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Regulation of TRPM2 channels by Fyn kinase

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Graduate Program in Physiology
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REGULATION OF TRPM2 CHANNELS BY FYN KINASE

(Thesis format: Monograph)

by

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Abstract

TRPM2 is a calcium-permeable non-selective cation channel that acts as a mediator of cell death in response to oxidative stress. It has been shown that oxidative stress increases TRPM2 tyrosine phosphorylation and activation – an effect that is blocked by PP2, a non-specific inhibitor of Src family kinases. However, the kinase and target TRPM2 tyrosine residue(s) involved have not yet been identified. Here, we investigated the potential regulation of TRPM2 by Fyn. Intracellular application of recombinant Fyn potentiated TRPM2 currents in HEK293 cells expressing inducible TRPM2 (TRPM2-HEK293 cells). Further, a physical interaction between Fyn and TRPM2 was demonstrated by co-immunoprecipitation in TRPM2-HEK293 cells. Additionally, tyrosine phosphorylation of TRPM2 was induced by Fyn in TRPM2-HEK293 cells, and the amount of phosphorylation detected was related to the activation state of Fyn. We propose that by augmenting TRPM2 activity, Fyn kinase potentiates cellular calcium overload and facilitates cell death in response to oxidative stress.

Keywords

TRPM2, Fyn kinase, tyrosine phosphorylation, protein-protein interaction, calcium

Co-Authorship Statement

Drs. John MacDonald and Michael Jackson supervised and contributed to the design of all experiments discussed in this work. Dr. Jillian Belrose contributed to the design of the biochemistry experiments conducted in this work. Further, Dr. Belrose conducted an experiment assessing the effect of Fyn on TRPM2 currents in cultured hippocampal neurons (Appendix A, Fig. S1). Dr. Fabiana Caetano conducted preliminary electrophysiology experiments assessing the effect of Fyn on TRPM2. These experiments contributed to the design of the electrophysiological experiments described in this work. Additionally, Dr. Hongbin Li performed an experiment assessing the effect of a Fyn-interfering peptide (Fyn(39-57)) on TRPM2 currents in cultured hippocampal neurons (Appendix A, Fig. S2).

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List of Abbreviations

AA	amino acid
AD	Alzheimer's disease
ADPR	adenosine diphosphate ribose
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
Aβ	β-amyloid
cADPR	cyclic adenosine diphosphate ribose
CaM	calmodulin
CCR	coiled-coil region
CNS	central nervous system
DNA	deoxyribonucleic acid
EC_{50}	half-maximal effective concentration
ECS	extracellular solution
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
H_2O_2	hydrogen peroxide
HEK293	human embryonic kidney 293
HEK293T	SV40 Large T-antigen-containing human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	hoseradish peroxidase
IC_{50}	half-maximal inhibitory concentration
ICS	intracellular solution
IQ	calmodulin binding motif
I-V	current-voltage

KO	knockout
LTRPC2	long transient receptor potential channel 2
MHR	transient receptor potential melastatin homology regions
mRNA	messenger ribonucleic acid
NAADP	nicotinic acid-adenine dinucleotide phosphate
NAD^+	nicotinamide adenine dinucleotide
NADase	nicotinamide adenine dinucleotide glycohydrolase
ND2	nicotinamide adenine dinucleotide dehydrogenase subunit 2
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NUDT9	nucleoside diphosphate-linked moiety X-type motif (NUDIX)
NUDT9-H	nucleoside diphosphate-linked moiety X-type homology motif
PARG	poly- adenosine diphosphate ribose-glycohydrolase
PARP	poly-adenosine diphosphate ribose-polymerase
PCR	polymerase chain reaction
PKA	protein kinase A
PSD-93	post-synaptic density-93
PTPL1	protein tyrosine phosphatase-L1
PTPα	protein tyrosine phosphatase α
qRT-PCR	quantitative real-tmie polymerase chain reaction
ROS	reactive oxygen species
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SFK	Src family kinase
SH domain	Src homology domain
STEP	striatal enriched phosphatase

TAT	transactivator of transcription
TRP	transient receptor potential channel
TRPC7	transient receptor potential canonical type 7 channel
TRPM	transient receptor potential channel melastatin subfamily
TRPM2	transient receptor potential melastatin type 2 channel
TRPM2-HEK293	HEK-293 cells expressing inducible FLAG-tagged human TRPM2
TRPM2-S	TRPM2 short splice variant, deletion of AA995-1503
TRPM2-SSF	TRPM2 striatum shorter form splice variant, deletion of AA1-214
TRPM2-ΔC	TRPM2 C-terminal splice variant, deletion of AA1292-1325
TRPM2-ΔN	TRPM2 N-terminal splice variant, deletion of AA538-557
TRPM2-ΔNΔC	TRPM2 N/C-terminal splice variant, deletion of AA538-557,1292-1325
UD	unique domain
WT	wild-type

Section 1

INTRODUCTION

1.1 General introduction

Transient receptor potential melastatin 2 (TRPM2) is a non-selective cation channel that is permeable to calcium (Ca^{2+}). TRPM2 is broadly expressed in the central nervous system (Nagamine et al., 1998), and expression of functional TRPM2 channels has been demonstrated in CA1 neurons of the hippocampus (Olah et al., 2009). A physiological role for TRPM2 in neurons was recently identified; TRPM2 was shown to be required for N-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (Xie et al., 2011). Moreover, TRPM2 has been shown to function as a mediator of cell death in response to oxidative stress in neurons (Kaneko et al., 2006) and other cell types (Hara et al., 2002; Wehage et al., 2002). As such, TRPM2 channel activation has been implicated in the pathogenesis of several oxidative stress-related neurodegenerative diseases. Mechanisms by which TRPM2 activation is regulated remain largely unexplored. However, it has been shown that tyrosine phosphorylation of TRPM2 is induced following oxidative stress (Zhang et al., 2007). This phosphorylation coincides with an increase in TRPM2 channel activation (Zhang et al., 2007). Further, this phosphorylation is reduced by protein tyrosine phosphatase-L1 (PTPL1), and PP2, a non-selective inhibitor of Src family kinases (SFK) (Zhang et al., 2007). Fyn kinase is a ubiquitously expressed tyrosine kinase, which belongs to the SFK family (Salter & Kalia, 2004). Notably, like TRPM2, Fyn expression has been demonstrated in postsynaptic densities, its activation is augmented in response to oxidative stress and Fyn signaling has been shown to contribute to cell death (Ittner et al., 2010; Sanguinetti et al., 2003). Further, preliminary evidence suggests that Fyn regulates TRPM2 activation through an NMDA receptor-independent mechanism (MacDonald/Jackson lab, unpublished). This thesis will investigate the regulation of TRPM2 channels by Fyn kinase.

1.2 Transient receptor potential melastatin 2 (TRPM2)

Transient receptor potential melastatin 2 (TRPM2), previously designated TRPC7 and LTRPC2, is a non-selective cation channel, which is permeable to calcium (Ca^{2+}).

TRPM2 belongs to the transient receptor potential (TRP) family of integral membrane ion channel proteins. This protein family was named based on structural homology of its members to the TRP protein of *Drosophila melanogaster* (Hardie et al., 2001). The *Drosophila* TRP protein, which was first isolated in 1989, was found to be essential for phototransduction (Montell & Rubin, 1989). To date, 28 mammalian TRP proteins have been identified (Nilius & Owsianik, 2011). Each TRP family member contains six transmembrane domains, forms a cation-permeable channel and has at least 20% sequence homology with other TRP family members (Montell, 2005; Clapham, 2003). However, within the TRP family, there is substantial diversity in expression patterns, activation mechanisms, regulatory mechanisms as well as cation permeability (Jiang et al., 2010; Sumoza-Toledo & Penner, 2011). For example, TRP channels are variably activated by specific ligands, voltage, temperature and/or mechanical force (Holzer, 2011). Further, permeability and selectivity of TRP channels to Na^+, K^+, Ca^{2+} and Mg^{2+} vary substantially between family members (Sumoza-Toledo & Penner, 2011). Although expression patterns and functions of TRP channels are highly diverse and vary for each family member, many TRP channels are expressed in the central nervous system and are involved in sensory processes (Montell, 2005). Mammalian TRP proteins have been divided into six subfamilies based on sequence homology: canonical or classic (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP), mucolipin (TRPML) (Nilius & Owsianik, 2011).

The TRPM subfamily, to which TRPM2 belongs, was named for melastatin (TRPM1), a tumor suppressor protein whose expression is inversely related to tumor size, aggressiveness and metastasis of human primary cutaneous melanomas (Duncan et al., 1998). The mammalian TRPM subfamily consists of eight members, denoted TRPM1–TRPM8. Six of the eight TRPM subfamily members can be further subdivided into pairs that display similar biophysical properties and high sequence homology (50-80%): TRPM1/3, TRPM4/5, TRPM6/7 (Nilius & Owsianik, 2011). With respect to sequence homology, the remaining two TRPM proteins, TRPM2 and TRPM8, are less closely related to other TRPM subfamily members (Montell, 2005). Like the TRP family as a whole, TRPM subfamily members exhibit substantial diversity in expression patterns,

activation mechanisms, regulatory mechanisms as well as cation permeability (Sumoza-Toledo & Penner, 2011).

The gene encoding human TRPM2 is located on chromosome 21q22.3, is 90 kb in length and contains 32 exons (Nagamine et al., 1998). The human *TRPM2* gene encodes an mRNA transcript of approximately 6.5 kb, which in turn encodes a 1503 amino acid (AA) protein with a molecular weight of approximately 170 kDa (Nagamine et al., 1998). Using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and *in situ* hybridization, expression of TRPM2 mRNA was shown to be nearly ubiquitous (Fonfria et al., 2006; Jiang et al., 2010). Notably, TRPM2 mRNA expression was highest in the brain and pituitary (Fonfria et al., 2006). Further, functional expression of TRPM2 protein has been demonstrated in several cell types, including neurons (Hill et al., 2006; Olah et al., 2009), microglia (Kraft et al., 2004), immunocytes (Sano et al., 2001), cardiomyocytes (Yang et al., 2006) and pancreatic β-islet cells (Togashi et al., 2006). Notably, functional TRPM2 channels have also been identified in hippocampal CA1 pyramidal neurons (Olah et al., 2009). Studies of TRPM2 channel function commonly use patch-clamp electrophysiology and/or Ca^{2+} imaging. To confirm that TRPM2 channels are specifically being studied, these techniques are often used in conjunction with established pharmacological agents (such as activators, antagonists and regulatory factors) and analysis of channel properties (such as reversal potential and current-voltage relationship). Patch-clamp studies commonly involve recording adenosine diphosphate ribose (ADPR)-induced TRPM2 currents. Alternatively, Ca^{2+} imaging typically involves using fluorescent Ca^{2+} indicators to monitor changes in intracellular Ca^{2+} concentration in response to oxidative stressors, such as hydrogen peroxide (H_2O_2). Although TRPM2 has been predominantly characterized as a cell surface protein, a recent study showed that TRPM2 also functions as a lysosomal Ca^{2+}-release channel in beta cells (Lange et al., 2009). Interestingly, in dendritic cells, TRPM2 functions as a lysosomal Ca^{2+}-release channel, but not as a plasma membrane Ca^{2+}-entry channel (Sumoza-Toledo et al., 2011). The physiological relevance and factors that determine cellular localization of TRPM2 are not clearly defined at present.

1.3 Structural and biophysical properties of TRPM2

TRPM2 channels are widely thought to consist of four subunits surrounding a central cation-conducting pore (Somoza-Toledo & Penner, 2011). TRP channels belong to the same ion channel superfamily as voltage-gated K^+, Na^+ and Ca^{2+} channels, and consequently, they have similar membrane topology (Yu & Catterall, 2004). Each subunit contains six transmembrane domains (S1-6), intracellular N- and C-terminal tails, and a pore-forming loop between S5 and S6 (Nagamine et al., 1998; Jiang et al., 2010). Figure 1 shows a schematic of TRPM2.

The N-terminal tail of human TRPM2 is cytosolic and consists of 752 amino acids (AA 1-752) (Maruyama et al., 2007). The N-terminus contains four TRPM homology regions (MHR), which consist of amino acid stretches totaling approximately 700 AA in length that are highly conserved among members of the TRPM subfamily (Fleig & Penner, 2004; Perraud et al., 2003). The functional importance of these MHR regions has not yet been established (Fleig & Penner, 2004). However, Perraud et al. (2003) have provided preliminary evidence that the MHR regions may contribute to tetrameric assembly and/or surface localization of TRPM2 channels. Also within the N-terminal tail of TRPM2 is a calmodulin (CaM)-binding IQ-like motif (AA 406-416), which contributes to TRPM2 channel facilitation and activation by intracellular Ca^{2+} (Tong et al., 2006) (see section 1.7 for more details on Ca^{2+}/CaM regulation of TRPM2 channels).

The transmembrane portion of human TRPM2 is 294 amino acids in length (AA 753-1046) and consists of six transmembrane domains interspersed with alternating extracellular and intracellular loops (Fig. 1; Jiang et al., 2010). Loops connecting S2 and S3, as well as S4 and S5 are intracellular, and loops connecting S1 and S2, S3 and S4, as well as S5 and S6 are extracellular (Jiang et al., 2010). The re-entrant extracellular pore-loop connecting S5 and S6 (AA 958-1025) forms a central cation-permeating pore (Sumoza-Toledo & Penner, 2011). Cysteine residues C996 and C1008 within the pore-loop region have been identified as essential for TRPM2 channel function; point

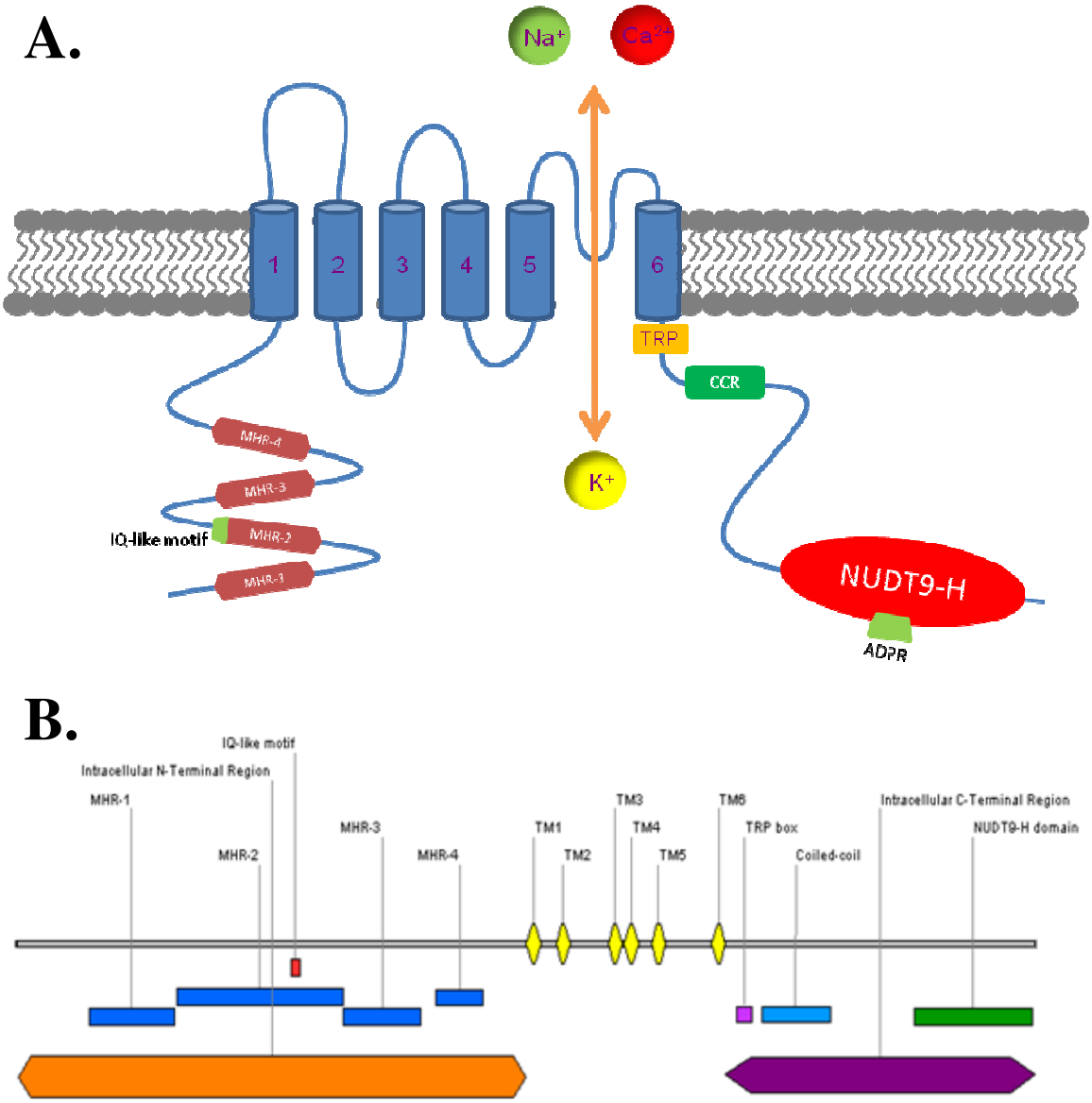

Figure 1.1: Schematic images of human TRPM2.

Structural (A) and scaled linear (B) schematics of TRPM2. TRPM2 channels consist of six transmembrane regions (TM1-6) as well as intracellular N- and C-termini. The N-terminus contains four conserved TRPM homology regions (MHR-1-4) and a Ca^{2+}/CaM-binding IQ-like motif. The C-terminus contains a TRP box motif, a coiled-coil region (CCR) and a NUDT9-H domain.

mutations at these sites result in nonfunctional channels (Mei et al., 2006a). Further, residues E960, Q981, D987 and E1022 are important determinants of divalent cation permeability (Xia et al., 2008). The other transmembrane domain-connecting loops are short (3-56 AA), and their physiological functions are not well described at present.

The C-terminal tail of human TRPM2 is cytosolic and consists of 457 amino acids (AA 1047-1503) (Jiang et al., 2010). Immediately distal to transmembrane segment S6, in the C-terminus of TRPM2, is a 23 amino acid TRP box motif (AA 1063-1085), which is a highly conserved region among members of the TRPM, TRPC and TRPV subfamilies (Clapham, 2003; Montell, 2005). The function of the TRP box remains unclear at present. Also within the C-terminal tail of TRPM2 is a coiled-coil region (CCR; AA 1167-1201) (Mei et al., 2006b). This CCR region is thought to contribute to the tetrameric assembly of TRPM2 subunits (Fleig & Penner, 2004). Accordingly, deletion of the CCR resulted in a loss of TRPM2 subunit interaction and TRPM2 currents (Mei et al., 2006b). Similarly, site-directed mutagenesis of specific residues within the CCR that have been implicated in protein-protein interactions also disrupted TRPM2 subunit interaction and TRPM2 currents (Mei et al., 2006b). TRPM2 also contains a unique C-terminal enzymatic domain called NUDT9-H (AA 1236-1503) (Jiang et al., 2010; Perraud et al., 2003). TRPM2 channels are gated by binding of intracellular ADPR to this NUDT9-H domain (Perraud et al., 2001) (more details on TRPM2 activation mechanisms are provided in section 1.4). The NUDT9-H domain is so named because it displays substantial sequence homology (39%) to the mitochondrial ADPR pyrophosphatase NUDT9, an enzyme that catalyzes the hydrolysis of ADPR to adenosine monophosphate (AMP) and ribose-5-phosphate (Shen et al., 2003). TRPM2 has been called a ‘chanzyme’ because it functions as both an ion channel and an enzyme (Perraud et al., 2003). Notably, when expressed in *E. coli*, the ADPR hydrolase activity of the NUDT9-H was over 100 times lower than NUDT9 (Perraud et al., 2001; Shen et al., 2003). However, both NUDT9-H and NUDT9 displayed identical affinities for ADPR and substrate specificity for ADPR compared to other nucleoside diphosphate derivatives tested (Perraud et al., 2001). Amino acids substitutions at residues 1405-1406 of TRPM2 have been implicated as critical for the reduction in activity of NUDT9-H relative to NUDT9 (Kuhn & Luckhoff, 2004). Interestingly, mutations I1405E/L1406F within

NUDT9-H fully restored the enzymatic activity of this region relative to NUDT9, but abolished ADPR-induced TRPM2 activation (Kuhn & Luckhoff, 2004). Perraud et al. (2005) suggested that the NUDT9-H domain may be an evolutionary adaptation of the ADPR-binding region of the NUDT9 enzyme into an ADPR-dependent gating mechanism for TRPM2. Although a specific role for the enzymatic activity of NUDT9-H, beyond gating of TRPM2, has not yet been identified, the possibility that this enzymatic activity may serve some physiological function should not be excluded. In addition to its role in TRPM2 channel gating, the NUDT9-H domain has also been implicated in TRPM2 channel assembly and surface trafficking (Perraud et al., 2003).

TRPM2 is a non-selective cation channel that displays significant permeability to Na^+, K^+, Ca^{2+} and Mg^{2+} (Xia et al., 2008). The relative permeabilities of these cations are 1 Na^+:1.1 K^+:0.9 Ca^{2+}:0.5 Mg^{2+} (Xia et al., 2008). Thus, the inward current mediated by TRPM2 is primarily attributable to Na^+ movement, but the channel is also significantly permeant to Ca^{2+}. The single-channel conductance of TRPM2 is approximately 50-80 pS, with a mean channel open time on the order of several seconds (Jiang et al., 2010; Perraud et al., 2001). Open TRPM2 channels display a linear current-voltage (I-V) relationship, which implies that TRPM2 channel gating is not voltage-dependent (Perraud et al., 2001; Sano et al., 2001). Further, TRPM2 has a reversal potential of approximately 0 mV, which is characteristic of non-selective cation channels (Perraud et al., 2001; Sano et al., 2001).

Several identified TRPM2 splice variants have provided insight into how TRPM2 structure relates to channel function. TRPM2-S (848 AA) is a splice variant that contains a premature stop codon, resulting in a non-functional truncated protein that contains only the N-terminal tail and the first two transmembrane domains (Zhang et al., 2003). TRPM2-SSF (1289 AA) is a striatum shorter form of TRPM2 with a deletion of AA 1-214 (Uemura et al., 2005). TRPM2-SSF displays substantially less channel activity than full-length TRPM2 (Uemura et al., 2005). TRPM2-ΔN (1483 AA) is a variant with deletion of AA 538-557, TRPM2-ΔC (1469 AA) has deletions of AA 1292-1325, and TRPM2-ΔNΔC has deletions of AA 538-557 and AA 1292-1325 (Wehage et al., 2002). Currents were not significantly stimulated by ADPR or H_2O_2 in TRPM2-ΔN or TRPM2-

ΔNΔC (Wehage et al., 2002). Interestingly, H_2O_2, but not ADPR, significantly stimulated currents in TRPM2-ΔC, which contains a partially disrupted ADPR-binding NUDT9-H domain (Wehage et al., 2002) (more details on TRPM2 activation mechanisms are provided in section 1.4).

1.4 Oxidative stress and TRPM2 activation

Oxidative stress refers to an imbalance in cellular redox state, which is marked by the generation of reactive oxygen species (ROS) (Miller & Zhang, 2011). ROS, such as H_2O_2, are highly reactive and can induce cellular damage by oxidation of DNA and proteins as well as lipid peroxidation (Miller & Zhang, 2011). Oxidative stress has been widely implicated as a causative factor in the pathogenesis of several diseases, including cancer (Halliwell, 2007), stroke (Allen & Bayraktutan, 2009), Alzheimer's disease (Saltana & Butterfield, 2010), atherosclerosis (Vogiatzi et al., 2009), and many others. However, the molecular signaling pathways underlying oxidative damage are complex. A common event following oxidative stress is an elevation in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Jiang et al., 2010). Increased $[Ca^{2+}]_i$ may result from activation of Ca^{2+}-permeable ion channels on the plasma membrane or release from intracellular Ca^{2+} stores, such as the endoplasmic reticulum (ER) (Clapham, 2007). Subsequently, intracellular Ca^{2+} exerts pleiotropic effects within cells through diverse signaling pathways (Berridge et al., 2000; Clapham, 2007). For example, Ca^{2+} signaling has been implicated in modulating cellular excitability and mediating cell death (Clapham, 2007). TRPM2 channels provide a means by which $[Ca^{2+}]_i$ is increased in response to oxidative stress.

As described in section 1.3, TRPM2 channels are activated by binding of intracellular agonist ADPR to the NUDT9-H domain of TRPM2 (Perraud et al., 2001). ADPR is the most potent activator of TRPM2, with half-maximal effective concentrations (EC_{50}) for TRPM2 activation by ADPR variably reported in the range of 10-90 μM (Jiang et al.,

2010), which is comparable to reported endogenous intracellular ADPR levels of 5-75 μM (Gasser & Guse, 2005; Heiner et al., 2006).

Nicotinamide adenine dinucleotide (NAD^+) has also been shown to activate TRPM2 channels (Sano et al., 2001). NAD^+ is a less potent TRPM2 activator than ADPR and has an EC_{50} of approximately 1-1.8 mM (Jiang et al., 2010). Although some sources have suggested that NAD^+ directly activates TRPM2 (Hara et al., 2002; Sano et al., 2001), others have suggested that NAD^+ is first converted to ADPR, which in turn gates TRPM2 (Perraud et al., 2005; Wehage et al., 2002). NAD^+ can be converted to ADPR by sequential enzymatic actions of polyADPR-polymerase (PARP), which converts NAD^+ into polyADPR, and polyADPR-glycohydrolase (PARG), which breaks down polyADPR into monomeric ADPR (Blenn et al., 2011). Alternatively, NAD^+ can be directly hydrolyzed by NAD^+ glycohydrolase (NADase) enzymes into ADPR (Knowles et al., 2013). Thus, NAD^+ may activate TRPM2 by a direct, ADPR-independent mechanism or through the production of ADPR. TRPM2 activation has also been demonstrated in response to several other molecules that are metabolically related to ADPR, such as cyclic ADPR (cADPR; EC_{50} = 0.7 mM), and nicotinic acid-adenine dinucleotide phosphate (NAADP; EC_{50} = 0.73 mM) (Jiang et al., 2010). The mechanisms by which such agents induce TRPM2 activation is unclear at present, but it has been proposed that they may bind to an alternate site on TRPM2 or be converted metabolically to ADPR, which in turn activates TRPM2 (Beck et al., 2006).

TRPM2 channel activation has also been demonstrated in response to ROS, such as H_2O_2 (Hara et al., 2002; Wehage et al., 2002). Activation of TRPM2 channels by H_2O_2 has been proposed to occur through ADPR-independent and ADPR-dependent mechanisms. Wehage et al. (2002) demonstrated that H_2O_2 significantly stimulated currents in the ADPR-insensitive TRPM2-ΔC splice variant. This finding supports the idea that H_2O_2 induces TRPM2 activation through an ADPR-independent mechanism. However, the mechanism underlying ADPR-independent TRPM2 activation is not clearly defined at present. On the other hand, an ADPR-dependent mechanism has been proposed: H_2O_2 stimulates activation of PARP/PARG and NADase enzymes, which results in conversion of NAD^+ to ADPR, which in turn activates TRPM2 (Takahashi et al., 2011).

Ca^{2+} ions also play an important role in TRPM2 activation. It is widely accepted that both intracellular and extracellular Ca^{2+} are critical regulators of TRPM2 activation by ADPR (McHugh et al., 2003; Tong et al., 2006) (more details on TRPM2 regulation by Ca^{2+} are provided in section 1.7). Other studies have shown that intracellular Ca^{2+} can actually gate TRPM2 channels through an ADPR-independent mechanism (McHugh et al., 2003; Du et al., 2009a). Du et al. (2009a) showed that intracellular Ca^{2+} can activate currents in TRPM2 mutants with disrupted ADPR binding motifs as well as the ADPR-insensitive splice variants TRPM2-ΔN, TRPM2-ΔC and TRPM2-ΔNΔC. Notably, activation of TRPM2 by Ca^{2+} alone (EC_{50} = 16.9 μM) was greatly facilitated by the presence of 10 μM ADPR (EC_{50} = 0.49 μM) (Du et al., 2009a). This suggests that TRPM2 is activated synergistically by both ADPR and Ca^{2+}. It follows that after oxidative stress, ADPR levels increase, TRPM2 channels are activated, and local $[Ca^{2+}]_i$ increases, which in turn potentiates TRPM2 activation via a positive feedback loop (McHugh et al., 2003). Because TRPM2 mediates susceptibility to cell death in response to oxidative stress, it has been implicated in the pathogenesis of several oxidative stress-related diseases (more details on TRPM2 in central nervous system (CNS) disease are provided in section 1.6).

1.5 Physiological roles of TRPM2

A variety of physiological roles have been ascribed to TRPM2. For example, TRPM2 activation has been implicated in insulin release from β-islet cells of the pancreas (Togashi et al., 2006). Uchida et al. (2011) showed that basal blood glucose levels were higher in TRPM2 knockout (TRPM2-KO) mice compared to wild-type (TRPM2-WT) mice. Further, in isolated β-cells, glucose-evoked cellular Ca^{2+} influx and insulin secretion were reduced in TRPM2-KO compared to TRPM2-WT (Uchida et al., 2011). TRPM2 has also been shown to contribute to cytokine production in immune cells (Yamamoto et al., 2008). For example, Yamamoto et al. (2008) showed that H_2O_2-induced production of the chemokine CXCL2 in murine monocytes is dependent on TRPM2 Ca^{2+} influx; CXCL2 production is impaired in TRPM2-KO mice. Additionally,

TRPM2 was shown to be essential for lipopolysaccharide-induced production of cytokines IL-6, IL-8, IL-10 and TNF-α (Wehrhahn et al., 2010). TRPM2 has also been implicated as a regulator of dendritic cell chemotaxis in response to cytokines; TRPM2-deficient dendritic cells display impaired cytokine-induced chemotaxis (Sumoza-Toledo et al., 2011). Further, TRPM2 has also been shown to mediate H_2O_2-induced increases in vascular endothelial permeability (Hequet et al., 2008). Although TRPM2 expression is most abundant in the CNS (Sumoza-Toledo & Penner, 2011) and several physiological roles of TRPM2 in peripheral tissues have been identified, the physiological functions of TRPM2 in the CNS were until recently unknown. However, Xie et al. (2011) showed that TRPM2 plays a role in hippocampal synaptic plasticity. Specifically, loss of TRPM2 expression in TRPM2-KO mice impairs NMDAR-dependent long-term depression (Xie et al., 2011). One of the best-described roles of TRPM2 is that its activation confers susceptibility to cell death in response to oxidative stress (Hara et al., 2002; Wehage et al., 2002). The contribution of TRPM2 to cell death is described in detail in section 1.4. However, when dysregulated, this function of TRPM2 may contribute to various pathologies.

1.6 TRPM2 channels in CNS disease

Several neurological pathologies are characterized by dysregulation of Ca^{2+} homeostasis in response to oxidative stress (Zundorf & Reiser, 2011). TRPM2 activation has been shown to mediate cellular Ca^{2+} influx and susceptibility to cell death in response to oxidative stress (Hara et al., 2002; Wehage et al., 2002). As such, excessive TRPM2 activation may contribute to the pathogenesis of several oxidative stress-related pathologies (Jiang et al., 2010). Details on activation of TRPM2 channels in response to oxidative stress are provided in section 1.4. The expression of TRPM2 in a variety of tissues as well as its role as a mediator of cell death makes it an attractive therapeutic target for several diseases.

TRPM2 has been linked to neurological disorders through identification of genetic variants. For example, TRPM2 has been linked to Western Pacific amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia (PD) (Hermosura & Garruto, 2007). A single nucleotide polymporphism, resulting in substitution of proline 1018 to leucine (P1018L), was found to be closely associated with a subset of Western Pacific ALS and PD (Hermosura et al., 2008). This mutation results in faster inactivation of TRPM2-P1018 relative to TRPM2-WT (Hermosura et al., 2008). Similarly, single nucleotide polymorphisms, such as aspartic acid 543 to glutamic acid (D543E), have been linked to bipolar disorder (McQuillin et al., 2006). However, in each case, the functional relevance of genetic variants in TRPM2 to disease pathology remains unclear at present.

TRPM2 activation has been implicated in the pathogenesis of Alzheimer's disease (AD). The amyloid hypothesis, a prominent theory of AD pathogenesis, proposes that that β-amyloid (Aβ), a peptide that accumulates in the brains of AD patients, is the neurotoxic agent that drives AD pathogenesis (Hardy and Selkoe, 2002). A recent study showed that activation of TRPM2 channels contributes to Aβ-induced death in rat primary striatal cultures (Fonfria et al., 2005). Transfection of cells with the TRPM2-S splice variant, which inhibits TRPM2 in a dominant negative fashion, blocked Aβ-induced increases in $[Ca^{2+}]_i$ and cell death (Fonfria et al., 2005). Further, mRNA knockdown of TRPM2 reduced Aβ toxicity (Fonfria et al., 2005). Notably, degeneration of striatal neurons is not a major characteristic of AD; hippocampal neurons are more important in this respect. Whether TRPM2 is involved in the degeneration of hippocampal pyramidal neurons remains an open question. Further, the molecular mechanism by which TRPM2 may contribute to Aβ toxicity in AD has not yet been identified.

Recent evidence also suggests that TRPM2 may play a role in stroke pathology. Fonfria et al. (2006) demonstrated that in the rat transient middle cerebral artery occlusion model of stroke, TRPM2 mRNA expression is elevated 1 and 4 weeks after ischemic injury. Further, during acute ischemia *in vivo* or oxygen glucose deprivation *in vitro*, a TRP-like channel is activated, resulting in increased $[Ca^{2+}]_i$ (Lipski et al., 2006). While this evidence doesn't conclusively implicate TRPM2 as a pathogenic factor in stroke, it does provide preliminary support for this notion.

1.7 TRPM2 regulation mechanisms

As previously described, a number of pharmacological agents have been shown to regulate TRPM2 activation. Several inhibitors of TRPM2 activation have been identified. For example, AMP, an enzymatic breakdown product of ADPR by NUDT9 and NUDT9-H, can antagonize TRPM2 activation (half-maximal inhibitory concentration [IC_{50}] = 10-70 μM) by ADPR, NAD^+, cADPR and NAADP (Beck et al., 2006; Kolisek et al., 2005; Lange et al., 2008). Mechanistically, AMP is widely thought to inhibit TRPM2 by competitively binding to the ADPR binding site in the NUDT9-H domain of TRPM2 (Sumoza-Toledo & Penner, 2011). Alternatively, 8-Br-cADPR inhibits TRPM2 activation by cADPR and H_2O_2, but can synergize TRPM2 activation by ADPR (Kolisek et al., 2005). Other non-specific TRPM2 antagonists include clotrimazole, flufenamic acid (FFA) and N-(p-amylcinnamoyl)anthranilic acid (ACA) (Kraft et al., 2006). However, no specific pharmacological inhibitors have been identified for TRPM2 to date.

As described briefly in sections 1.3 and 1.4, Ca^{2+} is an important regulator of TRPM2 function. Importantly, both intracellular and extracellular Ca^{2+} influence channel activation. In the absence of both extracellular and intracellular Ca^{2+}, ADPR is not sufficient to induce TRPM2 currents (Starkus et al., 2007). Further, in the absence of intracellular Ca^{2+}, ADPR alone could not activate TRPM2 (Perraud et al., 2001). In the absence of extracellular Ca^{2+}, ADPR induced TRPM2 activation when $[Ca^{2+}]_i$ was at least 30 nM (Starkus et al., 2007). TRPM2 activation by ADPR is markedly facilitated by intracellular Ca^{2+} (Perraud et al., 2001; Tong et al., 2006). Mechanistically, intracellular Ca^{2+} binds to the small Ca^{2+}-binding protein CaM, which in turn binds the IQ-like motif in the N-terminal tail of TRPM2 and facilitates channel activation (Sumoza-Toledo & Penner, 2011). Notably, Ca^{2+}/CaM binding to the IQ-like motif was shown to be essential for TRPM2 activation (Tong et al., 2006). TRPM2 channel inactivation has been observed for $[Ca^{2+}]_i$ lower than 100 nM (Starkus et al., 2007). Some studies have shown that intracellular Ca^{2+} can actually gate TRPM2 channels

through an ADPR-independent mechanism (McHugh et al., 2003; Du et al., 2009a) (more details on gating of TRPM2 by Ca^{2+} are provided in section 1.3). Notably, although Ca^{2+} may gate TRPM2 independently of ADPR, it has also been shown that elevated $[Ca^{2+}]_i$ also increases the sensitivity of TRPM2 for ADPR (Starkus et al., 2007). Extracellular Ca^{2+} is also an important modulator of TRPM2 function (Csanady & Torocsik, 2009; Starkus et al., 2007). Extracellular Ca^{2+} that enters cells through TRPM2 during channel activation can facilitate further channel activation through a positive feedback mechanism (Tong et al., 2006). Using fast and slow intracellular Ca^{2+} buffering and varying resting $[Ca^{2+}]_i$, McHugh et al., (2003) demonstrated that TRPM2 activation was ultimately regulated by intracellular Ca^{2+}. By extension, extracellular Ca^{2+} was proposed to modulate TRPM2 function by entering cells and acting on a TRPM2-associated Ca^{2+} sensor (McHugh et al., 2003).

TRPM2 channels are also regulated by acidic pH. Both intracellular and extracellular protons were shown to block TRPM2 channels (IC_{50}: pH 5.3 for external, pH 6.7 for internal) (Du et al., 2009b). However, the mechanism underlying TRPM2 regulation by acidification is unclear at present (Du et al., 2009b; Yang et al., 2010). Additionally, Togashi et al. (2006) demonstrated that TRPM2 gating exhibits temperature-dependence. Notably, the temperature sensitivity of TRPM2 is augmented by the presence of intracellular ADPR, NAD and cADPR, which suggests that temperature may facilitate TRPM2 activation by these agents (Togashi et al., 2006).

1.8 TRPM2 regulation by tyrosine phosphorylation

It was recently reported that tyrosine phosphorylation of TRPM2 increases channel activation (Zhang et al., 2007). In HEK293 cells expressing TRPM2, H_2O_2 treatment increased tyrosine phosphorylation of TRPM2, $[Ca^{2+}]_i$ and apoptotic cell death—effects that were mitigated by the tyrosine phosphatase PTPL1 (Zhang et al., 2007). This suggests that a tyrosine phosphorylation event is responsible for regulating TRPM2 activation and modulating susceptibility to cell death. Notably, this study used Ca^{2+}

imaging to study TRPM2 function. However, this technique does not specifically assess TRPM2 function, as rises in $[Ca^{2+}]_i$ could be attributable to release from intracellular stores or other Ca^{2+}-permeable membrane channels. Further, the kinase responsible for this phosphorylation and the tyrosine residue(s) phosphorylated remain unknown. PTPL1 was also shown to physically interact with TRPM2 by immunoprecipitation and glutathione-S-transferase pull-down assays (Zhang et al., 2007). Notably, PTPL1 has been implicated in cell survival and tumorigenesis (Freiss & Chalbos, 2011). Zhang et al. (2007) also showed that treatment of cells with genistein, a non-specific tyrosine kinase inhibitor, reduced H_2O_2-induced tyrosine phosphorylation of TRPM2, Ca^{2+} influx and cell death (Zhang et al., 2007). Further, treatment of cells with PP2, an inhibitor of Src family tyrosine kinases (SFKs), similarly reduced H_2O_2-induced tyrosine phosphorylation of TRPM2, Ca^{2+} influx and cell death. However, PP2 is a non-specific inhibitor of SFKs, and the specific SFK member involved has not yet been identified. Taken together, these findings support the idea that tyrosine phosphorylation of TRPM2 is important for regulation of channel activation. Zhang et al. (2007) proposed three mechanisms by which TRPM2 phosphorylation may affect channel activation: (1) phosphorylation of the NUDT9-H domain, which may affect ADPR binding, (2) phosphorylation of CaM binding sites, which may affect Ca^{2+}/CaM regulation, or (3) phosphorylation of sites critical for pore function, which may affect channel opening or permeability. Modulation of TRPM2 tyrosine phosphorylation, and consequently channel activation, may be a pathway by which susceptibility to cell death is modulated.

1.9 Fyn kinase

Fyn kinase is a cytosolic protein that phosphorylates substrate proteins on tyrosine residues. Fyn is a member of the Src family of non-receptor protein tyrosine kinases (SFK). This protein family was named based on structural homology of its members to Src, the prototypical member of the family, which was first identified in Rous sarcoma virus (Scales et al., 2011). The SFK family consists of eight members, which are subdivided into subfamilies based on amino acid sequence homology: Src-related (Src,

Fyn, Yes, Fgr) and Lyn-related (Lyn, Lck, Hck, Blk) (Ingley, 2008). There are also three Src-related kinases (Brk, Frk, Srm), which have a similar domain structure to Src kinases (Ingley, 2008). There is a high degree of amino acid sequence conservation among SFK members. Outside of an N-terminal domain whose sequence is almost completely unique to each member, SFKs share approximately 70% amino acid sequence identity (Hunter, 1997). However, within the SFK family, there is substantial diversity in expression patterns, regulatory mechanisms and implicated signaling pathways (Engen et al., 2008). Notably, five SFK members are expressed in the mammalian CNS: Src, Fyn, Yes, Lck and Lyn (Salter & Kalia, 2004).

The gene encoding human Fyn is located on chromosome 6q21, is 213 kb in length and contains 14 exons (Popescu et al., 1987). To date, three major alternatively spliced isoforms of Fyn have been identified (Cooke & Perlmutter, 1989; Goldsmith et al., 2002). Notably, only one of the isoforms (Fyn-B) is highly expressed in the central nervous system (Cooke & Perlmutter, 1989; Long et al., 2011). For isoform Fyn-B, the *Fyn* gene encodes an mRNA transcript of approximately 3.6 kb, which in turn encodes a 537 AA protein with a molecular weight of approximately 59 kDa (Resh, 1998).

Structurally, Fyn shares a conserved domain structure with the other SFK members. This structure consists of (from N- to C-terminus): a Src homology 4 (SH4) domain, unique domain (UD), SH3 domain, SH2 domain, linker region, catalytic domain and regulatory domain (Salter & Kalia, 2004). The N-terminal SH4 domain contains sites for lipid modifications, such as myristoylation, which are important for anchoring Fyn to the cytosolic face of membranes (Rawat & Nagaraj, 2010). The UD is an N-terminal region whose size and sequence are highly variable between SFK members (Pérez et al., 2009). It follows that the UD may play a role in determining specificity of SFKs, such as substrate selectivity, protein-protein interactions and/or regulation of activity. For example, UDs of some SFKs have been shown to mediate protein-protein interactions. Gingritch et al. (2004) showed that Src interacts with NADH dehydrogenase subunit 2 (ND2), which in turn interacts with NMDAR. Anchoring of Src to the NMDAR complex through Src's UD is critical for regulation of NMDAR activity and, resultantly, synaptic transmission and plasticity (Gingritch et al., 2004). However, the function of Fyn's UD

remains unknown. The SH3 domain is a highly conserved region that mediates interactions with other proteins (Hauck et al., 2001). Specifically, the SH3 domain binds proline-rich sequences containing a PxxP motif (where 'x' represents any amino acid) (Hauck et al., 2001). Similarly, the SH2 domain is a highly conserved region that also mediates protein-protein interactions (Nachman et al., 2010). SH2 binds to sequences containing phosphorylated tyrosine residues (Nachman et al., 2010). The linker region participates in intramolecular interactions and regulates Fyn activation (Pérez et al., 2013). The catalytic domain transfers a phosphate group from adenosine triphosphate (ATP) to specific tyrosine residue(s) on substrate proteins (Thomas & Brugge, 1997). The C-terminal regulatory domain is a short region involved in regulating kinase activity (Kim & Lee, 2005).

Activation of Fyn is primarily regulated by phosphorylation events and intramolecular interactions. Autophosphorylation of a tyrosine residue (Y420) within the catalytic domain induces Fyn activation (Thomas & Brugge, 1997). Importantly, phosphorylation of Y420 is the primary regulator of Fyn activation and is essential for Fyn activation (Cheng et al., 1991). In addition to self-phosphorylation, the phosphorylation state of Fyn-Y420 is regulated by kinases, such as ErbB4 (or an ErbB4-activated tyrosine kinase) (Bjarnadottir et al., 2007) and phosphatases, such as striatal enriched phosphatase 61 (STEP61) (Nguyen et al., 2002). The C-terminal regulatory domain contains a tyrosine residue (Y531) that, when phosphorylated, interacts with the SH2 domain and stabilizes an inactive conformation (Kim & Lee, 2005). Phosphorylation of Fyn-Y531 is regulated by kinases, such as Csk (Colognato et al., 2004) , and phosphatases, such as protein tyrosine phosphatase α (PTPα) (Gibb et al., 2011). Further, phosphorylation of an N-terminal serine residue (S21) by protein kinase A (PKA) is involved in regulating Fyn activity (Yeo et al., 2011). Site-directed mutagenesis of this serine to alanine (S21A) ablates phosphorylation at this site and decreases Fyn activity (Yeo et al., 2011). Notably, the linker region is a short sequence that contains an SH3 substrate motif (PxxP) that stabilizes an inactive conformation when bound to the SH3 domain (Pérez et al., 2013). Fyn activity is also increased by ROS such as H_2O_2 (Abe and Berk, 1999; Sanguinetti et al., 2003).

1.10 TRPM2, Fyn and NMDA receptor signaling

N-methyl-D-aspartate (NMDA) receptor (NMDAR) is a glutamate-gated ionotropic receptor. Like TRPM2, NMDAR is a non-selective cation channel, which is significantly permeable to Na^+, K^+ and Ca^{2+} (Sakurada et al., 1993). Notably, NMDARs display voltage-dependent blockage by Mg^{2+} at resting membrane potential (Sakurada et al., 1993). Under normal physiological conditions, NMDARs play a role in synaptic transmission at glutamatergic synapses. At these synapses, glutamate binds to α-amino-3-hyroxy-5-methyl-4-isoazoleproprionic acid (AMPA) receptors (AMPARs), activating them and causing depolarization of the postsynaptic cell. Such depolarization is sufficient to relieve the voltage-dependent Mg^{2+} block of NMDARs. Subsequently, binding of co-agonists glutamate and glycine induces NMDAR activation (Kew & Kemp, 2005). Such NMDAR activation induces an acute rise in $[Ca^{2+}]_i$. Cellular influx of Ca^{2+} through NMDARs has been implicated in a number of physiological processes, such as synaptic plasticity (Liu et al., 2004). However, under pathological conditions, excessive Ca^{2+} influx through NMDARs has been shown to contribute to neuronal death (Choi, 1992).

Co-activation of TRPM2 and NMDAR may cause excessive Ca^{2+} influx and as a result promote neuronal death. As described in section 1.7, activation of TRPM2 is dependent on extracellular and intracellular Ca^{2+} (McHugh et al., 2003). Intracellular Ca^{2+} has been shown to facilitate ADPR-mediated TRPM2 activation (McHugh et al., 2003). Interestingly, intracellular Ca^{2+} has also been shown to gate TRPM2 activation by an ADPR-independent mechanism (Du et al., 2009a). Notably, ADPR alone was insufficient to gate TRPM2 channels in CA1 pyramidal neurons; Olah et al. (2009) observed that an additional influx of Ca^{2+} through NMDARs was necessary to induce TRPM2 activation. Further, clotrimazole, a TRPM2 antagonist, was shown to reduce glutamate-induced Ca^{2+} overload and neuronal death in rat hippocampal pyramidal neurons (Isaev et al., 2002). Thus, there appears to be a functional connection between TRPM2 and NMDAR.

SFKs, including Fyn, have also been shown to regulate NMDAR function by tyrosine phosphorylation. For example, Src has been shown to phosphorylate NMDARs, and intracellular application of recombinant Src was shown to increase NMDAR currents (Wang & Salter, 1994). Further, phosphorylation of NMDAR by Fyn has been demonstrated (Salter and Kalia, 2004). This phosphorylation has been linked with increased NMDAR current and pathological levels of intracellular Ca^{2+} (Ittner et al., 2010). Notably, regulation of NMDARs by SFKs is subtype-specific. Specifically, Src selectively phosphorylates and regulates GluN2A subunit-containing NMDARs, whereas Fyn selectively phosphorylates and regulates GluN2B subunit-containing NMDARs (Yang et al., 2011). GluN2B subunit-containing NMDARs, whose activities are potentiated by Fyn, have been implicated in neuronal apoptosis (Hardingham et al., 2002; Yang et al., 2011). It follows that TRPM2, Fyn and NMDARs may contribute to Ca^{2+} dysregulation and cell death in neurons.

1.11 Rationale and hypothesis

As described in section 1.8, H_2O_2-induced tyrosine phosphorylation of TRPM2, which coincides with increased channel activation, was reduced by PP2, a non-selective SFK inhibitor (Zhang et al., 2007). This suggests that TRPM2 activation is regulated by a SFK, which has not yet been identified. TRPM2 currents can be activated in cultured hippocampal CA1 neurons by repeated applications of NMDA in the absence of exogenous ADPR (MacDonald lab, unpublished). Interestingly, with intracellular applications of recombinant Fyn, repeated NMDA applications resulted in increased TRPM2 currents over time—an effect that was abolished by inhibition of Fyn signaling (MacDonald/Jackson lab, unpublished). This potentiation of TRPM2 current could be due to an increase in NMDAR currents or an enhancement of TRPM2 activation or both. Taken together, these findings suggest that Fyn may be a SFK involved in regulation of TRPM2 activation. Thus, this thesis will investigate the potential regulation of TRPM2 channels by Fyn kinase.

We hypothesize that Fyn kinase phosphorylates TRPM2 channels and enhances their function, resulting in larger TRPM2 currents. Our specific objectives are **(1)** to demonstrate that Fyn regulates TRPM2 currents, **(2)** to determine whether Fyn induces tyrosine phosphorylation of TRPM2, and **(3)** to characterize the potential physical interaction between Fyn and TRPM2.

Section 2

MATERIALS AND METHODS

2.1 Cell culture

HEK293 cells stably expressing inducible FLAG-epitope-tagged TRPM2 (TRPM2-HEK293 cells) were generously provided by Dr. A.M. Sharenberg (University of Washington, and Children's Hospital and Medical Center, Seattle, WA). The T-REx™ System (Invitrogen, CA) used to generate this cell line is a tetracycline-regulated mammalian expression system. Transcriptional activation of FLAG-TRPM2 can be induced by binding of tetracycline or doxycycline to a Tet repressor controlling FLAG-TRPM2 expression. HEK293T cells were generously provided by Dr. H.H.M.Van Tol (University of Toronto, and Centre for Addiction and Mental Health, Toronto, ON). TRPM2-HEK293 cells and HEK293T cells were cultured at 37°C and 5% CO_2 in 100 mm culture dishes containing 10 mL Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). Cells were split every 3-4 days to a maximum of 35 passages.

2.2 Whole-cell voltage-clamp electrophysiology

For electrophysiological recordings, TRPM2-HEK293 cells were plated on 35 mm culture dishes 1-2 days prior to recording. TRPM2 expression was induced by administration of doxycycline (1.0 μg/mL) 18-24 hours prior to electrophysiological recordings. Whole-cell voltage-clamp recordings were carried out with TRPM2-HEK293 cells at 20-22°C using an Axopatch-1D amplifier (Molecular Devices, CA). Data acquisition was performed using pCLAMP Clampex 10.2 software (Molecular Devices). Recording electrodes, with a final resistance of 3-5 MΩ when filled with intracellular solution (ICS), were prepared from TW150-F3 borosilicate glass (World Precision Instruments, FL) using a PP-83 two-stage puller (Narishige, NY). Electrodes were filled with ICS, containing (in mM): 135 cesium gluconate, 10 HEPES buffer, 10 EGTA, 4 Mg-ATP, 2 $MgCl_2$, 1 $CaCl_2$, and 0.1 ADPR. Extracellular solution (ECS), consisting of (in mM) 140 NaCl, 25 HEPES buffer, 5.4 KCl, 33 glucose, 1 $MgCl_2$, and 2 $CaCl_2$, was applied using a SF77B multibarrel perfusion system (Warner Institute, CT). Since

TRPM2 activation requires extracellular Ca^{2+}, $CaCl_2$ in the ECS was replaced with equimolar $BaCl_2$ during recordings, as indicated, to abolish TRPM2 current and permit channel recovery, while maintaining the total extracellular divalent cation concentration. Osmolarity of ICS and ECS were 290-300 and 310-315 mOsm, respectively. Further, ICS and ECS were adjusted to pH 7.3 and 7.4, respectively. Holding current was recorded as a function of time with the membrane potential clamped at -60 mV for all recordings. TRPM2 currents were isolated by exploiting distinctive channel activation and inactivation properties. Currents were activated by ADPR (0.1 mM, in patch pipette). Further, abolition of currents was demonstrated by substitution of extracellular Ca^{2+} with equimolar Ba^{2+}. Currents were only evoked in doxycycline-induced cells. Peak TRPM2 current amplitudes were measured in cells treated with constitutively active recombinant Fyn kinase (1 U/mL, in patch pipette) and vehicle-treated control cells. TRPM2 currents were recorded using the following protocol: (1) TRPM2 channels were initially 'primed' by application of Ca^{2+}-containing ECS until 1 nA TRPM2 current was observed, (2) TRPM2 current was abolished for 3 minutes by applying Ca^{2+}-free ECS to permit channel recovery, (3) TRPM2 current was recorded during application of Ca^{2+}-containing ECS until TRPM2 current inactivation was observed, (4) TRPM2 channel recovery was permitted by abolishing TRPM2 current during a 5 minute application of Ca^{2+}-free ECS, (5) a second TRPM2 current was recorded during application of Ca^{2+}-containing ECS until TRPM2 current inactivation was observed, (6) TRPM2 current was again abolished by application of Ca^{2+}-free ECS. No difference was observed between the first and second TRPM2 current peaks. Results presented here reflect data gathered from the first TRPM2 peak current response.

2.3 Generation of TRPM2 constructs

Wild-type TRPM2 construct:

Wild-type human TRPM2 (accession number NP_003298.1) was amplified from a human cDNA library by polymerase chain reaction (PCR) using KOD Hot Start DNA

Polymerase (EMD Biosciences, CA). Forward and reverse primers for TRPM2 amplification (listed in table 2.1) were generated from a pIRESpuro-FLAG-TRPM2-WT template using Vector NTI software (Invitrogen). These primers contained NotI and AscI substrate sequences for subsequent sub-cloning. The following thermal profile was used for this PCR reaction: (1) 95°C for 2 minutes, (2) 10 cycles of 94°C for 15 seconds, 60°C (-1°C/cycle) for 15 seconds and 72°C for 120 seconds, and (3) 25 cycles at 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 120 seconds. The PCR product was resolved on a 1% w/v agarose gel containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich). The PCR product was extracted from the gel using a GenElute Gel Extraction Kit (Sigma-Aldrich) according to the manufacturer's protocol. A double restriction enzyme digest with NotI and AscI enzymes (New England Biolabs, MA) was carried out at 37°C for 2 hours. Subsequently, digested DNA was purified with a GenElute Gel Extraction Kit according to manufacturer's protocol. The purified DNA product was then ligated into NotI/AscI-digested pIRESpuro-FLAG vector at a 1:5 (vector:insert) molar ratio using T4 DNA ligase (New England Biolabs). The ligated product was transformed into JM109 competent cells (Promega, WI) and grown up on LB + ampicillin plates overnight at 37°C. Individual colonies were then expanded in LB + ampicillin selection medium, shaking at 250 RPM overnight at 37°C. Plasmid DNA was isolated from pellets using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to manufacturer's protocol. Incorporation of TRPM2 into pIRESpuro-FLAG vector was confirmed by triple restriction digest (AscI/NotI/XhoI, New England Biolabs). Positive clones were further confirmed by DNA sequencing (London Regional Genomics Centre).

Site-directed mutagenesis:

To identify potential tyrosine residue(s) through which Fyn may regulate TRPM2 phosphorylation and activation, GPS 2.1, a proteomic phosphorylation site prediction tool (Xue et al., 2008) was used. This software compares short amino acid sequence motifs on a substrate protein and kinase(s) of interest and predicts high-probability phosphorylation sites. The four identified TRPM2 tyrosine residues with the highest prediction scores were Y74, Y142, Y523 and Y1503. Four mutant human TRPM2

constructs were generated by site-directed mutagenesis. Primers (Sigma-Aldrich; listed in table 2.1) were designed using Vector NTI software to amplify wild-type human TRPM2 with replacement of the tyrosine residue at amino acid 74 with phenylalanine (TAT → TTT) for TRPM2-Y74F, tyrosine 142 to phenylalanine (TAC→TTC) for TRPM2-Y142F, tyrosine 523 to phenylalanine (TAC→TTC) for Y523F, and tyrosine 1503 to phenylalanine (TAC→TTC) for TRPM2-Y1503F. TRPM2-Y1503F was generated using a single PCR reaction, using KOD polymerase, the primers listed in table 2.1 and pIRESpuro-FLAG-TRPM2-WT as a template. The following thermal profile was used for this PCR reaction: (1) 95°C for 2 minutes, (2) 10 cycles of 94°C for 15 seconds, 60°C (-1°C/cycle) for 15 seconds and 72°C for 120 seconds, and (3) 25 cycles at 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 120 seconds. Two separate PCR reactions were used to generate two fragments of TRPM2-Y74F, TRPM2-Y142F and TRPM2-Y523F using KOD polymerase, the primers listed in table 2.1 (TRPM2-Y74F-1 and TRPM2-Y74F-2; TRPM2-Y142F-1 and TRPM2-Y142F-2; or TRPM2-Y523F-1 and TRPM2-Y523F-2) and pIRESpuro-FLAG-TRPM2-WT as a template. The following thermal profile was used for theses PCR reactions: (1) 95°C for 2 minutes, (2) 10 cycles at 94°C for 15 seconds, 60°C (-1°C/cycle) for 15 seconds, and 72°C for 10-120 seconds, and (3) 25 cycles at 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 10-120 seconds. Full-length TRPM2-Y74F, TRPM2-Y142F and TRPM2-Y523F were then generated using PCR reactions with KOD polymerase, the two fragments of each construct and primers listed in table 2.1 (TRPM2-Y74F-1 forward and TRPM2-Y74F-2 reverse; TRPM2-Y142F-1 forward and TRPM2-Y142F-2 reverse; or TRPM2-Y523F-1 forward and TRPM2-Y523F-2 reverse). The following thermal profile was used for these PCR reactions: (1) 95°C for 2 minutes, (2) 5 cycles at 94°C for 15 seconds, 60°C (-1°C/cycle) for 15 seconds, and 72°C for 120 seconds. The reactions were paused to add forward and reverse primers, and the PCR reactions were resumed with (3) 5 cycles at 94°C for 15 seconds, 55°C (-1°C/cycle) for 15 seconds, and 72°C for 120 seconds, and (4) 25 cycles at 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 120 seconds.

Following mutagenesis, PCR products of TRPM2-Y74F, TRPM2-Y142F, TRPM2-Y523F and TRPM2-Y1503F were resolved on a 1% w/v agarose gel containing 0.5 µg/mL ethidium bromide. The PCR products were extracted from the gel using a

Table 2.1: Primers used in generation of wild-type (WT) and mutant (Y74F, Y142F, Y523F and Y1503F) TRPM2 constructs.

Construct	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
TRPM2-WT	AAACTTAAGCTTGGTACCGAGCTCGG	TTACTAGTGGATCCGAAAAGGGCGA
TRPM2-Y74F-1	AAACTTAAGCTTGGTACCGAGCTCGG	TTTCCACAAAAAACACGCATTCTTTCT
TRPM2-Y74F-2	AAAGAATGCGTGTTTTTTGTGGAAAGTTC	TTACTAGTGGATCCGAAAAGGGCGAAT
TRPM2-Y142F-1	AAACTTAAGCTTGGTACCGAGCTCGG	AGACTCGGACGAACTTTTTCACCTTCTGG
TRPM2-Y142F-2	AGAAGGTGAAAAAGTTCGTCCGAGTCT	TTACTAGTGGATCCGAAAAGGGCGA
TRPM2-Y523F-1	AAACTTAAGCTTGGTACCGAGCTCGG	TCCAGGTTCTCGAACAGGTAGAGCA
TRPM2-Y523F-2	ACCTTGCTCTACCTGTTCGAGAACCTG	TTACTAGTGGATCCGAAAAGGGCGA
TRPM2-Y1503F	AAACTTAAGCTTGGTACCGAGCTCGG	TATCGAGAATTCTCAGAAGTGAGCCCCG

GenElute Gel Extraction Kit according to the manufacturer's protocol. A double restriction enzyme digest with NotI and AscI enzymes was carried out at 37°C for 2 hours. Subsequently, digested DNA was purified with GenElute Gel Extraction Kit according to the manufacturer's protocol. The purified DNA product was then ligated into NotI/AscI-digested pIRESpuro-FLAG vector at a 1:5 (vector:insert) molar ratio

using T4 DNA ligase. The ligated product was transformed into JM109 competent cells and grown up on LB + ampicillin plates overnight at 37°C. Individual colonies were then expanded in LB + ampicillin selection medium, shaking at 250 RPM overnight at 37°C. Plasmid DNA was isolated from pellets using the GenElute Plasmid Miniprep Kit according to manufacturer's protocol. Incorporation of TRPM2 mutants into pIRESpuro-FLAG vector was confirmed by triple restriction digest (AscI/NotI/XhoI). Positive clones were further confirmed by DNA sequencing (London Regional Genomics Centre).

2.4 Transfection

24 hours prior to transfection, TRPM2-HEK293 cells or HEK293T cells were plated on 100 mm culture dishes at approximately 30% cell confluence, resulting in approximately 60% cell confluence at the time of transfection. TRPM2 expression was induced in TRPM2-HEK293 cells by administration of doxycycline (1.0 µg/mL) to cells 18-24 hours prior to transfection. Cells were transfected with JetPRIME™ (Polyplus Transfection, NY) according to the manufacturer's protocol. Briefly, 10 µg total DNA (2 µg of pIRESpuro-HA, Fyn-WT, FynY531F or Fyn-K299M, where indicated; 2 µg TRPM2-WT, TRPM2-Y74F, TRPM2-Y142F, TRPM2-Y523F or TRPM2-Y1503F, where indicated; 1 µg green fluorescent protein (PLB-GFP); and 5-7 µg pcDNA3.1) was added to 500 µL JetPRIME™ buffer (Polyplus Transfection) in a sterile microtube and mixed by vortexing. 12.5 µL JetPRIME™ enzyme (Polyplus Transfection) was added to microtube and vortexed for 10 seconds. After a 10 minute incubation at room temperature, 500 µL transfection mix was added drop-wise to plates of cells, and plates were gently mixed and incubated for four hours at 37°C and 5% CO_2. Following this incubation, the transfection media was removed and replaced with DMEM + 10% FBS + 1 µg/µL doxycycline. Subsequently, cells were incubated at 37°C and 5% CO_2 for 48 hours prior to lysis.

2.5 Immunoprecipitation and Western blotting

After transfection, TRPM2-HEK293 cells or HEK293T cells were homogenized in lysis buffer (1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), Complete Mini protease inhibitor cocktail (Roche, Germany) and HALT phosphatase inhibitor cocktail (Pierce, IL)). Homogenates were centrifuged (16,100 rcf at 4°C for 30 min) to remove insoluble debris. Samples containing 1.0 mg of protein in 500 µL lysis buffer were incubated overnight with 20 µL packed anti-FLAG M2 affinity gel beads (Sigma, MO). Beads were subjected to three centrifugation-wash cycles with lysis buffer to remove elements not associated with beads. Proteins were eluted and denatured by incubating (30 min at 37°C) with 2x Laemmli sample loading buffer (4% SDS, 125 mM Tris-HCl (pH 6.8), 20% glycerol, 0.02% bromophenol blue and 5% β-mercaptoethanol). Subsequently, samples were centrifuged (5,000 rcf at 4°C for 5 min). Eluates were resolved by 8% SDS-PAGE and transferred to nitrocellulose membranes. Blots were subsequently probed with rabbit anti-Fyn (H-80; Santa Cruz, CA), mouse anti-phosphotyrosine (4G10; Upstate Biotechnology, NY), HRP-conjugated mouse anti-FLAG (Sigma-Aldrich) or mouse anti-β-actin (Sigma-Aldrich) antibodies followed by appropriate HRP-conjugated secondary detection antibodies. Blots were visualized using SuperSignal West Pico enhanced chemiluminscence (Thermo Scientific, IL) and VersaDoc^{TM} Imaging System (Bio-Rad, CA).

2.6 Drugs and peptides

The sources of materials for this study are as follows: NaCl, glucose, $CaCl_2$, $MgCl_2$, $BaCl_2$, gluconic acid, CsOH, ADPR, $Mg(ATP)_2$, Ponceau S stain, GenElute Gel Extraction Kit, GenElute Plasmid Miniprep Kit, GenElute Plasmid Midiprep Kit, Dulbecco's Modified Eagle Medium, Fetal Bovine Serum (Sigma-Aldrich), Supersignal West Pico Stable Peroxide Solution, Supersignal West Pico Luminal/Enhancer, BCA Protein Assay Kit (Thermo Scientific), KCl, HEPES, EGTA, EDTA, SDS, glycerol, bromophenol blue, β-mercaptoethanol, NP-40, sodium deoxycholate, Tris, HCl (Bioshop,

ON), Mini-complete protease inhibitor cocktail tablets (Roche), HALT™ phosphatase inhibitor cocktail (Pierce), JM109 bacteria (Promega), KOD polymerase (EMD Millipore), doxycycline (Pfizer, ON), JetPRIME (Polyplus), AscI, NotI, XhoI, CIP (New England Biolab), and recombinant active Fyn kinase (Upstate Biotechnology).

2.7 Data analysis and statistics

Electrophysiological data analysis was performed using pCLAMP Clampfit 10.2 software (Molecular Devices). Densitometric analysis of Western blots was conducted using Image Lab 3.0 (Beta 5) software (Bio-Rad). All data are presented as mean ± SEM Statistical analysis was conducted using GraphPad Prism® 5 (GraphPad Software, CA). Where indicated, means were compared using an unpaired Student's t-tests or one-way ANOVA followed by Tukey's post-hoc test. For all tests, $P<0.05$ was considered significant.

Section 3

RESULTS

3.1 Regulation of TRPM2 currents by Fyn kinase

Our first experimental objective was to assess whether Fyn functionally regulates TRPM2 channels. Preliminary evidence from the literature and our lab suggested a potential role for Fyn in regulation of TRPM2 channels. Zhang et al. (2007) showed that TRPM2 activation induced by H_2O_2 treatment could be reduced by PP2, a non-specific SFK inhibitor. Thus, one or more SFK is likely responsible for augmenting TRPM2 currents. We previously demonstrated that TRPM2 currents could be evoked in cultured hippocampal neurons by repeated applications of NMDA in the absence of ADPR (MacDonald/Jackson lab, unpublished). Further, repeated NMDA applications resulted in increased TRPM2 currents over time – an effect that was abolished by inhibition of Fyn signaling using a short interfering peptide, Fyn(39-57) (Appendix A, Fig. S2; MacDonald/Jackson lab, unpublished). This Fyn-mediated augmentation of TRPM2 currents could be due to an increase in NMDAR currents, a direct enhancement of TRPM2 activation or both. In this study, we assessed whether Fyn regulates TRPM2 currents in HEK293 cells expressing inducible TRPM2 (TRPM2-HEK293 cells; cell line described in section 2.1). Notably, HEK293 cells do not express endogenous NMDARs (Domingues et al., 2006), so enhancement of TRPM2 currents by NMDAR is not a confounding variable in this experimental paradigm.

A whole-cell voltage-clamp electrophysiological recording protocol was designed to record TRPM2 currents in TRPM2-HEK293 cells (detailed in section 2.2). TRPM2-HEK293 cells contain an inducible protein expression system, in which TRPM2 expression can be induced by treatment of cells with doxycycline 18-24 hours prior to recording. After inducing TRPM2 expression, TRPM2 currents were evoked in TRPM2-HEK293 cells. Characteristic of TRPM2 activation, inward currents were activated by ADPR (0.1 mM, in patch pipette) in the presence of extracellular Ca^{2+} (2 mM) (Fig. 3.1A). Further, these currents were abolished by application of Ca^{2+}-deficient ECS. Notably, because currents mediated by some cation channels, such as TRPM7, are

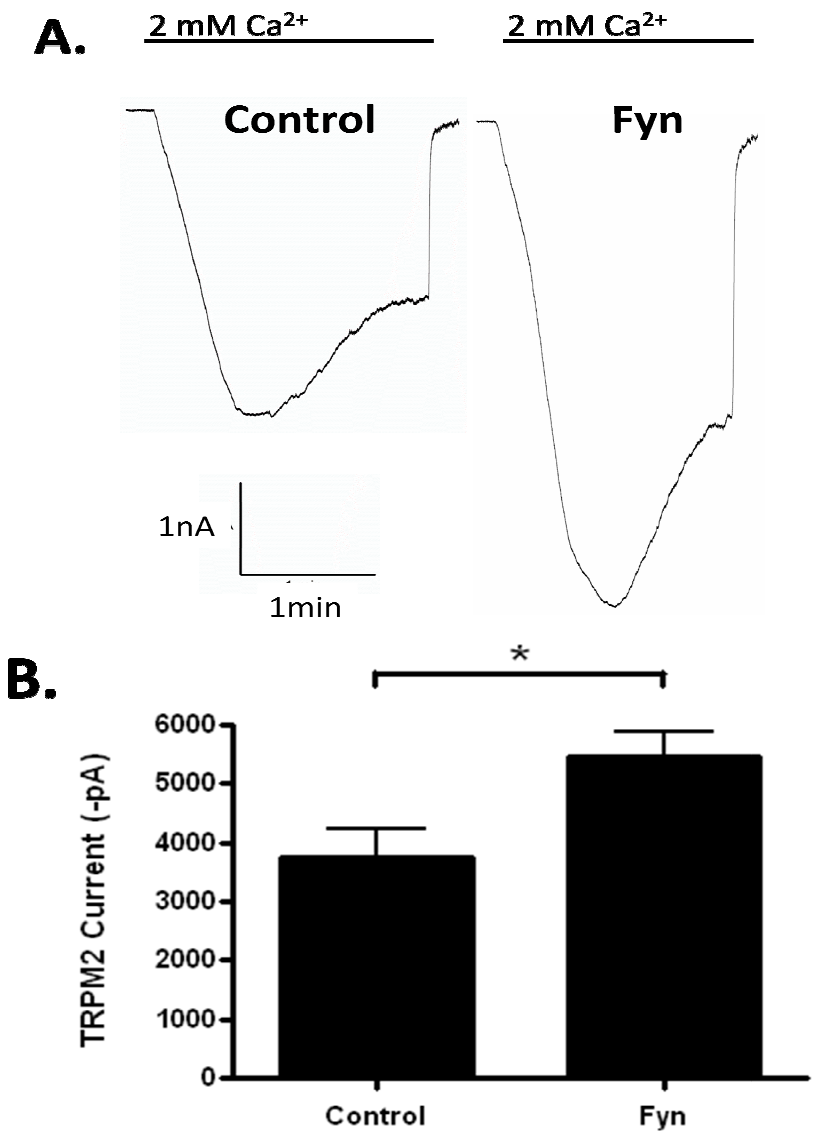

Figure 3.1: Fyn potentiates TRPM2 currents in TRPM2-HEK293 cells.

(A) Representative whole-cell recordings of TRPM2 currents activated by ADPR (0.1 mM, in patch pipette) in presence of recombinant Fyn (1 U/mL) or vehicle (in patch pipette). Horizontal bars above graphs indicate presence of Ca^{2+} (2 mM) in ECS. TRPM2 currents were abolished in absence of extracellular Ca^{2+}. Holding potential was -60 mV. **(B)** Summary bar graph showing potentiation of TRPM2 currents by Fyn. Data are presented as mean ± SEM (n=8 per condition). Statistical analysis: unpaired Student's t-test, *$P < 0.05$.

dependent on extracellular divalent cation concentration (Chen et al., 2010), Ca^{2+} (2 mM) was substituted with Ba^{2+} (2 mM) in Ca^{2+}-deficient ECS to maintain a constant total divalent cation concentration. These findings are consistent with previously reported activation and inactivation properties of TRPM2.

To determine whether Fyn regulates TRPM2 currents, recombinant Fyn kinase (1 U/mL) or vehicle was delivered to the cell interior by inclusion in the patch pipette. Peak TRPM2 current amplitudes were compared in the presence of Fyn or vehicle. TRPM2 current amplitudes were significantly increased in cells treated with intracellular Fyn (-5470 ± 425 pA, n=8) relative to vehicle-treated control cells (-3743 ± 478 pA, n=8) ($P<0.05$) (Fig. 3.1B). This result suggests that Fyn potentiates TRPM2 currents in an NMDAR-independent manner. However, the molecular mechanism underlying this regulation remains unclear. Subsequent experiments were focused on defining the mechanism through which Fyn regulates TRPM2 function.

3.2 Expression of Fyn and TRPM2 in TRPM2-HEK293 cells

Our next objective was to characterize the molecular mechanism by which Fyn may regulate TRPM2 function. Specifically, we investigated whether Fyn induces tyrosine phosphorylation of TRPM2 and whether Fyn physically interacts with TRPM2 (see sections 3.3 and 3.4, respectively). A combination of biochemistry and molecular biology techniques were employed to study these relationships between Fyn and TRPM2. In each of these experiments, TRPM2-HEK293 cells were induced to express TRPM2 by pre-treatment with doxycycline 24 hours before lysis. Additionally, in each experiment, these cells were transfected with one of three constructs designed to induce expression of wild-type Fyn (WT), constitutively active Fyn (Y531F) or inactive Fyn (K299M) or empty vector (transfection protocol detailed in section 2.4). The expression of Fyn and TRPM2 relative to β-actin was compared between each transfection condition.

First, Fyn expression was quantified relative to β-actin. No Fyn immunoreactivity was detected in empty vector-transfected cells (Fig. 3.2A,C). This suggests that endogenous

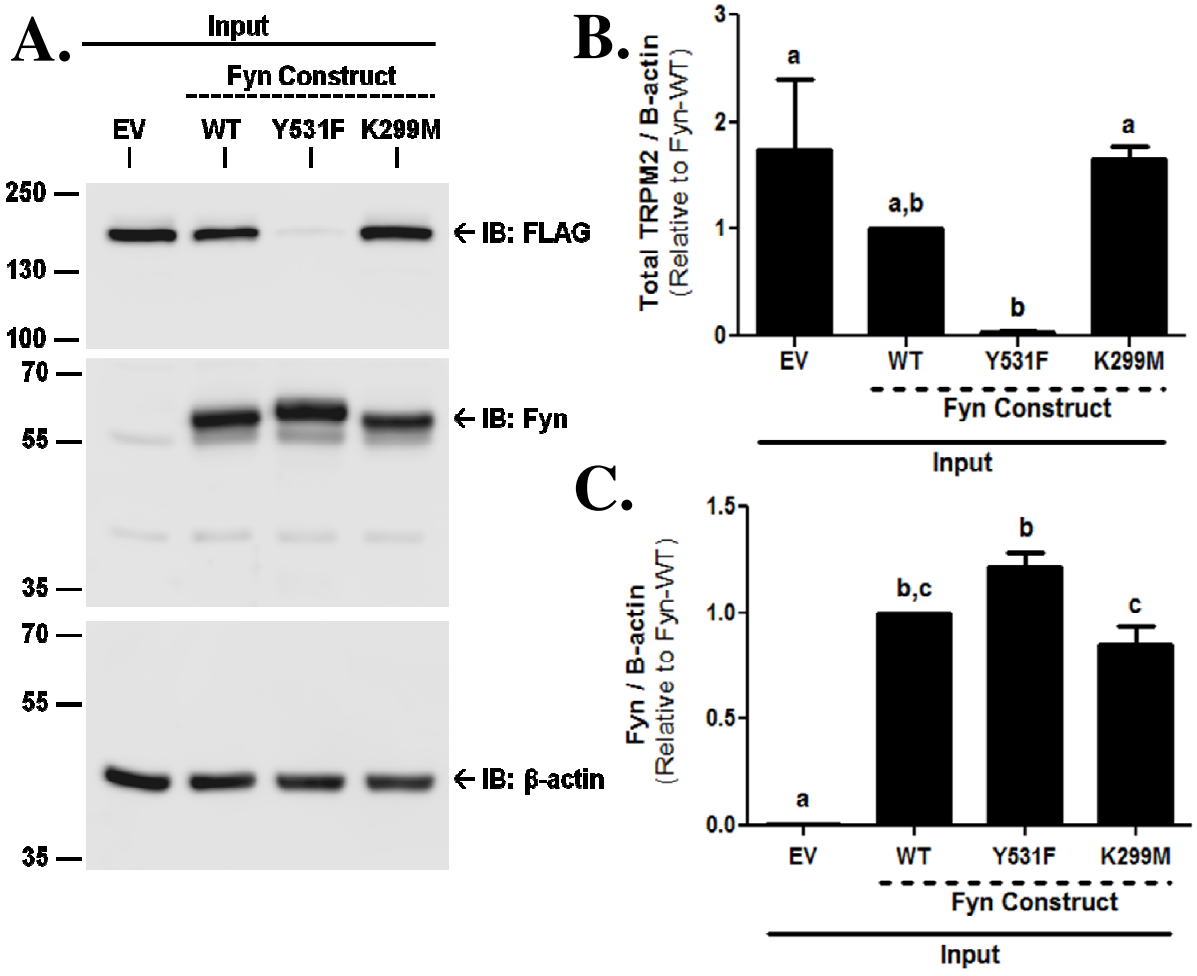

Figure 3.2: Expression of TRPM2 and Fyn in TRPM2-HEK293 cells.

Expression of TRPM2 (FLAG) and Fyn in TRPM2-HEK293 cells transiently transfected with Fyn constructs (wild-type, WT; constitutively active, Y531F; dominant negative, K299M) or empty vector (EV). **(A)** Representative blot showing input expression levels of Fyn, FLAG and β-actin. **(B)** Summary bar graph showing that TRPM2 expression is decreased in cells expressing Y531F relative to EV and K299M. **(C)** Summary bar graph showing that Fyn expression was not detected in EV-transfected cells. Further, Fyn expression was increased in cells expressing Y531F relative to K299M. Expression of FLAG and Fyn were quantified relative to β-actin. Data are presented as mean ± SEM (n=3 per condition). Statistical analysis: one-way repeated-measures ANOVA with Tukey's post-hoc test. Identical letters (a, b, c) indicate no significant difference between groups ($P < 0.05$).

Fyn is either absent from TRPM2-HEK293 cell lysates or below the detection threshold under these experimental conditions. In this regard, previous studies have suggested that HEK293 cells do express endogenous Fyn (Wu et al., 2007). Post-transfection Fyn expression levels in cells expressing constitutively active Fyn (Y531F), wild-type Fyn (WT) and inactive Fyn (K299M) were fairly comparable and all significantly higher than empty vector-transfected cells (Fig. 3.2A,C). Although Fyn expression was higher in cells expressing constitutively active Fyn (Y531F) relative to inactive Fyn (K299M) ($P<0.05$) (Fig. 3.2A,C), considering the inherent variability of post-transfection protein expression levels, a relatively consistent amount of Fyn was detected in cells expressing constitutively active Fyn (Y531F), wild-type Fyn (WT) and inactive Fyn (K299M).

Next, TRPM2 expression was quantified relative to β-actin. Such quantification allowed us to assess whether the activation status Fyn affects TRPM2 protein levels. A relatively high variability in TRPM2 expression levels was observed in cells expressing constitutively active Fyn (Y531F), wild-type Fyn (WT), inactive Fyn (K299M) and empty vector. TRPM2 expression was significantly decreased in cells expressing constitutively active Fyn (Y531F) relative to empty vector and inactive Fyn (K299M) ($P<0.05$) (Fig. 3.2A,B). Although not statistically significantly, wild-type Fyn (WT)-expressing cells exhibited TRPM2 expression levels higher than cells expressing constitutively active Fyn (Y531F) and lower than those expressing inactive Fyn (K299M) or empty vector (Fig. 3.2A,B). Taken together, these observations suggest that the expression level of TRPM2 in TRPM2-HEK293 cells depends on the activation state of Fyn. Specifically, increased Fyn activity corresponds with decreased TRPM2 expression. However, the mechanism underlying this phenomenon is unclear at present.

3.3 Tyrosine phosphorylation of TRPM2 induced by Fyn kinase

We were interested in determining whether Fyn induces tyrosine phosphorylation of TRPM2. Notably, tyrosine phosphorylation of TRPM2 has been shown to coincide with

increased TRPM2 activation, intracellular Ca^{2+} and apoptotic cell death in HEK293 cells expressing TRPM2 (Zhang et al., 2007). However the kinase responsible for this phosphorylation has not been identified. We have shown that Fyn augments TRPM2 channel function (Fig. 3.2). Given these findings and the fact that Fyn is a tyrosine kinase, it follows that the mechanism by which Fyn potentiates TRPM2 currents likely involves tyrosine phosphorylation of TRPM2 by Fyn. Here, we investigated whether Fyn induces tyrosine phosphorylation of TRPM2.

To determine whether Fyn induces tyrosine phosphorylation of TRPM2, we assessed the relative amount of TRPM2 tyrosine phosphorylation in TRPM2-HEK293 cells co-expressing TRPM2 as well as wild-type Fyn (WT), constitutively active Fyn (Y531F), inactive Fyn (K299M) or empty vector. First, TRPM2-HEK293 cells were induced to express TRPM2 by pre-treatment of cells with doxycycline 24 hours before lysis. These cells were also transfected with one of three constructs designed to induce expression of wild-type Fyn (WT), constitutively active Fyn (Y531F) or inactive Fyn (K299M) or empty vector. Tyrosine phosphorylation was subsequently analyzed by immunoprecipitation and Western blotting (detailed in section 2.5). Briefly, cells were lysed in the presence of protease and phosphatase inhibitors. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted for tyrosine phosphorylation and FLAG (total TRPM2).

Although lysate TRPM2 expression was substantially reduced in cells expressing constitutively active Fyn (Y531) relative to the other transfection conditions studied (Fig. 3.2), immunoprecipitation of FLAG-tagged TRPM2 constructs yielded a reasonably consistent amount of TRPM2 for each transfection condition (Fig. 3.3). Tyrosine phosphorylation of TRPM2 was detected for cells transfected with all Fyn constructs and empty vector. The fact that TRPM2 tyrosine phosphorylation was observed in cells transfected with empty vector and inactive Fyn (K299M) suggests that there is some basal TRPM2 phosphorylation that is attributable to endogenous Fyn or other tyrosine kinases. However, the physiological importance of this phosphorylation and whether it occurs at sites that are important for TRPM2 activation remain unknown. Tyrosine phosphorylation of TRPM2 was significantly increased in TRPM2-HEK293 cells

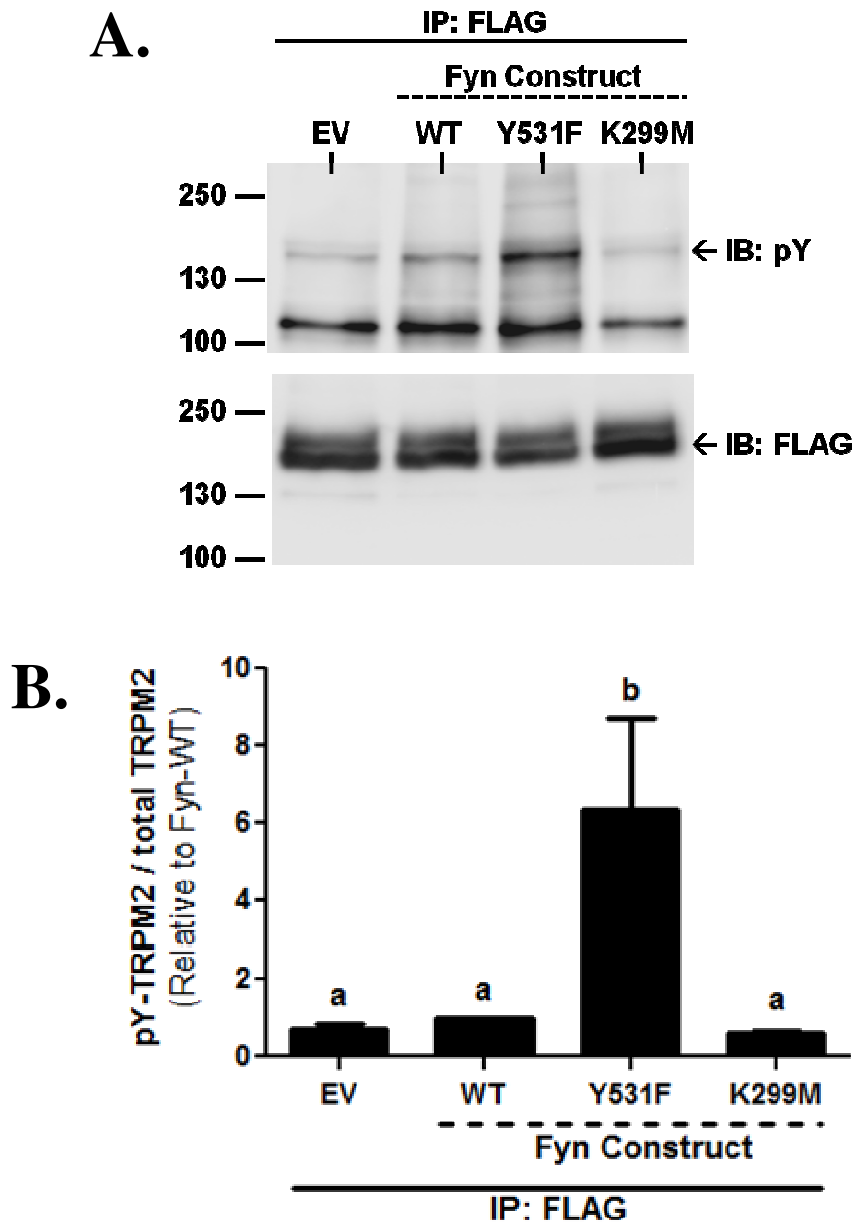

Figure 3.3: Fyn induces tyrosine phosphorylation of TRPM2.

Representative blot (**A**) and summary bar graph (**B**) showing that TRPM2 tyrosine phosphorylation was increased in TRPM2-HEK293 cells expressing constitutively active Fyn (Y531F) relative to empty vector (EV), wild-type Fyn (WT) and inactive Fyn (K299M). Data are presented as mean ± SEM (n=4 per condition). Statistical analysis: one-way repeated-measures ANOVA with Tukey's post-hoc test. Identical letters (a, b) indicate no significant difference between groups ($P < 0.05$).

expressing constitutively active Fyn-Y531F relative to wild-type Fyn-WT, inactive Fyn-K299M and EV ($P<0.05$) (Fig. 3.3). This observation suggests that Fyn induces tyrosine phosphorylation of TRPM2 in an activation state-dependent manner. Specifically, increased Fyn activity corresponds with increased TRPM2 phosphorylation. The simplest mechanism to explain this phenomenon is direct phosphorylation of TRPM2 by Fyn, but the actual mechanism remains unclear at present. Notably, the phosphorylated TRPM2 tyrosine residue(s) responsible for modulation of channel function by Fyn have not been identified.

3.4 Physical interaction between Fyn kinase and TRPM2

Next, we wanted to determine whether Fyn physically interacts with TRPM2. Interaction between these proteins may be necessary or facilitative for Fyn-induced tyrosine phosphorylation of TRPM2. This interaction may involve direct protein-protein binding of Fyn to TRPM2. Alternatively, both proteins could be part of a larger protein complex, in which Fyn anchors to TRPM2 through one or more adaptor proteins. Regardless of whether this interaction is direct or indirect, such physical interaction may facilitate Fyn regulation of TRPM2 by stabilizing a configuration in which Fyn is maintained in close physical proximity to TRPM2.

To determine whether Fyn physically interacts with TRPM2, we assessed the relative amount of Fyn that co-immunoprecipitated with Fyn in TRPM2-HEK293 cells co-expressing TRPM2 as well as wild-type Fyn (WT), constitutively active Fyn (Y531F), inactive Fyn (K299M) or empty vector. First, TRPM2-HEK293 cells were induced to express TRPM2 by pre-treatment of cells with doxycycline 24 hours before lysis. These cells were also transfected with one of three constructs designed to induce expression of wild-type Fyn (WT), constitutively active Fyn (Y531F) or inactive Fyn (K299M) or empty vector. Co-immunoprecipitation of Fyn with TRPM2 was subsequently assessed by immunoprecipitation and Western blotting (detailed in section 2.5). Briefly, cells were lysed in the presence of protease and phosphatase inhibitors. Lysates were

separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted for Fyn and TRPM2.

Immunoprecipitates from lysates expressing wild-type Fyn (WT), constitutively active Fyn (Y531F) and inactive Fyn (K299M) exhibited immunoreactivity for Fyn, whereas immunoprecipitates from empty vector-transfected cells displayed no Fyn immunoreactivity (Fig. 3.4). This is consistent with the finding that no endogenous Fyn was detectable in empty vector-transfected TRPM2-HEK293 cell lysates (Fig. 3.2). Interestingly, co-immunoprecipitation of constitutively active Fyn (Y531F) with TRPM2 was significantly higher than wild-type Fyn (WT), inactive Fyn (K299M) and empty vector ($P<0.05$) (Fig. 3.4). This suggests that the amount of interaction between Fyn and TRPM2 may be dependent on the activation status of Fyn. Importantly, co-immunoprecipitation is not capable of distinguishing whether the interaction between Fyn and TRPM2 is direct or indirect (i.e. mediated by one or more adaptor proteins). So long as the intermolecular binding stringency within a multi-protein complex is sufficiently high, immunoprecipitation of one protein in the complex may co-precipitate other physically associated proteins. Regardless, this observation suggests that Fyn physically associates with TRPM2, which may be required or facilitative for Fyn regulation of TRPM2 channels.

3.5 Expression of Fyn and TRPM2 in HEK293T cells

Our next objective was to identify potential regulatory TRPM2 tyrosine residues through which Fyn may regulate TRPM2 function. To identify such regulatory tyrosine residues, a combined bioinformatic and genetic approach was used (detailed in section 2.3). Briefly, GPS 2.1 phosphorylation prediction software (Xue et al., 2011) was used to identify probable phosphorylation sites for Fyn on TRPM2 based on kinase-substrate

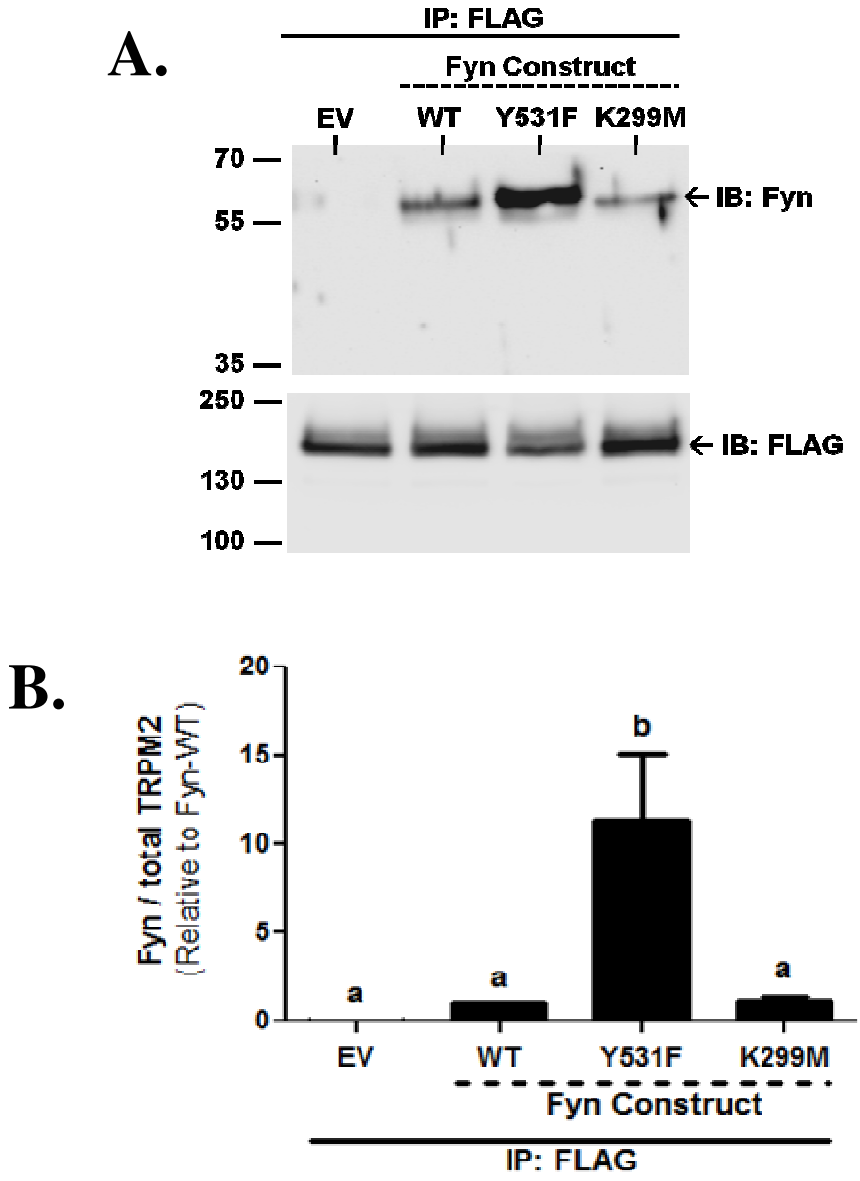

Figure 3.4: Fyn physically interacts with TRPM2.

Representative blot (**A**) and summary bar graph (**B**) demonstrating co-immunoprecipitation of Fyn (wild-type, WT; constitutively active, Y531F; dominant negative, K299M) with TRPM2. Co-immunoprecipitation of Y531F was significantly higher than empty vector (EV), WT and K299M. Data are presented as mean ± S.E.M. (n=3 per condition). Statistical analysis: one-way repeated-measures ANOVA with Tukey's post-hoc test. Identical letters (a, b) indicate no significant difference between groups ($P < 0.05$).

complimentarity of short sequence motifs. The four identified TRPM2 tyrosine residues with the highest prediction scores were Y74, Y142, Y523 and Y1503. Using site-directed mutagenesis, four mutant human TRPM2 constructs were generated in which each of these candidate tyrosine residues was mutated to phenylalanine to ablate possible phosphorylation at that residue (TRPM2-Y74F, -Y142F, -Y523F and -Y1503F). Subsequently, we investigated whether each of these TRPM2 mutant proteins (Y74F, Y142F, Y523F and Y1503F) exhibited reduced tyrosine phosphorylation relative to wild-type (WT) TRPM2 (section 3.6). Additionally, we assessed whether each of these TRPM2 mutations (Y74F, Y142F, Y523F and Y1503F) affected the physical interaction between Fyn and TRPM2 (section 3.7). A combination of biochemistry and molecular biology techniques were employed to study these relationships between Fyn and TRPM2. In each of these experiments, HEK293T cells were co-transfected to induce expression of a Fyn construct (constitutively active, Y531F; or empty vector) and a TRPM2 construct (wild-type, WT; or mutant, Y74F, Y142F, Y523F or Y1503F) (generation of TRPM2 constructs described in section 2.3). The expression of Fyn and TRPM2 relative to β-actin was compared between each transfection condition.

First, Fyn expression was quantified relative to β-actin. As observed in TRPM2-HEK293 cells, little or no Fyn immunoreactivity was detected in empty vector-transfected HEK293T cells (Fig. 3.2A,C). Post-transfection Fyn expression levels in HEK293T cells expressing constitutively active Fyn (Y531F) as well as wild-type (WT) or mutant (Y74F, Y142F, Y523F or Y1503F) TRPM2 were not significantly different from each other and all significantly higher than empty vector-transfected cells (Fig. 3.5A,C). Considering the inherent variability of post-transfection protein expression levels, a relatively consistent amount of Fyn was detected in cells expressing constitutively active Fyn (Y531F), regardless of which TRPM2 construct was expressed.

Next, TRPM2 expression was quantified relative to β-actin. Such quantification allowed us to assess whether the TRPM2 mutations examined or Fyn activation affects TRPM2 expression levels. Post-transfection TRPM2 expression were relatively consistent in HEK293 cells expressing constitutively active Fyn (Y531F) or empty vector as well as wild-type (WT) or mutant (Y74F, Y142F, Y523F or Y1503F) TRPM2 (Fig. 3.5A,B).

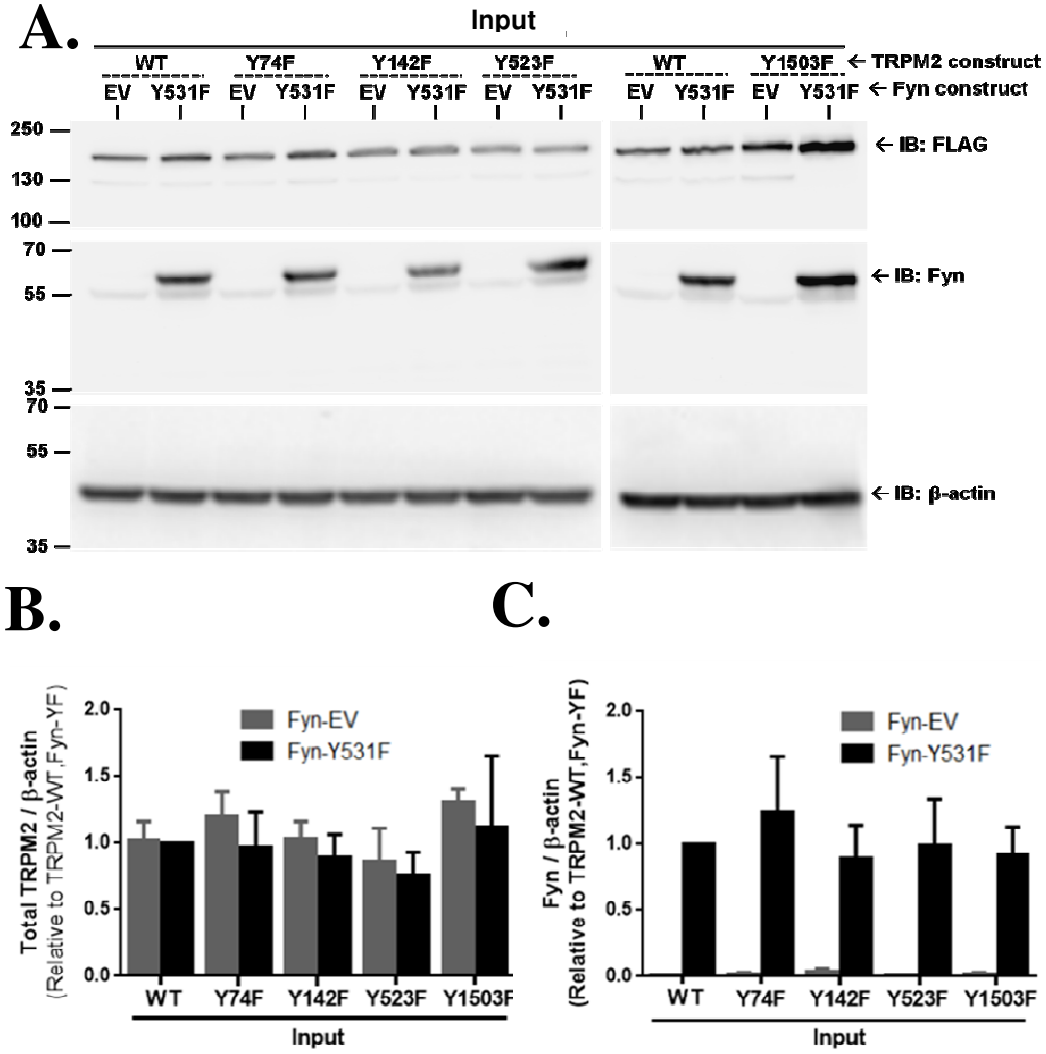

Figure 3.5: Expression of TRPM2 and Fyn in HEK293T cells.

Expression of TRPM2 (FLAG) and Fyn in HEK293T cells co-transfected with a TRPM2 construct (wild-type, WT; or mutant, Y74F, Y142F, Y523F, Y1503F) and a Fyn construct (constitutively active, Y531F; or empty vector, EV). **(A)** Representative blot showing input expression levels of FLAG, Fyn and β-actin. **(B)** Summary bar graph showing that TRPM2 expression was not significantly different between the transfection conditions examined. **(C)** Summary bar graph showing that Fyn expression was detected in Fyn-Y531F-, but not Fyn-EV-transfected cells. Further, expression of Fyn was not significantly different between the conditions transfected with Fyn-Y531F. Expression of FLAG and Fyn were quantified relative to β-actin. Data are presented as mean ± SEM (n=3 per condition). Statistical analysis: one-way repeated-measures ANOVA; $P < 0.05$.

Notably, TRPM2 expression levels were not significantly different between any of the transfection conditions studied (Fig. 3.5B). Thus, considering the inherent variability of post-transfection protein expression levels, a relatively consistent amount of TRPM2 was detected in HEK293T cells, regardless of which TRPM2 construct was expressed.

3.6 Tyrosine phosphorylation of wild-type and mutant TRPM2 by Fyn kinase

As previously described, tyrosine phosphorylation of TRPM2 coincides with increased TRPM2 activation, intracellular Ca^{2+} and apoptotic cell death in HEK293 cells expressing TRPM2 (Zhang et al., 2007). However, the phosphorylated tyrosine residue on TRPM2 that is responsible for its regulation by Fyn has not been identified. Here, we discuss an experiment that was designed to determine whether Fyn induces phosphorylation of four candidate TRPM2 tyrosine residues (TRPM2-Y74, -Y142, -Y523 and -Y1503) that were identified using GPS 2.1 phosphorylation site prediction software (Xue et al., 2011).

As previously noted, four mutant TRPM2 constructs were generated in which each of these four candidate tyrosine residues was individually mutated to a phenylalanine. Since this mutation effectively ablates phosphorylation of the corresponding residue, it is expected that if one or more of these four candidate tyrosine residues is phosphorylated by Fyn, then the corresponding mutant(s) should exhibit reduced Fyn phosphorylation relative to wild-type TRPM2. Thus, here we investigated whether phosphorylation of TRPM2-Y74, -Y142, -Y523 and -Y1503 are induced by Fyn. To determine whether Fyn induces tyrosine phosphorylation of these candidate TRPM2 tyrosine residues, we assessed the relative amount of TRPM2 tyrosine phosphorylation in HEK293T cells co-expressing constitutively active Fyn (Y531F) or empty vector as well as wild-type (WT) or mutant (Y74, Y142, Y523 and Y1503) TRPM2. First, HEK293T cells were co-transfected to induce expression of one Fyn construct (constitutively active, Y531F; or empty vector) and one TRPM2 construct (wild-type, WT; or mutant, Y74, Y142, Y523,

Y1503). Tyrosine phosphorylation was subsequently analyzed by immunoprecipitation and Western blotting (detailed in section 2.5). Briefly, cells were lysed in the presence of protease and phosphatase inhibitors. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted for tyrosine phosphorylation and FLAG (total TRPM2).

Tyrosine phosphorylation of TRPM2 was detected in all cells, regardless of which Fyn and TRPM2 constructs were expressed. The fact that TRPM2 tyrosine phosphorylation was observed in cells transfected with empty vector suggests that there is some basal TRPM2 phosphorylation that is attributable to endogenous Fyn or other tyrosine kinases. However, the physiological importance of this phosphorylation and whether it occurs at sites that are important for TRPM2 activation remain unknown. There were no significant differences in tyrosine phosphorylation of TRPM2 between the wild-type and mutant TRPM2 constructs examined (Fig. 3.6). Thus, Fyn-induced phosphorylation of wild-type and mutant TRPM2 was not detected under these experimental conditions. It is possible that this experimental paradigm was not sufficiently sensitive to detect phosphorylation of these candidate regulatory sites or that Fyn does not actually induce phosphorylation of these specific sites.

3.7 Physical interaction between Fyn kinase and wild-type or mutant TRPM2

Next, we wanted to determine whether the candidate regulatory TRPM2 tyrosine residues (Y74, Y142, Y523 and Y1503) are important for the physical interaction between Fyn and TRPM2. Interaction between these proteins may be necessary or facilitative for Fyn-induced tyrosine phosphorylation of TRPM2. Moreover, tyrosine phosphorylation could be necessary for the physical interaction between Fyn and TRPM2. However, the regions of Fyn and TRPM2 that are responsible for mediating their physical interaction have not yet been identified. Notably, phosphorylated tyrosine residues may provide potential binding sites for the SH2 domain of Fyn. Previously discussed results demonstrate that

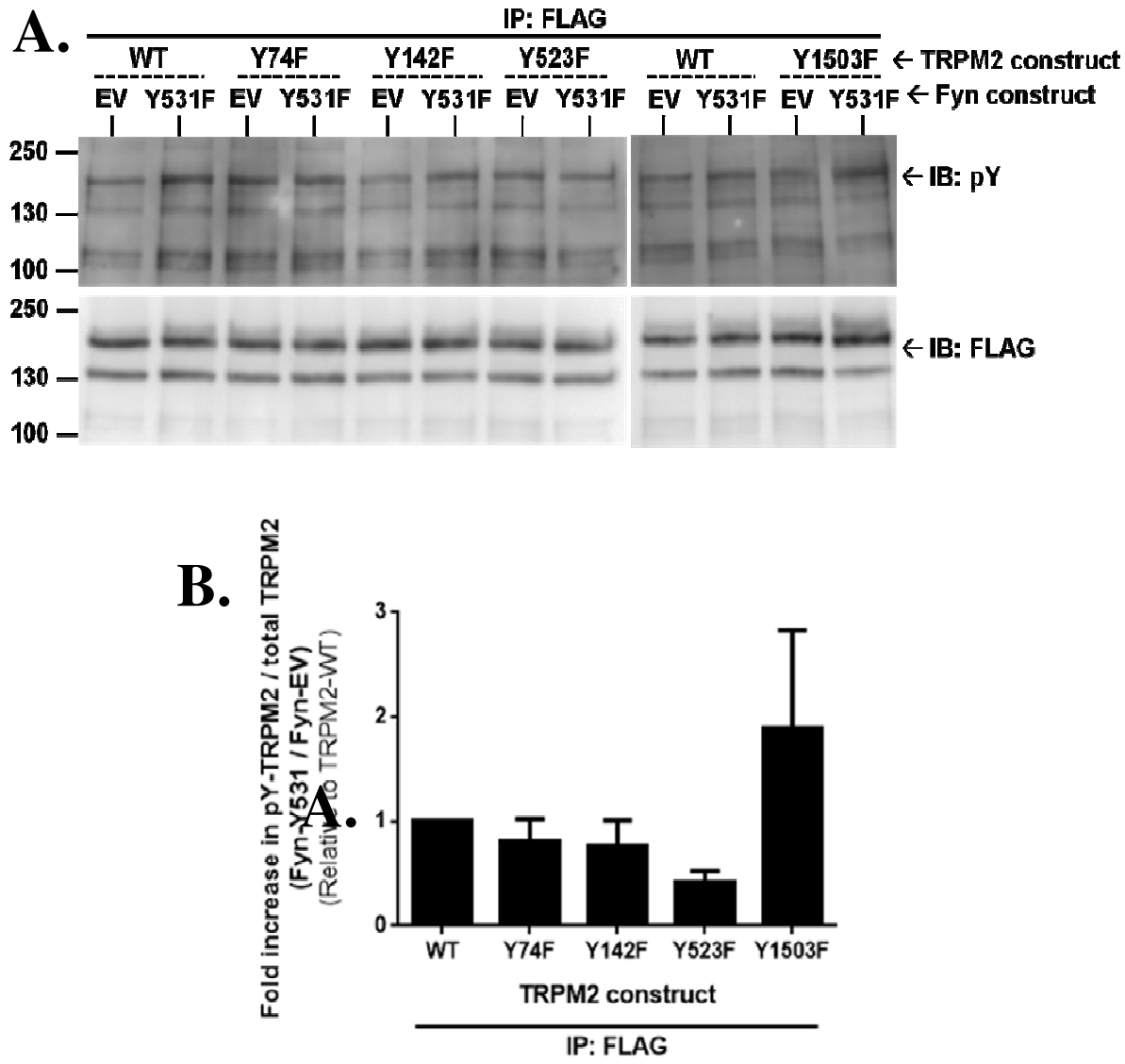

Figure 3.6: Fyn does not induce tyrosine phosphorylation of TRPM2-Y74, -Y142, -Y523 and -Y1503

Fyn-induced TRPM2 phosphorylation was not significantly different in mutant (Y74F, Y142F, Y523F, Y1503F) relative to wild-type (WT) TRPM2 in HEK293T cells. Representative blot (**A**) and summary bar graph (**B**) showing that TRPM2 tyrosine phosphorylation was not significantly different in HEK293T cells expressing the TRPM2 constructs examined (wild-type, WT; or mutant, Y74F, Y142F, Y523F, Y1503F). Data are presented as mean ± S.E.M. (n=3 per condition). Statistical analysis: one-way repeated-measures ANOVA; $P<0.05$.

Fyn co-immunoprecipitates with TRPM2, implying a physical interaction between these molecules (see Fig. 3.4). This experiment was designed to determine whether the four candidate TRPM2 tyrosine residues (TRPM2-Y74, -Y142, -Y523 and -Y1503) are important for the physical interaction between Fyn and TRPM2. As previously noted, four mutant TRPM2 constructs were generated in which each of these four candidate tyrosine residues was mutated to a phenylalanine. Since this mutation effectively ablates phosphorylation of the corresponding residue, it is expected that if phosphorylation of one or more of these four candidate tyrosine residues is important for the physical interaction of Fyn with TRPM2, then the corresponding mutant(s) should exhibit reduced Fyn co-immunoprecipitation relative to wild-type TRPM2. Thus, here we investigated whether phosphorylation of TRPM2-Y74, -Y142, -Y523 and -Y1503 are important for physical interaction between Fyn and TRPM2. In HEK293T cells co-expressing constitutively active Fyn (Y531F) or empty vector as well as wild-type (WT) or mutant (Y74, Y142, Y523 and Y1503) TRPM2, we assessed the relative amount of Fyn co-immunoprecipitation with TRPM2. First, HEK293T cells were co-transfected to induce expression of one Fyn construct (constitutively active, Y531F; or empty vector) and one TRPM2 construct (wild-type, WT; or mutant, Y74, Y142, Y523, Y1503). Fyn co-immunoprecipitation was subsequently analyzed by TRPM2 pull-down and Western blotting (detailed in section 2.5). Briefly, cells were lysed in the presence of protease and phosphatase inhibitors. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted for Fyn and FLAG (total TRPM2).

Immunoprecipitates from lysates expressing constitutively active Fyn (Y531F) showed immunoreactivity for Fyn, whereas immunoprecipitates from empty vector-transfected cells displayed no Fyn immunoreactivity (Fig. 3.7). There were no significant differences in the amount of Fyn that immunoprecipitated with TRPM2 between the transfection conditions examined (Fig. 3.7B). This observation suggests that the four candidate TRPM2 tyrosine residues identified are not essential for the interaction between Fyn and TRPM2. Further, these results provide no evidence that suggests that these residues play a role in mediating this interaction. It is possible that this experimental paradigm was not sufficiently sensitive to detect changes in the amount of

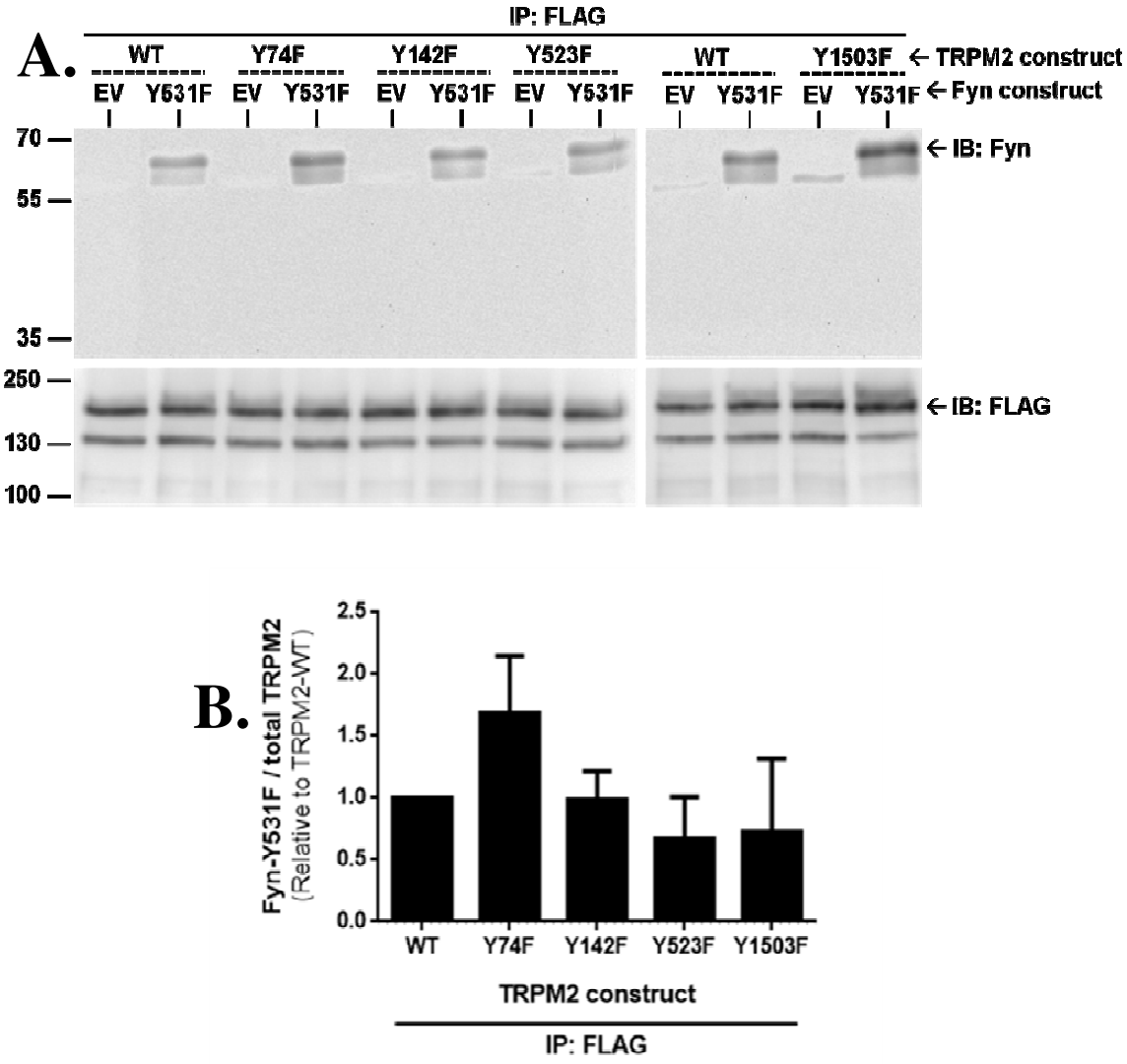

Figure 3.7: TRPM2-Y74, -Y142, -Y523 and -Y1503 are not critical for interaction between TRPM2 and Fyn.

Fyn physically interacts with wild-type (WT) and mutant (Y74F, Y142F, Y523F, Y1503F) TRPM2 in HEK293T cells. Representative blot (**A**) and summary bar graph (**B**) demonstrating co-immunoprecipitation of Fyn (constitutively active, Y531F) with wild-type and mutant TRPM2. The amount of Fyn that co-immunoprecipitated was not significantly different between the TRPM2 constructs examined. Data are presented as mean ± S.E.M. (n=3 per condition). Statistical analysis: one-way repeated-measures ANOVA. TRPM2 phosphorylation was not statistically different between cells transfected with different TRPM2 constructs ($P<0.05$)

protein-protein interaction between wild-type and mutant TRPM2 proteins or that these candidate tyrosine residues do not contribute to the physical interaction between Fyn and TRPM2.

Section 4

DISCUSSION

4.1 Summary of key findings

In this study, we investigated a potential physical and functional relationship between Fyn and TRPM2. Specifically, we investigated whether Fyn regulates TRPM2 currents, induces tyrosine phosphorylation of TRPM2 and physically interacts with TRPM2. Subsequently, we attempted to identify tyrosine residues on TRPM2 through which Fyn may regulate its channel activity. Notably, each experiment utilized a HEK293 cell variant (TRPM2-HEK293 or HEK293T) expressing both Fyn and TRPM2. Intracellular application of recombinant Fyn potentiated TRPM2 currents in TRPM2-HEK293 cells. Tyrosine phosphorylation of TRPM2 was increased in TRPM2-HEK293 cells transfected to increase Fyn expression, and the amount of phosphorylation detected was related to the activation state of Fyn. Further, Fyn directly co-immunoprecipitated with TRPM2, and the amount of Fyn that co-immunoprecipitated was related to the activation state of Fyn. Experiments designed to determine whether four candidate TRPM2 tyrosine residues (TRPM2-Y74, Y142, Y523 and Y1503) were phosphorylated by Fyn did not lead to identification of potential regulatory tyrosines. In summary, we have demonstrated that Fyn physically interacts with TRPM2, induces tyrosine phosphorylation of TRPM2, and increases currents mediated by TRPM2.

4.2 Regulation of TRPM2 by Fyn kinase

In this study, we have shown that Fyn physically interacts with TRPM2, induces tyrosine phosphorylation of TRPM2 and regulates TRPM2 function in HEK293 cell line variants (HEK293T cells and TRPM2-HEK293 cells) co-expressing Fyn and TRPM2. These results suggest that Fyn is an important regulator of TRPM2 in HEK293 cells. However, the molecular mechanism through which Fyn regulates TRPM2 remains an open question. Moreover, because protein expression, distribution and activation patterns differ between cell types, mechanisms of TRPM2 regulation may differ between HEK293

cells and other cell lines. However, results from parallel studies conducted in the MacDonald/Jackson lab (unpublished) suggest that Fyn also regulates TRPM2 function in neurons.

In these studies, cultured hippocampal neurons (21-28 days *in vitro*) were used to assess whether currents from endogenous TRPM2 are regulated by recombinant Fyn. In this experiment, TRPM2 currents were activated using a voltage ramp protocol. Briefly, TRPM2 currents were evoked by voltage ramps (±100 mV over 500 ms) applied every 10 s throughout the recording in the presence of exogenous ADPR (0.3 mM; in patch pipette). In cultured neurons, recombinant Fyn (1 U/mL; in patch pipette) significantly potentiated TRPM2 currents compared to vehicle-treated controls (Appendix A, Fig. S1). This result coincides with the finding that Fyn potentiates TRPM2 in TRPM2-HEK293 cells (Fig. 3.1), providing additional support for Fyn as a regulator of TRPM2 function.

To examine the role of Fyn's unique domain (Fyn-UD), a short peptide (Fyn(39-57)) was designed, whose sequence corresponds with part of Fyn-UD (AA 39-57). Fyn(39-57) presumably disrupts Fyn activity by binding to proteins that interact with Fyn-UD, and competitively displacing their interactions with Fyn. Notably, Fyn(39-57) and an analogous Src-interfering peptide (Src(40-58)), were shown to disrupt Fyn- and Src-mediated regulation of NR2B and NR2A subunit-containing NMDARs, respectively (Yang et al., 2011). Thus, Fyn(39-57) specifically interferes with Fyn and not Src. Fyn(39-57) was used to examine the role of Fyn in the regulation of TRPM2 function in neurons (MacDonald/Jackson lab, unpublished). In cultured hippocampal neurons, repeated applications of NMDA (0.1 mM; 1/60 s) in the absence of exogenous ADPR induced TRPM2 activation and a resultant increase in holding current over 25 minutes (Appendix A, Fig. S2). However, intracellular application of Fyn(39-57) (25 ng/mL; in patch pipette) significantly reduced this TRPM2 activation and increase in holding current (Appendix A, Fig. S2). This result reaffirms the notion that Fyn potentiates TRPM2 currents in cultured neurons. Further, this result suggests that the Fyn-UD is important for the regulation of TRPM2 by Fyn in neurons. The UD of Src has been implicated as an interaction-mediating domain (Gingrich et al., 2004). Similarly, Fyn-UD may mediate protein-protein interaction between Fyn and TRPM2, either directly or

through one or more adaptor proteins. Alternatively, Fyn-UD could be important for the interaction of Fyn with other related signaling proteins. However, the function of Fyn-UD has not yet been described.

4.3 Identification of TRPM2 tyrosine residue(s) responsible for Fyn modulation

Converging evidence suggests that TRPM2 activation is regulated by tyrosine phosphorylation. Zhang et al. (2007) showed that TRPM2 activation in response to oxidative stress was significantly reduced by the tyrosine phosphatase PTPL1 as well as by tyrosine kinase inhibitors, including PP2 – a non-specific SFK inhibitor. These results suggest that a tyrosine phosphorylation event, which is likely mediated by a SFK, regulates TRPM2 activation. However, the identity of the SFK member(s) that regulate TRPM2 has not previously been explored. We have shown here that Fyn induces tyrosine phosphorylation of TRPM2 and potentiates TRPM2 function. Further, the degree of TRPM2 phosphorylation is related to the activation state of Fyn. For example, TRPM2-HEK293 cells expressing constitutively active Fyn-Y531F exhibited significantly more TRPM2 tyrosine phosphorylation that cells transfected with empty vector, wild-type Fyn or inactive Fyn-K299M constructs (Fig. 3.3). This finding supports the notion that Fyn induces tyrosine phosphorylation of TRPM2.

However, the regulatory tyrosine residue(s) on TRPM2, whose phosphorylation results in increased TRPM2 currents, remain unidentified. Identification of these regulatory sites is critical for defining the activation properties of TRPM2. Because TRPM2 has been implicated as a mediator of cell death in a number of oxidative stress-related degenerative diseases, decreasing TRPM2 activation may be a therapeutic strategy to reduce cell death in such conditions. By extension, pharmacological agents that disrupt phosphorylation events that potentiate TRPM2 channel activation may reduce TRPM2 activation and associated cell death.

Several strategies have been employed for identifying phosphorylation sites for specific kinase-substrate combinations. For example, phosphorylation sites may be determined by Edman phosphate-release sequencing of ^{32}P-radiolabelled phosphoproteins. This technique involves purifying a phosphopeptide by antiphosphotyrosine antibody affinity chromatography and reverse phase high performance liquid chromatography followed by sequential release and sequencing of each amino acid from the N-terminus while monitoring for ^{32}P-radiolabelled tyrosine residues (Smart et al., 1981; Wagner et al., 1991). Alternatively, phosphorylation sites may be identified using mass spectrometry. In this approach, a phosphopeptide is enriched, using techniques such as immobilized metal affinity chromatography, followed by mass analysis using a mass spectrometer (Munton et al., 2007). Additionally, a bioinformatic prediction approach may be used to identify tyrosine phosphorylation sites. This approach utilizes phosphorylation site prediction software, which predicts high-probability phosphorylation sites based on substrate consensus sequences that have been established or identified in the literature for a kinase of interest (Phanstiel et al., 2011; Xue et al., 2008). Notably, as phosphorylation prediction software begins to incorporate three-dimensional structural information about kinases and substrates of interest into prediction algorithms, more accurate predictions of phosphorylation sites will be possible. Additionally, a genetic approach may be used to identify sites of tyrosine phosphorylation. This approach utilizes site-directed mutagenesis of candidate tyrosine residues followed by an assay to confirm loss of tyrosine phosphorylation, such as Western blotting with a phosphotyrosine-specific antibody or monitoring ^{32}P-radiolabelling of phosphoproteins (Daubner et al., 1992; Nakamura et al., 2000). A limitation of this approach is that a given protein may contain multiple tyrosine residues, which would require time-consuming mutagenesis of each tyrosine residue. For example, TRPM2 contains 31 intracellular tyrosine residues (13 N-terminal, 15 C-terminal and 3 intracellular loop) that could be phosphorylated by Fyn. Notably, each of these approaches has been used successfully in the literature and could be used to investigate which TRPM2 tyrosine residue(s) may be phosphorylated by Fyn.

In this study, a genetic approach was combined with a bioinformatic prediction approach to identify the TRPM2 tyrosine residue(s) responsible for channel regulation by Fyn. Rather than sequentially mutate all 31 intracellular TRPM2 tyrosine residues, GPS 2.1

phosphorylation site prediction software (Xue et al., 2010) was utilized to identify high-probability Fyn substrate sites, thus increasing the efficiency of identifying regulatory phosphorylation sites. To assess the ability of GPS 2.1 software to successfully predict tyrosine phosphorylation sites, predicted Fyn phosphorylation sites were compared to literature-established phosphorylation sites for known Fyn substrates: NMDAR subunit NR2B and microtubule-associated protein tau. GPS 2.1 predicted that Fyn phosphorylates NMDAR subunit NR2B at residues Y1472, Y1336 and Y1252, which were identified as the major Fyn phosphorylation sites on NR2B by Nakazawa et al. (2001) using site-directed mutagenesis and Western blotting. Further, GPS 2.1 predicted that Fyn phosphorylates tau (isoform 2) at residues Y18 and Y197, which were identified as the major Fyn phosphorylation sites on tau by Scales et al. (2011) using mass spectrometry and two-dimensional phosphopeptide mapping. Notably, GPS 2.1 software has been successfully used by several authors to make initial predictions of phosphorylation sites, which were subsequently confirmed by other techniques (Ishiguro et al., 2010; Wang et al., 2012). These findings provided some confidence in the ability of GPS 2.1 software to accurately predict Fyn substrate phosphorylation sites.

Using GPS 2.1 phosphorylation site prediction software, the four identified TRPM2 tyrosine residues with the highest prediction scores were Y74, Y142, Y523 and Y1503 (Fig. 4.1). For each of these potential regulatory tyrosine residues, a TRPM2 mutant construct was designed and synthesized using site-directed mutagenesis. For each mutant, the potential regulatory tyrosine was substituted with phenylalanine. Phenylalanine is chemically identical to tyrosine, except that it lacks an aromatic hydroxyl group, which is essential for phosphorylation. Thus, mutation of tyrosine to phenylalanine effectively ablates phosphorylation at the targeted residue. By extension, if phosphorylation of these residues is indeed important for Fyn regulation of TRPM2, such point mutations should abolish Fyn regulation of TRPM2. Thus, it is expected that if Fyn induces phosphorylation of one or more of these tyrosine residues, phosphorylation of the corresponding mutant TRPM2 protein(s) should be reduced or ablated. HEK293T cells co-expressing a Fyn construct (Fyn-Y531F or EV) and a TRPM2 construct (TRPM2-WT, -Y74F, -Y142F, -Y523F, or -Y1503F) were used to assess whether these TRPM2 mutations reduced or ablated Fyn-induced tyrosine

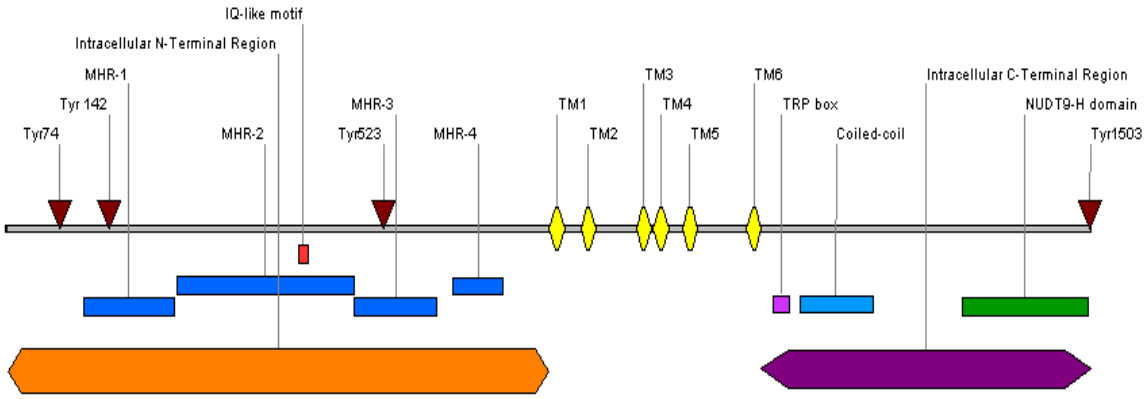

Figure 4.1: Potential regulatory TRPM2 tyrosine residues.

Linear schematic of human TRPM2 protein indicating four tyrosine residues (Y74, Y142, Y523 and Y1503; indicated by arrows), which were identified as probable phosphorylation targets of human Fyn kinase using GPS 2.1 software. Four mutant human TRPM2 constructs were prepared by site-directed mutagenesis, in which these tyrosine residues were individually substituted with phenylalanine.

phosphorylation of TRPM2. However, the amount of Fyn-induced phosphorylation of TRPM2 was not significantly different in any of the TRPM2 mutants relative to wild-type TRPM2 (Fig. 3.6).

There are several possible reasons that Fyn-induced TRPM2 phosphorylation was not found to be significantly different in mutant relative to wild-type TRPM2. Firstly, it is possible that phosphorylation of one or more of the candidate sites analyzed here is/are induced by Fyn, but this phosphorylation was not effectively detected in this study. Failure to detect phosphorylation of a specific tyrosine residue may be attributable to the use of a non-specific phosphotyrosine antibody (mouse anti-phosphotyrosine 4G10; Upstate Biotechnology, NY). This antibody does not specifically recognize the tyrosine residue of interest. As such, if phosphorylation of the site of interest is relatively low compared to the total phosphorylation of TRPM2, it may be difficult to resolve minute

differences in phosphorylation of an individual tyrosine of interest. This problem is compounded by the fact that Fyn may phosphorylate multiple tyrosine residues within TRPM2. Alternatively, Fyn may induce tyrosine phosphorylation of a residue that was not predicted by GPS 2.1 software. Supplementary use of another technique to assess tyrosine phosphorylation, such as mass spectrometry or amino acid sequencing (as described above), could be employed to better assess whether Fyn induces phosphorylation of these candidate regulatory TRPM2 tyrosine residues.

Zhang et al. (2007) demonstrated that phosphorylation of TRPM2 at an as-yet-unidentified tyrosine residue closely coincides with increased TRPM2 channel activation. However, the significance of such tyrosine phosphorylation has not yet been described. For example, TRPM2 phosphorylation could directly cause an increase in channel gating, facilitate channel activation by another protein or be unrelated to channel function. Details on potential mechanisms by which Fyn-induced tyrosine phosphorylation of TRPM2 may regulate TRPM2 function are discussed section 4.5.

To assess whether these point mutations of the four potential regulatory TRPM2 tyrosine residues (TRPM2-Y74F, -Y142F, -Y523F and -Y1503F) affect TRPM2 regulation by Fyn kinase, an additional experiment could be designed. Whole-cell voltage-clamp recordings could be conducted in HEK293T cells as previously described (section 2.2). Cells would be transfected with one of five TRPM2 constructs (TRPM2-WT, -Y74F, -Y142F, -Y523F, -Y1503F). Recombinant active Fyn or vehicle would be delivered intracellularly by inclusion in the patch pipette. It is expected that if Fyn regulates TRPM2 function through one or more of these tyrosine residues, Fyn-induced potentiation of TRPM2 currents would be reduced for the corresponding mutant TRPM2 protein(s). Taken together, these experiments may lead to the identification of TRPM2 tyrosine residue(s) whose phosphorylation by Fyn coincides with increased TRPM2 activation.

4.4 Physical interaction between Fyn and TRPM2

Protein-protein interaction between Fyn and TRPM2 may be important for regulation of TRPM2 function by Fyn. The results of the present study show that Fyn physically interacts with TRPM2 (Fig. 3.4). Notably, Fyn-TRPM2 interaction was demonstrated in cells expressing constitutively active Fyn-Y531F, wild-type Fyn and inactive Fyn-K299M, but not in empty vector-transfected cells (Fig. 3.4). Further, the extent of this interaction may be related to the activation state of Fyn; significantly more constitutively active Fyn-Y531F co-immunoprecipitated with TRPM2 than wild-type Fyn or inactive Fyn-K299M (Fig. 3.4). These results suggest that protein-protein interaction occurs between Fyn and TRPM2. As previously noted, potentiation of TRPM2 currents by Fyn may contribute to dysregulation of calcium homeostasis and cell death in several oxidative stress-related degenerative diseases. If the regulation of TRPM2 by Fyn is dependent on the physical interaction between these molecules, then pharmacologically disrupting this interaction may be a strategy to reduce the contribution of TRPM2 to such degeneration. Clearly, defining the nature of the interaction between these molecules would be critical for designing pharmacological interaction-disrupting agents. For example, protein-protein interaction between Fyn and TRPM2 could be disrupted using a short interfering peptide designed in a similar manner to Fyn(39-57), with an identical amino acid sequence to the interaction-mediating domain of Fyn or TRPM2. Such a peptide, if administered at a sufficiently high concentration, would be expected to competitively bind to Fyn or TRPM2 (depending on which protein was used as a template for the peptide sequence) and disrupt the physical interaction between Fyn and TRPM2. Consequently, if this interaction is important for the functional regulation of TRPM2 channel activity by Fyn, then Fyn-mediated potentiation of TRPM2 channel activity would also be disrupted by this peptide.

Whether the physical interaction between Fyn and TRPM2 is direct or indirect (i.e. mediated through one or more scaffolding proteins) is unclear at present. In this study, co-immunoprecipitation was used to assess whether or not there is a physical interaction between these proteins. This technique involves sequestering one protein with an agarose bead-linked antibody. Subsequently, the bead-antibody-antigen complex is separated

from any unbound components by several alternating centrifugation and wash cycles. The antigen of interest and any physically associated proteins are then eluted and analyzed by Western blotting. Importantly, this technique is not capable of distinguishing whether Fyn binds directly to TRPM2 or whether both proteins are part of a larger complex of physically associated proteins. However, the use of relatively high-stringency immunoprecipitation and wash buffers increases the probability that interaction detected by co-immunoprecipitation is direct. The immunoprecipitation and wash buffers used here were relatively stringent compared to those used in other co-immunoprecipitation studies (1% NP-40, 1% sodium deoxycholate; detailed in section 2.5). Whether the interaction is direct or indirect, provided the stringency of binding is sufficiently high, pull-down of antibody-sequestered TRPM2 would result in co-immunoprecipitation of Fyn. Along these lines, Sato et al. (2008) showed that post-synaptic density-93 (PSD-93) mediates the physical interaction between Fyn (but not Src) and NMDARs. PSD-93 was shown to mediate phosphorylation of NMDARs by Fyn as well as synaptic localization of Fyn (Sato et al., 2008). Similarly, Gingrich et al. (2004) showed that the physical interaction between Src (but not Fyn) and NMDARs was mediated by NADH dehydrogenase subunit 2 (ND2), which functions as an adaptor protein in this complex. In this complex, ND2 is essential for Src regulation of NMDAR function (Gingrich et al., 2004). Similarly, there may be one or more adaptor proteins that physically link Fyn to TRPM2. Alternatively, Fyn may associate directly with TRPM2.

The domains of Fyn and TRPM2 that are responsible for mediating their physical interaction have not yet been identified. As described previously, Fyn contains several domains that may mediate interactions with other proteins. For example, the SH3 domain of Fyn mediates interactions with proline-rich sequences that contain PxxP motifs (where ‘x’ represents any amino acid) (Salter & Kalia, 2004). Analysis of the amino acid sequence of TRPM2 reveals six such motifs: AA 544-547, 551-554, 575-578, 1078-1081, 1248-1251 and 1324-1327. However, whether these motifs mediate the interaction between Fyn and TRPM2 has not been investigated. The SH2 domain of Fyn mediates interactions with specific sequences that contain phosphotyrosine residues (Salter & Kalia, 2004). TRPM2 sequence analysis reveals 31 tyrosine residues (13 N-

terminal, 15 C-terminal and 3 intracellular loop) that could act as docking sites for Fyn-SH2 binding. As previously described, all SFK members contain an N-terminal unique domain, which shares a low degree of sequence homology with other SFK members (Salter & Kalia, 2004). Because of the low sequence homology, this region could be important for determining the unique actions of each SFK. The unique domain of Src has been shown to mediate its physical interaction with ND2 (Gingrich et al., 2004). By extension, it is possible that the unique domain of Fyn could similarly mediate protein-protein interaction with TRPM2. However, the role of Fyn's unique domain has not yet been characterized. As described above, in cultured hippocampal neurons, intracellular application of Fyn(39-57) (25 ng/mL) through its inclusion in the patch pipette significantly reduced the TRPM2 activation and increase in holding current evoked by repeated NMDA applications (Appendix A, Fig. S2). Since Fyn(39-57) specifically disrupts interactions mediated by Fyn's unique domain, it stands to reason that this domain is critical for regulation of TRPM2 function by Fyn. However, this does not necessarily imply that physical interaction between Fyn and TRPM2 is mediated by Fyn's unique domain. For example, the unique domain could mediate a physical association of Fyn to an adaptor protein or regulate Fyn activity or substrate specificity through an as-yet-undescribed mechanism.

Experiments could be designed to identify the binding domains that mediate the interaction between Fyn and TRPM2. As previously noted, Fyn(39-57) disrupts protein-protein interactions mediated by Fyn's N-terminal unique domain. Thus, it stands to reason that if Fyn(39-57) disrupted the interaction between Fyn and TRPM2, it is mediated by Fyn's unique domain. To identify Fyn's binding site on TRPM2, several short FLAG-epitope-tagged fragments of TRPM2 could be generated. HEK293 cells would then be transfected to induce expression of these constructs as well as constitutively active Fyn-Y531F. After lysis, SDS-PAGE and transfer to nitrocellulose membranes, FLAG immunoprecipitates would be immunoblotted for Fyn. The TRPM2 fragment that demonstrated immunoreactivity for Fyn would represent the domain that physically interacts with Fyn. Alternatively, if the interaction is mediated by Fyn-SH2, then sequentially mutating each tyrosine residue to phenylalanine and determining whether Fyn physically interacts with each TRPM2 mutant would lead to identification of

the interaction-mediating tyrosine residue. Similarly, if the interaction between Fyn and TRPM2 is mediated by Fyn-SH3, then sequentially mutating each proline-rich PxxP motif and determining whether Fyn physically interacts with each TRPM2 mutant would lead to identification of the interaction-mediating PxxP motif.

Physical interaction between Fyn and TRPM2 may be dependent on Fyn activation. Notably, significantly more constitutively active Fyn-Y531F co-immunoprecipitated with TRPM2 than wild-type Fyn or inactive Fyn-K299M (Fig. 3.4). Mechanistically, this observation could be attributable to the different conformations of active and inactive Fyn. In its inactive conformation, Fyn forms a closed, inaccessible bundle, which is stabilized by intramolecular interactions of its SH2 domain with tyrosine 531 and its SH3 domain with a PxxP motif within its linker region (Liu et al., 1993; Superti-Furga et al., 1993). Alternatively, in its active conformation, Fyn is characterized by an open, accessible structure (Cowan-Jacob et al., 2005). It is possible that the interaction between Fyn and TRPM2 is more stable when Fyn is in an active, open conformation. Alternatively, protein-protein interaction between Fyn and TRPM2 may be dependent on TRPM2 phosphorylation. As previously described, the SH2 domain of Fyn binds to amino acid sequences that contain phosphorylated tyrosine residues. By extension, it is possible that tyrosine phosphorylation of TRPM2 by Fyn may regulate the interaction of Fyn with TRPM2 by providing SH2 binding sites. This mechanism would also explain the finding that constitutively active Fyn-Y531F interacts with TRPM2 to a greater degree than wild-type Fyn or inactive Fyn-K299M (Fig. 3.4); increased Fyn activation results in increased TRPM2 tyrosine phosphorylation, which may provide more binding sites for Fyn and consequently, more interaction between Fyn and TRPM2. A similar mechanism was proposed for the interaction between Fyn and tau. Specifically, Usardi et al. (2011) showed that the SH2 domain of Fyn interacts with tyrosine 18 of tau – a reported target of Fyn phosphorylation (Hernandez et al., 2009). Thus, Fyn regulates its own interaction with tau through tyrosine phosphorylation. Whether the candidate regulatory TRPM2 tyrosine residues identified using GPS 2.1 software (TRPM2-Y74, -Y142, -Y523 and -Y1503) are important for the physical interaction between Fyn and TRPM2 remains an open question. Notably, if one or more of these tyrosine residues are important for the physical interaction between Fyn and TRPM2, the corresponding

tyrosine to phenylalanine mutants should exhibit reduced co-immunoprecipitation of Fyn with TRPM2. To assess the importance of the these candidate TRPM2 regulatory tyrosines, HEK293T cells were co-transfected with active Fyn (Fyn-Y531F) or empty vector as well as each of five TRPM2 constructs (TRPM2-WT, -Y74F, -Y142F, -Y523F, -Y1503F). Immunoprecipitation of TRPM2 and immunoblotting were carried out as described previously (see section 2.5). The amount of Fyn that co-immunoprecipitated with each mutant TRPM2 construct was not significantly different for each of the four mutant TRPM2 constructs relative to wild-type TRPM2 (Fig. 3.7). Thus, point mutations of the proposed regulatory TRPM2 tyrosine residues had no discernible effect on the physical interaction between Fyn and TRPM2. This observation suggests that these candidate TRPM2 phosphorylation sites are not critical for the interaction between Fyn and TRPM2. Post-transfection lysate expression levels of TRPM2 and Fyn relative to β-actin in HEK293T cells are shown in Fig 3.5.

The physiological significance of the physical interaction between Fyn and TRPM2 remains unclear at present. It is likely that anchoring of Fyn to TRPM2 facilitates channel regulation. However, it is also possible that the regulation of TRPM2 by Fyn is unrelated to the physical interaction between these proteins.

4.5 Mechanism of TRPM2 regulation by Fyn kinase

We have shown that TRPM2 currents are increased by intracellular application of recombinant Fyn. Fyn-mediated augmentation of TRPM2 activity could result from increased expression, increased surface localization and/or altered gating properties of TRPM2. Characterizing the mechanism through which Fyn regulates TRPM2 is important for understanding the basic functional properties of TRPM2 channels and for designing means to disrupt the functional regulation of TRPM2 by Fyn.

To assess whether TRPM2 expression is modulated by Fyn, lysate TRPM2 expression levels were compared in TRPM2-HEK293 cells co-expressing TRPM2 as well as wild-type Fyn, constitutively active Fyn (Y531F), inactive Fyn (K299M) or empty vector.

Interestingly, despite the finding that TRPM2 currents are augmented by Fyn (Fig. 3.1), expression of active Fyn-Y531F resulted in a decrease in TRPM2 expression relative to inactive Fyn-K299M and empty vector (Fig. 3.2). Notably, there are important differences between the experimental paradigm used to study TRPM2 currents and that used to study TRPM2 expression, phosphorylation and protein-protein interaction. In the electrophysiological protocol used to study TRPM2 currents, recombinant active Fyn was delivered transiently to the intracellular compartment for the duration of each recording by inclusion in the patch pipette. Alternatively, in the biochemistry protocol used to study TRPM2 expression, phosphorylation and protein-protein interaction, Fyn expression was induced by transfecting cells 48 hours prior to cell lysis with constructs that drive Fyn expression. Thus, the electrophysiology technique solely accounts for transient effects of Fyn on TRPM2, whereas the biochemistry technique accounts only for more long-term effects of Fyn on TRPM2. To study the short-term effects of Fyn on TRPM2 expression, TRPM2-HEK293 cells could be induced to express TRPM2, transfected to induce Fyn expression, and lysed at various time points after transfection. Subsequent Western blotting for Fyn and TRPM2 would reveal a profile of TRPM2 and Fyn expression levels over time.

It is possible that Fyn influences TRPM2 activity by altering TRPM2 surface expression. Surface biotinylation could be used to assess whether Fyn modulates the surface localization of TRPM2. Surface expression of TRPM2 would be compared in HEK293 cells expressing biotinylated TRPM2 as well as wild-type Fyn, constitutively active Fyn (Y531F), inactive Fyn (K299M) or empty vector. If Fyn influences TRPM2 activity by altering its surface localization, it is expected that cells expressing active Fyn-Y531F would exhibit higher TRPM2 surface expression than the other conditions.

Alternatively, it is possible that Fyn influences TRPM2 activity by altering the gating properties of TRPM2 channels. Experiments could be designed to investigate such mechanisms. For example, whole-cell voltage-clamp electrophysiology could be used to determine whether Fyn affects ADPR sensitivity, Ca^{2+} sensitivity or Ca^{2+}-dependent inactivation of TRPM2. Notably, the roles of TRPM2 tyrosine phosphorylation and

physical interaction in the regulation of TRPM2 function are discussed in sections 4.3 and 4.4, respectively.

4.6 Specificity of TRPM2 regulation by Fyn kinase

Since there is a high degree of sequence homology and common domain structure between SFK members (Salter and Kalia, 2004), it is possible that other SFKs may also regulate TRPM2 function. Notably, Zhang et al. (2007) demonstrated that PP2 reduced H_2O_2-induced TRPM2 phosphorylation, Ca^{2+} influx and cell death. However, because PP2 is a non-specific SFK inhibitor, this effect cannot be attributed to any specific SFK member. To determine whether the observed regulation of TRPM2 channels is Fyn-specific, experiments could be designed to determine whether Src, another SFK member, also regulates TRPM2. First, whether intracellular application of recombinant active Src (via the patch pipette), regulates TRPM2 currents in TRPM2-HEK293 cells could be assessed. Additionally, the Fyn- and Src-specific interfering peptides Fyn(39-57) and Src(40-58) could be applied intracellularly (via the patch pipette) to determine whether each influences TRPM2 currents regulated by endogenous SFKs, which are expressed in HEK293 cells.

To determine whether the increase in TRPM2 tyrosine phosphorylation observed is Fyn-specific, a construct that drives expression of constitutively active Src (Src-Y527F) could be utilized. Tyrosine phosphorylation of TRPM2 would be compared in FLAG-immunoprecipitates of TRPM2-HEK293 cells co-expressing TRPM2 as well as Src-Y527F, Fyn-Y531F or empty vector. If phosphorylation of TRPM2 is Fyn-specific, cells expressing Fyn-Y531F will exhibit more TRPM2 tyrosine phosphorylation than those expressing Src-Y527F or empty vector. Alternatively, if Src also induces tyrosine phosphorylation of TRPM2, then it is expected that Src-Y527F would exhibit more tyrosine phosphorylation than empty vector-transfected cells.

To determine whether the interaction between Fyn and TRPM2 is Fyn-specific, TRPM2-HEK293 cells expressing TRPM2 could be transfected with a construct driving

expression of Src-Y527F or Fyn-Y531F. If the physical interaction observed is Fyn-selective, FLAG-immunoprecipitates of cells expressing Fyn-Y531F should exhibit immunoreactivity as previously demonstrated, and cells expressing Src-Y527F should not show Src immunoreactivity.

4.7 TRPM2, Fyn and NMDA receptor signaling

Fyn kinase, TRPM2 and NMDARs may form a functional signaling complex whose activity is augmented in several oxidative stress-related degenerative diseases. Co-activation of these molecules may synergistically increase Ca^{2+} influx, resulting in activation of cell death pathways. Functional relationships between Fyn and NMDAR, as well as TRPM2 and NMDARs have been previously described in the literature. In this study, we describe a novel relationship between Fyn and TRPM2. We propose that co-activation of TRPM2, Fyn and NMDARs could lead to pathological dysregulation of intracellular Ca^{2+} homeostasis and neuronal death in oxidative stress-related degenerative diseases.

Functional relationships between Fyn and NMDAR, as well as TRPM2 and NMDARs have been described previously. Fyn has previously been shown to phosphorylate NMDARs, resulting in cytotoxic Ca^{2+} overload (Ittner et al., 2010). Intracellular Ca^{2+} associated with NMDAR activation has also been shown to activate TRPM2 (Olah et al., 2009). Thus, TRPM2 channels may be co-activated with NMDARs.

In this study, we have described a novel relationship between Fyn and TRPM2. Specifically, we have shown that Fyn physically interacts with TRPM2 (Fig. 3.4), induces tyrosine phosphorylation of TRPM2 (Fig. 3.3) and potentiates currents mediated by TRPM2 (Fig. 3.1). Importantly, Fyn was also shown to enhance NMDAR-activated TRPM2 currents in cultured hippocampal neurons (Appendix A, Fig. S1; MacDonald/Jackson lab, unpublished). In this experimental context, Fyn may have potentiated TRPM2 currents by directly phosphorylating TRPM2 currents or by augmentation of NMDARs. Since HEK293 cells do not express endogenous NMDAR

(Domingues et al., 2006), in this system, TRPM2 currents were augmented by Fyn through an NMDAR-independent mechanism. Increased intracellular Ca^{2+} resulting from activation of Ca^{2+}-permeable NMDAR and TRPM2 channels may further potentiate TRPM2 current since TRPM2 is activated in a concentration-dependent manner by intracellular Ca^{2+} (Du et al., 2009a). This is compounded by the fact that Fyn can potentiate currents mediated by both TRPM2 and NMDAR. Taken together, these results suggest that co-activation of TRPM2, Fyn and NMDAR may result in pathological Ca^{2+} overload and consequent cell death in response to oxidative stress.

4.8 Oxidative stress and signaling of TRPM2, Fyn and NMDAR

In independent studies, activation of TRPM2, Fyn and NMDAR have been demonstrated in response to oxidative stress. Two independent studies published in 2002 showed that in HEK293 cells transfected to induce expression of TRPM2, treatment with oxidative stressors, such as H_2O_2, induces activation of TRPM2 channels and consequent increases in $[Ca^{2+}]_i$ (Hara et al., 2002; Wehage et al., 2002). Sanguinetti et al. (2003) demonstrated that Fyn autophosphorylation and kinase activation occur in response to oxidative stressors, such as H_2O_2. Activation of Fyn (but not Src or Yes) results in tyrosine phosphorylation of caveolin-1 in response to oxidative stress (Sanguinetti et al., 2003). Further, treatment of rat hippocampal slices with H_2O_2 results in a transient reduction in glutamatergic synaptic transmission, which is followed by a delayed increase in NMDAR activation (Avshalumov & Rice, 2002). Moreover, sustained activation of NMDARs can induce activation of NADPH oxidase 2 (NOX2), an enzyme that produces superoxide and induces oxidative stress and cell death in local cells (Reyes et al., 2012). Taken together, TRPM2, Fyn and NMDAR are all activated in response to oxidative stress.

Further, TRPM2, Fyn and NMDAR activation have been independently implicated as mediators of cell death. TRPM2 activation in response to extracellular H_2O_2 treatment and intracellular ADPR administration has been shown to mediate cell death in HEK293

cells expressing TRPM2 (Hara et al., 2002; Wehage et al., 2002). Fyn has been also been implicated as a mediator of cell death. For example, in a cytolytic hybridoma cell line, Fyn was shown to mediate cell death signal transduction of the apoptosis-inducing Fas receptor (Atkinson et al., 1996). Additionally, in a T cell hybridoma cell line, Fyn activation was shown to be critical for T-cell receptor-induced caspase activation and consequent apoptosis (Ricci et al., 2001). Excessive activation of NMDAR has also been shown to induce cellular influx of pathological levels of intracellular Ca^{2+} and evoke cell death (Ittner et al., 2010). Specifically, GluN2B subunit-containing NMDARs have been implicated in neuronal apoptosis (Hardingham et al., 2002). Thus, TRPM2, Fyn and NMDARs have all been implicated as contributors to cell death.

TRPM2, Fyn and NMDAR overactivation have been implicated in oxidative stress-related degenerative diseases. For example, each of these molecules have been implicated in the pathogenesis of Alzheimer's disease (AD). Although somewhat controversial, one theory of AD maintains that increased oxidative stress in the brains of affected patients is critical for the pathogenesis of AD (Christen, 2000). Overactivation of TRPM2 has been suggested to contribute to cell death in a variety of oxidative stress-related degenerative diseases, such as AD (Jiang et al., 2009). Mechanistically, oxidative stress causes mitochondrial dysfunction, resulting in production of ADPR, which in turn activates TRPM2 (Perraud et al., 2005). Fyn has also been implicated in AD pathogenesis. Shirazi and Wood (1993) showed that Fyn expression is increased in subsets of hippocampal neurons in AD patients relative to age-matched unaffected controls. Fyn-deficient mice display reduced Aβ-induced neuronal death (Lambert et al., 1998). Further, overexpression of Fyn results in increased Aβ-induced synaptic damage and cognitive deficits (Chin et al., 2005). Additionally, NMDAR has been implicated in AD pathogenesis. For example, Shankar et al. (2007) showed that activation of NMDAR is absolutely required for Aβ-induced synaptic damage (Shankar et al., 2007). Therefore, TRPM2, Fyn and NMDAR have all been implicated as pathogenic factors in oxidative stress-related degenerative diseases, such as AD. Although no positive relation between Fyn, TRPM2 and NMDAR has been demonstrated, we propose that co-activation of these molecules could result in pathological increases in $[Ca^{2+}]_i$ and cell death in response to oxidative stress through the mechanism described in section 4.7.

Whether the findings of these *in vitro* experiments are physiologically meaningful depends on whether they accurately represent what occurs *in vivo*. All of the experiments conducted in this study utilized HEK293 variant cell lines to study TRPM2. These cell lines are useful for studying TRPM2 for several reasons. Firstly, TRPM2 activation has been demonstrated in response to stimulation of NMDA receptors (Olah et al., 2009). Because HEK293 cells do not express NMDARs, their potential confounding influence on TRPM2 function is nonexistent. Also, because there are few effective commercially available antibodies for TRPM2, it is difficult to study neuronal TRPM2 using biochemical techniques. However, using a HEK293 cell expression system, such as the inducible TRPM2-HEK293 cell line or transfection of HEK293T cells with plasmids driving TRPM2 expression, TRPM2 can be linked to a FLAG epitope tag and detected using an antibody directed against the FLAG epitope. Although HEK293 cell lines facilitate the study of TRPM2 channels, they may function differently than cells in an *in vivo* context. That Fyn was also shown to potentiate TRPM2 currents in cultured hippocampal neurons supports the regulatory role for Fyn on TRPM2 that is described in this study (Appendix A, Fig. S1). However, because these neurons were isolated from the hippocampus and cultured, they may also function differently than they would *in vivo*.

If Fyn does augment TRPM2 function *in vivo* as described here *in vitro*, and this augmentation of TRPM2 currents significantly contributes to cell death in oxidative stress-related degenerative diseases, then pharmacologically disrupting this regulation could provide a potential therapeutic strategy. This regulation could be disrupted by designing a short interfering peptide that mimics an interaction-mediating domain of Fyn or TRPM2. Such a peptide would function by competitively binding to Fyn or TRPM2 and interfering with the normal interaction between these proteins. Notably, such a peptide could be tagged with a transactivator of transcription (TAT)-tag, which allows a protein of interest to be effectively taken up from the extracellular medium into the intracellular compartment.

TRPM2 was previously shown to be critical for NMDAR-dependent long-term depression (LTD) (Xie et al., 2011). To assess whether Fyn-mediated regulation of

TRPM2 contributes to synaptic plasticity, the effect of this peptide on LTD and long-term potentiation (LTP) in hippocampal slices could assessed. Further, the effect of this peptide in animal models of oxidative stress-related diseases could be assessed.

To study the potential role of TRPM2 in AD, $TRPM2^{+/-}$ mice were crossed with APPswePS1dE9 mice, a transgenic model of AD that expresses the Swedish amyloid precursor protein (APP) mutation and a deletion of presinilin 1 (PS1) in exon 9 (MacDonald/Jackson lab, unpublished; Malm et al., 2011). It was demonstrated that knockout of TRPM2 rescued cognitive deficits observed in Barnes Maze and Morris Water Maze in the AD mouse model after 12 and 15 months (MacDonald/Jackson lab, unpublished). To determine whether the interaction between Fyn and TRPM2 contributes to degeneration in AD, the effect of administering the Fyn/TRPM2-interfering peptide to APPswePS1dE9 mice could be assessed.

4.9 Significance of work

The present study was designed to characterize a signaling pathway through which Fyn may modulate TRPM2 function. Fyn was shown to physically interact with TRPM2 (Fig. 3.4), induce tyrosine phosphorylation of TRPM2 (Fig. 3.3) and potentiate currents mediated by TRPM2 (Fig. 3.1). Further, an attempt was made to identify regulatory tyrosine residues through which Fyn may regulate TRPM2 function. However, the latter experiment did not yield any potential regulatory sites.

We propose that by augmenting TRPM2 activity, Fyn kinase may potentiate cellular Ca^{2+} overload and neuronal death in oxidative stress-related degenerative diseases. TRPM2 is expressed in neuronal subpopulations (Olah et al., 2009) as well as several other cell types, including immune cells, endothelial cells, cardiomyocytes, pancreatic β-cells and others (Jiang et al., 2010). TRPM2 overactivation has been implicated in the pathogenesis of several oxidative stress-related neurodegenerative, cardiovascular, inflammatory and endocrine disorders (Jiang et al., 2010). Further, Fyn expression is nearly ubiquitous (Sanguinetti et al., 2003). It follows that, in several cell types, potentiation of TRPM2

activation by Fyn may contribute to cell death. Pharmacologically disrupting Fyn regulation of TRPM2 may be a useful therapeutic strategy to reduce degeneration such pathological conditions.

4.10 Overall conclusions

In the present study, we have characterized a novel functional and physical relationship between TRPM2 channels and Fyn kinase. Fyn was shown to physically interact with TRPM2, and the degree of interaction between these molecules was dependent on the activation state of Fyn (Fig. 3.4). Fyn was also shown to induce tyrosine phosphorylation of TRPM2, and the amount of phosphorylation was related to the activation state of Fyn (Fig. 3.3). Lastly, Fyn was shown to augment currents mediated by TRPM2 channels (Fig. 3.1). Under conditions of oxidative stress, co-activation of Fyn and TRPM2 may result in excessive activation of TRPM2 channels, pathological increases in $[Ca^{2+}]_i$ and consequent cell death.

Section 5

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Appendices

Appendix A: Supplementary Figures

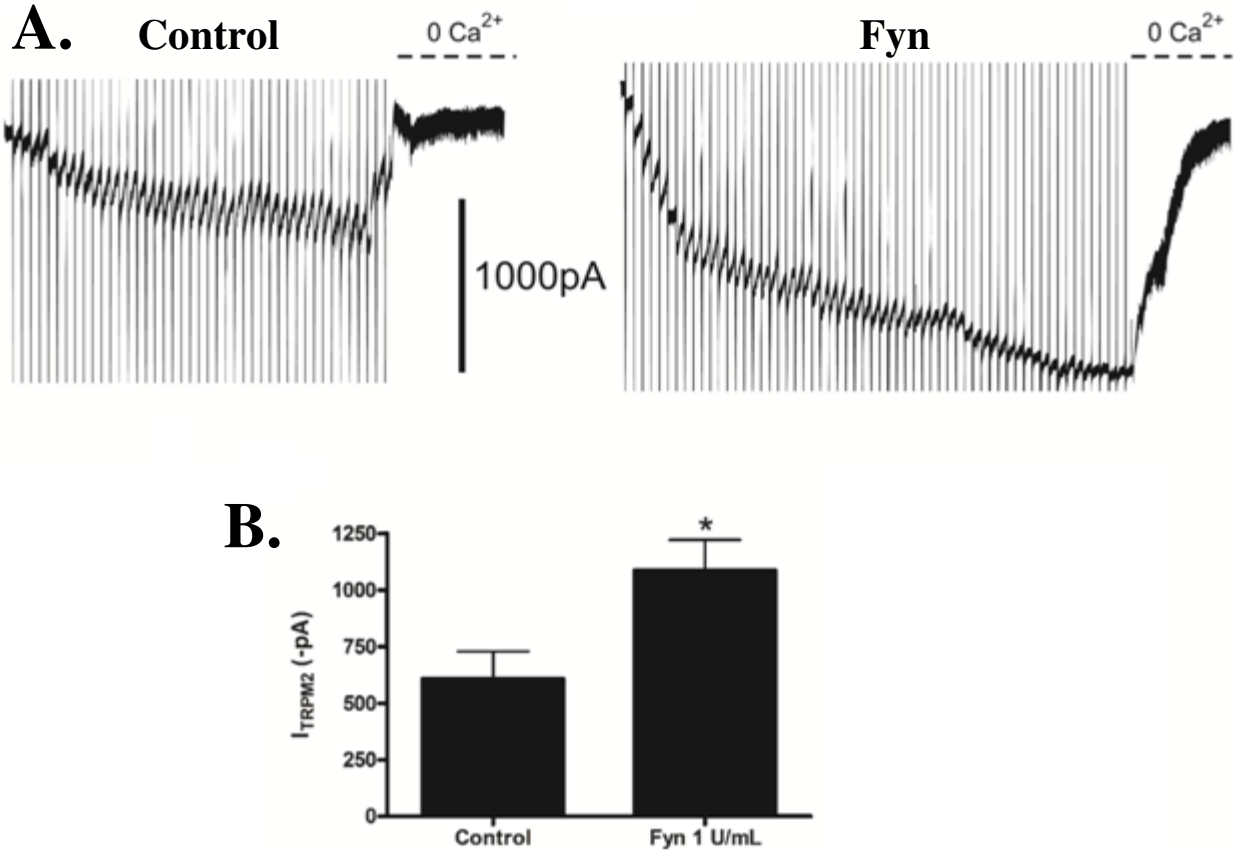

Figure S1: Fyn potentiates TRPM2 currents in cultured hippocampal neurons.

(A) Representative whole-cell recordings of TRPM2 currents activated by voltage ramps in the presence of ADPR (0.3 mM, in patch pipette) as well as recombinant Fyn (1 U/mL) or vehicle (in patch pipette). Dashed bars above graphs indicate times at which Ca^{2+} (2 mM)-containing ECS was replaced with Ca^{2+}-deficient (0 Ca^{2+})-ECS. TRPM2 currents were abolished in the absence of extracellular Ca^{2+}. Holding potential was -60 mV. **(B)** Summary bar graph showing potentiation of TRPM2 currents by Fyn. Data are presented as mean ± SEM (control, n=9; Fyn, n=10). Statistical analysis: unpaired Student's t-test, *$P < 0.05$. (Dr. Jillian Belrose, unpublished).

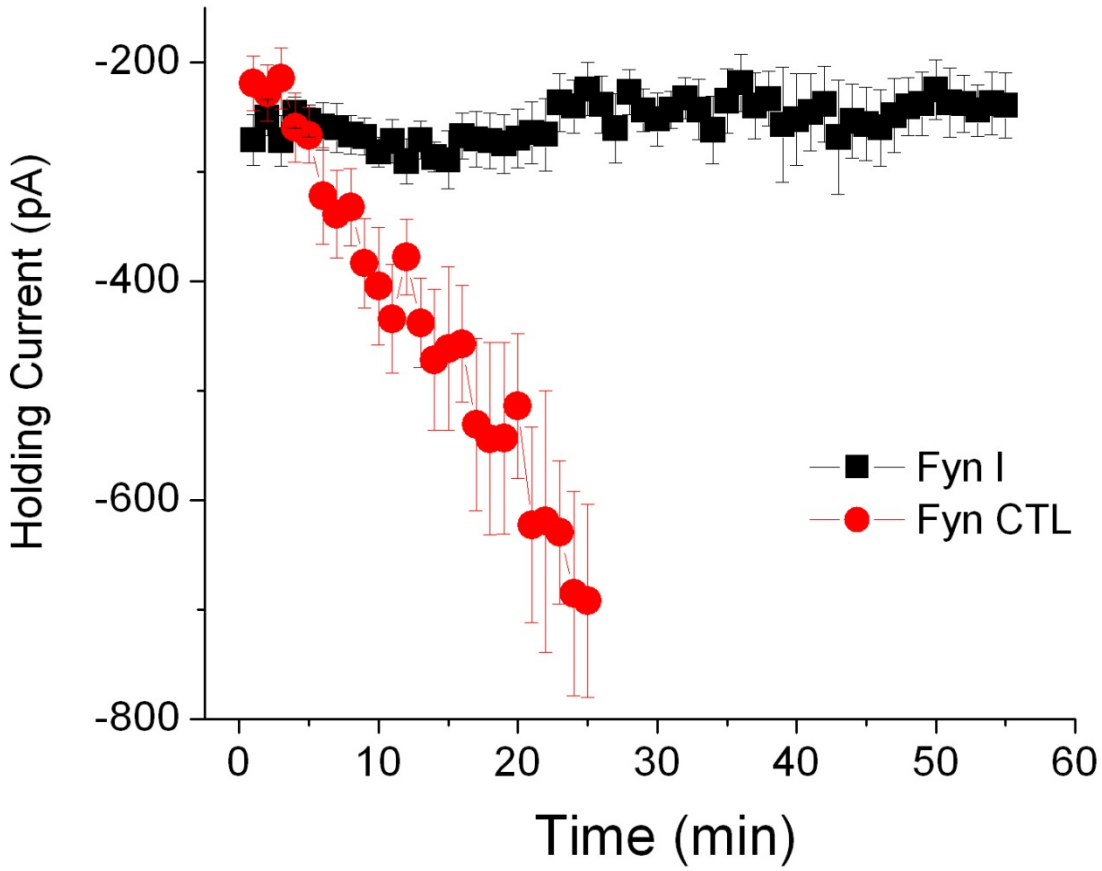

Figure S2: Fyn(39-57) attenuates TRPM2 currents in cultured hippocampal neurons.

In cultured hippocampal neurons (21-28 days *in vitro*), repeated applications of NMDA (0.1 mM; 1/60 s) in the absence of exogenous ADPR caused TRPM2 current activation and a resultant increase in holding current over 25 minutes (Fyn CTL). Moreover, intracellular application of Fyn(39-57) (25 ng/mL; in patch pipette) attenuated this TRPM2-mediated increase in holding current for 60 minutes (Fyn I). (Dr. Hongbin Li, unpublished).

Appendix B: Curriculum Vitae

EDUCATION

2013/09- **Doctor of Medicine (M.D.) Candidate**
2017/06 *Queen's University, Kingston, ON*

2011/05- **Master of Science (M.Sc.) Candidate, Physiology**
2013/06 *University of Western Ontario, London, ON*
Thesis: Regulation of TRPM2 channels by Fyn kinase
Supervisors: Dr. John F. Macdonald and Dr. Michael F. Jackson

2007/09- **Bachelor of Medical Sciences (B.M.Sc.), Physiology and Pharmacology**
2011/04 *University of Western Ontario, London, ON*
Honors thesis: Regulation of TRPM2 current by Fyn kinase
Thesis supervisors: Dr. John F. MacDonald and Dr. Michael F. Jackson

HONOURS AND AWARDS

2012-2013 Western Graduate Research Scholarship (WGRS) in Physiology
2011-2012 ($7,750 per academic year)

2011-2012 Schulich Ontario Graduate Scholarship ($15,000)

2011 Western Scholar Distinction

2010 Heart and Stroke Foundation of Ontario Summer Student Stipend ($3,500)

2007 Western Scholarship of Excellence ($2,000)

2010-2011 Queen Elizabeth II – Aiming for the Top Scholarship
2009-2010 ($3,500 per academic year)
2008-2009
2007-2008

2010-2011 University of Western Ontario Faculty of Science Dean's Honor List
2009-2010
2008-2009
2007-2008

TEACHING EXPERIENCE

2012-2013 **Teaching Assistant**, Department of Physiology and Pharmacology
2011-2012 *University of Western Ontario, London, ON*
Third-year Physiology Laboratory (3130Y); course coordinator: Tom Stavraky

PRESENTATIONS

- **Johnston, M.L.**, Belrose, J.C., Caetano, F., Jackson, M.F., MacDonald, J.F. Regulation of TRPM2 by Fyn kinase. Oral presentation delivered at Robarts Research Institute Molecular Brain Research Group seminar series, London, ON (2013/02/06).
- **Johnston, M.L.**, Belrose, J.C., Caetano, F., Jackson, M.F., MacDonald, J.F. Regulation of TRPM2 channels by Fyn kinase. Poster presented at University of Western Ontario Physiology and Pharmacology Research Day, London, ON (2012/11/06).
- **Johnston, M.L.**, Belrose, J.C., Caetano, F., Jackson, M.F., MacDonald, J.F. Regulation of TRPM2 channels by Fyn kinase. Poster presented at Great Lakes G Protein Coupled Receptor Retreat, London, ON (2012/10/18). [Poster award winner: $200].
- Belrose, J.C., **Johnston, M.L.**, Li, H., Jackson, M.F. MacDonald, J.F. Regulation of TRPM2 function by glutathione and fyn kinase: Implications for neurotoxicity following stroke. Poster presented by Jillian Belrose at the Canadian Stroke Congress, Calgary, AB (2012/10/01). [Contributed electrophysiology and biochemistry data].
- Belrose, J.C., **Johnston, M.L.**, Chen, M., Li, H., Martyn, A.C., Prado, M.A., Prado, V.F., Jackson, M.F., MacDonald, J.F. TRPM2 is implicated in cellular senescence and Aβ-mediated toxicity in hippocampal neurons. Poster presented by Jillian Belrose at the Canadian Association for Neuroscience conference, Vancouver, BC (2012/05/22). [Contributed electrophysiology and biochemistry data].
- **Johnston, M.L.**, Belrose, J.C., Caetano, F., Jackson, M.F., MacDonald, J.F. Regulation of TRPM2 by Fyn kinase. Poster presented at London Health Research Day, London, ON (2012/03/20).
- **Johnston, M.L.**, Andrade, F., Jackson, M.F., MacDonald, J.F. Role of Fyn kinase as a regulator of TRPM2-mediated current. Poster presented at University of Western Ontario Physiology and Pharmacology Poster Day, London, ON (2012/03/28).
- **Johnston, M.L.**, Jackson, M.F., MacDonald, J.F. Evaluating Fyn kinase as a regulator of TRPM2-mediated current. Oral presentation delivered at University of Western Ontario Physiology and Pharmacology Seminar Series, London, ON (2012/10/25).