Sodium Channels: Mutants and Post-transcriptional Regulation Part I
Instructor: David Deitcher
TA: Kristy Lawton

The $\alpha$ subunit of a sodium channel is composed of 4 homology domains of 6 transmembrane segments to yield a 24 transmembrane segment structure. Positive charges in S4 of each repeat sense voltage and portions marked “I” are involved in sodium ion selectivity.

Sodium channels are a fundamental component of the neuron. As a neuron depolarizes and reaches threshold, the voltage gate of the $\text{Na}^+$ channel moves, initiating the opening of the sodium channel. The large excess of sodium ions outside the neuron flows rapidly through the ion selective pore into the cytoplasm to further depolarize the neuron and eliciting an action potential. The $\text{Na}^+$ channel then inactivates preventing further ion flow and $\text{K}^+$ channels open in response the depolarization allowing $\text{K}^+$ to flow outward to repolarize the neuron.

Sodium channels must be targeted to the right place and only be active under the proper conditions. Sodium channels associate with $\beta$ subunits that regulate their trafficking in the cell and their association with cytoskeletal elements such as ankyrin and spectrin. In Drosophila the $\beta$ subunit is believed to be the product of the $\text{tipE}$ gene. In mammals, the position of sodium channels within the neuron is also crucial as they are concentrated at initial segments of axons and at nodes of Ranvier. Both the activation and inactivation of $\text{Na}^+$ channels must occur only under the proper conditions for a neuron to function properly. Point mutations in the $\text{Na}^+$ channel gene SCN1A lead to a variety of epilepsies and seizure related conditions. The syndromes are often resistant to anti-epileptic drugs.

Recently, the gene responsible for the epileptic-like seizures in the $\text{Drosophila}$ mutant $\text{bangsenseless}$ was identified. The responsible gene, $\text{paralytic (para)}$, encodes a sodium channel. The mutation identified is in exon 30 of the gene. The resulting amino acid change is a Leucine to Phenylalanine change in S3 of the fourth repeated portion of the channel. This change appears to alter the inactivation of the
channel. In this weeks lab we will perform PCR on $bss^1$ genomic DNA and see if we can identify the mutation.

Modified from Parker et al., 2011

Alternative splicing and RNA editing also regulate sodium channels. In the Drosophila para gene, alternative splicing is quite extensive. There is predicted to be hundreds of different splice variants and more the 25 have been positively identified. Some splice variants have been implicated in altering channel function. In particular, one paper reported that persistent sodium current is elevated in certain splice variants (Lin et al, 2009). RNA editing has also been found in para mRNAs. In this weeks lab we will try to detect one of these editing sites. The editing site results in a change of one nucleotide - see below (modified from Hanrahan et al., 2000).
In mammals, RNA editing has been shown to be crucial in the functioning of the GluR2, in which the edited site results in an amino acid change that controls calcium sensitivity. In para, the situation is not completely clear whether the edited sites are crucial. The site we are looking at in the lab has not been examined yet for its effect on Na+ channel function. Presumably, neurons do not go to this extra level of regulation for no reason and one edited site is thought to increase the persistent sodium current. The persistent sodium current is a small current that continues after most of the Na+ channels have inactivated. It is thought to be important in regulating how excitable a neuron is. In this weeks lab we will try to detect the presence of RNA editing at the Ssp site in homology domain III.

Procedure

Preparation of genomic DNA- the squish prep

1) Pipet 50 µl of squish buffer into two eppendorf tubes. Add 1 µl of the proteinase K solution to each tube.
2) Into 1 tube place an unconscious w1118 fly and crush it with the pestle. Similarly, in the other tube crush a bss1 fly.
3) Close the tube up and incubate at 37C for 30 minutes. Then incubate tube at 95C for 5 minutes. [During the 37C step, proteinase K digests much of the protein in the squish prep and the 95C step is included to inactivate the proteinase K before doing PCR]

Preparation of RNA

1) In the hood, pipet 0.5 mls of Trizol [you must where gloves and eye protection] into the provided homogenizer. Add 5 w1118 flies directly into the homogenizer with the Trizol. Insert glass pestle and twist gently to completely crush the flies.

2) Pipet 0.5 mls of mixture into one 1.5 ml tube. Let sit 5 minutes. Then spin the tube for 1 minute to pellet undissolved fly debris. Transfer supernatant to a new tube and add 0.1 ml of chloroform. Vortex for 15 seconds (till milky pink in appearance), then let sit 3 minutes at room temperature.

3) Centrifuge at 12,000g for 15 minutes at 4C. Be sure to use a balance tube.

4) Pipet the top (aqueous) phase into a fresh 1.5 ml tube. Avoid the interface between the two layers. Add 0.25 ml of isopropanol, MIX WELL. Failure to mix well will result in a low yield of RNA. Incubate 10 minutes at room temperature. Centrifuge at 12,000g for 15 minutes at 4C. Direct the hinge of the tube to the outside of the rotor during centrifugation. This will aid you in finding your RNA pellet later.
5) Inspect bottom of tube on side of hinge for a white pellet. Pipet off supernatant while making sure you don't disturb the pellet. Add 1 ml of 70% ethanol, spin 12,000g for 5 minutes at 4C. Pipet off ethanol, spin briefly again and attempt to remove remaining ethanol. Let pellet air dry for a few minutes. Resuspend the pellet in 30 µl of DEPC treated water. Let sit for 5 minutes and determine if your pellet has gone into solution.

6) In a fresh tube add 995 µl of water. Add 5 µl of RNA, then mix well (but do not introduce air bubbles). Use spectrophotometer to measure the amount of RNA at 260 nm. To calculate RNA yield use the conversion of 40 µg/ml = 1 OD 260 unit.

**Reverse Transcription**

You will prepare 1 reverse transcription reaction

1) To each tube add 1-5 µg of RNA (calculated from above)

1 µl of random primer (2 µl if you use 5 µg of RNA)

1 µl of 10 mM dNTPs

Add water to 14 µl, mix.

2) Heat to 65C for 5 minutes, then place on ice. Then spin down briefly.

3) Add 4 µl of 5X 1st strand buffer
   1 µl of 0.1 M DTT, mix.
   1 µl of Superscript III (reverse transcriptase). Make sure you have actually pipetted the RT by looking at your pipet tip and make sure you have expelled it in the tube. Mix gently but well. Spin down briefly.

4) Incubate at room temp. for 5 minutes. This allows the random primers to be extended by RT.

5) Transfer tube to heat block set at 50C. Incubate 60 minutes.

6) Inactivate by heating tube at 70C for 15 minutes. Spin briefly and then place tube on ice.

**PCR**

**Detection of bss1 mutation**

1) Set up two 0.5 ml tubes on ice, label them w1118 and bss1.

2) Add in order the following to each tube:
37.5 µl water
5 µl 10X buffer
1 µl dNTPs
2.5 µl exon 30- sense primer
2.5 µl exon 30 antisense primer

MIX gently, then add 1 µl of DNAzyme, mix gently, then add 1 µl of the squish prep to the appropriate tube (bss1 into bss1, w1118 to w1118)

Detection of Ssp RNA editing site

1) Set up two 0.5 ml tubes on ice, label them w1118 genomic and w1118 cDNA.

2) Add in order the following to w1118 genomic tube:
37.5 µl water
5 µl 10X buffer
1 µl dNTPs
2.5 µl Ssp genomic sense primer
2.5 µl Ssp genomic antisense primer

MIX gently, then add 1 µl of DNAzyme, mix gently, then add 1 µl of the w1118 squish prep to the appropriate tube.

3) 2) add in order the following to w1118 cDNA tube:
33.5 µl water
5 µl 10X buffer
1 µl dNTPs
2.5 µl Ssp cDNA sense primer
2.5 µl Ssp Ssp cDNA primer

MIX gently, then add 1 µl of DNAzyme, mix gently, then add 5 µl of the w1118 cDNA.

The above samples will be placed in a thermocycler for amplification. Next week we will prepare samples for sequencing (bss1 detection) and for digestion with SspI to detect the RNA editing site.