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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Temperature-Dependent Modulation Of Axonal Growth In Neurons Via TRPV4 Activation

Femke Cornelissen

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

Prof. dr. Bert BRONE

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*Running title: *TRPV4 in Neuronal Growth*

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ABSTRACT

Traumatic brain injury (TBI) is characterized by an alteration in brain function caused by an external force. It is accompanied by both neuroinflammation and neurodegeneration. It is known that an increase in inflammation correlates with an increase in the brain's temperature. This, in turn, influences the brain's neuronal activity. One of the receptors that have a distinct role in thermosensation in the brain is Transient Receptor Potential Vanilloid 4 (TRPV4). Research is yet to agree on how TRPV4 activation affects neuronal growth. Here, we investigated the effect of TRPV4 activation on neuronal growth and filopodia/lamellopodia dynamics of primary cortical neurons. Cells were subjected to increasing temperatures ranging from 37°C to 39°C. Both live cell imaging and laser-scanning technology were used to determine the effects. Results showed that increasing the temperature led to a reduction in neuronal growth. Moreover, it was seen that in the absence of TRPV4, neuronal growth increased compared to growth at a physiological temperature of 37°C. Nevertheless, the rate of growth did not change. We can conclude that temperature has a distinct effect on neuronal growth and filopodia/lamellopodia dynamics and that the effect is caused by TRPV4 activation. In this work, we also showed the first steps towards a technology that allows for real-time and controlled modulation of temperature.

INTRODUCTION

Nowadays, traumatic brain injury (TBI) is still one of the leading causes of morbidity and mortality worldwide. Globally, the annual incidence of TBI is variably estimated at 27 to 69 million, and the injury continues to pose a significant challenge to health systems due to the major socioeconomic burden a TBI patient carries (1). TBI is characterized by an alteration in brain function caused by an external force (2). The injury is accompanied by neurodegeneration and neuroinflammation, and the pathophysiology of TBI comprises two distinct phases: the primary injury, which is directly related to the external impact on the brain and has a first cerebral damaging effect; the secondary injury, which can occur hours or days later and comprises biochemical cascades that further damage the brain (3). A crucial event accompanying the primary injury is the increase in blood flow and the recruitment of leukocytes, which both correlate with an increase in neuroinflammation and this, in turn, leads to a rise in the brain's temperature (4, 5).

The neuronal activity in the brain is highly sensitive to temperature differences, and even changes of 1°C or less can result in functional

alterations in the nervous system. The impact of this thermal increase can be seen in several physiological activities of the brain, such as action potential firing and synaptic transmission (5). Under normal conditions, the brain temperature depends on three main factors: cerebral blood flow, the temperature of the blood perfusing the brain and local heat production (6). Hyperthermia, a body temperature greater than 38°C, is frequently experienced by patients in the acute period following TBI. Various studies have shown a correlation between hyperthermia and worsened neurologic status in patients. In contrast to hyperthermia, hypothermia, a body temperature below 35°C, has been found to limit brain damage after injury. Marion *et al.* concluded that using mild hypothermia treatment (33° for 24h) improved neurologic recovery (7, 8).

It has to be noted that increased brain temperature does not always relate to a disease condition (Figure 1). In healthy individuals, the average brain temperature (36.1 to 40.9°C) is two or more degrees higher than the average body temperature (36,8 ± 0.4 °C). Various factors such as brain region, sex, age and menstrual cycle have been found to influence the brain temperature (5, 9, 10).

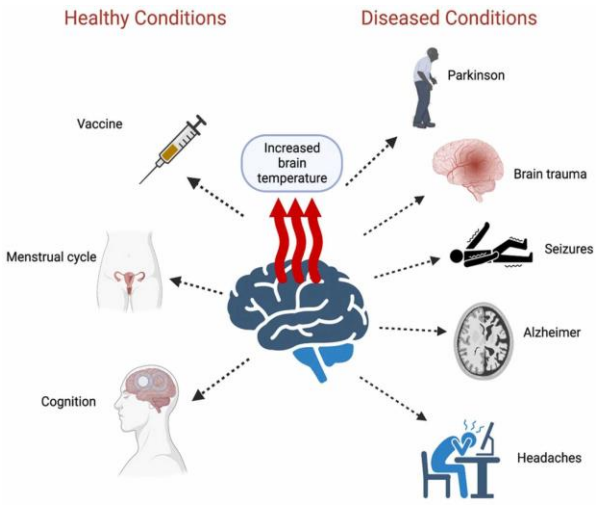

Figure 1: schematic overview of the correlation between increased brain temperature and various health conditions. *A rise in the brain's temperature can be observed in both healthy (as receiving a vaccine, the menstrual cycle, and cognitive activities) and diseased conditions (Parkinson's disease, brain trauma, seizures, Alzheimer's disease, and headaches) (5).*

After injury in the brain, the regeneration of neurons depends on axonal pathfinding, a process in which the axon's growth cone plays a crucial role. This highly motile structure, specialized with lamellipodia and filopodia, scans the extracellular environment for guidance cues, which determine the direction of growth (11, 12). Two types of guidance cues can be distinguished, namely, repulsive and attractive cues, which can be either chemical or physical cues. Until recently, the focus of most axonal guidance studies has been towards understanding the response of a neuronal system to different chemical cues (e.g. cytokines and neurotransmitters). This implies a lack of detailed investigation and evidence of the mechanism involved in the guidance of axons by physical cues such as temperature or force (13). As stated before, temperature increases will be found in the brain after injury occurs, and therefore, the increase can be seen as a guidance cue for regenerating axons.

An important group of receptors that play an important role in the physiology and pathophysiology of the central nervous system (CNS) and the peripheral nervous system (PNS) are the Transient Receptor Potential or TRP channels. These cationic channels act as signal transducers by either altering the membrane potential or the intracellular Ca^{2+} concentration and are activated by several chemical, mechanical, and thermal stimuli (14). The TRP superfamily can be divided into six subfamilies in mammals: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPA (Ankyrin), TRPML (Mucolipin), and TRPP (Polycystic). Here, the focus will be on the TRPV subfamily receptors, specifically TRPV4 (14).

TRPV4 is a Ca^{2+} - permeable non-selective ion channel that plays a distinct role in thermosensation in the brain. In addition, TRPV4 is also sensitive to osmolarity and mechanical changes (15). In thermosensation, activation of the channel occurs when temperatures rise above 34°C. Watanabe *et al.* reported that the activation of TRPV4 occurs at the physiological temperature range and that it is thus activated at normal body temperature. This may indicate that the channel serves as a constitutively activated Ca^{2+} entry channel sensitive to a small rise or fall in temperature (16). TRPV4 localizes in the growth cone, where it regulates axonal motility via influences on microtubules and actin. In turn,

microtubule dynamics are also an important regulator of TRPV4 activity. Moreover, TRPV4 forms a supra-molecular complex containing cytoskeletal proteins and regulatory kinases. This allows it to integrate signaling from various intracellular second messengers and signaling-cascades and to regulate cytoskeleton dynamics.

Very contradictory results have been found concerning the effect of TRPV4 on axonal growth. Goswami *et al.* reported that TRPV4 could both stabilize and destabilize microtubule structures in the cytoskeleton of rat dorsal root ganglion (DRG) neurons, depending on the activation state of the receptor. When activated, TRPV4 binds directly to the actin and microtubules of the cytoskeleton, leading to changes in the dynamics and, ultimately, to destabilization and retraction of the growth cone. Furthermore, in transfected F11 cells, TRPV4 activation led to rapid changes in the morphology of lamellipodia and filopodia and disrupted axonal integrity with varicosity formation and neurite retraction (17). In the study of Tian *et al.*, the effect of TRPV4 agonist GSK1016790A on the dendrite morphology of newborn neurons in the adult hippocampal dentate gyrus was tested. Results showed that the number of newly generated neurons was significantly decreased after TRPV4 activation (18). However, a positive effect of TRPV4 activation was found by Jang *et al.*, who stated that activation of TRPV4 is essential for axonal growth of neonatal rat DRG neurons in early development. TRPV4 was found to be highly expressed in peripheral sensory/motor nerves, and its activation was found to regulate neurite outgrowth through Neurotrophic Factor-derived mechanisms (19). The positive effect of TRPV4 activation is also supported by the findings of Shibasaki *et al.*, which indicate that TRPV4 activation in hippocampal neurons at the physiological temperature is important for regulating neuronal excitability and behaviors in mammals. Results show that TRPV4-deficient mice exhibit reduced depression-like and social behaviors compared to wild-type mice and that disruption of the brain temperature-detection system through TRPV4 poses a high risk of losing fine neuronal excitability and might cause abnormal behaviors. A fine line can be distinguished between the physiological and pathophysiological effects of TRPV4. It should be noted that the studies cited above did not consider the activation-temperature (<34) of TRPV4 in their research. Therefore, it is possible that the effects seen are related to other functionalities of the receptor than temperature.

Lastly, not only interactions with the receptor, but also the Ca^{2+} influx mediated by the activation of TRPV4 has shown to be important. It is proven that Ca^{2+} is an important regulator of axonal growth and that neurite outgrowth only occurs within the optimal range of Ca^{2+}. Therefore, it is possible that the change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), due to activation of TRPV4, inhibits neuronal growth (18).

In this work, we evaluated the characteristics of axonal growth of primary cortical murine neurons after subjecting them to increasing temperatures. We hypothesize that activation of TRPV4 by thermal increases (<37°C) will lead to deceleration or even inhibition of axonal outgrowth. More insights into the process of axonal growth in high temperatures related to pathogenesis are necessary to better understand the event of axonal regrowth after brain injury.

EXPERIMENTAL PROCEDURES

Animals – TRPV4 wild-type (WT) C57BL/6 mice were obtained from Charles River, and TRPV4 KO mice were kindly provided by the Laboratory of Ion Channel Research at KU Leuven. All mice were maintained in a temperature-controlled room (22±2°C) with food and water available ad libitum on a 12h light/dark cycle. The housing and experiments were conducted in accordance with the guidelines of the Belgian Law and the European Council Directive and approved by the Institutional Animal Care and Use Committee of Hasselt University.

Genotyping – Newborn pups were genotyped for TRPV4 on postnatal day 1 using PCR technology and gel electrophoresis. DNA from tail tissue was extracted using 10x Kapa Express extraction buffer, 1 U/µl Kapa Express extraction enzyme (KAPA Biosystems, USA) and Milli-Q water. DNA samples were diluted in a PCR mix solution containing 10 µM of each primer for TRPV4 (Table S1) in Kapa2G Fast genotyping mix. The used primers and PCR program can be found in the supplementary (Table S2). After PCR, samples were loaded on a 3% agarose gel (UltraPure Agarose, (Invitrogen), TAE 1x, 1:10000 GelRed (Biotium)) together with a control (DNA

SmartLadder, Eurogentec). DNA migration was started at a constant voltage (V) of 160. Gels were evaluated using the D-Digit Gel Scanner (LI-COR Biosciences). TRPV4 KO animals were used in filopodia/lamellipodia dynamics experiments.

Primary neuron isolation – Cortical neurons from TRPV4 WT or KO C57BL/6 mice were isolated. In brief, brains of postnatal day 0 to 2 (P0 – P2) mice pups were dissected and the midbrain, cerebellum, and meninges were carefully removed. The remainder of the brains was mechanically and enzymatically digested using a glass pipette and Neurobasal (NB, Gibco) supplemented with B27 (Gibco), Penicillin/Streptomycin (P/S, Invitrogen), glutamine (Sigma-Aldrich) and DNase (75 mg/15 mL, Roche). The cell suspension was filtered through a 70µm cell strainer. After, 5 mL fetal bovine serum (FBS) was added. This was washed by centrifuging (10 min, 1000 RPM) and the pellet was resuspended in NB (supplemented with B27, P/S and glutamine). Lastly, cells were seeded onto pre-coated (poly-D-lysine, 24h) 96-well plates (6.7 x 10^3 cells/well, Greiner), µ-slide 8-well plates (28.5 x 10^3 cells/well, Ibidi) or 35 mm MatTek glass-bottom dishes (15 x 10^4 cells/dish, MatTek) and incubated in a humidified incubator at 37°C, 5% CO_2.

Incucyte live cell imaging - Neuronal growth of primary neurons was visualized using the Incucyte S3 live cell imager (Sartorius). Primary cortical neurons cultured in 96-well plates (Greiner, 6.7 x 10^3 cells) were subjected to an ambient temperature of 37°C or 39°C and 5% CO_2. Neurons were imaged for 24 hours, and every 30 minutes, a picture was taken with an average scanning time of 5 minutes (whole plate). The agonist (GSK1016790A, Tocris) and antagonist (GSK2193874, Tocris) of TRPV4 were added as positive and negative control, respectively. The added concentration for the agonist equaled 0.03, 0.06, 0.1 and 0.3 µM. For the antagonist, concentrations of 0.03, 0.06, 0.1, 0.3, 1 and 10 µM were used. Branch length was measured by manually tracking branches of individual cells every 2h using NeuronJ (ImageJ, National Institute of Health). The total branch length per time point was calculated as the sum of branch lengths per time point per condition divided by total number of cells. The total speed of growth was calculated as the difference between timepoint 24h and timepoint 0h. The experiment was repeated three times, and the results shown are all three experiments combined.

Fluorescence microscopy optimization - Optimizing of the imaging time, agonist/antagonist concentrations and the overall experimental setup was done using a Zeiss Elyra PS.1 fluorescence microscope (with a Plan-Apochromat 63x/1.40 Oil DIC M27 objective, NA 1.4) provided with a 488 nm laser. Cells in a pre-coated 35 mm MatTek glass bottom dish (15 x 10^4 cells/dish, MatTek) were stained with Cellmask green membrane dye (1/10.000, Invitrogen) and incubated for 30 minutes. After, cells were washed 3x with Neurobasal without phenol red (NB without phenol red, Gibco). Cells were imaged in NB without phenol red and all images were processed using Fiji software.

Fluorescence microscopy – Growth cone dynamics were visualized using a Zeiss LSM880-Airyscan confocal microscope (with a Plan-Apo Chromat 63x/1.40 Oil DIC M27 objective, NA 1.4) provided with a CW Argon Laser tuned at 488 nm. Cells in pre-coated µ-slide 8-well plates (28.5 x 10^3 cells/well, Ibidi) were stained with Cellmask green membrane dye (1/10.000, Invitrogen) and incubated for 30 minutes. After, cells were washed 3x with NB without phenol red. Cells, either TRPV4 WT or KO, were first imaged at a baseline temperature of 37°C in NB without phenol red. After, cells were subjected to an increased temperature of 39°C. Neuronal growth was captured within 20-minute time-lapses (60 frames, 30-second intervals). All images were processed using Fiji software. Overlay images were obtained by overlapping timepoint 0 min (T0) and timepoint 20 min (T20). To calculate the area of the cells, the mean gray value of the cell was taken after thresholding for both T0 and T20. After, the mean gray values of both time points were compared to visualize growth dynamics.

Fabrication of thermal sensor – In collaboration with the Institute for Materials Research (imo-imomec, Uhasselt), a thermal sensor/actuator was designed for the simultaneous actuation and measurement of temperature within a

microfluidic space (figure 2). The sensor was fabricated, as stated in Oudebrouckx *et al.* (20)

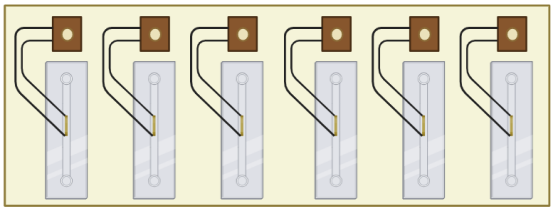

Figure 2: Simplified scheme of the design of the thermal sensor. *The sensor is composed of a glass coverslip, a polyimide structure covered with insulating Kapton tape containing microfluidic channels, and a plexiglass cover. Within the microfluidic channel, a copper wire is deposited which allows a rapid increase of temperature. Temperature is controlled by the influx of electric current mediated by a digital multimeter.*

Temperature calibration test – 100 µM Laser Grade rhodamine B (99% purity, Thermo Fisher Scientific) was dissolved in a carbonate buffer (pH 8) and filtered before use with a syringe filter (0.2 µm pore size). After, the dye was added to the thermal sensor. Fluorescence imaging of the rhodamine dye was performed using the Nikon Eclipse Ti2-E microscope (with a CFI Plan Fluor 4x/0.13 and CFI Plan-ApoChromat Lambda 20x/0.75 objective, Kinetix sCMOS camera and CoolLED pE-800 LED excitation) provided with a 550 nm laser. Fluctuations in the fluorescence intensity were captured within a 5-minute timelapse (125 frames, 400-millisecond intervals). Analysis of the fluorescent images was done using Fiji software. The increment/decrement of fluorescent intensity was visualized by plotting the Z-axis profile.

Optimization of cell growth within the thermal sensor – Cell viability within the thermal sensor was tested using the SH-SY5Y cell line. Briefly, the individual sensor parts were sterilized by UV radiation (1500 W, 15 min for each side). After, the sensor was assembled as stated elsewhere (20). SH-SY5Y cells were cultured in T75 flasks as follows. Old medium was discarded and left over medium was washed away using phosphate buffered saline (PBS). After, trypsin was added and spread evenly in the flask. Left over trypsin was discarded and the flask was incubated for 5 min. After, cells were detached by tapping the flask on the bench. 10 mL of Dulbecco′s Modified Eagle′s Medium (DMEM, supplemented with 10% FBS, 1% P/S, 1% l-glutamine) was added. The flask was washed with the aspirated medium containing the cells. Cells were collected in a 15 mL falcon tube and 0.5 mL of cells were added to a new T75 flask to maintain the cell line. SH-SY5Y cells were plated in both PDL-coated and non-coated sensors, and cell viability was assessed by eye. Different conditions of cell density (20.000, 25.000, 30.000 and 50.000 cells) and different methods of plating were tested. Additionally, cell viability was tested on the insulating Kapton tape within the sensor. Here, Kapton tape was pasted into the wells of a 24-well plate. Various conditions were tested: Kapton tape with coating, Kapton tape without coating, Kapton tape on glass coverslip with coating and Kapton tape on glass coverslip without coating. Viability was again assessed by eye.

Statistics - Statistical analysis and graphs were produced using Prism 9 (GraphPad Software). Data distributions were assessed for normality (Shapiro–Wilk). To compare multiple groups, a two-way ANOVA, followed by Tukey's multiple comparison test, was used if data was normally distributed. If not, the Kruskal-Wallis test was used. Details about the sample size and specific statistical analyses can be found in the figure legends. P-values smaller than .05 were considered significant.

RESULTS

Neuronal growth modulation induced by TRPV4 agonist GSK101 and antagonist GSK21 - To examine the effect of increasing temperature on neuronal growth, we must be able to compare the effect with an already established one. Therefore, we first determined how growth is modulated when TRPV4 is inhibited or activated. Figure 3 shows the dose-response curves of both the agonist GSK1016790A (referred to as GSK101) and the antagonist GSK2193874 (referred to as GSK21). In this first experiment, primary cortical neurons (referred to as neurons) were subjected to different concentrations of GSK101 (0.03, 0.06, 0.1 and 0.3 µM) and GSK21 (0.03, 0.06, 0.1, 0.3, 1 and 10 µM). Neurons were allowed to grow for 24 hours (24h) at a temperature of 37°C, and live cell imaging was used to visualize growth. Activation of TRPV4 occurred when the agonist GSK101 was added (figure 3A). Here, concentrations of 0.03, 0.1 and 0.3 resulted in a reduction of the average branch

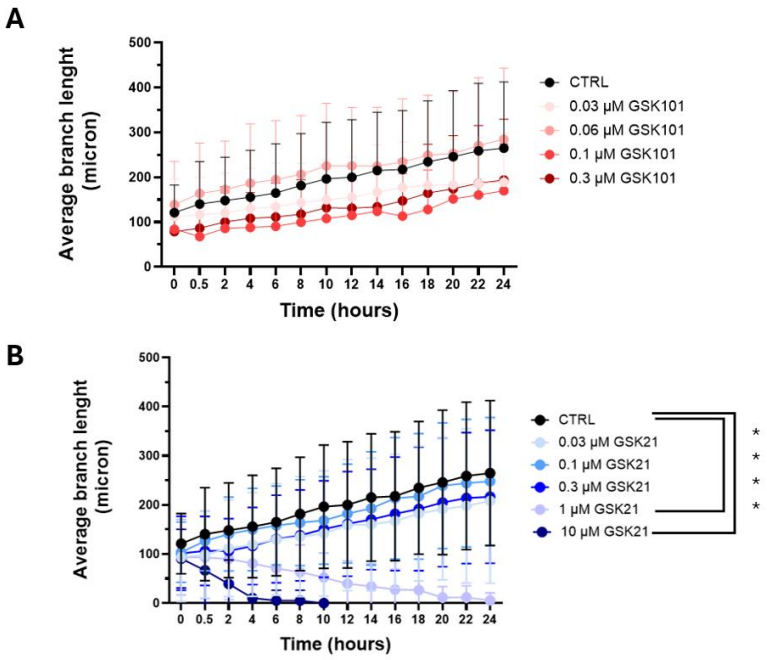

Figure 3: Dose-response curve of TRPV4 agonist and antagonist at 37°C. *Live-cell imaging was performed to obtain images of growing neurons over a period of 24h. Images of every two hours were analysed and neuronal growth, visualized as the average branch length of cells at distinct time points, was calculated. Neuronal growth of the various conditions was then compared to the control condition.* ***A:*** *Dose-response curve of agonist GSK101. Primary cortical neurons were subjected to various concentrations of GSK101 (0.03, 0.06, 0.1 and 0.3 µM) and were allowed to grow for 24h at a stable temperature of 37°C. No significant differences were seen when comparing the GSK101 concentrations with the control.* ***B:*** *Dose-response curve of antagonist GSK21. Primary cortical neurons were subjected to various concentrations of GSK21 (0.03, 0.06, 0.1, 0.3, 1 and 10 µM) and were allowed to grow for 24h at a stable temperature of 37°C. A significant difference (P <0,0001) was found when comparing 1 and 10 µM GSK21 to the control condition. However, due to the high amount of cell death found in these conditions, they were not taken into consideration. An ANOVA, followed by a Tukey multiple comparison test was used. In further experiments, 0.03 µM GSK101 and GSK21 will be used. TRPV4; transient-receptor potential vanilloid 4, **** = p < 0.0001*

length over 24h when compared to the control condition. However, the reduction was not significant. Inhibition of TRPV4 occurred when the antagonist GSK21 was added (figure 3B). A significant difference (P < 0.0001) was seen when comparing 1 and 10 µM GSK21 with the control condition. However, both conditions were not taken into consideration due to the high amount of cell death in these conditions. In further experiments, 0.03 µM was used as the optimal concentration of GSK101 because of its closeness to the maximum effective concentration (EC50, 0.018 nM) of the agonist and the expected result seen in figure 3 (21). For GSK21, the half maximal inhibitory concentration (IC_{50}) values are 2 and 40 nM for rat and human receptors, respectively (22). The exact value in mice is not yet determined, and therefore, we opted to take the same concentration as will be used for GSK101.

Neuronal growth modulation induced by temperature increases – Average branch lengths of neurons subjected to 37°C and 39°C were evaluated to determine the effect of temperature on neuronal growth. Figure 4A shows the average branch lengths at 37°C. A reduction of growth can be seen after both inhibiting and activating TRPV4 by adding GSK21 and GSK101, respectively. However, no significant difference could be found. When comparing 39°C (figure 4B), we saw that activation of TRPV4 did not have an effect on the average branch length. On the other hand, inhibiting TRPV4 increased the average branch length. Again, no significant difference could be

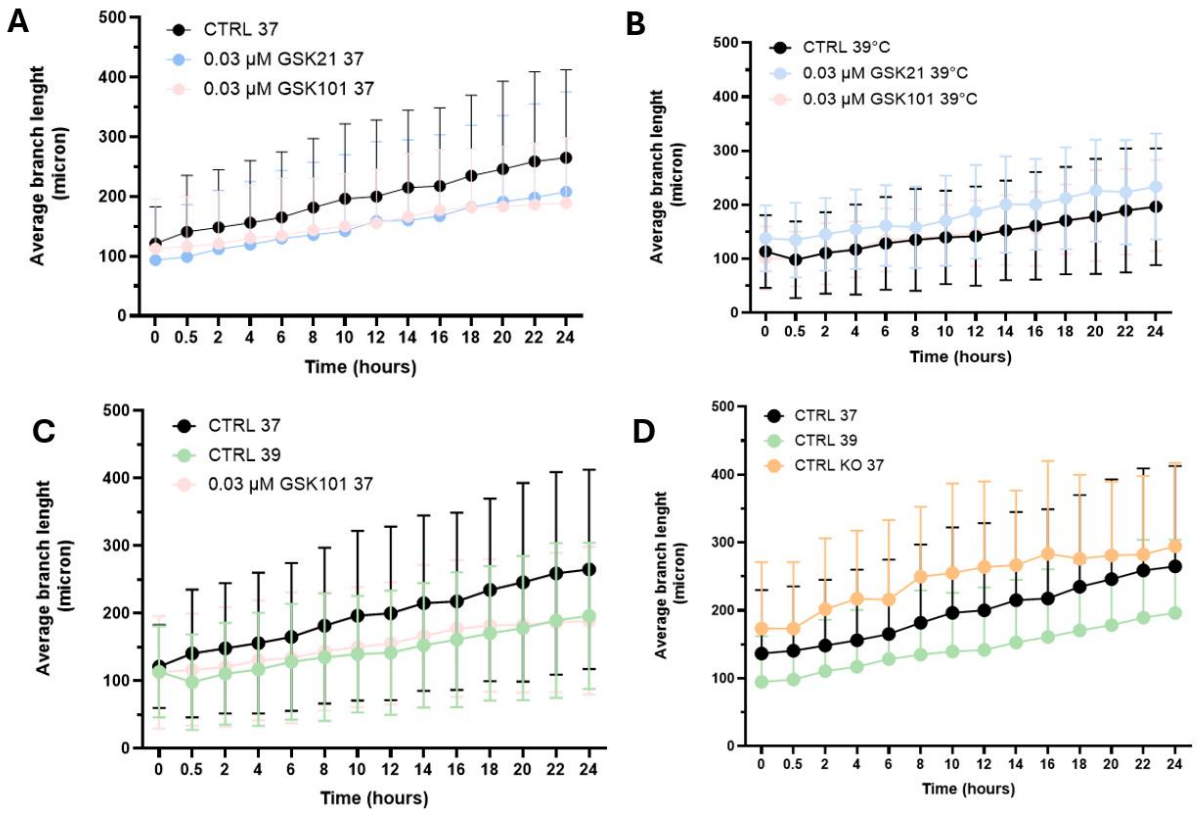

Figure 4: Neuronal growth modulation after temperature induction. *Neurons were allowed to grow for 24h at a stable temperature of 37°C or 39°C with and without the addition of GSK21 or GSK10. Black represents neurons without the interference of GSK21 or GSK101; Blue neurons where TRPV4 was inhibited by GSK21 (0.03 µM) and pink where TRPV4 was activated by GSK101(0.03 µM). Live-cell imaging was performed to obtain images over a period of 24h. Images of every two hours were analyzed, and neuronal growth, visualized as the average branch length of cells at distinct time points, was calculated.* ***A:*** *Neuronal growth at 37°C. A reduction of growth can be seen after adding both GSK101 and GSK21.* ***B:*** *Neuronal growth at 39°C. Inhibiting TRPV4 resulted in higher neuronal growth.* ***C:*** *Temperature vs TRPV4 activation. Neurons subjected to a temperature of 39°C were compared to neurons subjected to GSK101. Here, we see that the temperature effect observed in neurons that grew at 39°C is comparable with the effect when TRPV4 is activated in neurons at 37°C.* ***D:*** *Neuronal growth in control conditions. In all conditions (CTRL 37, CTRL 39 and CTRL KO 37), neurons were able to grow for 24h in either a stable temperature of 37°C (black and orange) or 39°C (green). Here, conditions were compared to the control condition of neurons without the TRPV4 receptor. Lower growth can be seen for both 37°C and 39°C. Statistical testing did not result in significant differences between the compared conditions for all graphs. TRPV4; transient receptor potential vanilloid 4*

found. What is striking is that the control condition at 39°C corresponds to the effect caused by activation of the receptor by GSK101 at 37°C (figure 4C). Despite the trend that can be seen, the results show no significant difference between the control at 37°C and the control at 39°C. Lastly, a comparison was made between TRPV4 wild-type (WT) mice and TRPV4 knock-out (KO) mice (figure 4D). A trend can be observed in which the average branch length reduces when activation of the receptor increases. Overall, no significant differences could be found for the various conditions.

Decreased speed of growth induced by GSK101 - The average speed of neuronal growth was examined to determine whether the lower growth of neurons at a temperature of 39°C or after activation of TRPV4 by GSK101 was due to a slower rate of growth (figure 5). When comparing

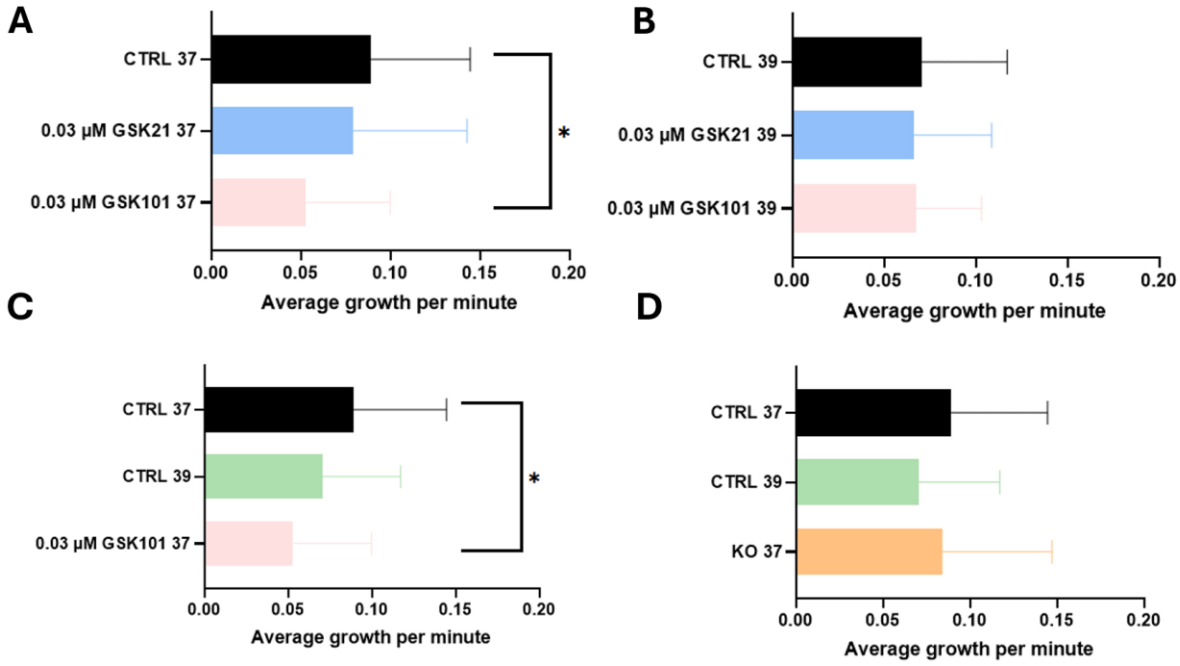

Figure 5: Average speed of growth of neurons subjected to different temperatures. *Neurons were allowed to grow for 24h at a stable temperature of 37°C or 39°C with and without the addition of GSK21 or GSK101. The speed of growth is visualized as the averaged total growth at time point 24h (T24) minus total growth at time point 0 (T0). Black represents neurons without the interference of GSK21 or GSK101; Blue neurons where TRPV4 was inhibited by GSK21 (0.03 µM) and pink where TRPV4 was activated by GSK101(0.03 µM).* ***A:*** *Average growth per minute at 37°C. Inhibiting TRPV4 slightly diminished the speed of growth, activating TRPV4 reduced the speed almost by half compared to the control. Statistical testing showed a significant difference (P = 0,0349) between the control and 0.03 µM GSK101 at 37°C.* ***B:*** *Average growth per minute at 39°C. Both inhibiting and activating TRPV4 did not change the speed of growth.* ***C:*** *Temperature vs TRPV4 activation. Neurons subjected to a temperature of 39°C were compared to neurons subjected to GSK101. Here, we see that increasing temperature does not have a significant effect on the speed of growth in neurons.* ***D:*** *Speed of growth in control conditions. In all conditions (CTRL 37, CTRL 39 and CTRL KO 37), the speed of growth was calculated for neurons, which were able to grow for 24h in either a stable temperature of 37°C (black and orange) or 39°C (green). Here, conditions were compared to the control condition of PCNs without the TRPV4 receptor. No significant differences were seen when comparing conditions. The Welch t-test was used to compare the control and 0.03 µM GSK101 at 37°C. TRPV4; transient receptor potential vanilloid 4, CTRL; control*

the different temperature conditions (37°C vs 39°C), no significant difference could be found (figure 5D). Moreover, absence of TRPV4 did not influence the speed of growth. A significant difference was seen in the average speed of growth when TRPV4 was activated by the addition of GSK101 (0.03 µM) at 37°C (figure 5A). The same effect was not observed when the temperature increased to 39°C (figure 5B).

Modulation of filopodia and lamellipodia dynamics by temperature increases - Growth dynamics were visualized more in-depth by using confocal laser-scanning microscopy. Here, neuronal growth was captured within a 20-minute timelapse. Figure 6A shows the area %, which correlates to the mean gray value of pixels, at the start (0 min, T0) and at the end (20 min, T20) of the timelapse. All conditions (37 WT, 39 WT, 37 KO and 39 KO) showed an increase in filopodia and/or lamellipodia growth. However, no significant differences were seen between T0 and T20 in all conditions. A very contradictory result was found for WT neurons subjected to a temperature of 39°C. Here, a decrease in area % was expected. Yet, neuronal growth was increased, and the area % was even higher when compared to WT neurons at 37°C (figure 6B). The increase in area % was the highest

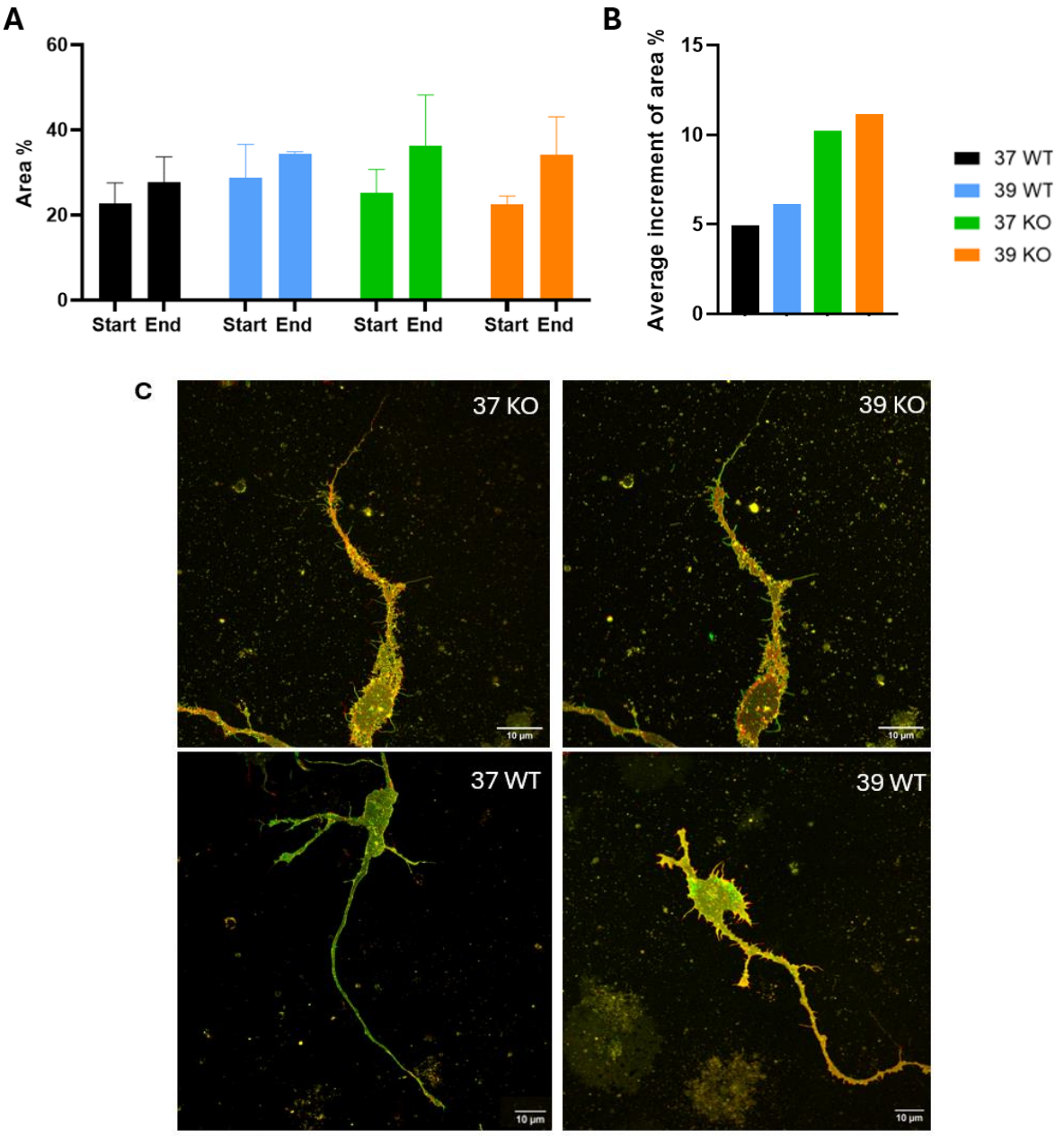

Figure 6: Filopodia and lamellipodia dynamics of neurons subjected to different temperatures. *Neurons were imaged using confocal laser-scanning microscopy, and filopodia/lamellipodia dynamics were visualized within a 20-minute timelapse. Start refers to the beginning of the timelapse (0 min, T0), and end refers to the end of the timelapse (20 min, T20).* ***A:*** *The percentage of the area (area %) occupied by the whole cell. The area % of both TRPV4 WT and KO mice subjected to different temperatures (37°C and 39°C) was measured at T0 and T20. All conditions showed an increase in filopodia and/or lamellipodia growth. No significant differences were seen between the T0 and T20 in all conditions.* ***B:*** *Average increment of area %. The area % at T0 was subtracted from the area % at T20. The increment was calculated for all conditions (37 WT, 39 WT, 37 KO and 39 KO). No significant differences were seen when comparing the different conditions.* ***C:*** *Confocal images of neurons (TRPV4 WT and KO) subjected to different temperatures (37°C or 39°C). T0 and T20 images were overlapped to visualize changes in filopodia and lamellipodia dynamics. Red represents filopodia/lamellipodia at T0,green at T20 and yellow represents the areas that remained static throughout the timelapse. TRPV4; transient receptor potential vanilloid 4, PCNs; primary cortical neurons, WT; wild-type, KO; knock-out*

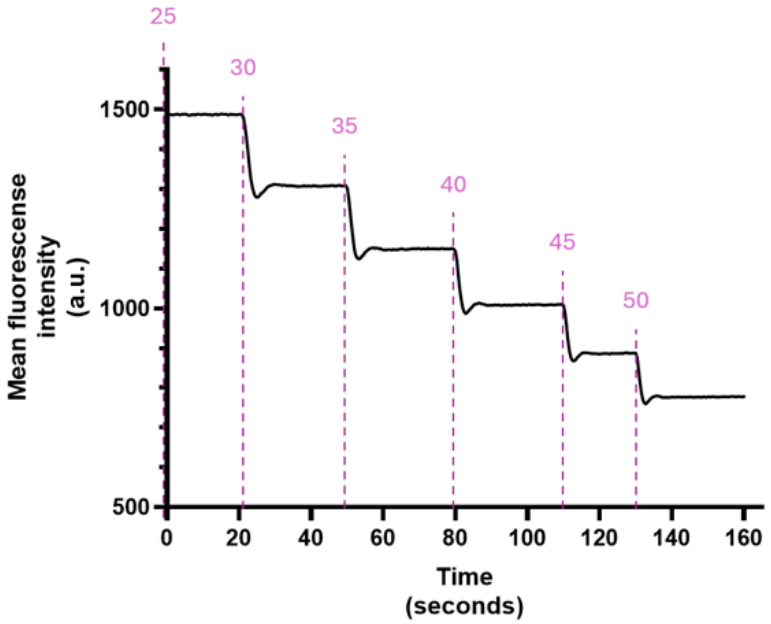

Figure 7: Z-axis profile of rhodamine fluorescence. *The figure shows the Z-axis profile of rhodamine fluorescence in function of temperature. Temperature increases of 5°C were induced every 20 to 30 seconds by changing electrical parameters such as current and resistance, and the rhodamine fluorescence intensity was allowed to stabilize over a period of 30 seconds. The pink values correspond to the induced temperature in °C. Fluorescent images were analyzed using ImageJ, and an inverse relation could be found between the fluorescence and the temperature. Within 2 to 3 seconds, the temperature was increased 5°C, confirming the rapid increase possible with the thermal sensor.*

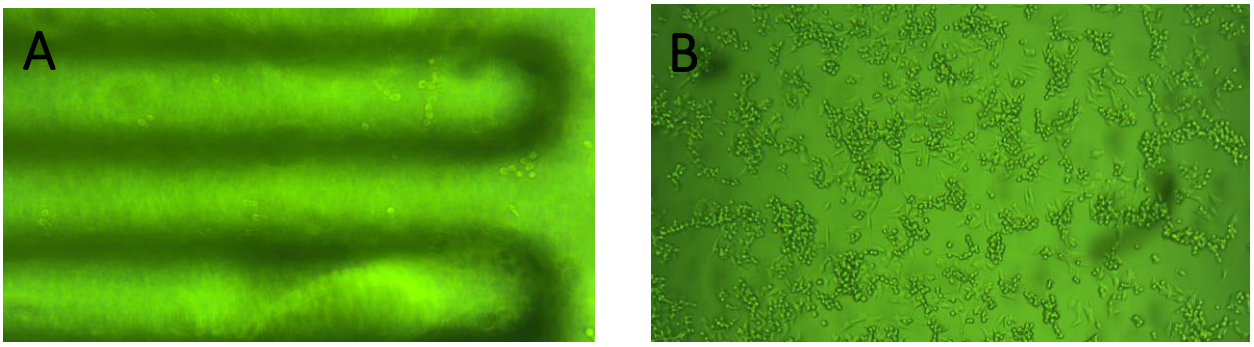

Figure 8: Cell viability of SH-SY5Y cells. *Cell viability of SH-SY5Y cells was tested within the microfluidic channel and on the insulating Kapton tape to assess biocompatibility.* ***A:*** *Cell viability within the microfluidic channel. SH-SY5Y cells were plated in various densities (20.000, 25.000, 30.000 and 50.000 cells) within the channels of the thermal sensor. Cell viability was assessed by eye, and cells were checked every hour for one day. No cell growth was seen within the microfluidic channel.* ***B:*** *Cell viability on insulating Kapton tape. SH-SY5Y cells were plated, and various conditions were tested: Kapton tape with coating, Kapton tape without coating, Kapton tape on glass coverslip with coating and Kapton tape on glass coverslip without coating. Cell viability was assessed by eye, and cells were checked every day for three days. After 3 DIV, cells were still alive and exhibited growth. DIV; days in vitro*

for the KO condition at 39°C. Despite the big differences between all conditions when comparing the increase in area %, no significant differences were found.

Rapid temperature modulation using a state-of-the-art thermal sensor – To be able to regulate temperature fluctuations in a more controlled manner, a state-of-the-art thermal sensor was fabricated in close collaboration with the Institute of Material Research (IMO-IMMEC) at Hasselt University. The sensor (figure 2, materials and methods) contains a copper wire within its microfluidic channels, which allows for a rapid change of temperature when electric parameters such as current and resistance are changed using a digital multimeter. The possible rapid increases in temperature within the sensor were visualized using rhodamine. The dye was added into the microfluidic channel of the sensor and fluorescence images were taken within a 5-minute timelapse. The fluorescence of rhodamine is inversely proportional to the temperature. Figure 7 shows the Z-axis profile of rhodamine subjected to increasing temperatures. Temperature increases of 5°C were induced every 20 to 30 seconds, and the rhodamine fluorescence intensity was allowed to stabilize over a period of 30 seconds. Within 2 to 3 seconds, the temperature was increased 5°C, confirming the rapid increase possible with the thermal sensor.

Decreased cell viability of SH-SY5Y cells with the thermal sensor – To visualize neuronal growth when temperature changes are within the range of 0.1°C, a state-of-the-art thermal sensor was created. In the last experiment, SH-SY5Y cells were cultured within the microfluidic channel of the receptor and cell viability was assessed by eye (figure 8A). A cell line was used as it is a faster and more efficient way to examine biocompatibility. In case of biocompatibility primary neurons will be used as a next step. When plated in the microfluidic channel, survival of the cells was low to zero after 1 day in vitro (DIV). To verify that it was not due to the cell line, HEK 293 cells (human embryonic kidney cells) were used for culturing (image not shown). Unfortunately, this also did not yield a positive result. The biocompatibility of all components was checked through a thorough literature search. However, little was known about the biocompatibility of the insulating Kapton tape used to trap heat within the sensor. To clarify this, the biocompatibility of the Kapton tape was tested. The tape was stuck to the bottom of wells in a 24-well plate. Different test conditions were created: Kapton tape with poly-D-lysine (PDL) coating, Kapton tape without PDL coating, Kapton tape on glass coverslip with PDL coating and Kapton tape on glass coverslip without PDL coating. SH-SY5Y cells were cultured on all test conditions, and cell viability was again assessed by eye (figure 8B). Viability was checked every day for 3 days, and still, after 3 DIVs, cells were alive and growing.

DISCUSSION

Neurons, the key players in our CNS, have the unique ability to receive and transmit information throughout the whole body. Neuronal dysfunction caused by TBI disrupts key functions of the CNS and is associated with various complications, such as behavioral changes and speech problems (23). Not only TBI but also pathologies such as fever can influence neuronal functions, but this perhaps to a lesser extent (24). What factors play a distinct role in neuronal dysfunction is not well understood. One of the factors we are interested in is the increased temperature in the brain during pathophysiological processes. It is known that temperature affects neural activity and neural functions, yet little is known about the normal and pathological fluctuations of brain temperature and the mechanisms underlying brain thermal homeostasis and its changes. Therefore, we investigated the effect of temperature on neuronal growth and growth dynamics. Additionally, we examined how activation of the thermosensitive TRPV4 might be the underlying reason for the displayed effect.

Dose-response curves of GSK101 and GSK21 reveal 0.03 µM as the optimal dose for inhibiting/activating TRPV4

Before examining the effect of temperature on neuronal growth, we first determined the optimal concentration of the TRPV4 agonist GSK101 and antagonist GSK21 (figure 3). For both, the EC_{50}/IC_{50} was kept in mind. The dose-response curves revealed that the addition of 0.03 µM for GSK101 resulted in the desired effect (figure 3A). For GSK21, the lowest tested concentration was seen as optimal (figure 3B). Further research should determine if other concentrations of GSK21

result in a different effect. Concentrations lower than or equal to 0.03 µM should be examined to determine if the reduction seen in figure 3B is indeed due to this low concentration. But also, concentrations between the range of 0.1 and 0.3 µM have to be investigated as the former showed no effect. It should be noted that the IC_{50} of GSK21 for mice has not been determined yet and that we did not take it into account to determine the optimal concentration.

Activation of TRPV4, caused by temperature increases, reduces neuronal growth in primary cortical neurons

Next, the effect of increasing temperatures on neuronal growth was investigated. Neurons grown at a stable temperature of 37°C or 39°C were compared to neurons which grew under the influence of the agonist or antagonist (figure 4). When comparing neuronal growth at 37°C, a reduction in growth was seen for both GSK101 and GSK21. A first explanation may be that both inhibition and activation lead to disruption of the calcium homeostasis of the cell. As described before, Ca^{2+} is a key regulator of neuronal growth, and neurite outgrowth only occurs within the optimal range of Ca^{2+} (18). Recently, Bagnell *et al.* suggested a link between TRPV4 activation and the activation of the Ras homolog family member A (RhoA) pathway. They demonstrated direct TRPV4-RhoA binding interactions that lead to bidirectional regulation that dynamically controls actin cytoskeletal changes (25). Activation of TRPV4 leads to the release of RhoA and calcium-mediated RhoA activation. Moreover, it has been said that in the context of neuronal injury, RhoA and Rho-associated protein kinase (ROCK) activation inhibit axonal regeneration. In animal models of TBI, activation of RhoA and ROCK signalling promoted dendritic spine retraction, whereas inhibition of RhoA signalling pathways led to improved motor and cognitive outcomes (26). This finding can be an explanation for why neuronal growth is improved when TRPV4 is inhibited by GSK21 at 39°C (figure 4B).

Increasing the temperature to 39°C showed a reduction of growth comparable to the reduction seen when TRPV4 is activated by GSK101 at 37°C (figure 4C). The result was not significant. However, this does suggest that temperature may play a role in neuronal growth and that TRPV4 may be a key driver of neuronal growth modulation. This last remark is supported by the results of Goswami *et al.*, who reported that TRPV4 interacts directly with actin, tubulin, and neurofilament proteins through its C-terminus and that the receptor colocalizes with actin-rich structures such as filopodia and lamellipodia. Both contribute to the regulation of cell morphology and net movement of neurites (17). Additionally, a temperature increase to 39°C can correlate to an increased activation of RhoA-dependent signaling leads to actin stress fiber formation and cellular contraction (25).

As mentioned before, TRPV4 is a Ca^{2+} permeable ion channel, and therefore, it is possible that neuronal growth modulation can be linked to the Ca^{2+} influx caused by TRPV4 activation. The study of Tsai *et al.* was suggested that local Ca^{2+}-pulses, if generated by local receptor signals, can serve as a local signaling mechanism that regulates speed, spatial sensing, turning, and chemotaxis of migrating cells by triggering local lamellipodia retraction and adhesion (27).

In addition to increasing temperature, neuronal growth was also examined in the absence of the TRPV4 receptor. Here, neuronal growth was higher compared to the growth at 37°C (figure 4D). But again, the difference was not significant (28). A possible explanation for the increased neuronal growth may be the absence of direct TRPV4-RhoA binding interactions. As mentioned before, RhoA and ROCK activation inhibits axonal regeneration. However, in the absence of TRPV4, it may be that RhoA activation does not occur and that down-stream signaling cascades may not be activated, resulting in an increased growth (25).

The finding that neuronal growth is improved in the absence of the receptor supports the speculations reported by Watanabe *et al.* that the channel is already activated at body temperature (<37°C) and that it serves as a constitutively activated Ca^{2+} entry channel sensitive to a small rise or fall in temperature.

TRPV4 activation by GSK101 lowered the speed of growth significantly in primary cortical neurons

To determine whether the lower growth of neurons at a temperature of 39°C or after activation of TRPV4 by GSK101 was due to a slower rate of growth, the average speed of neuronal growth was examined (figure 5). A significant reduction was

seen when TRPV4 got activated by GSK101 at 37°C (figure 5A). This result is in accordance with the finding that TRPV4 activation by GSK101 reduced neuronal growth. Also here, the explanation can be linked to the local Ca^{2+}-pulses generated by local receptor signals, which in turn will regulate speed.

Filopodia and lamellipodia show greater dynamics in absence of TRPV4

Lastly, filopodia and lamellipodia movements were observed to investigate the effect of temperature at a higher spatial resolution (figure 6). No clear differences were found in the dynamics of filopodia and lamellipodia when subjected to different temperatures or in the absence of TRPV4 due to the heterogeneity of the samples. However, cells subjected to 39°C showed slower dynamics when visually observed. This, too, can be explained using the results of Tsai *et al.* Activation of TRPV4 may result in local receptor signaling that influences local lamellipodia retraction and adhesion.
Experiments should be repeated, and the process should be optimized to minimize technical (incubation time, etc.) and biological (different litters,...) variation.

Real-time controllability of temperature using a state-of-the-art thermal sensor

In this work, a state-of-the-art thermal sensor was used that allows controlled temperature changes. This sensor was able to induce temperature increases of 5°C within 2 to 3 seconds, showing its potential to rapidly increase temperature.

In the initial experiments, cell viability was checked using SH-SY5Y cells (figure 8). Unfortunately, cells did not survive. The biocompatibility of all components was checked through a thorough literature search, except for the tape (3M, Belgium) used to combine all parts. However, little was known about the biocompatibility of the insulating Kapton tape used. Therefore, biocompatibility was tested using different test conditions (figure 8B). Results showed good biocompatibility of the Kapton tape. The next step would be to try an alternative for the 3M tape. One idea would be to use medical-grade tape. Despite the possible improvements, microfluidics is a challenging technology and optimization of the sensor is still needed to enhance cell viability.

CONCLUSION

Within this research project, the effect of temperature on neuronal growth was studied. Here, we identified a possible link between temperature and neuronal growth. However, our findings need to be confirmed by more research. Furthermore, the first step towards a state-of-the-art thermal sensor that allows for real-time and controlled modulation of temperature was made. A key issue in this work is optimization. Further experiments should be more optimized to result in significant results and variability within each experiment should be lowered. Overall, this work helped gain insight into a possible pathologic effect of temperature occurring within traumatic brain injury.

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Author contributions – BB and SZ conceived and designed the research. FC and SZ performed experiments and data analysis. JG, LJ and TV provided the thermal actuator device used in experiments. FC wrote the paper. All authors carefully edited the manuscript.

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SUPPLEMENTARY

Table 1: Primers. The table shows the primers used in the TRPV4 genotyping PCR program. Depending on the position of the bands, we can distinguish three genotypes. A band at 1200 bp indicates the knock-out genotype and at 950 bp the wild-type genotype. If both bands are observed the genotype is heterozygous.

WT forward	5' CTG TCC CAG CCT CCC CTC CT 3'
Mutant forward	5' CAT GAA ATC TGA CCT CTT GTC CCC 3'
Reverse	5' GCT CCT GTT GAA CAT GCT TAT CG 3'

PCR; polymerase chain reaction, TRPV4; transient receptor potential vanilloid 4, bp; base pairs, WT; wild-type

Table 2: PCR program. The table shows the PCR program that was used to genotype for TRPV4.

	Temperature	**Time**	**Repeats**
1	95°C	1'	1x
2	95°C	10''	1x
	64°C	10''	30x
	72°C	10''	1x
3	72°C	1'	1x
4	12°C	∞	

PCR; polymerase chain reaction, TRPV4; transient receptor potential vanilloid 4

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