With the generous support from IETF, we have been making significant progress over the past six months on investigating the roles of the calcium-activated ion channels in ET. Our main progress is summarized below.

**Aim 1. Dissecting the contributions of the Ca\(^{2+}\)-activated ion channels to the membrane excitability of the IO neurons *in vitro***

1. **Clarification of the molecular identities of the voltage gated calcium channels in IO neurons.**

In order to understand the physiological functions of the Ca\(^{2+}\)-activated ion channels in IO neurons, it is critical to have a comprehensive understanding of the molecular identities of the voltage-gated Ca\(^{2+}\) channels (Cav), which are upstream of the Ca\(^{2+}\)-activated ion channels. It is well established that the T-type Cav channels are responsible for the low-threshold Cav current observed in the IO neurons. Nevertheless, the molecular identities of the high threshold Cav channels are still unclear; and people have vaguely attributed all the IO high threshold Cav current to P/Q type Cav (Cav2.1) channels. In order to identify the identities of the high threshold Cav channels, we applied sub-type specific high threshold Cav channel blockers to IO neurons; and utilized TMEM16B Ca\(^{2+}\)-activated Cl\(^{-}\) channel (CaCC), which is activated by the Cav channels as a current amplifier (*Fig. 1*). To our surprise, We found that P/Q type Cav channels are not the sole contributor of the high threshold calcium conductance in IO neurons because ω-agatoxin IVA (AGTX) only blocked ~30% of the calcium sensitive tail current in wildtype IO neurons. To further verify this, we co-applied 200 μM Ni\(^{2+}\), a non-specific R type Cav blocker, with AGTX. We found that Ni\(^{2+}\) blocked additional 40% of the tail current. There were still ~30% of residual tail current in the presence of these two Cav channel blockers. This remaining tail current may derive from CaCC activation by another high threshold Cav channel (presumably N-type Cav channels, (Urbano, et al, *PNAS* (2006) 103, 16550-16555)). Therefore, multiple Cav channel types are responsible for the high threshold Cav conductance in IO neurons, which activate TMEM16B CaCC. We are applying the N-type Cav channel blocker, ω-conotoxin to...
see if we can completely block the remaining Cav current.

Our findings are important to clarify the ionic basis of IO neuron excitability and provide more potential therapeutic targets for ET treatment. This work is expected to be submitted as a manuscript by the end of the funding cycle.

2. Examined SK channel blocker apamin and BK channel blocker paxilline on IO subthreshold oscillation (STO).

STO is one of the key features of IO neuron membrane excitability (Fig. 2A). It has been proposed that the enhancement of IO STO is an important mechanism of harmaline induced ET. We set out to test if two Ca2+-activated K+ channels, BK and SK channels play important roles in IO STO. Our preliminary data showed that blocking BK channels by paxilline had no effect on STO (data not shown), while blocking SK channels by apamin dramatically suppressed STO (Fig. 2B). More electrophysiology characterization is undergoing to understand the mechanism.

**Figure 2.** Apamin effects on IO subthreshold oscillation (STO). STO of an IO neuron before (A) and after (B) 20 nM application of SK channel blocker, apamin.
Aim 2. Elucidating the contributions of the Ca\textsuperscript{2+}-activated ion channels to the pathogenesis of ET in vivo.

1. Successfully built a custom designed tremor detection device and used it to record the harmaline-induced tremor in WT and TMEM16-KO mice. Our assay can continuously monitor the initiation, development and extinction of harmaline-induced tremor (Fig. 2). Distinct from the commercial tremor detection device, our assay is compatible with videotaping so that animal behavior can be correlated with tremorgenesis. Different dosages of harmaline have been used in the field to test tremorgenesis in mice. To find the optimal dosage for our experiments, we tried 10, 20 and 30 mg/Kg harmaline on the WT mice and chose the lowest dosage 10 mg/Kg, which has the smallest adverse effects on animals, for our future experiments.

We reliably detected the development of harmaline induced tremor at ~13 Hz (Fig. 2C). Based on our preliminary data, our TMEM16B KO mice exhibited significant enhancement of motion power and early development of tremor after harmaline injection (Fig. 2B-D). This enhancement of harmaline induced action tremor in TMEM16B KO mice is consistent with our video recording observations, where the TMEM16B KO mice demonstrated more mobility during tremor. On the other hand, the WT mice exhibited much less action tremor as they were frequently immobilized. Interestingly, the frequency of the harmaline tremor in TMEM16B mice was not changed. The underlying mechanism is under further investigation.

![Figure 2](image.png)

**Figure 2. Establishing a custom-made tremor detection assay to detect harmaline induced ET in mice.** A) The diagram of our custom-made tremor detection assay. An accelerometer is attached to the bottom of a hanging cage to detect tremor. B) Representative raw tremor trace recorded in wildtype and TMEM16B KO mice in response to systemic IP injection of 10 mg/Kg harmaline. C) Time course of harmaline-induced tremor after spectral analysis in 10-minute intervals after injection. D) TMEM16B KO mice exhibited significantly enhanced tremor power than WT mice at 13 Hz.
2. **Validated a new IO-specific cre line.**

According to the Allen Mouse Brain Atlas and literature, corticotropin releasing hormone (crh) is highly expressed in the IO. We obtained the newly generated crh cre mice and crossed it with a fluorescent reporter line, Ai14. We successfully observed robust the crh-driven fluorescent signal in IO neurons and climbing fibers (**Fig. 3**). There are only few other brain regions also express fluorescent signal. These regions in general do not have motor control functions. Thus, our experiment suggested that we can use the crh-cre linke to generate IO specific knockout (TMEM16B, BK and SK) or overexpression (optogenetics: channelrhodopsin and Ca2+ imaging: GCaMP) mice.

![Figure 3. Validation of the IO-specific crh-cre line. Crh induced RFP (Ai14) expression in the IO neurons (top) and their climbing fibers targeting to the cerebellar cortex (bottom). Hoechst labeled nuclei and GFAP labeled glial cells.](image)

3. **Generation of IO-specific TMEM16B KO mice.**

Due to harmaline’s potential inhibitory effect on TMEM16B and TMEM16B’s expression in a few brain regions other than the IO, we are currently generating TMEM16B floxed mouse line. We are planning to cross TMEM16B floxed mice with the crh-cre line to generate IO-specific TMEM16B KO mice and use these animals to test TMEM16B’s contribution in ET.

4. **Generated IO specific BK channel knockout mice by crossing BK floxed mice with the crh-cre.**

The electrophysiology recording and harmaline-induced tremor characterization of the IO-specific BK KO mice is currently undertaking.

5. **Establish a novel in vivo Ca2+ imaging method to directly monitor neuronal activities of the IO.**

Cal-520-AM and Calbryte-520-AM are two new Ca2+ probes with superior sensitivity, dynamics and stability (**Fig. 4**). When these probes were injected into the PC layer of the cerebellum or
sensory cortex and incubated for 1.5h, the neuronal activity of GCs in GC layer and PC dendrites in molecular layer of mice can be reliably monitored using two-photon microscopy \textit{in vivo}. These results suggest that the method is feasible for directly monitor climbing fiber activity \textit{in vivo} if these Ca2+probes are injected into the IO.

In addition to the Ca2+-dye, we are planning to genetically express Ca2+ probes in the IO driven by the crh-cre. We will inject fluorescent Ca2+ probe AAV-GCaMP6 into the IO of the crh-cre mice or cross a genetically engineered GCaMP6 mouse line with the crh-cre.

Figure 4. \textit{In vivo} two-photon Ca2+ imaging (A) of granule cells (B) and Purkinje cells primary dendrites in cerebellum (B). (C) The Ca2+ dynamics of sensory neurons in cortex.

In summary, we have made great progress as we initially planned. The assays, tools and reagents that we have established and continue to build will greatly facilitate further understanding of ET pathogenesis. In the next 6 months, we expect to submit at least one manuscript derived from this project. With accumulation of more preliminary data during the 2\textsuperscript{nd} half of the funding cycle, we expect to submit an R01 application that is directly based on this project by the end of 2018.