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## Identification of HCN Channel Proteins in the Rat Retina

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### Introduction

#### GENERAL BACKGROUND

Hyperpolarization-activated cyclic nucleotide-gated (HCN) protein channel activity has been observed in mammalian cells for over 30 years—though genes encoding these channels were not identified until 1997.<sup>1</sup> HCN channels conduct ionic currents across the cellular membrane where activation by hyperpolarization generates a stabilizing depolarization. HCN channels regulate cell excitability in mammalian cardiac and neuronal tissue. Dubbed “pacemaker” channels, they are responsible for initiation of rhythmic activity and control of spontaneous firing in cardiac pacemaking activity in the sinoatrial node (SAN).<sup>2</sup> HCN channels are also thought to be involved in the stabilization of any inherent cycle length variability of the SAN and in protecting the SAN from excess hyperpolarization by surrounding atrial muscle.<sup>3</sup> In the brain, HCN channels play a fundamental role in regulating the autonomous rhythmic activity of single neurons such as thalamocortical relay neurons and the periodic oscillatory spindle wave activity in the thalamus during early and late stages of sleep.<sup>4, 5</sup>

#### PHYSIOLOGICAL EXPRESSION OF MAMMALIAN HCN CHANNELS

Past research has found HCN channels in cardiac tissue (e.g., in the sinoatrial node and Purkinje fibers) and has determined their properties (e.g., threshold activity voltage, gene sequence, etc.). In the brain, HCN channels are found in the hippocampal CA1 and CA3 pyramidal neurons and *stratum oriens* and *stratum lucidum* regions, the pyramidal neurons of the neocortex, the inhibitory basket cells and Purkinje neurons of the cerebellum, the excitatory thalamocortical relay neurons, and the inhibitory thalamic reticular neurons.<sup>6</sup>

#### EXPERIMENTAL OBJECTIVES

Four isoforms of the HCN channel protein have been characterized in mammalian tissue. HCN channels have already been characterized in retinal photoreceptors and bipolar cells, but there are no published studies identifying the channels in cells that were anatomically confirmed to be retinal ganglion cells. Current research in this laboratory studies the distribution of HCN channels in mammalian retinal

ganglion cells. In this study, cross sectional antibody staining showed location of the proteins within the retina and electrophysiological measurements confirmed HCN channel activity. In order to show that the antibodies used in the lab were indeed staining HCN channel proteins, this project used Western blots to characterize the bound proteins by their molecular weights. Protein samples were extracted from rat retina tissue and separated by gel electrophoresis. Commercially-available isoform-specific antibodies, also used in the cross sectional staining, were then used to selectively bind to the proteins within the retinal protein sample. Using Western blot analysis, the molecular weights of the bound proteins were then compared with published values for mammalian HCN channel proteins.

## Methodology

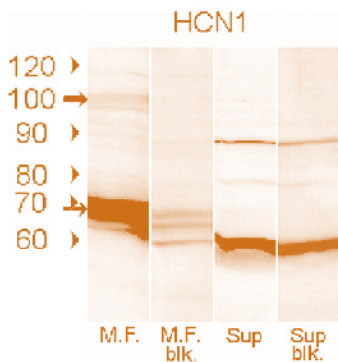
### PROTEIN PREPARATION

Freshly isolated retinas were individually frozen by dropping them into liquid nitrogen and then storing them in Eppendorf tubes. Twenty retinas were then put into a tissue grinder and homogenized in an ice cold homogenization buffer containing 50mM tris, 5mM EDTA, 300mM NaCl, 0.3 M sucrose, 50 mM HEPES, 1 mM ethylenediamine tetraacetic acid (EDTA), and a protease and phosphatase inhibitor cocktail (Sigma, P8340)[50 mM NaF, 50 mM beta glycerol phosphate (pH 8.0), 0.2 mM sodium orthovanadate]. After centrifuging the homogenate at 13000g for 15 min at 4°C, the supernatant was collected and the pellet re-suspended in the homogenization buffer; these two steps were repeated twice. The final pellet was discarded and the supernatant from all three spins was centrifuged at 45000g for 1 hr at 4°C in an ultracentrifuge. The membrane-enriched pellet from the final spin was re-suspended in 500 µl of homogenization buffer and assayed for total protein by the Bradford method. The protein extract was mixed with NuPage LDS sample buffer (4x) and NuPage Reducing agent (10x) and heated to 70°C for 10 min. This suspension was loaded at 50-100 µg of protein per lane onto a 10% bis-tris polyacrylamide gradient gel (NuPage, Invitrogen) and run with MOPS running buffer (membrane fraction). The ultracentrifugation supernatant was also analyzed (cytoplasm fraction). Protein standards Benchmark and See Blue were run in lanes adjacent to the samples.<sup>7</sup>

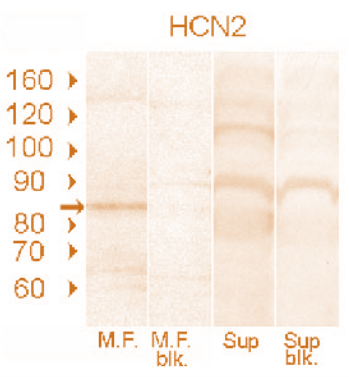
### WESTERN BLOT ANALYSIS

After electrophoretic separation, proteins were transferred from the polyacrylamide gradient gel to a nitrocellulose membrane (0.2 µm pore diameter). The membrane was blocked in TBST [0.1% (v/v) Tween-20 in Tris buffered saline] containing 5% dry non-fat milk (w/v) for 1 hr at room temperature. The membrane was then divided and incubated overnight at 4°C with either an anti-HCN antibody or an anti-HCN antibody that had been pre-adsorbed overnight at 4°C with a two-fold higher concentration of HCN peptide. After several rinses in TBST, species appropriate secondary antibodies conjugated to horseradish peroxidase were applied for 1 hr at room temperature and again rinsed in TBST. Protein bands were visualized using ECL detection with SuperSignal West Pico Chemiluminescent Substrate. The primary antibody and the peptide-blocked antibody were diluted in TBST containing 3% BSA (w/v) to concentrations of 1:200 for HCN2 and HCN1, 1:500 for HCN3, and 1:1000 for HCN4. The secondary antibody was diluted to a concentration of 1:5000 in TBST containing 3% BSA (w/v).<sup>7</sup>

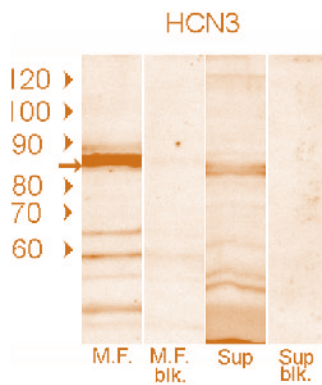
**Data**



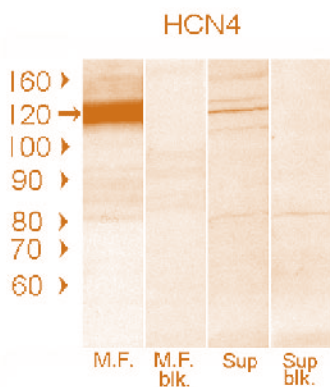
**Figure 1.** The HCN1 antibody specifically bound protein at molecular weights of 61 kDa and 102 kDa in the membrane fraction (M.F., arrows). Peptide blocking of the antibody greatly decreased the intensity of both band signals (M.F. blk.). Antibody binding in the cytoplasm fraction was non-specific (Sup and Sup blk.).



**Figure 2.** The HCN2 antibody specifically bound protein at a molecular weight of 83 kDa in the membrane fraction (M.F., arrow). Peptide blocking of the antibody eliminated the band signal (M.F. blk.). Antibody binding in the cytoplasm fraction was non-specific (Sup and Sup blk.).



**Figure 3.** The HCN3 antibody specifically bound protein at a molecular weight of 85 kDa in the membrane fraction (M.F., arrow). Antibody binding in the cytoplasm fraction was specific and similar to that of the membrane fraction, though the amount of bound protein was greatly decreased (Sup and Sup blk.). In both cases, peptide blocking of the antibody greatly decreased the intensity of the band signal (M.F. blk.).



**Figure 4.** The HCN4 antibody bound protein at a molecular weight of 120 kDa (arrow) in the membrane fraction (M.F.). Antibody binding in the cytoplasm fraction was specific and similar to that of the membrane fraction, though the amount of bound protein was greatly decreased (Sup and Sup blk.). In both cases, peptide blocking of the antibody greatly decreased the intensity of the band signal (M.F. blk.).

## Results and Conclusion

### MOLECULAR WEIGHT ANALYSIS

Antibodies against HCN1 labeled two protein bands (61 kDa and 102kDa), whereas only the 102 kDa protein weight was expected. The 61 kDa band might represent a truncated form of the protein. The experimental HCN2 protein weight (83 kDa) was lower than the expected 95kDa. The molecular weight results for HCN3 (85 kDa) and HCN4 (120kDa) matched closely with the published values of 87 kDa for HCN3 and 129 kDa for HCN4. HCN3 antibodies were also specifically labeled in three faint bands at lower molecular weights. Differences between published values (derived from rat brain tissue) and the experimental results (from rat retinal tissue) might reflect slightly different amino acid sequences of HCN channel proteins in the rat brain and retina, or else post-translational modifications giving rise to differences in distances migrated in the PAGE gels.

### MEMBRANE FRACTION vs. CYTOPLASM FRACTION

HCN channel proteins were expected to reside in the cellular membrane and thus the experimental membrane fraction. The cytoplasm fraction was run, and in the case of HCN3 and HCN4 showed specific antibody binding—the antibodies against HCN3 and HCN4 bound to proteins in the cytoplasm. The proteins in these cytoplasmic fractions were possibly HCN channel proteins stored in the cytoplasm before being delivered to the membrane. Alternatively, they could be a separate class of proteins that also bind specifically to the HCN antibodies. The latter theory could be tested by treating the retinal tissue with additional HCN antibodies, thus increasing the specificity of protein binding.

## Acknowledgments

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