Amino acid substitution in the S1 or CH1-CH2 linker domain of TRPM4: Two new TRPM4 variants found in complete heart block patients lead to gain of expression and gain of current

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ABSTRACT

Background: TRPM4 is a Ca\textsuperscript{2+}-activated ion channel permeable to monovalent cations. Its expression in the heart has been confirmed, with most data supporting its presence in the conductive cardiac tissue. Numerous investigations have linked TRPM4 mutations to conduction disorders such as right bundle branch block and complete heart block. More than 25 different TRPM4 variants have been reported in patients with conduction abnormalities. Interestingly, both gain- and loss-of-function variants were shown to exhibit similar pathological phenotypes.

Aims: To study the effect of two new TRPM4 variants found in patients suffering from complete heart block.

Methods: We transfected HEK-293 cells either with wildtype (WT) TRPM4 or one of these new variants: p.Tyr790Cys or p.Leu1113Val. We analysed total and membrane protein expression 48 hours after transfection using biotinylation assay and western blotting. To assess TRPM4 function, electrophysiological recordings were performed in the inside-out patch-clamp configuration.

Results: We found both variants (p.Tyr790Cys and p.Leu1113Val) to show a strong overall gain-of-expression, specifically at the plasma membrane. This correlated with an increased current in patch-clamp recordings.

Conclusions: Two novel TRPM4 variants (p.Tyr790Cys and p.Leu1113Val) responsible for complete heart block in young patients show a significant gain-of-function secondary to enhanced cell membrane cellular expression.

INTRODUCTION

The transient receptor potential melastatin 4 (TRPM4) channel belongs to the transient receptor potential (TRP) channel family. Its expression has been detected in the heart, kidney, immune, pancreatic, and muscle cells in mice and humans (1-4). The TRPM4 gene codes for a 1214-amino-acid long protein, located on chromosome 19 (5). While it is permeable only to monovalent cations, such as Na\textsuperscript{+}, K\textsuperscript{+}, Li\textsuperscript{+}, and Cs\textsuperscript{+}, the channel's activity is gated by intracellular calcium and is voltage modulated (6,7). Contrarily to other TRP channels, TRPM4 is not permeable to Ca\textsuperscript{2+} but plays an important role in
Ca\textsuperscript{2+}-dependent mechanisms such as muscular contraction, insulin secretion, immune response, and tumour development via membrane depolarization(5). The functional ion channel TRPM4 is a protein complex with a homo-tetrameric structure. Guo and others recently resolved its structure using Cryo-EM (8). Accordingly, each protein consists of the six transmembrane domains S1-S6, embedded in the lipid layer of the membrane. S1-S4 are the voltage-sensitive domains, whereas ion conduction occurs via pore domains S5-S6. The middle part contains the linker helix domain with twelve helices that allow interactions between domains of a subunit. The intracellular tier consists of the N-terminal nucleotide-binding domain, the ankyrin repeat domain, and the C-terminal coiled-coil helix.

In humans, cardiac TRPM4 mRNA is highly expressed in the Purkinje fibres of the heart, whereas it is much less expressed in the ventricles and His bundle (9,10). In Purkinje fibres of bovine hearts, TRPM4 protein has been detected as well (11). The precise role of TRPM4 in the heart remains to be elucidated. Several studies have identified patients with TRPM4 mutations leading to gain-of-expression, such as p.E7K, p.R164W, p.A432T, p.I376T, p.Q854R and p.G844D, or loss-of-expression, such as p.A432T, p.A101T, p.S1044C, and p.A101T/P1204L (11-16). Strikingly, the resulting gain- or loss-of-function electrophysiological measurements have both been associated with similar phenotypes ranging from atrioventricular block, right bundle branch block (RBBB) to complete heart block (11). Given the pathogenic role of TRPM4 in progressive familial heart block type I, it is likely that these reports of different types of cardiac conduction delays are different stages of the same disease (i.e. PFHB). In four of these mutants, p.A101T, p.S1044C, the double variant p.A101T/P1204L, and p.Q854R, Bianchi et al. showed that loss-of-expression was associated with increased TRPM4 protein turnover, whereas gain-of-expression TRPM4 variants displayed an extended protein half-life compared to WT(17).

This study uncovers two new TRPM4 variants and respectively presents two pedigrees of patients with permanent pacemaker requirements due to high-grade heart block at an early age. We further identify and characterize the biochemical properties of these two new TRPM4 variants.

MATERIAL AND METHODS
Patient participation and genetic screening
The patients enrolled were both patients that required pacemaker implantation due to high-grade heart block. State-of-the-art diagnostic screening did not yield positive results for any known putative genetic mutations. Thus, a targeted exome panel sequencing was performed by Next Generation Sequencing using the TrueSight One Sequencing Panel (Illumina). The results were compared with the reference sequence of the human TRPM4 gene (MIM# 606936; RefSeq NM_017636.3; Ensembl gene reference: ENSG00000130529). Written informed consent was obtained prior to the study according to the declaration of Helsinki.

Patient DNA
Remaining blood samples that had been drawn for other diagnostic purposes were used to extract DNA. The DNA was then amplified and subsequently sequenced.

Cells
All experiments were performed using human embryonic kidney 293 (HEK-293) cells. For use in cell culture, 1X Dulbecco’s Modified Eagle Medium 500 ml (1X DMEM) was always supplemented with 10% heat-inactivated Foetal bovine serum (FBS), 5 mM L-glutamine, 100 μg/ml streptomycin and 100 IU penicillin. Cells were kept at 37 °C and 5% CO₂.

Transfection
The reagent LipoD293™ was used for chemical transfections. The cells were transfected at 70-80% confluency for optimal transfection efficiency and minimal toxicity. For transfection, complementary DNA (cDNA) carrying either the patients’ mutation or the empty backbone vector was used. The concentration and purity of the two samples were determined using NanoDrop One (Thermo Fisher). The samples were then diluted to 500 ng/μL in nuclease-free water.
For each sample to analyse using biochemistry, 1000 ng of DNA or empty vector was transfected into 100 mm dishes according to the manufacturer’s manual. For each sample to analyse using patch-clamp, 300 ng of DNA or empty vector was transfected into 35 mm dishes according to the manufacturer’s manual.

Protein extraction
We used biotinylation to enrich cell surface proteins. Briefly, 48 hours after transfection, cells were washed with ice-cold PBS 1X (pH 8.0) and then incubated with
biotin (EZ-link Sulfo-NHS-SS-Biotin, Thermofischer scientific) for 35 min at 4°C. After the incubation period, cells were washed with ice-cold 50 mM Tris-HCl, followed by ice-cold PBS 1X (pH 8.0), and finally, pH 7.4. After the final wash step, cells were scraped and resuspended in lysis buffer containing 150 mM NaCl, 1 mM EGTA, 50 mM HEPES, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100 and complete protease inhibitor cocktail (Roche, Germany). After cells were subsequently solubilized for 2h at 4°C on a turning wheel, protein concentrations were measured using the Bradford assay. For the biotinylated fractions, 160 µl of magnetic Dynabeads™ MyOne™ Streptavidin T1 (Thermofischer scientific) were used per sample. The beads were first washed 6 times with lysis buffer using at least a 1:6 volume ratio and magnetic separation. After the last wash step, the supernatant was removed, and the biotinylated samples were added onto the beads. Depending on the available protein concentrations, we loaded 1 to 2 mg protein of the biotinylated sample onto the beads (same amounts for the same experimental runs). This was incubated overnight at 4°C on a turning wheel. After incubation, the coated beads were washed 4 times using magnetic separation. Finally, 50 µL of 2X NuPage sample buffer (Invitrogen) containing 100 mM dithiothreitol (DTT) was added onto the beads to elude the membrane proteins. For the input fractions, we used 4x NuPage sample buffer. All samples were denatured for 30 min at 37 °C. Samples were then centrifuged at 2’000 rcf for 2 min at room temperature to pellet remaining nuclei contamination as well as beads. The supernatant was used as protein sample to load onto the gel.

SDS-PAGE
For Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) we used a separation gel with a concentration of 9% acrylamide. Thirty-five µl of protein samples were loaded onto the gels, which were run at 50 V for the first 30 min, followed by 60 min at 180 V.

Western Blot
Proteins from the gel were transferred to a 0.2 µm nitrocellulose membrane using the Trans-Blot Turbo Transfer Pack and the Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer’s instructions. After transfer, the membrane was washed once with water. To ensure that the transfer of proteins worked properly, the
membrane was briefly immersed in the Ponceau-S solution. The remaining Ponceau dye was then washed away using water and 1X Tris-buffered saline (TBS). In a next step, the membrane was blocked with 5% milk for 1 hour at room temperature (RT) and subsequently washed three times with 0.05% TBS-Tween (TBS-T). Then, the membrane was incubated with the primary antibodies for 2 h and 30 min at RT. Table 1 lists all primary antibodies used with their respective dilutions. Incubation was followed by 5 washing steps in TBS-T. Then, the membrane was incubated with the secondary antibodies (dilution 1:15’000) for 90 min at RT. The membrane was washed three times with TBS-T and three times with 1X TBS. The membrane was scanned using the Odyssey fluorescence scanner (LI-COR).

<table>
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<th>Host species</th>
<th>Reference and Company</th>
<th>2° Antibody</th>
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<td>mouse</td>
<td>ab7671 (Abcam)</td>
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**Inside-out patch clamp**

Electrophysiological recordings were performed in the inside-out patch clamp configuration with patch pipettes (1–2 μm tip opening) pulled from 1.5 mm borosilicate glass capillaries (Zeitz-Instruments GmbH) using micropipette puller P 97 (Sutter Instruments). The tips were polished to have a pipette resistance of 2–4 MΩ in the
bath solution. The pipette solution contained (in mM) 150 NaCl, 10 HEPES, and 2 CaCl2 (pH 7.4 with NaOH). The bath solution contained (in mM) 150 NaCl 10 HEPES, 2 HEDTA (pH 7.4 with NaOH) as 0 Ca2+ solution. Solutions containing 300 μM Ca2+ were made by adding the appropriate concentration of CaCl2 without buffer to a solution containing (in mM) 150 NaCl, 10 HEPES (pH 7.4 with NaOH) as reported previously (18). Bath solution with 0 and 300 μM Ca2+ concentrations were applied to cells by a modified rapid solution exchanger (Perfusion Fast-Step SF-77B; Warner Instruments Corp.). Membrane currents were recorded with a Multiclamp 700B amplifier (Molecular Devices) controlled by Clampex 10 via a Digidata 1332A (Molecular Devices). Data were low-pass filtered at 5 kHz and sampled at 10 kHz. Experiments were performed at room temperature (20–25°C). For measuring steady-state currents, stimulation protocol consisted of a single 200 ms voltage step to −100 and then to +100 mV.

**Statistical Analyses**

Data are represented as the mean ± standard deviation. Unless otherwise stated Kruskal-Wallis test was used to compare the samples, with a \( p < 0.05 \) considered significant. Electrophysiology data were exported and analyzed using IGOR PRO 6 (Wavematrix, London, UK).

**RESULTS**

**Clinical phenotypes and genetic pedigrees**

{Figure 1}

The first index patient, a now 19-year-old boy, presented with syncope at the age of 13 years and had documented complete heart block (CHB) leading to pacemaker implantation. He displayed a heterozygous missense mutation (NM_017636.3:c.2369 A>G), leading to a replacement of tyrosine at the position of amino acid 790 by cysteine (p.Tyr790Cys). His father and 14-year-old sister, both carrying the same heterozygous mutation, have intermittent RBBB and persistent RBBB, respectively.

The second patient, a 4-month-old boy who presented with episodes of extreme lethargy, was noted to have a complete heart block, also requiring pacemaker implantation. Genetic analyses identified a novel \textit{TRPM4} heterozygous mutation (NM_017636.3:c.3337 C>G) leading to a replacement of leucine at the position of amino acid 1113 by valine. His 37-year-old mother was asymptomatic but
demonstrated complete RBBB and was an heterozygous carrier of the same mutation. Both identified TRPM4 variants are absent from large public databases, including gnomad.

The ECGs show cardiac conduction disorders
(Figure 2)
All ECGs from individuals carrying the TRPM4 mutations showed ECG evidence of cardiac conduction disease. The index patient carrying the mutation p.Tyr790Cys displayed sinus rhythm with a heart rate of 78 bpm and complete right bundle branch block (RBBB). Right axis deviation is noted possibly reflecting additional left posterior fascicular block (Figure 2A). His sister’s ECG (Figure 2B) showed a similar pattern, with sinus rhythm at a heart rate of 60 bpm, right axis deviation, and complete RBBB. Figure 2C shows the ECG of the 4-months-old index patient who carries the mutation p.Leu1113Val, with a complete heart block, an atrial rhythm of 156 bpm likely reflecting a junctional rhythm with intrinsic RBBB. His mother’s ECG, displayed in Figure 2D, shows sinus rhythm with complete RBBB.

Localization of amino acid substitution on TRPM4 structure
(Figure 3)
Figure 3A displays both amino acids affected by p.Tyr790Cys or p.Leu1113Val respectively. While p.Tyr790Cys is an amino substitution in transmembrane domain S1, p.Leu1113Val is located in the cytosolic, 19 residue long CH1-CH2 linker and is a substitution of the amino acid immediately proximal of CH2.(19) Figure 3B shows the close proximity of the tyrosine 790 residue to the Ca²⁺ binding pocket and neighbouring aromating rings of phenylalanine residues. Valine 1113, on the other hand, is located on the outmost lateral part of the channel and is surrounded by less potential interaction partners within immediate proximity. Moreover, as visualized in Figure 3C the amino substitution undergone in p.Tyr790Cys is substantial: The substituted residue no longer has an aromatic ring prone to pi-stacking, but instead has a thiol group capable of forming disulfide bridges. The substitution of p.Leu1113Val illustrated in Figure 3 E is more subtle: Leucine has a branched aliphatic side chain with three methyl groups, while valine has a similar side chain with two methyl groups.
**TRPM4 protein expression in HEK-293 cells**

{Figure 4}

To assess the functional implications of these genetic variants at the cellular level, we transfected HEK-293 cells with cDNA according to the one of these patients and performed biotinylation and western blot assays to quantify total, cell surface and intracellular protein expression. In Figure 4A, the empty vector and WT-TRPM4 samples display two TRPM4 bands. The TRPM4 detected in the empty vector corresponds to endogenous TRPM4, as demonstrated by Amarouch et al. (20) However, we can see that the expression of TRPM4 is stronger in the WT-transfected cells. The upper band (black arrow) is the fully glycosylated form of TRPM4, while the lower band is the core glycosylated (white arrow) form of the protein (21). Given their strong signal intensities, it is impossible to distinguish these two bands in the p.Tyr790Cys and p.Leu1113Val samples. Indeed, both variants induced a gain of TRPM4 expression in total cellular fraction, as shown in 4C, and even more strikingly in the biotinylated fraction, representing the channels expressed at the plasma membrane, as quantified in 4E. The presence of actin in the input fraction and its absence in the biotinylated fraction confirm the successful enrichment of cell surface proteins in the biotinylated fraction.

**TRPM4 functional activity**

{Figure 5}

These findings begged the question of whether these genetic variants leading to a gain of TRPM4 protein at the cell membrane also resulted in functional TRPM4 channels. Thus, we performed patch-clamp recordings using the inside-out configuration to measure the activity of the TRPM4 channels. Figure 5 shows that both variants induce approx. 4-fold increase in TRPM4 current compared to WT, indicating that these mutations preserve activity of the ion channel and, thus, the gain-of-expression induces a gain-of-function.

**DISCUSSION**

The present study identifies two novel heterozygous TRPM4 mutations leading to highly pathological complete heart block at a young age. The family members carrying one of these mutations all displayed a RBBB at the time of the study. This study further
characterizes these variants' biochemical and biophysical properties, demonstrating that both variants lead to a gain-of-expression of functional TRPM4 channels at the membrane, thereby inducing a gain-of-function.

TRPM4 mutations were first described in individuals with right bundle branch block (RBBB) in 2009 (16), while complete heart block has been first associated with TRPM4 mutations in 2017 (22). The patients' clinical presentations with syncope, complete heart block and healthy relatives displaying RBBB was also present in the identified gain-of-function mutation, p.Q854R (17).

Indeed, the western blots on whole-cell protein lysates (input), as well as membrane fraction (biotinylated) confirmed a strong increase in TRPM4 protein expression in both variants compared to WT. There was a trend suggesting the p.Tyr790Cys variant induced a stronger gain-of-expression, especially at the cell surface than the p.Leu1113Val. Compared to the WT channel, the average 10 to 40-fold increase (input and biotinylated fraction respectively) in the p.Tyr790Cys variant is, to our knowledge, the strongest gain-of-expressions ever observed caused by TRPM4 mutations. While two carriers of the mutation were healthy, merely displaying RBBB, the index patient presented with sudden, unexplained syncope at age 13. Since this mutation is located in the transmembrane domain, the striking gain-of-expression and function raises the question whether the mutation alters the binding properties of TRPM4 to membrane proteins and promotes a stronger accumulation at the membrane. Another mechanism that has been proposed for gain-of-function mutations, is a diminished TRPM4 endocytosis and degradation via altered SUMOylation (16). Nevertheless, this has only been demonstrated for variant TRPM4 p.E7K, which is situated between the N-terminus and the MHR1 (N-terminal homology region), making it more accessible to post-translational modifications. A similar mechanism could be postulated for variant p.Leu1113Val as the shift from leucine to valine in its unobstructed position could affect post-translational modifications or other interactions with binding partners. However, while many other characterized gain-of-expression mutations such as p.R164W, p.I376T, p.A432T, p.Q854R and p.G844D respectively occur in the domains MHR1-H3, MHR3-H2, MHR3-H5, S2-S3 linker or the sequence between S2 and S2-S3 linker, p.Tyr790Cys is the first that is located on a transmembrane helix (S1). Its sheltered location renders it less likely to be subject to post-translational modifications.
Furthermore, the patch clamp recordings showed a correlated gain-of-function indicating a preserved activity of the ion channel. However, this current increase was only approx. 4-fold that of WT (compared to 40x-fold increased protein surface expression). Therefore, it is possible that the activity of the single channels is altered by this mutation. This would be compatible with its location near the hydrophilic Ca\(^{2+}\) binding pocket. Dual et al. specifically speculated that binding of Ca\(^{2+}\) causes arginine 905 to move up toward tyrosine 790 in order to prime the channel for voltage-dependent opening (23). Hence, a shift from tyrosine to cysteine could potentially disturb the voltage-sensitivity of the channel. Thus, a compensatory upregulation of TRPM4 expression due to an impaired function would be a plausible alternative mechanism. This hypothesis is supported by the correlated increase in TRPM4 expression in the unbiotinylated intracellular fraction. Performing single-channel recordings to further confirm this hypothesis was beyond the scope of this study.

Despite our focus on gain-of-expression mutations, it has to be acknowledged that TRPM4 loss-of-expression variants have often displayed identical phenotypes (17). The leading hypothesis for this phenomenon is that these altered TRPM4 channels may influence supernormal conduction by modulating the number of open or recruitable Na\(_v\)1.5 channels (17,24). This voltage-gated sodium current is the driver of the depolarization phase in cardiac action potentials. While TRPM4 gain-of-function mutants may slightly depolarize the plasma membrane resting potential, leading to an increased number of inactivated Na\(_v\)1.5 channels precipitated in their refractory period, TRPM4 loss-of-function mutations could lead to a slight hyperpolarization of the resting membrane potential, causing an increased membrane depolarization that needs to be overcome to activate the Na\(_v\)1.5 channels, hence leading to a reduced excitability (14). Strikingly, Ozhathil et al. recently showed that Trpm4 deletion reduced the peak Na\(^+\) current in mouse cardiomyocytes (25). However, in-vivo mouse studies did not find altered resting membrane potentials in Trpm4 KO mice (26), nor in AAV9-induced overexpression of WT Trpm4(27). On the contrary, a recent study by Vandewiele et al. used different pathological pro-arrhythmic mouse models to demonstrate that TRPM4 was partially responsible for a long-lasting inward background current induced by Ca\(^{2+}\)-overload in cardiomyocytes(28). Using Trpm4 KO mice or newly proposed TRPM4 inhibitor and the anti-inflammatory drug meclofenamate, these authors could suppress these arrhythmias without overt...
adverse effects. This underscores the pathogenicity of gain-of-function TRPM4 mutations. However, in the clinical setting discussed in the present study, there is no reason to assume a Ca\(^{2+}\) overload. Thus, the pathogenic role of TRPM4 mutations is most likely trigger-dependent and multi-factorial and requires further mechanistic investigations.

In summary, we here characterize two new TRPM4 mutations, both leading to a gain-of-expression and gain-of-current in HEK-293 cells. Both mutations affect at least two members of each family. Their phenotype varied from RBBB to complete heart block. This study further demonstrates the role of TRPM4 in cardiac conduction disease (29). Thus, it underlines the importance for physicians to consider and test for mutations in the TRPM4 gene when faced with such phenotypes.

REFERENCES

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DISCLOSURES
None.
Figure 1. Pedigree of the families carrying the TRPM4 p.Tyr790Cys<sup>+</sup>/− and p.Leu1113Val<sup>+</sup>/− mutations respectively. Squares and circles indicate males and females, respectively; black symbols represent mutation carriers; open symbols represent mutation negative/unaffected persons; symbols with a slash represent deceased persons. Probands are indicated by an arrow.
Figure 2. 12-lead electrocardiogram (ECG) recorded with 25 mm/s, 10 mm/mV and 100Hz. 1 small red square represents 1 mm. (A) is the ECG of 19-year-old index patient with mutation p.Tyr790Cys. (B) shows the ECG of the 14-year-old sister of A. (C) represents the ECG of 4-months-old index patient with mutation p.Leu1113Val. (D) shows the ECG of the mother of C.
Figure 3. A-C are images from the RCSB PDB (rcsb.org) of PDB DOI (10.2210/pdb6BQV/pdb) from Autzen HE, Myasnikov AG, Campbell MG, Asarnow D, Julius D, Cheng Y. Structure of the human TRPM4 ion channel in a lipid nanodisc. Science. 2018;359:228-232. (A) shows the Ca\(^{2+}\) bound closed state structure of human TRPM4 with membrane prediction. The black rectangles signal the site of mutation around tyrosine at position 790 (pink) or leucine at position 1113 (green). (B) Expanded view on the rectangle around TYR 790 in A. (C) Expanded view on the rectangle around Leu 1113 in A. (D) is a visualization of the amino acid substitution caused by the missense mutation in p.Tyr790Cys. (E) is a visualization of the amino acid substitution caused by the missense mutation in p.Leu1113Val.
Figure 4. Protein expression measured by western blot. MW stands for molecular weight and is given in kilodalton (kDa). (A) shows a representative blot. The black rectangles represent distinct pictures of the membranes. While the top and middle rectangles, both revealed using the same secondary antibody, are two parts of the same membrane that has been cut between 70 and 55 kDa, the bottom membrane represents the top membrane revealed with a different secondary antibody. Samples loaded were protein lysates from HEK-293 cells either transfected with empty vector, wildtype TRPM4 (WT), p.Tyr790Cys or p.Leu1113Val. The left panel displays the protein expression in the input (= total) fraction, whereas the middle panel shows membrane-bound proteins in the biotinylated fraction, and the right panel represents the unbiotinylated intracellular fraction. Na+/K+-ATPase and actin were used as loading and biotinylation controls. (B) shows the quantified band intensity of TRPM4 normalized to the Na+/K+-ATPase band of p.Tyr790Cys and p.Leu1113Val relative to WT. * shows p-value <0.05. ** shows p-value <0.01 using the Kruskal-Wallis test. N=4 for all samples except p.Leu1113Val, where N=3. (C) shows the analogous quantification of (B) for the biotinylated fractions. * shows p-value <0.05. ** shows p-value <0.01 using the Kruskal-Wallis test. N=4 for all samples except p.Leu1113Val, where N=3.
Figure 5. Inside-out patch-clamp recordings. (A) shows representative traces recorded in patches excised from HEK-293 cells transfected with empty vector, wildtype TRPM4, p.Tyr790Cys or p.Leu1113Val variants. (B) Quantification of TRPM4 currents. * p-value <0.05 using non-parametric t test. N=6 cells per condition recorded on 3 different days.