims to overcome these limitations by building a construct from therapeutic cells and biomaterials as a means to mimic and replace the autograft. In this study engineered neural tissue (EngNT) was manufactured using human stem cells (CTX) to bridge a critical-length gap injury. When compared to the autograft in an animal model the EngNT-CTX construct restored function to an equivalent or greater extent.

**Key words**

Nerve regeneration; Functional recovery; Tissue engineering; Stem cells.

## Introduction

Peripheral nerve injuries (PNI) are associated with a damaging socioeconomic impact because the resulting loss of function can affect quality of life due to disability and chronic pain *[1, 2]*. Peripheral nerves have the natural capacity to regenerate, however, the rate is remarkably slow and functional recovery is often incomplete [3, 4]. Current treatments mainly employ microsurgical interventions, with direct (primary) repairs used for nerve transections which can be sutured with no tension and the autograft remaining the clinical gold standard for the repair

of larger gaps. Alternative therapies are limited to hollow tubular conduits or decellularised nerve allografts [5, 6], but these have not become widely adopted into the clinic. Unlike the autograft these therapies lack the presence of columns of living Schwann cells which provide support and guidance to regenerating axons [1].

Tissue engineering aims to address this limitation through providing a combination of therapeutic Schwann cells and biomaterials to build a construct that could recreate the supporting properties of the autograft [7]. There is significant interest in developing therapeutic cells that mimic the Schwann cell phenotype and an extracellular matrix architecture equivalent to that which an autograft provides.

Autologous Schwann cells are an obvious source of cells for the manufacture of a therapeutic construct, however, their availability is limited by the same factors as an autograft since they are found only in nerve tissue. This creates a requirement for an allogeneic source that can be used as an 'off-the-shelf' product [8]. Stem cells provide a useful potential source of Schwann cells or their therapeutic equivalents and various options have been tested [9], although challenges remain due to host immune responses, variability, tumorigenicity risks and ethical concerns [9-11]. Our previous study demonstrated a means to overcome many limitations through the use of a differentiated clinical-grade clonal human stem cell line (CTX0E03) which is conditionally immortalised with the c-mycER$^{TAM}$ transgene which allows cell proliferation only in the presence of 4-hydroxytamoxifen (4-OHT) [7, 12]. This provides a cell line that can be expanded efficiently *in vitro* but the cells will not proliferate in the body in the absence of 4-OHT [12]. CTX0E03 cells have been used in clinical trials for stroke [13] and critical limb

ischaemia [14] and a portfolio of preclinical and clinical data therefore exists to provide evidence of safety following transplantation.

In addition to supporting the therapeutic cells, engineered tissue constructs need to integrate physically with the nerve repair environment and it has been suggested that the mechanical properties should match those of native nerve [15]. Repair constructs therefore need to have tensile strength and stiffness equivalent to nerve tissue, be able to accommodate sutures or adhesives, and degrade in a controlled manner that preserves the mechanical integrity of the repair [15]. Furthermore the entire construct needs to be biocompatible, minimally immunogenic and amenable to production according to Good Manufacturing Practice (GMP) standards [16]. To achieve these requirements, the cellular component that actively supports and guides regeneration tends to be delivered within an outer sheath material, mimicking the endoneurium and epineurium of natural nerve tissue respectively [15, 16].

Probably the most commonly used biomaterial for PNI repair is collagen, which is the dominant extracellular matrix protein present in natural nerve tissue [16]. Several products made of cross-linked Type-I collagen for PNI such as; NeuraGen®, Neuroflex™, NeuroMatrix™, NeuraWrap™, and NeuroMend™ have been approved by the U.S Food and Drugs Administration (FDA) and European Medicines Agency (EMA) and are commercially available [5]. However, since these medical devices are designed to be implanted as empty tubes to bridge nerve gaps they tend to be relatively stiff (compared to natural nerve tissue) in order to maintain a patent lumen.

Our previous study demonstrated that EngNT-CTX could be used inside a stiff collagen tube (NeuraGen) to support regeneration when implanted into a 12 mm injury gap in the sciatic nerve of athymic nude rats for 8 weeks [7]. However, regeneration was also observed in the empty conduit group, indicating that the injury model may not have represented a critical sized defect equivalent to a long gap in humans. Therefore, to support translation to the clinic, additional preclinical work must test how EngNT-CTX performs in relation to the current clinical gold standard autograft in a longer gap model over a longer time, and an outer sheath component needs to be developed to match nerve tissue mechanical properties. The aim of this study was to optimise and test repair constructs containing EngNT-CTX made using materials suitable for mechanical integration and GMP manufacture, in a definitive long gap nerve injury model using a comprehensive set of outcome measures.

## Materials and Methods

### Study design

The objective of this study was to assess the performance of EngNT-CTX in the repair of a critical length nerve injury in athymic nude rats (15 mm gap).

Athymic nude male rats (200-220 g, Hsd:RH-Fox1nu, Envigo) were randomised to eight groups and subjected to 1 of 4 gap repairs (autograft, EngNT-CTX, EngNT-F7 or empty tube) for 8 or 16 weeks. These endpoints were chosen to enable a comprehensive set of outcome measures investigating nerve regeneration and functional recovery to be conducted.

Group sizes (N=7) for the main comparison (autograft v EngNT-CTX) were statistically powered with an effect size of 1.3, a significance of α 0.05 and power ß 0.8. Additional control groups

(empty tube and EngNT-F7) were included with N=3 and were not used in the main statistical analysis, which involved a two-tailed unpaired t-test comparing autograft with EngNT-CTX.

The investigator was blinded during the repair microsurgery and harvest, throughout the functional and FOB measurements and the histological and functional analysis.

**Cell Culture**

Conditionally immortalised clonal human neural stem cells (CTX0E03 level P25-P33, ReNeuron Ltd, UK) were expanded using previously described methods [12]. CTX0E03 cells were cultured in Dulbecco's Modified Eagle's Medium:F12 (DMEM:F12, Gibco) supplemented with human albumin solution (0.03%; Grifols); Glutamax (2 mM; Gibco); human transferrin (5 µg/ml; Sigma), putrescine dihydrochloride (16.2 µg/ml; Sigma), human insulin (5 µg/ml; Sigma), progesterone (60 ng/ml; Sigma), sodium selenite (40 ng/ml; Sigma), epidermal growth factor (20 ng/ml; Sigma), basic fibroblast growth factor (10 ng/ml; Invitrogen), and 4-hydroxytamoxifen (4-OHT) (100 nM; Sigma) in laminin-coated (10 µg/ml in DMEM:F12 for 2 hrs at 37°C; Amsbio) 175 $cm^2$ flasks. CTX0E03 cells were passaged using TrypZean/EDTA (Lonza Bioscience) for 3 minutes at 37°C followed by defined trypsin inhibitor (Thermofisher). Cells were spun at 300 *g* and resuspended in DMEM: F12, counted, and reseeded for expansion or differentiation for experimental use. For differentiation, CTX0E03 cells were seeded at 7000 cells/$cm^2$ and left for 1 week in a 37 °C incubator with 5% $CO_2$ following the above protocol but growth factors and 4-OHT were omitted and 10% fetal bovine serum was added to the media.

Schwann cells derived from neonatal rats (SCL 4.1/F7; level P15-25, Health Protection Agency, UK) were used as an alternative to CTX0E03 cells in some conduits in order to serve as a control

for the cellular component as they have shown beneficial effects on nerve regeneration in previous studies [7, 17-23]. These were maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with penicillin and streptomycin (100 U/ml and 100 mg/ml, respectively; Sigma) and 10% v/v foetal calf serum (Gibco - DMEM-complete) in standard cell culture flasks. The cultures were maintained at sub-confluent levels in a 37 °C incubator with 5% $CO_2$ and passaged with trypsin/EDTA (Life Technologies) when required.

Dissociated dorsal root ganglion (DRG) neurons were prepared from adult Sprague Dawley rats (250 – 280g) as described previously [17]. DRGs were cleaned of surrounding tissue then incubated in collagenase IV (0.125%; Sigma Aldrich) for 90 minutes at 37 °C then dissociated by trituration and washed twice with 20 ml culture medium before being incubated for 24 h with cytosine arabinoside (0.01mM) to deplete glia. The resulting neuron-enriched cultures were then immediately used in assays.

**Assessment of dCTX phenotype**

Analysis was carried out via immunocytochemistry (ICC) after differentiating CTX0E03 cells for 7 or 14 days by removal of growth factors and 4-OHT. For ICC, cells were seeded at 7000 cells/$cm^2$ and left to differentiate in a T175 flask for 7 or 14 days. The differentiated CTX (dCTX) cells were transferred to laminin coated coverslips, and stained before fluorescence microscopy (AxioLab A1, Zeiss, UK) and image capture. Undifferentiated CTX (uCTX) cells were used as a comparator.

Contraction profiles of CTX cells in collagen gels

Contraction profiles were conducted to determine the cell density needed to allow self-alignment within collagen gels as described in detail previously [24]. UCTX and dCTX cells at densities of $4x10^6$, $3x10^6$, $2x10^6$, $1x10^6$ and $0.5x10^6$ per ml of collagen were assessed in 75 µl rat tail collagen hydrogels in a 96 well plate. Gels were detached from the wells after being immersed in the appropriate culture medium and left to contract in a humidified incubator at 37 °C with 5% $CO_2$. After 24 h, media was removed from the wells and images were captured of each gel. Percentage contraction was calculated using ImageJ by comparing the area of the well occupied by each gel after 24 h to its original area.

**Fabrication of EngNT**

Cellular hydrogels containing Schwann cells (F7) ($4x10^6$ cells/ml) or dCTX cells ($2x10^6$ cells/ml) were produced using rat tail collagen (2 mg/ml, First Link) according to previously established protocols and stabilised using plastic compression [17]. To produce collagen hydrogels, 80% v/v of collagen was mixed with 10% v/v 10x minimum essential medium (MEM; Sigma) and neutralised drop wise with sodium hydroxide (1:20 and 1:100 dilutions using deionised water, Fisher Scientific) until visual colour change of yellow to red. Then, 5% v/v of culture media containing cells was added and cellular hydrogels were cast by adding 400 µl per tethering mould and setting for 15 mins at 37 °C. Moulds were then immersed in 8 ml of the appropriate culture media, left to contract and align [3, 4] in a humidified incubator at 37 °C with 5% $CO_2$ for 24 h, then stabilised for 15 mins using a RAFT™ absorber [5] (Lonza Bioscience) to produce EngNT. Acellular gels were made in the same way without cells being included in the culture media component of the collagen gel mixture.

For the assessment of EngNT in co-culture with neurons, DRG cultures were seeded onto the surface of EngNT-CTX or EngNT-Schwann cell sheets in 50 µl DMEM-complete. Samples were incubated for 1 h at 37 °C to allow attachment of neurons then 2 ml of DMEM-complete was added to each well. After 72 h incubation at 37 °C with 5% $CO_2$ samples were fixed with 4% paraformaldehyde (PFA) and stained using ICC. Five predetermined fields were imaged using fluorescence microscopy (AxioLab A1, Zeiss, UK) and neurite length was measured using ImageJ.

For *in vivo* testing the resulting sheets of EngNT were rolled via their short axis to form rods (15 mm length keeping the longitudinal alignment). Each rod was placed on a pre-wetted (5 mins in phosphate buffered saline (PBS)) previously dry sterilised (UV 20 mins/side) 40 mm x 18 mm (w x h) collagen membrane (Collagen Solutions, Glasgow, UK). 200 µl of fibrin glue (1:1 mix of 10% fibrinogen 90% basal DMEM:F12, and 25% thrombin 75% basal DMEM:F12, TISSEEL, Baxter) was then added to the surface of this sheet and a 40 mm long cylinder was produced by rolling the sheet around the EngNT. After 5 min, the ends of the conduit were trimmed transversely using a scalpel to produce a 16 mm length conduit containing EngNT + 0.5 mm overhanging sheath at each end. Fibrin glue was then used to seal the edge of the wrap. After a further 5 mins, the conduit was placed into a universal tube containing 1 ml of Hibernate-A (Gibco) and stored at 4°C for up to 3 hours [19].

Immunocytochemistry

All samples were fixed in 4% PFA for 24 hrs at 4 °C and then stored in PBS. Monolayer dCTX and uCTX cells on coverslips were permeabilised with 0.5% Triton-X for 15 min and non-specific

binding was blocked using 5% serum for 15 min (species matched to secondary antibody). Samples were then incubated overnight at 4 °C using antibodies to detect GFAP, S100 (both rabbit; DAKO, UK), and βIII-tubulin (mouse; Sigma, UK; all diluted to 1:400 using PBS). Samples were washed 3 times with PBS and the appropriate secondary antibody (Dylight anti-mouse 488, 1:400; Dylight anti-rabbit 549, 1:300 diluted in PBS; IgG; Vector Laboratories, UK) was used to detect the primary antibody for 45 minutes at ambient temperature (21 °C). Hoechst 33258 (1 µg/ml; Sigma, UK) was used for 10 minutes to label cell nuclei. Negative immunostaining controls where primary antibody was omitted were included for comparison. The monolayer cell immunostaining protocol was adapted for the EngNT co-culture constructs; all incubation times (other than for the primary antibody) were doubled and each PBS wash was 10 minutes. Analysis was conducted by capturing 5 predetermined fields using fluorescence microscopy.

**Surgical repair of rat sciatic nerve**

All experimental procedures that involve animals were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986)/the European Communities Council Directives (2010/63/EU) and approved by the UCL Animal Welfare and Ethics Review Board.

**Implanting EngNT-CTX.**

Athymic nude male rats (200-220 g, Hsd:RH-Fox1nu, Envigo) were randomised to eight groups and subjected to a gap repair with an autograft, EngNT-F7 or EngNT-CTX in a collagen membrane or empty silicone tube for 8 or 16 weeks (Fig. 1). Rats were deeply anesthetised by inhalation of isoflurane, and the left sciatic nerve of each animal was exposed at mid-thigh level, transected and then either an empty silicone tube (Syndev, UK; 1.57 mm inner diameter,

0.42 mm wall thickness), a nerve graft (autologous nerve tissue reversed and replaced), an EngNT-CTX or an EngNT-F7 conduit was positioned between the stumps to produce an inter-stump distance of 15 mm. Conduits or grafts were retained in place using two 10/0 epineurial sutures at the proximal stump and one at the distal stump, then wounds were closed in layers and animals were allowed to recover then maintained for 8 or 16 weeks. Animals were culled after 8 weeks to assess histological outcomes, or 16 weeks to assess functional recovery, as the primary outcome measure.

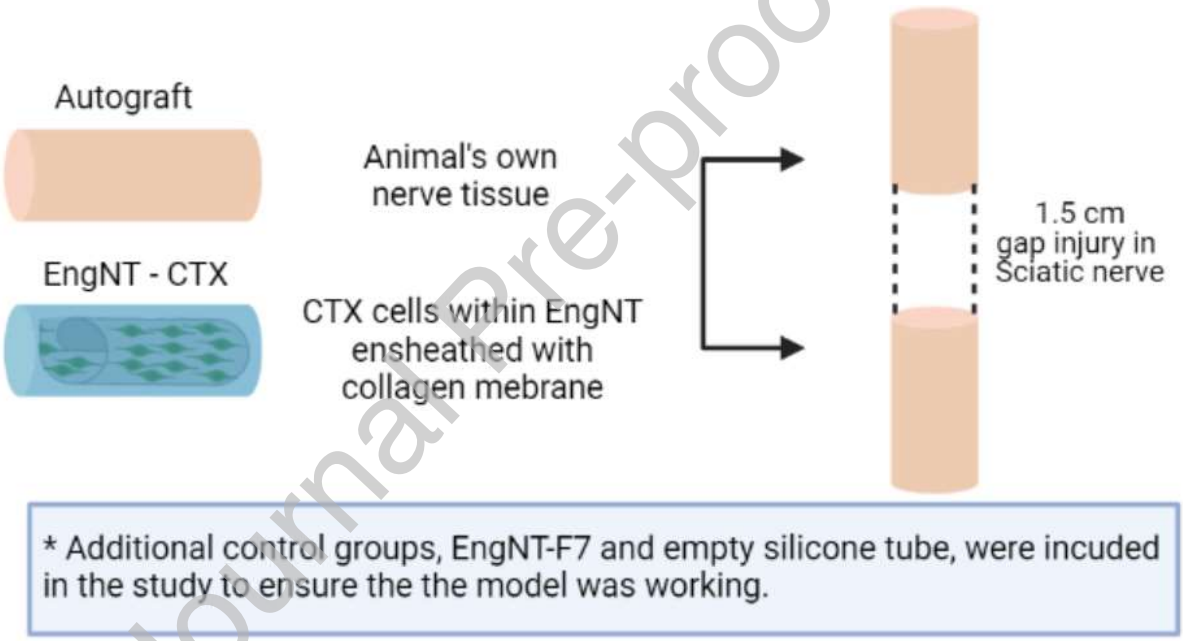





*Fig 1: Study design including the details of the experimental and control groups. The main study compared the use of EngNT-CTX to an autograft (N=7) which were implanted into a 1.5 cm gap injury in the sciatic nerve in athymic nude rats. Two additional control groups, EngNT-F7 and empty silicone tube (N=3), were used to ensure the model worked and represented a critical-sized gap injury model.*

**Epineurium pouch**

In order to determine the effect of sheath material on nerve regeneration an initial study was conducted. This was to compare the relatively stiff collagen-based material NeuraGen® to a softer collagen membrane and to natural nerve epineurium. The left sciatic nerve of nine male Sprague-Dawley rats (250-280 g) were exposed as described above. The animals were then subjected to a 15 mm gap injury which was treated with either EngNT-F7 delivered in NeuraGen®, collagen membrane, or nerve epineurium. For the epineurium group the animal's own tissue was used to form an epineurium pouch [25]. This was done by making a 15 mm incision in the epineurium, removing a 15 mm length of the endoneurium by gently teasing it away from the surrounding epineurium and replacing it with 15 mm EngNT-F7 (Supplementary Fig 1A). Four 10/0 sutures were used to close the epineurium. The NeuraGen® and collagen sheath constructs were implanted as described above.

**Testing functional reinnervation.**

Nerve function was assessed weekly using Static Sciatic Index (SSI) and von Frey and at the end-point using electrophysiology, then animals were culled with phenobarbital. Nerve tissue was removed and fixed in 4% paraformaldehyde for processing and histological analysis.

***Static Sciatic Index***

Functional recovery was analysed using SSI. The animal's hind paws were imaged from below with the animal standing on a Perspex platform and the toe spread (TS), between the 1st and 5th toe, and the intermediary toe spread (IT), between the 2nd and 4th toe, were measured and equation 1 was used to calculate SSI.

*Equation 1:* $SSI = (108.44 \times TSF) + (31.85 \times ITSF) - 5.49$.

$TSF = (TS_{injury} - TS_{control})/ TS_{control}$ $ITSF = (ITS_{injury} - ITS_{control})/ ITS_{control}$

***Von Frey***

The animals were placed on a grid and von Frey filaments made of nylon, which all have the same length but vary in diameter to provide a range of forces (0.008 g - 300 g), were applied through the underside of the grid to stimulate the centre of the animal's hind paws. A response was measured by the retraction of the animal's paw. The threshold response was recorded by decreasing the stimulus until no response was detected.

***Electrophysiology***

Animals were anaesthetised using isoflurane and nerve function was assessed by electrophysiology (Synergy Ultrapro 3) by comparing the repaired nerve to the contralateral undamaged nerve in each animal. Monopolar needle electrodes were attached to the animal; a grounding electrode was placed in the tail of the animal and a reference electrode was placed above the hip bone. A stimulating electrode was placed against the proximal nerve 2 mm above the injury site and a recording needle was placed into the gastrocnemius muscle. The distance between the stimulating and recording electrodes was standardised. The nerve was stimulated using a bipolar stimulation constant voltage configuration and the muscle response recorded. The stimulation threshold was determined by increasing the stimulus amplitude in 0.1 V steps (200 μs pulse), until both a reproducible, stimulus-correlated muscle action potential was recorded and a significant twitch of the animal's hind paw was seen. The amplitude (mV) of the compound muscle action potential (CMAP) was measured from baseline to the greatest peak and the latency was measured from the time of stimulus to the first deviation from the baseline. Muscle action potentials were conducted in triplicate for both the injured nerve and contralateral control nerve in each animal.

**Histological analysis of tissue repair.**

Following functional assessment, repaired nerves were excised under a dissecting microscope, fixed in 4% paraformaldehyde (Fisher Scientific) at 4 °C for at least 24 h and then washed in PBS (Sigma). Transverse cryostat sections (Thermo scientific HM525 NX, 10 µm thick) were prepared from the remaining proximal and distal parts of the device and the nerve stumps. Proximal and distal samples were immersed in 15% sucrose PBS solution at room temperature (21 °C) until samples submerged, after which they were immersed in 30% sucrose PBS solution for at least 16 h at 4 °C. Samples were then immersed in a 1:1 ratio of 30% sucrose/PBS and frozen section compound (Leica) for 2 h at room temperature, before being snap frozen in liquid nitrogen. The transverse sections that were used for analysis were from positions 2 mm into the proximal and distal stumps, or 2 mm into the proximal and distal parts of the repair site, measured from the proximal or distal sutures in each case. Sections were mounted onto microscope slides (Superfrost™ Plus, Thermo Fisher Scientific) and stored at -20 °C.

Sections were permeabilised with 0.3% Triton X-100 (Sigma) for 30 mins, blocked using 10% horse serum (Dako) for 1 h and then incubated in primary antibody containing 10% horse serum for 2 h at room temperature. After washing 3x5 mins with PBS, sections were incubated with DyLight-conjugated secondary antibodies containing 10% horse serum (1:400, Vector Laboratories) at room temperature for 1 h. Sections were washed 3x5 mins with PBS and mounted using VECTASHIELD HardSet mounting medium (Vector Laboratories) and confocal microscopy (Zeiss, LSM710 Germany) was used to tile scan the whole nerve section using a 20x lens. Volocity software (Perkin Elmer, Waltham, MA) was used to count all of the neurofilament

positive axons present in each transverse section by running automated image analysis protocols.

**Sheath material Characterisation**

***Uniaxial tensile testing***

The mechanical properties of rat sciatic nerve tissue and the collagen membrane selected as a potential sheath material were measured using uniaxial tensile testing (TA Instruments Bose Electroforce 3200, 225N load cell, WinTest® 7). Nerve samples were clamped between titanium grips at *in situ* tension with a 5 mm gauge length and subjected either to a ramp test to failure at 10 mm.min$^{-1}$, or to dynamic mechanical analysis (DMA). Sheath material samples were cut to a standard tensile testing shape with flared ends for clamping and a narrower central segment of 5 mm and gauge length of 10 mm. Cross-sectional area was measured histologically using adjacent nerve tissue. Fresh tissue was transported in Aqix® solution on ice and testing was conducted at 21.0 ± 0.5°C within 1 h post mortem. For DMA, a strain sweep was conducted to determine the linear viscoelastic range. Following this, a frequency sweep was conducted over a range of 1-30 Hz, at a constant strain amplitude of 0.75%.

**Statistical analysis**

Normality tests were conducted on all data to determine appropriate statistical tests, and one-way analysis of variance (ANOVA) or two-tailed t-tests were performed, as data followed a normal distribution. A one-way ANOVA was followed by a Tukey's or Dunnett's post hoc test.

For all tests, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 were considered to be significant.

Table 1: List of primary antibodies used for histological analysis.

| Antibody | Target | Dilution | Source |
|---|---|---|---|
| βIII-Tubulin | Neurons | 1:400 | Sigma Aldrich T8660 |
| S100 | Schwann cells | 1:400 | Dako Z0311 |
| GFAP | Immature Schwann cells | 1:400 | Merck C9205 |
| Neurofilament-H | Axons | 1:1000 | Eurogentec SMI-35-050 |
| ChAT | Motor axons | 1:50 | Merck AB144P |



## Results

### *Neuronal regenerative capacity of CTX cells in vitro*

Differentiated (dCTX) and undifferentiated (uCTX) CTX cells were both able to contract free-floating collagen gels in a standard contraction assay, demonstrating a density-dependent contraction profile over 24 h (Fig 2A, B). The dCTX cells showed greater contraction than the uCTX cells at every cell density, with 2 x $10^6$ cells/ml giving an optimal contraction of more than

60%. The optimal seeding density enabled EngNT to be made using dCTX cells since previous work has shown that contraction of more than 60% in the assay will result in cellular alignment in tethered gels [26]. Immunocytochemistry revealed that dCTX cells have an elongated morphology aligned to the longitudinal axis of EngNT (Fig 2C).

Co-culture assays were used to assess the capability of EngNT-CTX in supporting neurite outgrowth compared to EngNT-Schwann and acellular constructs *in vitro*. This was done by seeding DRG neurons on the top of each of these constructs and analysing neurite growth after 72 hours (Fig 2D). Both EngNT-CTX and EngNT-Schwann supported robust neuronal growth which was significantly longer than in acellular constructs. Mean neurite length for EngNT-Schwann was approximately 55 µm greater than for EngNT-CTX (Fig 2E). The same co-culture assay was used to determine whether the differentitation period of CTX cells had an effect on neurite outgrowth. It was found that the mean neurite length was the same whether the CTX cells were differentiated for 7, 11 or 14 days (Fig 2F).

Immunocytochemistry was used to determine CTX differentiation to a neuronal (βIII-Tubulin) or glial (s100B and GFAP) phenotype following 2 weeks of differentiation (Fig 2G). More dCTX were positive for GFAP compared to uCTX control cells (Fig 2G). A similar number of dCTX and uCTX cells were positive for βIII-Tubulin and S100. On further investigating the differentiation time of CTX cells, no difference was seen in the number of GFAP positive CTX cells following 7 and 14 day differentiation (Fig 2H). For the remainder of the study a 7 day differentiation period was used to minimise manufacture time and reduce cost, with a view to facilitating future commercial-scale production.

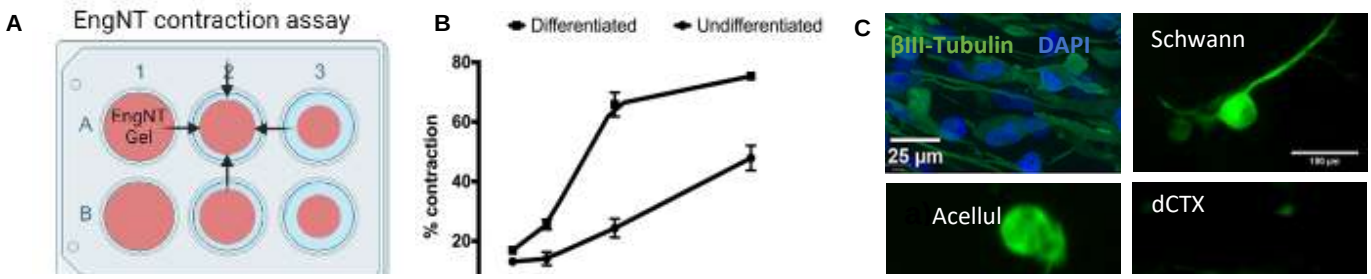


*Fig 2: Assessment of EngNT constructs in vitro. The ability of CTX and dCTX cells to contract collagen was assessed in a free-floating gel assay (A), with % contraction assessed after 24 h, N=3, mean ± SEM (B). Aligned EngNT constructs were then formed using dCTX cells (C) and seeded with DRG neurons to form co-cultures (D), diagram adapted from [27]. Neurite outgrowth was assessed using βIII-Tubulin immunofluorescence which stained DRG neurites brightly. Neurite length was compared in acellular gels, EngNT-dCTX, and EngNT-Schwann after 72 h (E), N=6, mean ± SEM, one-way ANOVA with Tukey's post-test, **p<0.01, ***p<0.001. There was no difference in mean DRG neurite length after 72 h in co-culture with EngNT-dCTX containing CTX cells differentiated for 7, 11 or 14 days (F). Expression of neuronal and glial markers in CTX cells in monolayer culture following 2 weeks of differentiation (dCTX) was compared to undifferentiated CTX cells (uCTX) using immunofluorescence. Total cell numbers were assessed using Hoechst. All uCTX and dCTX cells expressed βIII-Tubulin and s100, with relatively fewer cells expressing GFAP, N=3, mean ± SEM, no significant difference (G). Expression of GFAP in dCTX cells was no different after 7 or 14 days differentiation (H) N=3, mean ± SEM.*

***Sheath material characterisation***

**Tensile strength**

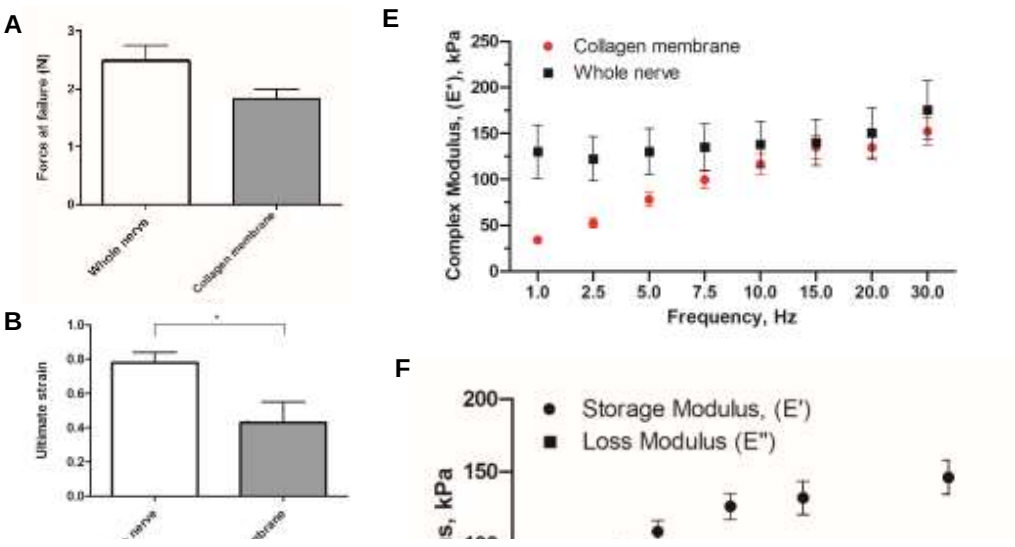


*Fig 3: Tensile testing of collagen membrane material. Ultimate tensile force (A), ultimate strain (B) and Young's modulus (C) of collagen membrane compared to rat sciatic nerve. Layering sheets of collagen material increased tensile strength (D) to above that for nerve tissue (represented by the blue dotted line). Tensile DMA revealed complex modulus of whole nerve and collagen membrane (E), storage and loss modulus for collagen membrane (F) and for nerve (G). N=6 for whole nerve and N=9 for collagen membrane, mean ± SEM, T-test (A). N=6, mean ± SEM, T-test *p<0.05 (B-C). N=3, mean ± SEM, one-way ANOVA with Dunnett's post-test *p<0.05, ***p<0.001 (D). N=4 collagen sheath, N=12 whole nerve, mean ± SEM (E-G).*

The collagen membrane had not been used previously in nerve repair so it was important to test some mechanical properties, particularly strength and stiffness, so that these could be compared with those of natural nerve tissue. For the purposes of this application the material was assessed alongside rat sciatic nerve tissue to give an indication of the overall target properties. A single sheet of collagen membrane (thickness 64 ± 12 µm) cut to a 5 mm width at the narrowest point for testing had a slightly lower break strength (force at failure) than rat sciatic nerve tissue (Fig 3A). The strain at which the collagen sheet ruptured was approximately half that at which nerve tissue failed (Fig 3B), but prior to tensile failure the collagen sheet exhibited a similar Young's modulus to nerve tissue. When used as a sheath around EngNT, the 5 mm width of the collagen sheet would become the circumference of a single-layer wall around a cylinder with a diameter of ~1.6 mm, which is approximately the diameter of the rat sciatic nerve and therefore the target size for the EngNT constructs. Based on the required tensile strength being greater than nerve tissue, it was determined that a 15 mm wide strip of the collagen sheet should be wrapped 3x around the EngNT in order to provide the appropriate outer sheath for a nerve repair construct (Fig 3D), mimicking the epineurium and providing a final wall thickness of ~190 µm. DMA was used to compare the viscoelastic properties of the collagen material to those of nerve tissue by applying a sinusoidal strain within the linear viscoelastic region and measuring the corresponding response. Fig 3 (E-G) shows that the collagen sheet and nerve tissue both showed viscoelastic behaviour with complex modulus increasing with higher frequency, and over the range tested both showed a similar dominantly elastic behaviour, with storage modulus being greater than loss modulus. The collagen membrane had similar tensile properties to the epineurium of the native nerve tissue,

indicating that it could be used as a thin flexible outer sheath for EngNT which may provide better mechanical integration than stiffer materials traditionally used as hollow nerve conduits.

**Testing the effect of different sheath materials on axon growth *in vivo***

An initial 8-week study was conducted to determine what effect different sheath materials had on nerve regeneration *in vivo*. Nine animals were subjected to a 15 mm gap injury and received treatment with EngNT-F7 delivered within one of three sheath materials; NeuraGen®, collagen membrane or nerve epineurium. For the epineurium group the animal's own tissue was used to form an epineurium pouch. This was done by making an incision in the epineurium, removing a 15 mm length of the endoneurium and replacing it with 15 mm EngNT-F7 (Supplementary Fig 1A). It was found that all materials supported axonal regeneration (Supplementary Fig 1B). This suggested that the collagen membrane could be used as an alternative to the commercially available NeuraGen®.

***Critical gap length in athymic nude rats***

A pilot study was conducted to test whether a 15mm gap length in an athymic nude rat sciatic nerve injury at 6 or 8 weeks represents a critical length injury model, wherein an empty tube supports poor regeneration compared with a nerve autograft. Repaired nerves were dissected and 10 micron transverse sections were taken at the distal stump and assessed for neurite growth by determining the number of neurofilament-positive axons (Supplementary Fig 2). The number of axons present was considerably higher in the autograft group, with minimal axons observed in the distal stump of the empty tube group after 6 or 8 weeks, indicating that 15 mm represents a critical gap length at these time points in terms of axonal growth.

***EngNT-CTX supported nerve regeneration and functional recovery***

Athymic nude rats were used in this study to eliminate a host immune response following the implantation of human CTX0E03 cells. The animals were closely monitored and all remained healthy throughout the experiment. A functional observational battery (FOB) of regular health checks was conducted to ensure the wellbeing of the animals, with no issues observed. One animal died during an early part of the surgical repair procedure and was reallocated to the empty tube control group in order to maintain the EngNT-CTX and autograft group sizes for statistical power (this is why there are only 2 animals in the 16 week empty tube control group).

**Neurite regeneration**

The primary histological outcome measure used in the study was the number of axons present in transverse sections through the distal stump of the repaired nerves (Supplementary Fig 3). This provides an indication of the extent to which axons have been able to traverse the repair site and penetrate the downstream nerve segment. The number of neurofilament-positive neurites in the distal stump as a percentage of the proximal stump was significantly higher in the autograft group than in the EngNT-CTX group at 8 weeks, however, by 16 weeks the number of neurites was similar between the two groups (Fig 4A). The number of neurofilament-positive neurites in the distal stump as a percentage of the proximal stump were similar between the EngNT-F7 and EngNT-CTX groups and minimal neurites were seen in the empty tube group (Supplementary Fig 4). Neurofilament staining detects the total population of axons (motor and sensory) within the nerve cross-section, whereas choline acetyltranferase (ChAT) detects motor fibres specifically. Fig 4B shows that a significantly greater proportion of neurites

present in the distal stump of the EngNT-CTX treated animals were ChAT-positive motor fibres in comparison to the autograft group at 8 weeks. By 16 weeks the number of motor fibres as a proportion of the total axons present was equivalent between the two groups.

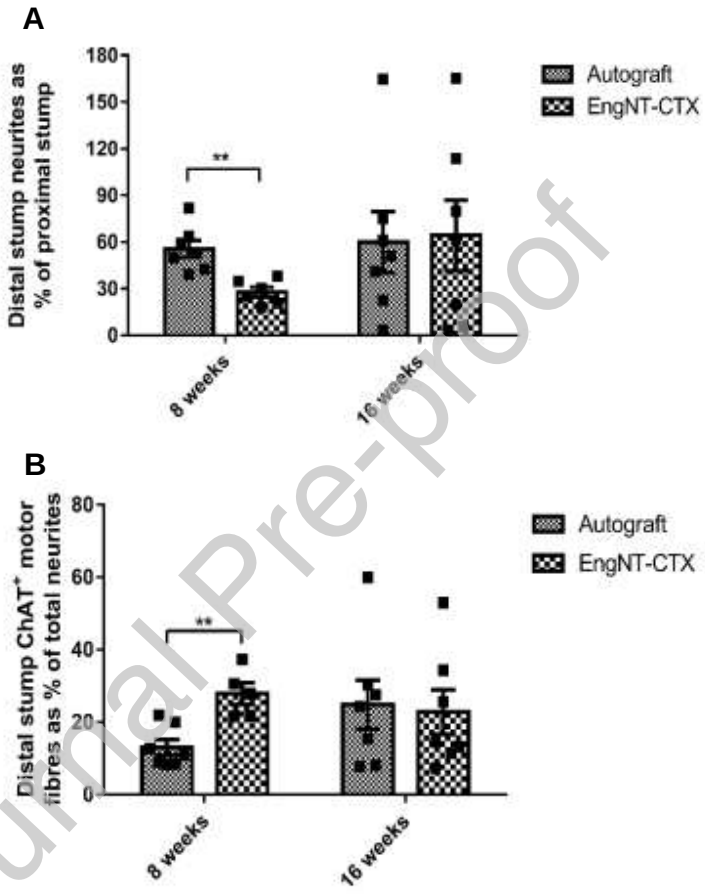
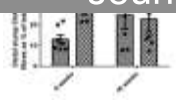





*Fig 4: Quantification of neurofilament-positive axons in the distal stump as a percentage of the proximal stump of a 15 mm gap repaired with an autograft or EngNT-CTX in a collagen membrane at 8 and 16 weeks (A). The number of axons seen in the proximal stumps at 8 weeks were 18384 ± 3066 and 20301 ± 4077 for the autograft and EngNT-CTX groups respectively, and 3389 ± 1357 and 4600 ± 2190 at 16 weeks (Mean ± SEM). A significantly higher number of axons*



*were seen in the autograft group at 8 weeks but a similar number of axons were seen between the groups at 16 weeks. Quantification of ChAT-positive motor fibres in the distal stump as a percentage of the total number of axons (B). N=7. Data are means ± SEM. Two-tailed unpaired T-test, **p<0.01.*

**Functional recovery**

Electrophysiology was performed under terminal anaesthesia, using the contralateral and repaired nerve in each animal (Fig 5). Recordings were taken from both gastrocnemius muscle and tibialis anterior at 16 weeks in order to provide two separate measures of functional reinnervation at the endpoint, which was the primary functional outcome measure for the study. Additionally, electrophysiology was performed using the gastrocnemius muscle at 8 weeks to provide an early indication of regeneration. At 8 weeks, an electrophysiological response (stimulation-linked target muscle contraction) was obtained in 5 out of 7 animals that received an autograft, whereas all 7 animals in the EngNT-CTX group showed a response. All animals in the two groups showed an electrophysiological response to stimulation at 16 weeks. All EngNT–F7 treated animals responded whereas none of the empty tube animals responded at 8 weeks and only 50% responded at 16 weeks (Fig 6A). This response to nerve stimulation provides an indication of whether there was functional axonal continuity between the stimulation site (proximal to the repair) and the target muscle. Where continuity was detected, CMAP and latency were measured (Fig 5 and 6).

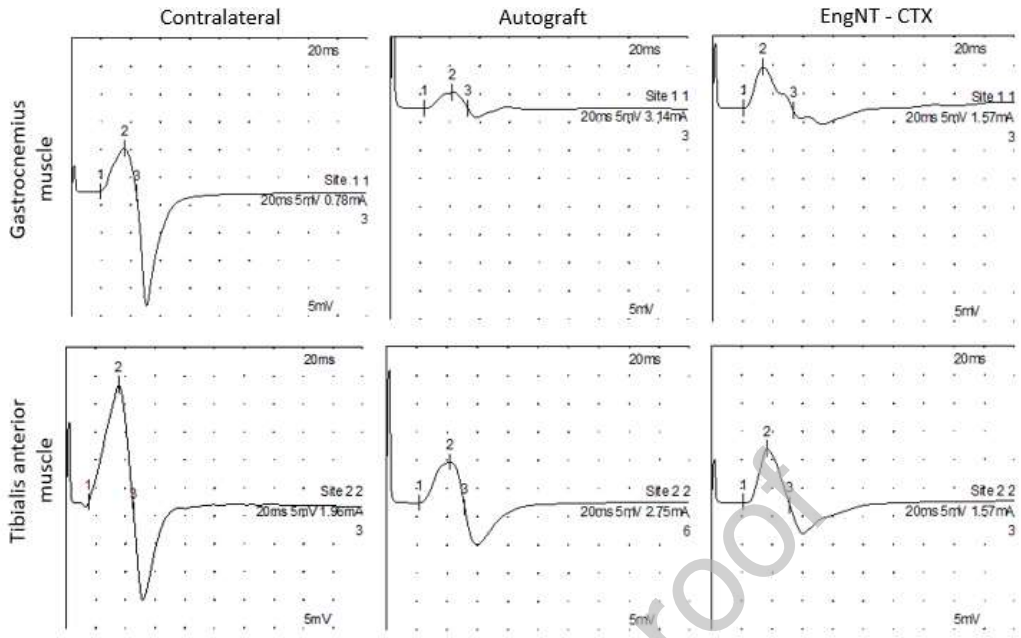
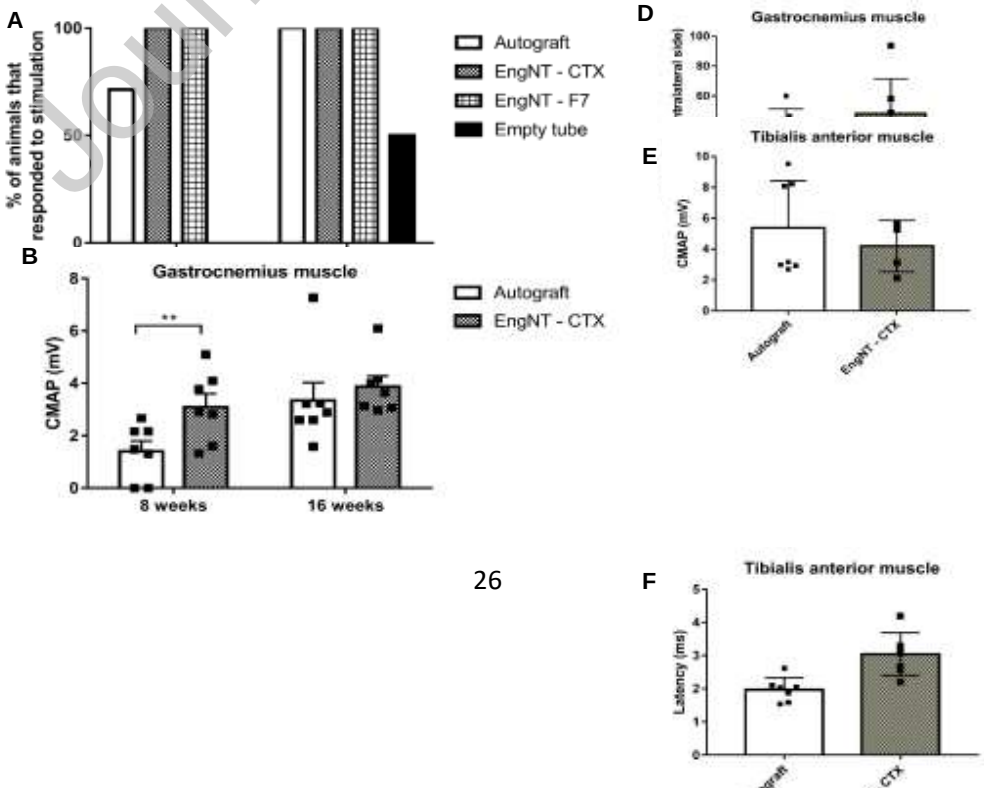



*Fig 5: Representative electrophysiology traces recorded from the gastrocnemius and tibialis anterior muscle at 16 weeks for the contralateral nerve and the autograft and EngNT-CTX treated groups.*



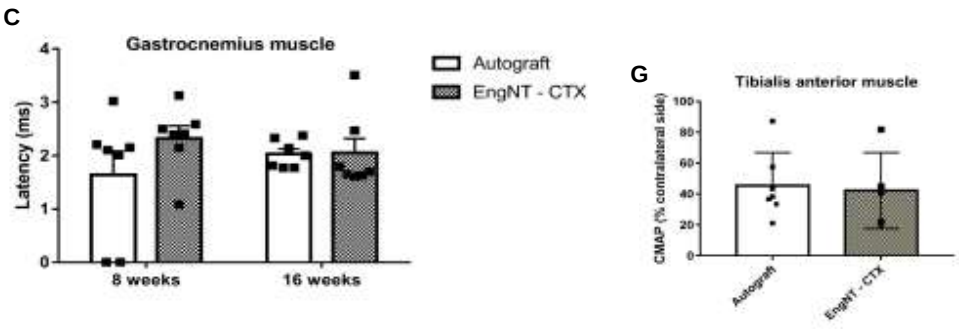



*Fig 6: Recovery of muscle function assessed using electrophysiology. Percentage of animals that demonstrated a muscle response to proximal electrical stimulation in all four groups at 8 and 16 weeks, indicating electrophysiological continuity (A). Compound muscle action potential (CMAP) (B) and latency (C) recordings from gastrocnemius muscle in the autograft and EngNT-CTX groups at 8 and 16 weeks. CMAP presented as a percentage of the corresponding contralateral CMAP at 16 weeks (D). CMAP (E) and latency (F) recordings from tibialis anterior muscle at 16 weeks (G). N=7. Data are means ± SEM. T-test, *p<0.05 when all animals in the autograft group for CMAP were included (using a value of zero for the two animals with no response).*

CMAP amplitude and latency recordings from the contralateral nerves were consistent between animals (data not shown). In the repaired nerves there was a trend towards greater muscle CMAP amplitude in the EngNT-CTX group in comparison to the autograft group at both 8 and 16 weeks (Fig 6B). The EngNT-F7 group showed a similar gastrocnemius muscle CMAP amplitude to the EngNT-CTX and autograft groups at 8 and 16 weeks, whereas no CMAP was elicited in

any of the animals that received an empty tube repair at 8 weeks and a minimal response was seen in one animal at 16 weeks (Supplementary Fig 5). The CMAP amplitudes recorded from the tibialis anterior muscle at 16 weeks were similar between the autograft and EngNT-CTX groups (Fig 6E). There were no differences in latency recorded between the groups at either 8 or 16 weeks in the gastrocnemius muscle (Fig 6C) and tibialis anterior (Fig 6F). In addition to presenting CMAP amplitude values (Fig 6B, E), in order to control for inter-animal variability the data were expressed as a percentage of contralateral CMAP for both gastrocnemius and tibialis anterior at 16 weeks and the same trends were observed (Fig 6D, G).

Static sciatic index (SSI) recording showed an initial loss of function immediately after surgery, with some gradual recovery over time although there was considerable variability between individual animals (Supplementary Fig 6A). There was no significant difference in SSI over 16 weeks between the autograft and EngNT-CTX groups (Supplementary Fig 6B), however, the empty tube group was consistently lower than the autograft, EngNT-CTX and EngNT-F7 groups over most time points (Supplementary Fig 6C).

Recovery of sensory function was assessed weekly using von Frey analysis. Filament diameter is related to the force applied and this was converted to a von Frey score on a scale of 1 to 10. If no response was recorded with the maximum force then a score of 11 was assigned. There was a clear loss of function in all animals 1 day following injury repair and the sensory function returned towards baseline by 13 weeks post-surgery. No difference was seen in the sensory recovery between the autograft and the EngNT-CTX groups (Supplementary Fig 7A) or the EngNT-F7 and empty tube groups (Supplementary Fig 7B).

## Discussion

The results presented demonstrate preclinical evidence that EngNT-CTX within a collagen membrane supported neuronal regeneration and functional recovery to the same extent as a nerve autograft in a critical length gap (15 mm) in athymic nude rats. The primary outcome measures were (1) histology showing regenerated motor fibres in the distal stump and (2) electrophysiology showing functional reinnervation of downstream muscles. With both outcome measures at 8 and 16 weeks there was equivalent regeneration in EngNT-CTX repairs and nerve autografts.

This finding is consistent with our initial study that indicated EngNT-CTX could support regeneration, but importantly here for the first time this has been demonstrated in a critical length nerve gap model [7]. The definition of a critical length nerve gap is where no regeneration will occur over the gap without the use of a nerve graft or bridge. This has been reported to be ~15 mm in rats and ~ 40 mm in humans [17, 28], however the strain of rat and the time point at which regeneration is measured can affect this. There is little previous literature in which athymic nude rats have been used with a critical length nerve gap, so prior to the main experiment we demonstrated in a pilot study that a 15 mm gap represented a critical length defect, with no neurite growth through empty conduits when compared to autografts at 6 or 8 weeks post injury. This was also confirmed by the additional control groups included in the main pre-clinical study, where there were clear differences observed in regeneration and functional recovery between the empty conduit and treatment groups at 8 and 16 weeks. Similar effects have been reported in previous studies which used Sprague-Dawley rats [17, 18, 23] and our data therefore indicate that this is a suitable model in which

critical gap length repair can be conducted in athymic animals which tolerate implantation of human cells. While the focus of this study was to test the effectiveness of EngNT-CTX in nerve repair, it would be interesting in future studies to explore the fate of the implanted cells and the mechanisms by which they support regeneration. This could be achieved in the rat model by using human-specific immunostaining to identify the CTX cells at various time points following implantation [14]. This could provide information about the similarities and differences in terms of host cell infiltration and the interaction between host cells and transplanted cells in EngNT-CTX compared to autografts in the first few weeks following nerve gap repair.

The collagen membrane wrapped around the EngNT was selected to have similar mechanical properties to host nerve tissue which made handling and implanting the manufactured EngNT constructs similar to nerve autografting. The collagen membrane maintained the EngNT at the repair site throughout the regeneration period and there were no adverse effects such as additional fibrosis or inflammation observed during tissue harvest. This is the first report of the Collagen Solutions collagen membrane material being characterised mechanically and used in a nerve repair context. It showed broadly similar tensile strength and stiffness properties to nerve tissue and by wrapping multiple layers around the EngNT it was able to withstand similar forces to nerve tissue, mimicking the epineurium functionally. The methodology for wrapping EngNT with the collagen membrane was a manual procedure which would benefit from automation in order to make this approach scalable and suitable for clinical translation.

Histology in sections through the distal stump of repaired nerves was used to indicate the number of neurites which had traversed the injury site. In all cases there were fewer neurites present in the distal stump than the proximal stump, so data were expressed as % of proximal stump in order to accommodate any differences between individual animals in terms of the number of neurons present in the sciatic nerve. There were more neurites overall detected in the distal stumps of the autograft group compared to the EngNT-CTX group at 8 weeks, but a greater proportion of these were motor fibres in the EngNT-CTX group. By 16 weeks the number of total neurites and proportion of motor fibres was similar between the two groups.

The greater number of motor fibres seen at 8 weeks with the EngNT-CTX repair is consistent with the electrophysiology data, which showed a correspondingly greater gastrocnemius muscle CMAP amplitude in the EngNT-CTX group compared to the autograft group. This pattern of more ChAT negative neurites present in the autograft group along with a lower CMAP at 8 weeks could indicate a larger population of sensory neurites or non-conducting neurite sprouts [29]. The 8 week results indicate differences in the extent to which autograft and EngNT-CTX support regeneration, with sub-populations of neurons possibly responding at different rates in the two environments. It would be interesting to explore this further, for example by histological analysis of neuromuscular junction reinnervation in the target muscles at a series of time points. By 16 weeks the electrophysiology and histological data were equivalent between the two groups. CMAP recording was performed using two separate downstream muscles, the gastrocnemius and the tibialis anterior, which provides robust evidence of equivalent reinnervation in the EngNT-CTX and autograft groups.

Previous reports of electrophysiology data for a long gap defect in athymic nude rats is lacking, however, studies in wild-type rats have shown that the CMAP returns to ~40-60% of the contralateral after 12 weeks in shorter gap models (~10 mm) [30, 31]. This is broadly in line with the CMAP reported here in a longer gap, where autograft CMAP recovered to approximately 19% at 8 weeks and 34% at 16 weeks, with the EngNT-CTX group 12-14% higher at each time point.

While recovery of motor function was clear from the electrophysiological data, sensory analysis (SSI and von Frey) indicated that all animals recovered sensory function to a similar extent by the 16 week end-point. This contrasts with a previous study, involving a 13 mm gap defect in athymic nude rats bridged using autografts or silicone tubes filled with cell suspensions which showed differences in sensory function recovery between treatment groups within a time period of 12 – 16 weeks post injury [32]. While there are numerous differences between our study and that one, since sensory recovery was also seen in the empty conduit group where there was virtually no nerve tissue regeneration, it is likely that that the apparent sensory recovery detected was due to collateral sprouting.

**Conclusions**

In conclusion, the results from this study indicate that an EngNT-CTX construct can support nerve regeneration equivalent to the clinical gold standard autograft across a critical gap based on both histological and electrophysiological outcome measures. Furthermore, it can be implanted within a collagen membrane wrap to provide a replacement tissue with similar mechanical and handling properties to natural nerve. This preclinical data will therefore

support progression through the necessary manufacture and regulatory steps to testing in human patients.

**Acknowledgments:**
Funding for this project was provided by the UCL Technology Fund, UK Innovation and Science Seed Fund and an Investment Accelerator grant from Innovate-UK to Glialign Ltd. The CTX0E03 cells were kindly provided by ReNeuron Ltd (Pencoed, UK) and the collagen membrane was kindly supplied by Collagen Solutions (Glasgow, UK).

Thanks goes to Dr Celia Murray-Dunning for her involvement in conducting the gel contraction analysis in Fig 2B.

The contributions of the authors are as follows; MLDR – Conceptualisation; methodology; validation; formal analysis; investigation; writing-original draft and visualisation, AGED – Conceptualisation; methodology; validation; and investigation, KSB – formal analysis and investigation, JS –Conceptualisation; methodology; resources; supervision; project administration and funding acquisition, JBP – Conceptualisation; methodology; validation; resources; writing-original draft; supervision; project administration and funding acquisition.

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**Graphical abstract**