NEUROPROTECTIVE MODULATION OF PRO-APOPTOTIC POTASSIUM CURRENTS BY THE HEPATITIS C VIRUS NON-STRUCTURAL PROTEIN 5A

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Callie A. Norris, M.S.

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We and others have shown that potassium loss is a requisite apoptotic event in mammalian cortical and midbrain neuronal systems. Potassium loss after oxidative injury is mediated through an increase in the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor-mediated channel insertion of the delayed rectifier voltage-gated potassium channel Kv2.1. This occurs through the dual phosphorylation mechanism of S800 and Y124, mediated by p38 and Src respectively. Inhibiting the potassium current surge is enough to block neuronal cell death in models of neuronal injury. Thus, targeting this cellular apoptotic pathway could provide novel therapeutic strategies in neuroprotection for diseases such as Alzheimer's, Parkinson's, and ischemic stroke. Recently the hepatitis C virus (HCV) non-structural 5A (NS5A) protein was found to confer anti-apoptotic properties through the same pathway in hepatocytes, contributing to viral persistence. Here we show that expression of NS5A (genotype 1b) in neurons limits Kv2.1 functional expression, modulates the pro-apoptotic potassium current surge, and is neuroprotective. Importantly, we define a mechanism for this regulation whereby NS5A1b inhibits Src phosphorylation at the Y124 residue of Kv2.1. These anti-apoptotic effects are not as profound in cells expressing NS5A of the 1a genotype, suggesting that these effects are genotype-selective. Preliminary results inhibiting Casein Kinase II, known to phosphorylate NS5A, lead us to speculate that post-translational modifications are required for K^+ current modulation. Our results indicate that NS5A1b maintains neuronal viability after injury via Kv2.1

current modulation, and that this could translate to improved therapies in neuroprotection. It is therefore likely that this pathway is critical for NS5A-induced adaptation of host cell activity for maximizing and perpetuating hepatic viral infection.

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1.0 INTRODUCTION

1.1 POTASSIUM HOMEOSTASIS IN APOPTOSIS

Neuronal apoptosis via oxidative damage occurs in a myriad of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and ischemic injury leading to stroke. Many advances have been made including the development of therapies leading to clinical trials, although successful neuro-protective treatments for these conditions remain elusive. Seeking a solution to this problem, two groups discovered that maintaining decreased potassium levels inside cortical and midbrain neurons contributes to a unique anti-apoptotic environment. They also demonstrated that potassium loss is an essential signal in many cell death pathways [1,2]. The loss of intracellular K^+, the most abundant cation within the cell, is a good candidate for the ionic regulation of apoptosis as it can deliver effective cell death signals without turning on propagated suicide programs that could be extensively deleterious. Levels can drop as low as 35 mM from established concentrations of ~140 mM, providing a suitable environment for caspase and nuclease activation, as well as playing a critical role in the apoptotic volume decrease [3]. Importantly, interfering with the processes responsible for the apoptotic K^+ current surge can effectively block neuronal cell death following oxidative injury [2,4-7]. Thus, targeting this cellular apoptotic pathway could provide novel therapeutic strategies in neuroprotection.

Voltage-gated K^+ (Kv), outward delayed rectifier (I_K) channels are specifically poised to efflux potassium in large quantities due to their voltage dependence, slow-inactivation in response to depolarization, and ability to conduct K^+ well [3]. Indeed, Yu et al (1997) found that in cortical neurons, the apoptotic, caspase 3 activator staurosporine was associated with K^+ current enhancement in as early as 3 hours after treatment, and that keeping intracellular levels of potassium high blocked this cell death. Similar studies were confirmatory of this phenomenon in other apoptotic pathways [8]. One such cell death pathway, which our laboratory has worked to characterize, is described in Figure 1. An oxidative insult such as reactive oxygen species (ROS), an oxidizing agent or peroxynitrite leads to the liberation of intracellular zinc from metal binding proteins such as metallothionein [9]. Through either mitochondria [10] or 12-lipoxygenase (an enzyme with a byproduct of superoxide) [11]. Zinc then leads to additional ROS production which then activates the apoptosis signal regulating kinase (ASK-1) [6,12]. ASK-1 phosphorylates the mitogen activated protein kinase (MAPK) p38, which leads to the enhancement of K^+ currents [7] and downstream caspase activation [6,11-13].

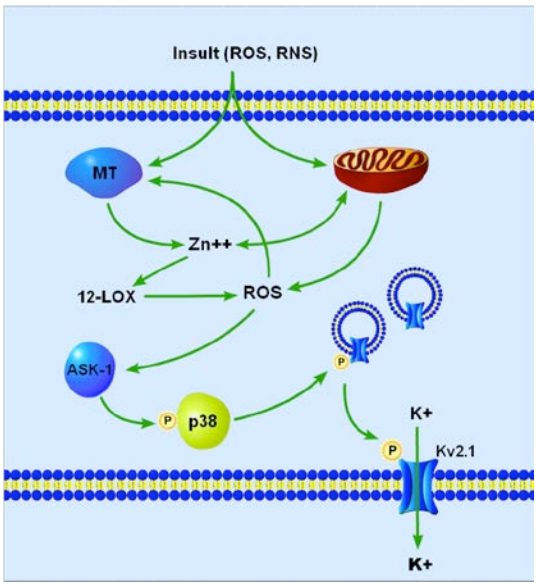


Figure 1. Schematic diagram of proposed pathway of enhancement of Kv2.1-mediated K^+ currents.

Oxidants trigger the liberation of intracellular zinc from metal binding proteins, which activates the p38 MAPK pathway [6]. P38 directly phosphorylates Kv2.1, promoting membrane SNARE-dependent insertion of the channel [14], a subsequent surge of Kv2.1 activity, and leads to neuronal apoptosis [15]. This is a necessary step in apoptosis, as caspases are inhibited by physiological levels of the cation. ROS, reactive oxygen species; RNS, reactive nitrogen species (adapted from Redman et al 2009a).

Kv2.1, the channel responsible for most I_K current in cortical neurons [16,17], is responsible for the distinctive surge in these cells, demonstrated in our laboratory in part by using Kv2.1 dominant negative constructs [7]. This process is triggered by the dual phosphorylation mechanism of Kv2.1 residues S800 and Y124, mediated by p38 and Src respectively (Figure 2) [15,18]. This promotes membrane insertion of the channel via soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent channel insertion, rather than in gating characteristics of channels already present in the plasma membrane [7,14,15]. Inhibiting the potassium surge, through the blockade of channel membrane insertion, for example, is enough to block neuronal cell death in certain models of injury [6,7,19].

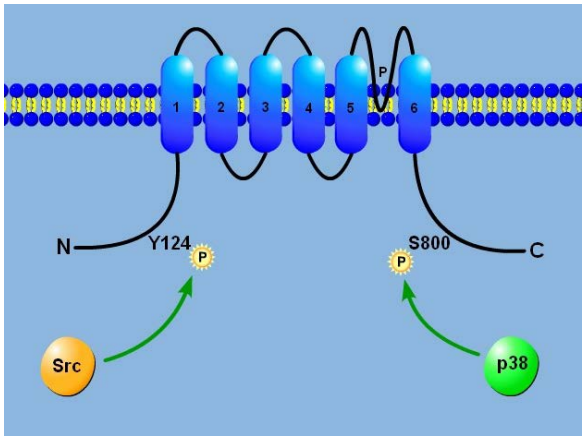


Figure 2. Schematic of Kv2.1 residues required for K^+ current enhancement during neuronal apoptosis.

The trans-membrane Kv2.1 protein is phosphorylated at n-terminal Y124 and c-terminal S800 residues by Src and p38 MAPK respectively. Both N and C termini are cytoplasmic (adapted from Redman et al 2009b).

1.2 HCV NS5A BLOCKS THE K^+ CURRENT SURGE IN LIVER CELLS UNDERGOING APOPTOSIS

It was recently shown that this K^+ channel-dependent pathway is observed in hepatocytes undergoing apoptosis [20]. Hepatocyte cell death happens constantly as these cells turn over under normal conditions, but infection with the hepatitis C virus (HCV) interferes with normal cell death processes. To mediate persistent infection, the virus must replicate its genome, as well as disrupt normal cell physiology to hinder cellular apoptosis, produced either by normal cell turnover or by the virus itself [21]. Still, there is a scarcity of published data detailing the molecular mechanisms by which this occurs. Because ion channels have been implicated in viral apoptotic

programs [22], HCV may act to disrupt channel function during this process. Indeed, Mankouri *et al*. (2009) established that delayed rectifying K^+ current activity was diminished in hepatoma cells expressing the non-structural 5A (NS5A) HCV protein. They also showed that these currents did not demonstrate a surge following oxidative treatment with the thiol oxidant 2,2' dithiodipyridine (DTDP), a well-defined pathway [4,6]. Mankouri et al. (2009) found the primary channel involved in protection was Kv2.1, and proposed that it occurred through a p38 MAPK dependent mechanism. However, NS5A can carry out robust RNA replication of the virus *in vitro* when expressing a full replicon, which was used by Mankouri et al. (2009) in most experimental procedures. However, importantly, NS5A alone was sufficient to suppress K^+ current enhancement in these cells. It is therefore likely that this pathway is critical for NS5A-induced adaptations perpetuating hepatic viral infection, perhaps by increasing viral persistence. Thus, we hypothesized that cortical neurons may respond to NS5A through a similar mechanism.

We report here that NS5A1b modulates basal neuronal K^+ currents, limits the current surge, and protects neurons from apoptosis after injury. Further, we have elucidated the mechanism of action, demonstrating that this protein interferes with Src-mediated phosphorylation of Y124 on Kv2.1. The modulation of apoptosis, however, is not as pronounced in NS5A of the 1a genotype, which led us to speculate that this is due to genotypic divergence, perhaps related to differential modifications as a result of interactions with host proteins.

2.0 METHODS

2.1 MATERIALS

The NS5A-expression vectors were kindly provided by Dr. Mark Harris (Institute of Molecular and Cellular Biology, University of Leeds, Leeds, UK; [20]. The Kv2.1 mammalian expression vector was given by J. Trimmer (UC Davis), and the Kv2.1 (Y124) mutant from A. Elson (Weizmann Institute, Rehovot, Israel). All other Kv2.1 mutants were generated as described [15]. Chemicals and other materials were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted.

2.2 CELL CULTURE AND TRANSFECTION PROCEDURES

Cortical neuronal cultures were prepared in accordance with The University of Pittsburgh Institutional Animal Care and Use Committee, and as previously described from embryonic day 16 Sprague-Dawley rats [23]. Cortices were dissociated with trypsin and cells were plated on 11-mm poly-L-ornithine or poly-L-lysine-treated coverslips in a growth medium composed of 80% Dulbecco's modified minimal essential medium, 10% Ham's F12-nutrients, and 10% bovine calf serum (heat-inactivated and iron-supplemented) with 25 mM HEPES, 24 U/ml penicillin, 24 µg/ml streptomycin. Cells were maintained in 5% CO_2 at 37^{o}C, and medium was partially

replaced three times a week. Non-neuronal cell proliferation was inhibited after 2 weeks in culture with 1–2 mM cytosine arabinoside, and the medium was reduced to 2% serum without F12-nutrients. At 3–5 weeks in vitro these cultures contain 10–20% neurons [24]. Electrophysiology was performed on 3 to 4-week-old cultures (19-26 days in vitro), and toxicity assays were performed on 4-week-old cultures (25-29 days in vitro). Neurons were transfected as previously described [7] using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's instructions with a plasmid encoding NS5A1b-eGFP, NS5A1a, NS5A1a (PA2) or their parent vectors eGFP or PSG5, as noted, and with enhanced GFP to increase the ability to select transfected cells for electrophysiology. For toxicity assays, cells were transfected with a luciferase-expressing vector [7,25-27] along with the above plasmids, as noted in the text. Cells were transfected with 1.5 µg of plasmid DNA in a 24-well plate, and media was replaced 3-5 hours after transfection. Cells were used for electrophysiology or treated with microglia for toxicity studies 24 hours after transfection.

Chinese hamster ovary (CHO) cells were maintained as described with minor alterations [15]. Maintenance media consisted of F12 nutrient medium with 10% FBS and cells were cultured at 5% CO_2, 37^{o}C. 24 hours before transfection (as above, with 1.4 µg DNA per 6-well plate containing 5 cover slips) cells were plated at 2.8 x 10^5 cells per well. After transfection, media was replaced with serum free, containing 10 mM HEPES for 2-4 hours. Cells were then maintained with serum-containing media, and recordings were performed 24 hours after transfection. For biochemical experiments, CHO cells were seeded at a density of 1.7 x 10^6 in 100 mm dishes and transfected as described with 7.334 µg DNA per dish. CHO cells were

transfected for electrophysiology or biochemistry with WT Kv2.1 or mutants S800E, S800A, Y124F; NS5A1b, 1a, PA2, or parent vectors; and Src or p38 as noted.

Immortalized rat brain microglia (HAPI cells) were cultured, plated and activated as previously described [27-29]. Microglia were maintained in Dulbecco's modified MEM supplemented with 10% heat-inactivated fetal bovine serum. For electrophysiological studies, cells were plated in trans-well inserts at a density of 0.5×10^6 cells/well for 24 hours prior to placing the inserts on top of neuronal cultures and activating the microglia with 10 U/mL interferon-γ (IFN-γ) and 1 μg/mL lipopolysaccharide (LPS) for 60 min. This leads to oxidative stress conditions from activated iNOS and NADPH oxidase [28,30,31]. For toxicity assays, cells were plated directly onto neuronal cultures at a density of 0.25×10^6 cells per well and activated with IFN-γ and LPS for 24 hours before viability assessment.

2.3 DRUG TREATMENT

For inhibition of Casein Kinase II 5-Oxo-5,6-dihydroindolo-[1,2-a]quinazolin-7-acetic acid (IQA, Casein Kinase II Inhibitor IV, Calbiochem, Gibbstown, NJ), was used; a indoloquinazoline derivative that suppresses CK2 activity in vitro with an IC_{50} of 0.39 μM. This inhibitor, specific for CK2 with minimal activity with 44 other kinases, takes advantage of a relatively smaller ATP active site within CK2, as IQA competes with ATP for binding there [32]. In indicated experiments, IQA (10 μM) was added with conditioned media 4 hours after transfection, and was present in the external recording solution for electrophysiological measurements.

2.4 ELECTROPHYSIOLOGICAL MEASUREMENTS

Recordings were performed on transfected neurons or CHO cells, as evidenced by eGFP-positive cells at room temperature using the whole cell patch clamp technique with 2–3 MΩ patch electrodes [6]. Electrodes were pulled with a model P-97 mechanical pipette puller (Sutter Instruments, Novato, CA) from 1.5 mm borosilicate glass (Warner instruments, Hamden, CT). Statistical analysis was performed using InStat software along with Origin for graphic design.

For voltage clamp recordings extracellular recording solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 10 HEPES, 10 d-glucose, pH 7.2; 0.25 μM tetrodotoxin was added to block Na^+ channels. The electrode (internal) solution consisted of (in mM): 100 K-gluconate, 11 EGTA, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 2.2 Mg_2ATP, 0.33 GTP; pH 7.2. Osmolarity was adjusted to 280 mOsm with sucrose. Currents were measured with an Axopatch 200B amplifier (Axon Instruments) and pClamp software (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz, and digitized at 10 kHz. Following a depolarizing pre-pulse to inactivate A-type, transient outward K^+ currents, potassium currents were evoked with 80-ms voltage steps from −70 mV in 10-mV increments to 50mV. For analysis and determination of current densities, steady state current amplitudes were measured at + 10 mV and normalized to cell capacitance. Series resistance was compensated (~80%). Neurons previously exposed to activated microglia (AMG) were washed and media was replaced with media containing butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF; 10 μM) to prevent downstream caspase-induced cell death, and the inability to perform electrophysiological recordings. Cells were recorded from 2-5 hours after AMG addition.

For current clamp recordings the extracellular solution contained (in mM): 146 NaCl, 7.8 glucose, 20 HEPES, 4.7 KCl, 0.6 $MgSO_4$, 1.6 $NaHCO_3$, 0.13 NaH_2PO_4, 2.5 $CaCl_2$; pH 7.3, bubbled with 100% O_2. The electrode solution contained (in mM): 94 K^+-gluconate, 30 KCl, 10 Phosphocreatine di tris salt, 10 HEPES, 0.2 EGTA, 4 Mg_2ATP, 0.3 Na_2GTP; pH 7.3. Measurements were performed with an Axoclamp 2B amplifier and LabVIEW-RT 8.0 software. Voltage responses were induced with 1 sec current steps ranging from -120 pA to 120 pA. Virtual excitatory synapses were implemented according to $I_{syn}(t) = k \times g_{syn}(t) \times (V_M - E_{rev})$. Synaptic conductance, $g_{syn}(t)$, was modeled after the sum of two exponentials, with time constants of 1 ms for the rising phase and 7 ms for the falling phase. The synaptic reversal potential, E_{rev}, was set to 0. Synaptic strength was adjusted to have a peak amplitude of 1, by scaling the dimensionless factor *k*. Thresh-g_{syn}, which is the minimal amount of conductance needed to cause an action potential, was determined with an automated binary search routine that systematically adjusted the strength of the virtual EPSP until threshold-g_{syn} was determined [33].

2.5 VIABILITY ASSAYS

In transfected, viable neurons Luciferase reporter genes were used as a marker of cell viability [7,25,26]. Twenty-four hours after transfection with luciferase and a plasmid of interest, cells were exposed to activated microglia to induce neuronal toxicity [27]. Then cells were assessed for viability using Steadylite luciferase substrate (Perkin Elmer, Boston, MA) per manufacturer's instructions. In this assay, viability is proportional to luciferase luminescence.

2.6 IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Twenty-four hours after transfection, CHO cells were lysed (Upstate buffer, Lake Placid, NY), centrifuged and supernatants were collected and stored at -80^{o}C. Protein concentration was measured using a bicinchoninic acid assay (Pierce, Rockford, IL), equal protein was pre-cleared with protein A plus G agarose beads (Santa-Cruz, Santa Cruz, CA), and incubated with Kv2.1 monoclonal antibody (NeuroMab, Davis, CA) and the bead slurry. Samples consisting of beads bound with immuno-complexes were rinsed and prepared for reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) by addition of sample buffer (625 mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol), and boiling at 100^{O}C for 5 minutes.

Samples were then separated on 8% SDS PAGE gels (Mini Protean 3 System, Bio-Rad, Hercules, CA), transferred to 0.2 μm nitrocellulose membranes (Bio-Rad), blocked with 1% Bovine Serum Albumin (BSA) in PBS with 0.05% Tween 20 (PBST) at room temperature for 1 hour and probed with either Kv2.1, phosphotyrosine antibodies (BD Biosciences, San Diego, CA), or immunoaffinity-purified rabbit serum directed at phosphorylated S800 of Kv2.1 [15]. Infrared fluorescent goat secondary antibodies (Li-Cor, Lincoln, NE) were used to visualize proteins. Optical density was measured using an Odyssey infrared imaging system (Li-Cor) with its improvement over film techniques in non-linear measurement and ability to more accurately measure and elicit quantitative results.

3.0 RESULTS

3.1 NS5A1B SUPRESSES DELAYED RECTIFIER K^+ CURRENTS IN CORTICAL NEURONS AND IS NEUROPROTECTIVE

HCV NS5A1b modulates Kv2.1 activity in cortical neurons. Mankouri et al. (2009) found that a hepatocyte-derived cell line expressing HCV NS5A of the 1b genotype demonstrated significantly reduced (~70%) K^+ currents as compared to untransfected or vector-expressing cells. This effect was not restricted to cells that support HCV RNA replication, as HEK293 cells transfected with virus protein along with Kv2.1 showed similar effects. To assess whether suppressed Kv2.1 activity is also seen in neurons transfected with NS5A1b, we used whole-cell electrophysiological recordings to measure K^+ currents in cortical cultures. As in the hepatoma cells, there was a significant decrease (~60%) in voltage-gated, delayed rectifier K^+ current densities in neurons transfected with a NS5A1b-GFP fusion protein under basal conditions (Figure 3A). This indicates that NS5A1b expression inhibits Kv2.1 channel activity in cortical cultures, as the channel is the primary mediator of delayed rectifier K^+ currents in this cell type [7]. It also suggests that the viral protein is interfering with expression or with normal trafficking of channels at the surface of neurons.

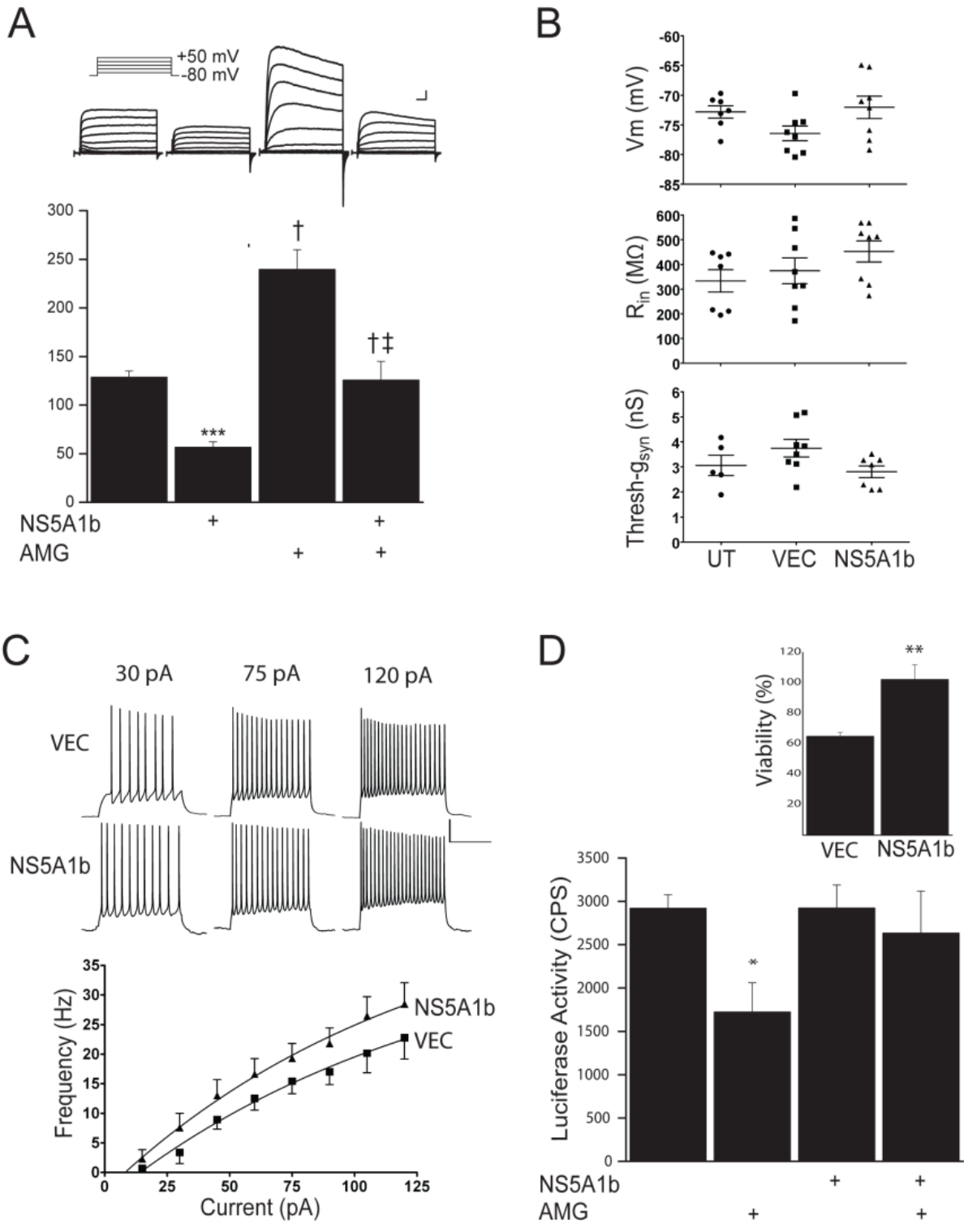
NS5A1b limits the K^+ current surge during injury. During oxidant-induced apoptosis, neurons undergo a large Zn^{2+}- and p38-dependent efflux of potassium via delayed rectifying channels, signified by a large current enhancement [4,6]. Mankouri et al (2009) reported a limited K^+ current surge in NS5A-expressing hepatoma cells compared to vector-expressing controls in the presence of 2,2'-dithiodipyridine (DTDP), an oxidant apoptogen that leads to potassium current enhancement [4]. Therefore, we investigated whether neurons transfected with NS5A would be protected from induced oxidative stress and intracellular Zn^{2+} release, as demonstrated by a decreased K^+ current surge. For these studies we used activated microglia (AMG), which produces comparable K^+ current enhancement to DTDP through a similar cell death pathway, but within a more physiologically based system. Knoch *et al.* (2008) reported that AMG co-cultured in trans-wells with cortical neurons induce oxidative stress from reactive oxygen and nitrogen species, subsequent neuronal intracellular zinc release, and robust apoptotic activation, including a pronounced voltage-gated K^+ current surge. Here we show that vector-expressing neurons exposed to AMG show robust K^+ current enhancement (Figure 3A). Similarly, AMG-exposed cells transfected with NS5A1b showed an increased average current density compared to NS5A1b-expressing cells not exposed to the oxidant. Nevertheless, currents evoked by NS5A1b-expressing neurons exposed to microglia were significantly lower than in activated, vector-transfected cells, and were nearly identical to currents seen in neurons expressing vector without AMG (Figure 3A). Thus, NS5A limited the overall extent of K^+ current enhancement, which could be neuroprotective.

NS5A expression does not alter intrinsic neuronal properties. Since we saw significant changes in K^+ currents under basal cellular conditions in the presence of NS5A1b, we next

assessed changes in intrinsic electrical properties, in collaboration with Dr. John P. Horn and Mitchell G. Springer. Because Kv2.1 activation is voltage-dependent, we expected that resting membrane potential, input resistance, and threshold synaptic conductance in neurons transfected with NS5A would be consistent with control cells. Indeed, levels in NS5A1b-expressing cortical cells were comparable to vector or untransfected cells in each of these parameters (Figure 3B). However, as is consistent with decreased Kv2.1 activity, we saw slightly increased firing frequencies as a function of current in cells transfected with NS5A1b as compared to those transfected with its parent vector (Figure 3C). Thus, the actions of the viral protein may affect integrative characteristics of cortical neurons.

NS5A1b maintains viability in neuronal cultures exposed to activated microglia. Injured neurons expressing NS5A demonstrate limited overall current enhancement in voltage-gated K^+ channels, required for neuronal apoptosis. Thus, we investigated whether this translated into protection from cell death following microglial-induced toxicity. In order to perform this study, we utilized a luciferase reporter assay in which luciferase activity correlates with viability, as previously confirmed with random field counts within GFP transfected cells [7,25,26]. Neurons were transfected with a luciferase-expressing plasmid and either NS5A1b or vector. In this assay, cells are injured with either kainic acid, a control non-apoptogenic stimulus to produce the maximum neuronal cell death [34] (results not shown), or with LPS and IFN-γ activated microglia that induced 45-50% cell death in control cells within 24 hours. In contrast to vector-expressing cells, we found that cells transfected with NS5A1b were virtually immune to AMG insult (Figure 3D). Along with our electrophysiological data, and previous studies linking viability to limitation of the K^+ current surge [2,6,7], these studies demonstrate that limiting threshold levels of K^+ current

enhancement is sufficient to confer neuroprotection. These data further support a mechanism by which NS5A inhibits neuronal apoptosis by interfering with Kv2.1 channel function.


A
+50 mV
-80 mV
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200
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0
***
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NS5A1b
AMG
B
Vm (mV)
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-65
-70
-75
-80
-85
R_{in} (MΩ)
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500
400
300
200
100
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Thresh-g_{syn} (nS)
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5
4
3
2
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UT
VEC
NS5A1b
C
30 pA
75 pA
120 pA
VEC
NS5A1b
Frequency (Hz)
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30
25
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15
10
5
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25
50
75
100
125
Current (pA)
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VEC
D
Viability (%)
120
100
80
60
40
20
**
VEC
NS5A1b
Luciferase Activity (CPS)
3500
3000
2500
2000
1500
1000
500
0
*
NS5A1b
AMG

Figure 3. NS5A interferes with K^+ channel function and is neuroprotective.

(A) (Top) Representative whole-cell K+ currents in rat cortical neurons in culture, evoked by 10 mV incremental steps (-80 to +50 mV). Neurons were transfected with vector (VEC) or NS5A (genotype 1b) plasmids and exposed to vehicle or activated microglia (AMG). Scales are 1 nA and 5 ms. (Bottom) Mean (± s.e.m.; n=7-20 cells per group, 49 cells total) current densities (at +10 mV) from cells such as those shown above (normalized to cell capacitance). ANOVA/Bonferroni, all $p<0.001$; ***VEC vs. NS5A, $^{+}$VEC vs. VEC/AMG, $^{+}$NS5A vs. NS5A/AMG and $^{++}$VEC/AMG vs. NS5A/AMG. (B) Current clamp was used to measure the resting membrane voltage (Vm), input resistance (R_{in}) and threshold synaptic conductance (Thresh-g_{syn}) in untransfected (UT) and vector-expressing cells (VEC) or NS5A1b-transfected neurons (mean ± s.e.m.; n=5-8 cells per group). Scales are 20 mV and 500 ms (C) Repetitive firing was recorded for VEC and NS5A1b transfected neurons using 1 sec depolarizing current steps of increased amplitudes. (Top) Traces are representative of neurons in each group at three different depolarizing current stimuli (n=8). (Bottom) Frequency-current relationships were fit to nonlinear regressions and compared using an F test. NS5A1b transfected neurons have a significantly higher repetitive firing frequency as a function of current when compared to VEC transfected neurons ($P<0.01$). (D) Rat cortical neurons expressing NS5A1b, but not empty vector (VEC), are protected from exposure to AMG. Transfection is performed, and cells are exposed to AMG after 24h. Cells are then harvested and assayed for luciferase activity as an index of cell viability [35] 24h post-AMG exposure. Shown are mean (± S.D.) luciferase values (counts per second, CPS) of a representative experiment, performed in quadruplicate; *$p < 0.05$, ANOVA/Dunnet. Inset, mean ± s.e.m. of viability for a total of 7 independent experiments (each performed in quadruplicate), expressed as a percent of control; **$p<0.01$, paired t-test.

3.2 NS5A1B INHIBITS SRC BUT NOT P38 PHOSPHORYLATION OF KV2.1-ENCODED K^+ CHANNELS

3.2.1 *P38-dependent Kv2.1 S800 phosphorylation is not affected by NS5A1b.*

To elucidate the specific molecular mechanism of modulation of the Kv2.1-dependent apoptotic pathway by NS5A1b, we used Chinese hamster ovary (CHO) cells which do not possess voltage-gated K^+ channels [8]. Thus, any changes in voltage-gated currents that we detected could be attributed to exogenously expressed channels. Importantly, CHO cells, which are normally resistant to oxidative injury, follow an apoptotic pathway similar to cortical cells

when expressing Kv2.1, including the potassium current surge [7,14]. Indeed, as in neurons, we found that CHO cells co-expressing NS5A1b, along with Kv2.1, have depressed basal K^+ currents compared to those transfected with Kv2.1 and vector alone (Figure 4A).

Mankouri et al. (2009) suggests that NS5A confers its effects through the modulation of p38 MAPK phosphorylation of Kv2.1 S800 (see Figure 2), a process known to be required for the potassium current surge [14,15]. We thus began testing this putative mechanism using CHO cells expressing a mutant Kv2.1 channel. When the serine at the 800 residue is mutated to a positively charged amino acid (such as glutamate), it mimics constitutive phosphorylation at this site, independent of p38 activity [15]. Based on results by Mankouri et al (2009), . However, although Kv2.1 (S800E) currents were increased, mimicking the oxidant-induced neuronal current surge, we noted similar inhibitory effects with NS5A1b as were seen in Kv2.1 wild-type channels (Figure 4A). This seemed to indicate a different mechanism of NS5A modulation of K^+ channels than was proposed by Mankouri et al. (2009).

Consequently, we moved to a biochemical approach to investigate the level of Kv2.1 S800 phosphorylation after p38 induction, in the presence or absence of NS5A1b. After immunoprecipitation with a Kv2.1 antibody, we measured p38 phosphorylation by using a phospho-specific antibody that our laboratory had previously generated toward the S800 site of Kv2.1 [15]. Indeed, collaborative studies with Kai He demonstrated that NS5A1b did not block phosphorylation at the S800 site in CHO cells expressing Kv2.1, regardless of p38 over-expression. A non-phosphorylatable mutant, Kv2.1 (S800A) [15], was used as a control for the specificity of the antibody (Figure 4B). These, along with electrophysiological data in Figure 4A,

led us to the conclusion that the modulation by NS5A1b of neuronal and Kv2.1-expresssing CHO cell K^+ currents is not likely due to interference with p38 activation at Kv2.1 S800.

3.2.2 *P38 NS5A interferes with Src-mediated phosphorylation of Kv2.1 Y124.*

A variety of kinase systems interact with Kv2.1 to affect its activity [36-38]. For example, phosphorylation by Src and dephosphorylation by protein tyrosine phosphatase work in concert to up and down-regulate activity of the K^+ channel at a specific tyrosine [38,39]. Redman et al. (2009) demonstrated that this tyrosine (Y124) is required, in addition to the S800 residue, for K^+ current enhancement leading to oxidant-induced apoptosis in cortical neurons. Thus, the surge is triggered, in part, by concurrent activation of Src and p38 kinase pathways.

There are two class II polyproline motifs conserved throughout all NS5A genotypes that interact with Src-homology 3 (SH3) domains to suppress the host Src-like kinases Hck, Lck and Lyn [40]. Consequently, we investigated whether the viral protein inhibited Src-mediated processes. To test this, we expressed channels carrying a mutation that replaces the tyrosine with a phenalaline, rendering the channel non-phosphorylatable at the Y124 site (Kv2.1 (Y124F); note that phospho-mimicking mutants are non-functional) [18]. Unlike the current inhibition seen in NS5A1b-expressing CHO cells harboring wild type Kv2.1 or Kv2.1 (S800E) mutants, potassium currents of cells expressing Kv2.1 (Y124F) are not inhibited by NS5A1b (Figure 4A). This suggests that the viral protein interferes with Src phosphorylation of residue Y124 - a signaling step that is necessary for the large surge of Kv2.1 currents during apoptosis [15], and which could also be necessary for channel trafficking under basal conditions.

As a result, we investigated whether NS5A 1b can block Src phosphorylation of Kv2.1. Again, in collaboration with Kai He, we used Kv2.1 immunoprecipitation in CHO cells over-expressing Src in the presence or absence of NS5A1b. We then probed with a phosphotyrosine antibody, since measures to generate a phospho-specific Kv2.1 Y124 antibody had previously proven unsuccessful [18]. The phosphotyrosine signal, upregulated with overexpression of Src, was significantly decreased in those cells transfected with NS5A1b (Figure 4C). This is in accordance with our electrophysiological results (Figure 4A), as well as findings that suggest over 80% of Src phosphorylation of the channel is targeted to this residue [38]. Taken together, these data demonstrate a likely Y124, Src-dependent mechanism for NS5A1b Kv2.1 inhibition.

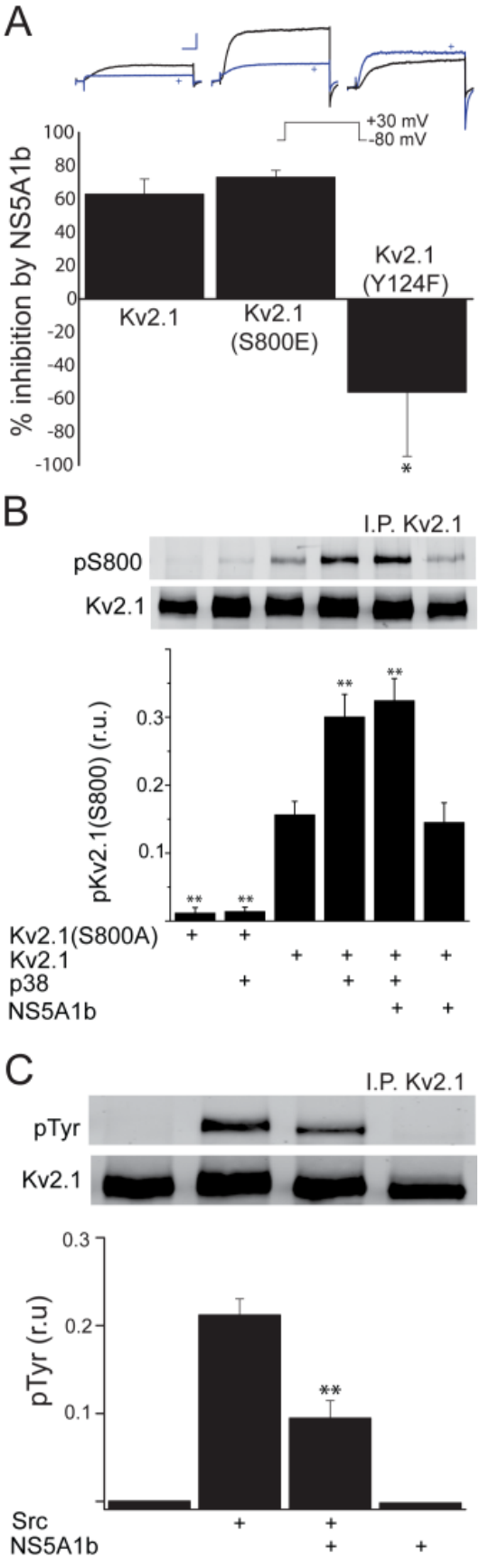
A
+30 mV
-80 mV
% inhibition by NS5A1b
100
80
60
40
20
0
-20
-40
-60
-80
-100
Kv2.1
Kv2.1
(S800E)
Kv2.1
(Y124F)
*
B
I.P. Kv2.1
pS800
Kv2.1
pKv2.1(S800) (r.u.)
0.3
0.2
0.1
**
**
**
**
Kv2.1(S800A)
Kv2.1
p38
NS5A1b
C
I.P. Kv2.1
pTyr
Kv2.1
pTyr (r.u)
0.3
0.2
0.1
**
Src
NS5A1b

Figure 4. NS5A1b inhibits Src, but not p38, phosphorylation of Kv2.1.

(A) (Top) Representative whole-cell currents (-80 to +30 mV step) in Chinese hamster ovary (CHO) cells expressing Kv2.1, Kv2.1(S800E), or Kv2.1 (Y124F) together with either VEC or NS5A1b (marked by a +). Note decreased currents in NS5A1b-expressing cells, except in the case of the Kv2.1(Y124F) mutant. Scales are 5 nA and 5 ms. (Bottom) Average K^+ current suppression by NS5A1b co-expression, expressed as a % of the average currents present in vector expressing cells; n=9-19 cells per group (82 cells total); *p<0.05, significantly different from the other two groups, ANOVA/Dunnet. (B) CHO cells were co-transfected with Kv2.1(S800A), Kv2.1, p38, NS5A1b or vector. Kv2.1 immunoprecipitates were run on SDS-PAGE and probed with a phospho-specific antibody recognizing the phosphorylated form or residue S800 [41]. Kv2.1 phosphorylation at S800 was increased by p38 over-expression in wild-type, but not in mutant channels. Co-expression of NS5A1b was ineffective in blocking the actions of p38. (Top) Representative immunoblot; (Bottom) summary and quantification of results in three independent experiments, normalized to total Kv2.1 protein; **p<0.01, ANOVA/Dunnet vs. Kv2.1 control (third bar). (C) CHO cells were co-transfected with Kv2.1, Src, NS5A1b or vector. Kv2.1 immunoprecipitates were run on SDS-PAGE and probed with a phosphotyrosine. Tyrosine phosphorylation of Kv2.1 was increased by Src over-expression. Co-expression of NS5A1b in this case significantly inhibited the actions of Src. (Top) Representative immunoblot; (Bottom) summary and quantification of results in three independent experiments; **p<0.01, ANOVA/Bonferroni vs. Src/vector group (second vs. third bar).

3.3 NS5A ACTIONS ARE GENOTYPE-SPECIFIC.

As we found the Src-phosphorylated Kv2.1 residue Y124 to be the likely target of NS5A, and as the viral protein is known to interact with host Src-like proteins through a conserved polyproline motif, we next utilized an NS5A mutant in which the conserved prolines had been changed to alanines, termed NS5A (PA2). Hepatoma cells harboring an HCV replicon with this same mutation (replicon^{PA2}) have normal potassium currents, compared to cells harboring the non-mutated replicon [20]. Electrophysiological results in cortical neurons with NS5A (PA2) mutants showed no current inhibition (Figure 5A), apparently suggesting that abolishing Src-like binding is sufficient to render NS5A ineffective at modulating K^+ currents in this system. However, we noted that this NS5A (PA2) mutant is of a different genotype (1a vs. 1b) [42], and

when comparing currents generated from cells transfected with the wild type NS5A1a to those expressing the NS5A1a (PA2) mutant, there was no significant difference, i.e. the current inhibition was absent in even the wild type cells (Figure 3A). In further collaboration with Kai He, NS5A1a also did not inhibit Src-mediated tyrosine phosphorylation of Kv2.1 channels expressed in CHO cells (Figure 5B) as we had seen in NS5A1b-expressing cells (Figure 4C). These results suggest that there are differences between NS5A genotypes that account for the lack of suppression of the basal potassium currents. Indeed, as the genotypes share only 80% sequence homology, there is 20% genotypic disparity that could account for observed differences [43]. Moreover, NS5A1a did not confer similar neuroprotection as its 1b counterpart in cortical neurons exposed to AMG (viability was 69.8 $\pm$ 2.3% of control; n=3; compare to Figure 3B). Together, these data indicate that the inhibitory effects of NS5A on Kv2.1-mediated currents are genotype-specific, and that the K^+ channel inhibitory effect of NS5A1b is a critical component of its anti-apoptotic effects.

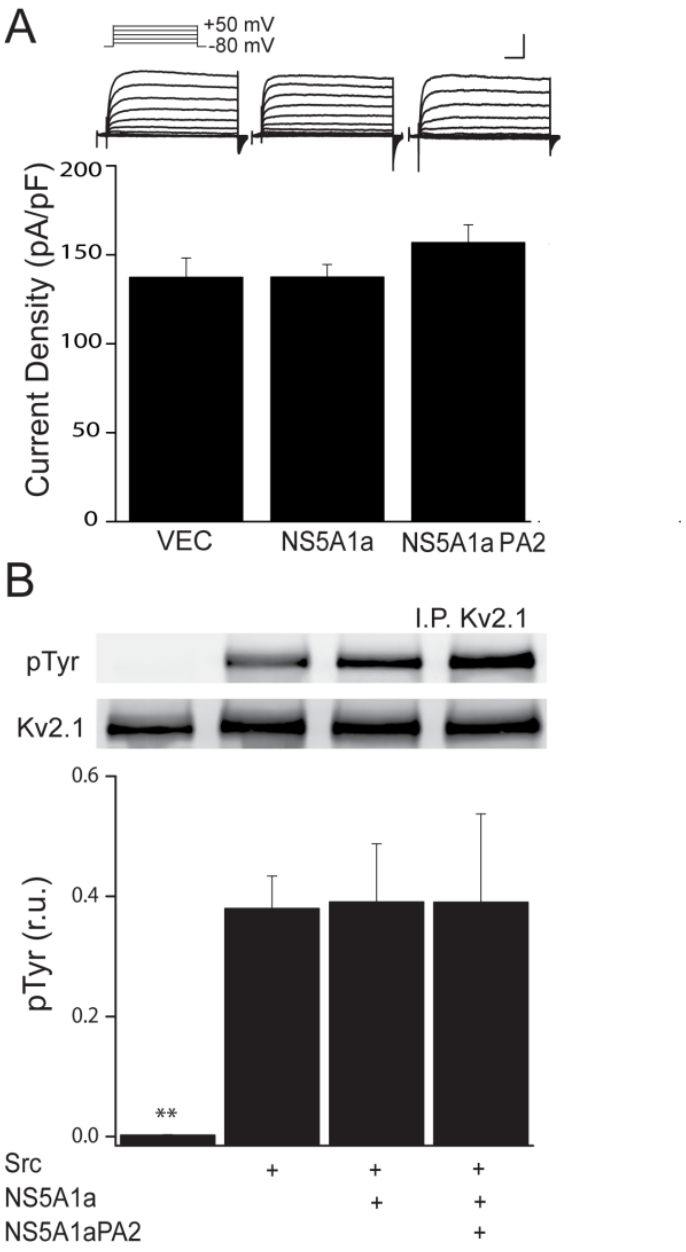

Figure 5. Genotype 1a of NS5A is ineffective in blocking K+ currents in neurons, or preventing SRC phosphorylation of Kv2.1.

(A) Representative whole-cell K+ currents in rat cortical neurons in culture, evoked by 10 mV incremental steps (-80 to +50 mV). Neurons were transfected with vector (VEC), NS5A1a, or NS5A1a(PA2) plasmids (Top). Scales are 3 nA and 25 ms. (Bottom) Mean (± s.e.m.; n=14-16 cells per group; 44 total cells) current densities (at +10 mV) from neurons transfected with empty vector, NS5A (genotype 1a) or a mutant (PA2) of NS5A1a lacking the polyproline motif that has been shown to interact with Src-like kinases. (B) Experiments similar to those shown in Figure 2C, illustrating the lack of inhibition of Src phosphorylation of Kv2.1 channels in CHO cells

expressing NS5A1b (n=3); **p<0.01, ANOVA/Dunnet; all groups are significantly different from Kv2.1+vector group (first bar).

3.4 CK2 PHOSPHORYLATION OF NS5A MAY BE REQUIRED FOR K^+ CURRENT MODULATION.

The modulation of K^+ channel activity, Src-mediated phosphorylation, and apoptosis in NS5A1b but not 1a-expressing cells led us to speculate that these discrepancies are due to genotypic divergence. Moreover, genotype disparity of HCV correlates with differences in virus virulence and cancer incidence [44,45], as well as phosphorylation profile of the NS5A protein [46]. Likewise, functions of the viral protein are dependent on specific phosphorylation events [47]; however, the precise role phosphorylation plays in NS5A's subsequent interactions with host proteins has not been fully elucidated. In vitro cell-free and transfected cell models show that the constitutively active, pleiotropic kinase CK2 (formerly known as casein kinase 2) phosphorylates NS5A [46,48], and it is thought to be the primary kinase targeting the viral protein for basal phosphorylation [43,49,50]. Intriguingly, there is some heterogeneity in the phosphorylation profiles of CK2 between genotypes 1a and 1b, trending toward more predicted CK2 residues in the 1b protein [46].

Due to the apparent constitutive activation of CK2 [48], we used the specific inhibitor 5-oxo-5,6-dihydroindolo-[1,2-a]quinazolin-7-acetic acid (IQA) to reduce expression of the protein, and to see if this translates to reduced K^+ current inhibition previously seen in cells with NS5A1b expression. IQA (10 μM) was added with conditioned media after transfection of neurons with

NS5A1b or vector, and was present in the external recording solution for electrophysiological measurements. In the presence of IQA, there was no significant difference in K^+ current densities in NS5A1b-expressing or vector-expressing cortical cells (Figure 6). Thus, CK2 suppression blocked basal current modulation previously seen in NS5A-expressing cells. This preliminary result suggests that phosphorylation of the viral protein by CK2 is necessary for conferred anti-apoptotic properties by NS5A1b.

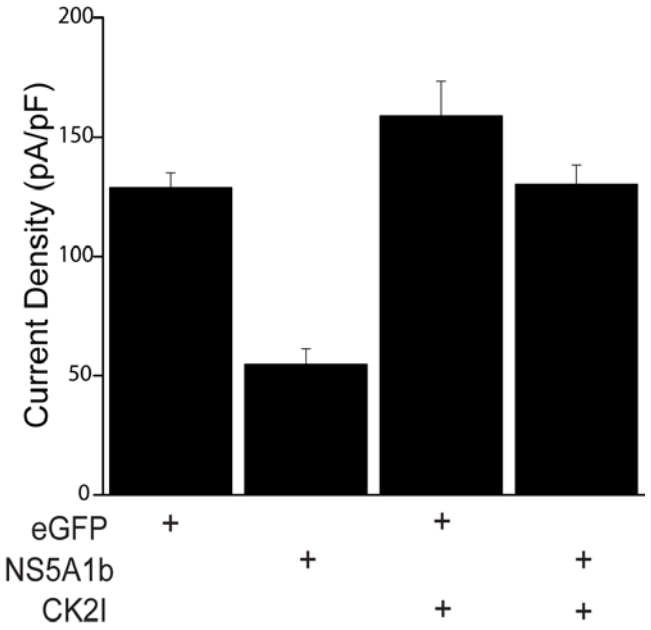

Figure 6. CK2 inhibibtion blocks current modulation by NS5A1b.

Mean (± s.e.m.; n=6-20 cells per group) current densities (at +10 mV) from neurons transfected with empty vector (eGFP) or NS5A1b, and treated with 10 µM 5-oxo-5,6-dihydroindolo-[1,2-a]quinazolin-7-acetic acid (IQA; CK2I). First two bars are experiments from Figure 3A.

4.0 DISCUSSION

The results in this study show that the HCV-derived protein NS5A genotype 1b modulates Kv2.1 channel activity in cortical neurons and CHO cells, two cell types which lack the ability to replicate the virus. Furthermore, NS5A1b limits the magnitude of the pro-apoptotic K^+ current surge, and confers neuro-protection following AMG-induced toxicity. These findings are genotype-specific, as NS5A1a does not confer the same anti-apoptotic effects. Finally, host-cell modifications of the virus protein, such as phosphorylation, may play a role in anti-apoptotic properties, as treatment with a specific kinase inhibitor disrupts NS5A1b basal K^+ current inhibition.

HCV, which affects over 3% of the world's population, induces a chronic liver infection that causes serious and persistent liver disease in the majority of people infected. In many cases, it leads to cirrhosis and hepatocellular carcinoma [51]. The HCV non-structural protein 5A (NS5A) is a highly studied, potential therapeutic target for treatment of this disease, as it is required for virus replication. Nevertheless, the full spectrum of NS5A function is unknown. Viral proteins must be adaptable to different host cell environments and be able to complete varying tasks at specific times in the life cycle of both the virus, and host. Additionally, virus infection that translates to host cell death is not well suited for propagation of the virus itself. NS5A is one of 10 proteins processed from the HCV RNA genome, and has previously been linked to anti-

apoptotic properties [40]. Recently, a mechanism was defined [20] that may explain how this protein promotes viral replication and persistence. Moreover, interference with K^+ channels is currently the only proposed mechanism for how NS5A blocks apoptosis in the viral system.

NS5A, a 60 kDa protein required for viral replication, is divided into 3 domains. Domain I is the only domain to have been crystallized [52], and thus more is known about this N-terminal domain of the protein. Within this domain, and most proximal to the N-terminus, sits a membrane-anchoring amphipathic alpha helix necessary for viral replication complex formation [53-55], and likely necessary for membrane trafficking of the epidermal growth factor receptor [56]. Domain I also contains a zinc binding region that is required for HCV replication and is conserved throughout all HCV genotypes. However, the full functional range of NS5A as a zinc metalloprotein is not well understood. Contained within this region are a number of cysteine residues important for viral replication complex assembly [52,57]. Other salient features of the protein are caspase/calpain cleavage sites in domain I [58,59], and the aforementioned class II polyproline motifs, located between domains II and III. Additionally, within NS5A there is a highly conserved area of hyperphosphorylation within domain II of the protein, and two basal phosphorylation motifs. The most conserved of the latter among genotypes is the more amino-terminal region, and the least conserved lies in domain III near the c-terminus [60,61]. Hyperphosphorylation is required for viral RNA replication, and is also dependent upon other HCV proteins, namely NS4 [62,63]; however, basal phosphorylation is less well understood. This is generally due to the fact that, as mentioned, little is known about the more c-terminal domains, particularly domain III [64]. Figure 7 highlights some important features of NS5A.

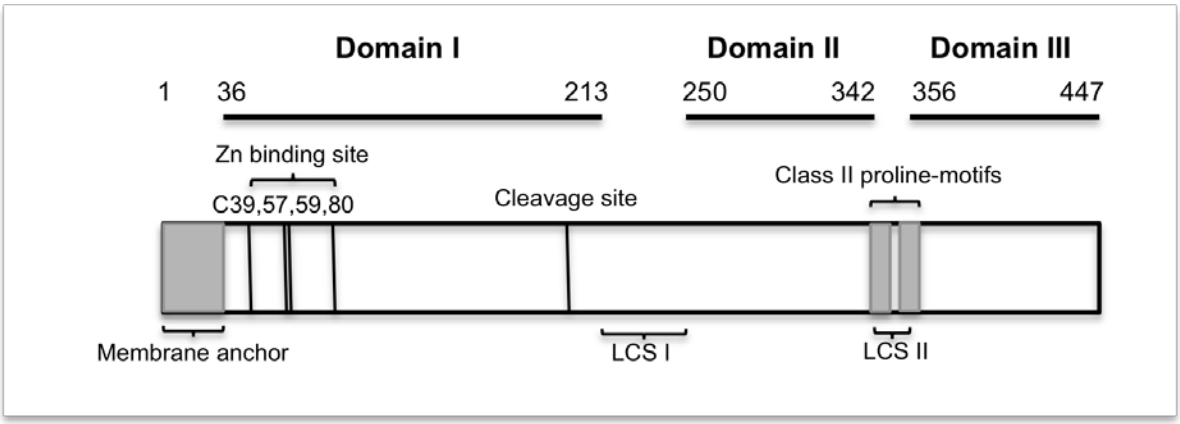

Figure 7. Features of the NS5A protein.

NS5A contains three domains, separated by two low complexity sequences consisting of repetitive amino acids. Motifs include a membrane-anchoring alpha helix, a zinc-binding motif, a cleavage site targeted by proteases, and Class II poly-proline motifs (adapted from Moradpour et al., 2005 and He et al., 2006 by Elias Aizenman).

Defining the molecular domains of NS5A necessary for restricting Kv2.1 function is of primary importance in the potential development of small molecule inhibitors that mimic the actions of the viral protein as a neuro-therapeutic strategy. The work presented herein helps to define areas of interest based on genotype disparity and presence or absence of effects that block cell death pathways. Of interest, genotype 1 (including 1a and 1b) accounts for most forms of HCV, especially in the US, and has been associated with more severe liver disease, more aggressive disease progression, resistance to therapy and recurrence, and importantly, increased incidence of hepatocellular carcinoma in patients exhibiting chronic, active HCV infection [65]. The anti-apoptotic properties of NS5A1b via its inhibition of Kv2.1 channel function could be important contributors towards its increased persistence and association with liver cancer. Notably, cell death related to viral persistence is one of the greatest disparities among genotypes, particularly between the two type 1 viruses, which share only 80% sequence homology [43]. Most

sequence differences are found near the C-terminal domain of the protein in domain III [66,67], immediately adjacent to the SH3-binding polyproline motifs, which themselves are highly conserved among all HCV genotypes [68]. Until recently there was no demonstrated function of domain III of the NS5A protein, although exchange of multiple serines to unphosphorylatable residues disrupts the efficiency of virus production [50,64]. Moreover, a specific phosphorylation site was discovered to be crucial for virion production, assembly and release, [47], and thus, other functions of this region are likely to rely on phosphorylation as well. It has been suggested that different phosphorylation profiles are present in NS5A1a and NS5A1b, particularly within the 70 amino acids that lie most proximal to the C-terminus [69]. Because the functions thus far attributed to this domain of NS5A are required for the virus life cycle and are well conserved, there is likely another function to account for the discrepancy among genotypes, such as viral persistence relating to the newly proposed mechanism of apoptotic inhibition through a K^+ channel [20]. Post-translational modifications such as phosphorylation events are one potential avenue to accomplish this [70].

Much is known about how host proteins interact with NS5A, but there is a paucity of information about how this interaction affects the functions of NS5A within the cell. Whether the genotype-specific effects of NS5A1b on K^+ channel modulation are due to specific amino acid differences, phosphorylation levels, or both, remains to be established. NS5A is known to extensively interact with host proteins, and the kinase CK2 has been predicted to phosphorylate specific sites on the C-terminus of the protein, differentially by genotype [46,49,50,71]. In addition to intrinsic viral proteins that process NS5A, such as the NS3/NS4A serine proteinase complex [72-74], host proteins also play a role in its processing. As mentioned, one way in which this virus

protein is acted upon by host proteins is through phosphorylation by the kinase CK2 [46,49,50,71]. Although there are many conserved phosphoacceptor sites between viral genotypes, one of which is required for effective virion production [47], there is some heterogeneity in the phosphorylation profiles of CK2, between genotypes 1a and 1b, trending toward more predicted CK2 residues in the 1b protein [46]. It is not known how phosphorylation of NS5A proceeds in our system;, the kinase is present in the brain at levels higher than any other organ, and is localized specifically to neurons within the CNS [43,71,75]. It is thought to play a role in a variety of processes including p53 regulation and learning and memory [76]. CK2 is a pleiotropic protein with over 300 known substrates, and is constitutively active within the cell [48]. Thus, differences in phosphorylation profile have more to do with the number and location of specific sites, rather than activity of the kinase itself. Preliminary results herein showed a dependence on the full expression of CK2 for modulatory effects on basal delayed rectifier K^+ channels (Figure 6). CK2 is also thought to modify other virus proteins [43,49,50] and thus its activity may be required for significant viral actions within multiple cell types. The functional significance of NS5A phosphorylation by CK2 has remained elusive despite years of study. Continued work in this system could elucidate further mechanisms and requirements for the anti-apoptotic properties of NS5A via this pathway our laboratory has defined over the past 10 years (Figure 1).

Phosphorylation events are known to regulate viral-host cellular events such as caspase cleavage. As mentioned, there are caspase/calpain cleavage sites in domain I of NS5A that may be used to target portions of the viral protein to various cellular positions during injury [58,59]. A recent paper by Turowec et al (2011) described overlapping caspase cleavage and CK2 phosphoylation motifs. It has been previously established that kinase substrates become more or

less likely to be cleaved by caspase, dependent on their phosphorylation profile in overlapping motifs, and that these interactions play a role in cell survival and cancer [77-79]. In fact, CK2 phosphorylation residues often overlap with caspase cleavage sites, and phosphorylation can prevent or increase the likelihood of protease cleavage at specific sites [80]. This might be a link between two post-translational modifications of the virus protein, and could be required for cell death prevention. Of note, sequence observations confirm that the cleavage site contained within NS5A is a predicted site of CK2 phosphorylation. Furthermore, this CK2 phosphorylation site is only predicted in viral protein sequences of the 1b genotype [46].

Understanding the pathways and specific mechanisms of how NS5A blocks neuronal apoptosis is crucial to the design of small molecule or peptide mimetics that could be used to block the Kv2.1 K^+ surge following neuronal injury and to treat oxidative stress in neurons. NS5A is known to disturb various signaling pathways in cells, including MAPK inhibition [56,81,82] and PI3K activation [42]. It was thought to mediate these effects primarily through a polyproline motif (PxxPxR) at the C-terminus, which binds to SH3 domains of proteins, including Src-family kinases, amphiphysin II, as well as other kinases [73,83,84]. However, distinct to the mechanism proposed by Mankouri et al. (2009), our results suggest that the K^+ current modulatory effects of NS5A are not mediated by the SH3-binding, polyproline motifs in this protein, since both the 1a and 1b genotypes contain these sequences, and NS5A1a lacked function in our assays. In addition, while the SH3-binding domain of NS5A effectively binds and inhibits the action of the Src-family kinases Hck, Lck and Lyn, it has not been shown to bind Src itself [85]. However, it was recently reported that Src is required for the interaction between NS5A1b and the HCV RNA-dependent polymerase NS5B. NS5A1b can bind Src, not at the kinase SH3 domain, but at

a different, SH2 domain, although the specific NS5A1b-SH2 binding interaction region has yet to be defined [86].

A recent study describes two additional Kv2.1 tyrosine residues phosporylated by Src [87] which likely account for the minimal phosphotyrosine signal in cells transfected with Kv2.1 (Y124F) after Src induction [38]. Indeed, studies with Kai He in our laboratory in CHO cells transfected with Kv2.1 (Y124F) and Src confirm a consistent, but slight (~17% of wild type cells) tyrosine phosphorylation signal (phosphotyrosine levels were 0.037 ± 0.003 r.u., n=3; compared to 0.212 ± 0.019 r.u., n=3, from Figure 4C; $p<0.001$, unpaired t test; results not shown). Co-expression with NS5A also inhibited this non-Y124 Src-induced phosphorylation of Kv2.1 (Y124F) (0.015 ± 0.004 r.u., n=3; significantly different from vector-expressing cells, $p<0.05$, t test; not shown). This suggests that the effects of the viral protein may represent a general repressive mechanism of the actions of Src. What this means in regards to therapies using drugs mimicking NS5A Src-inhibition of the channel is yet to be determined and further analysis is critical. The likely requirement for phosphorylated tyrosine residues, in combination with the known phosphorylation differences between NS5A1b and NS5A1a [88], could provide important clues at defining the mode of action of the viral protein for modulating K^+ channel function. This mechanism, which evolved in a virus to prevent cell death, may provide critical information for the development of novel neuroprotective strategies to block K^+ current-mediated following injury.

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