Characterizing the P2X3 and CASK Interaction

A Major Qualifying Project Report

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Abstract

Chronic pain accompanies many diseases and conditions. The molecular pathways transmitting pain are not completely understood. Investigating the mechanisms at the pre-synapse has revealed involvement of P2X3 receptors and calcium-dependent scaffolding protein kinases (CASK). While involvement of these two molecules and their interaction have been described in pain transmission in rats, much less is known about the existence of this interaction in humans. This was the aim of our studies. Through overexpression of human P2X3 and CASK in HEK cells, co-immunoprecipitation, Western blotting, and immunostaining techniques, we studied the P2X3 and CASK interaction, focusing on the receptor C-terminal sequences. Overexpression of P2X3 and CASK was stable along with protein subcellular co-localization. CASK proteins were pulled down with human P2X3 receptors verifying an interaction. The mechanism of interaction remains under investigation and is a new target for the development of pain relief medication.

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1. Introduction

Chronic pain has become an increasing problem worldwide and impacts over 1.5 billion people ("Global Pain," 2011). Chronic pain is diagnosed as pain that can span from inconvenient to extreme, either continuous or periodic, and lasts longer than six months. It is difficult to treat chronic pain due to the symptoms experienced by the individual, ranging from aching, sharp, or throbbing feelings to burning, tingling, stabbing, or the pins and needles sensation ("Types of Pain," 2007). Pain severity is arbitrary and complicated to quantify because everyone registers pain differently ("Evaluation of Pain," 2007).

Because chronic pain is intricate and the pain pathways are not fully understood, pharmaceutical companies have created general medications to regulate pain by attempting to subsidize the symptoms, rather than blocking the pathways involved. Medications such as Vicodin and Percocet have been created that contain either hydrocodone or oxycodone, both of which are opioids. Opioids are a group of compounds that bind to opiate receptors, mostly located in the brain, and are known for decreasing pain severity for the patient ("What are Opioids?," 2011). These drugs are mainly to treat chronic pain, while Ibuprofen and Tylenol are medications that are used to treat acute pain, which is pain that lasts only a short period of time before subsiding ("Prescription Drug Abuse," n.d.).

Unfortunately, these medications have led to many problems such as addiction and drug efficacy. Even though opioids have the potential in minimizing pain that people experience, there is a high risk of addiction to these drugs because of the euphoric effect that is caused by the flood of dopamine and serotonin in the brain. In addition to this, the body becomes desensitized to the drug after several doses of the same amount, so the amount must be increased in order to achieve the same efficacy (Dugdale, 2010). Based on these reasons, further research should develop a novel way to treat pain rather than relying on medications with a chance of drug dependency.

There have been strides in understanding the molecular mechanisms of the sensitization in pain pathways in order to treat the source of pain, instead of the symptoms. We analyzed a specific purinergic receptor called P2X3, which is believed to have a modulatory role in transduction of pain stimuli and in chronic pain progression. Another component arguably related to P2X3 is calcium/calmodulin-dependent serine kinase (CASK), which was recently identified as a novel scaffold protein kinase that co-localizes with P2X3 (Gnanasekaran et al., 2013). However, the P2X3/CASK complex is still not completely understood, especially regarding the nature of the interaction and any molecular and structural determinants that regulate the complex. Despite the homology, significant differences have been observed between human and rat P2X3 receptors (Sundukova et al., 2012),

therefore it is necessary to confirm the P2X3 receptor and CASK protein complex with the human receptor.

At the Center for Biomedical Sciences and Engineering at the University of Nova Gorica, we researched the P2X3 receptor and CASK protein, their interaction, and differences found between human and rat P2X3 sequences in relation to CASK. P2X3's modulation and control to suppress pain signals hold potential for the development of novel pain relief medications.

2. Background

2.1 Chronic Pain Pathways

Pain can be distinguished into two different types: acute and chronic. Acute pain is defined as a sharp, quick pain that is felt when the body is injured and then subsides after the injury is healed. Chronic pain can be divided into many different sections due to its complexity in area, severity, and persistence. Chronic pain can turn into neuropathic pain if damage or dysfunction of nervous structures is observed and the pain inhibitory pathway is much weaker as well. When a painful stimulus is felt by nociceptive receptors found in the skin, the pathway to the brain is extremely similar in both cases (Hall, 2010).

When the body receives a stimulus from sensory receptors found in the skin, open nerve endings are activated. This creates a cascade of impulses throughout the peripheral nervous system (PNS) and central nervous system (CNS). The sensory neurons, also called Aδ- and C-fibers, transmit the signal towards the ganglia with different speeds due to thin or poorly myelinated fibers (Weiss et al., 2008). After passing the ganglion, the impulse enters the dorsal horn of the spinal cord where synapse interactions occur. Presynaptic voltage-gated calcium channels release vesicles containing glutamate into the synapse to activate the postsynaptic neuron via N-methyl-D-aspartate (NMDA) receptors. This occurs until the signal is relayed to the thalamus in the lower brainstem, which can then be transmitted to the sensory and motor cortices or the hypothalamus. From there, the sensation of pain is felt by the individual and either a motor or emotional response occurs.

Individual differences in the pain threshold can occur at the different neuronal levels, PNS or CNS. A motor response occurs within the spinal cord, without reaching the upper central neuronal components, and allows the individual to move away from the painful stimuli. Emotional responses occur when the signal is passed into the hypothalamus. Overall, once the stimulus begins, the pathway occurs instantaneously (Hall, 2010). Figure 2-1 below shows this pre- and post-synaptic interaction.

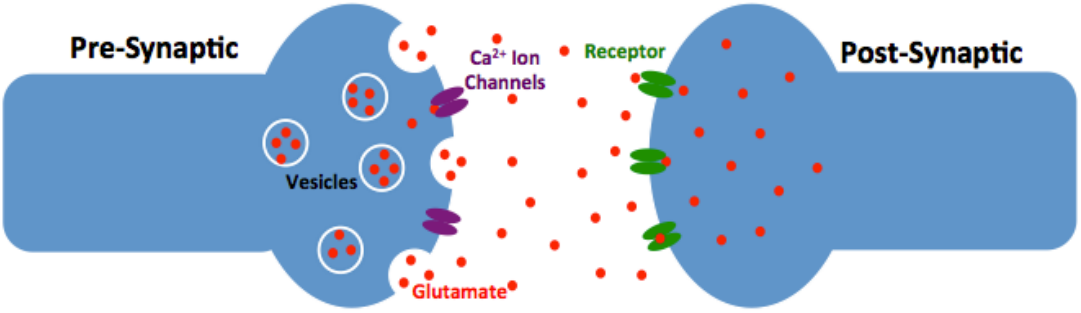

Figure 2-1: Pre and Postsynaptic Interaction

Additionally, there are also other ligand- and voltage-gated channels, including purinergic receptors, which are present in the periphery and synapse that facilitate in transferring the pain signal.

2.2 Current Trends in Pain Medication

Over-the-counter analgesic drugs, or OTCs, are non-prescription drugs that are used to treat different types of pain. The market for analgesic drugs has become present over the years with the increase of drug use in the population. Among these sorts of drugs are NSAIDs, or nonsteroidal anti-inflammatory drugs, which are also antipyretic, or fever-reducing. Some of the most effective NSAIDs known include aspirin and ibuprofen. This class of drugs is a safe, common, and inexpensive alternative to many pain medications (Abbott & Fraser, 1998).

NSAIDs work by inhibiting enzymatic activity that participates in physiological processes of the PNS and CNS. COX 1, or cyclooxygenase 1, and COX 2 are involved in the synthesis of prostaglandins, which can be autocrine or paracrine hormones. COX 1 is normally expressed in many tissues in the human body, while COX 2 is expressed in the presence of inflamed tissues, which accounts for redness in inflamed tissue regions. NSAIDs inhibit this action as antagonists, inhibiting the production of prostaglandins, thereby reducing redness and inflammation (Abbott & Fraser, 1998).

Opioids are a class of prescription analgesic drugs that have become generally used by the population. The most popular opioid is morphine, an alkaloid that originates from the juice of the poppy flower. Other opioids are naturally occurring and can be harvested, such as morphine. Opioids are mostly administered orally, but can also be introduced intravenously, subcutaneously, nasally, and through epidural means. Despite these modes of administration, their mechanism of action is the same (Schäfer, 2010).

Analgesic opioids, such as morphine and codeine, work by reducing neuronal excitement. This excitement is indicative of a pain signal, and they are defined as full opioid agonists, a term reserved for compounds that activate opioid receptors. Opioid receptors are a family of receptors that signal neurons to decrease the pain signal. The signal is usually minimized in the presence of endogenous opioids and takes the form of peptides which bind and activate the opioid receptors. Through the use of these agonists, the pain signal is decreased and pain is inhibited. This is their primary mechanism of action, yet nearly all drugs contain side effects. Side effects of opioid users include sedation and nausea (Schäfer, 2010).

Despite the efficacy of these two classes of drugs, they come with their disadvantages. NSAIDs, while lacking addictive properties, can be abused very easily. Since OTCs are available in supermarkets, drug stores, and pharmacies, they are available to the general population without prescription. Drug abuse, which may come with a lack of the effects of overdose and prolonged use, is a widespread and challenging problem that pharmacologists continue to tackle today. Perhaps the most destructive somatic disease that related to prolonged NSAID abuse is analgesic nephropathy, a kidney disease (Vadivel et al., 2006). Opioids are also addictive compounds due to their narcotic properties. Over time, those with prescriptions for opioids, such as hydrocodone or dihydrocodeine, may develop dependencies which can lead to full blown addictions (Veilleux et al., 2010). While these drugs do satisfy those suffering from pain, the side effects and risks still pose dangers. These drugs minimize the pain signal by nullifying neuronal excitement and impairing production of prostaglandins, yet do not directly inhibit the pain signal. Further research of the molecules and mechanisms that direct the pain signal ought to be a priority, rather than searching for new methods to avoid the onset of nephropathy or narcotic addictions.

2.3 Purinergic Receptors

Purinergic receptors, also named purinoceptors, are located in the plasma membrane and are known to be participants in many organism functions such as learning, locomotion, and feeding behavior. In addition, purinergic receptors have specific cellular functions such as proliferation and migration of neural stem cells, changes in blood vessels, apoptosis, and cytokine secretion (Burnstock, 2013). These receptors are active in a wide range of roles in the body.

Purinergic receptors include the P1 and P2 classes. The P1 family contains adenosine receptors and the P2 family contains P2X and P2Y subfamilies. The latter family provides affinities only for nucleosides. P2Y receptors have a slight affinity for adenosine triphosphate nucleoside (ATP), but accept

uracil triphosphate nucleoside, uracil diphosphate nucleoside (UDP), UDP-glucose, and adenosine diphosphate nucleoside as well. The P2X family binds to ATP with different affinities, from nM for P2X3 to mM ATP concentrations for P2X7 receptors (Jellinger, 2003).

2.3.1 P2X Receptors

P2X receptors are not found solely in mammals, but also in other organisms such as amoebas and zebrafish. They are ATP ligand-gated ion channels, meaning the presence of ATP opens the channel which subsequently regulates the passage of ions. Each P2X receptor consists of three subunits and each subunit is formed by two domains spanning the plasma membrane, a large extracellular loop, and intracellular carboxyl and amino termini. Multimers of P2X subunits, either homomeric or heteromeric, create the ion channels and different receptor subtypes are expressed in different tissues. In particular, homotrimeric P2X3 is being researched for its importance in pain, especially in chronic pain because the P2X3 receptor is highly expressed in sensory fibers (North, 2002).

2.3.2 P2X3 Receptor

Of the seven P2X receptors, P2X3 is suggested to be involved in pain transduction in sensory neurons. These receptors are specifically found on the pre-synapse in C-fiber afferent sensory neurons (Ford, 2012). Extracellular ATP rapidly activates the extracellular domain of P2X3 receptors, which are sensitive to nanomolar ATP concentrations, inducing an ion channel to form between three subunits. Calcium, potassium, and sodium flow through the non-specific channel and the resulting action potentials are interpreted as pain (Fabbretti & Nistri, 2012).

Researchers looked to identify negatively-charged residues owing to the extracellular activation of P2X3 receptors in the presence of the P2X3-specific agonist, alpha,beta-methylene-ATP (α,β-meATP), which is more stable than ATP and not hydrolysable. The use of this agonist, which induced enzymatic activity with an artificial molecule, rapidly desensitized P2X3. However, when Ca^{2+} flooded the extracellular domain, P2X3 receptors regained potential to activate in the presence of ATP. They found that P2X3 receptors rapidly activate and recover in the presence of high levels of extracellular Ca^{2+} (Fabbretti et al., 2004).

Recent advances have shown that human and murine P2X3 receptors differ in activity based on the phosphorylation state in C-terminus residues (Sundukova et al., 2012).

2.3.3 Comparison of Human and Rat P2X3 Sequences

Human and rat P2X3 sequences are extremely similar with their amino acid sequences being 97% identical (Garcia-Guzman et al., 1997). Focusing on the intracellular C-terminal region, there are few differences in residues between the two species.

Rat P2X3 receptors are inhibited when a C-terminal Src inhibitory kinase (Csk) phosphorylates tyrosine-393. However, human receptors have a phenylalanine at this residue position and tyrosine is located at residue 376. Since human receptors generate higher currents, slower desensitization, and faster recovery than rat receptors, research suggests that P2X3 activity results from this amino acid alteration (Sundukova et al., 2012).

When rat P2X3 receptors are mutagenized and tyrosine-393 is altered to a phenylalanine, there are significantly larger currents than seen in the wild type. When phenylalanine-376 is changed to tyrosine, there is no substantial difference in responses for the receptors (Sundukova et al., 2012). Therefore, tyrosine-393 and its interaction with Csk must alter the P2X3 response in rats (D'Arco et al., 2009).

When human receptors are mutagenized and tyrosine-376 is mutated to phenylalanine, there is a decreased current response when compared to the wild type. There is also a decrease when phenylalanine-393 is changed to tyrosine, however, the decrease is not as great indicating that tyrosine-376 is more vital in P2X3 response. These results conclude the importance of the different tyrosine residues found between humans and rats (Sundukova et al., 2012).

Figure 2-2 below shows a schematic representation of the human and rat C-terminal sequences and the two mutants used in this project, ManRat and RatMan, previously characterized in published work (Sundukova et al., 2012).

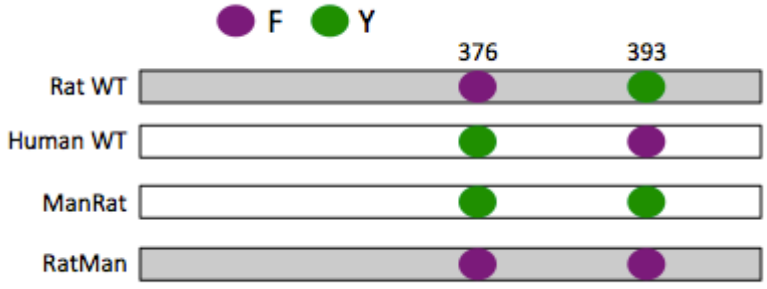

Figure 2-2: Rat, Human, ManRat, and RatMan P2X3 Sequences Compared

RatMan consists of a rat P2X3 receptor backbone with a Y393F single point mutation while ManRat contains a human P2X3 receptor backbone with a F393Y mutation. These mutants allow for experimentation to observe the role of these C-terminal residues in receptor function.

2.4 CASK

Calcium/calmodulin-dependent serine kinase (CASK) is a phosphorylating scaffold enzyme that is capable of altering the expression and function of P2X3 (Volonté & Burnstock, 2013). Recombinant expression of P2X3 and CASK in human embryonic kidney (HEK) 293T cells showed an increase in serine phosphorylation on the receptor protein, which has been shown to upregulate expression. CASK knock-out (KO) mutants were also found to enhance P2X3 expression, while silencing CASK resulted in decreased expression. Because of this, CASK has shown to exercise control over P2X3 receptors and may be an intracellular target for studying purinergic signalling in nociceptors (Gnanasekaran et al., 2013).

The interaction is postulated to have a phosphorylating mechanism. CASK has a pseudokinase domain suggested to phosphorylate neurexin (Mukherjee et al., 2008); however, no other CASK target has been identified. Being a scaffold protein, CASK could interact with adaptor proteins that may contain phosphorylating enzymes. This in turn extends the capabilities of CASK when it forms a complex with other proteins. Preliminary binding experiments suggest that the N-terminal P2X3 domain is not involved in the interaction. The interaction could be thus centralized at the intracellular C-terminus. CASK could modulate the activity of P2X3 in health or disease, resulting in higher or lower receptor opening efficiency. The postulated interaction between CASK and P2X3 is illustrated below in Figure 2-3. As the illustration suggests, CASK creates a complex with P2X3. When the channel opens, non-specific ions flow into the cell. The CASK/P2X3 complex may then disassemble (Gnanasekeran et al., 2013), suggesting that the interaction has a functional nature and is controlled by extracellular ATP. The complex disassembly is also important to control effectors downstream in purinergic signaling (Fabbretti, 2013).

Also, there is the possibility for more protein-protein interactions in this complex (Fabbretti, 2013; Volonté & Burnstock, 2013).

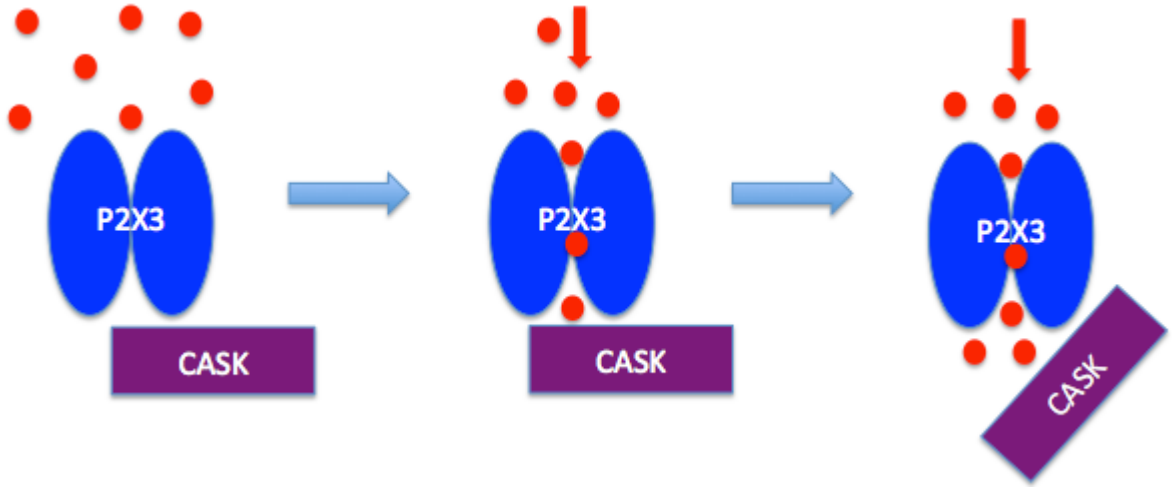

Figure 2-3: P2X3 and CASK Interaction. The P2X3 and CASK proteins are labeled. When ions, such as calcium and sodium, flow through the P2X3 receptor, the complex disassembles.

To analyze the importance of CASK in the CNS, CASK KO and knock-down mice were created, and the CASK KO mice died within one day of birth. In the same study, CASK expression was knocked-down to about 30 percent and these mice showed less bodily growth yet sustained viability. Further research examined that CASK was not necessary for evoked neurotransmitter release, but does play some extraordinary role in modulating spontaneous release of neurotransmitters essential to maintain the synaptic strength (Atasoy et al., 2007). Also, CASK mutants have been associated to diseases determined in altered synaptic function, such as mental retardation (Hackett, 2010).

2.5 P2X3 Antagonists

By targeting molecules involved in chronic pain, drug therapeutics can be configured to block the pain pathways. The pharmaceutical industry is interested in providing small molecular antagonists. These agents would be capable of blocking out pain signaling provoked through P2X3 and other similar ion channels (Ford, 2012).

One company is specifically targeting the P2X3 receptor and undergoing clinical trials. Afferent Pharmaceuticals is exploring the practical use of controlling P2X3's activity by developing novel therapeutics to treat chronic pain and other painful diseases. In 2011, they produced 9 different P2X3 antagonists that led to one compound that is currently in clinical studies with human patients to treat osteoarthritis pain, chronic cough, and interstitial cystitis. Orally administered AF-219 will complete its second phase of clinical trials in 2014 ("P2X3 Antagonism", 2012).

3. Materials and Methods

Cell Culture and Transfection

Human embryonic kidney (HEK) 293T cells were obtained from the ATCC (American Tissue Culture Collection, Maryland). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM of L-glutamate, and antibiotics (1X penicillin and 1X streptomycin). Cells were passaged once the cells were more than 70% confluent. To passage the cells, cells were incubated for 5 minutes in a solution containing 0.025% Trypsin and 0.01% EDTA in phosphate buffer solution (PBS) to digest extracellular matrix and cell-cell surface adhesion contacts. The proteolytic digestion was blocked by DMEM, supplemented with 10% FBS. Cells were recovered in a pellet by centrifugation (5 min). The supernatant was removed and a cell count was performed with a hemocytometer. The number of cells was calculated with the following equation:

$$\text{Cells/mL} = (\text{Live Cells} * 10^4) / (\text{Number of Squares} * \text{Dilution factor})$$

The desired number of cells was moved into a new flask containing new complete medium.

For the first half of the experiments, cells were plated in 35 mm Petri dishes and transfected with the calcium/phosphate method (D'Arco et al., 2009) and 0.5 µL of each plasmid. The plasmids and their concentrations are listed in Table 1.

Plasmid Concentrations for Transfections

Plasmid	Concentration
GFP	0.082 µg/µL
pcDNA3	0.088 µg/µL
CASK myc	~0.1 µg/µL
Human P2X3	~0.1 µg/µL
Rat P2X3	0.76 µg/µL
RatMan	0.1 µg/µL
ManRat	1.124 µg/µL
Human 220	0.210 µg/µL

Table 1: Plasmids and Their Concentrations used in Transfection Experiments. Concentrations were measured with the Quanti-iT dsDNA HS Assay kit (Q32851, Invitrogen) and samples were read in the Qubit™ fluorometer.

After calculating the plasmid concentrations and to obtain higher transfection efficiencies, the transfection protocol was changed. Cells were washed with PBS and a mixture containing 200 µL of Opti-MEM media (containing minimal essential media, HEPES, sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, and growth factors), a total of 1 µg of plasmid DNA, and 2 µL of X-tremeGene HP Transfection Reagent (365752001, Complete, Roche, Basel, Switzerland) was added drop by drop to the petri dishes. The following plasmids, already present in the lab (Gnanasekeran et al., 2013) were used: pcDNA3-human P2X3 (A. North, Sheffield, UK; NCBI accession number: AAH74793), pcDNA3-rat P2X3 (A. North; NCBI accession number: CAA62594), pcDNA3-CASK (L. Tsai, Cambridge, MA, USA), pEGFP (Clontech, Mountain View, CA, USA), and pcDNA3 (Invitrogen Life Science, Carlsbad, CA, USA) as an empty vector. The plasmids (Figure 3-1) were verified by agarose gel electrophoresis.

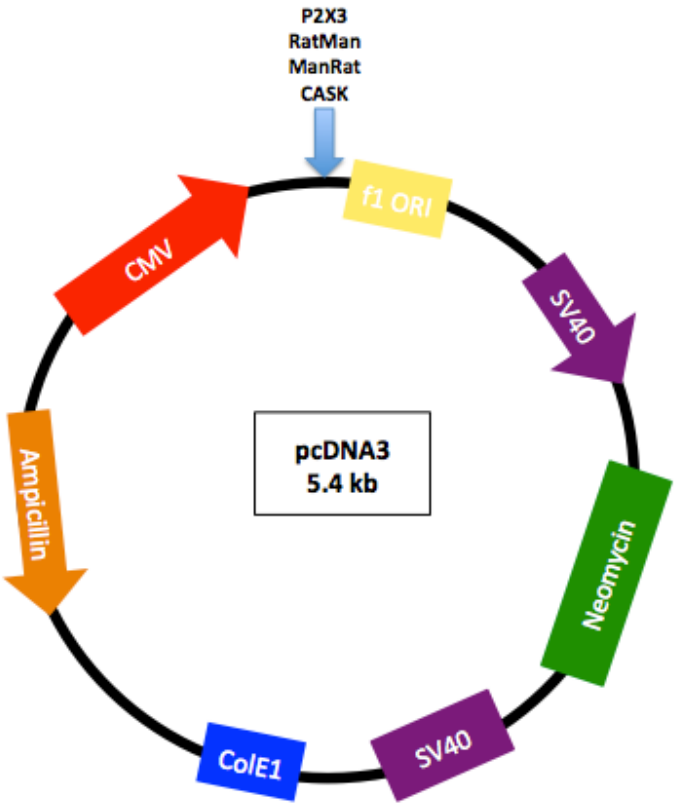

Figure 3-1: The pcDNA3 plasmid with the cut site labeled for the wild-type and mutant P2X3 plasmids and CASK plasmid.

Western Blotting

Transfected HEK 293T cells were lysed in TNE buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM EDTA), 0.1M NaF, 20 mM orthovanadate, and a mixture of protease inhibitors (Roche), scraped into Eppendorf tubes, homogenized with a syringe, and centrifuged for 20 minutes at 4°C. An aliquot from these samples were then mixed with bicinchoninic acid solution and 4% copper sulfate (Sigma) to determine the protein concentrations with a spectrophotometer (reading at 562 nm). To increase efficiency for determining the protein concentrations, the Quanti-iT dsDNA HS Assay kit (Q32851, Invitrogen) was used and concentrations were determined by the Qubit™ fluorometer.

From each sample lysate, proteins were diluted 1:1 with the loading dye buffer (Laemmli, 1972). Proteins were separated by electrophoresis at 75V for 20 minutes, and then 130V for approximately an hour and a half. While the concentration of the stacking gel is constant at 4%, the concentration of the running gel is chosen depending on the predicted molecular weights of proteins to be separated, and can range from 6-12%.

In our experiments, cell lysates were separated on 8% SDS-PAGE gels (running), containing 40% acrylamide-bis, 1.5M Tris pH 8.8, 10% SDS, 10% ammonium persulfate (APS), and TEMED. The gel was then transferred to a nitrocellulose membrane using Towbin's transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol in water) at 100V for an hour. The membrane was stained with a Ponceau solution (Sigma, Milan, Italy) to evaluate the transfer efficiency. The membrane was washed and blocked in a 5% milk solution with 1X Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) on a shaker for an hour.

The membrane was probed with the primary rabbit anti-P2X3 antibodies (0.8 µg/mL, APR016, Alomone, Jerusalem, Israel) and rabbit anti-CASK antibodies (0.4 µg/mL, 10777, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was incubated with the secondary antibody, rabbit HRP-conjugated anti-Ig (2 µg/mL, A0545, Sigma). The anti-P2X3 antibody antigen corresponds to the peptide (C)VEKQSTDSGAYSIGH, from a rat C-terminus P2X3 receptor region, and has been validated to detect human, rat, and mouse P2X3 receptors (Alomone; Sundukova et al., 2012). The signals from the Western blot were detected by using the ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore, Billerica, USA) and images acquired using Kodak MM4000.

Co-Immunoprecipitation

Cell lysates were prepared as previously described for the Western blot and protein concentrations were determined by using a spectrophotometer. For immunoprecipitation, the protein

lysate samples were added to a solution containing PureProteome Protein A Magnetic Beads (Millipore) and rabbit anti-P2X3 antibodies (0.4 µg/mL, sc-25694, Santa Cruz). The rabbit anti-P2X3 antibody immunogen corresponds to the 60 aa peptide: (C)LCDIILLNFLKGADQYKAKKFEEVNETTLKIAALTNPVYPSDQTTAEKQSTDSGAFSIGH from a human P2X3 receptor. It was designed to detect human, rat, and mouse P2X3 receptors (Santa Cruz).

After rotation of the samples for an hour at 4°C, the immune complexes were purified by magnetic separation and a series of washing steps in 1X TBS-T. Samples were eluted from magnetic beads by heating at 95°C for 10 minutes with help of the magnet. Immunopurified protein samples were then loaded onto a 8% SDS-PAGE polyacrylamide gel, transferred to nitrocellulose, and processed for Western blotting as previously described.

Immunofluorescence

HEK 293T cells grown on (untreated) glass coverslips were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature and blocked with 5% BSA, 1% FBS, and 0.1% Triton X-100 in PBS for an hour at room temperature. Cells were incubated for 45 minutes with rabbit anti-rat P2X3 antibodies (4 µg/mL, Alomone, Jerusalem, Israel) and anti-CASK antibodies (10 µg/mL, Millipore, Milan, Italy) added to the blocking solution and then washed. The cells were then incubated with secondary antibodies AlexaFluor 488 (4 µg/mL, Invitrogen) and AlexaFluor 594 (4 µg/mL, Invitrogen). Nuclei were counterstained with 4',6-Diamidino-2-phenylin-dole (DAPI) (5 µg/mL , Invitrogen). Samples were observed with the Zeiss Axio Observer.Z1 inverted microscope and images were collected with Axiovision (Zeiss) software.

4. Results

4.1 Plasmid Analysis

An agarose gel electrophoresis was performed to verify the quality of the plasmids used in subsequent experiments (Figure 4-1).

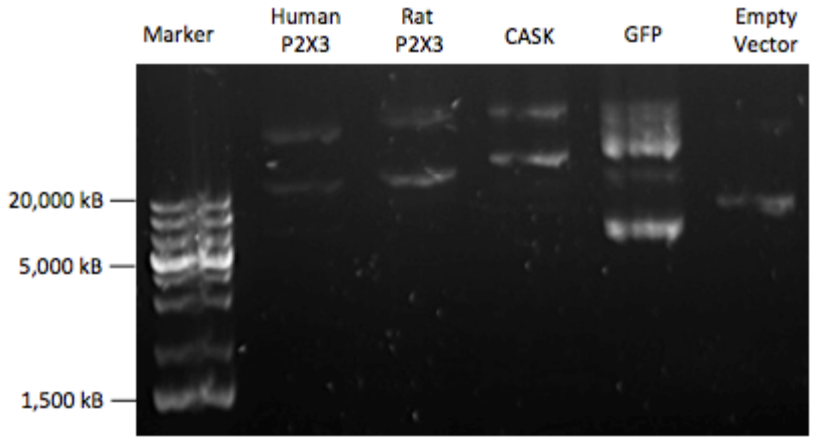

Figure 4-1: Human and rat P2X3, CASK, GFP, and an empty vector's plasmid DNA. Plasmids were resolved on 1% agarose gel, supplemented with ethidium bromide, and visualized under UV. Plasmids were of expected size.

4.2 Plasmid Expression

Initial experiments, including immunofluorescence, were performed to test the expression of the P2X3 receptor and CASK protein separately and together in transfected HEK cells. For immunofluorescence, cells were transfected for 24 hours with plasmids containing different combinations of the P2X3 receptor and CASK cDNA. Human P2X3 was transfected in two cell cultures: one with co-transfection of CASK and one without. The same was performed for rat P2X3. In addition, controls were created and cells were transfected with CASK alone and an empty vector. The results of this experiment showed that CASK co-expresses and co-localizes with human P2X3 and rat P2X3 in HEK cells (Figures 4-2 and 4-3).

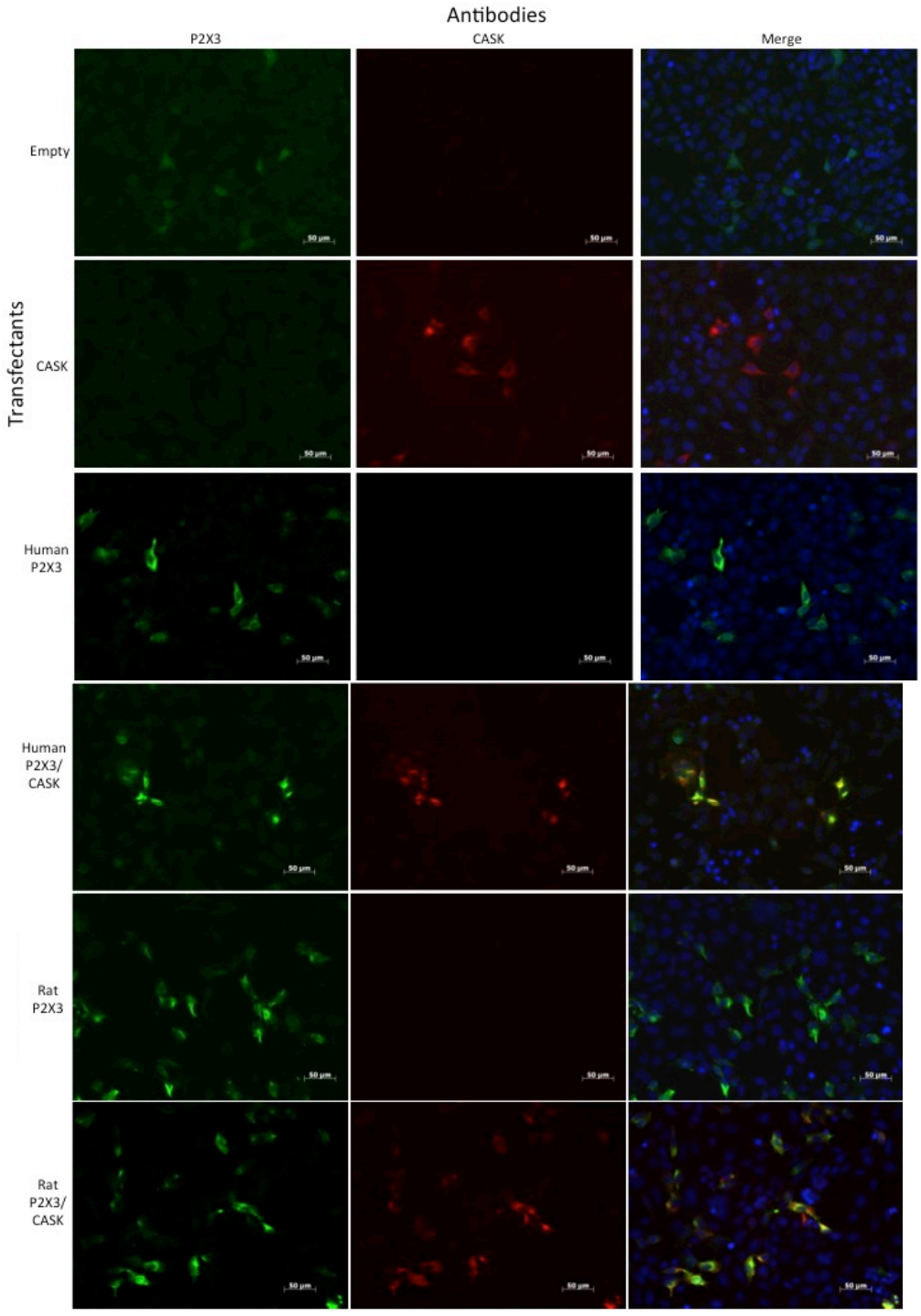

Figure 4-2: Expression of human and rat P2X3 receptor and CASK in HEK cells 24 hours after transfection. Cells were transfected with a control plasmid (Empty), plasmid containing CASK, human P2X3, human P2X3 and CASK, rat P2X3, or rat P2X3 and CASK. Both human and rat receptors were detected by immunocytochemistry with secondary antibodies conjugated to AlexaFluor 488 (green) and

CASK with secondary antibodies conjugated to AlexaFluor 594 (red). Nuclei were labeled with DAPI (blue). Images were acquired on a Zeiss Axio Observer.Z1 inverted microscope and processed using Axiovision (Zeiss) software. Bar = 50 µm.

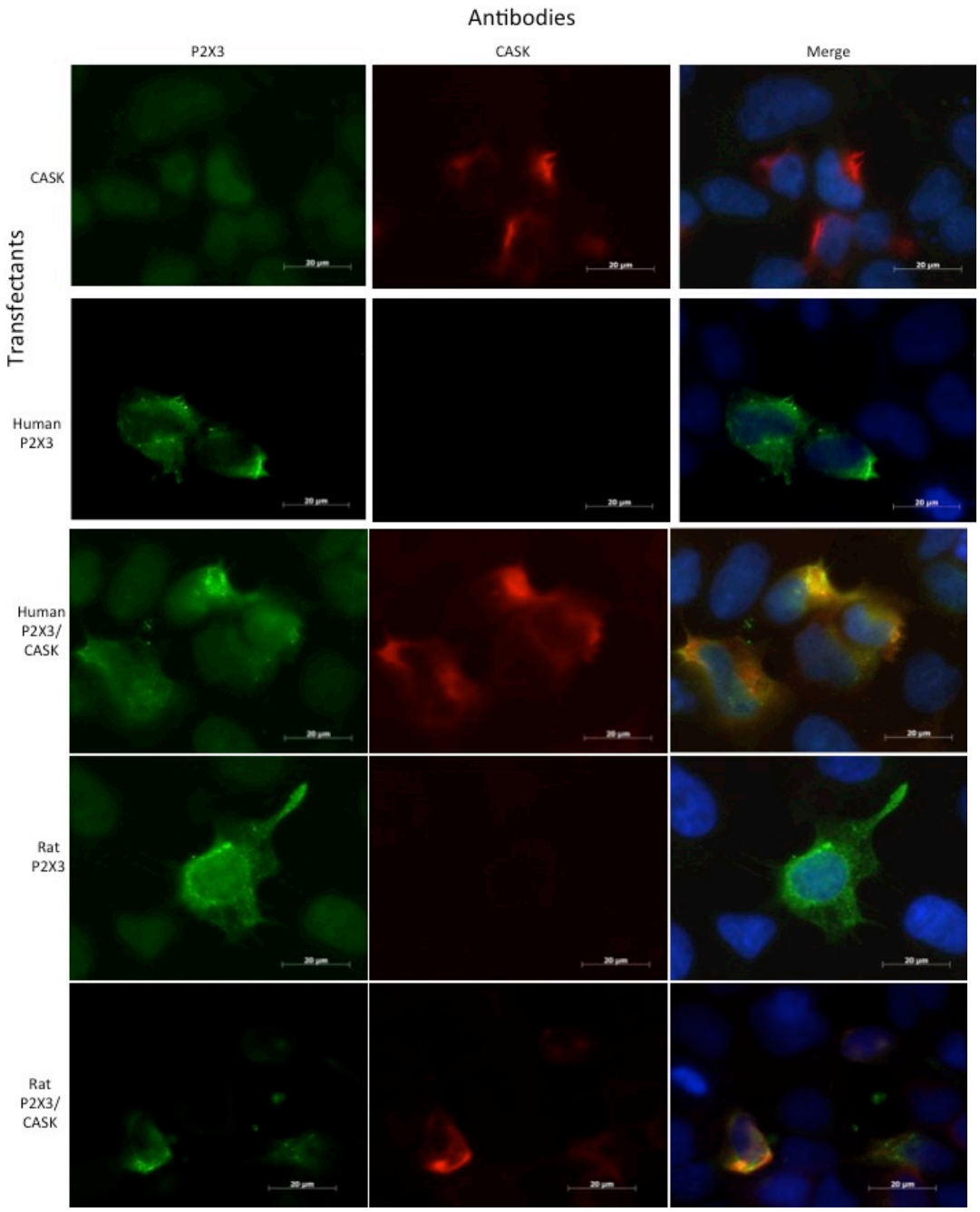

Figure 4-3: Expression of human and rat P2X3 receptor and CASK in HEK cells 24 hours after transfection. Cells were transfected with a plasmid containing CASK, human P2X3, human P2X3 and CASK, rat P2X3, or rat P2X3 and CASK. Both human and rat receptors were detected by

immunocytochemistry with secondary antibodies conjugated to AlexaFluor 488 (green) and CASK with secondary antibodies conjugated to AlexaFluor 594 (red). Nuclei were labeled with DAPI (blue). Images were acquired on a Zeiss Axio Observer.Z1 inverted microscope and processed using Axiovision (Zeiss) software. Bar = 20 µm.

P2X3 signal intensity was strong for all human and rat P2X3 transfections and CASK signal was well represented in all CASK and P2X3/CASK transfections. DAPI staining tagged the DNA in each cell and its signal was seen abundantly in the last column as a representation of all cells. A co-localization of the P2X3 receptor and CASK was seen as yellow since both protein signals, red and green, overlapped each other.

Transfection efficiencies were calculated for each transfection seen above in Figures 4-2 and 4-3 (Figure 4-4).

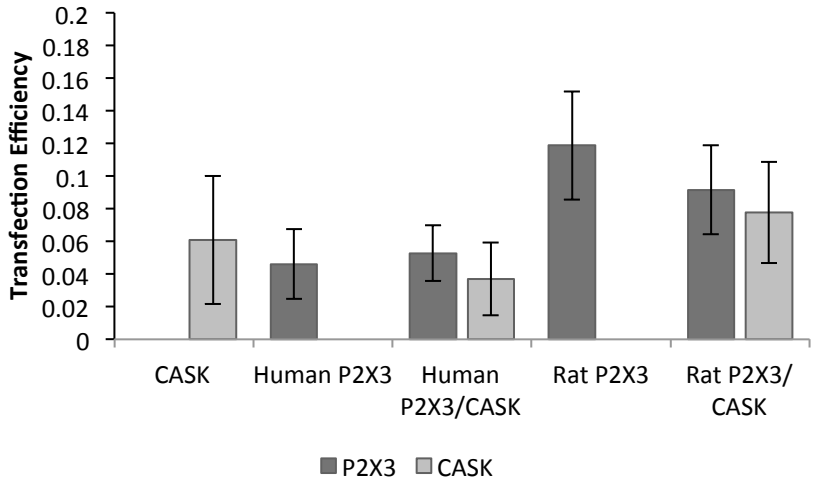

Figure 4-4: Transfection efficiency after 24 hours. Transfection efficiency was quantified for ten different fields by dividing the number of cells expressing transfected plasmids by the total number of cells stained with DAPI (n = 10).

The rat P2X3 single transfection had the greatest efficiency at 11.88 ± 3.32%. The rat P2X3/CASK sample also had a greater efficiency than either human P2X3 sample. CASK was expressed in all cells transfected with the CASK plasmid and its efficiency average was 5.85%.

An immunofluorescence experiment was repeated 48 hours after transfection (Figures 4-5 and 4-6) to compare the results to the post-24 hours of transfection.

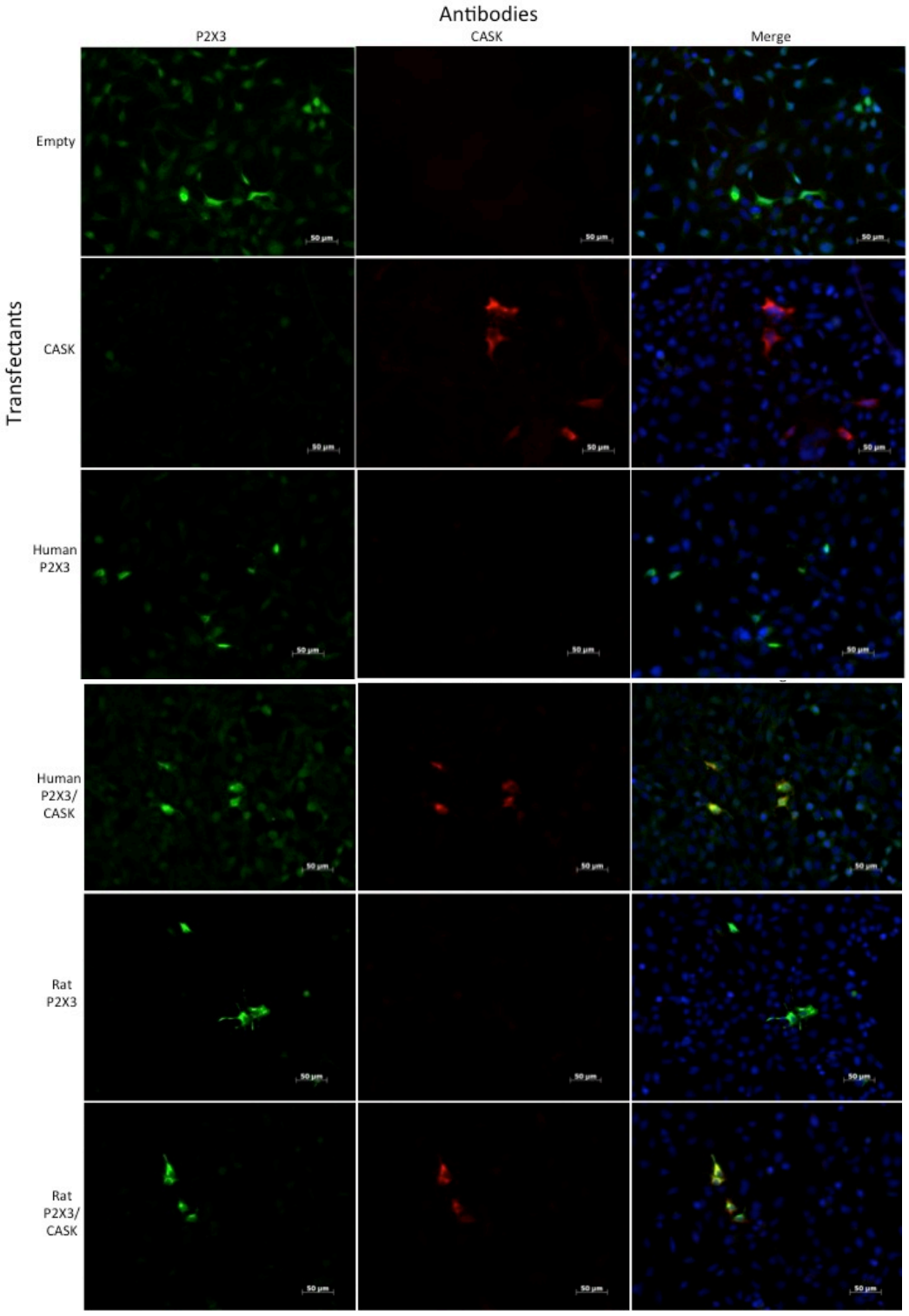

Figure 4-5: Expression of human and rat P2X3 receptor and CASK in HEK cells 48 hours after transfection. Cells were transfected with a control plasmid (Empty), plasmid containing CASK, human P2X3, human P2X3 and CASK, rat P2X3, or rat P2X3 and CASK. Both human and rat receptors were

detected by immunocytochemistry with secondary antibodies conjugated to AlexaFluor 488 (green) and CASK with secondary antibodies conjugated to AlexaFluor 594 (red). Nuclei were labeled with DAPI (blue). Images were acquired on a Zeiss Axio Observer.Z1 inverted microscope and processed using Axiovision (Zeiss) software. Bar = 50 µm.

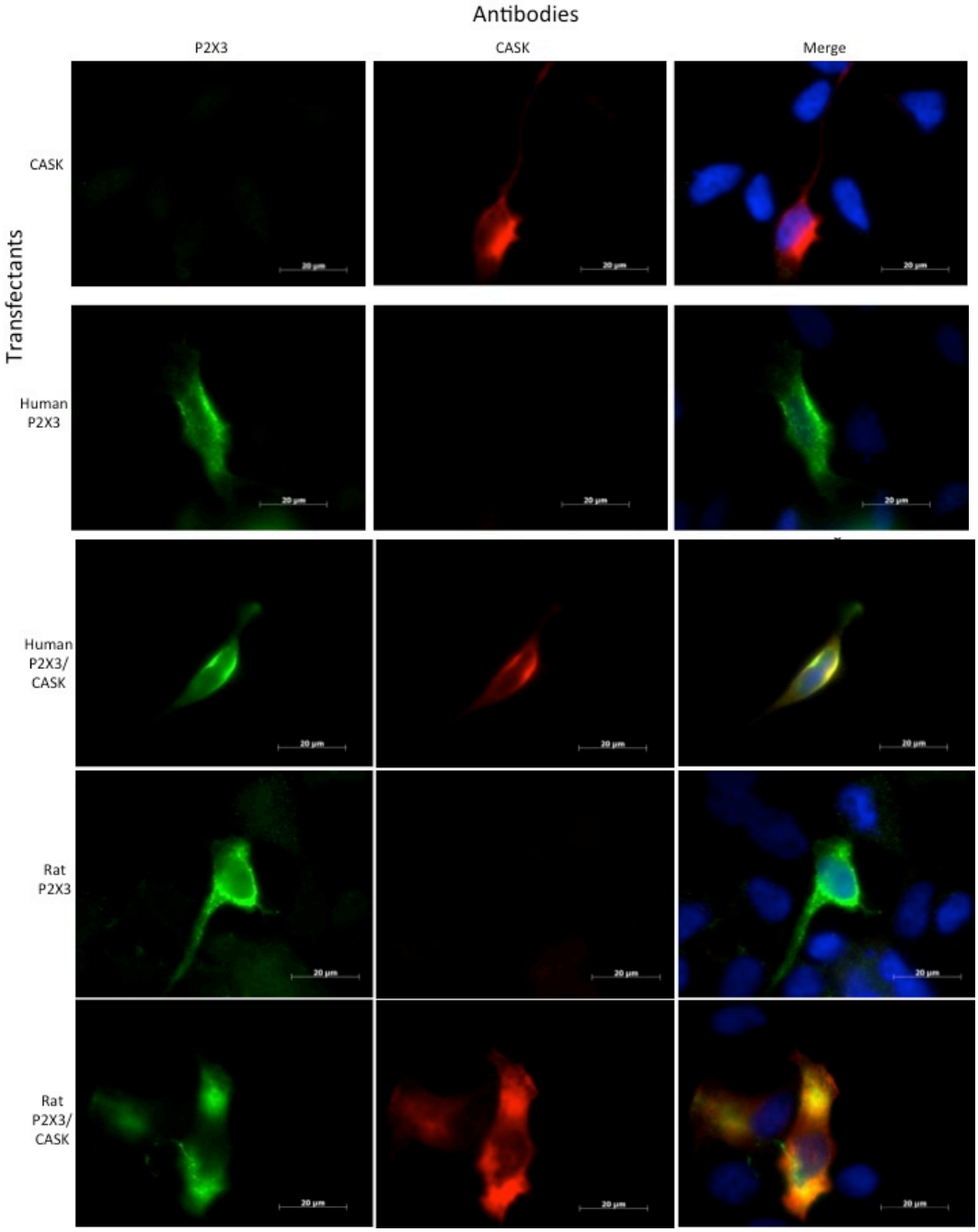

Figure 4-6: Expression of human and rat P2X3 receptor and CASK in HEK cells 48 hours after transfection. Cells were transfected with a plasmid containing CASK, human P2X3, human P2X3 and

CASK, rat P2X3, or rat P2X3 and CASK. Both human and rat receptors were detected by immunocytochemistry with secondary antibodies conjugated to AlexaFluor 488 (green) and CASK with secondary antibodies conjugated to AlexaFluor 594 (red). Nuclei were labeled with DAPI (blue). Images were acquired on a Zeiss Axio Observer.Z1 inverted microscope and processed using Axiovision (Zeiss) software. Bar = 20 µm.

Similar to Figures 4-2 and 4-3, P2X3 signals were strongly observed in all human and rat P2X3 transfections after 48 hours. CASK signals were evident in all CASK transfections, with a better observation of this in Figure 4-6. Co-localization between the P2X3 receptor and CASK was visible in the merge column, represented by yellow since both P2X3 and CASK signals, red and green, overlapped each other. Transfection efficiencies were calculated again for the cells seen in Figures 4-5 and 4-6 (Figure 4-7).

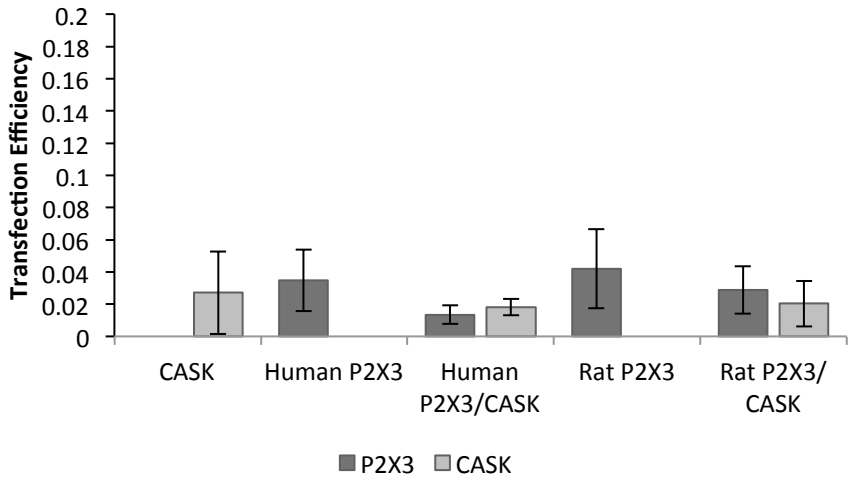

Figure 4-7: Transfection efficiency after 48 hours. Transfection efficiency was quantified for ten different fields by dividing the number of cells expressing transfected plasmids by the total number of cells stained with DAPI (n = 10).

The post-48 hours samples had similar efficiencies to each other for both proteins. Both P2X3 receptors were successfully transfected into an average of 2.5% of the HEK cells. The CASK protein was successfully observed in around 2% of all HEK cells. No outliers were found.

Another immunofluorescence experiment was performed to check co-expression of wild-type and mutant P2X3 receptors with CASK (Figure 4-8). Two different mutants were used, RatMan and

ManRat, as previously described in the Background section. RatMan is a rat P2X3 receptor with a single point mutagenesis at residue 393, mutating a tyrosine into a phenylalanine (Y393F). ManRat is a human P2X3 receptor with a single point mutagenesis also at residue 393, which mutates a phenylalanine into a tyrosine (F393Y).

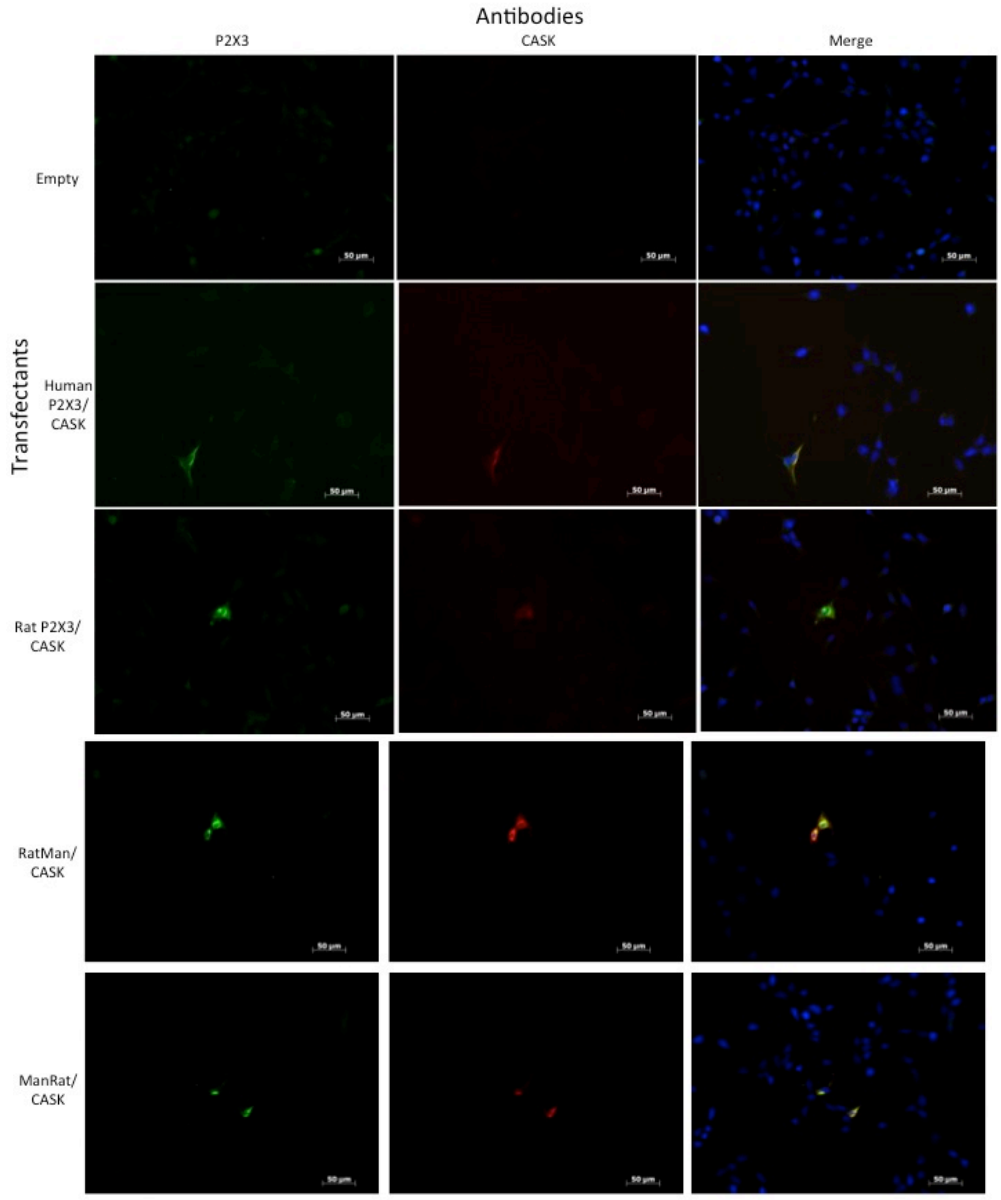

Figure 4-8: Comparative expression of wild-type and mutated P2X3 receptors and CASK in HEK cells. Cells were transfected with a control plasmid (Empty), plasmid containing human P2X3 and CASK, rat P2X3 and CASK, RatMan P2X3 and CASK, or ManRat P2X3 and CASK. All receptors were detected by immunocytochemistry with secondary antibodies conjugated to AlexaFluor 488 (green) and CASK with secondary antibodies conjugated to AlexaFluor 594 (red). Nuclei were labeled with DAPI (blue). Images

were acquired on a Zeiss Axio Observer.Z1 inverted microscope and processed using Axiovision (Zeiss) software. Bar = 50 µm.

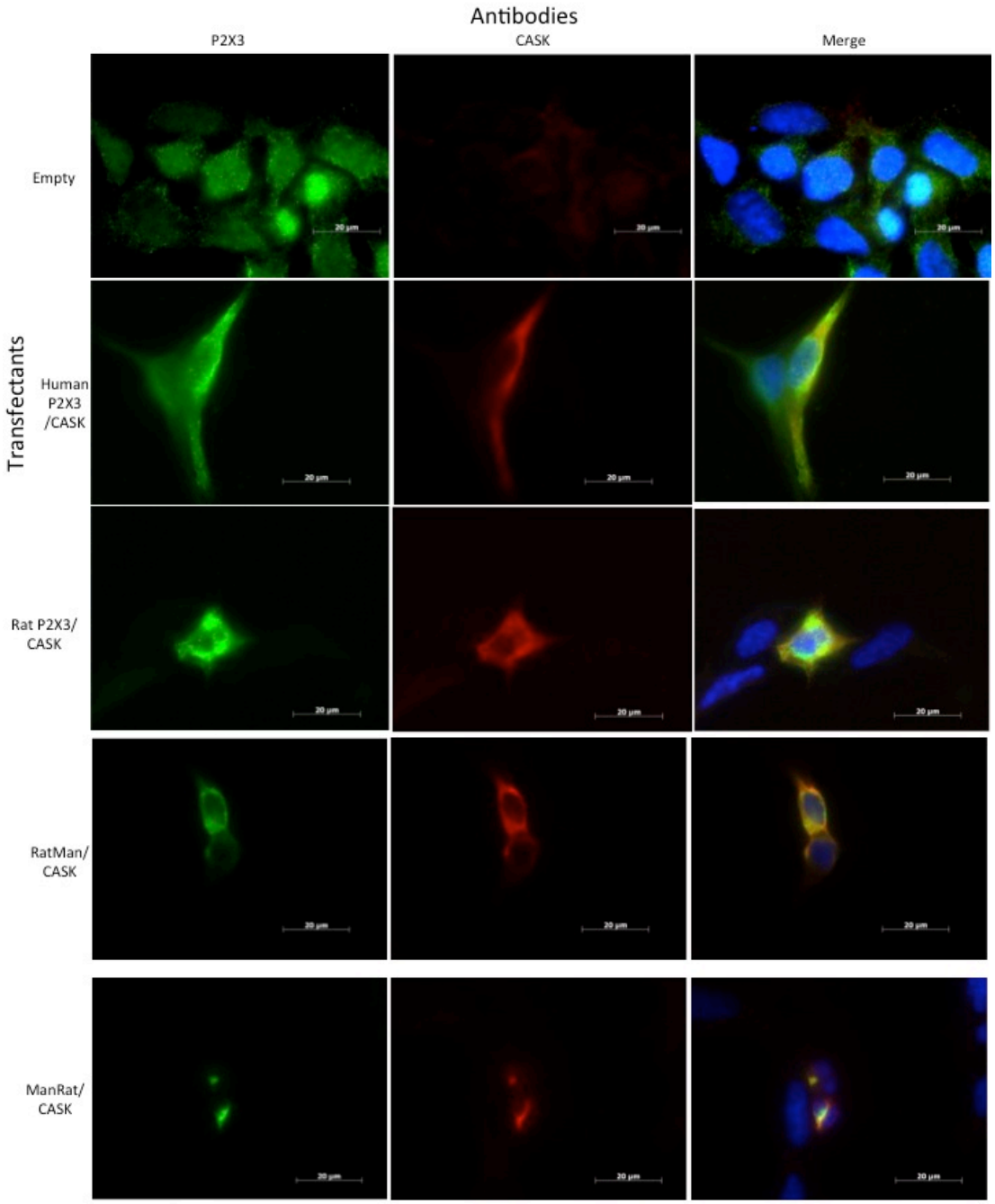

Figure 4-9: Comparative expression of wild-type and mutated P2X3 receptors and CASK in HEK cells. Cells were transfected with a control plasmid (Empty), plasmid containing human P2X3 and CASK, rat P2X3 and CASK, RatMan P2X3 and CASK, or ManRat P2X3 and CASK. All receptors were detected by immunocytochemistry with secondary antibodies conjugated to AlexaFluor 488 (green) and CASK with

secondary antibodies conjugated to AlexaFluor 594 (red). Nuclei were labeled with DAPI (blue). Images were acquired on a Zeiss Axio Observer.Z1 inverted microscope and processed using Axiovision (Zeiss) software. Bar = 20 µm.

Transfection efficiencies were calculated for the human, rat, RatMan, and ManRat P2X3 receptors and CASK co-transfections (Figure 4-10).

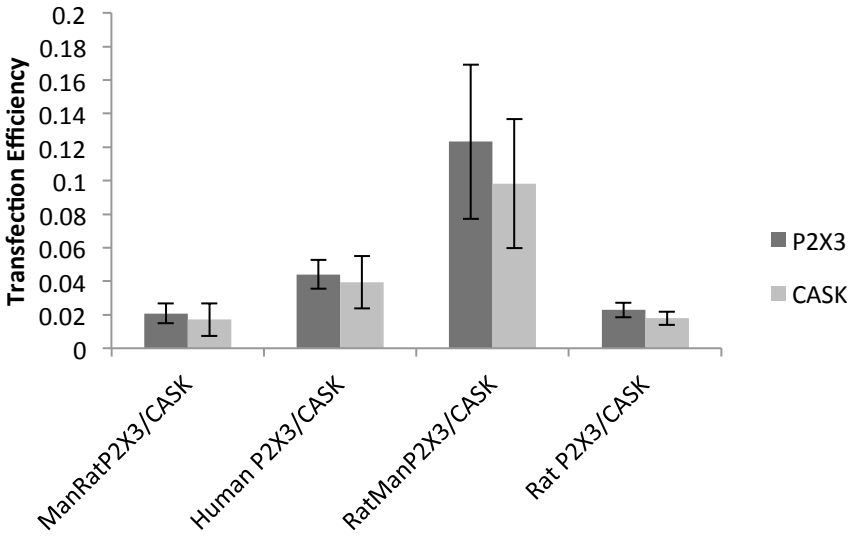

Figure 4-10: Comparison of transfection efficiencies for the human and rat wild-type P2X3 receptors and mutant receptors, ManRat and RatMan, when co-expressed with CASK. Transfection efficiency was quantified for ten different fields by dividing the number of cells expressing transfected plasmids by the total number of cells stained with DAPI (n = 4).

The P2X3 and CASK proteins seemed to transfect at similar efficiencies in each sample. Besides the RatMan P2X3 receptor at 12.32 ± 4.60% efficiency, the other samples showed lower transfection efficiencies. Both ManRat and rat P2X3 transfected cells had the lowest efficiencies at 2.08 ± 0.59% and 2.28 ± 0.43%, respectively.

Western blots were performed to further characterize plasmid expression. Resulting bands of both proteins, P2X3 and CASK, confirmed their presence and expression. A western blot was performed with HEK cells transfected with human, rat, RatMan, and ManRat P2X3 sequences and CASK. An empty vector was also used to serve as a negative control (Figure 4-11).

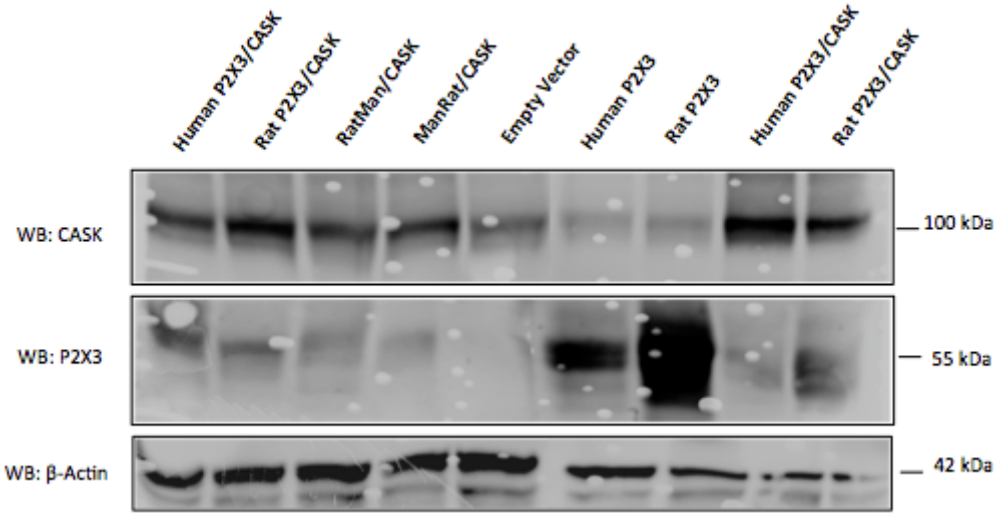

Figure 4-11 – Western blot showing the expression of human, rat, RatMan, and ManRat P2X3 receptors and CASK in HEK cells. The left four lanes represent samples of HEK cells transfected with plasmids containing human, rat, RatMan, or ManRat P2X3 receptors and CASK. The right four lanes are samples of HEK cells transfected with human or rat P2X3 plasmids, with and without a CASK plasmid. After running the samples on 8% SDS-PAGE gels, the membrane was probed with primary rabbit anti-P2X3 (Alomone) and anti-CASK (Santa Cruz) antibodies. Rabbit HRP-conjugated anti-Ig (Sigma) secondary antibody was then added. Bands were detected using ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore) and images were taken with Kodak MM4000. Actin was used as a loading control.

To provide a raw quantification approach, band intensities for each sample represented in Figure 4-11 were normalized against their respective actin band and then compared to the control, or empty vector (Figure 4-12).

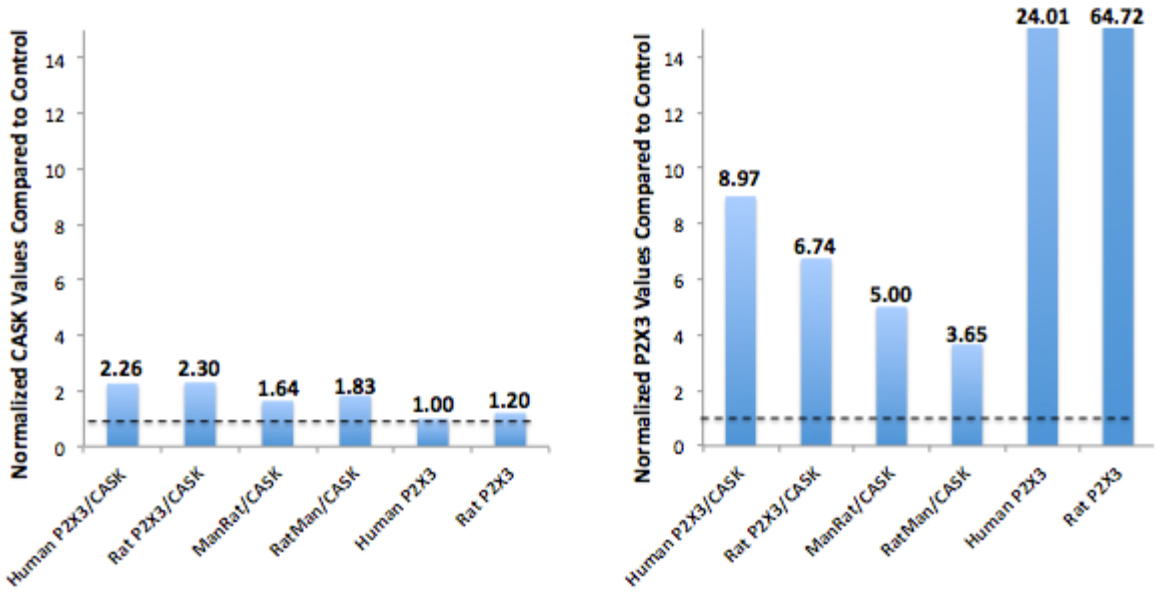

Figure 4-12: CASK and P2X3 Band Intensities Quantified from Figure 4-11. Raw intensity values, expressed as absolute units, for P2X3, CASK, and actin were calculated by ImageJ software. The P2X3 and CASK values were then normalized against each sample's respective actin value. Both sets of data were compared to the control, or empty vector. The control is represented as the dashed line at a value of 1.

For the CASK values graph, all samples with a CASK transfection had more expression than the control. Those without a CASK plasmid, human and rat P2X3, had CASK expression levels equal to or just above the control. For the P2X3 values graph, all samples expressed more P2X3 protein than the control. The human and rat P2X3 single transfections had exceptionally high expression at values of 24.01 and 64.72 units, respectively.

To standardize the protein lysates and Western blot procedure, multiple Western blots were performed. The intensity values were calculated for samples run in all Western blots performed and the values were normalized against each respective actin band (Figure 4-13).

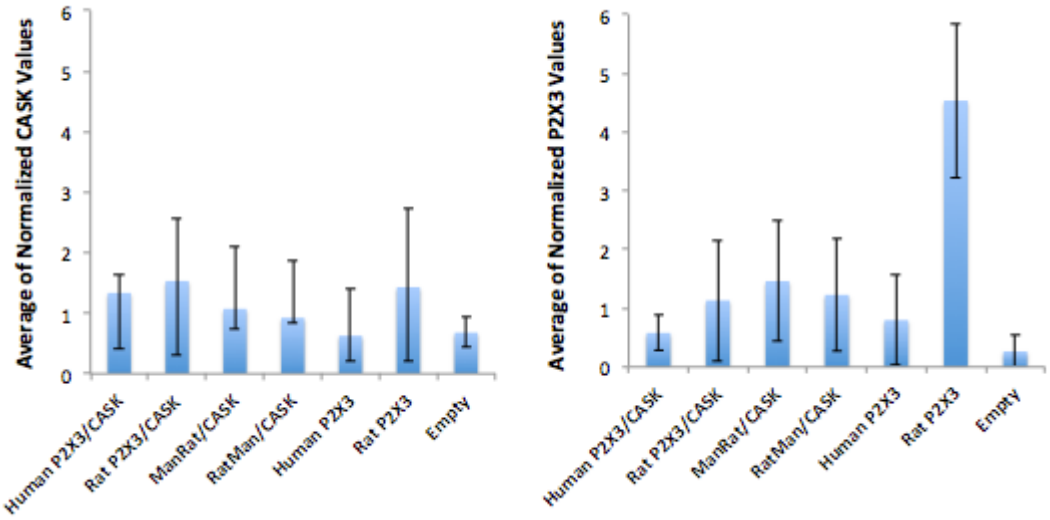

Figure 4-13: CASK and P2X3 Band Intensities Quantified from All Western Blots. Raw intensity values for P2X3, CASK, and actin were calculated by Image J software. The P2X3 and CASK values were then normalized against each sample's respective actin value. Standard deviation was calculated and is shown by error bars (n = 6 for human P2X3/CASK and rat P2X3/CASK; n = 5 for empty; n = 3 for ManRat/CASK, RatMan/CASK, human P2X3, and rat P2X3).

For the CASK values, all samples had similar intensity values. The human P2X3 and empty plasmid transfections have the lowest CASK expression levels. For P2X3 values, the empty vector had the lowest expression level and the rat P2X3 sample had significantly higher P2X3 expression.

4.3 Interaction between P2X3 and CASK

Rat P2X3 and CASK have been previously shown to pull down together with an anti-P2X3 antibody, meaning they are part of the same molecular complex. Co-immunoprecipitation was performed to test whether human P2X3 receptors and CASK proteins also pull down together, in order to provide a rationale for future biomedical applications.

The co-immunoprecipitation was performed with HEK cells transfected with human or rat P2X3 and CASK plasmids. An empty vector served as a control (Figure 4-14). Anti-P2X3 antibodies attached to the P2X3 receptor, thereby pulling down proteins attached to the receptor.

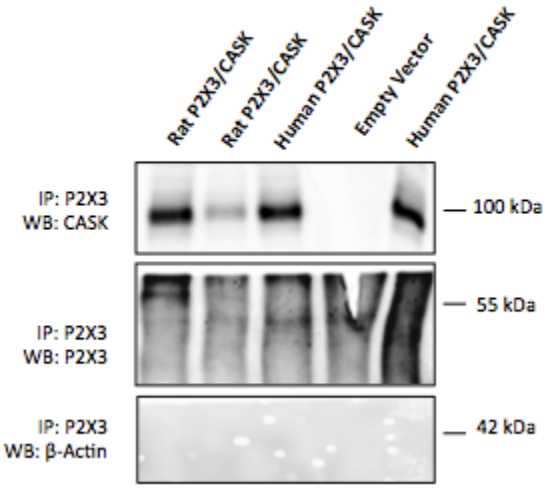

Figure 4-14: Pull down of CASK with human and rat P2X3 receptors. Cells were transfected with a control plasmid (Empty Vector), plasmid containing rat P2X3 and CASK, or human P2X3 and CASK. Co-transfections were done in duplicate to test reproducibility. Rabbit anti-P2X3 (Santa Cruz) antibodies were used to pull down the molecular complex. After the samples were run on 8% SDS-PAGE gels, the membrane was probed with primary rabbit anti-P2X3 (Alomone) and anti-CASK (Santa Cruz) antibodies. Rabbit HRP-conjugated anti-Ig (Sigma) secondary antibody was then added. Bands were detected using ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore) and images were taken with Kodak MM4000.

CASK protein was pulled down in all samples except the empty plasmid. Less expression was observed for the second rat P2X3/CASK transfection. The P2X3 receptor had a very low expression level. A band was observed in the first human P2X3/CASK sample around 55 kDa. No actin signals appeared, as expected.

4.4 Identification of Residues Responsible for Interaction between P2X3 and CASK

A co-immunoprecipitation was performed for both wild-type and mutant P2X3 receptors to analyze if mutations at residues 376 and 393 were involved in the CASK interaction (Figure 4-15). Anti-P2X3 antibodies attached to the human, rat, and mutant P2X3 receptors, pulling down proteins associated to the receptors.

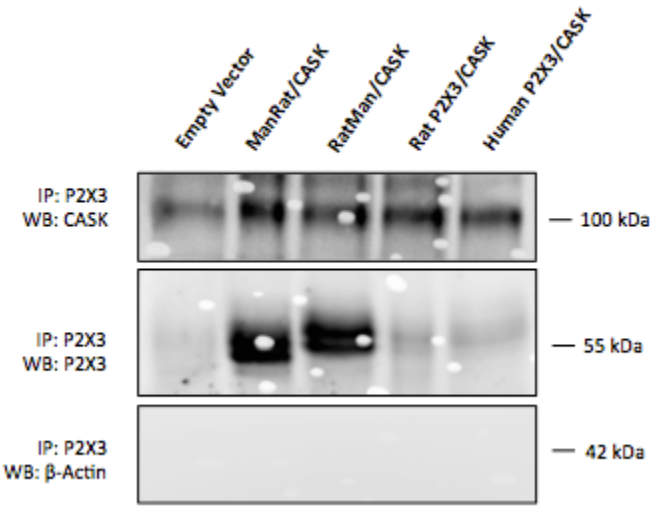

Figure 4-15: Pull down of CASK with human, rat, ManRat, and RatMan P2X3 receptors. Cells were transfected with a control plasmid (Empty Vector), plasmid containing rat P2X3 and CASK, human P2X3 and CASK, ManRat P2X3 and CASK, or RatMan P2X3 and CASK. Rabbit anti-P2X3 (Santa Cruz) antibodies were used to pull down the molecular complex. After the samples were run on 8% SDS-PAGE gels, the membrane was probed with primary rabbit anti-P2X3 (Alomone) and anti-CASK (Santa Cruz) antibodies. Rabbit HRP-conjugated anti-Ig (Sigma) secondary antibody was then added. Bands were detected using ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore) and images were taken with Kodak MM4000.

CASK protein signals appear in each lane, with a lower expression for the empty vector sample. Strong P2X3 expression is observed for both mutants, ManRat and RatMan. Very weak P2X3 expression is seen for the rat and human P2X3/CASK samples. Actin signals were not detected, as expected.

5. Discussion

The overall goal for this project was to further understand the relationship between the P2X3 receptor and the CASK protein. Through the use of different methods, the group was able to transfect and measure the overexpression of CASK protein and visualize its co-localization with P2X3 receptors in microscopy and Western blot experiments. In addition, it was also determined that the human P2X3 receptor is in the same molecular complex as CASK, which was previously seen for the rat P2X3 receptor.

Through the use of immunofluorescence (Figures 4-2, 4-3, 4-5, & 4-6), it was confirmed that P2X3 and CASK plasmids are expressed in HEK cells, and the proteins co-localize in the same cells. Although this is not sufficient to conclude that these molecules are a part of the same complex, it proves that they could function in close proximity. The images suggest the proteins are co-localized in or around the cell membrane, or at least in the same cellular compartment. This experiment also proved that the cells were correctly transfected with the confirmed plasmids (Figure 4-1).

When comparing samples post 24 and 48 hour transfections (Figure 4-4 and Figure 4-7 respectively), a clear time-dependent change in P2X3 expression was observed, which could be due to the cytotoxicity of P2X3 in the presence of a high concentration of ATP from high density cultures (Apicella et al., 2012). Some efforts have been made to titrate the plasmid concentrations before reading spectrophotometer measurements, in order to render them comparable. Also, the transfection efficiency is the result of a balance between the P2X3 receptor expression, the time after transfection, and the concentration of extracellular ATP present (Apicella et al., 2012).

The expression efficiency for the rat P2X3 samples after 24 hours was significantly greater than the human P2X3 transfections. This could be due to the higher plasmid concentration used for rat P2X3 during the transfection. However, previous research (Sundukova et al., 2012) suggests that human P2X3 receptor expression is significantly greater than the rat receptor when transfected with equal amounts of the plasmids. Therefore, transfecting with more rat P2X3 plasmid should not have shown such a considerably greater expression level over the human receptor.

Similarly, the immunofluorescence experiments that used cells transfected with mutants (Figures 4-8 & 4-9) showed correct plasmid expression for P2X3 and CASK. However, for the empty vector, a small amount of green fluorescence appears in the 20x magnification and a large amount of green fluorescence appears in the 100x magnification. This could be attributed to potential background

noise that could have been picked up during the imaging process because there was a lack of manual control of auto-exposure mode. Also, it should be noted that for the ManRat P2X3/CASK sample, the merge of both signals seems to appear co-localized inside the cell (Figure 4-9). The proteins seemed to co-localize around the cell in all other samples.

In addition, the transfection efficiency for the RatMan mutant was more than double the human P2X3 efficiency, which was the second highest value. The comparison, however, is not simple since microscopy quantifies expression at single cellular level, while biochemistry data report the entire lysates. Therefore, it is also suggested that there may have been problems with the transfection rather than the immunofluorescence. Importantly, the transfection protocol and plasmid preparations were changed in order to improve the receptor expression yield, rendering it very complex to compare initial microscopy experiments with Western blot data. Finally, it was suspected to obtain higher efficiency values in all transfected samples because of the protocol change to use the X-treme Gene protocol, which should have greatly increased the results. Unfortunately, this did not occur, and lower transfection efficiencies were observed instead (Figure 4-10), most likely due to the cytotoxic potential of overexpressed P2X3 receptors (Apicella et al., 2012).

To further analyze the plasmid expression of the HEK cells, a Western blot of both the wild-type samples and the mutants showed correct transfection of proteins of the expected molecular size (Figure 4-11). For the samples that were transfected with CASK, the bands' intensities (Figure 4-12) were higher than the empty vector, which proves that more protein was being expressed compared to the empty vector and the human and rat P2X3, which only contained endogenous CASK. For P2X3 expression, all samples showed higher intensities as compared to the control. The rat and human P2X3 samples that were not co-transfected with CASK show much greater expression of P2X3 than those that were co-transfected with CASK, suggesting that CASK controls the P2X3 receptor stability or that it influences P2X3-mediated cytotoxicity. These results contradict research showing that CASK stabilizes the P2X3 receptor, which would yield higher expression (Gnanasekaran et al., 2013). A double transfection of the cells could have inhibited the other expression levels, while the singular transfection did not. However, there is no explicit data proving this theory.

Also, the stain for β-actin shows two bands that were stained rather than one. This could be a result of the quality of the lysates and some protease activity that could have been active throughout the samples. Lastly, Figure 4-13 shows that throughout all of the Western blots that were performed, not much difference was shown in CASK values, while there was a variety in the P2X3 values. The empty

vector overall contained the least amount of P2X3, which was expected because it served as a negative control, while the rat P2X3 values were extremely higher than the others. This was due to the high variability between different experiments that showed high expression levels in one of the Western blot values, which skewed the overall average for the other Western blot values. Because the original Western blot experiments were loaded with different plasmid concentrations, the data from these experiments were extremely diverse, which caused the error bars to be large in the figure.

Through the use of co-immunoprecipitation, an anti-P2X3 antibody was used to test if the human P2X3 receptor would pull down with the CASK protein, which was previously noted with the rat P2X3 receptor. As seen in Figure 4-14, CASK shows similar band intensities in the transfected cells, except for one of the rat P2X3 samples, which could have been due to a decreased concentration in the cell lysate. Unfortunately, the P2X3 receptor did not appear, which may have been due to P2X3 degradation. However, since the structure of the P2X3 receptor is complex, the purification of these proteins is not easy because they are highly embedded in the membranes. While CASK is a soluble protein and external to the membranes, P2X3 receptors could be more resistant to extraction, which could explain why little was resolved in the SDS-PAGE. Nevertheless, because CASK is present, this indirectly indicates that the P2X3 receptor must have pulled down the CASK protein because it was directly connected to the anti-P2X3 antibody and magnetic bead complex. Although it is unknown whether CASK and P2X3 are directly attached, it can be concluded that P2X3 receptors from both human and rat origin are a part of the same molecular complex containing CASK. The membrane was stained for β-actin in addition to serve as a loading control; however, β-actin was not identified since it is not associated with the P2X3/CASK complex, which provided a specificity control.

For the co-immunoprecipitation that involved the mutant P2X3 plasmids (Figure 4-15), CASK expression is similar for both mutant and wild-type samples, which indicated that it cannot be inferred which residue plays a more influential role in the interaction. Ideally, there would have been varying CASK expression levels between the different conformations, which would have highlighted one residue over another. Also, CASK expression appeared in the empty vector sample, although endogenous CASK could not have been present without the aid of P2X3. This expression could be explained by human error in the protocol. Furthermore, P2X3 showed a strong intensity for both of the mutants, including duplex bands for each, but also showed a weak intensity for the wild types. All human and rat P2X3 and CASK co-transfection samples in both co-immunoprecipitation experiments showed low expression levels, which could be due to the degradation of the P2X3 receptor in the cell lysates.

Overall, the experiments confirmed the co-expression and co-localization of the P2X3 receptor and CASK protein. It confirmed a complex between human P2X3 and CASK, which had not been previously discovered. Also, HEK cells were shown to stably overexpress the P2X3 receptor and CASK protein, which can now be used as a model cell line to further study their interactions.

6. Recommendations

The experiments performed were useful in visualizing the co-localization of the P2X3 receptor and the CASK protein, as well as proving that these molecules interact directly or indirectly in the same complex. To further understand this complex, it would be beneficial to perform silver staining and proteomic experiments to determine other potential molecules involved. This could lead to understanding more about the chronic pain pathway and how pain signals could be blocked or altered.

In addition, it would also be interesting to look at P2X3 activity by using siRNA or a similar small RNA interfering technique to terminate possible adaptor proteins or kinases. This would leave P2X3 and CASK interacting exclusively, and the consequences of these interfering RNAs could be observed. Knock-down experiments could also modulate the P2X3 and CASK complex to explore its functionality. Also, the CASK role in the P2X3 receptor biology is also poorly known, even though there has been published data that report their role in receptor stability, mobility and trafficking to/from the surface membrane. The expression of both CASK and P2X3 from human origin in a human context (i.e. HEK cells) can open new interesting avenues in the study of the interactions and modulations of the P2X3 receptors.

HEK cells in culture are prone to secrete high concentrations of ATP, even after mechanical stress. It is known that ATP activates and opens P2X3 receptor channels, which could induce long-lasting cytotoxicity (Apicella et al., 2013), as well as influence the CASK/P2X3 interaction state (Gnanasekeran et al., 2013). Further experiments, eventually done in the presence of selective inhibitory drugs (receptor antagonists) are necessary to understand the time course of these events. Furthermore, it is now clear that single point mutations can change the overall stability of the receptor and their functional properties, and this could be further explored because human and rat receptors show 98% homology. Currently, nothing is known regarding differences in trafficking and stability of human receptors and how this is influenced by CASK.

Phosphorylation between P2X3 and CASK could also be researched. CASK is named for its serine kinase activity, and while the protein interaction with P2X3 is unknown, it is possible to determine the phosphorylation activity of CASK on the receptor. CASK could assemble a complex of adaptor proteins or kinases capable of altering the phosphorylation state of P2X3. Also, using anti-phosphoserine antibodies to bind to the C-terminal intracellular domain could reveal further information.

If the functionality of the P2X3/CASK complex is known, then pharmaceutical companies can begin to create drugs that can target this complex, which could stop the pain signal at the pre-synapse in

the dorsal horn, rather than allowing the pain signal to travel to the brain and create a sensory response. Although this could only be administered in specific cases due to the potential hazards of pain desensitization, further uses for this type of medication could be explored.

The use of FRET, or Förster resonance energy transfer, could help with the drug development process, as well as provide more information on the P2X3 and CASK interaction. This technique exposes a fluorescent tag to light which produces a color and signal. However, if two fluorescent tags were close in proximity, then the colors and signals would be different. Signals would fluoresce a different color due to resonance energy transfer from the donor fluorophore to the other proximal acceptor fluorophore. If P2X3 was tagged with a fluorescent tag, such as CFP, and CASK was tagged with a different fluorescent tag, such as YFP, then a direct interaction would fluoresce yellow due to YFP. If the interaction were indirect, then it is likely that the fluorescence would take a cyan color, due to CFP. Since the P2X3 and CASK interaction is dynamic and transient, this method could be applied to test different compounds added to a solution containing the P2X3/CASK complex to observe whether the complex disassembles or not, based on the signals given by the fluorophores. Then conclusions could be inferred as to the efficiency of each drug to alter or inhibit the pain signal, leading to the discovery of new pain medications.

In conclusion, our work explored the requirements of the CASK and P2X3 interaction, confirming the validity of previously discovered mechanisms in a human context. This supports the importance of further investments to design biomedical research applications based on fluorescence biotechnologies or synthetic biology based on this interaction mechanism.

6. Bibliography

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