EXAMINING EXCITABILITY: FROM ELECTROPHYSIOLOGY TO MESOSCALE

CALCIUM IMAGING

by

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B.H.Sc., The University of Calgary, 2018

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2023

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Abstract

Preclinical drug discovery relies on the identification of appropriate target compounds and usage of effective animal model screening tests. In this work excitability across systems as well as spatial and temporal scales were evaluated to understand long QT syndrome (LQTS) therapeutic targets and develop a behavioral assay that could sensitively characterize motor phenotype onset in a Huntington Disease (HD) mouse model.

**Case I:** The pairing of the tetrameric voltage-gated potassium channel, KCNQ1, with an accessory β-subunit, KCNE1, gives rise to the slow delayed cardiac rectifier current ($I_{Ks}$), known to play an important role in the physiological shortening of the cardiac action potential. With loss-of-function mutations in both subunits found to be associated with LQTS, enhancing $I_{Ks}$ has been identified as a therapeutic approach. Here, the NSAID, mefenamic acid, was found to dose- and rate-dependently activate $I_{Ks}$. More KCNE1-saturated $I_{Ks}$ channel complexes had a greater response to mefenamic acid treatment. KCNE1 residue K41 was identified as critical for mefenamic acid action, suggesting a potential binding site.

**Case II:** HD is a dominantly inherited neurodegenerative disease with characteristic motor symptoms. Animal models with increasingly better face and construct validity have been developed to understand HD pathophysiology. Although HD gross motor defects have been extensively characterized, less is known about forelimb motor deficits. Using a high-throughput alternating reward/non-reward water-reaching task, HD forelimb movement defects and associated aberrant cortical activity were examined. HD heterozygous-zQ175 mice displayed an event sequence defect at ~5.5 months with progressive forelimb deficits starting at ~6 months.
Cortical activity associated with water-reaching increased over time in HD but not wildtype mice. Gross motor defects characterized using the tapered beam and rotarod tasks, as well as post-hoc striatal immunostaining, confirmed HD pathology at ~8 months.

Overall, at the nanoscale level, a biophysical and pharmacological characterization of mefenamic acid’s effect on $I_{Ks}$ highlighted a binding site and the potential of the NSAID to act as a precursor compound for LQTS therapeutic development. At the mesoscale level, a water-reaching task was developed and used to characterize HD phenotype demonstrating the potential of the behavioral task to examine therapeutic efficacy and intervention windows.
Lay Summary

Excitation underlies synaptic and axon transmission of action potentials in the brain and excitation-contraction coupling in the heart. In this work, excitability was examined across scales and systems to uncover therapeutic targets and develop screening tests. At the nanoscale, a biophysical and pharmacological characterization revealed that mefenamic acid can enhance the slow delayed cardiac rectifier current ($I_{Ks}$) in a dose- and rate-dependent fashion that is regulated by interactions with its β-subunit, KCNE1. Mefenamic acid was identified as a promising precursor compound in long QT syndrome therapeutic development. At the mesoscale level, a forelimb water-reaching task was developed and revealed event sequence and forelimb motor defects as well as changes in cortical activity in a Huntington Disease mouse model. The behavioral task could be used in the future to determine the onset and manifestation of other movement disorders, therapeutic intervention windows, and test drug efficacy.
Preface

Introduction

A version of the Introduction has been published in *Frontiers in Physiology* and *Neuron*:

- **Wang, Y., J. Eldstrom, and D. Fedida.** 2020. Gating and regulation of KCNQ1 and KCNQ1+KCNE1 channel complexes.


I wrote the text and designed and drafted the figures found in this chapter. Dr. Jodene Eldstrom and Dr. David Fedida revised the Kv7.1 / *I*<sub>Ks</sub> section (a version is published). Dr. Timothy H. Murphy and Jeffrey LeDue revised Sections 1.2.1-1.2.2 (a version is published).

Chapter 2: *In vitro* characterization of mefenamic acid modulation of Kv7.1

A version of Chapter 2 has been published in *Molecular Pharmacology*:

- **Wang, Y., J. Eldstrom, and D. Fedida** 2019. The *I*<sub>Ks</sub> ion channel activator, mefenamic acid, requires KCNE1 and modulates channel gating in a subunit-dependent manner.

Both myself, Dr. Jodene Eldstrom and Dr. David Fedida conceptualized and designed the study. I acquired, analyzed, and interpreted all the data found within this chapter. Except for the “Discussion” where I provided the initial draft which was edited by Dr. David Fedida, I wrote the remaining text found in this chapter. I drafted and designed all the figures found within this chapter. Mutant constructs were synthesized by Dr. Emely Thompson, Fariba Ataei and myself. Cell culture and transfections were performed by Fariba Ataei and myself, respectively.
Chapter 3: *In vivo* progressive monitoring of altered cortical activity and fine motor impairment in a Huntington Disease mouse model

A version of Chapter 3 has been published in *eNeuro*:


Both myself and Dr. Lynn A. Raymond conceptualized and designed the behavior and post-mortem experiment testing scheme. Dr. Dongsheng Xiao and Dr. Timothy H. Murphy designed the water reaching task. I acquired all the data. Except for Figures 3.14A and 3.15C which were analyzed by Dr. Marja D. Sepers as well as Days 9 and 23 which were scored by Dr. Marja D. Sepers and Evan Fung, respectively, I analyzed all other data found in this chapter. Both myself and Dr. Marja D. Sepers interpreted all the data. Dr. Marja D. Sepers and I wrote the custom Matlab code used for widefield cortical GCaMP activity analysis with assistance from Daniel Ramandi and Jeffery LeDue. I drafted and designed all figures and wrote the text found in this chapter which was revised by Dr. Timothy H. Murphy and Dr. Lynn A. Raymond. Surgeries were performed by Pumin Wang. Mouse genotyping was performed by Lily Zhang. All experiments and procedures were carried out in accordance with the Canadian Council on Animal Care and approved by the University of British Columbia Committee on Animal Care (protocols A18-0036 and A19-0076).
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<th>Definition</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4′-Diisothiocyanato-2,2′-stilbenedisulfonic acid</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>EC&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum effective concentration</td>
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<tr>
<td>EQ</td>
<td>Fully saturated I&lt;sub&gt;Ks&lt;/sub&gt; channel complex</td>
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<td>EQQ (2:4 KCNE1 to KCNQ1 ratio)</td>
<td>Unsaturated I&lt;sub&gt;Ks&lt;/sub&gt; channel complexes</td>
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<tr>
<td>EQQQQ (1:4 KCNE1 to KCNQ1 ratio)</td>
<td></td>
</tr>
<tr>
<td>ExLLSM</td>
<td>Expansion lattice light sheet microscopy</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>fUS</td>
<td>Functional ultrasound</td>
</tr>
<tr>
<td>F-V</td>
<td>Fluorescence-voltage</td>
</tr>
<tr>
<td>GECI</td>
<td>Genetically encoded calcium indicator</td>
</tr>
<tr>
<td>GEI</td>
<td>Genetically encoded indicator</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus</td>
</tr>
<tr>
<td>G-V</td>
<td>Conductance-voltage</td>
</tr>
<tr>
<td>HCP</td>
<td>Hexachlorophene</td>
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<td>HD</td>
<td>Huntington Disease</td>
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<tr>
<td>HMR1556</td>
<td>(3R,4S)-(+)-(3-hydroxy2,2-dimethyl-6-(4,4,4-trifluorobutoxy) chroman-4-yl)-N-methylmethanesulfonamide</td>
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<tr>
<td>I&lt;sub&gt;Ks&lt;/sub&gt;</td>
<td>Delayed cardiac rectifier potassium current</td>
</tr>
<tr>
<td>I&lt;sub&gt;max&lt;/sub&gt; – I&lt;sub&gt;min&lt;/sub&gt;</td>
<td>Peak to steady state difference current</td>
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<tr>
<td>I&lt;sub&gt;max&lt;/sub&gt; = peak current amplitude</td>
<td></td>
</tr>
<tr>
<td>I&lt;sub&gt;min&lt;/sub&gt; = minimum current amplitude</td>
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</tr>
<tr>
<td>I-V</td>
<td>Current-voltage</td>
</tr>
<tr>
<td>JLNS</td>
<td>Jervell and Lange-Nielson Syndrome</td>
</tr>
<tr>
<td>k</td>
<td>Slope factor</td>
</tr>
<tr>
<td>KCNE1</td>
<td>β-subunit (formally known as minK)</td>
</tr>
<tr>
<td>KCNQ1</td>
<td>α-subunit (Kv7.1)</td>
</tr>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage-gated potassium</td>
</tr>
<tr>
<td>LQTS</td>
<td>Long QT Syndrome</td>
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<tr>
<td>M1</td>
<td>Primary motor cortex</td>
</tr>
<tr>
<td>M2</td>
<td>Secondary motor cortex</td>
</tr>
<tr>
<td>mHtt</td>
<td>Mutant form of Huntington protein</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neuron</td>
</tr>
<tr>
<td>$n''$</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>PBA</td>
<td>Phenylboronic acid</td>
</tr>
<tr>
<td>PD</td>
<td>Pore domain</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Poly-unsaturated fatty acids</td>
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<tr>
<td>ROIs</td>
<td>Regions of interest</td>
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<td>rsplagl</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>ssptl</td>
<td>Somatosensory cortex trunk</td>
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<tr>
<td>ssplun</td>
<td>Somatosensory cortex area unassigned</td>
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<tr>
<td>STD</td>
<td>Standard deviation</td>
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<tr>
<td>$V_{1/2}$</td>
<td>Voltage at half maximal activation</td>
</tr>
<tr>
<td>VCF</td>
<td>Voltage clamp fluorimetry</td>
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<td>visp</td>
<td>Primary visual cortex</td>
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<td>VSD</td>
<td>Voltage sensor domain</td>
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Amino acid table

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<td>Ala</td>
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Acknowledgements

Thank you to Dr. Timothy H. Murphy, Dr. Lynn A. Raymond, Dr. Filip Van Petegem, Dr. Eric Accili and Dr. David Fedida for your expertise in multiscale techniques, mesoscale imaging, behavioral neuroscience, Huntington Disease and/or $I_{Ks}$ structure, function, and gating and/or scientific discussions.

Dr. Accili, thank you for chairing my committee meetings and for your mentorship. Dr. Murphy and Dr. Raymond, thank you to the both of you for your understanding, support, and mentorship throughout my degree. Dr. Van Petegem, thank you for your kind encouragement, support and mentorship. Thank you to everyone for scientific discussions as well as for inspiring me to continue pursuing research.

Thank you to the Canadian Institutes of Health Research for their grant support of the labs and for a personal CGS-M and Vanier-CGS award. Thank you also to the University of British Columbia for a 4Y Doctoral Fellowship and Faculty of Medicine Graduate Award as well as the Society for Neuroscience and Biophysical Society for conference grants.

Thank you to all my colleagues in the labs I have had the privilege of working in. In particular, thank you to Marja, Emely and Ying for teaching me whole-cell patch clamp in acute slice, culture and two-electrode voltage clamp, respectively. Thank you to Marja and Jeff for guiding me through the HD mesoscale paper and multiscale imaging technologies, respectively. Special thanks also go out to all the undergraduate and graduate students, post-doctoral fellows, colleagues, instructors, professors, and mentors I met through my extracurricular involvements.
In particular, thank you to Kelli, Matt and Elaine for your support of my professional goals and collaboration on several projects.

Most importantly, thank you Marja for your mentorship, friendship and never-ending generosity, enthusiasm, and encouragement. Thank you for believing in me even when I did not believe in myself. This thesis would not be possible without you. Thank you for standing by my side and supporting me through the darkest and brightest of moments. From the bottom of my heart thank you for all that you do - you are absolutely and fantastically inspirational!
Chapter 1: Introduction

The emergence of directed preclinical drug discovery and clinical development during the late 19th and first half of the 20th centuries, have spurred a plethora of advancements in medicine and patient well-being (Bosch and Rosich, 2008; Sakula, 1988). The journey through to final registration however, can be long consisting of multiple interacting phases (Kirsch, 2020). The transition from preclinical discovery to human phase trial testing and eventual registration, referred to here as ‘Clinical Development’, will not be examined or discussed.

**PRECLINCIAL DISCOVERY**

- Target selection & validation
- Assay development
- Synthesis, screening and optimization
- Animal model testing

**CLINICAL DEVELOPMENT**

- Clinical transition
- Phase 1
- Phase 2
- Phase 3
- Registration

*Figure 1.1 Preclinical discovery and clinical development timeline.* Timeline highlights common phases during drug development. Figure is adapted from (Kirsch, 2020).

The timeline highlights common phases during preclinical drug discovery: 1) target selection and validation, 2) assay development, 3) screening and chemical analog synthesis and optimization of the lead compound and finally, 4) animal model testing of the lead compound (Figure 1.1)(Hughes et al., 2011; Kirsch, 2020). Largely due to their high throughput and low cost, most screening and analog optimization for desired potency and efficacy is done using *in vitro* assays. After sufficient *in vitro* testing, the lead compound is selected for examination of effectiveness,
absorption, distribution, metabolism, elimination, and toxicity in animal models of disease with appropriate biochemical and behavioral assay(s). These later phases in drug discovery depend heavily on the validity and reliability of prior steps. Put in another way, the drug discovery project will likely fail in later stages if: 1) the selected target is not a pathway driving the underlying pathophysiology of the disease, 2) the selected target affects other critical processes in the body that when altered, prove fatal or produce severe side effects or 3) the assay used to quantify and compare effectiveness is not selective for the activity of interest relevant to the disease. Here in this thesis, I detail two example cases that examine different phases of preclinical drug discovery which span the cardiovascular and nervous systems as well as spatial and temporal scales.

**Example Case 1:** The delayed cardiac rectifier potassium current, $I_{Ks}$, plays an important role, especially at high heart rates, in the physiological shortening of the cardiac action potential (Sanguinetti et al., 1996). Unsurprisingly, mutations in KCNQ1 ($\alpha$-subunit, Kv7.1: voltage-gated potassium channel) and KCNE1 ($\beta$-subunit), which when paired together give rise to the $I_{Ks}$ current (Barhanin et al., 1996; Bendahhou et al., 2005; Sanguinetti et al., 1996), have been implicated in various cardiac arrhythmia syndromes such as atrial fibrillation and long QT syndrome (LQTS) (Y. H. Chen et al., 2003; Eldstrom and Fedida, 2011; Jervell and Lange-Nielsen, 1957; Olesen et al., 2014; Wang et al., 1996). With nearly all mutations seen in LQTS patients identified as loss-of-function and 50% of those loss-of-function mutations identified in the KCNQ1 subunit (Ackerman et al., 2011; Hedley et al., 2009), enhancing and activating $I_{Ks}$ currents has been suggested as a promising therapeutic approach for treating LQTS.
Several naturally occurring biomolecules and synthetic drugs are presently known to modify $I_{Ks}$ gating (Bollmann et al., 2020; Corici et al., 2013; Eldstrom et al., 2021; Hiniesto Iñigo et al., 2022; Sara I. Liin et al., 2018; Zheng et al., 2012) and may serve as the foundation for the synthesis and optimization of chemical analogs to produce a lead compound. To determine the potential of these known compounds, the binding site and mechanism of action (including how channel gating kinetics and channel structure are modified) needs to first be understood utilizing in silico molecular dynamic simulations combined with in vitro verification using assays such as whole-cell patch clamp as well as in silico modeling of channel gating kinetics. Accurate in silico models of $I_{Ks}$ channel gating may also be combined with other ion channel models such as hERG ($I_{Kr}$) to better describe and predict the consequences of specific ion channel modulations on the overall cardiac action potential, normal sinus rhythm and excitation-contraction coupling.

**Example Case 2:** Synaptic and circuit changes which precede progressive striatal medium spiny neuron (MSN) and cortical neuronal loss, in the case of Huntington Disease (HD) results in characteristic motor dysfunction, cognitive impairment, and neuropsychiatric symptoms (Cepeda and Levine, 2022; McColgan and Tabrizi, 2018). These characteristic motor symptoms of HD include chorea, bradykinesia, rigidity and difficulties with balance and gait. Although several assessments such as rotarod, balance beam and gait tasks such as the footprint test are commonly used to assess motor defects in HD mice (Abada et al., 2013; Brooks et al., 2012; Pallier et al., 2009; Southwell et al., 2016), bodyweight is a major confound of these tasks (Batka et al., 2014; McFadyen et al., 2003) necessitating the development and usage of other behavioral assessments. Given reaching towards a target and manipulating objects persist in our daily lives and the general ‘reach-to-grasp’ features of forelimb movements has been shown to be similar
between humans and mice (Galiñanes et al., 2018), preclinical behavioral assessment of skilled forelimb reaching tasks such as food pellet and water reaching tasks could improve our understanding of HD movement defects. Water reaching tasks further enable longitudinal multi-trial assessment providing a high throughput system for behavioral monitoring and phenotyping throughout the duration of HD progression. Combining \textit{in vivo} electrophysiology, single or multiphoton imaging with water reaching tasks would then enable capture of changes in functional brain activity linked to behavioral deficits. Longitudinal analysis will also inform disease onset and aid in the examination of therapeutic intervention windows. The development of the water reaching task as an \textit{in vivo} functional assay to potentially assess lead compound efficacy is therefore needed.

The two cases described above represent examples of the first two phases of preclinical discovery: target selection and validation as well as assay development (Hughes et al., 2011; Kirsch, 2020).

\textbf{In Example Case 1,} \(I_{K_s}\), the presently known synthetic activator compounds (Bollmann et al., 2020; Corici et al., 2013; Eldstrom et al., 2021; Hiniesto Iñigo et al., 2022; Sara I. Liin et al., 2018; Zheng et al., 2012) need to first be validated and characterized using whole-cell patch clamp, molecular dynamic simulations, structural biochemistry and \(I_{K_s}\) models of gating kinetics before downstream synthesis and optimization of chemical analogs can begin. \textbf{In Example Case 2,} HD, novel fine motor assessment tools need to be developed and validated first before their potential to inform downstream therapeutic efficacy can be explored. The remaining portions of this “Introduction” will first review Kv7.1 and \(I_{K_s}\) mechanisms of gating and known natural and synthetic modulators followed by a review of the importance of multiscale imaging approaches with a focus on HD.
1.1 EXAMPLE CASE 1: Gating and regulation of KCNQ1 and KCNQ1+KCNE1 ($I_{Ks}$)

1.1.1 The world of Kv7.1 and its accessory subunits

Potassium ion channels are categorized structurally and functionally into four major classes; calcium-activated, inward rectifying, tandem two-pore domain, and voltage-gated potassium (Kv) channels, and are known to play an essential role in cell signaling in both excitable and non-excitable cells. Within the Kv7 channel family, the tetrameric voltage-gated KCNQ potassium channel subfamily is comprised of five known isoforms, Kv7.1-7.5 (KCNQ1-5). Recent research has improved our understanding of the gating and regulatory mechanisms of the first isoform, Kv7.1, commonly referred to as KCNQ1, alone and in complex with its regulatory subunit KCNE1.

Expression of KCNQ1 has been detected throughout the body including in the heart, inner ear, pancreas, kidney, colon and intestine, stomach and thyroid gland (Cui, 2016; Liin et al., 2015; Nakajo and Kubo, 2015; Robbins, 2001). When expressed alone, KCNQ1 produces a fast activating and deactivating current which undergoes a mostly hidden inactivation, revealed as a hook in the tail current (Hou et al., 2017; Pusch et al., 1998; Sanguinetti et al., 1996; Tristani-Firouzi and Sanguinetti, 1998). The inactivation is quite apparent when KCNQ1 is expressed in mammalian cells (Meisel et al., 2018; Westhoff et al., 2019), but much less apparent when expressed in oocytes (Hou et al., 2017). This fast activating and deactivating KCNQ1 current has a very low conductance (Hou et al., 2017; Yang and Sigworth, 1998), but has yet to be positively identified with any endogenous current(s) in the body (Abbott, 2014). Unlike other members of the KCNQ channel subfamily, KCNQ1 has been reported to associate with all five single
transmembrane domain KCNE β-subunits, KCNE1-5, which modify channel kinetics to a greater
or lesser extent (Barhanin et al., 1996; Eldstrom and Fedida, 2011; Melman et al., 2002). The
sometimes dramatic modulation of KCNQ1 kinetics by specific KCNE subunits, and the
expression of different KCNE subunits in various tissues to a large degree underlie the diverse
physiological roles that KCNQ1 plays throughout the body (Sanguinetti, 2000).

KCNE1 co-expression with KCNQ1 increases current expression and greatly slows activation and
deactivation kinetics. Both KCNE2 and KCNE3 in combination with KCNQ1 induce
constitutively open channels not only across, but also beyond both ends of the physiological
voltage range, with KCNE3 producing currents with much larger current amplitudes (Schroeder
et al., 2000b). KCNQ4 and KCNQ5 on the other hand have an inhibitory effect when combined
with KCNQ1 and decrease current amplitude under physiological conditions (Angelo et al., 2002;
Grunnet et al., 2002; Schroeder et al., 2000a). As well as the KCNEs, KCNQ1 is also regulated by
several other proteins, intracellular signaling molecules and co-factors including
phosphatidylinositol 4,5-bisphosphate (PIP2), adenosine 5′-triphosphate (ATP), calmodulin
(CaM) and protein kinase A (PKA) (Loussouarn et al., 2003; Matavel and Lopes, 2009; Thompson
et al., 2017; Zaydman et al., 2013) (Figure 1.2) which in turn further increases the flexibility of
current complexes and contributes to their importance throughout body systems.
Figure 1.2 Binding site, signaling pathway and functional effect of various intracellular signaling molecules and co-factors on $I_{Ks}$.

Left Table: Functional effect of PKA phosphorylation, ATP, Ca$^{2+}$, CaM, CaM$_{1234}$, and PIP2 on $I_{Ks}$ where “+” and “−” denotes a stimulatory and inhibitory effect, respectively. “Obligatory” indicates ATP is required for channel conductance. Left panel: Cartoon of the single
transmembrane β-subunit, KCNE1, and the α-subunit, KCNQ1. KCNQ1 consists of 6 transmembrane domains (TM1-4 and TM5-6 form the voltage sensor and pore domain, respectively) and 4 helices (A–D). Four KCNQ1 subunits come together to form the channel with 1–4 KCNE1 subunits (variable stoichiometry). Binding sites for ATP, CaM/CaM_{1234} and PIP2 as well as sites of PKA phosphorylation are depicted using colored arrows (for binding sites) or directly on KCNQ1 (for PKA phosphorylation). Ca^{2+} is known to bind to CaM but not CaM_{1234} however, Ca^{2+} may modulate the channel by binding and interacting with other proteins and/or other locations on the I_{Ks} channel complex which are presently unknown. Right Table: The impact of KCNE1:KCNQ1 stoichiometry on the phosphorylation of residues S27 and S92 by PKA and the consequent functional effect. Checkmarks indicate PKA phosphorylation occurs, or a functional effect is seen on the respective stoichiometrically fixed KCNE1:KCNQ1 complexes (EQ, EQQ, EQQQQ, and KCNQ1). The “X” indicates a functional effect is not seen despite PKA phosphorylation of KCNQ1. Right panel: Signaling pathway for β-adrenergic enhancement of I_{Ks} current through PKA phosphorylation.
1.1.2 KCNE1 modulation of KCNQ1 kinetics in health and disease

The impact of KCNE1 co-assembly on KCNQ1 is the most studied and best understood of the accessory subunits. KCNE1 co-expression depolarizes the voltage dependence of activation (G-V) of KCNQ1 by about +50 mV, slows activation 1000-fold, and delays deactivation kinetics (Sanguinetti et al., 1996). A fully saturated 4:4 octameric complex of KCNE1 and KCNQ1 (EQ) has the slowest activation kinetics compared with KCNQ1 alone and the most positive half-activation potential (G-V V½). KCNE1 increases the macroscopic current by increasing the underlying single-channel conductance and stabilizing the open pore (Werry et al., 2013), while eliminating the inactivation seen in KCNQ1 channels (Hou et al., 2017; Meisel et al., 2018; Tristani-Firouzi and Sanguinetti, 1998). These pore effects of KCNE1 are accompanied by changes in the Rb⁺/K⁺ selectivity (Pusch et al., 2000; Zaydman et al., 2014) and in the pharmacological effects of various drugs (Abitbol et al., 1999; Hou et al., 2019; Wang et al., 2020; Yu et al., 2013) and fatty acid analogues (Larsson et al., 2018; S. I. Liin et al., 2018). Functionally, the co-assembly of KCNE1 with KCNQ1 reproduces most of the characteristics of the delayed rectifier potassium current, $I_{K_s}$, in the heart, although the occurrence of mutations in the other KCNE subunits (2-5) in clinical cases of LQTS (Eldstrom and Fedida, 2011) and lone atrial fibrillation (Olesen et al., 2014) indicate that the full exposition of $I_{K_s}$ likely requires a more complete understanding of the contributions of KCNE subunits other than KCNE1 to the channel complex.

$I_{K_s}$ together with $I_{Kr}$ (the hERG channel, and possible accessory subunits of its own) (Abbott et al., 1999) form the main repolarizing currents of the cardiac action potential (Barhanin et al., 1996;
Specifically, $I_{\text{Ks}}$ has been reported to create a “repolarization reserve” at fast heart rates, when channels can open and current summates between beats to generate a large repolarizing current which shortens the action potential and facilitates diastolic filling of the ventricles. As KCNE1 delays activation to such a large degree, the open probability of the channel reaches only about 0.2 after 4 seconds at room temperature (Werry et al., 2013), which suggests that $I_{\text{Ks}}$ channels remain 99.6% closed during the normal heartbeat (Ruscic et al., 2013) and limits their major contribution to action potential repolarization to sympathetic activation during times of stress.

Due to the critical role of $I_{\text{Ks}}$ in heart rhythm regulation, unsurprisingly, many mutations in KCNQ1 and KCNEs have been functionally linked to life-threatening long and short QT syndromes as well as atrial fibrillation (Y. H. Chen et al., 2003; Moss and Kass, 2005; Olesen et al., 2014; Peng et al., 2017; Steffensen et al., 2015; Wang et al., 1996). $I_{\text{Ks}}$ current has however, also been reported in the inner ear where it plays an important role in maintaining endolymph K$^+$ homeostasis. The flow of K$^+$ into hair cells within the cochlea depolarizes them and results in downstream hearing transduction to the brain. Unsurprisingly, some homozygous mutations in KCNE1 have been found to cause Jervell and Lange-Nielson syndrome (JLNS), a LQTS with deafness (Chouabe et al., 1997; Jervell and Lange-Nielsen, 1957). Other homozygous LQTS mutations are not associated with deafness (Jackson et al., 2014).

1.1.3 Structural properties of KCNQ1

The KCNQ1 $\alpha$ subunit like that of all other Kv channels is comprised of six transmembrane segments, S1-S6 (Lee et al., 2009)(Figure 1.2), and together four KCNQ1 monomers co-assemble
as a tetramer to form the KCNQ1 channel. Transmembrane helices S1-S4 form the VSD with the S5-S6 helices forming the pore domain (PD) of the channel in a domain-swapped manner – that is the VSD of one domain regulates the PD of the adjacent subunit (Long et al., 2005; Sun and MacKinnon, 2017). In particular, the S4 segment of KCNQ1 contains a net total charge of +3 which arises from positively charged arginine residues, and this is unlike other Kv channels which contain two extra gating charges.

During gating, salt bridges between a conserved glutamate (E160) in S2, and positive charges in S4 (R228) present at rest, are broken and reformed with R231 and R237 as the S4 moves outward (Cui, 2016). Aqueous clefts allow extracellular or intracellular accessibility to most of these charges at rest and during channel activation as the gating charges in the S4 segment sense and move in response to changes in transmembrane voltage. The PD on the other hand contains an ion selectivity filter made up of a series of highly conserved amino acids in potassium channels, GYGDX. In addition to the six transmembrane segments which make up the VSD and PD, the KCNQ1 monomer also contains four intracellular helices (Figure 1.2).

In order to open the pore of the KCNQ1 tetramer, alone, or in complex with KCNE1, the VSD must activate, and this means that upon membrane depolarization the S4 segment moves in the extracellular direction with some degree of rotation to transfer charges across the electric field from the inside to the outside across a charge transfer centre (Tao et al., 2010). How the four VSDs within the tetramer act, whether independently, or together to open the channel pore is controversial, but it is generally understood that this translation of the S4 segment is first transferred to the S4-S5 linker. In response to this movement in the linker, the cytoplasmic lower
halves of the S6 segment, which normally form a barrier to ion passage, pull apart at the PXG motif, which leads to pore opening (reviewed in (Labro and Snyder, 2012)). Potassium ions can then enter the selectivity filter and dehydrate, transiting the filter in coordination with backbone carbonyl oxygen atoms, along what is referred to as a “low resistance pathway” for efficient conduction of $K^+$ (Doyle et al., 1998).

1.1.4 Co-assembly of KCNQ1 with KCNE1

The subunit assembly ratio of KCNE1 to KCNQ1 has been a matter of debate over the past several years, with some groups reporting a fixed KCNE1:KCNQ1 ratio of 2:4 and others reporting a variable stoichiometry between 1:4 and 4:4 depending on the concentration of KCNE1. In biochemical studies involving subunit counting and also in fluorescence photobleaching, the suggestion is that the stoichiometry is fixed at 2:4 (H. Chen et al., 2003; Morin and Kobertz, 2008; Plant et al., 2014). However, there are also suggestions that the number of KCNE1 subunits bound to KCNQ1 can be variable, based on the fact that the gating of KCNQ1 is dependent on the expression level of KCNE1 (Cui et al., 1994; Wang et al., 1998; Yu et al., 2013), the pharmacological properties (Yu et al., 2013) and other single subunit counting experiments which support a variable stoichiometry of up to 4:4 (Nakajo et al., 2010). Experiments using linked constructs of KCNE1 and KCNQ1 which force the stoichiometry of KCNE1 to KCNQ1 from between 1:4 to 4:4 (EQQQQ, EQQ, EQ) have definitively established the ability of the two proteins to assemble in different stoichiometries (Murray et al., 2016; Nakajo et al., 2010).

Current activation becomes progressively slower as more KCNE1 subunits are added into the KCNQ1 ion channel tetramer compared with KCNQ1 alone. Changes in macroscopic channel
kinetics show the G-V curve of \( I_{Ks} \) becoming progressively depolarized with more KCNE1 subunits in the \( I_{Ks} \) complex, and increased single channel conductance and latency to first opening in 4:4 (EQ) compared with 1:4 (EQQQQ), 2:4 (EQQ), and 0:4 (KCNQ1 alone) complexes (Hou et al., 2017; Murray et al., 2016). Co-expression of KCNE1 along with the fixed 2:4 (EQQ) and 1:4 (EQQQQ) constructs leads to incorporation of free KCNE1 subunits into spare sites in the channel complex and recapitulation of the whole cell and single channel kinetics, G-V curve and conductance of the 4:4 (EQ) fully saturated heteromeric channel. The use of targeted unnatural amino acid expression to incorporate different numbers of cross-linkable KCNE1 subunits into \( I_{Ks} \) complexes and predictably change the UV-induced current decay rate provides further strong support for the variable stoichiometry model of KCNQ1 and KCNE1 assembly (Murray et al., 2016; Westhoff et al., 2017). In support of these conclusions, the cryo-EM structure of KCNQ1+KCNE3 was captured in a 4:4 stoichiometry (Sun and MacKinnon, 2020).

Having established the potential for different numbers of KCNE1 forming complexes with KCNQ1 in oocytes and mammalian cell expression systems, the stoichiometry of association is by no means as clear physiologically in various mammals, including humans. The half-activation voltage and deactivation rates for \( I_{Ks} \) vary between dog, guinea-pig and rabbit (Chinn, 1993; Lei and Brown, 1996; Liu and Antzelevitch, 1995), all mammals used to model human cardiac electrophysiology, which suggests the possibility that different species have different KCNQ1:KCNE1 stoichiometries underlying their \( I_{Ks} \). The kinetics in human myocytes (Bosch et al., 1998; Virag et al., 2001) and drug sensitivity of iPSC-CM to ML277 suggests an unsaturated ratio of 2:4 KCNE1:KCNQ1 (Yu et al., 2013), although the maturity and uniformity of iPS cells is not fully understood (Pourrier and Fedida, 2020). As well, different primary sequences for the
KCNE1 subunits may contribute to the different physiological expression and kinetics of $I_{Ks}$ in different species.

An understanding of the stoichiometry of $I_{Ks}$ is however, extremely important, since determining the exact numbers of regulatory subunits impacts the efficacy of pharmacological drugs, which has consequences for the treatment of cardiac arrhythmic disorders associated with mutations in KCNQ1 and KCNE1. Most activators of the $I_{Ks}$ channel complex such as ML277, L-364,373 and zinc pyrithione, with the exception of mefenamic acid (Abitbol et al., 1999; Doolan et al., 2002; Magyar et al., 2006; Wang et al., 2020), phenylboronic acid (Mruk and Kobertz, 2009), and some polyunsaturated fatty acids (Bohannon et al., 2020; Larsson et al., 2018; S. I. Liin et al., 2018) are reported to have limited efficacy on both partially and fully saturated $I_{Ks}$ complexes (Yu et al., 2013). Post-translational modification of KCNQ1 during β-adrenergic stimulation is also stoichiometrically graded with a response to cyclic adenosine monophosphate (cAMP) requiring at least one KCNE1 (Thompson et al., 2018b). A variable stoichiometry model for $I_{Ks}$ therefore allows for great flexibility in the modulation of KCNQ1 kinetics and the $I_{Ks}$ current.

### 1.1.5 Activation models

Mechanistically, our understanding of the gating properties of voltage-dependent potassium channels such as KCNQ1 and $I_{Ks}$ originates from analyses carried out on Shaker channels and the squid axon. The Markov state models used to simulate the activation of KCNQ1 and KCNQ1+KCNE1 channels originates in the Eyring rate theory models of (Eyring, H., Lumry, R., and Woodbury, J. W., 1949; Hodgkin and Huxley, 1990). The basic assumptions of their model for the squid axon potassium channel included the idea of four identical and independent gating
particles that, if positively charged, would cross the electric field from the interior of the axon to
the outside before the channel could open. This led to a five-state sequential gating scheme
comprising four closed states and an open state, many key features of which are still relevant
almost 70 years later, not least because the cloning of potassium channels has revealed a tetrameric
structure of VSDs that fits into the Hodgkin-Huxley model scheme (Jan and Jan, 1989).

In voltage-dependent Shaker channels it is generally accepted that, like in the original Hodgkin
and Huxley model, the delay seen in Shaker channel activation requires that more than one VSD
transition must occur before channel opening, and thus that all four identical VSDs must undergo
independent conformational changes when the channel is closed. Unlike the original model, each
VSD in the case of Shaker channels is preferred to undergo two independent conformational
changes (resting to intermediate to activated) before the VSD becomes activated (Zagotta et al.,
1994). Once all four VSDs are activated, the channel undergoes a final or multiple concerted
transition(s) (Ledwell and Aldrich, 1999; Schoppa and Sigworth, 1998) from closed to open. This
final transition(s) is required because channel opening after the activation delay is slower than
predicted from a strict Hodgkin-Huxley model.

These concepts from squid axon and Shaker potassium channels were first adapted into KCNQ1
and \( I_{Ks} \) channels by (Silva and Rudy, 2005). The activation delay observed suggests that the VSDs
of KCNQ1 and \( I_{Ks} \), much like those of Shaker, must also undergo at least two transitions during
VSD activation, prior to PD opening. As such, both KCNQ1 and \( I_{Ks} \) models assumed a scheme
where four VSDs transition independently through two conformational changes prior to full
activation, and then undergo a single concerted step to allow PD opening. To simulate slow
activation in $I_{Ks}$, the first VSD transition in the KCNQ1 model from resting to intermediate states was slowed. Both the second VSD transition rate as well as the transition between the first and second open state were further modified to be rapid. Together, these modifications ensured that more channels would remain in the resting state for a longer time and slow $I_{Ks}$ activation. Experimentally, a delay in KCNQ1 deactivation was also seen, suggesting the presence of multiple open states between the inactivated state and the last closed state for the KCNQ1 gating model.

In KCNQ1 and $I_{Ks}$ this led to models with 15 closed states, and different open states, and in the case of KCNQ1 alone, an inactivated state. Five open states were introduced for KCNQ1 alone, and two for $I_{Ks}$ to account in part for activation delay and for multi-exponential deactivation kinetics. The Silva and Rudy $I_{Ks}$ gating model was later briefly modified in 2013 to account for single channel conductance behaviors seen in $I_{Ks}$ (Werry et al., 2013).

The Silva and Rudy models, like the Hodgkin and Huxley models, are classical in the sense that data from direct interrogation of VSD movement (either gating current measurements or voltage clamp fluorimetry (VCF)) and from single channel measurements were not available at the time the models were developed. In the past decade, data from VCF and other techniques has been collected improving our understanding of the underlying molecular events during KCNQ1 and $I_{Ks}$ activation.

VCF tracks changes in emission from fluorophores attached to the extracellular end of the S4 segment as its environment changes during voltage sensing (Cha and Bezanilla, 1997; Claydon and Fedida, 2007; Horne et al., 2010; Mannuzzu, L. M., Moronne, M. M., and Isacoff, E. Y., 1996),
and while the signals from different fluorophores can vary, it has generally been shown to give a reliable measure of S4 displacement in a variety of Kv channels (Larsson, H. P., Baker, O. S., Dhillon, D. S., and Isacoff, E. Y., 1996). Fluorescence-voltage (F-V) relationships for KCNQ1 and $I_Ks$ have usually been obtained after removal of extracellular cysteines in the S3 and S6 domains, and the mutation and labeling of G219C in the S3-S4 linker with Alexa Fluor 488 C5-Maleimide (Alexa-488) (Barro-Soria et al., 2014; Hou et al., 2017; Osteen et al., 2010; Westhoff et al., 2019). The C214A/G219C/C331A construct shows a $V_{1/2}$ of ionic current activation that is $\sim 10$ mV hyperpolarized to wildtype (WT), but otherwise has unchanged current kinetics. Tetramethyl rhodamine (TMR) has also been used as a fluorophore, as have other locations in the S3-S4 loop (e.g. K218, and V221). A comparative study showed that these give similar, but not identical F-V relationships, with TMR exhibiting a fluorescence quenching upon depolarization, and the Alexa-488 at the 221C site giving the most hyperpolarized F-V relationship (Barro-Soria et al., 2014).

What is generally accepted in all studies is that KCNE1 dramatically increases the voltage separation between the voltage sensor fluorescence F-V and the G-V, and the fluorescence signal itself is clearly divided into two activating components, $F_1$ and $F_2$ (Barro-Soria et al., 2014; Nakajo and Kubo, 2014; Osteen et al., 2012, 2010; Westhoff et al., 2019; Zaydman and Cui, 2014). The $V_{1/2}$ of the $F_1$ component, which comprises about 2/3 of the fluorescence signal, is hyperpolarized to close to -100 mV in the presence of KCNE1, while the $V_{1/2}$ of the $F_2$ component is at $\sim +20$ mV, close to the $V_{1/2}$ of the G-V which is depolarized 40-50 mV in the presence of KCNE1. Gating charge movement shows a similar hyperpolarization when KCNQ1 is co-expressed with KCNE1, overlaying the voltage dependence of $F_1$ and confirming that the hyperpolarized $F_1$-V represents
S4 displacement (Barro-Soria et al., 2014). It has not yet been possible to resolve gating currents that correlate with the F2 movement, and this is attributed to the slow time course of F2-dependent charge movement. However, external MTSET modification of cysteine residues placed near the top of S4 show two-step modification with depolarizations to 0 mV or above, and thus support the idea of two VSD movements during $I_{Ks}$ activation (Barro-Soria et al., 2014).

KCNE1 therefore imposes a discordance between the time course of current and fluorescence signals and steady-state changes, which together with the F-V to G-V separation, overall implies that KCNE1 induces a requirement for movement of multiple voltage sensors before channel opening (Barro-Soria et al., 2014; Osteen et al., 2010). A sequential gating scheme requiring all four VSDs to be activated prior to PD opening, with a single open state has been introduced. The overlap of the F2 component of the F-V curve with the G-V suggests that a concerted activation step involving all four VSDs from the intermediate to activated state occurs, after which the channel opens. This model is similar to cooperative gating schemes described for Shaker channels, and early $I_{Ks}$ models (Silva and Rudy, 2005) which also invoked a concerted VSD step prior to channel opening.

Other groups have explained the presence of F1 and F2 components in the F-V and the wide separation of the F1-V and the G-V in KCNQ1+KCNE1 with a model that not only includes two state transitions for the VSDs, but also assumes independent VSD movement (Zaydman et al., 2014). Although constitutive currents are seen in the KCNQ1 charge reversal mutants (E1R/R2E and E1R/R4E which arrest VSDs in the intermediate and activated states, respectively), when these mutants are co-expressed with KCNE1, the E1R/R2E $I_{Ks}$ currents are eliminated whereas the
E1R/R4E $I_{K_S}$ currents increase 10-fold. The absence of E1R/R2E $I_{K_S}$ currents suggests that in the presence of KCNE1, the PD cannot open when VSDs are in the intermediate state but can open when 0–4 VSDs are in the activated state. Thus, the second fluorescence step is equated with VSD transitions and not PD opening. From these experiments intermediate-open (IO) states were omitted from the 30-state KCNQ1 model, to generate a model for $I_{K_S}$ where at least one VSD must be activated for the PD to open regardless of the state of the other VSDs (Zaydman et al., 2014).

The development of KCNQ1 gating models since that proposed by Silva and Rudy and KCNQ1 inactivation will not be discussed at length due to the lack of physiological relevance of the KCNQ1 current (Abbott, 2014). Briefly, it is generally accepted that when expressed without KCNE1 the fluorescence waveforms and the F-V relationship (representing VSD movement) of KCNQ1 overlaps quite well with the time course of current activation and better with the G-V (representing pore opening) curves, respectively (Nakajo and Kubo, 2014; Osteen et al., 2010). This overlap suggests that each VSD movement contributes in a 1:1 ratio to channel conductance (Osteen et al., 2010) such that KCNQ1 channels can open when only a single VSD is activated.

An updated 30-state allosteric model has been proposed where the VSD could either be in the resting, intermediate or activated states and the PD could either be closed or open (Zaydman et al., 2014). Allosteric coupling between the voltage sensor and PD was also further decomposed in this model to its elementary components, “k” which represents PD opening and VSD activation and, “θ” which represents VSD-PD coupling. A recent reinterpretation of inactivation in KCNQ1 channels has also been proposed based on a KCNQ1 activation model with two open states and is cognizant of changes in Rb$^+$/K$^+$ conductance ratio (Hou et al., 2017) since selectivity differences
also exist between KCNQ1 which undergoes inactivation and $I_{Ks}$ which does not undergo inactivation (Pusch, 1998; Pusch et al., 2000; Seebohm et al., 2003).

KCNQ1 alone has been suggested to undergo two inactivation processes. A fast inactivation phase is sometimes seen after the initial current activation, but is most clearly revealed and studied as a transient increase in conductance after membrane repolarization to potentials which exceed −50 mV (Hou et al., 2017; Meisel et al., 2018; Pusch et al., 1998). An additional voltage-dependent slow inactivation phase has also been reported primarily in mutant KCNQ1 channels including the LQTS-associated mutations S338W (S339 in hKCNQ1) and L273F (Gibor et al., 2007; Hou et al., 2017; Meisel et al., 2018). Although various molecular mechanisms for inactivation have been proposed, the two KCNQ1 inactivation processes are in contrast to the classical N-, C- and U-type mechanisms of inactivation.

1.1.6 Modulators of KCNQ1 and $I_{Ks}$

The kinetics of KCNQ1 are not only affected by co-assembly with KCNE1, but also by modulation induced by several intracellular signaling molecules and co-factors such as PIP2, CaM, ATP, and PKA. Figure 1.2 graphically summarizes the signaling pathway for β-adrenergic enhancement of $I_{Ks}$ (right panel) as well as the binding sites (left lower panel) and impact of these intracellular proteins on $I_{Ks}$ (top tables). Beyond these naturally occurring modulators are a number of synthetic compounds which act on either KCNQ1 and/or $I_{Ks}$. 
1.1.7 PIP2 is essential for VSD-PD coupling

PIP2, a co-factor of various channels such as voltage-gated potassium and calcium channels, transient receptor potential channels and epithelial sodium channels, is also an essential co-factor of KCNQ1. Molecular dynamics simulation and other experimental results previously mapped the binding site of PIP2 to residues in the S2-S3 and S4-S5L and the cytoplasmic end of the S6, placing PIP2 in a location in which it can interact with both the VSD and PD and thus affect VSD-PD coupling (Eckey et al., 2014; Thomas et al., 2011; Zaydman et al., 2013). In support of this, a cryo-EM structure with PIP2 bound shows where the lipid is located: near positive charges in the N-terminal domain (R116) and S2-S3L (R181, K183, R195, K197) but also L234 in S4, Q244, W248 and R249 in the S4-S5L as well as K87 of KCNE3 (Sun and MacKinnon, 2020).

PIP2 depletion is known to decrease $I_{Ks}$ channel currents (Loussouarn et al., 2003; Park et al., 2005) but this depletion does not prevent S4 movement indicating the loss of PIP2 uncouples VSD movement from opening of the pore (Barro-Soria et al., 2017; Zaydman et al., 2013). In support of this, mutations that prevent pore closure and show instantaneous currents during step potential changes are insensitive to PIP2 depletion (e.g. KCNQ1-L353K; (Zaydman et al., 2013)) or lose their voltage-dependent component (KCNQ1-I268A; (Barro-Soria et al., 2017). The cryo-EM structure of KCNQ1 in the absence of PIP2 further demonstrated the importance of PIP2 in VSD-PD coupling revealing a KCNQ1 structure with activated VSDs but closed PD (Sun and MacKinnon, 2017).
Molecular dynamic simulations and an NMR structure have shown that KCNE1 sits in the lipid-filled cleft between two adjacent VSDs and the PD, probably not particularly dissimilar to where KCNE3 is found in cryo-EM structures, (Sun and MacKinnon, 2020), and based on this positioning is well placed to affect VSD-PD coupling. Consistent with this, mutations in the S4-S5L of KCNQ1 alone such as L251A, V255W, H258A and T247A, have produced similar changes to the activation waveform and G-V as those seen when KCNE1 is present in the complex (Labro et al., 2011). In the presence of KCNE1, the channel complex has also been shown to become more sensitive to PIP2 as a significantly lower concentration of exogenous PIP2 was required to prevent current rundown upon excision of membrane patches.

1.1.8 CaM modulates current amplitude and assembly

Calmodulin (CaM), a highly conserved calcium binding protein has been recognized as a key modulator of various channels such as Cav1.2 and Nav1.5, all of which are involved in the regulation of the cardiac action potential (Sorensen et al., 2013). CaM consists of two EF hand pairs capable of binding a total of four Ca$^{2+}$ ions (Babu et al., 1985). Asp to Ala mutations at position one of each EF hand have been used to disrupt Ca$^{2+}$ binding to CaM (CaM$_{12}$: Ca$^{2+}$ binding sites in the N-lobe EF hand are disrupted; CaM$_{34}$: Ca$^{2+}$ binding sites in the C-lobe EF hand are disrupted; CaM$_{1234}$: all four Ca$^{2+}$ binding sites are disrupted). These Ca$^{2+}$ insensitive CaM constructs (CaM$_{12}$, CaM$_{34}$ and CaM$_{1234}$) have previously been deployed to determine the functional role of Ca$^{2+}$ and CaM.

One of the first pieces of evidence that calcium and potentially calcium binding proteins could modulate $I_{Ks}$ was the sensitivity of the current to calcium in guinea pig cardiomyocytes (Tohse,
Since mutations in CaM, KCNQ1 and KCNE1 have all been linked to LQTS, it is not surprising that CaM plays a diverse role in both \( I_{Ks} \) assembly and gating. Using metal affinity chromatography, CaM was found to co-purify with a 6xHis-tagged C-terminus portion of KCNQ1 (Ghosh et al., 2006). Binding of CaM to the C-terminus portion of KCNQ1 was later confirmed using immunoprecipitation in both the presence and absence of \( \text{Ca}^{2+} \) (Shamgar et al., 2006) as well as structurally using x-ray crystallographic data (Dvir et al., 2014b). This C-terminus structure was also further confirmed in the cryo-EM structure of KCNQ1 (Sun and MacKinnon, 2017). Specifically, CaM was found to bind to the two proximal cytosolic helices (helices A and B) in a 1:1 ratio of KCNQ1 to CaM. In addition to this C-terminal binding site however, the cryo-EM structure also revealed a novel CaM binding site located at the S2-S3 loop of the VSD not present in the original crystallographic structural representation which did not contain the transmembrane domains of the channel (Sachyani et al., 2014; Sun and MacKinnon, 2020, 2017). Thus, by interacting with both the VSDs at the S2-S3 loop and helices A and B (Figure 1.2 left panel), CaM was proposed to provide a potential link between the VSD and PD. In support of this hypothesis, PIP2, previously found to be critical in VSD-PD coupling has been shown to compete with CaM for binding at helix B at specifically residues K526 and K527 (Tobelaim et al., 2017). As such, CaM, much like PIP2, may also act to stabilize the channel in its open state.

Beyond this recent discovery that CaM may play a role in VSD-PD coupling, classically, CaM has more consistently been reported to be important for channel assembly (Ghosh et al., 2006; Sachyani et al., 2014; Shamgar et al., 2006). Point mutations in helix A (1375D) and helix B (V516D), which altered CaM binding, have been shown to reduce protein expression and decrease current density suggesting that CaM may be required for proper channel assembly, folding and
tetramerization (Haitin et al., 2008; Sachyani et al., 2014). In addition, KCNQ1 has also been reported to traffic to the cell surface in a process dependent on Ca$^{2+}$ and CaM. Jiang et al. have shown that, following Angiotensin II type 1 receptor (AT1R)-mediated KCNQ1 trafficking, the channel may then combine with KCNE1 which in turn increases $I_{Ks}$ current amplitude (Jiang et al., 2017). The Ca$^{2+}$-insensitive CaM mutant, CaM$_{1234}$ however, was shown to prevent AT1R stimulation from increasing $I_{Ks}$ current density, suggesting that CaM not only plays a role in channel assembly and tetramerization but also in trafficking.

Electrophysiological studies have also provided consistent evidence supporting the important roles calcium and CaM play in enhancing both KCNQ1 and $I_{Ks}$ current. Increasing intracellular Ca$^{2+}$ concentrations has been found to enhance KCNQ1 current amplitude (Shamgar et al., 2006). Consistent with this, chelation of Ca$^{2+}$ by BAPTA has been reported to decrease KCNQ1 current amplitude (Ghosh et al., 2006) with the previously reported increase in current amplitude following administration of intracellular Ca$^{2+}$ (Shamgar et al., 2006) possibly explained by the ability of CaM to relieve KCNQ1 inactivation in a Ca$^{2+}$ dependent manner (Ghosh et al., 2006). In the presence of KCNE1, the role of CaM in enhancing current amplitude is preserved. Both the co-assembly with CaM$_{1234}$ (Sachyani et al., 2014) and bath application of the CaM antagonist W7 (Shamgar et al., 2006) have been reported to decrease $I_{Ks}$ current amplitude. Interestingly, CaM$_{12}$ but not CaM$_{34}$ was also shown to decrease $I_{Ks}$ current amplitude, suggesting that not only is CaM able to modulate $I_{Ks}$, but its modulation is highly dependent on Ca$^{2+}$, and specifically where Ca$^{2+}$ binds in the KCNE1-KCNQ1-CaM complex (Sachyani et al., 2014).
From a cell signaling point of view, the stimulatory action of CaM has been shown to be modulated by calcium/calmodulin-dependent protein kinase II (CaMKII). Intracellular application of the CaMKII inhibitor, autocamtide-2 as well as another inhibitor, KN-93, reduced $I_{Ks}$ current amplitude. KN-93 preincubation was also able to prevent any stimulatory action of intracellular CaM administration suggesting that CaMKII may act to turn off CaM enhancement of $I_{Ks}$ (Xie et al., 2015). However, more studies to confirm and uncover the full signaling pathway of CaM-mediated $I_{Ks}$ enhancement and channel assembly are still needed.

### 1.1.9 ATP is essential for channel opening

Several studies have demonstrated that the removal of ATP decreases or completely abolishes $I_{Ks}$ activity, with current rescued by the reintroduction of ATP (Bezanilla et al., 1986; Loussouarn et al., 2003). Using photo-crosslinking, the phosphates of ATP were suggested to electrostatically interact with a cluster of basic residues (R380, K393 and R397), whereas the nucleoside moieties of ATP were suggested to interact with hydrophobic aromatic residues such as W379 in the helix A to helix B region downstream from the S6 segment (Li et al., 2013) (Figure 1.2 left panel). Functional studies have indicated that, although both W379S and the double mutant R380S/R397W abolish KCNQ1 currents, F-V signals are still present and are identical to that of WT KCNQ1, which indicates that ATP binding is not required for VSD movement. Moreover, in a prior study, PIP2, known to be essential for VSD-PD coupling, was found to hyperpolarize the F-V curve of a constitutively open KCNQ1 mutant, L353K (Zaydman et al., 2013). This shift was abolished in the absence of PIP2, but removal of ATP sites in the background of L353K did not eliminate the displacement of the F-V curve, suggesting that ATP is perhaps not involved in VSD-PD coupling (Li et al., 2013). These results suggest that ATP plays a role in PD opening through
interactions with the KCNQ1 C-terminus, but other ATP binding sites may exist which could in the future reveal a role for ATP in either VSD movement and/or VSD-PD coupling.

1.1.10 β-adrenergic enhancement of current through PKA phosphorylation

Under stressful conditions, circulating sympathetic hormones such as epinephrine bind to and activate G-protein coupled β-adrenergic receptors leading to the release of the α-subunit of the G-protein (Terrenoire et al., 2005)(Figure 1.2 right panel). This α-subunit in turn activates adenyl cyclase 9 which leads to an increase in the intracellular levels of cAMP. The binding of cAMP then activates PKA, which phosphorylates the C-terminus of KCNQ1 in the presence of Yotiao (Marx, 2002). Specifically, PKA phosphorylation has previously been described to occur at residues S27 and S92 (Lopes et al., 2007; Lundby et al., 2013; Marx et al., 2002). Although KCNQ1 alone has been found to be largely unresponsive to the functional transduction of PKA phosphorylation (Kurokawa et al., 2003), the phosphorylation of the N-terminus of KCNQ1 in the presence of KCNE1 produces a left-shift in the voltage dependence of activation, a slowing of deactivation kinetics and an increase in current amplitude (Dilly et al., 2004; Terrenoire et al., 2005). Together these changes in kinetics increase repolarizing current and shorten the cardiac action potential to allow sufficient time for ventricular filling. PKA-mediated phosphorylation is later removed by protein phosphatase 1. cAMP is degraded by the cAMP-specific phosphodiesterase, PDE4D3.

Using linked constructs which fix the KCNE1:KCNQ1 stoichiometry at different ratios (Murray et al., 2016), the hyperpolarizing shift in the voltage dependence of activation following cAMP exposure was progressively increased with increasingly saturated $I_{Ks}$ complexes: the more KCNE1
in the complex, the greater the hyperpolarization, which showed that not only is the action of cAMP on \( I_{Ks} \) stoichiometrically dependent, but also that only one KCNE1 subunit is required for a basal response of the \( I_{Ks} \) complex to cAMP (Thompson et al., 2018b). In support of this latter finding, shortening of the first latency to opening in the presence of cAMP was seen in \( I_{Ks} \) constructs with KCNE1:KCNQ1 ratios of 1:4 (EQQQQ) and 2:4 (EQQ), similar to that seen in fully saturated \( I_{Ks} \) complexes (EQ). Using a phosphomimetic KCNQ1 mutant, this shortening of the first latency following cAMP treatment seen in WT channels was reduced but not completely abolished in the mutant S27D channel, indicating that this residue is only partly responsible for the shortening of first latency seen post-cAMP (Thompson et al., 2018a). Although phosphorylation of residue S27 plays a partial role in shortening of the first latency, it appears to be largely responsible for cAMP mediated changes in open probability. The double phosphomimic mutant S27D/S92D on the other hand, completely abolished any changes in first latency, single-channel conductance and subconductance distribution as a result of cAMP exposure. Mechanistically, these effects of phosphorylation are largely mediated by enhancement of VSD activation, allowing the channel to open more frequently, quicker, and to higher sublevels, as mutant channels with fixed-activated VSDs showed little further response to cAMP (Thompson et al., 2017).

1.1.11 Synthetic compound modulation of KCNQ1 and \( I_{Ks} \)

To date, although several activators have been reported, many however, are only effective on KCNQ1 alone with limited efficacy on physiologically relevant saturated and unsaturated \( I_{Ks} \) complexes. These activators include ML-277, zinc pyrithione and L-364,373 (Gao et al., 2008; Magyar et al., 2006; Willegems et al., 2022; Yu et al., 2013).
The known activators of $I_{Ks}$ are rottlerin, phenylboronic acid (PBA) (Matschke et al., 2016; Mruk and Kobertz, 2009) and hexachlorophene (HCP) (Zheng et al., 2012), as well as stilbenes such as 4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid (DIDS) and 4-acetamido-4’-isothiocyanatostilbene-2,2’-disulfonic acid (SITS), fenamates such as mefenamic acid (Abitbol et al., 1999), and fatty acids such as lauric acid (Doolan et al., 2002). PBA and HCP have been shown to increase both KCNQ1 alone and $I_{Ks}$ current amplitudes, although they are more potent on $I_{Ks}$ (Mruk and Kobertz, 2009; Zheng et al., 2012). In contrast, lauric acid, DIDS and SITS have been shown to mainly increase $I_{Ks}$ current (Abitbol et al., 1999; Doolan et al., 2002). Presently, no binding site has been proposed for PBA and HCP. Rottlerin is suspected to bind in the same spot as known KCNQ1 activators such as L-364,373 (Matschke et al., 2016). Residue E43 has been proposed to critically impact DIDS and mefenamic acid (Abitbol et al., 1999). PUFAs in contrast are known to bind at the voltage sensor and pore with critical residues identified as R218, R221, K316 and Y268 (Yazdi et al., 2021).

With the exception of PUFAs, a complete biophysical characterization of the effects of $I_{Ks}$ activators - drug concentration and rate-dependent changes to current waveforms, the conductance-voltage (G-V) relationship, single channel conductance and consideration of potency on different stoichiometric $I_{Ks}$ complexes - are largely unavailable. The binding site and mechanism of action of $I_{Ks}$ activators are also largely unknown. Given 50% of loss-of-function LQTS mutations are identified in the KCNQ1 subunit (Ackerman et al., 2011; Hedley et al., 2009), understanding the mechanism of action of known $I_{Ks}$ activators is readily needed and could uncover promising LQTS therapeutic approaches. The aim of Example Case 1 is therefore to characterize mefenamic acid effects on Kv7.1 and $I_{Ks}$.
1.2 EXAMPLE CASE 2: Multiscale imaging informs Huntington Disease

Since Roman physician Galen’s support of the encephalocentric theory, the localization of brain function is a de rigueur component of any neuroscience investigation (Dunn, 2003). Matching pathological findings with patient symptoms by Broca and other researchers defined early neuroanatomical principles and led to the idea that select brain areas mediate specific functions (Penfield and Boldrey, 1937). However, the search for the “engram”, the physical traces of memory using lesion studies in animal models, failed to find a localized spot or engram in the brain (Lashley, 1950). Lashley instead proposed that memory of a task was stored in multiple brain regions and damage to one area would not knock out memory, but rather lead to compensatory function of other brain areas. Since then, this idea of distributed functional connectivity has gained support in neuroscience and now requires the use of multiscale tools for its assessment.

Recent imaging and electrophysiological findings support dynamic wide-scale functional connectivity. Wide field imaging of voltage sensitive dyes and intrinsic signals have demonstrated that a single whisker deflection evokes activity first in the primary somatosensory barrel cortex then the whisker motor cortex and eventually affecting activity over wide regions that encompass both cortical hemispheres (Ferezou et al., 2007; Frostig et al., 2017; Mohajerani et al., 2013; Pinto et al., 2019). This wide scale activation is also required for task performance as the inactivation of multiple disparate cortical sites was found to degrade performance on tasks of increased cognitive complexity (Pinto et al., 2019). Examination of activity propagation during a water reward task combining two-alternative forced choice and Go/No-Go designs has also demonstrated that activity associated with successful selection first emerged in the contralateral classical visual regions such as the visual cortex and superficial superior colliculus and then spread to nearly all
remaining recorded regions such as the hippocampus, caudoputamen and motor cortices (Steinmetz et al., 2019). Human cognitive studies have further confirmed this idea of distributed functional connectivity. Employing functional magnetic resonance imaging (fMRI), the activation of cerebellar circuits typically thought to be most closely involved with motor phenomenon were found to be engaged in non-motor cognitive tasks (King et al., 2019). The involvement of multiple brain areas in functions such as sensation, cognition, movement, and choice in both animal models and humans necessitates the questions: How can we simultaneously examine brain activity and structure both in relation to behavioral function on a multiregional scale? How are these multiregional functions impacted in brain disorders, injury, and disease?

1.2.1 Hypothesis testing – scales matter

Given strong evidence that neural processing is widely distributed, it is important that imaging assessments match these fields of view. The study of neuronal activity and structure is often divided into various scales: nano, micro, meso and macro that help to classify the many roles instruments and approaches play (Bohland et al., 2009)(Figure 1.3).

At the level of microscale connectomics, electron microscopy is often used to characterize dendritic and axonal projections of individual reconstructed neurons. Microscale circuits are then reconstructed leading to high-resolution connectivity data (Helmstaedter et al., 2013; Lee et al., 2016). Mesoscale connectomics in contrast, generally involve cell-type specific approaches which take advantage of genetic targeting of neuronal subpopulations allowing for high spatiotemporal mapping of inter-regional connectivity in relation to behavioral function (Bohland et al., 2009; Oh et al., 2014). On the largest end of the spectrum, macroscale connectome mapping examines projections across different organ systems and brain regions using non-invasive imaging
approaches such as magnetic resonance imaging (MRI) (including diffusion-weighted MRI and fMRI), electroencephalography, positron emission tomography (PET) and magnetoencephalography (Logothetis, 2008; Lu et al., 2021).

1.2.2 Tailoring imaging to scientific questions

Despite all the methods listed above, even the nanoscale structure of entire cortical columns obtained from electron microscopy (Markram et al., 2015) misses the big picture and will fail to take into account distributed functional connectivity across different brain regions. In contrast, many less-invasive macroscale imaging approaches (Devor et al., 2013) lack the ability to obtain more cell-type specific information which is critical in uncovering mechanistic pathways explaining disease pathophysiology. High spatiotemporal resolution and wide field of view tools that enable multiscale and multi-area examinations of cellular structure and function are needed in the context of live animals and high-resolution histology (MICrONS Consortium et al., 2021).

Currently, many *in vivo* imaging and electrophysiological techniques such as functional ultrasound (fUS) (Macé et al., 2018; Urban et al., 2015), fiber photometry (Kim et al., 2016; Sych et al., 2019), 3-photon microscopy (Ouzounov et al., 2017; Wang et al., 2018), ultra-wide field of view 2-photon mesoscope (Sofroniew et al., 2016; Yu et al., 2020), wide field conventional epifluorescence imaging (Barson et al., 2020; Ferezou et al., 2007; Lake et al., 2020; Mohajerani et al., 2013), and ultrahigh density multielectrode recording (Jun et al., 2017; Steinmetz et al., 2021) are rapidly emerging to help fill this technical and multiscale gap for functional assessment while improvements to microscopy and histology such as expansion lattice light sheet microscopy (ExLLSM) (Gao et al., 2019) has facilitated post-mortem work (Figure 1.3). By understanding
activity within pathways at multiple levels, circuit-based and/or pharmacological therapeutics can be better informed to control neuronal activity directly or indirectly and ultimately reduce aberrant movement, thought, and processing of sensory stimuli.

**Figure 1.3 Schematic overview of the temporal and spatial resolution of multiscale techniques.**

Temporal resolution scale for major multiscale techniques (above) denotes sampling time for live *in vivo* chronic imaging and recording. Dotted lines denote duration beyond a week. Asterisk denotes that although live *in vivo* imaging and recording durations beyond a week have been reported (and can be as long as months), the limit for chronic implantation, imaging and recording is either unknown or largely dependent on factors such as implantation and/or window location and surgical procedures. Spatial resolution of multiscale techniques (below) divided into micro, meso and macroscale.
1.2.3 Huntington disease: Genetics, symptomatology, and pathophysiology

Huntington disease (HD) is a neurodegenerative disorder characterized by a triad of symptoms: motor dysfunction, cognitive impairment and neuropsychiatric symptoms (Cepeda and Levine, 2022; McColgan and Tabrizi, 2018). In healthy individuals, the CAG triplet repeat in exon 1 of the huntingtin (HTT) gene is between 17 and 18 repeats in length but may be expanded to >39 resulting in full penetrance of the disease (Walker, 2013). Since the disease is inherited in a dominant fashion, HD is one of the most common genetic neurodegenerative disorders (Fisher and Hayden, 2014). Diagnosis is based on familial history, genetic testing and presence of motor symptoms defined in the Unified HD Rating Scale (UHDRS) (“Unified Huntington’s Disease Rating Scale: reliability and consistency. Huntington Study Group,” 1996).

Initially, patients will experience a hyperkinetic phase characterized by involuntary movement known as chorea symptoms (Bates et al., 2015; Cepeda and Levine, 2022; McColgan and Tabrizi, 2018). This will develop into slowed voluntary movement (bradykinesia) and eventual rigidity (akinesia). Cognitively, executive functions are affected leading to intellectual decline, memory loss and speech difficulties (Ho et al., 2003). Apathy, dysphoria, irritability, depression and anxiety are also common neuropsychiatric symptoms reported in HD patients (McColgan and Tabrizi, 2018).

The triad of symptoms experienced are a result of neurodegeneration in largely the striatum although neurodegeneration in the cortex also occurs with ~30% of the brain mass lost by late stages of the disease (Rosas et al., 2008, 2002; Vonsattel et al., 1985). The Vonsattel grading system is commonly used to classify the extent of striatal degeneration (Vonsattel et al., 1985).
The striatum is made up of primarily medium spiny neurons (MSNs) which can be classified based on their projection and expression pattern (Surmeier et al., 2007). MSNs that express substance P and the D1-type DA (dopamine) receptor are known as direct-pathway MSNs and are positively modulated by dopamine release. These neurons project directly to output structures of the basal ganglia (internal segment of the globus pallidus (GPe) and substantia nigra pars reticulata). MSNs that express enkephalin (ENK) and the D2-type DA receptor and are inhibited by dopamine release, are known as indirect-pathway MSNs and possess multi-synaptic indirect connections to the basal ganglia (external segment of the GPe and subthalamic nucleus). The loss of indirect-pathway MSNs which are known to suppress cortical excitation and competing actions is thought to give rise to involuntary and irregular muscle movements (chorea) (Albin et al., 1992; Reiner et al., 1988). In later disease stages, both the indirect-pathway MSNs, which suppress undesired movement, and the direct-pathway MSNs, which promote desired movement, are affected to the same degree resulting in rigidity (akinesia)(Reiner et al., 1988).

At the molecular level, mutant forms of the Huntington protein (mHtt) are prone to cleavage, fragmentation, misfolding and aggregation (Folger and Wang, 2021). mHtt plays a role in mitochondrial dysfunction, calcium dysregulation as well as increasing oxidative stress and reactive oxygen species by impacting various transcription factors, heat shock proteins, proteasome components, calcium-binding proteins and mitochondrial membranes (Jurcau, 2022; Kolobkova et al., 2017). Expression of mHTT is further known to affect N-methyl-D-aspartate receptor (NMDAR) trafficking (Parsons and Raymond, 2014). Increased interaction between 2B-NMDARs and postsynaptic density protein 95 (PSD-95) at extrasynaptic sites increase signaling of cell stress pathways. Impaired endocytosis of GluN3A-containing (Glutamate [NMDA]
receptor subunit 3A) NMDARs further leads to their increased surface expression, making striatal neurons more susceptible to dendritic spine loss. Together all these changes result in neurodegeneration giving rise to the triad of symptoms.

1.2.4 Animal models of HD

Since the discovery that dominantly inherited expansions >39 of a CAG triplet repeat in exon 1 of the HTT gene causes HD (MacDonald et al., 1993), over 50 distinct mouse and rat models with increasingly better face and construct validity have been developed (Menalled et al., 2014; Pouladi et al., 2013).

R6/1 and R6/2, were the first HD mouse models and express a truncated N-terminal fragment of the human HTT gene (Mangiarini et al., 1996). Although this model provides rapid disease onset and an aggressive phenotype including cognitive defects (detected using procedural and spatial learning and memory tests), jerky movements similar to chorea, hypoactivity as well as balance, coordination and gait impairments (Cayzac et al., 2011; Menalled et al., 2009; Pallier et al., 2009), the model has limited construct validity and may therefore not reflect the pathophysiology of the disease. For instance, although N-terminal mHtt fragments tend to accumulate in the nucleus (unlike WT HTT which reside in the cytosol), the fragmentation and splicing of mHtt is variable which is not reflected by the usage of a truncated fragment of the gene in the R6/1 and R6/2 HD models (Neueder et al., 2017).

The second class of HD mouse models consist of full-length transgenic mouse models including the YAC128 and BACHD mouse models (Gray et al., 2008; Slow et al., 2003). Similar to the N-
terminal transgenic mouse models, these full-length transgenic mouse models also recapitulate HD symptomatology albeit now with gradual phenotype progression and a normal lifespan. Although this is a clear improvement in construct validity, both the native murine Htt and the human mHtt are expressed resulting in protein overexpression (Pouladi et al., 2013). Overexpression of Htt has been reported to increase body weight (Kudwa et al., 2013; Moreno et al., 2016) which is known to be a confounding factor in many motor assessment tasks highlighting a drawback of these HD animal models.

Lastly, knock-in heterozygous mouse models of HD have recently been introduced such as the HdhQ111, CAG140 and zQ175 models (Lin et al., 2001; Southwell et al., 2016; Wheeler et al., 1999). These mouse models express one WT copy and one copy of mHtt under control of the endogenous Htt promoter. Knock-in models, despite having the advantage of having the greatest construct validity, a normal lifespan (except for homozygous zQ175 mice (Southwell et al., 2016)) and ability to recapitulate HD symptomatology, are often difficult to work with due to their slow onset of disease symptoms. For this reason, some investigators have opted to work with homozygotes or choose to backcross onto a FVB/N background (known to increase neurodegeneration)(Southwell et al., 2016).

1.2.5 Behavioral assessment: focus on motor assessments

For mouse researchers, cognitive and memory impairment (associative learning, spatial memory and recognition), psychiatric disturbances (aggression/agitation, depressive-, anxiety- and apathy-like behaviors) and alterations to circadian rhythm have been extensively characterized using classical assessments such as social preference, open field, forced swim, tail suspension test,
various water and maze tasks, fear conditioning as well as three-chamber and novel object tests (Brooks and Dunnett, 2009; Brown et al., 2000; Kosel et al., 2020). Motor assessments, however, will be the topic of focus.

Locomotor activity is commonly assessed using the open field test. In this test, the mouse is placed into an open arena with total distance and velocity traveled, turning, rearing and other behaviors examined. Although these measurements are often correlated with hyper- or hypoactivity, decreased locomotion and exploration due to anxiety has been reported as a confounding variable (Brooks and Dunnett, 2009). In this case, the mouse will tend to spend a decreased amount of time in the center.

The gold standard for gross motor balance and coordination assessment in HD is the rotarod test (Brooks and Dunnett, 2009). During this task, mice are required to balance while walking or running on a rotating rod. The ability of the mouse to stay on the rod without falling despite changes in speed is thought to be correlated to their balance and coordination as well as cognitive ability to learn the task. Task engagement and fatigue remain as issues (Oakeshott et al., 2012). Further to this, body weight is known to negatively impact task performance and is a confounding variable (McFadyen et al., 2003).

Assessments such as beam, and gait testing are also very popular in assessing gross motor function (Brooks and Dunnett, 2009). During either balance or tapered beam tasks, mice are placed on one end and are trained to walk / run across to the other side. During the task, the time to cross the beam and the number of falls or slips are examined and related to balance and coordination (Liu
et al., 2021a; Peng et al., 2016). The assessment of gait is often done using the footprint test which involves painting the paws of mice and having the mice walk. Alternatively, automatic treadmill gait assessments (e.g. Catwalk system (Noldus IT)) have also been used. In all circumstances, measurements such as stride length and feet placement are assessed and related to balance and coordination. Similar to the rotarod, factors such as task engagement, fatigue and/or body weight have been reported as problematic confounds for beam and gait testing (Batka et al., 2014; McFadyen et al., 2003; Oakeshott et al., 2012).

Due to the confounds of many gross motor assessments, examination of fine motor dysfunction has been identified as an attractive alternative (Klein et al., 2012). Fine motor tasks may enable the characterization of subtle motor phenotypes and inform disease onset. These skilled forelimb tasks include automated home-cage lever pulling tasks (Woodard et al., 2017a) which have been explored with HD mouse models using PiPaw cages (Woodard et al., 2021). Briefly, mice were trained in their homecage to pull a lever to receive water rewards with the hold-duration and target position of the lever pull altered thereby increasing the difficulty of the task. Young YAC128 HD mice were found to have slower learning through the various difficulty levels of the task compared to WT mice whereas, older YAC128 mice, although did not experience slower learning, tended to overshoot the target position then move back into the target position (Woodard et al., 2017a). Q175-FDN HD mice in contrast displayed slower learning to a different lever pulling task and were found to never achieve performance levels of WT mice (Woodard et al., 2021).

Although the advantages of PiPaw cages include minimization of experimenter impact, the reach-to-grasp behavior typically performed by humans in daily life is more accurately reflected in object
reaching and grasping as opposed to lever pulling. Presently, several skilled forelimb tasks have been developed including food pellet reaching which requires mice to reach, grasp and retrieve food through a narrow opening as well as water reaching which requires mice to reach, grasp and retrieve drops of water available on a spout (Galiñanes et al., 2018; Klein et al., 2012; Mohammed et al., 2020; Quarta et al., 2022). To date, only one study has examined forelimb motor deficits in YAC128 mice demonstrating that unlike WT mice which are able to increase their success rate at the pellet reaching task across the 8 days of testing, the success rate remained unchanged in YAC128 mice (Glangetas et al., 2020). Object reaching and grasping requires supination, pronation, and balance of the object within the hand and fingers. This is especially important in the case of water reaching tasks since without fine coordination of the hand, the water would slip through the digits (Becker et al., 2020; Galiñanes et al., 2018; Whishaw et al., 1992, 2018b). The development of a longitudinal, high-throughput water reaching task to examine progressive fine motor dysfunction throughout HD disease progress is readily needed. Combining such a task with simultaneous electrophysiology recording or single / multi-photon imaging will also enable the capture of associated aberrant circuit changes overtime.

1.2.6 Genetically encoded calcium indicators

Organic dyes either injected or loaded into cells (e.g. Fluo-3 AM) offer the ability to detect changes in calcium dynamics (Paredes et al., 2008). Genetically encoded indicators (GEIs) however, are less invasive and can be expressed constitutively or induced in animal lines using cre/loxp and/or Flp-FRT systems (Lin and Schnitzer, 2016). Plasmids encoding GEIs can also be packaged into viral constructs such as an AAV (adeno-associated virus) and injected into regions of interest in any animal. In both cases, stable expression and selective labelling of neuronal subpopulations is
possible allowing for the study of changes across development stages and disease progression.

GEIs have since been developed for vesicle release (genetically encoded pH indicators e.g. pHuji) (Shen et al., 2014) as well as changes in neurotransmitter concentrations (genetically encoded neurotransmitter indicators e.g. iGluSnFR) (Marvin et al., 2013), transmembrane voltage (genetically encoded voltage indicators e.g. Mermaid2) (Tsutsui et al., 2013) and intracellular calcium concentrations (genetically encoded calcium indicators (GECIs) e.g. GCaMP) (Chen et al., 2013).

Unlike action potential generation and neurotransmitter receptor opening which are transient, calcium entry is a slower biochemical change (Lin and Schnitzer, 2016). Although this amplification contributes to the brightness of GECIs, making them one of the most popular GEIs, the kinetics of calcium transients are ~10x slower reducing their temporal precision. Currently, the most commonly used GECIs are the GCaMP6 series. The response of GCaMP6f to mouse cortical action potentials is ~20% ΔF/F more than organic dyes with GCaMP6m, 6s and 7 producing even larger response but with longer decay time (Chen et al., 2013; Podor et al., 2015).

Structurally, the GCaMP protein consists of: a circularly permuted enhanced green fluorescent protein (cpEGFP), CaM and the CaM-interacting M13 peptide (chicken smooth muscle M13 fragment of myosin light chain kinase) (Akerboom et al., 2009; Wang et al., 2008). Ca\(^{2+}\) binding to CaM results in GCaMP conformational changes that deprotonate the chromophore resulting in green fluorescence. Several mutations have been introduced to CaM and cpEGFP to improve calcium sensitivity and dynamic range (Chen et al., 2013). Further improvements to GECIs include accelerating calcium binding and the development of red GECIs (Lin and Schnitzer, 2016). Red
GECIs although dimmer, have the advantage of deeper tissue penetration, improved signal-to-noise ratio over autofluorescence and can allow for simultaneous usage with green GECIs enabling the ability to combine calcium imaging with optogenetic stimulation or simultaneous recording from two neuronal subpopulations in the same region of interest.

1.2.7 Application of multiscale imaging approaches to HD mouse models and motor behavioral assessments

To date, most neurological disease research has largely concentrated on either discrete microscale studies isolated to a particular cell-type in a set location or macroscale examinations both with little emphasis on multiscale network level activity across brain regions. Fine motor function such as pellet or water reaching (Prsa et al., 2017), or other homecage motor tasks such as skilled lever pulling (Silasi et al., 2018; Woodard et al., 2017b) or joy-sticks (Bollu et al., 2019) however, could be conducted using head-fixed animals and would therefore be ideal to employ with longitudinal wide field imaging or other multiscale readouts. These techniques include fUS (Macé et al., 2018; Urban et al., 2015), 3-photon microscopy (Ouzounov et al., 2017; Wang et al., 2018), ultra-wide field of view 2-photon mesoscope (Sofroniew et al., 2016; Yu et al., 2020), wide field conventional epifluorescence imaging (Barson et al., 2020; Ferezou et al., 2007; Lake et al., 2020; Mohajerani et al., 2013), and ultrahigh density multielectrode recording (Jun et al., 2017; Steinmetz et al., 2021). Ultimately, live in vivo imaging and electrophysiological techniques combined with novel fine motor assessment tools present the opportunity to 1) uncover the connectivity and function of interbrain regions throughout the lifespan of a mouse model of disease and 2) evolve therapeutics in such a way that it entails precise and personalized cell and circuit manipulation that restores
motor and cognitive function as well as psychological wellbeing to patients with neurological
disease. The aim of Example Case 2 is therefore to characterize forelimb motor defects and
associated aberrant cortical activity in a Huntington Disease mouse model.

1.3 Dissertation research aims
In summary, the examination of how synthetic compounds such as DIDS and mefenamic acid
affect $I_{Ks}$ may uncover important therapeutic precursor compounds for the treatment of LQTS.
With almost all mutations in LQTS patients identified as loss-of-function and 50% of those loss-of-function mutations identified in the KCNQ1 subunit (Ackerman et al., 2011; Hedley et al.,
2009), enhancing and activating $I_{Ks}$ currents has been suggested as a promising LQTS therapeutic
approach. The biophysical and pharmacological characterization of mefenamic acid’s effect on
Kv7.1 and $I_{Ks}$ will be carried out using whole-cell patch clamp.

In turn, assessments that examine simultaneous widefield cortical activity changes and fine motor
defects are needed in HD research to better characterize these motor deficits in mice and in the
future, test drug efficacy. Although gross motor defects have been extensively characterized in HD
mouse models using tasks such as rotarod, balance beam and gait tasks (Abada et al., 2013; Brooks
et al., 2012; Pallier et al., 2009; Southwell et al., 2016), less is known about skilled forelimb deficits
and underlying cortical circuit changes. HD phenotype characterization will be carried out using a
custom water-reaching behavioral task with simultaneous widefield cortical recording and placed
in the context of known gross motor defect and HD pathology onset using tapered beam, rotarod
and immunohistochemistry staining.
As such, the aims of this thesis are as follows:

**AIM I (Chapter 2):** Characterization of mefenamic acid modulation of Kv7.1 and $I_{Ks}$.

**AIM II (Chapter 3):** Characterization of skilled forelimb motor defects and associated cortical activity changes in a Huntington Disease mouse model using the water-reaching task.
Chapter 2: *In vitro* characterization of mefenamic acid modulation of Kv7.1

2.1 Introduction

The potassium voltage-gated KCNQ channel subfamily is comprised of five known isoforms, KCNQ1-5 (Abbott, 2014). Expression of the first isoform, KCNQ1, has been detected throughout the body including in the heart, stomach and ear (Liin et al., 2015). When by itself, KCNQ1 produces a fast activating and deactivating current that has not yet been found to underlie any specific endogenous currents in the body (Abbott, 2014). KCNQ1 however, also co-assembles with several β-subunits, KCNE1-5, which modulate KCNQ1 current kinetics (Bendahhou et al., 2005; Eldstrom and Fedida, 2011; Manderfield and George, 2008). In the heart, the co-assembly of KCNQ1 with KCNE1 and perhaps other KCNE subunits, produces a slowly activating and deactivating delayed cardiac rectifier K⁺ current (\(I_{Ks}\)), which contributes significantly to cardiac repolarization (Lundquist et al., 2005; Sanguinetti et al., 1996).

There is no general agreement on the stoichiometric ratio of KCNE1 to KCNQ1 subunits underlying \(I_{Ks}\) *in vivo* (Morin and Kobertz, 2008; Murray et al., 2016; Nakajo et al., 2010; Plant et al., 2014), though we know a variable stoichiometry of 4:1 up to 4:4 is possible (Murray et al., 2016). Given that the kinetics of \(I_{Ks}\) are greatly affected by the number of KCNE1 subunits, great flexibility in the expressed physiological and pharmacological properties of \(I_{Ks}\) channel complexes is expected from a variable stoichiometry.

The complex also has clinical importance in disease syndromes including cardiac arrhythmia, with the severity ranging from syncope to sudden death (Splawski et al., 2000). Approximately 50% of
the mutations seen in long QT syndrome (LQTS) patients are in the KCNQ1 subunit (LQTS type 1) (Hedley et al., 2009), with mutations in KCNE1 causing LQTS type 5.

Activators of $I_{Ks}$ that can act on the relevant saturated and unsaturated $I_{Ks}$ complexes are of particular interest as they may have therapeutic potential in the treatment of LQTS types 1 and 5. To date, although several activators have been reported, some are only effective on KCNQ1 alone with limited efficacy on $I_{Ks}$. These include ML-277, zinc pyrithione and L-364,373 (Gao et al., 2008; Magyar et al., 2006; Yu et al., 2013). The known activators of $I_{Ks}$ are phenylboronic acid (PBA) (Mruk and Kobertz, 2009) and hexachlorophene (HCP) (Zheng et al., 2012), as well as stilbenes such as 4,4'-diisothiocynano-2,2'-stilbenedisulfonic acid (DIDS) and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), fenamates such as mfenamic acid (Abitbol et al., 1999), and fatty acids such as lauric acid (Doolan et al., 2002). PBA and HCP have been shown to increase both KCNQ1 alone and $I_{Ks}$ current amplitudes, although they are more potent on $I_{Ks}$ (Mruk and Kobertz, 2009; Zheng et al., 2012). In contrast, lauric acid, DIDS and SITS have been shown to only increase $I_{Ks}$ current (Abitbol et al., 1999; Doolan et al., 2002).

The fenamate, mfenamic acid (Fig. 1A), is a nonsteroidal anti-inflammatory drug primarily prescribed to treat menstrual pains (U.S.FDA, 2008). Originally identified as a chloride channel blocker, mfenamic acid has since been shown in various expression systems (Xenopus oocytes, rat mesenteric arteries, canine ventricular myocytes, guinea-pig ventricular myocytes, CHO cells and COS-7 cells) to increase mammalian $I_{Ks}$ current amplitudes as well as produce a variable amount of instantaneous current and inhibit tail current decay (Abitbol et al., 1999; Busch et al., 1994; Chadha et al., 2012; Magyar et al., 2006; Toyoda et al., 2006; Unsöld et al., 2000).
In the present study, using transiently expressed human $I_{Ks}$ in mammalian cells, we have carried out a more complete biophysical characterization of the effects of mefenamic acid than has been attempted to date. We show that mefenamic acid has minimal effect on KCNQ1 in the absence of KCNE1 and have quantified drug concentration- and rate-dependent changes in the $I_{Ks}$ current waveforms and the conductance-voltage relationship. As the stoichiometry of $I_{Ks}$ may vary and affect its pharmacology and current kinetics (Murray et al., 2016; Nakajo et al., 2010), we have analyzed the dependence of mefenamic acid actions on the stoichiometry of $I_{Ks}$ channel complexes. Lastly, through mutational analysis we identify a specific regulatory site for mefenamic acid on KCNE1. The results suggest that residue K41 on KCNE1 is of particular importance in mediating the effect mefenamic acid has on $I_{Ks}$.

2.2 Materials and methods

2.2.1 Solutions and drugs

Unless otherwise stated, all drugs and chemicals used to make solutions were obtained from Sigma-Aldrich (Mississauga, ON, Canada). The control bath solution for whole-cell experiments contained: 135 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2.8 mM NaAcetate and 10 mM HEPES, pH 7.4 with NaOH. The pipette solution for whole-cell experiments contained: 130 mM KCl, 5mM EGTA, 1 mM MgCl$_2$, 4 mM Na$_2$-ATP, 0.1 mM GTP and 10 mM HEPES, pH 7.2 with KOH. Mefenamic acid and HMR1556 ((3R,4S)-(+-)N-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy) chroman-4-yl]-N-methylmethanesulfonamide) (Tocris Bioscience, Oakville, ON, Canada) were prepared as stock solutions (50 mM, 200 mM or 500 mM for mefenamic acid and, 2 mM for HMR1556) dissolved in 100% dimethyl sulfoxide. Stock mefenamic acid solutions were diluted in control whole-cell bath solution to obtain final mefenamic acid concentrations of 10 µM,
30 µM, 100 µM, 300 µM, 500 µM or 1 mM which were perfused onto mammalian cells for whole-cell experiments. Stock HMR1556 solutions were pipetted directly into the chamber to obtain a final HMR1556 concentration of 1 µM for whole-cell experiments. Concentrations of dimethyl sulfoxide in final bath solutions never exceeded 0.2% (V/V). The maximum concentration of mefenamic acid (1 mM) lowered the pH of the final bath solution by 0.15 ± 0.02 (n=3). This was not corrected.

2.2.2 Constructs, cell culture and transfection

$I_{Ks}$ is generally understood to be functionally comprised of combinations of KCNQ1 and KCNE1 subunits. The stoichiometry of the two subunits may be variable in heterologous expression systems (Murray et al., 2016), and may also vary in vivo (Dvir et al., 2014a). In the initial experiments (Figs. 2.1-2.4), the initial stoichiometric ratio of KCNQ1:KCNE1 was set at the maximum, 4:4. This was achieved through transfection of a linked KCNE1 and KCNQ1 cDNA (Murray et al., 2016), which is expected to assemble as a tetramer with four KCNQ1 and four KCNE1 subunits. For simplicity this will be denoted as EQ. In later experiments where the ratio was varied (Fig. 2.5), cells were transfected with KCNQ1 without KCNE1 (Q1), or linked constructs containing one KCNE1 linked with two KCNQ1s (EQQ, expected to assemble in a 2:4 ratio), or one KCNE1 linked with four KCNQ1s (EQQQQ, expected to assemble in a 1:4 ratio). EQ, EQQ and EQQQQ constructs were generated as previously described (Murray et al., 2016). In all cases we consider the currents that result from different combinations of KCNQ1 and KCNE1 (except KCNQ1 alone, Q1) to be $I_{Ks}$ and we use this name interchangeably with the constructs themselves.
tsA201 transformed human embryonic kidney 293 or ltk- mouse fibroblast cells were cultured in modified Eagle’s medium supplemented with 10% fetal calf serum and 100 μg/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin and, plated for whole-cell patch clamp experiments as previously described (Murray et al., 2016; Westhoff et al., 2019). Cells were transiently transfected with either: 1) GFP tagged Q1 (Q1-GFP); 2) EQ, EQQ or EQQQQ and GFP in a 1.5-2.5:1.0 μg ratio or; 3) mutant KCNE1 and Q1-GFP in a 4.5-6.0:1.5 μg ratio using Lipofectamine2000 (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer’s protocol. All KCNE1 mutations were generated using site-directed mutagenesis and Pfu Turbo (Agilent Technologies, Santa Clara, CA, USA) followed by sequence confirmation of all mutations. Whole-cell patch clamp experiments were conducted 24 to 48 hrs post transfection.

2.2.3 Electrophysiology

An Axopatch 200B amplifier, Digidata 1440A and pClamp 9 or 11 software was used to conduct all experiments. Electrodes ranging from 1-3 MΩ resistance were first pulled from thin-walled borosilicate glass (Sutter Instruments, Cal. USA) using a linear multistage electrode puller (Sutter Instruments) and then fire polished before use. Series resistance compensation of 70-80% was used for all whole-cell experiments. Data were sampled at 20 kHz and filtered at 5 kHz during acquisition. For voltage clamp protocols, interpulse intervals refer to start-to-start times between sweeps.

2.2.4 Data analysis

GraphPad Prism 8.1.1 software was used to analyze all data. Where applicable, One-way Anova followed by the Bonferroni multiple comparison post-hoc test was used to determine statistical
significance. A p-value less than 0.05 was considered statistically significant. All data in the figures are shown as mean ± SEM (standard error of the mean) and reported in the tables as mean ± STD (standard deviation).

**Whole-cell experiments:** Mefenamic acid dose-response diary plots (Figs. 2.1B-C right panels) and curves (Figs. 2.2A-B and 2.5D) were obtained from measurement of the activating $I_{Ks}$ current. Specifically, the peak to steady state difference currents ($I_{\text{max}} - I_{\text{min}}$) were calculated by subtracting the minimum amplitude of the activating current ($I_{\text{min}}$) from the peak amplitude of the activating current ($I_{\text{max}}$). This value was then plotted against either the corresponding sweep number or log$_{10}$ concentration of mefenamic acid (Figs. 2.1 and 2.2A). Where applicable, the difference current in mefenamic acid was normalized to the maximum control (in the absence of mefenamic acid) difference current, and subtracted from 1.0 to obtain the ‘Normalized Response’, which was plotted against the corresponding log$_{10}$ concentration of mefenamic acid (Figs. 2.2B and 2.5D). Dose- and Normalized-response curves were fit with a specific binding equation in order to obtain the EC$_{50}$ and Hill coefficients ($n^H$) as in Figures 2.2A-B and 2.5D. The HMR1556 response diary plot in the presence of mefenamic acid (Fig. 2.1D right panel) was obtained by plotting the initial peak current amplitude against the corresponding sweep number. All current-voltage (I-V) and conductance-voltage (G-V) plots in Figures 2.3, 2.5, 2.6 and 2.7 were obtained from the normalized peak of the 4 s depolarizing pulses ($I/I_{\text{max}}$) and normalized peak of the initial tail current ($G/G_{\text{max}}$), respectively, and plotted against the corresponding voltage. G-V plots were fitted with a Boltzmann sigmoid equation in order to obtain the $V_{1/2}$ and slope ($k$) values (Tables 2.1 and 2.2). In the case of the mutant EQ $I_{Ks}$, the change in $V_{1/2}$ of activation ($\Delta V_{1/2} = V_{1/2}$ in the presence of mefenamic acid – $V_{1/2}$ control) was further determined (Fig. 2.6D; Table 2.2). In some cells, G-V
relationships in the presence of mefenamic acid for WT EQ, K41R and G40C were essentially linear, and consequently the $V_{1/2}$ of activation was read from the plots and included in the calculations of the mean values in Tables 2.1 and 2.2. All deactivation traces in Figures 2.4A-B were fitted with a single exponential equation in order to obtain the time constants of deactivation ($\tau_{\text{deact}}$) which were plotted against the corresponding membrane potential (Fig. 2.4C).

2.3 **Results**

2.3.1 **Mefenamic acid increases EQ $I_{Ks}$ current expressed in mammalian cells**

Initially, to ensure that mefenamic acid (Fig. 2.1A) had no effect on the endogenous currents in $ltk$-mouse fibroblast (LM) cells, different concentrations of mefenamic acid (10 µM, 30 µM, 100 µM, 300 µM, 500 µM and 1 mM) were perfused onto untransfected LM cells (Fig. 2.1B). At all concentrations of mefenamic acid, no changes were observed in the waveform (Fig. 2.1B left panel) and peak to steady-state difference currents ($I_{\text{max}} - I_{\text{min}}$; Fig. 2.1B right panel) compared to untreated cells (control). A single concentration of mefenamic acid (100 µM) has previously been shown to enhance $I_{Ks}$ activity leading to a variable amount of instantaneous current and inhibition of tail current decay in *Xenopus* oocytes (Busch et al., 1997, 1994) and various mammalian cells (Magyar et al., 2006; Toyoda et al., 2006; Unsöld et al., 2000). The concentration dependence of this enhancement of $I_{Ks}$ activity using different concentrations of mefenamic acid perfused onto LM cells transiently transfected with EQ is shown in Figure 2.1C. With increasing concentrations of mefenamic acid, the control sigmoidal waveform (indicative of slow activation kinetics), was transformed into an almost linear waveform with significant instantaneous current (Fig. 2.1C left panel). The corresponding difference current diary plot of increasing concentrations of mefenamic acid shows the gradual transformation to an instantaneous current
over time (Fig. 2.1C right panel). Consistent with previous findings, the decay in tail current seen in control was also inhibited in a dose-dependent manner with increasing concentrations of mefenamic acid (Fig. 2.1C left panel).

To confirm that this instantaneous current was produced by mefenamic acid enhancement of $I_{Ks}$ activity specifically, we employed the $I_{Ks}$ blocker, HMR1556 (Gögelein et al., 2000). In these experiments, LM cells transiently transfected with EQ were first preincubated in 100 µM mefenamic acid for approximately 30 min (data not shown), then when an $I_{Ks}$-positive cell was identified, 1 µM HMR1556, was applied to the bath (Fig. 2.1D). As is evident in the representative traces (Fig. 2.1D left panel) and diary plot (Fig. 2.1D right panel), following HMR1556 treatment, the instantaneous current amplitude decreased over time to that of endogenous current amplitudes, suggesting that the instantaneous current was in fact flowing through $I_{Ks}$ channels.
Figure 2.1 Mefenamic acid increases EQ current expressed in mammalian cells.

(A) Molecular structure of mefenamic acid. Nitrogen and oxygen atoms are depicted in blue and red, respectively. (B) Response of an untransfected cell to mefenamic acid (abbreviated as ‘Mef’ in this and all other figures). Left panel shows representative current traces in the absence of mefenamic acid (control; black) and after the addition of 10 µM - 1 mM mefenamic acid as indicated (grey). Right panel shows $I_{\text{max}} - I_{\text{min}}$ (peak to steady-state difference currents) vs. sweep number during the addition of different concentrations of mefenamic acid (solid grey lines). (C) Dose response of EQ currents to mefenamic acid. Left panel shows currents in control (black) and in response (grey) to different concentrations of mefenamic acid. Right panel shows the data in a diary plot. (D) Response of EQ to 1 µM HMR1556 in the presence of mefenamic acid (100 µM mefenamic acid preincubation for approximately 30 min, data not shown). Complete block by HMR1556 is indicated by the red arrows. Left panel shows 100 µM mefenamic acid alone (black), and in response to 1 µM HMR1556 (grey). Right panel shows the data in an initial peak amplitude vs sweep number.
diary plot. The solid grey line indicates addition of HMR1556. All currents (Figs. 2.1B-D) were obtained by pulsing to +60 mV for 4 s followed by a pulse to -40 mV for 0.9 s. The interpulse interval was 15 s. Holding potential was -80 mV. Dotted baselines denote the zero current level.

2.3.2 Mefenamic acid dose-response curves for EQ $I_{Ks}$

Increasing concentrations of mefenamic acid gradually transformed the sigmoidal activation waveform of EQ $I_{Ks}$ into an almost linear waveform, and this change was quantified by measuring peak to steady-state difference currents (Fig. 2.2A upper panel; see Methods). More specifically, difference currents were calculated by subtracting the initial amplitude of the activating current ($I_{min}$) from the peak amplitude of the activating current ($I_{max}$) and plotting it against the corresponding log concentration of mefenamic acid (Fig. 2.2A). Normalized Response relationships for EQ were obtained by transformation of $I_{max}$-$I_{min}$ data (see Methods) and fit with a specific binding equation (Fig. 2.2B). The EC$_{50}$ and Hill Coefficient ($n^H$) for mefenamic acid were 60 µM and 0.49, respectively. To ensure consistent results and to allow for comparison with previous literature, all subsequent experiments to characterize the gating properties, the subunit stoichiometry, and specific regulatory residues were conducted using 100 µM mefenamic acid, unless otherwise stated.
Figure 2.2 Mefenamic acid dose-response curves for EQ.

All currents used to calculate dose-response curves were obtained using the same protocol described in Fig. 2.1. (A) Representative dose-response calculation and curve. Upper panel shows representative $I_{\text{max}} - I_{\text{min}}$ (peak to steady-state difference current) measurements in the absence of mefenamic acid (black) and in the presence of 300 µM mefenamic acid (grey). Lower panel shows $I_{\text{max}} - I_{\text{min}}$ vs log concentration of mefenamic acid, data from Fig. 2.1C.

(B) Mean log concentration-response curve for EQ ($n = 3-5$ at each concentration); EC$_{50} = 60$ [38, 89; 95% CI] µM; $n^H = 0.49$ [0.39, 0.60; 95% CI]). Responses were normalized to the maximum peak to steady-state difference current in the absence of mefenamic acid and subtracted from 1.0 (see Methods).

2.3.3 Mefenamic acid rate-dependently hyperpolarizes EQ $I_{Ks}$ current-voltage (I-V) and conductance-voltage (G-V) relationships

To investigate what happens to EQ $I_{Ks}$ I-V and G-V relationships following treatment with 100 µM mefenamic acid, a 4 s activation protocol with varying interpulse intervals was used (Fig. 2.3).

In Figure 2.3A are representative waveforms of EQ $I_{Ks}$ both in the absence (control; upper panel) and presence of mefenamic acid (lower panel) and, show the characteristic delay of current activation in control and the appearance of an instantaneous current with mefenamic acid
treatment. The corresponding I-V (Fig. 2.3B) and G-V relationships (Fig. 2.3C) were obtained by plotting the normalized peak amplitudes at the end of the 4 s depolarizing pulses (\(I/I_{\text{max}}\)) or the normalized peak of the initial tail current (\(G/G_{\text{max}}\)), respectively, against the corresponding voltage. During exposure to mefenamic acid, the I-V relationship became more linear and hyperpolarized (Fig. 2.3B). The G-V relationship following treatment with mefenamic acid was also hyperpolarized (control: \(V_{1/2} = 24.0 \text{ mV}; k = 24.0 \text{ mV}\); mefenamic acid: \(V_{1/2} = -2.4 \text{ mV}; k = 65.4 \text{ mV}\); Fig. 2.3C).

Visually, all the I-V relationships at different interpulse intervals (30 s, 20 s, 15 s and 7 s) in the presence of mefenamic acid appeared almost linear and more hyperpolarized (Fig. 2.3D). The degree of hyperpolarization was graded with increasingly shorter intervals and I-V relationships for interpulse intervals of 7 s and 30 s were respectively, the most and least hyperpolarized. In contrast, altering the interpulse interval dramatically affected the G-V relationships of mefenamic acid-treated EQ (Fig. 2.3E). The degree of hyperpolarization of the G-V plots and \(V_{1/2}\) of activation was also greater with shorter interpulse intervals. The G-V relationship and \(V_{1/2}\) for an interpulse interval of 7 s (-108 mV) was the most hyperpolarized followed by the G-Vs and \(V_{1/2}\)s at interpulse intervals of 15 s (-86.5 mV), 20 s (-80.5 mV) and, finally, 30 s (-13.1 mV) which was the least hyperpolarized (Table 2.1). The \(V_{1/2}\) of activation at all tested interpulse intervals (30 s, 20 s, 15 s and 7s) in the presence of mefenamic acid was significantly different from control (Table 2.1). The slope of the G-V relationship was significantly decreased when the interpulse interval was either 20 s (53.3 mV; \(p = 0.0045\)) or 30 s (57.2 mV; \(p = 0.0035\)) in the presence of mefenamic acid when compared to control (20.4 mV) (Table 2.1). The slope of the G-V relationship, however,
was not significantly different when the interpulse interval was 15 s (23.2 mV) or 7 s (6.9 mV) in the presence of mefenamic acid when compared to control (Table 2.1).

Altering the interpulse interval of the activation protocol to 7 s however, did not significantly affect the control (in the absence of mefenamic acid) I-V and G-V relationships (Figs. 2.3D-E), although the G-V relationship did become steeper. Overall, these results show that mefenamic acid hyperpolarizes the I-V and G-V relationships of EQ $I_{Ks}$ in a rate-dependent fashion.

![Figure 2.3 Mefenamic acid rate-dependently hyperpolarizes EQ I-V and G-V relationships.](image)

Currents were obtained using a 4 s step protocol with pulses from -150 mV to +100 mV, followed by a repolarizing step to -40 mV for 1 s. (A) Currents are shown for EQ in the absence (control; interpulse interval was 15 s; upper panel), and the presence of 100 µM mefenamic acid (interpulse interval was 30 s; lower panel). (B) I-V plots of current at the end of the 4 s depolarizing pulses in control (black triangles), and mefenamic acid (grey triangles),
data from Fig. 2.3A. (C) G-V relationships obtained from peak initial tail currents in control ($V_{1/2} = 24.0 \text{ mV}; k = 24.0 \text{ mV};$ black circles) and mefenamic acid ($V_{1/2} = -2.4 \text{ mV}; k = 65.4 \text{ mV};$ grey circles), data from Fig. 2.3A. (D) and (E) Effect of different interpulse intervals on I-V (triangles) and G-V (circles) plots in control and mefenamic acid. Intervals were 7 s (n=1; orange), and 15 s (n=4; black) in control; and, 7 s (red), 15 s (green), 20 s (blue) or 30 s (purple) in mefenamic acid (n=4-6). For G-V plots, Boltzmann fits were, for 7 s ($V_{1/2} = 17.8 \text{ mV}; k = 12.9 \text{ mV}$) and 15 s ($V_{1/2} = 23.9 \text{ mV}; k = 20.4 \text{ mV}$) in control; and, for 7 s ($V_{1/2} = -108 \text{ mV}; k = 6.9 \text{ mV}$), 15 s ($V_{1/2} = -86.5 \text{ mV}; k = 23.2 \text{ mV}$), 20 s ($V_{1/2} = -80.5 \text{ mV}; k = 53.3 \text{ mV}$), and 30 s intervals ($V_{1/2} = -13.1 \text{ mV}; k = 57.2 \text{ mV}$) in mefenamic acid (see Table 2.1). $V_{1/2}$ values for mefenamic acid compared with control were significantly different at all intervals (Table 2.1).

2.3.4 **Mefenamic acid slows EQ $I_K$ deactivation**

To investigate changes in rates of EQ deactivation following treatment with 100 µM mefenamic acid, tail currents were obtained in the absence (Fig. 2.4A) and presence of mefenamic acid (Fig. 2.4B) and fit with single exponential decay curves. Deactivation time constants were obtained from these fits and plotted against the membrane potential (Fig. 2.4C). The $K^+$ reversal potential was found to be approximately -80 mV, and therefore the rate of deactivation at -80 mV was omitted. Treatment with mefenamic acid significantly decreased the rate of deactivation at -70 to -140 mV.
Figure 2.4 Mefenamic acid slows EQ deactivation.

Tail currents were obtained by pulsing to +60 mV for 4 s to activate $I_{Ks}$ current, followed by a 4 s pulse to a range of potentials from -40 mV to -150 mV in 10 mV steps. Holding potential was -90 mV. (A) Representative tail currents (black) fit with a single exponential curve (blue lines) for EQ $I_{Ks}$ in the absence of mefenamic acid. (B) Representative tail currents (black) fit with a single exponential curve (blue lines) for EQ $I_{Ks}$ in the presence of 100 µM mefenamic acid. (C) Time constants of deactivation ($\tau_{\text{deact}}$) vs different membrane potentials for EQ $I_{Ks}$ in the absence (n=3, black circles) and in the presence of 100 µM mefenamic acid (n= 5, grey circles). **, *** and **** denotes significance where $p = 0.0089$, $p = 0.0003$ and $p < 0.0001$, respectively.

2.3.5 Effect of $I_{Ks}$ stoichiometry on response to 100 µM mefenamic acid

To investigate whether the effect of mefenamic acid on $I_{Ks}$ dose-response and G-V relationships was dependent on the E1:Q1 stoichiometry, mammalian cells (LM and tsA201 cells) were transiently transfected with either EQ $I_{Ks}$, EQQ $I_{Ks}$, EQQQQ $I_{Ks}$ or KCNQ1 alone (Fig. 2.5). These $I_{Ks}$ constructs fix the ratio of E1:Q1 to either 4:4, 2:4 or 1:4 through linking the C-terminus of KCNE1 to the N-terminus of one, two or four KCNQ1 sequences, respectively.

No change in the KCNQ1 waveform was seen following treatment with 100 µM mefenamic acid (control: Fig. 2.5A left upper panel; mefenamic acid: Fig. 2.5A left lower panel). Mefenamic
acid also did not significantly shift the $V_{1/2}$ of activation or slope of the G-V relationship (Table 2.2), supporting the conclusion that mefenamic acid has no effect on KCNQ1 alone.

When one KCNE1 subunit was present (EQQQQ $I_{Ks}$), the instantaneous current characteristic of mefenamic acid’s effect on EQ $I_{Ks}$ no longer occurred (Fig. 2.5B left panel). The EQQQQ $I_{Ks}$ waveform was sigmoidal both in the absence (Fig. 2.5A left upper panel) and presence of 100 μM mefenamic acid (Fig. 2.5A left lower panel). Despite mefenamic acid not having a dramatic transformative effect on the activation waveform of EQQQQ, inhibition of EQQQQ tail current decay following mefenamic acid treatment still occurred. A significant leftward shift in the $V_{1/2}$ of activation also occurred (control: -1.5 mV; mefenamic acid: -20.8 mV; p = 0.0298; Table 2.2). There were no significant changes in the slope of the G-V relationships.

Similar to EQQQQ $I_{Ks}$, there was no dramatic transformative effect on the EQ $I_{Ks}$ activation waveform following mefenamic acid treatment (Fig. 2.5C left panel). Inhibition of EQ $I_{Ks}$ tail current decay however, still occurred. A significant leftward shift in the $V_{1/2}$ of activation also occurred (control: 15.4 mV; mefenamic acid: -1.6 mV; p = 0.0472; Table 2.2). Again, the slope of the G-V relationships did not change. Overall, the leftward shift in the $V_{1/2}$ of activation ($\Delta V_{1/2}$) for EQQQQ (-19.3 mV) and EQ (-17.1mV) was less dramatic than the $\Delta V_{1/2}$ of activation for EQ (-110 mV) (Table 2.2). The normalized responses of the different $I_{Ks}$ stoichiometries (normalized difference currents) to different concentrations of mefenamic acid are plotted in Figure 2.5D. At all concentrations, the normalized responses of EQQQQ $I_{Ks}$ and EQ $I_{Ks}$ were significantly reduced compared to the response of EQ $I_{Ks}$. The EC$_{50}$ and $n^H$ were, respectively: 902 μM and 0.66 for EQQQQ $I_{Ks}$; 615 μM and 0.47 for EQ $I_{Ks}$; and, 60 µM and 0.49 for EQ $I_{Ks}$. 59
Since no change in the KCNQ1 waveform or significant shift in the \( V_{1/2} \) was seen following mefenamic acid treatment (Fig. 2.5A), these data were not included in the log concentration-Normalized Response plot in Figure 2.5D.

**Figure 2.5 Effect of \( I_{Ks} \) stoichiometry on response to 100 \( \mu \)M mefenamic acid.**

The currents and conductance-voltage plots (Figs. 5A-C) were obtained using the protocols described in Fig. 2.3 with a 15 s interpulse interval. For each stoichiometry (see Methods), representative currents are shown in the absence of (control; upper left panel) and in the presence of 100 \( \mu \)M mefenamic acid (lower left panel). Right panels show the corresponding G-V plots in control (black circles) and presence of mefenamic acid (grey circles).

Boltzmann fits were: (A) for Q1 in control (n = 5): \( V_{1/2} = -20.5 \) mV, \( k = 8.8 \) mV; and, in mefenamic acid (n = 4): \( V_{1/2} = -25.6 \) mV, \( k = 14.9 \) mV; (B) for EQQQ Q in control (n = 4): \( V_{1/2} = -1.5 \) mV, \( k = 18.1 \) mV; and, in mefenamic acid (n = 4): \( V_{1/2} = -20.8 \) mV, \( k = 18.3 \) mV; (C) for EQQ \( I_{Ks} \) in control (n = 5): \( V_{1/2} = 15.4 \) mV, \( k = 20.6 \) mV; and in mefenamic acid (n = 4) \( V_{1/2} = 15.4 \) mV, \( k = 20.3 \) mV (See Table 2.2). (D) Normalized log concentration-response relationships for EQ, EQQ and EQQQ Q \( I_{Ks} \) were obtained using the analysis method described in Fig. 2.2.
For EQ $I_{Ks}$: EC$_{50} = 60 \ [38, 89; 95\%\ CI] \ \mu M$, $n^H = 0.49 \ [0.39, 0.60; 95\%\ CI]$, green diamonds, data from Fig. 2.2B; for EQQ $I_{Ks}$: EC$_{50} = 615 \ [422, 955; 95\%\ CI] \ \mu M$, $n^H = 0.47 \ [0.37, 0.60; 95\%\ CI]$, blue diamonds; and for EQQQQ $I_{Ks}$: EC$_{50} = 902 \ [663, 1383; 95\%\ CI] \ \mu M$, $n^H = 0.66 \ [0.49, 0.87; 95\%\ CI]$, red diamonds. For each construct, $n = 3-5$ at each concentration. **, *** and **** denotes a significantly different response when compared to EQ $I_{Ks}$ and, where $p<0.05$, $p<0.0005$ and $p<0.0001$, respectively.

2.3.6 Mapping the mefenamic acid regulatory sites on KCNE1

Since our data indicate that mefenamic acid has minimal effect on KCNQ1 alone and the effect of mefenamic acid is dependent upon channel stoichiometry, we further examined potential mefenamic acid regulatory sites on KCNE1. The binding site for mefenamic acid has previously been suggested to lie between residues 39-43 on KCNE1 (Abitbol et al., 1999), however, the importance of each residue to mefenamic acid’s subsequent effect on $I_{Ks}$ have not previously been characterized. Using mutational analysis, we therefore characterized how singularly mutating residues in this region would affect mefenamic acid’s ability to alter the waveform and G-V relationship of EQ $I_{Ks}$.

Similar to WT EQ $I_{Ks}$, all mutant EQ $I_{Ks}$ showed the characteristic delay of current activation in the absence of mefenamic acid (L42C, Fig. 2.6A; K41C, Fig. 2.6B; K41R, Fig. 2.6C; G40C, Fig. 2.7; current data not shown for K41E and E43C). Additionally, the characteristic appearance of instantaneous current and inhibition of tail current decay induced by mefenamic acid on WT EQ $I_{Ks}$ was preserved in the G40C (Fig. 2.7B), L42C (Fig. 2.6A) and E43C (data not shown) EQ $I_{Ks}$ mutants. When an interpulse interval of 15 s was used, mefenamic acid also significantly altered the shape of the G-V relationship (Fig. 2.7C) and left shifted the $V_{1/2}$ of activation for G40C ($\Delta V_{1/2}: -76.1$ mV) (Table 2.2, Fig. 2.7C). This effect on the G-V relationship and $V_{1/2}$ of G40C was also
dependent on the interpulse interval – with the shortest interpulse interval (interpulse intervals examined include: 7 s, 15 s and 30 s) producing the most dramatically altered G-V relationship (Fig. 2.7C) and visually the most leftward shift in $V_{1/2}$. In contrast, the effect mefenamic acid had on the G-V relationships of L42C and E43C were not as dramatic (L42C: Fig. 2.6A right panel; data not shown for E43C). When an interpulse interval of 15 s was used, the shape of the G-V relationship in both the absence and presence of mefenamic acid was sigmoidal for L42C and E43C. As well, the slope of the G-V relationship was not significantly altered following mefenamic acid treatment, however a significant hyperpolarizing shift in the $V_{1/2}$ of activation occurred ($\Delta V_{1/2}$ for L42C: -37.2 mV; $\Delta V_{1/2}$ for E43C: -24.4 mV) (Table 2.2, Fig. 2.6D) for both mutants. Mutating residues L42, E43 and, especially G40 on KCNE1 therefore only minimally reduced mefenamic acid’s effect on EQ $I_{Ks}$.

Mutations made at K41 on KCNE1 had a different result. Unlike WT EQ $I_{Ks}$, after treatment with 100 $\mu$M mefenamic acid, the waveform of K41C still showed delayed current activation and WT tail current decay (Fig. 2.6B lower left panel). Moreover, there was also no change in the slope and shape of the G-V relationship, (Fig. 2.6B right panel) or significant shift in the $V_{1/2}$ of activation (Table 2.2, Fig. 2.6D). Even in the presence of 1 mM mefenamic acid, there was still no effect on the slope and shape of the G-V relationship (Fig. 2.6B right panel) or significant shift in the $V_{1/2}$ of activation when compared to control (Table 2.2, Fig. 2.6D). Mutating residue K41 therefore drastically reduced mefenamic acid’s effect on EQ $I_{Ks}$. We hypothesized that neutralization of the positively charged K41 was responsible for the loss of efficacy of mefenamic acid. In support of this idea, similar to K41C, after treatment with 100 $\mu$M mefenamic acid, the waveform of K41E still showed WT-like delayed current activation and tail current decay (data
not shown). Although there was once again no change in the slope and shape of the G-V relationship (data not shown), a significant right shift in the V_{1/2} of activation compared to control was seen with 100 µM mefenamic acid (ΔV_{1/2} for K41E with 100 µM: +24.9 mV) (Table 2.2, Fig. 2.6D). Even in the presence of 1 mM mefenamic acid, there was no effect on the slope and shape of the G-V relationship but still a right shift in V_{1/2} of activation compared to control (ΔV_{1/2} for K41E with 1 mM: +38.1 mV) (Table 2.2, Fig. 2.6D).

In contrast, much like G40C, treatment with 100 µM mefenamic acid transformed the slowly activating waveform of K41R (Fig. 2.6C upper left panel) into one which has an instantaneous current and inhibited tail current decay (Fig. 2.6C lower left panel) characteristic of mefenamic acid’s effect on WT EQ I_{Ks}. Mefenamic acid also significantly altered the shape of the K41R G-V relationship (Fig. 2.6C right panel) when an interpulse interval of 15 s was used. This effect on the shape of the K41R G-V relationship however, was visually less dramatic than that of G40C and WT EQ I_{Ks} (WT EQ I_{Ks} G-V plots in control and 100 µM mefenamic acid both with an interpulse interval of 15 s is overlaid on Fig. 2.6C right panel for comparison; ΔV_{1/2}: -110 mV). Despite this, the effect mefenamic acid had on the G-V relationship of K41R was also dependent on the interpulse interval (data not shown). A shorter interpulse interval of 7 s produced a more dramatically altered G-V relationship and more leftward shift in V_{1/2} than when the interpulse interval was 15 s (data not shown). Additionally, mefenamic acid also resulted in a significant leftward shift in the V_{1/2} of activation of K41R (ΔV_{1/2}: -46.2 mV) (Table 2.2, Fig. 2.6D). These results clearly show that residue K41, and especially the charge on this residue is important in facilitating mefenamic acid’s modulation of I_{Ks} gating kinetics.
Figure 2.6 Mapping the mefenamic acid regulatory sites on KCNE1.

The currents and conductance-voltage (G-V) plots (Figs. 2.6A-C) were obtained using the protocols described in Fig 2.3. The interpulse interval was 15 s. For each EQ $I_{Ks}$ mutant, representative currents are shown in the absence of (control; upper left panel) and in the presence of 100 µM mefenamic acid (lower left panel). Right panels show the corresponding G-V plots in control, (black circles) and presence of 100 µM (grey circles) and 1 mM (purple circles) mefenamic acid. WT EQ $I_{Ks}$ in control (blue triangles) and presence of 100 µM mefenamic acid (red triangles), both with a 15 s interpulse interval is overlaid in Fig 2.6C (data from Fig 2.3E). Boltzmann fits were: (A) for L42C in control (n = 3): $V_{1/2} = 68.9$ mV, $k = 21.5$ mV; and, in 100 µM mefenamic acid (n = 3): $V_{1/2} = 31.8$ mV, $k = 14.8$ mV; (B) for K41C in control (n = 6): $V_{1/2} = 17.1$ mV, $k = 19.7$ mV; and, in 100 µM mefenamic acid (n = 4): $V_{1/2} = 11.3$ mV, $k = 19.3$ mV; and, in 1 mM mefenamic acid (n = 4): $V_{1/2} = 14.0$ mV, $k = 18.3$ mV; (C) for K41R in control (n = 3): $V_{1/2} = 72.7$ mV, $k = 20.8$ mV; and in 100 µM mefenamic acid (n = 4) $V_{1/2} = 26.6$ mV, $k = 49.3$ mV (Table 2.2). (D) Change in $V_{1/2}$ ($\Delta V_{1/2}$) for each EQ $I_{Ks}$ mutant in control vs. mefenamic acid (n = 3-6 at each concentration). *, **, *** and **** denotes a significant change in $V_{1/2}$ comparing control to the presence of mefenamic acid, where $p < 0.03$, $p = 0.0011$, $p = 0.0003$ and $p<0.0001$, respectively.
Figure 2.7 Effect of mefenamic acid is preserved in the G40C \( I_{Ks} \) mutant.

The currents and conductance-voltage (G-V) plots were obtained using the protocols described in Fig. 2.3. (A) Currents are shown for G40C in the absence of mefenamic acid (control; interpulse interval was 15 s). (B) Currents are shown for G40C in the presence of 100 µM mefenamic acid (interpulse interval was 15 s). (C) Effect of different interpulse intervals on G-V plots in control and 100 µM mefenamic acid. Intervals were 15 s in control (n = 4; black), and 7 s (n = 3; red), 15 s (n = 3; green) or 30 s (n = 3; purple) in mefenamic acid.

2.4 Discussion

Previous studies have shown that mefenamic acid increases activation of \( I_{Ks} \) current and slows tail current decay, but all of these have only used a single concentration of drug (100 µM). Generally, mefenamic acid effects are restricted to complexes of KCNQ1 and KCNE1 (Busch et al., 1994, 1997; Unsöld et al., 2000), although Abitbol et al. (1999) suggested that mefenamic acid may also
facilitate KCNQ1 expressed alone in oocytes. Most studies have not shown that facilitation of \( I_{K_s} \) is accompanied by dramatic changes in the current activation time course, except for Unsöld (2000). In this study we confirm that mefenamic acid enhances \( I_{K_s} \) activity (Fig. 2.1C), but not KCNQ1 alone (Fig. 2.5A), and that this effect is specific to \( I_{K_s} \) given that the large instantaneous current was blocked by HMR1556 (Fig. 2.1D), and no effect on endogenous currents was seen in untransfected cells treated with increasing concentrations of mefenamic acid (Fig. 2.1B). Following validation of these previous findings we further defined the concentration dependence, effect of interpulse interval, stoichiometry dependence and the KCNE1 regulatory sites for mefenamic acid actions on \( I_{K_s} \).

2.4.1 Mefenamic acid actions on saturated complexes of \( I_{K_s} \) (EQ)

Increasing concentrations of mefenamic acid (1, 10, and 30 µM) have been used to confirm the functional effect of activating \( I_{K_s} \) in pre-constricted rat mesenteric arteries (\( E_{\text{max}} \) of 96.1%) (Chadha et al., 2012), but as noted above, regardless of the expression system, a single concentration of 100 µM has been used to characterize the electrophysiological actions of mefenamic acid on \( I_{K_s} \). One striking effect is the induction of instantaneous current and reduction in the overall time-dependent slow activation of \( I_{K_s} \) (Fig. 2.1C). These current changes were used to define the concentration-dependence of mefenamic effects on \( I_{K_s} \), giving an EC\(_{50}\) and \( n^H \) of 60 µM and 0.49, respectively (Fig. 2.2). An \( n^H \) of <1 does not suggest multiple sites of action or positive cooperative binding of mefenamic acid to the channel complex, which is supported by the similar values of \( n^H \) for different stoichiometric ratios of KCNQ1:KCNE1 (\( n^H = 0.47 \) and 0.66 for EQQ and EQQQQ, respectively). The more commonly reported (in all prior studies) action of mefenamic acid is to slow current deactivation (Busch et al., 1994; Magyar et al., 2006; Toyoda et al., 2006) and our experiments
also confirmed the marked slowing of tail currents with time constants increasing across the range of repolarizing potentials (e.g. from 0.52 s to 1.24 s at –90 mV, Fig. 2.4C).

Mefenamic acid has a hyperpolarizing effect on the $I_{Ks}$ I-V and G-V relationships which, in turn, is reflected by a leftward shift in the $V_{1/2}$ of activation. Quantitatively, this hyperpolarization of the $V_{1/2}$ has previously been inconsistently reported (-15 mV in CHO cells and -26 mV in canine ventricular myocytes) (Magyar et al., 2006; Unsöld et al., 2000). In Figure 2.3, we demonstrated that this inconsistency may be related to mefenamic acid’s striking effect on the G-V relationship that results in part from slowed channel deactivation at shorter pulse intervals. Prior studies often did not state intervals between pulses so this cannot be verified.

With a voltage protocol that lasted 5 s and an interpulse interval of 7 s, there was not enough time to allow for complete $I_{Ks}$ deactivation between voltage clamp pulses in the presence of mefenamic acid. As such, an accumulation of current occurred which partly explains the dramatically altered G-V relationship and instantaneous current at this rate (Fig. 2.3E). We initially hypothesized that if enough time were given to allow for $I_{Ks}$ deactivation, the G-V relationship in the presence of mefenamic acid would mirror that seen in control (in the absence of mefenamic acid). However, even with an interpulse interval of 30 s in the presence of mefenamic acid, when deactivation should be complete, the G-V relationship still showed a significantly large departure in shape, slope and $V_{1/2}$, when compared to control. At interpulse intervals of 15, 20 and, especially 30 s, there is a flattening of the voltage dependence of the G-V relationship caused by mefenamic acid that suggests a fundamental modification in the way that $I_{Ks}$ senses and/or responds to changes in the transmembrane potential in the presence of the drug.
2.4.2 Mefenamic acid action is diminished with fewer KCNE1 subunits in the $I_{Ks}$ channel complex

As the KCNQ1:KCNE1 stoichiometry of $I_{Ks}$ is likely variable in vivo (Dvir et al., 2014a), understanding the effect of mefenamic acid on different subunit ratios is extremely important, and the use of fixed stoichiometry constructs in the present experiments allowed a quantitative comparison of the action of mefenamic acid on different stoichiometries of $I_{Ks}$ (Murray et al., 2016). The $V_{1/2}$ of activation for KCNQ1 alone was not altered by mefenamic acid, but with the partially saturated $I_{Ks}$ complexes, EQQ and EQQQQ, the $V_{1/2}$ of activation was significantly hyperpolarized (Fig. 2.5). This hyperpolarization was less dramatic than that seen when $I_{Ks}$ was fully saturated (EQ, Fig. 2.3E). Similarly, in the dose response curves, the responses of EQQ and EQQQQ to mefenamic acid at all concentrations were significantly less than that of EQ, which further supports the idea that the effect of mefenamic acid on $I_{Ks}$ is stoichiometrically graded.

2.4.3 Mefenamic acid binding to the $I_{Ks}$ complex

The binding site for DIDS and mefenamic acid on $I_{Ks}$ has previously been suggested to lie between residues 39-43 on KCNE1, with residue E43 specifically identified as critical for the binding of DIDS (Abitbol et al., 1999). Whether this site and/or other residues in the mapped region are critical for the binding of mefenamic acid to KCNE1, and/or $I_{Ks}$, was not studied. Through mutational analysis, we now show that although mutation of residues E43 and L42 in KCNE1 results in a reduced response to mefenamic acid, residue K41 is critical for the action of mefenamic acid (Fig. 2.6).
Most of the mutations themselves do have variable effects on the gating of $I_{Ks}$ in the absence of mefenamic acid (Table 2.2), but importantly K41C has little effect on the position or slope of the $I_{Ks}$ G-V relationship compared to WT EQ. This suggests that K41C does not itself destabilize the normal interactions between KCNQ1 and KCNE1 in the $I_{Ks}$ channel complex, or its ability to respond to applied changes in potential. These results are particularly interesting, taken in the context of the known importance of interactions between KCNQ1 and this region of KCNE to the pathophysiology of short QT syndrome, in which extremely slow deactivation is a feature (Dvir et al., 2014a).

The E43C, L42C, and K41E mutations shift the $V_{1/2}$ of $I_{Ks}$ to approximately +70 mV (Table 2.2), which is opposite to the direction expected if they were inhibiting the interactions between KCNQ1 and KCNE1 (Murray et al., 2016), but which may explain their lesser response to mefenamic acid (Fig. 2.6D). The G40C mutant responds almost like WT to mefenamic acid (Fig. 2.7) and, so defines a proximal limit of the critical region. Taken together, the data indicate the primary importance of K41 in the binding of mefenamic acid to the $I_{Ks}$ channel complex, and the response of K41R but not K41E to mefenamic acid suggests the importance of electrostatic rather than steric interactions in this effect.

2.4.4 Relevance of mefenamic acid activation of $I_{Ks}$ channel currents

Unlike most other activators that have little effect on $I_{Ks}$ channels with increasingly saturated stoichiometries (Gao et al., 2008; Magyar et al., 2006; Yu et al., 2013), we have shown that mefenamic acid can enhance all $I_{Ks}$ channel complexes of different stoichiometries, suggesting that this molecule or others like it may represent a therapeutic approach to treating LQTS types 1 and
5. Although this is well beyond the scope of the present study, we note that mefenamic acid is presently prescribed at a recommended dosage of 500 mg per day which has been reported to equate to a mean plasma concentration of 82.9 µM (Cryer and Feldman, 1998). This provides little clue towards the amount needed therapeutically to treat LQTS, but such concentrations cause a potent activating effect of mefenamic acid that is also dependent on the stimulus rate, which is important as \( I_{Ks} \) primarily contributes to cardiac repolarization at high heart rates. The definitive stoichiometry of \( I_{Ks} \) in humans as well as the degree of \( I_{Ks} \) channel activation required for a therapeutically beneficial shortening of the QT interval are presently unknown, so we cannot know whether compounds like mefenamic acid could have a beneficial effect in LQTS. We do, however, know that due to the known adverse gastrointestinal effects of COX1 inhibition, and block of other channels such as TRPC, TRPM and TREK channels (Jiang et al., 2012; Klose et al., 2011; Takahira et al., 2005), mefenamic acid itself may not be a suitable candidate unless modifications are made.

2.5 Conclusion

The KCNQ1 channel alone is insensitive to up to 1 mM mefenamic acid, and the drug increases \( I_{Ks} \) channel complex currents dependent upon the number of KCNE1 subunits present, unlike most other activators that have little effect on \( I_{Ks} \) channels with increasingly saturated stoichiometries. The instantaneous currents in the presence of mefenamic acid and the prolonged deactivation of tail currents are caused by a voltage-dependent shift of channel gating kinetics towards more negative potentials, and a marked decrease in the voltage sensitivity of the channel. The ability of mefenamic acid to mediate these gating changes relies on binding to \( I_{Ks} \) regulated through K41 on KCNE1 and potentially other residues surrounding it. The presence of mefenamic acid bound to
the $I_{Ks}$ channel complex fundamentally alters the ability of the KCNQ1/KCNE1 gating machinery to respond to the transmembrane potential gradient.

<table>
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<tr>
<th>EQ Control: Interpulse Interval 15 s</th>
<th>$V_{1/2}$ 23.9 ± 3.7</th>
<th>$k$-factor 20.4 ± 2.9</th>
<th>n 4</th>
<th>p-value</th>
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<tr>
<td>EQ + Mefenamic Acid: Interpulse Interval 7 s</td>
<td>-108 ± 9.0</td>
<td>6.9 ± 7.7</td>
<td>6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EQ + Mefenamic Acid Interpulse Interval 15 s</td>
<td>-86.5 ± 14.8</td>
<td>23.2 ± 11.2</td>
<td>4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EQ + Mefenamic Acid Interpulse Interval 20 s</td>
<td>-80.5 ± 18.2</td>
<td>53.3 ± 15.3</td>
<td>5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EQ + Mefenamic Acid Interpulse Interval 30 s</td>
<td>-13.1 ± 14.9</td>
<td>57.2 ± 15.4</td>
<td>6</td>
<td>0.0057</td>
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Table 2.1 Effect of interpulse interval on EQ $I_{Ks}$ G-V after treatment with mefenamic acid

$V_{1/2}$ of activation (mV) and slope value ($k$-factor, mV) in the absence and presence of 100 µM mefenamic acid for EQ $I_{Ks}$ at different interpulse intervals. ± denotes standard deviation with the p-value indicating statistical difference in $V_{1/2}$ compared to control as determined using a One-way Anova and Bonferroni multiple comparisons test.
Table 2.2 Effect of cystine-scanning and varying KCNE1 stoichiometry on $I_{K_s}$ G-V after treatment with mefenamic acid

$V_{1/2}$ of activation (mV) and slope value ($k$-factor, mV) in the absence and presence of mefenamic acid for mutant EQ $I_{K_s}$ and different stoichiometrically saturated WT $I_{K_s}$. ± denotes standard deviation with the p-value indicating statistical difference in $V_{1/2}$ compared to control as determined using a One-way Anova and Bonferroni multiple comparisons test. NS, not significant. * Mefenamic acid dose was either 100 µM (where applicable upper row values) or 1 mM (where applicable lower row values). Given the dramatic effect mefenamic acid has on the G-V relationship for some constructs, Boltzmann curves could not be properly fit in some cases.

<table>
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<th>Control</th>
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<th>Δ$V_{1/2}$</th>
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Chapter 3: Water-reaching platform for longitudinal assessment of cortical activity and fine motor coordination defects in a Huntington Disease mouse model

3.1 Introduction

Synaptic and circuit changes that precede progressive striatal medium spiny neuron (MSN) and cortical neuronal loss in Huntington Disease (HD) result in a characteristic triad of clinical manifestations, including: motor dysfunction, cognitive impairment, and neuropsychiatric disorders (Cepeda and Levine, 2022; McColgan and Tabrizi, 2018). The hallmark motor symptoms of HD include fine motor incoordination, chorea, bradykinesia, rigidity and difficulties with balance and gait.

Since the discovery that dominantly inherited expansions >39 of a CAG triplet repeat in exon 1 of the \textit{HTT} gene causes HD (MacDonald et al., 1993), over 50 distinct mouse and rat models with increasingly better face and construct validity have been developed (Menalled et al., 2014; Pouladi et al., 2013). Although several assessments such as rotarod, balance beam and gait tasks (such as the footprint test) are commonly used to assess motor defects in HD mice (Abada et al., 2013; Brooks et al., 2012; Pallier et al., 2009; Southwell et al., 2016), bodyweight remains a major confound of these tasks (Batka et al., 2014; McFadyen et al., 2003), necessitating the development and usage of other behavioral assessments.
In clinical settings, impairments in reaching and/or skilled hand movements have been observed in HD patients (Klein et al., 2011). Skilled forelimb movement learning and performance has also been examined in HD mice using automated home-cage lever pulling systems (Woodard et al., 2021, 2017a), demonstrating that HD motor learning deficits are related to impaired striatal neuronal plasticity (Woodard et al., 2021). Given reaching towards a target and manipulating objects is commonly used in our daily lives and the general ‘reach-to-grasp’ features of forelimb movements has been shown to be similar between humans and rodents (Galiñanes et al., 2018), preclinical behavioral assessment of skilled forelimb reaching tasks could improve our understanding of HD movement defects. Water-reaching tasks further enable longitudinal multi-trial assessment providing a high throughput system for behavioral phenotyping throughout the duration of disease progression, and may detect more subtle motor learning and movement defects. Combining water-reaching assessment with simultaneous recording of widefield cortical activity further enables examination of pathophysiological circuit changes underlying HD movement defects.

To date, few studies exist examining these wide-scale cortical circuit changes in HD mouse models. Using 3D magnetic resonance imaging, arteriolar cerebral blood volume level changes in the striatum and motor cortex were observed in HD mice beginning at 3 months of age which worsened overtime (Liu et al., 2021b). Hemodynamic measurements are, however, indirect indicators of neuronal activity. Using mesoscale voltage-sensitive dye imaging, our group has shown that hindlimb stimulation evokes a larger area and longer lasting cortical response in anesthetized HD compared to WT mice (Sepers et al., 2021). Given recent neurophysiology studies have demonstrated the involvement of multiple brain regions in sensation, cognition and
movement (Pinto et al., 2019; Steinmetz et al., 2019), widefield functional assessment of cortical circuit changes during task performance can provide longitudinal read-outs of HD pathology across brain networks. Accordingly, we used a water-reaching task to demonstrate progressive changes in widefield cortical activity and skilled forelimb movement defects. The full-length HTT knock-in heterozygous zQ175 mouse model was employed due to its greater construct validity over other HD mouse models (Pouladi et al., 2013).

3.2 Methods

3.2.1 Animals

All experiments and procedures were carried out in accordance with the Canadian Council on Animal Care and approved by the University of British Columbia Committee on Animal Care (protocols A18-0036 and A19-0076). Mice were group housed with 2 to 4 mice per cage under a controlled 12 hr light/dark cycle (7:00 lights on, 19:00 lights off). Standard laboratory mouse diet was available ad libitum. Water was available ad libitum except during the duration of head-fixed water-reaching behavioral testing and when mice were tapered back to ad libitum water consumption. Surgery and subsequent behavioral testing was performed on 6 female heterozygous zQ175 knock-in C57BL/6 mice expressing GCaMP6s and 6 female wildtype (WT) littermates as controls starting at ~5 months of age. zQ175 C57BL/6 mice and WT littermates, both expressing GCaMP6s, were obtained by first crossing heterozygous zQ175 C57BL/6 mice with homozygous transgenic Thy-1 GCaMP6s line 4.3 C57BL/6 mice (https://www.jax.org/strain/029928) with homozygous transgenic Thy-1 GCaMP6s line 4.3 C57BL/6 mice (HHMI Janelia Research Campus)(Dana et al., 2014; Sofroniew et al., 2016) then crossing subsequent offspring to obtain homozygous GCaMP6s expression. Animal tissue was
collected through ear clipping at weaning. DNA extraction and PCR analysis were subsequently used to determine genotype. Health status and weight of all animals was assessed daily. A limitation of our study is that only female mice were employed. Learning and task non-compliance in water reaching (Galiñanes et al., 2018) has been reported in WT mice. Female mice were selected since mice performing the task (that were originally from different cages) could be co-housed.

3.2.2 Animal surgery

All mice were subjected to head-bar and chronic transcranial window surgery as previously described (Murphy et al., 2016; Silasi et al., 2016) and allowed to recover for a week before commencement of behavioral testing. Briefly, an incision and skin retraction over the cortex was made enabling a glass coverslip to be applied using Metabond clear dental cement (Parkell, Edgewood, NY, USA; Product: C&B Metabond) onto un-thinned bone. A steel head-fixation bar was also placed 4 mm posterior between bregma and the bar edge.

3.2.3 Behavioral testing timeline

During the handling period, mice were first habituated to daily human contact for three weeks. Mice then underwent surgery and were allowed to recover for one week before initial tapered beam assessment (5 days). After 2 days of initial tapered beam testing, mice were also habituated to first the confinement tube only, then to the confinement tube and head fixation and, finally the confinement tube, head fixation and experimental setup. The duration of head fixation was progressively increased at a rate of ~7 min/day for 5 days.
Mice were then water restricted for skilled forelimb head-fixed water-reaching behavioral training and testing. From the water-reaching task, mice had the potential to receive ~1 mL/day of water. Given variation in weight due to *ad libitum* food consumption and excrement, mice who either performed poorly or lost more than 0.5 g in weight compared to the previous day were given up to ~1.1 mL of task-independent water. All mice received ~100 µL of additional water daily. As such, all mice received ~1.1 mL of task-independent and/or behavioral test-derived water daily. The humane endpoint was defined as a maximal weight loss of 15% from a pre-water-restricted baseline weight. No mice reached the humane endpoint during the duration of the study.

After at least 60 days (maximum 67 days) of water restriction and water-reaching behavioral training and testing, mice were tapered back to *ad libitum* access to water. During this readjustment period, mice received 1.1 mL of task-independent water on the first day. In subsequent days mice received progressively increased water at a rate of ~500 µL/day for ~4 days. This additional water was administered at three different times during the light cycle to prevent water intoxication. After stabilization of mouse weight, *ad libitum* access to water was restored for the duration of the behavioral testing.

Accelerating rotarod (4 days) and final tapered beam testing (7 days) was then conducted with a 1 day recovery period between the two behavior assessments. All animals were sacrificed with intraperitoneal injection of pentobarbital sodium (240 mg/kg) and transcardially perfused with first 10 mL phosphate-buffered saline (PBS) then 10 mL 10% neutral buffered formalin (NBF). Whole brains were removed and post-fixed in NBF for post-mortem immunohistochemistry.
3.2.4 Head-fixed water reaching test

Mice underwent head-bar and chronic transcranial surgery and were trained to reach for water under head-fixed conditions following in part a previously described protocol (Galiñanes et al., 2018). All mice were water restricted after habituation to the confinement tube, head-fixation and experimental set up. A platform which extends 1.5 cm from the base of the confinement tube allowed the mice to rest their forepaws while not reaching for water. The water spout was fashioned using a blunted 22G needle bent at a 90° angle. The starting position of the water spout was ~0.75-1 cm posterior from the tip of the snout and positioned laterally so that the water drop made minimal contact with the whiskers. At this position, mice could touch the water spout with their paws and feel the water drop if they groomed which transitioned to reaching. For mice which did not groom, the water drop was allowed to touch their whisker pad which promoted grooming and transition into reaching toward the spout. Once mice started reaching, the distance of the water spout was gradually increased until a final distance of ~1.5 cm lateral and ~0.5 cm posterior to the tip of the snout was achieved. Only mice that reached the final distance with a success rate of at least ~80% on Day 15 were included for further analysis. Although all 12 mice underwent the entire behavioral testing timeline, two WT mice did not engage to the extent and/or meet this predetermined criteria and were not included in subsequent water-reaching analysis.

Trial structure included alternating reward (water drop presented) and no reward (no water drop, despite identical presentation of visual and auditory cues) trials. Unless an electronic failure occurred, all trials started with a rewarded trial. The experimental setup was illuminated with infrared LED illuminator lights. A Raspberry Pi single-board computer and custom Python script was used to control camera recording, blue and green light used to illuminate the cortex, cue light
signal, cue buzzer signal, water solenoid to deliver the water reward and capacitive touch sensor connected to the water spout.

At the start of reward and no reward trials, a Raspberry Pi infrared night vision camera (320 x 320 pixels; 60 Hz) and a 1M60 Pantera CCD camera (Dalsa) enabled behavioral and GCaMP activity recording, respectively. The cortex was illuminated using alternating green and blue light providing information about hemodynamic changes and exciting GCaMP, respectively and collected as 12-bit images through the Dalsa camera using XCAP imaging software (120 Hz). Binning camera pixels (8 x 8) produced a resolution of 68 µm/pixel. These imaging parameters have been used previously for widefield cortical GCaMP imaging (Vanni and Murphy, 2014; Xiao et al., 2017).

A 0.1 s duration green LED light flash 2 s after the start of camera recording was followed by a 0.1 s buzzer tone in the case of non-rewarded trials or a buzzer tone combined with simultaneous ~20 µL water reward in the case of rewarded trials 6 s after the start of camera recording. For rewarded trials, if a spout touch was detected by the capacitive touch sensor (Adafruit Industries, New York, NY, USA) after delivery of the water reward, the Picamera and Dalsa camera recording would cease 4 s after the time of spout touch. If a touch was not detected or it was a non-rewarded trial, camera recording would cease 10 s after delivery of the reward. Rewarded trials therefore ranged from 10-16 s and non-rewarded trials were 16 s in length. The intertrial interval was 4 s. A total of ~120 trials over a duration of ~39 min were conducted daily for 60-67 days. Since the capacitive touch sensor was found to not accurately determine reaches, touches and/or contact with the water spout, all trials were manually blind scored with 6 categories for rewarded
trials (disregard trial, no reach, groom, success, partial fail and complete fail) and 3 categories for non-rewarded trials (disregard trial, no reach and unrewarded reach). Trials which were disregarded included trials where there was either an electronic failure (e.g. water solenoid delivered too much or too little water, truncated behavioral and/or cortical activity imaging video was recorded, etc.), experimenter intervention (e.g. during training, after the animal would cause the position of the water spout to move due to vigorous grooming/reaching, etc.) or when the trial was deemed too difficult to score. No reach trials referred to those wherein the mouse did not groom or lift either both or one paw off of the resting platform in a forward reaching motion. Groom trials referred to the mouse engaging in grooming behavior. Complete fail trials consisted of the mouse reaching forward but being unable to make contact with or obtain the water drop. Partial fail trials consisted of the mouse reaching forward and making contact with the water drop but then being unable to bring the water to its mouth to drink. No rewarded trials scored with the category ‘unrewarded reach’ referred to either the animal engaging in grooming behavior or reaching behavior even when no water was present on the water spout. Grooming behavior was included in this category to reduce scoring subjectivity between natural grooming behavior and groom-to-reach behavior. Mice were also observed to switch between grooming and reaching the spout. Successful trials on Day 8 were also scored for the absence and presence of early reaches defined as reaches that occurred before water reward presentation. Early reaches and unrewarded reaches are referred to as impulsive reaches and reflect an event sequence defect.
3.2.5 Accelerating rotarod test

Mice were tested as previously described (Woodard et al., 2021). Briefly, mice were tested for 4 consecutive days on the rotarod (Ugo Basille) accelerated from 5 to 40 RPM over a total time period of 300 s. Mice received 3 trials per day with a 2 hr inter-trial interval (ITI). A fall was defined as the mouse falling from the rotarod or completing a rotation holding onto the rod and not trying to right itself at any point during the rotation. If a fall or full rotation occurred, the trial was ended and the time recorded. Mice that reached the maximum allowed time were scored as 300 s and the trial ended. The average latency to fall for the 3 trials was scored.

3.2.6 Tapered beam test

Mice were tested using an automated touch sensing tapered beam test (Ardesch et al., 2017). Briefly, conductive paint surfaces serving as input electrodes to four 12-channel capacitive touch sensors (Adafruit Industries, New York, NY, USA) connected to a Raspberry Pi single-board computer recorded the start and finish times to traverse the beam using a custom Python script. The beam measured 100 cm in length tapering from 3.5 cm to 0.5 cm with a wider 1 cm base component extending to the left and right 1 cm below the upper surface of the beam. Mice received 4 trials per day for 5 and 7 consecutive days during the first and second round of tapered beam testing. Average time required to traverse the beam across the 4 trials was scored.

3.2.7 Immunohistochemistry

Coronal brain sections were cut on a vibratome at 50 µm thickness (Leica VT1000S, Leica Microsystems GmbH). Slides were then boiled in sodium citrate (10 mM sodium citrate, 0.05% Tween20, pH 6) to allow antigen retrieval. After washing, slices were permeabilized with 0.3%
Triton X-100, blocked with BlockAid Blocking Solution (Molecular Probes) and Image-iT FX Signal Enhancer (Molecular Probes) before 1:100 primary antibody labeling overnight (rabbit monoclonal anti-DARPP-32 (Abcam; ab40801) and mouse anti-NeuN (MilliporeSigma; ZMS377)). Phosphate-buffered saline, 0.1% Tween 20 (PBST) washing was then followed by 1:1000 secondary antibody labeling (rhodamine (TRITC) AffiniPure goat anti-mouse IgG(H+L) (JacksonImmuno Research Laboratories Inc.; 115-025-003) and AlexaFluorTM647-R-phycoerythtin goat anti-rabbit IgG(H+L) (A20991, Thermo Fisher)). Sections were washed with PBST and mounted on glass coverslips with Prolong™ Gold Antifade Mountant (Thermo Fisher; P36930) for subsequent imaging. Sections were imaged with a 10x and 63x objective using an upright Leica imaging system (SP8 DIVE). Staining intensity was determined using ImageJ software. Relative intensity values are expressed relative to background.

3.2.8 Kinematic and mesoscale GCaMP analysis

To accommodate for varying rewarded trial lengths, the first 10 s were examined for all rewarded and non-rewarded trials.

Kinematic analysis of forelimb skilled reaching behavior Deeplabcut as described in (Mathis et al., 2018) was used to track body parts (right and left forepaws and mouth) and equipment landmarks of interest (platform and spout). Subsequent analyses were conducted using a custom Matlab code. For Figures 3.4 and 3.5, the distance from the height of the platform to the height of the spout was calculated and represents the distance to the spout (spout distance). The euclidean distance of the left paw trajectories was calculated from the time of water reward delivery to 1.1 s afterwards for all successful rewarded trials. This time period was selected since it corresponded
to the time needed to complete a successful reach. Euclidean distances traveled by the left paw were then binned. Histogram bin size reflects multiples of spout distance. For example, bin 4 contains successful rewarded trials where the euclidean distances of the left paw trajectories were 4x that of the spout distance. Euclidean distances are reported as multiples of spout distance since this distance represents the most efficient route the left paw could take to reach the spout. The average euclidean distance and standard deviation were calculated for each mouse then genotype averaged. For Figure 3.14 the y-direction traces of left paw movement for each trial are depicted.

**GCaMP image processing and analysis** All GCaMP image processing and analysis were conducted using custom Matlab codes. All GCaMP responses were movement and hemodynamic artifact corrected by subtracting changes in green reflectance signals from observed green epifluorescence (Vanni et al., 2017) and expressed as percentages relative to baseline responses ($F - F_0/F_0$)*100 where $F_0$ is the baseline from the start of the trial to water reward delivery. For region-based analysis, the brain-to-atlas approach in MesoNet (Xiao et al., 2021) was used to register cortical images to a common atlas using predicted cortical landmarks to determine regions of interest (ROIs). A 5 x 5 pixel region centered in each ROI was used for examination of peak amplitude and baseline standard deviation. Peak amplitude was calculated from the baseline (defined as 1-5 s from the start of the trial) to the peak. Cortical area activated was determined as pixel intensities greater than 4x standard deviation of the baseline (1-5 s). Contralateral (right) and ipsilateral (left) hemisphere ROIs include the primary motor (M1), secondary motor (M2), somatosensory mouth (sspm), somatosensory forelimb (sspfl), somatosensory hindlimb (ssphl), somatosensory area unassigned (sspun), somatosensory nose (sspn), somatosensory barrel field domain (sspbfd), somatosensory trunk (ssptr), primary visual (visp), retrosplenic lateral agranular
part (rspbgl) and retrosplenic dorsal (rspd) cortices. Forelimb movements after presentation of the water drop in rewarded trials were classified as successful or failed reaches. As such, subsequent ΔF/F analyses for these trial types were concentrated during the time period after the water drop (until 4 s afterwards). However, reaching during non-rewarded trials could occur throughout the trial and was not necessarily associated with a cue. Therefore, each ROI’s peak amplitude for unrewarded reach trials was evaluated across the entire trial duration (total 10 s).

3.2.9 Experimental design, statistical analysis and code accessibility

All experimenters were blinded to the genotype during the analysis. Unless otherwise stated, Two-way Anova and Šídák’s multiple comparisons post-hoc test were used. Statistical analysis was calculated using Graphpad Prism. Alpha level for all tests was p = 0.05. The code used for the analysis is available from the corresponding authors upon request.

3.3 Results

3.3.1 Overview of experimental assessment timeline

The behavioral testing timeline is depicted in Figure 3.1A. After ~1 week of chronic window and head-fixation bar surgical recovery, all mice underwent tapered beam training and testing to examine baseline gross motor function. Mice were then water restricted and trained to perform skilled forelimb water-reaching (Fig. 3.1B). Behavioral camera recording combined with markerless pose estimation (Mathis et al., 2018) enabled tracking and assessment of progressive forelimb coordination defects. Simultaneous recording of cortical activity using GCaMP6 mesoscale imaging further enabled assessment of progressive cortical circuit changes. After the completion of water-reaching assessment, mice were allowed to rest and were tapered back to ad
libitum water consumption. Mice then underwent rotarod testing and a second round of tapered beam testing to examine HD gross motor defects. Finally, HD pathology was determined using immunohistochemistry staining for DARPP-32, a striatal medium spiny neuron (MSN) marker. Mice were weighed daily to monitor health (data not shown). No mouse reached the humane endpoint of more than 15% loss of body weight.

Figure 3.1 Scheme of behavioral testing.

(A) Animals were allowed to recover from surgery (~1 week) before initial tapered beam testing (5 days) which was followed by water-restricted forelimb water-reaching testing (60-67 days). Mice were tapered back to ad libitum water consumption (~5 days) before rotarod testing (4 days) and final tapered beam testing (7 days) with one day in between rotarod and tapered beam testing to allow stamina recovery. (B) Side-view image of a representative head-fixed mouse in the water-reaching task. (C) Trial structure for the water-reaching task where alternating rewarded and non-rewarded trials were performed. The intertrial interval was 4 s. A visual cue 2 s after initiation of camera recording was followed by an auditory cue and water drop reward for rewarded trials and only an auditory cue for non-rewarded trials.
non-rewarded trials both 6 s after initiation of camera recording. If a spout touch was detected after water reward delivery, the rewarded trial ended 4 s after the spout touch was detected. In cases where a spout touch was not detected, the rewarded trial timed out 10 s after water reward delivery. All non-rewarded trials ended 10 s after the auditory cue. Total trial length was therefore 16 s for non-rewarded trials and could range from 10-16 s for rewarded trials depending on if a water spout touch was detected. For consistency, all trial lengths were truncated to 10 s in total for subsequent analyses.

3.3.2 Progressively reduced forelimb motor performance in HD mice

Trial structure for the water-reaching task with alternating rewarded and non-rewarded trials is depicted in Figure 3.1C. Briefly, a visual cue was delivered 2 s after initiation of camera recording for all trials. For rewarded trials, an auditory cue and water drop was delivered 4 s later (6 s since start of the trial). For non-rewarded trials, only an auditory cue was given with no water delivered. Water-reaching performance in both genotypes was quantified over 60 days (Fig. 3.2). Over time mice were trained during rewarded trials to reach forward towards the spout from their resting position (reach-to-grasp behavior), grasp the water drop then successfully bring the water to their mouth to drink (grasp-to-drink behavior)(Fig. 3.4A). We refer to this overall as the ‘reach-grasp-drink’ movement. Although no aversive punishment was given for reaching the spout during non-rewarded trials, no water reward was available on the spout making any attempts futile.

On Day 1 there were minimal successful trials as both groups were learning the task (Fig. 3.2A). In both genotypes (Day 1) the largest proportion of rewarded trials were spent not engaging with the task (no reach)(WT: 58.7 ± 4.7%; HD: 67.9 ± 5.0)(Fig. 3.2C) with the second largest proportion of trials spent reaching the water spout to swat the water away (partial fail)(HD: 22.3 ± 5.1%; WT: 33.4 ± 4.2%)(Fig. 3.2B). After performing successful trials for the first time on Day
WT mice engaged in frequent unrewarded reaching and/or groom-to-reach behavior on Day 5 (41.8 ± 10.0%) (Fig. 3.2D). WT mice however, quickly decreased unrewarded reaching behavior and by Day 8 performed minimal unrewarded reach trials (9.4 ± 2.7%). WT mice achieved a near perfect success performance rate by Day 8 (WT: 89.2 ± 3.7%) (Fig. 3.2A).

Similar to WT mice, HD mice also after performing successful trials for the first time on Day 3 (61.6 ± 14.3%) (Fig. 3.2A), engaged in frequent unrewarded reaching and/or groom-to-reach behavior on Day 5 (42.6 ± 11.6%) (Fig. 3.2D). The frequency of unrewarded reaching however, persisted. Although HD mice also reached near perfect success performance rates for rewarded trials by Day 8 (HD 92.3 ± 2.5%) (Fig. 3.2A), significantly more unrewarded reaching was still present on this day compared to WT (WT: 9.4 ± 2.7%; HD: 43.9 ± 12.3%; p=0.0169). By Day 11, the unrewarded reach trials in HD mice decreased to the same frequency as seen in WT mice.

Over time WT mice were able to maintain their high success rate until at least Day 60 (Fig. 3.2A). HD mice however, experienced a progressive decline in successful trials. By Day 60 the successful performance rate was 31.1 ± 10.0% for HD mice. No significant changes in weight were seen throughout the entire behavioral testing timeline indicating water restriction was not the cause of HD mice performance decline (data not shown).

Unsuccessful trials were divided and scored as either no reach, groom, partial fail and complete fail trials (Fig. 3.3). Partial fail scores denote trials where the mouse made contact with the spout and removed the water drop from the spout but was unable to retain and drink the water drop (successful reach-to-grasp performance but failed grasp-to-drink performance). Complete fail
scores denote trials where the mouse lifted their paw in a reaching behavior but the paw did not make contact with the spout (failed reach-to-grasp performance).

The low prevalence of complete fail trials for the duration of behavioral testing for HD mice (Fig. 3.3A) suggest that failure to perform the reach-to-grasp segment of the forelimb movement does not explain the decline in successful performance. HD mice, however, developed a significant increase in partial fail trials compared to WT mice by Day 30 (WT: 9.6 ± 2.2%; HD: 41.3 ± 4.2%; p=0.0024)(Fig. 3.2B) suggesting instead the grasp-to-drink segment of the movement was impaired. By Day 60 HD mice also developed a significant increase in no reach trials compared to WT mice (WT: 6.5 ± 6.5%; HD: 34.6 ± 8.1%; p=0.0007)(Fig. 3.2C). Throughout the whole duration of behavioral testing, mice in both genotypes spent a minimal number of rewarded trials grooming (Fig. 3.3). In all, behavior categorization revealed deficits in event sequence and forelimb function in HD mice.
Figure 3.2 Water reaching task behavioral categorization.

HD (n = 6) and WT (n = 4) mice are denoted in teal and gray, respectively. (A-C) Percent of successful (A), partial fail (B) and no reach (C) trials to the total number of rewarded trials over time. (D) Percent of unrewarded reach trials (reaching occurs despite there being no reward) to the total number of non-rewarded trials over time. Shaded intervals denote standard error of the mean (SEM). ***, ** and * denotes p <0.005, <0.01 and <0.05, respectively. See Figure 3.3 for additional data.
Figure 3.3 Categorization of unsuccessful rewarded trials.

Proportion of unsuccessful rewarded trial types (no reach: blue; groom: purple; partial fail: green; complete fail: dark teal) and total unsuccessful trials (line) to the total number of rewarded trials for HD (n = 6)(A) and WT (n = 4)(B) mice overtime. Error bars denote SEM. Grooming and complete fail trials in both genotypes were minimal with no statistical differences between genotypes.
3.3.3 Increased distance and variable forelimb reaching movement in HD mice

On average, WT mice were able to obtain the water drop 1.0 ± 0.1 s after reward delivery. As such, markerless pose estimation was used to track the left paw from the time of water reward delivery to 1.1 s afterwards (Fig. 3.4). Euclidean distance traveled by the left paw during successful trials is presented as multiples of the spout distance over this fixed period of time. Sample paired distribution of reaching trajectory distances for two WT (gray) and three HD (teal) mice on Day 8 (top panels) and Day 45 (bottom panels) with corresponding average euclidean distance and trial-to-trial standard deviation are shown (Fig. 3.4B)(all mice are shown in Fig. 3.5). Unlike on Day 8 when the average euclidean distance traveled by the left paw was the same in both genotypes (WT: 2.1 ± 0.1; HD: 2.1 ± 0.1 spout distances; p=0.9811), the left paw of HD mice traveled a greater distance during the reach on Day 45 than WT mice (WT: 1.9 ± 0.2; HD: 2.4 ± 0.2 spout distances; p=0.0320)(Fig. 3.4D). Sample left paw reaching trajectories on Day 45 are shown for WT and HD mice (Fig. 3.4C). The variability in reaching distances for all successful trials on Day 8 (measured as the standard deviation) was not statistically different between genotypes (WT: 0.62 ± 0.14; HD: 0.84 ± 0.12 spout distances; p=0.3397)(Fig. 3.4E). On Day 45 however, HD mice displayed a greater variability in reaching trajectory distances than WT mice (WT: 0.51 ± 0.05; HD: 0.95 ± 0.11; p=0.0360).
Figure 3.4 Kinematic analysis of successful trials.

HD (n = 6) and WT (n = 4) mice are denoted in teal and gray, respectively. (A) Representative images depicting the mouse at rest and the reach-grasp-drink water-reaching movement. Dots represent either different body parts or equipment labeled for use in markerless pose estimation. The spout distance (calculated from the height of the platform to the height of the spout; see Methods for more details) is depicted in purple. (B) Distribution of euclidean distance traveled (water reward delivery to 1.1 s afterwards) by the left paw during successful rewarded trials on Day 8 (top graphs) and Day 45 (bottom graphs) for representative WT and HD mice. The distance traveled in each trial was binned with intervals reflecting how many more times the path taken was compared to the spout distance (see Methods for more details). Relative frequencies (%) of each bin are reported. Average euclidean distance traveled (\( \bar{x} \)) and standard deviation (STD) are indicated and reflect multiples of spout distance. (C) Representative left paw X, Y trajectories of WT (top trace) and HD (middle and bottom traces) mice performing a successful water-
reaching movement during a rewarded trial on Day 45. Arrows denote path of trajectory. Purple arrows indicate the start of the trajectory. (D-E) Average euclidean distance traveled across all successful trials (D) and trial-to-trial variability (Standard deviation; STD) of successful reaching trajectories (E) on Day 8 and 45. Measurements are given in multiples of spout distance; D and E are per mouse averages. Error bars denote SEM. * denotes \( p<0.05 \). See Figure 3.5 for additional data.

**Figure 3.5 Euclidean distance distribution for successful trials on Day 8 and Day 45.**

Distribution of euclidean distance traveled by the left paw (water reward delivery to 1.1 s afterwards) during successful rewarded trials on Day 8 (top graphs) and Day 45 (bottom graphs) for all WT (gray) and HD (teal) mice. The distance traveled in each trial was binned with intervals reflecting how many more times the path taken was compared to the spout distance (calculated from the height of the platform to the height of the spout; see Methods
for more details). Relative frequencies (%) of each bin are reported. Average euclidean distance traveled ($\bar{x}$) and standard deviation (STD) are indicated in multiples of spout distance.

### 3.3.4 Changes in cortical activity dynamics during reaching over time in HD mice

The brain-to-atlas approach in MesoNet (Xiao et al., 2021) was used to register cortical images to a common atlas using predicted cortical landmarks. Regions of interest (ROIs) were then defined (Fig. 3.6A; ROIs are color and number labeled). Sample heat maps of trial-to-trial cortical $\Delta F/F$ for select ROIs during successful, unrewarded reach and/or partial fail trials on Day 8 and/or 45 from a WT and HD mouse are shown (Fig. 3.6B)(all mice are shown in Fig. 3.7-3.9). Across genotypes, widespread activation of M1, M2, rspagl and to a lesser degree sspfl was consistently apparent for all successful and partial fail trials after water drop presentation (Fig. 3.6B and Fig. 3.7-3.9). In successful trials, activation increased by Day 45 compared to Day 8 for HD but not WT mice.

Sample time series of GCaMP6 cortical wide-field imaging on Day 8 and 45 are shown in Figure 3.10A for a representative WT and HD mouse. On Day 8, despite both HD and WT mice having comparable success rates (Fig. 3.2A) and reaching distances (Fig. 3.4D), genotype differences in cortical activity were apparent (Fig. 3.10B-C). Examining specific ROIs further revealed that the peak amplitude across all ROIs in both the contralateral and ipsilateral hemisphere was greater in WT compared to HD mice (contralateral: $F_{1,8}=5.925$, $p=0.0409$, ANOVA; ipsilateral: $F_{1,8}=5.967$, $p=0.0404$, ANOVA)(Fig. 3.10B-C). Together this indicates that on Day 8, more extensive cortical activation associated with reaching was seen in WT compared to HD mice.
When examined longitudinally within genotypes (comparison of Day 8 to Day 45), the peak amplitude across all ROIs in the contralateral hemisphere increased in HD mice ($F_{1,10}=5.521$, $p=0.0407$, ANOVA) (Fig. 3.10E) with no significant changes in WT mice ($F_{1,72}=0.006$, $p=0.9388$, ANOVA) (Fig. 3.10D). In particular, the pixel regions centered in the contralateral secondary motor cortex (M2) and retrosplenial cortex lateral agranular part (rspagl) displayed significantly greater peak amplitude over time in HD mice (Fig. 3.10E). No significant changes in peak amplitude across all ROIs in the ipsilateral hemisphere were seen over time for WT ($F_{1,6}=0.026$, $p=0.8770$, ANOVA) and HD ($F_{1,10}=0.709$, $p=0.4193$, ANOVA) mice when comparing within genotypes (Fig. 3.11).

When comparing genotypes (WT to HD) a significant difference in contralateral M2 peak activity was seen on Day 45 (WT: $2.6 \pm 0.7$; HD: $5.8 \pm 0.9$; $p=0.0455$) (Fig. 3.10G). Figure 3.10F shows the average time course of contralateral M2 $\Delta F/F$ activation on Day 8 and 45 for all mice. Genotype differences were also seen in contralateral sspm, sspbfd and visp cortices on Day 8 (Fig. 3.12). Comparing WT to HD mice overtime further revealed differences in ipsilateral M2, rspagl and retrosplenial cortex, dorsal part (rspd) (Fig. 3.13). Overall, HD mice exhibited reduced peak cortical activity associated with successful reaching compared to WT mice on Day 8. When compared over time, peak cortical activity increased in HD but not WT mice.
Figure 3.6 Representative trial-to-trial GCaMP6 cortical activity in regions of interest.

(A) Cartoon depicts regions of interest investigated in subsequent analyses. (B) Representative trial-to-trial heat-map of GCaMP (ΔF/F) cortical activity in contralateral M1 (primary motor), M2 (secondary motor), sspfl (somatosensory forelimb) and rsplagl (retrosplenial lateral agranular) from a WT and HD mouse on Day 8 and Day 45 for success, unrewarded reach and/or partial fail trials. Individual trials are stacked in rows. Time of the water reward (for rewarded trials) and tone (for non-rewarded trials) is denoted with a black line. See Figure 3.7 to 3.9 for additional data.
Figure 3.7 WT trial-to-trial GCaMP6 heat-map for successful trials.

Success trial-to-trial heat-map of GCaMP ($\Delta F/F$) cortical activity in contralateral M1 (primary motor), M2 (secondary motor), sspfl (somatosensory forelimb) and rspagl (retrosplenial lateral agranular) for all WT mice on Day 8 and Day 45. Individual trials are stacked in rows. Time of the water reward is denoted with a black line.
Figure 3.8 HD trial-to-trial GCaMP6 heat-map for successful trials.

Success trial-to-trial heat-map of GCaMP (ΔF/F) cortical activity in contralateral M1 (primary motor), M2 (secondary motor), sspfl (somatosensory forelimb) and rspagl (retrosplenial lateral agranular) for all HD mice on Day 8 and Day 45. Individual trials are stacked in rows. Time of the water reward is denoted with a black line.
Figure 3.9 HD trial-to-trial GCaMP6 heat-map for unrewarded reach and partial fail trials.

Partial fail and unrewarded reach trial-to-trial heat-map of GCaMP (ΔF/F) cortical activity in M1 (primary motor), M2 (secondary motor), sspfl (somatosensory forelimb) and rspagl (retrosplenial lateral agranular) for all HD mice on Day 45 and Day 8, respectively. Individual trials are stacked in rows. Time of the water reward (for partial fail trials) and tone (for unrewarded reach trials) is denoted with a black line.
Figure 3.10 Longitudinal mesoscale GCaMP6 imaging of the cortex during water reaching.

HD (n = 6) and WT (n = 4) mice are denoted in teal and gray, respectively. 5x5 pixel regions are centered in regions of interest (ROIs) and examined. (A) Time series of cortical wide-field GCaMP imaging (ΔF/F) from a representative WT (left panels) and HD (right panels) mouse on Day 8 (top panels) and Day 45 (bottom panels) during successful trials. (B-C) Peak amplitude of regions of interest on Day 8 for successful trials in the contralateral hemisphere. (D) Peak amplitude of regions of interest on Day 8 and Day 45 for WT contralateral hemisphere. (E) Peak amplitude of regions of interest on Day 8 for successful trials in the HD contralateral hemisphere. (F) Time series of cortical wide-field GCaMP imaging (ΔF/F) for M2 on Day 8 and Day 45 during successful trials. (G) Peak amplitude of regions of interest on Day 8 for successful trials in the contralateral hemisphere.
(F_{1,8}=5.925, p=0.0409, ANOVA; across genotype)(B) and ipsilateral (F_{1,8}=5.967, p=0.0404, ANOVA; across genotype)(C) hemisphere. (D-E) Peak ΔF/F amplitude of ROIs in the contralateral hemisphere on Day 8 and Day 45 for WT (F_{1,72}=0.0059, p=0.9388, ANOVA)(D) and HD (F_{1,10}=5.521, p=0.0407, ANOVA)(E) mice. (F) Time course of M2 (secondary motor cortex) activation on Day 8 (top panel) and Day 45 (bottom panel). Vertical dotted line denotes time of water reward delivery. Horizontal dotted line denotes zero ΔF/F level. Significance reflects a difference in genotype peak response. (G) Corresponding change in peak ΔF/F amplitude of M2 over time. Error bars and shaded region denotes SEM. *** and # denotes p<0.005, <0.01 and <0.05, respectively. See Figure 3.11 to 3.13 for additional data.

Figure 3.11 Ipsilateral mesoscale GCaMP6 imaging of the cortex during successful trials on Day 8 and 45.

Peak ΔF/F amplitude of ROIs in the ipsilateral hemisphere on Day 8 and Day 45 for WT (F_{1,6}=0.0261, p=0.8770, ANOVA)(A) and HD (F_{1,10}=0.7094, p=0.4193, ANOVA)(B) mice.
Figure 3.12 Change in contralateral hemisphere ROI peak ΔF/F amplitude over time.

Peak ΔF/F amplitude of ROIs in the contralateral hemisphere over time for WT (n = 4)(gray) and HD (n = 6)(teal) mice. ** and * denotes p<0.01 and <0.05, respectively.
Figure 3.13 Change in ipsilateral hemisphere ROI peak ΔF/F amplitude over time.

Peak ΔF/F amplitude of ROIs in the ipsilateral hemisphere over time for WT (n = 4)(gray) and HD (n = 6)(teal) mice. ** and * denotes p<0.01 and <0.05, respectively.
3.3.5 Impulsive and failed trials performed by HD mice

Although both genotypes learned to perform successful trials by Day 8 (Fig. 3.2A), HD mice also engaged in non-cued impulsive reaching behavior during both rewarded and non-rewarded trials. Successful rewarded trials with early reaches – defined as reaches that occurred before presentation of the water drop – were more prevalent in HD than WT mice on Day 8 (WT: 1.8 ± 0.1%; HD 32.0 ± 8.2%; p=0.0188; % of total successful trials)(Fig. 3.14A-B). Representative left paw lift (y-direction traces of left paw movement) in Figure 3.14A depicts trial-to-trial prevalence of early reaches in a WT (gray) and HD (teal) mouse. HD mice also continued to engage in reaching behavior during non-rewarded trials (Fig. 3.14A, red panel) for more days than WT mice (Fig. 3.2D).

Cortical activity underlying unrewarded reach trials was compared to successful reaches for HD mice on Day 8. The peak amplitude of the GCaMP6 ROIs was first determined after the cue associated with a water reward for both trial types and was found to be reduced in unrewarded reach compared to successful trials (data not shown). Although successful trials sometimes included early reaches in HD mice, all these trials were characterized by a reach movement timed after the water drop (Fig. 3.14A, teal); however, reaches during non-rewarded trials occurred throughout the trial duration (Fig. 3.14A, red). Therefore, cortical activity underlying these unrewarded reaches was also examined across the entire trial duration and compared to successful reaches which occurred after the water reward (see Methods for more details)(Fig. 3.14C-D). In HD mice on Day 8, the peak amplitude of all the ROIs in the contralateral (F1,10=7.783, p=0.0191, ANOVA)(Fig. 3.14C), but not the ipsilateral (F1,10=1.531, p=0.2442, ANOVA)(Fig. 3.14D) hemisphere was significantly increased in unrewarded reach trials compared to successful reaches.
In particular, the peak cortical activity at the contralateral sspm and sspbfd cortices was significantly increased in unrewarded reach trials (Fig. 3.14C).

Overtime, unlike WT mice which maintained their successful performance at the water-reaching task, HD mice experienced a decline in performance (Fig. 3.2A). No differences in cortical GCaMP6 peak amplitude or area activated was seen when comparing successful to failed reach trials performed by HD mice on Day 45 (Fig. 3.15). Consistent reaching during non-rewarded trials ceased by Day 11 in HD mice (Fig. 3.2D). In all, HD mice at ~5.5 months have an event sequence defect as evident by impulsive reaches.
Figure 3.14 Impulsive reaches performed by HD mice on Day 8.

WT (n = 4) successful trials are denoted in gray. HD (n = 6) successful and unrewarded reach trials are denoted in teal and red, respectively. (A) Representative trial-to-trial traces of left paw lift (y-direction movement of the left paw) from a WT and HD mouse on Day 8 for successful and/or unrewarded reach trials. Individual trials are stacked in rows. Time of the water reward (for rewarded trials) and tone (for non-rewarded trials) is denoted with a black line. (B) Percent of early reaches (reaches which occurred before water drop reward delivery) to the total number of successful trials. (C-D) Peak amplitude of regions of interest in the contralateral ($F_{1,10}=7.783, p=0.0191$, ANOVA)(C) and ipsilateral ($F_{1,10}=1.531, p=0.2442$, ANOVA)(D) hemisphere for different HD trial types. Error bars denote SEM. * denotes p<0.05. See Figure 3.15 for additional data.
Figure 3.15 Mesoscale GCaMP6 imaging of the cortex during success and fail trials performed by HD mice on Day 45.

HD (n = 6) success and fail trials are denoted in green and blue, respectively. (A-B) Peak amplitude of regions of interest in the contralateral (F₁,₁₀=2.540, p=0.1421, ANOVA)(A) and ipsilateral (F₁,₁₀=0.2573, p=0.6230, ANOVA)(B) hemisphere for different trial types. (C) Area activated across the entire trial duration for successful and failed trials. The threshold was set at 4x standard deviation (STD) of the baseline. No significance between trial types.

3.3.6 Phenotyping of gross motor defects and HD pathology

HD motor phenotype assessment using the water-reaching task was compared to gross motor tests (Fig. 3.16A-D). Before the water-reaching assessment, both genotypes with the exception of Day 1, spent on average the same time traversing the tapered beam (Fig. 3.16A) indicating HD mice
likely do not have a gross motor deficit at ~5 months of age. However, persistent unrewarded reach trials (Fig. 3.2D), early reaches during successful trials (Fig. 3.14B) and reduced peak cortical activity associated with successful trials (Fig. 3.10B-C) were revealed in the water-reaching assessment in HD but not WT mice at the same age.

All mice were also subjected to a second round of tapered beam testing after water-reaching assessment at ~8 months of age (Fig. 3.16B). During this second testing phase, with the exception of Day 1, WT mice traversed the beam significantly faster than HD mice. We then compared the performance of the mice during the first round of testing (mice aged ~5 months) with the second round (mice aged ~8 months). Day 4 has previously been used as the first testing day after successful learning of the task (Ardesch et al., 2017). Comparison of Day 4 during the first round of testing to the last assessment day (Day 7) during the second round of testing, revealed that WT mice traversed the beam in a faster time by the last assessment day (first testing round Day 4: 4.728 ± 0.532 s; second testing round Day 7: 2.097 ± 0.077 s; p=0.0044)(Fig. 3.16C). For HD mice, the time to traverse the beam did not change between the first (Day 4: 5.541 ± 0.614 s) and second (Day 7: 5.550 ± 1.192 s) round of testing (Fig. 3.16C). After water-reaching training HD mice also displayed impaired performance on the accelerating rotarod task as determined by a decreased latency to fall compared to WT (Fig. 3.16D). Together, the second round of tapered beam and rotarod testing suggest HD mice have reached the gross motor manifest stage of disease. This is consistent with the decreased success rate seen by the end of the water-reaching task in HD mice (Fig. 3.2A).
Finally, striatal MSNs which make up 95% of all neurons in the striatum were immunostained for DARPP-32 (Fig. 3.16E-G). Consistent with previous literature (Peng et al., 2016; Southwell et al., 2016), a significant decrease in DARPP-32 (relative intensity WT: 0.531 ± 0.035; HD: 0.162 ± 0.014; t=9.765; df=6; p<0.0001) but not NeuN (relative intensity WT: 0.86 ± 0.07; HD: 0.81 ± 0.05; t=0.5644; df=6; p=0.5930) intensity was seen in HD compared to WT mice (Fig. 3.16G). Overall, at ~5 months, early HD phenotype was apparent in the water-reaching task but not the tapered beam test. Gross motor HD deficits determined using rotarod and tapered beam tests, as well as HD pathology revealed through reduced striatal DARPP-32 expression were consistent with forelimb reaching deficits at ~8 months.
Figure 3.16 Tapered beam and rotarod gross motor assessment and post-mortem immunohistochemistry staining.

HD and WT mice are denoted as teal and gray, respectively. (A-B) Time to traverse the tapered beam determined before (~5 months)(A) and after (~8 months)(B) water-reaching testing for HD (n = 6) and WT (n = 6) mice. (C) Time to traverse the tapered beam on the first day after completion of tapered beam learning (mice age: ~5 month; day of testing: 4) compared to the last testing date (mice age: ~8 month; day of testing: 7) for HD and WT mice. (D) Latency to fall from the rotarod determined after water-reaching testing (~8 months) for HD (n = 6) and WT (n = 6)
mice. (E) Representative Thy1-GCaMP6s coronal slice. DARPP-32 intensity was quantified in the striatum. (F) Representative images of DARPP-32, NeuN and DAPI staining in the striatum with a merged overlay from a WT and HD mouse. (G) DARPP-32 and NeuN intensity in the striatum of HD (n = 4) compared to WT (n = 4) mice. Error bars and shaded intervals denote SEM. ****, **, * and N.S. denote p <0.0001, <0.01, <0.05 and statistically non-significant, respectively as determined by Two-way Anova and Šidák’s multiple comparisons post-hoc test for tapered beam and rotarod tests and unpaired T-test for immunohistochemistry staining.

### 3.4 Discussion

The shared evolutionary origin and characteristics of skilled forelimb movements between humans and rodents (Galiñanes et al., 2018; Whishaw et al., 1992) enable translational parallels to be drawn from preclinical mouse studies. In HD patients and pre-symptomatic carriers, deficits in motor learning, temporal sequencing and coordination of voluntary movements have been reported (Feigin et al., 2006; Klein et al., 2011; Shabbott et al., 2013). Using a water-reaching task, we reveal the presence of event sequence defects and progressive increases in cortical activity underlying forelimb deficits in the zQ175 HD mouse model (see Figure 3.17 for a summary of the results).
Timeline of HD (top) and WT (bottom) learning and performance in the water-reaching task. Gross motor tapered beam and rotarod tasks are included. WT mice learn both the reach-grasp-drink movement and task event sequence (alternating reward then non-rewarded trial) by Day 8 (gray). Although HD mice also learn the reach-grasp-drink movement by Day 8 (gray) HD mice show reduced cortical activation compared to WT mice. HD mice also have an event sequence defect as evident by impulsive reaches (green). Over time the peak cortical activity, euclidean distance and variability of the reaching trajectory increases in HD mice but little to no change was seen in WT mice. Unlike WT mice, HD mice also do not maintain their rate of successful performance overtime. HD mice experience first a progressive increase in partial fail trials then an increase in no reach trials reflecting failed grasp-to-drink then low task engagement, respectively. Overall, this indicates a progressively worsening forelimb motor coordination defect (light to dark teal) in HD mice that was captured daily.

### 3.4.1 Task acquisition and performance across genotypes

For most motor tasks, initial learning is accompanied by trial-to-trial variability, enabling spatial exploration and progress towards efficient task execution (Dhawale et al., 2019). Variability is
subsequently reduced after strategy formation (Churchland et al., 2006). We observe similar features since by Day 8, both HD and WT mice were able to successfully learn the reach-to-grasp movement. Although the movement was successfully learned by both genotypes, cortical activity underlying successful reaches was reduced in HD mice compared to WT. HD mice also required more days to learn the alternating reward/non-reward event sequence as evident by initial impulsive reaching. We speculate that the extended continuation of reaching behavior during non-rewarded trials until Day 11 and the occurrence of early reaches on Day 8, both in HD mice, could be explained by underlying cognitive defects that slow learning due to an inability to remember when to reach or failure to suppress motor movement. Disconnection of the cortex and striatum as well as overactivation of the striatal direct pathway have previously been reported in early symptomatic HD mice (Barry et al., 2022) and may contribute to the hyperactivity and this event sequence defect.

Over time HD mice experienced a significant drop in successful reaches compared to WT. The increased trial-to-trial variability seen in HD mice compared to WT mice on Day 45 suggests that HD mice are attempting compensatory changes in reaching strategy at a time when they experienced a drop in performance. Consistent with this, positional error correction of the forelimb has previously been observed in consecutive reach trials (Becker et al., 2020). The significant increase in partial fail trials but not complete fail trials further suggests HD mice fail to engage in proper end-point fine motor corrections during the grasp-to-drink segment of the task (Elliott et al., 2001). Semi-flexed or closed paws have been shown to result in failed target reaching trials (Whishaw et al., 2018b) and could explain the decline in successful performance rates seen in HD mice. By Day 60, decreased task engagement was seen indicating that movement defects in HD
mice increased in severity and alternative reaching strategies were no longer sufficient to mediate continued motivation and task engagement.

3.4.2 Bilateral engagement of mesoscale cortical circuits during reaching

Consistent with other studies that report global activation of the cortex and involvement of the ipsilateral hemisphere during limb movement (Brunner et al., 2020; Heming et al., 2019; Quarta et al., 2022; Soma et al., 2019), our results also revealed widespread cortical activation across both hemispheres during water-reaching. Although we did not see wide-spread enhanced cortical activity in HD mice as some work indicates (Arnoux et al., 2018; Burgold et al., 2019; Sepers et al., 2021) compared to WT (except in M2), global cortical activation associated with reaching increased over time in HD mice (but not WT). The lack of increased cortical activity may be due to differences in reaching task-performing awake versus anesthetized animals, HD mouse models and/or cortical areas examined. We speculate that this increase in cortical activity seen over time in HD mice may be driven by increases in average euclidean distance of the reaching trajectory. The increased euclidean distance seen in HD mice was a result of multiple sub-reach attempts that eventually led to successful task execution. Another explanation could involve changes in local inhibitory inputs (Cummings et al., 2009), spontaneous firing rates and/or activity of the striatum (Donzis et al., 2020) over time during water-reaching assessment.

Examining cortical regions of interest revealed genotypic differences in peak GCaMP6 cortical responses in M2, sspm, sspbfd and visp of the contralateral hemisphere and rspagl, rspd and M2 of the ipsilateral hemisphere. Differences have been reported in tongue protrusions during freely moving pellet reaching between individual mice and trial types (Whishaw et al., 2018a). Although
tongue protrusions were not evident in either WT or HD mice, adjustments to the tongue within the mouth, the mouth itself or whisking could explain the differences seen in sspm and sspbfd. Chemosensory, but not spatial or visual cues have been shown to guide water-reaching behavior (Galiñanes et al., 2018). The significance of visual area GCaMP activity differences observed across genotypes seen in this study necessitates further investigation into other cortical areas not typically examined in the context of forelimb reaching and other motor tasks. The retrosplenial cortex also showed genotype-specific changes and has been linked to spatial memory and navigation (Czajkowski et al., 2014; Milczarek et al., 2018). Retrosplenial cortices may be involved in learning and maintaining correct spatial orientation of the paw towards the target (water reward). Further work is needed to fully understand the role retrosplenial cortices play in forelimb reaching and its contributions to the HD phenotype.

Optogenetic cortical silencing has revealed the motor cortex is critical for the adjustment of complex grasping movements (Mohammed et al., 2020). Specifically, M2 has also been reported to encode movement distance and smoothness (Quarta et al., 2022). Our findings that HD mice fail to perform the grasp-to-drink portion of the movement (increased partial fail trials compared to WT) and have an increased average euclidean distance in their reaching trajectory compared to WT likely explains the genotypic hyperactivity seen in HD contralateral M2 compared to WT and is consistent with the previously reported roles M2 plays in forelimb reaching. This M2 hyperactivity evident at the motor manifest, but not premanifest stage is analogous to increased striatal activity seen with phenotype progression in HD (YAC128) mice compared to age-matched WT mice during rotarod performance (Koch et al., 2022).
We acknowledge that epifluorescence wide-field calcium imaging which we use to assess excitability has reduced temporal resolution compared with voltage sensitive dyes and is sensitive to artifacts associated with light scattering, hemodynamics and movement. ROIs generated using cortical image and landmark registration to a common atlas (Xiao et al., 2021) may also not represent the same regions as those determined functionally. To mitigate some of these limitations, strobing of green reflectance light was used to correct hemodynamic artifacts (Vanni et al., 2017; Wekselblatt et al., 2016; Xiao et al., 2017). The head-fixed set-up further reduced movement. Despite some limitations, our study has demonstrated the water-reaching task can reliably characterize forelimb motor defects and reveal aberrant cortical activity in HD mice.

Consistent with other studies examining HD gross motor defects (Liu et al., 2021b; Southwell et al., 2016), we report no significant differences in tapered beam traverse time in ~5 month aged zQ175 HD compared to WT mice. Although HD and WT mice achieved similar success rates at the water-reaching task at this age, event sequence defects and reduced peak cortical activity associated with successful trials were apparent suggesting the increased sensitivity of the water-reaching task to detect early HD motor phenotype at ~5.5 months of age. Later, when HD mice experienced reduced performance at the water-reaching task, we and others also reported increased time to traverse the tapered beam, decreased latency to fall from the rotarod, and decreased DARPP-32 expression in striatal MSNs (Liu et al., 2021b; Peng et al., 2016; Smith et al., 2014; Southwell et al., 2016).

Future studies could examine the contribution of diverse cortical areas (such as retrosplenial, visual and somatosensory) and subcortical regions (such as the striatum (Brunner et al., 2020),
cerebellum (Guo et al., 2021) and thalamus (Sauerbrei et al., 2020)) to forelimb tasks in mouse models of HD and other movement disorders. Therapeutic rescue of the HD phenotype using optogenetics and parsing the contribution of direct-indirect striatal (Albin et al., 1992; Barry et al., 2018; Reiner et al., 1988) and M2 cortico-striatal pathways (Fernández-García et al., 2020) would also enable mechanistic understandings of HD forelimb defects. The knock-in mice containing HD expanded triplet repeats used in our study would provide an ideal vehicle for these future experiments involving circuit-based manipulation by targeted expression of optogenetic agents. Through crossing with existing transgenic channelrhodopsin or halorhodopsin lines, or through the introduction of red-shifted opsins by AAVs, the role of specific interneuron classes could be determined and therapeutically modulated during water-reaching assessment. Overall, the ability of the water-reaching task to characterize HD phenotype especially at early stages of disease suggests it could be used to inform the onset of other movement disorders, therapeutic intervention windows and test drug efficacy.
Chapter 4: Conclusion

At the nanoscale level, a biophysical and pharmacological characterization of mefenamic acid’s effect on $I_{Ks}$ highlighted the NSAID as a precursor compound for LQTS therapeutic development. At the mesoscale level, a water-reaching task was developed and used to characterize HD phenotype. In conclusion, the two cases described above represent examples of preclinical target selection and validation as well as assay development at different spatial scales in the cardiovascular and nervous systems (Hughes et al., 2011; Kirsch, 2020).

4.1 $I_{Ks}$ activators: mefenamic acid

Mutations in KCNQ1 and KCNE1 which together form the delayed cardiac rectifier potassium current, $I_{Ks}$, have been implicated in many cardiac arrhythmia syndromes such as atrial fibrillation and LQTS (Jervell & Lange-Nielsen, 1957; Wang et al., 1996; Chen et al., 2003; Eldstrom & Fedida, 2011; Olesen et al., 2014). With nearly all mutations seen in LQTS patients identified as loss-of-function and 50% of those loss-of-function mutations identified in the KCNQ1 subunit (Hedley et al., 2009; Ackerman et al., 2011), enhancing and activating $I_{Ks}$ currents has been suggested as a promising therapeutic approach for treating LQTS. Of interest, mefenamic acid, has previously been identified by numerous groups to enhance $I_{Ks}$ currents in expression systems such as canine and guinea-pig ventricular myocytes (Magyar et al., 2006; Toyoda et al., 2006) as well as heterologous expression systems such as *Xenopus laevis* oocytes and CHO, COS-7, tsA201 and LM cells (Busch et al., 1994; Abitbol et al., 1999; Unsöld et al., 2000; Toyoda et al., 2006; Wang et al., 2020).
In Chapter 2, a biophysical and pharmacological characterization of mefenamic acid’s effect on \( I_{Ks} \) was provided. Consistent with previous studies (Busch et al., 1994, 1997), mefenamic acid was found to enhance \( I_{Ks} \) activity but not KCNQ1 alone. The enhancement was specific to \( I_{Ks} \) given the large instantaneous current was blocked by the specific \( I_{Ks} \) inhibitor, HMR1556, and no effect on endogenous currents was seen in untransfected cells. The rate-dependent effect of mefenamic acid on \( I_{Ks} \) may in part be explained by slowed channel deactivation and suggests an explanation for the inconsistent hyperpolarizing effects mefenamic acid has been reported to have on the \( V_{1/2} \) (Unsöld et al., 2000; Magyar et al., 2006).

More KCNE1-saturated \( I_{Ks} \) channel complexes were also found to have a greater response to mefenamic acid treatment suggesting the binding of mefenamic acid may be dependent on KCNE1. In support of this proposal, residue K41 located on the extracellular end of KCNE1, was found to be critical in mediating mefenamic acid’s activating effect on the fully saturated \( I_{Ks} \) channel complex (EQ). Cysteine scanning revealed that although other extracellular KCNE1 residues in the same region impacted the effect of mefenamic acid to varying degrees, only the K41C mutation completely abolished mefenamic acid effect up to a concentration of 1 mM. Previous cross-linking studies have identified key interactions between this extracellular region of KCNE1 and KCNQ1’s transmembrane segments, S6 and S1, suggesting that residues in either the S6 and/or S1 region could also provide critical clues to explain mefenamic acid’s mechanism of action and/or uncover its binding site.

In close proximity to residue K41 on KCNE1 and this proposed drug binding pocket are known gain-of function S1 mutations, S140G and V141M, which have previously been reported to slow
deactivation in the presence of KCNE1 (Peng et al. 2017). Since the current waveforms of S140G and V141M are qualitatively similar to those seen after exposure of WT EQ $I_{Ks}$ to mefenamic acid, the same mechanism may underlie the effects of mefenamic acid and the $I_{Ks}$ S1 mutations.

The co-evolved interface between the extracellular end of S1 and the pore domain is thought to be important for bracing the VSD, to allow efficient force transmission to the pore (Lee et al., 2009), but can also impact permeation as the S140G and V141M mutations in S1 enhance rubidium permeation through $I_{Ks}$ complexes (Peng et al. 2017). The importance of this S1-pore coupling to channel function is supported by mutational analyses of S1 residues (Chen et al., 2003; Hong et al., 2005; Wang et al., 2011b) which displays current waveforms with instantaneous onset. Slowing of deactivation by S1 mutations and $I_{Ks}$ activators such as mefenamic acid may therefore be the result of slowing the dissociation of the S1/KCNE1/pore domain complex by providing steric hindrance to dissociation or stabilizing the complex.

To further confirm this proposed binding site and mechanism of action, structural examinations using cryo-electron microscopy to visualize the binding of mefenamic acid and corresponding molecular dynamic simulations of drug binding are needed. Cysteine-scanning and other mutagenesis scanning (that take advantage of the residue size differences of alanine and tryptophan) of nearby residues on KCNE1, the pore-domain and S1 will also be important in shedding further insight on the mechanism of action. Such scanning may uncover additional critical residues to the action of mefenamic acid.
If mefenamic acid indeed binds at this KCNE1-KCNQ1 interface, a total of four identical mefenamic acid binding sites may be available on the $I_{K_S}$ channel complex (one at each inter-subunit interface). To confirm this, the introduction of different numbers of K41C mutations to a fully saturated $I_{K_S}$ channel complex can be used to better understand whether cooperative binding of mefenamic acid to the $I_{K_S}$ channel complex exists and how many mefenamic acid compounds must bind to induce the full effect seen on WT EQ $I_{K_S}$ channels.

Lastly, molecular dynamic simulations have revealed that L-364,373 and zinc pyrithione potentially bind at a common site (Gao et al., 2008; Magyar et al., 2006; Yu et al., 2013). Although the binding site for these KCNQ1 activators is not the same as that proposed here for mefenamic acid, the stilbene DIDS has been suggested to bind to the same site as mefenamic acid (Abitbol et al., 1999). Residue E43 on KCNE1 was identified as critical for DIDS binding. DIDS has further been identified as an $I_{K_S}$ activator suggesting a common binding site for activators may exist surrounding residues K41 and E43.

Hexachlorophene has also been identified as an $I_{K_S}$ activator (Zheng et al., 2012). Although no information is known about its binding site, the $I_{K_S}$ current waveforms after hexachlorophene (HCP) treatment are qualitatively similar to that post-mefenamic acid treatment and those of S1 mutations suggesting HCP may also bind at the proposed S1/KCNE1/pore domain interface. Further electrophysiological (including cysteine-scanning) characterization, molecular dynamic simulations and structural determination of HCP’s binding site on $I_{K_S}$ by cryo-electron microscopy are needed to determine whether DIDS and HCP also bind to this proposed site revealed in this study.
Limitations to the study include the usage of linked KCNQ1-KCNE1 constructs with a fixed α- and β-subunit stoichiometry as well as expression of such channels in heterologous systems (e.g. ltk- mouse fibroblast cells). In both cases this does not reflect the $I_{Ks}$ channel known to have a variable stoichiometry or the environment of human cardiac myocytes. Future studies could seek to examine how $I_{Ks}$ activators such as mefenamic acid, DIDS and hexachlorophene affect the cardiac action potential in isolated cardiomyocytes from animal models such as guinea pigs and determination of the degree of $I_{Ks}$ channel activation required for therapeutically beneficial shortening of the QT interval in humans using \textit{in silico} cardiac action potential modeling.

4.2 Huntington Disease forelimb defect phenotyping

Reaching and manipulating objects are performed daily by HD patients and pre-symptomatic carriers (Klein et al., 2011). The onset of fine skilled motor discoordination may precede that of gross motor deficits necessitating the examination and characterization of forelimb defects in animal models of HD.

In Chapter 3, using a high-throughput alternating reward/non-reward water-reaching task and simultaneous cortical imaging, WT mice were found to learn the event sequence and reach-grasp-drink movement by Day 8. Although HD mice learned the reach-grasp-drink movement by Day 8, reduced cortical activation was found underlying successful reaches performed by HD compared to WT mice. HD mice were also found to have an event sequence defect as evident by impulsive reaching at ~5.5 months of age when gross motor defects were not apparent. Over time the peak cortical activity, Euclidean distance and variability of the reaching trajectory increased in HD mice. Little to no changes were seen in WT mice. Unlike WT mice, HD mice also do not maintain
their rate of successful performance overtime indicating a progressive forelimb motor coordination defect. Gross motor assessment using rotarod and tapered beam as well as post-mortem immunohistochemistry confirmed the motor manifest stage of HD at ~8 months.

Improvements in cranial window preparations including the use of curved custom coverslips and preparations that preserve the intact overlying bone in a transparent state - as used in this study - have enabled imaging of mesoscale function across the cortex (Silasi et al., 2016). Potential drawbacks; however, include artifacts due to blood volume, changes in oxygenation and movement (Bumstead et al., 2017; Chan et al., 2015; Ma et al., 2016). The use of voltage sensitive dyes will also provide greater temporal resolution (Lin and Schnitzer, 2016).

Presently, various adaptations of wide field GCaMP imaging include automated cortical imaging within home cages (Murphy et al., 2020, 2016), combination with fMRI imaging (Lake et al., 2020), and wide field scopes that can travel with freely moving rats and mice (Rynes et al., 2021; Scott et al., 2018). In the future, the water-reaching task may be combined with these adaptations of wide field imaging to provide insight into: 1) the effects of limiting experimenter intervention/training in the case of home cage adaptation, 2) the role subcortical regions such as the striatum may play in reaching and HD pathology in the case of fMRI imaging and 3) whether free moving water reaching will reveal similar phenotypes to head-fixed water reaching in HD mice (free movement may allow behavioral and/or associated cortical compensation in HD mice).

Overall, a limitation to the current study includes the lack of further mechanistic exploration. Chronic implantation of photometry fibers (Kim et al., 2016a; Sych et al., 2019) into HD transgenic mouse models will enable continued monitoring of the progressive imbalance between D1-expressing (direct pathway) medium spiny neurons (D1-MSNs) versus D2-MSNs (indirect...
pathway) degeneration thought to cause chorea during early stages of HD (Albin et al., 1992; Barry et al., 2018; Reiner et al., 1988) and may provide mechanistic insight into HD forelimb defects. Briefly, Drd1a-Cre (https://www.jax.org/strain/028298) and Drd2-Flpo (https://www.jax.org/strain/034419) mice can first be crossed with each other and then with zQ175 HD mice. When injected with AAVdj-EF1α-DIO-GCaMP6f-WPRE in the striatum, D1 neuronal activity recording is then possible using green fluorescence (Muir et al., 2018). Simultaneous injection of a custom AAV controlled by the EF1α promoter with Flp-dependent-jRCaMP expression into the striatum, will allow recording of D2 neuronal activity using red fluorescence. Closed-loop multicolor fiber photometry (Kim et al., 2016a) could then be used to modulate the relative strength of striatal activity in these two pathways during pre- and motor manifest stages of HD in efforts to correct the direct-indirect pathway imbalance and delay and/or reduce movement deficits.

Similarly, stimulation of the M2 corticostriatal pathways as reported by Fernández-García et al in 2020 may be conducted to understand the role M2 plays in forelimb reaching and examine potential phenotype rescue. Beyond optogenetic and other pharmacologic treatment interventions are emerging gene-therapy approaches. Therapeutic efficacy such as that of CRISPR gene editing may be assessed with behavioral tasks such as water-reaching and/or tapered beam or rotarod combined with fiber photometry (Liu et al., 2020; Morelli et al., 2022).

Pharmacological agents and ex vivo slice electrophysiology may also be used to identify whether changes in local inhibitory inputs (Cummings et al., 2009), spontaneous firing rates and/or activity of the striatum (Donzis et al., 2020) over time may explain HD forelimb motor defects and the
increase in cortical activity underlying successful reaching overtime in HD mice. Post-mortem expansion microscopy in combination with specific markers could further provide a more detailed evaluation of perisomatic inhibitory synaptic contact reduction in the cortex (Burgold et al., 2019) and degradation of corticostriatal projections over time in water-reaching HD mice.

4.3 Conclusion

In Example Case 1 (Chapter 2), mefenamic acid was found to activate $I_{Ks}$. A potential binding site and mechanism of action was further proposed for mefenamic acid and potentially other $I_{Ks}$ activators such as DIDS and hexachlorophene. The S1-KCNE1-pore domain interface and mefenamic acid may serve as critical targets and/or precursors in the development of LQTS therapeutics. In Example Case 2 (Chapter 3), the water-reaching task was used to characterize aberrant cortical activity underlying progressive forelimb defects. The behavior assessment may be used to gain insight into direct-indirect striatal mechanisms which may contribute to forelimb defects or to explore M2-corticostriatal stimulation rescue. The water-reaching task can also potentially be used to inform the onset of other movement disorders, therapeutic intervention windows and test drug efficacy.

The two cases described above therefore represent examples of target selection and validation (mefenamic acid) as well as assay development (water-reaching task)(Hughes et al., 2011; Kirsch, 2020), both critical phases in preclinical drug development. Later phases of drug development such as animal model testing of the lead compound and Clinical Development (Phased trials) depend heavily on these prior steps of precursor selection and behavior assay development. Preclinical research at the protein nanoscale, whole-animal behavioral and mesoscale cortical
circuit level therefore has the potential to inform and improve arrhythmic and movement disorder morbidity and mortality through the discovery of novel therapeutics and development of novel assays.
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