Magnetothermal Modulation of Calcium-Dependent Nerve Growth

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Nerve injuries are common, and the available treatments including invasive surgeries do not guarantee complete regeneration of the injured nerves and restoration of function. Despite the ability of peripheral nerves to regenerate, the slow rate of axonal growth hampers the functional recovery. Development of new approaches to discover underlying mechanisms that may accelerate axonal growth is needed to overcome these limitations and augment available treatments of nerve injury. In addition to chemical factors, recent studies suggested the use of optogenetics and electrical stimulation to promote axonal growth. The underlying mechanisms of these approaches, however, require further investigation. Furthermore, their application relies on invasive hardware, which may not be compatible with injured nerves under significant mechanical deformation. Here, it is shown that thermal activation of a heat sensitive ion channel TRPV1 promotes axonal growth in a calcium-dependent manner. By leveraging heat dissipation of magnetic nanoparticles in alternating magnetic fields, the calcium influx through TRPV1 channels endogenously expressed in dorsal root ganglion explants is triggered remotely. The accelerated axonal growth through elongation of neurofilaments and increased Schwann cell migration following magnetothermal stimulation is observed. These findings suggest future applications of magnetothermal modulation of axonal growth as a minimally invasive approach to accelerate nerve regeneration.

1. Introduction

Nerve injuries lead to lifelong disabilities with significant implications on the quality of life. Regenerative ability of peripheral nerves is hindered by the severity of the damage, the delay between the injury and intervention, and the patient age and medical history. Surgical interventions are common treatments for nerve injury and involve implantation of autografts or nerve guiding scaffolds intended to bridge the gaps between the proximal and distal nerve endings. However, surgical interventions alone, without additional biochemical cues, do not achieve complete regeneration, due to muscle atrophy, axonal denervation, and the slow rate of axonal growth.

In addition to surgical interventions, recent studies have explored chemical and biochemical cues such as controlled drug delivery or stem cell therapy to elucidate mechanistic principles underlying axonal growth and accelerate functional recovery following injury. Other approaches including optogenetics and electrical stimulation elicit neural excitation, which is hypothesized to trigger the secretion of factors such as brain-derived neurotrophic factor (BDNF), which supports axonal regeneration. The precise mechanisms underlying the growth-promoting effects of these approaches remain an area of active investigation, and their dependence on implanted hardware poses additional challenges to applications in delicate injured tissues. Furthermore, the use of transgenes in optogenetics currently limits its translational utility.
The emerging field of magnetic neuromodulation mediated by nanoparticles takes advantage of the negligible magnetic susceptibility and low conductivity of biological tissues to wirelessly deliver stimuli to target organs and cells deep within the body.\textsuperscript{23–26} Magnetic nanoparticles (MNPs) and their composites can be engineered to transduce magnetic fields into a variety of physical stimuli including heat, force, and chemical changes perceived by the cellular signaling machinery.\textsuperscript{27} Among these transduction modalities, hysteretic heating of MNPs in rapidly alternating magnetic fields (AMFs) has been most extensively applied across life science studies including cancer hyperthermia,\textsuperscript{28} neuromodulation,\textsuperscript{29–31} control of gene expression,\textsuperscript{32} hormone release,\textsuperscript{33} drug delivery,\textsuperscript{34–35} and stimulation therapy for neurological disorders.\textsuperscript{36} In the context of neuronal excitation and hormone release, magnetothermal transduction relied on triggering of calcium (Ca$^{2+}$) permeable heat sensitive ion channels, such as the capsaicin receptor transient receptor potential vanilloid family member 1 (TRPV1).\textsuperscript{37–39} TRPV1 is a non-selective cation channel activated by temperatures >41.5 ± 1.1 °C\textsuperscript{40} and pH values < 5.9.\textsuperscript{41} Interestingly, balancing of intercellular and extracellular calcium (Ca$^{2+}$) levels, has been hypothesized to promote axonal growth via regulation of the secretion of BDNF\textsuperscript{42} and netrin-1\textsuperscript{43} and the formation of the growth cone.\textsuperscript{44} Consistent with this hypothesis, activation of neurons by capsaicin was shown to accelerate axonal growth, which was attributed to activation of the protein kinase A pathway due to TRPV1-mediated Ca$^{2+}$ influx.\textsuperscript{45}

Considering the mediating role of Ca$^{2+}$ in promoting axonal growth and the permeability of TRPV1 to Ca$^{2+}$ influx, we hypothesized that a magnetothermal triggering of TRPV1 due to MNP heating would promote axonal growth. We employed dorsal root ganglion (DRG) explants as a model of peripheral nerve tissue to evaluate the possibility of accelerated axonal growth in response to TRPV1 activation in vitro (Figure 1a). We synthesized and functionalized MNPs and applied them as magnetothermal transducers in weak (40 kA m$^{-1}$) rapidly-alternating (165.7 ± 0.4 kHz) AMFs. TRPV1 expression and co-localization with neuronal markers was demonstrated in the DRG explants. DRGs were exposed to AMFs applied by a custom apparatus, and the magnetothermal modulation was optimized for applications in DRG cultures. We demonstrated that TRPV1 activation promoted accelerated axonal growth as revealed via neurofilament (NF) elongation and Schwann cell (SC) migration. This study offers insights into the role of TRPV1 in axonal growth and informs future applications for remote modulation of nerve regeneration.

2. Results and Discussion

2.1. Synthesis and Characterization of Magnetic Nanoparticles

Using previously reported organometallic synthesis, monodispersed chemically inert oleic-acid capped MNPs were produced with a diameter of 21 ± 1 nm (Figure 1b; Figure S1, Supporting Information).\textsuperscript{46} These MNPs exhibited saturation magnetization of 94 ± 14 emu g$^{-1}$\textsubscript{Fe3O4} as measured via vibrating sample magnetometry (VSM) (Figure 1b). Subsequently, surface coating with an amphiphilic block-co-polymer of poly(maleic anhydride-alt-1-octadecene) (PMAO) and poly(ethylene glycol) (PEG) was performed to render the oleic-acid capped hydrophobic MNPs biocompatible and stable in aqueous physiological conditions. The resulting PMAO-PEG layer had an average thickness of 4 nm as confirmed by transmission electron microscopy (TEM) (Figure 1b). Calorimetric evaluation of the heat dissipation by the MNPs was performed in an AMF with an amplitude $H_0 = 35$ kA m$^{-1}$ and a resonance frequency $f = 152$ kHz delivered by an electromagnet driven by a resonant circuit. We determined the specific loss power (SLP, heating efficiency per gram of iron) to be 639 ± 72 W g$^{-1}$Fe ($n = 3$, mean ± standard deviation, Figure 1b).

2.2. Design of the Magnetothermal Approach to Stimulate DRGs In Vitro

To enable magnetothermal activation of DRG explants, we designed a custom apparatus capable of delivering AMFs with amplitudes up to 40 kA m$^{-1}$ at a frequency of $f = 165.7 ± 0.4$ kHz to a working volume of ≈200 cm$^3$, which was suitable for simultaneous stimulation of four samples in a 4-well plate (Figure 2a–c; Figure S2, Supporting Information). Infrared (IR) camera imaging confirmed consistent increase in temperature in all four locations of a 4-well plate in the presence of MNPs (Figure 2b,c; Figure S2, Supporting Information). Similar instruments were previously applied for magnetothermal and chemomagnetic modulation in live subjects,\textsuperscript{31–34,36} indicating the possibility of future applications to promote nerve growth in vivo, including at a scale of human patients.\textsuperscript{47} Using this AMF apparatus, we verified the heating efficiency of our MNPs over repeated cycles of stimulation. Under AMF stimulation the temperature of the MNP solution (5 mg mL$^{-1}$) consistently increased from 37.5 to 43.5 °C in ≈40 sec across 3 cycles, as measured via a nonconductive optical temperature probe (Figure 2d,e). We further verified that the AMF coil itself was not producing excessive heating by repeating the measurements with culture media without MNPs (Figure 2d,e).

2.3. TRPV1 Expression in DRG Explants

Although TRPV1 expression in DRGs is well documented,\textsuperscript{37} we sought to further confirm the expression of the channel in our preparation of the DRG explants from the neonatal rats (Figure S1, Supporting Information). In addition, we aimed to characterize TRPV1 expression in the neuronal and glial cells within the explants. Using immunohistochemistry, we observed membrane TRPV1 expression in DRG neuronal cell bodies and in axons. (Figure 3a; Figure S3, Supporting Information). Interestingly, in addition to neurons, TRPV1 expression was also found to be associated with a marker of SCs ($100$).

2.4. The Response of DRG Explants to TRPV1 Activation

To assess whether the TRPV1 activation indeed yields Ca$^{2+}$ influx in our DRG explants, we exposed them to the TRPV1 agonist capsaicin (CAP, 10 μm in dimethyl sulfoxide, DMSO and Tyrode’s solution). Using fluorescent imaging with a
synthetic Ca\textsuperscript{2+} indicator Fluo-4 we found capsaicin-evoked Ca\textsuperscript{2+} influx in 41.5 ± 9.6% (mean ± standard error of the mean) of the cultured cells (Figure 3c,d; Figure S4, Supporting Information). This fraction of responsive cells decreased to 14.23 ± 7.4% in cultures incubated with the TRPV1 antagonist capsazepine (CZP, a final concentration of 5 µm), prior to exposure to CAP (Figure 3e,f; Figure S4, Supporting Information). Negligible responses were found upon exposure to DMSO alone (0.1% in Tyrode’s solution) (Figure S4, Supporting Information). We then applied magnetothermal stimulation to the DRG explants by incubating them in MNP solutions (5 mg mL\textsuperscript{-1}) and exposing them to ≈90 sec AMF stimulus. At these conditions the solution temperature increased above 42 °C, sufficient to trigger Ca\textsuperscript{2+} influx mediated by TRPV1 channels. Note that spontaneous neuronal excitation was observed in ≈5% of cells prior to the onset of magnetothermal stimulus. However, an additional ≈22% of cells were responsive to the magnetothermal stimulus commensurate with reaching the TRPV1 threshold (Figure S4, Supporting Information).

2.5. Magnetothermal Stimulation of TRPV1 to Promote Axonal Growth

The expression of TRPV1 in DRG explants, their response to magnetothermal activation manifested in Ca\textsuperscript{2+} influx, and the
role of Ca\textsuperscript{2+} in directing axonal growth, motivated us to test the hypothesis that the exposure to AMF in the presence of MNPs to increase the local temperature above TRPV1 threshold would promote neurite outgrowth. The latter can be quantified by measuring the elongation of cytoskeletal NF.

Prior to testing our hypothesis, we examined the biocompatibility of MNPs in the presence and absence of repeated AMF stimuli in DRG explants. We found that 3 cycles of temperature elevation reaching the TRPV1 threshold with the total stimulation duration of up to 10 min did not have a significant effect on cell viability, as determined via a Live–Dead assay (Figure 2f; Figure S1, Supporting Information). Biocompatibility of magnetite MNPs with a similar surface chemistry was verified previously by our group and others\cite{33,49}, which was further confirmed in this study. Based on prior findings, we did not anticipate significant internalization of the MNPs by the DRG cells\cite{33}.

To maintain sterile environment during stimulation experiments, we first measured via an optical thermometer the temperature increase in the MNP media in the presence of AMF prior to the start of each experiment. We then applied identical cycles of AMF to achieve the calibrated temperature increase in each trial (Figure S2, Supporting Information).

The efficacy of the magnetothermal stimulation on promoting axonal growth was examined via immunooanalysis of the expression of NF, performed 24 h following stimulation. To quantify NF elongation, we collected mosaic scans of entire DRGs using confocal microscopy. We developed an image analysis algorithm that quantifies the fluorescence intensity of NF expression in radial sections centered on DRG bodies (Figure 4: Figure 1c; Figure S5, Supporting Information). When comparing cells exposed to AMF stimulation in the presence or absence of MNPs (Figure 4a,b), we observed a significant increase in the elongation of NF in the MNP group. By dividing the radial sections around the DRG body into 50 bins, we found a 3.22 increase in NF expression in bins 21–40 in the MNP group versus the control group (Figure 4e,f). The accelerated axonal growth was diminished when DRGs were stimulated with MNPs in the absence of the TRPV1 antagonist CZP (Figure 4c,e,f). Incubation with DMSO (0.1% in medium), the CZP vehicle, alone in the presence of MNPs, yielded a significant increase in NF elongation compared to DRG incubation with CZP with a 13-fold increase in NF expression (Figure 4d,g,h). Furthermore, no significant difference was observed when comparing DRGs stimulated in the presence of MNPs, with and without incubation with DMSO.
Live–dead viability assay confirmed that exposure to CZP and DMSO (in cell media for 20 min) does not affect DRG viability (Figure S7, Supporting Information). Similarly, incubation with MNPs did not yield increases in axonal growth in the absence of AMF stimulation (Figure 4i,j; Figure S8, Supporting Information).

We then investigated whether accelerated axonal growth in response to magnetothermal stimulation can be detected over shorter time scales. We found that 3 h following stimulation, there was no significant difference in the NF elongation in DRGs exposed to AMFs in the presence or absence of MNPs in the media (Figure 4k,l; Figure S8, Supporting Information).

Figure 3. TRPV1 expression in DRG explants. a) TRPV1 expression in DRG explants visualized via immunofluorescence. Red – TRPV1; blue – DAPI. Scale bar = 40 µm. White arrows mark TRPV1 staining on cell bodies and axons b) Co-staining of TRPV1 (red) with i) neurofilament (NF, green), ii) NeuN (green), iii) S100 (green). Blue – DAPI in all images. Scale bar = 40 µm. c–f) Fluorescence change in response to capsaicin (CAP) in DRG explants: c,e) Before and after fluorescence images of the Ca²⁺ indicator Fluo-4 demonstrating Ca²⁺ influx into the DRG explants in response to c) 10 µM CAP or e) 10 µM CAP with addition of capsazepine (CZP, 5 µM). Scale bar = 100 µm. d,f) Average fluorescence intensity change ∆F normalized to the average baseline fluorescence F₀ during the first 20 s of recording (∆F/F₀) with exposure to d) CAP or f) CAP with addition of CZP. Solid line = mean, shaded area = standard error of the mean (s.e.m.)

We then investigated whether accelerated axonal growth in response to magnetothermal stimulation can be detected over shorter time scales. We found that 3 h following stimulation, there was no significant difference in the NF elongation in DRGs exposed to AMFs in the presence or absence of MNPs in the media (Figure 4k,l; Figure S8, Supporting Information). This suggests that boosting Ca²⁺ influx induces prolonged changes in cell signaling and cytoskeletal structure, which in our setup could only be observed after an extended period of recovery and growth. This observation inspires the future development of more sensitive readout strategies for early effects of magnetothermal stimulation.

Although our findings are consistent with prior in vitro studies indicating that TRPV1-mediated calcium influx may accelerate neurite outgrowth, the role of this channel in mediating axonal growth in vivo demands further investigation. For instance, previous studies in a rat model of a sciatic nerve injury observed that blocking TRPV1 could accelerate axonal growth. Such differences might be attributed to the timeline of the TRPV1 blockade in relation to the moment of injury, as effects of TRPV1 signaling are likely to differ between acute and chronic injury models. Given that MNPs have been previously shown to persist in tissues over several months, we anticipate that our technology will be well suited to investigate such differences by enabling stimulation at different timescales in animal models of injury.

As compared to pharmacological manipulations of TRPV1, MNPs eliminate side effects of systemic drug administration and offer enhanced spatiotemporal resolution. Additionally, by using MNPs with different magnetic coercivities that exhibit efficient heat dissipation in distinct AMF conditions, multiplexed magnetothermal stimulation may permit independent control over different cell populations. In future studies, this effect can be exploited to delineate contributions of different cells to TRPV1-mediated calcium influx and modulation of nerve regeneration in vivo.
2.6. Schwann Cell Migration Triggered by TRPV1 Activation

The number of SCs at the nerve injury site is known to decrease over time stemming from and contributing to continuing denervation. However, these cells play a critical role in modulating axonal regeneration via dedifferentiation to progenitor-like cells, Wallerian degeneration, and breaking of the myelin sheaths.\[53\] We therefore sought to examine whether triggering TRPV1-mediated Ca\(^{2+}\) influx in DRG explants would accelerate SC migration. Using immunoanalysis performed 24 h post-stimulation we found that SC migration from the DRG explants was indeed significantly accelerated by AMF stimulation in the presence of MNPs as compared to a control group without MNPs (Figure 5).

3. Conclusion

Motivated by prior work,\[45\] we hypothesized that Ca\(^{2+}\) influx mediated by the endogenously expressed capsaicin receptor TRPV1 contributes to accelerated neurite outgrowth in a DRG model of peripheral nerve growth. To test this hypothesis,
we applied a remote magnetothermal stimulation previously shown to trigger TRPV1 and evoke Ca\textsuperscript{2+} influx. We quantified neurite elongation in response to AMF stimuli in the presence of heat-dissipating MNPs and found increased outgrowth 24 h but not 3 h following stimulation. Moreover, increased SCs migration was observed following TRPV1 activation. In agreement with previous studies,\textsuperscript{55–56} we observed co-localization of TRPV1 not only with neuronal but also with SC markers, which suggests that TRPV1-mediated Ca\textsuperscript{2+} influx could directly promote SC migration. Further mechanistic studies are necessary.

Figure 5. The effect of magnetothermal stimulation on Schwann cells (SCs) migration. a–d) A mosaic scan (20×) of the entire DRG explants with S100 expression (a marker for SC cells) 24 h following AMF stimulation a,b) with MNPs and c,d) without MNPs. Green- NF. Blue- DAPI. Scale bar = 500 µm. b,d) enlargement of the white square in (a,c) respectively. Scale bar = 200 µm. e) Higher resolutions images of representative areas around the DRG body to quantify S100 expression in the presence of MNPs (upper panel) or without MNPs (lower panel). Scale bar = 40 µm. f) Average fluorescence intensity (mean ± standard error of the mean) of S100 expression in DRG explants cells, quantified out of the total cells in the image. MNPs with stimulation (blue, n = 5). NoMNPs with stimulation (purple, n = 5). Significant difference was observed between the two groups (one-sided Student’s t-test, *P = 0.031).
to delineate the effects of neuronal factors versus direct Ca\textsuperscript{2+} influx into the SCs on their migration.

While we predominantly focused on the contribution of TRPV1 to the observed modulation of neurite growth, which was supported by the diminished stimulation effects in the presence of a TRPV1 antagonist CZP, other heat-sensitive ion channels may similarly be activated. For example, transient receptor potential ankyrin 1 (TRPA1) is a non-selective cation channel that can be activated by allyl isothiocyanate, temperatures <17 °C, and temperature transients.\textsuperscript{[27]} We found TRPA1 expression in the DRG explants suggesting its contribution to Ca\textsuperscript{2+} influx due to temperature transients associated with AMF cycles (Figure S9, Supporting Information). Moreover, the effect can also be attributed to thermal fluctuations on the cell membrane.\textsuperscript{[58]}

Although our study in vitro aimed at demonstrating the potential of magnetothermal stimulation as a means to accelerate nerve growth and offering mechanistic insights underlying this effect, future research is expected to benefit from applying this technology in vivo in injury models. In these studies, MNPs would likely be delivered to the injured nerve, and a remote application of AMF would enable repeatable excitation of TRPV1 activity and Ca\textsuperscript{2+} influx in untherted subjects. Such chronic magnetothermal stimulation mediated by TRPV1 has previously been shown safe and effective in longitudinal in vivo studies of adrenal hormone release and therapeutic brain stimulation.\textsuperscript{[33,36,59]} The MNPs properties and AMF conditions employed in these prior studies were similar to those used here for modulation of nerve growth, indicating that this approach can be safely deployed in peripheral nerves in vivo. Additionally, applications of thermal transport models may enable precise calibration of parameters to deliver the necessary magnetothermal dosage to trigger thermal receptors in vivo while avoiding tissue erosion from heat shock.\textsuperscript{[33]}

Although envisaged as a tool for fundamental studies of the role of Ca\textsuperscript{2+} signaling in nerve regeneration, magnetothermal modulation may advance future therapeutic approaches for peripheral nerve\textsuperscript{[33]} and spinal cord\textsuperscript{[60]} injury by complementing an array of methods to accelerate axonal growth. MNPs and weak magnetic fields offer a possibility to excite neural activity remotely and with minimal invasiveness granting access to tissues where implantation of hardware poses a risk of further injury. While clinical translation of magnetothermal technology awaits thorough safety studies, applications of this approach in animal models of injury may rapidly inform therapeutic paradigms aimed at modulating calcium signaling in neurons and glia during regeneration.

### 4. Experimental Section

All solvents and reagents were purchased from Sigma-Aldrich unless otherwise mentioned in the text.

**Dorsal Root Ganglion (DRG) Culture:** DRG extraction was approved by the MIT Committee on Animal Care (0820-080-23).

DRGs explants were extracted from P0 Sprague Dawley rat pups. After extraction the DRG roots were cut and only the DRG body was seeded on a 12 mm round coverslips coated with 70 µL of Matrigel\textregistered solution (Corning®) diluted 1:30 in cell media, and placed within 24 and 4-well plates. On each coverslip, one DRG explant was seeded and grown for 2 days in incubator before the stimulation. For magnetothermal stimulation with calcium imaging, DRCs were seeded on 5 mm round coverslips fitting the dimensions of the gap of the coil (~7.5 mm). The media used was Neurobasal medium supplemented with serum free B27 (ThermoFisher).

**Magnetic Nanoparticle (MNP) Synthesis:** MNPs were synthesized according to the previously published protocol.\textsuperscript{[46]} Briefly, iron-oleate complex was prepared by heating to reflux at 60 °C, 92 mmol of sodium oleate (95%, TCI America), and 30 mmol of FeCl\textsubscript{3}·6H\textsubscript{2}O (99%, Acros) with a combination of solvents: 100 mL hexane, 50 mL ethanol, and 50 mL CH\textsubscript{3}CO\textsubscript{2}H and then heating to 170 °C and drying overnight on a hotplate. The mixture was then digested at 90 °C in 2:1 (volume ratio) of 1-octadecene (90%, 10 mL) and benzyl ether (98%, 5 mL) and heated to 200 °C. Mixture was heated to reflux at ~325 °C for 30 min followed by washes in hexane and ethanol solution (1:1 volume ratio). This synthetic procedure returns MNPs coated with oleic acid that can be stored in chloroform.

To render these particles water soluble and biocompatible, two polymers were grafted together and coated with the MNPs via intercalation of the grafted polymer. Two mixtures of 625 mg poly(ethylene glycol) amine (PEG, M.W. 5000) in 2 mL chloroform and 500 mg poly(maleic anhydride-alt-1-octadecene) (PMAO) in 2 mL chloroform were stirred overnight followed by chloroform evaporation overnight. The PEG-PMAO powder was re-suspended in chloroform (10 mg mL\textsuperscript{-1}) and sonicated with MNPs (10–20 mg mL\textsuperscript{-1}) for 1–2 h. Chloroform was evaporated overnight and the MNPs were sonicated for 1 h in Tris-Borate-EDTA (TBE) buffer (1x) and washed three times with milli-Q H\textsubscript{2}O. MNPs were stored in water at 4 °C until used.

**MNP Characterization:** To verify the polymeric coating and size of MNPs they were imaged using transmission electron microscopy (FEI Tecnai G2 Spirit TWIN). Hysteresis curves were generated using a vibrating sample magnetometer (VSM, Digital Measurement Systems Model 880A) in room temperature. The elemental concentration and iron content of MNPs was determined via inductively coupled plasma-optical emission spectroscopy ICP-OES (Agilent 7700 DV).

**Calorimetric Measurements of MNPs Heating Efficiency:** Heating efficiency, i.e., the specific loss power (SLP) of the MNPs was estimated according to temperature change measurements using an optical fiber temperature probe (Omega HFTFO-101) inserted to MNP solutions in water with a concentration of 2 mg mL\textsuperscript{-1} (n = 3). MNPs and the probe were placed in a small glass vial in a 7.5 mm gap of toroidal ferromagnetic core. A function generator was used to generate a voltage signal amplified by a 200 W amplifier (1020L, Electronics & Innovation). Field amplitude was determined using an inductive pickup coil. The circuit was calibrated to perform at frequency f = 152 kHz and field amplitude H\textsubscript{0} = 35 kA m\textsuperscript{-1}.

SLP was estimated by:

\[
\text{Specific Loss Power (SLP)} = \frac{C_w \Delta T \Delta m}{m} \quad (1)
\]

\(C_w = \text{Specific heat capacity of water, 4.184 } \frac{J}{m^3 K} \)

\(\Delta T/\Delta m = \text{Temperature increase under AMF} \)

\(m = \text{Concentration of MNPs diluted in water (g mL\textsuperscript{-1} ref)} \)

**Calcium Influx Imaging in DRG Explants:** For calcium imaging, calcium dye indicator Fluo-4 (Invitrogen) was used, incubated in the cell media for 45 min in 37 °C (1:1000 of 1 mm of Fluo-4 in dimethyl sulfoxide, DMSO). Cells media was replaced with Tyrode’s solution (at 37 °C) and imaged in inverted microscope (Olympus).

TRPV1 activation was examined using the TRPV1 agonist capsaicin (CAP, TCI America). Final concentration of 10 µm (total 0.1% DMSO) CAP was applied to the cells rapidly after baseline imaging (20 s). To verify the effect of DMSO on calcium influx, the CAP experiment was repeated with DMSO only. To verify the specificity to TRPV1 activation, the cells were incubated with capsazepine (final concentration of 5 µm CZP, #0464, TOCRIS), a TRPV1 antagonist for 20 min in the cell media, and repeated the same procedure for CAP administration in Tyrode’s solution.
Magnetotherm Stimulation During Calcium Imaging: Using the same coil and same circuit that was described in the calorimetry measurement, experiment was performed for simultaneous AMF stimulation and calcium imaging in the fluorescent microscope (Olympus) with x20 objective. The coil was mounted on the inverted microscope stage and driven-circuit was connected to the coil. Coverslip (5 mm) seeded with DRG was placed inside a sample holder covered with MNPs diluted in Tyrode’s solution. Temperature probe was used to measure the solution’s temperature during AMF stimulation. Fluo-4 was used as calcium indicator with the same procedure described above. A baseline of 20 s imaging was first imaged, followed by applying AMFs with frequency of 152 kHz and field amplitude H₀ = 35 kA m⁻¹.

Image Analysis of Calcium Influx into DRG Cells: All videos were taken using the Olympus CellSens software in a frame rate of 1 Hz and analyzed in ImageJ using the Bioformats package. Cells were marked in ImageJ using the functions “Threshold”, “Watershed”, and “Analyze Particles” resulting in automatic selection of the cells nuclei in the first frame. The mean value of the marked areas (cells) was then used and created a csv file that was analyzed in a custom MATLAB script. F₀ was calculated as the average fluorescence intensity during the first 20 s for each cell (baseline). ΔF/F₀ per cell in each frame was then calculated in MATLAB R2018b, and ΔF/ΔF₀ was plotted. A cell was defined as responsive when ΔF/F₀ value exceeded 15 times of the standard deviation calculated for the baseline.

AMF Stimulation of DRG Growth Within a Custom Apparatus: Two days following seeding within the 4-well plates, the DRGs (within plates) were placed within the coil and AMF stimulation was applied with the field amplitude of 40 kA m⁻¹ and frequency of 165.7 ± 0.4 kHz. Cell media was replaced with MNPs diluted in media for the stimulation duration (5 mg mL⁻¹). Following stimulation cells continued to grow in culture media without MNPs. The same height was retained in the coil for all experiments as the field amplitude can change in different heights within the coil. The ability to repeat the stimulation in physiological conditions across multiple cycles and trials was confirmed. Moreover, to keep sterile conditions, the temperature was not measured directly during the cell stimulation. Instead, a temperature profile was created for every experiment where the temperature increase was timed over two consecutive stimulations and used the exact same profile for experiments with the DRG cultures.

Stimulation was performed on day 2 from seeding and fixation of the cells for immunolocalization was performed at two time points: 3 and 24 h post-stimulation. The examined conditions included: DRGs incubated with MNPs with and without AMF stimulation, DRGs incubated with media only with and without AMF stimulation.

Additionally, the stimulation under the effects of capsazepine (CZP), the TRPV1 antagonist to examine the specificity to TRPV1 activation, and CZP vehicle DMSO was performed. DRGs were incubated with CZP (final concentration of 5 μM) for 20 min or with DMSO (same volume dilution) followed by AMF stimulation in the presence of MNPs and CZP or DMSO.

Viability Assay: To examine the cell viability in the presence of MNPs in their medium and under AMF stimulation, a Live–Dead Viability assay (Invitrogen) was performed. The percentage of live cells (via Calcein AM, green) and dead cells (via ethidium homodimer-1, red) were quantified in the DRG culture. Three hours post-stimulation, DRGs were incubated with the assay reagents and after 20 min of incubation at 37 °C, DRGs were imaged in a fluorescent microscope.

Similar assay was carried to examine the effect of DMSO and CZP on DRG cells viability. After 20 min incubation with either CAP/DMSO, similar procedure as described above was performed.

Immunohistochemistry: At the end of the experiment (3 or 24 h post-stimulation) the entire DRG explant was fixed for 15 min in 4% paraformaldehyde (PFA). After three washes with PBS, cells were permeabilized with 0.3% (vol/vol) Triton X-100 for 10 min at room temperature. Then, 2.5% donkey serum in PBS was used and incubated overnight in 4 °C. The next day, cells were incubated with primary antibody solution for 2 h, followed by three washes with PBS. Then, secondary antibody was added for another 2 h followed by another three washes with PBS. VECTASHIELD with DAPI (4’-6-diamidino-2-phenylindole) was used for staining the cell nuclei and for fluorescent preservation. The antibodies and dilutions are summarized in Table 1.

| Target | Primary antibody | Secondary antibody |
|--------|-----------------|-------------------|
| TRPV1 | Guinea pig (Gp) Anti-Capsaicin (AB5566, Millipore), 1:100, Lot# 3729344 | Cy3 AfiniPure Donkey Anti-Gp IgG (H+L) (Jackson ImmunoResearch Laboratories), 1:500 |
| TRPA1 | Rabbit anti-TRPA1 (NB110, Novus), 1:1000 | Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21206, Invitrogen), 1:1,000, Lot#2156521 |
| NeuN | Mouse Anti-NeuN antibody [187] (ab104224, Abcam), 1 μg mL⁻¹, Lot# GR3408621-2 | Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21202, Invitrogen), 1:1,000, Lot# 2309193 |
| S100 | Mouse Monoclonal Anti-S-100 (β-Subunit) antibody (S2532, Sigma-Aldrich), 1:100, Lot# 000131551 | Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21202, Invitrogen), 1:1,000, Lot# 2309193 |
| Neurofilament | Rabbit Anti-Neurofilament 200 antibody (N4142, Sigma-Aldrich), 1:500 | Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21206, Invitrogen), 1:1,000, Lot#2156521 |

For TRPV1/TRPA1 and neuronal/glial cells immunostaining, DRGs were grown for 3–5 days on coverslips and immuno-stained following the same procedure.

Imaging of Immunostained DRG Explants: A laser scanning confocal microscope (Fluoview FV1000, Olympus) was used for imaging of DRG whole explants with ×10, ×20, and ×60 objectives. Mosaic images of the entire DRG explant were generated by the FV1000 software (Olympus) with ×10 (for NF) or ×20 (for S100) objectives. Higher resolution images were taken with ×20 objective for S100 quantification.

Image Analysis of Neurofilaments Elongation: Matlab custom scripts were written for quantification of the fluorescent intensity across the different staining antibodies. NF quantification is summarized in Figure S5 (Supporting Information) and the following steps were carried: thresholding all the images at the same intensity level and making a binary image. The body of the DRG was then selected for each DRG image and defined the starting radius. The fluorescent intensity was then counted across 50 radial bins equally distributed around the DRG Body. Bins 21–40 were identified as significantly different between the groups of MNP stimulation versus no MNPs stimulation. To assess the differences in the axonal length between the different experimental conditions, bins were focused that were located far from the DRG perimeter. Additionally it was found that in DRG samples that were incubated with MNPs solutions, traces of particles remained at the DRG perimeter. The optical scattering and absorption of the MNPs interfered with visualization of immunofluorescence, and thus these bins (1–20) were eliminated from quantitative analyzes across all experimental groups.

For quantifying the cell viability using the Live–Dead assay, the total fluorescence intensity of live cells were counted normalized to the total intensity of all cells.
For quantifying the $100$ expression in DRG explants the cell nuclei (DAPI) was first identified. Then an area for interrogation around each nuclei was defined as a circle with a radius $1.5$ of the original nuclei, quantifying the positive expression (fluorescence intensity) in this area.

Statistical Analyses: For statistical quantification of fluorescent images the same threshold was applied to all immunofluorescence images across all conditions (MATLAB R2018b). Data were analyzed using Prism GraphPad 9 and MATLAB R2018b. Data were presented as mean ± standard error of the mean (S.E.M.). For all comparisons two-sided Student's t-test was used (except for the Schwann cells immunostaining analysis where one-sided Student's t-test was used) with threshold $*P < 0.05$, **$P < 0.01$. The results were obtained from at least four independent samples in each group. Power analysis was used to verify the required group size for analyzing the axonal growth based on the NF elongation quantitative image analysis of stimulation with MNPs versus without MNPs. DRG images were excluded from the analysis only if observable detachment of the DRG was noticed in the image or during the immunostaining process.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
D.R. and P.A. designed the experiments and conducted the analyses. D.R. and H.F. performed the experiments. D.R., Y.K., and K.N. synthesized, functionalized, and characterized the nanoparticles. F.K. and H.F. assembled and operated the magnetic apparatus. K.P. contributed to all the immunohistochemistry experiments and analyzes. All co-authors have contributed to writing of the manuscript.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
alternating magnetic fields, calcium, dorsal root ganglion, magnetic nanoparticles, nerve growth, TRPV1

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