Inhibition of HCN1 currents by norquetiapine, an active metabolite of the atypical anti-psychotic drug quetiapine

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Abstract

Background and Purpose Quetiapine is a second-generation atypical antipsychotic drug that has been commonly prescribed for the treatment of schizophrenia, major depressive disorder (depression), and other psychological disorders. Targeted inhibition of hyperpolarization-activated cyclic-nucleotide gated (HCN) channels, which generate Ih, may provide effective resistance against schizophrenia and depression. We investigated if HCN channels could contribute to the therapeutic effect of quetiapine, and its major active metabolite norquetiapine. Experimental Approach Two-electrode voltage clamp recordings were used to assess the effects of quetiapine and norquetiapine on currents from wild-type and mutant HCN1 and HCN2 expressed in Xenopus laevis oocytes. Key Results Norquetiapine, but not quetiapine nor 7-hydroxy quetiapine, has an inhibitory effect on HCN1 channels. Norquetiapine selectively inhibited HCN1 currents by shifting the voltage-dependence of activation to more hyperpolarized potentials in a concentration-dependent manner with an IC50 of 13.9 $\pm$ 0.8 μM for HCN1 and slowing channel opening, without changing the kinetics of closing. Inhibition by norquetiapine primarily occurs from in the closed state. Norquetiapine inhibition is not sensitive to the external potassium concentration, and therefore, likely does not block the pore. Norquetiapine inhibition also does not dependent on the cyclic-nucleotide binding domain. Norquetiapine had no effect on HCN2 channels. Conclusions and Implications HCN channels are key targets of norquetiapine, the primary active metabolite of quetiapine. These data help to explain the therapeutic mechanisms by which quetiapine aids in the treatment of anxiety, major depressive disorder, bipolar disorder, and schizophrenia, and may represent a novel structure for future drug design of HCN inhibitors.

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Keywords: HCN channel, norquetiapine, quetiapine, depression,

Abbreviations: MMD, major mood disorder; HCN, hyperpolarization-activated cyclic-nucleotide gated; CNBD, cyclic-nucleotide binding domain; WT, wild-type; FL, full-length; QTP quetiapine; NQTP, norquetiapine;

DATA AVAILABILITY

Data will be made available upon request.

FUNDING

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AUTHOR CONTRIBUTIONS

Experiments were performed and analyzed by AJJ, ACA, and ND. The manuscript was prepared by AJJ and ND.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Quetiapine is a second-generation atypical antipsychotic drug that has been commonly prescribed for the treatment of schizophrenia, major depressive disorder (depression), and other psychological disorders. Targeted inhibition of hyperpolarization-activated cyclic-nucleotide gated (HCN) channels, which generate I_h, may provide effective resistance against schizophrenia and depression. We investigated if HCN channels could contribute to the therapeutic effect of quetiapine, and its major active metabolite norquetiapine.

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Two-electrode voltage clamp recordings were used to assess the effects of quetiapine and norquetiapine on currents from wild-type and mutant HCN1 and HCN2 expressed in *Xenopus laevis* oocytes.

Key Results

Norquetiapine, but not quetiapine nor 7-hydroxy quetiapine, has an inhibitory effect on HCN1 channels. Norquetiapine selectively inhibited HCN1 currents by shifting the voltage-dependence of activation to more hyperpolarized potentials in a concentration-dependent manner with an IC_{50} of 13.9 ± 0.8 μM for HCN1 and slowing channel opening, without changing the kinetics of closing. Inhibition by norquetiapine primarily occurs from in the closed state. Norquetiapine inhibition is not sensitive to the external potassium concentration, and therefore, likely does not block the pore. Norquetiapine inhibition also does not dependent on the cyclic-nucleotide binding domain. Norquetiapine had no effect on HCN2 channels.

Conclusions and Implications

HCN channels are key targets of norquetiapine, the primary active metabolite of quetiapine. These data help to explain the therapeutic mechanisms by which quetiapine aids in the treatment of anxiety, major depressive disorder, bipolar disorder, and schizophrenia, and may represent a novel structure for future drug design of HCN inhibitors.

Bullet Point Summary

What is already known: Quetiapine antagonizes serotonin, dopamine, histamine, and adrenergic receptors, and blocks hERG and Nav1.5 channels.

What this study adds: HCN1 but not HCN2 channels are inhibited by norquetiapine; the major metabolite of quetiapine.

Clinical significance: This study contributes to understanding the mode of action of the atypical antipsychotic drug quetiapine.

Introduction

Hyperpolarization-activated cyclic-nucleotide gated (HCN) channels are the molecular correlate of I_h, and are widely expressed in the central and peripheral nervous systems. All four isoforms (HCN1-4) are expressed in the brain (Ludwig, Zong, Jeglitsch, Hofmann, & Biel, 1998; Moosmang et al., 2001; Pape, 1996; Santoro, Grant, Bartsch, & Kandel, 1997; Santoro et al., 1998) where they play a role in setting the resting membrane potential, modulating dendritic integration of synaptic inputs, reducing neuronal input resistance, neuronal pacemaking, and establishing action potential threshold (Pape, 1996). HCN channels are important for learning and memory, pain sensation, sour taste sensation, and vision (Chaplan et al., 2003; Knop et al., 2008; Nolan et al., 2004; Nolan et al., 2003; Paspalas, Wang, & Arnsten, 2013; Stevens et al., 2001). $HCN1^{-/-}$ mice show impaired motor learning but enhanced spatial learning and memory (Nolan et al., 2004; Nolan et al., 2003) and enhanced resistance to depression (Huang, Walker, & Shah, 2009; Lewis et al., 2011). HCN1 expression increases in the CA1 region of the dorsal hippocampus in a chronic unpredictable stress rat model. Notably, shRNA knockdown of HCN1 reduces the stress response in this model (C. S. Kim, Brager, & Johnston, 2018). Targeted viral knockdown HCN1 in the CA1 hippocampal region also enhanced mobility in the Porsolt swim test (C. S. Kim, Chang, & Johnston, 2012). Similarly, genetic ablation of *Trip8b*, an auxiliary protein that regulates HCN1 and HCN2 expression, also increases resistance to depression (Lewis et al., 2011). Furthermore, altered HCN-cAMP signaling in prefrontal cortex networks also appears to contribute to the working memory deficits in schizophrenia and stress (Arnsten, 2011; Gamo et al., 2015; Paspalas et al., 2013), while mutations in SHANK3 linked to schizophrenia (Gauthier et al., 2010; Guilmatre, Huguet, Delorme, & Bourgeron, 2014) may induce an HCN channelopathy (Yi et al., 2016). Furthermore, polymorphisms in HCN4 channels were associated with mood disorders and/or obsessive compulsive disorder (Szabo et al., 2011).

Quetiapine fumarate (Seroquel®) (QTP) is a second-generation atypical antipsychotic drug that has been commonly prescribed for the treatment of schizophrenia (Dev & Raniwalla, 2000; Small, Hirsch, Arvanitis, Miller, & Link, 1997), acute bipolar mania (Janicak & Rado, 2012), insomnia (Lin, Chiang, Tseng, Tam, & Loh, 2023), major depressive disorder (depression) (Ravindran et al., 2022), anxiety (Bandelow et al., 2010; Crapanzano, Damiani, & Guiot, 2021; Ravindran et al., 2022), Post-traumatic

stress disorder (Crapanzano, Damiani, Casolaro, & Amendola, 2023) and other psychological disorders (Saller & Salama, 1993). Like other atypical antipsychotics, QTP is structurally similar to clozapine and acts as an antagonist to serotonin, dopamine, histamine, and adrenergic receptors (Burns, 2001; Saller & Salama, 1993). QTP is primarily metabolized by hepatic cytochrome P450 3A4 (Dev & Raniwalla, 2000), with norquetiapine (NQTP) and 7-hydroxycholoroquine (7-OH QTP) as its major active metabolites. NQTP exhibits pharmacological activity that differs from QTP (Bakken, Molden, Knutsen, Lunder, & Hermann, 2012; DeVane & Nemeroff, 2001) and also exhibits antidepressant activity (Jensen et al., 2008; Lopez-Munoz & Alamo, 2013). In fact, NQTP shares structural similarities with several antidepressants including amoxapine and desipramine, and its physicochemical properties confer greater potential for its use as an antidepressant agent (D. W. Kim, Weon, Hong, Chung, & Lee, 2016; Lopez-Munoz & Alamo, 2013). Indeed, the effect of QTP in major depressive disorder is probably mediated, at least in part, by NQTP, which selectively inhibits norepinephrine transporter reuptake (Bandelow et al., 2010; Lopez-Munoz & Alamo, 2013).

In addition to antagonizing serotonin, dopamine, histamine, and adrenergic receptors, and inhibiting norepinephrine transporter reuptake, QTP and NQTP have been shown to block the hERG (human-Ether-a-go-go-Related Gene) potassium channel (Kongsamut, Kang, Chen, Roehr, & Rampe, 2002; Lee, Choi, Choi, & Hahn, 2018), and the sodium channel, Nav1.5 (D. H. Kim, Park, Park, Hahn, & Choi, 2020). Given the role of HCN channels in major depressive disorders, anxiety, and schizophrenia, examining the effects of antipsychotic drugs on this current is worthwhile. In the present study, we investigated the inhibitory mechanisms of norquetiapine on the HCN channels expressed in *Xenopus laevis* oocytes.

Methods

Group size

The number of cells recorded for each experimental group (n) are presented. Data for each group were collected from oocytes harvested on at least 2 separate occasions. Data subjected to statistical analysis had an n of at least five for each group.

Randomization

Randomization was not performed as this is not a standard procedure in electrophysiological recordings.

Blinding

Blinding of experiments is not applicable. Analysis was performed by an operator who was blinded to the recording conditions.

Cloning, oocyte isolation and channel expression

cDNA coding for the mouse HCN1 gene and mHCN1-CX5 containing a stop codon at residue F472 (denoted as HCN1-ΔCNBD in this article) was sub-cloned into the expression vector pGH19, while mouse HCN2 was sub-cloned into the pGEM vector. All clones were verified by PCR sequencing of the complete ORF. To obtain RNA, cDNA was linearized using NheI (New England Biolabs) for mHCN1 or SphI for mHCN2 and ~1.0 μg of linearized cDNA was used for *in vitro* transcription synthesis using the mMESSAGE mMACHINE™ T7 Transcription kit (Thermo Fisher Scientific, Life Technologies, USA).

All experiments were preformed using unfertilized *Xenopus* oocytes, extracted from anaesthetized female *Xenopus laevis*. Once extracted, oocytes were injected with 4.6 ng of mHCN1 using a Drummond Nanoject II injector (Drummond Scientific Company). Prior to injection oocytes were subject to a controlled temperature of 17 – 19 °C and placed in vials containing Barth antibiotic solution (mM): 90 NaCl, 3 KCl, 0.82 $MgSO_4.7H_2O$, 0.41 $CaCl_2.2H_2O$, 0.33 $Ca(NO_3)_2.4H_2O$ and 5 HEPES supplemented with 100 U/mL of penicillin-streptomycin and 10 mg/mL of kanamycin stock (10 mg/mL). Post injection cells were incubated in Barth antibiotic serum solution supplemented with ~5% horse serum. Cells were expressed and ready to be used in electrophysiological recordings 1 – 3 days post injection.

Electrophysiological recordings

Electrophysiological studies were conducted using the two-electrode voltage clamp (TEVC) technique. Borosilicate rapid fill microelectrode pipettes (FHC Inc., USA) were filled with filtered 1 M

KCL solution. Macroscopic currents were recorded from oocytes expressing full-length HCN1, HCN2, or HCN1-ΔCNBD in a bath solution containing (in mM): 89 KCl, 15 HEPES, 0.4 CaCl2, and 0.8 MgCl2, pH = 7.4 using OC-725C amplifier (Warner Instruments, USA) and digitized using a Digidata 1322A (Molecular Devices, Sunnyvale, CA, USA). Quetiapine (QTP) or norquetiapine (NQTP) (Toronto Research Chemicals, Toronto, ON, Canada) were dissolved in DMSO to make 100 mM stock solutions that were stored at −20°C. On the day of the experiments, the stock solutions were diluted in extracellular solution to the final desired concentrations. All data were acquired using the software Clampex 10.5 at a sampling rate of 5 KHz with a filter of 1 KHz. HCN1 activation was assessed by 1.7 s test-steps between −130 to −30 mV (ΔV = +10 mV) from a V_H = -30 mV, followed by a 1.5 s step to -130 mV. Deactivation was assessed by applying a 1.75 sec pre-pulse to −130 mV, followed by test pulses from +50 to −70 mV (ΔV = -10 mV). Since HCN2 channels activate slower and at more negative potentials, the protocol was altered to 2 s test-steps between −160 to −20 mV (ΔV = +10 mV) from a V_H = -30 mV, followed by a 1.5 s step to -160 mV. In all recordings, cells were held at the holding potential for an inter-pulse time of 27s to allow the channels to fully recover between sweeps. Control recordings (0 µM) were performed 2 mins after impaling the cells, to allow stabilization of currents, and then QTP or NQTP were added to the bath solution for at the defined concentration for 7.5 mins (or 15 mins in some cases as indicated) to enable pair-wise experiments. Experiments were also performed with equimolar quantities of DMSO used to solvate the drugs to their listed concentrations were used as additional controls. All recordings were conducted at room temperature (20 – 23 ºC).

Open-state inhibition was assessed using a constant pulse to -130 mV and adding 30 µM NQTP once HCN1 currents reached steady-state. Closed state inhibition was assessed using a repetitive pulse protocol with 2s pulses to −130 mV every 30 s from a holding potential (V_H) of -10 mV. Inhibition by 30 µM NQTP was compared when repetitive pulses were continuously applied during NQTP addition, or cells were held at V_H for 7.5 mins.

Data analysis and statistics

All recordings were analyzed offline using the Clampfit (Molecular Devices) software. Data was analyzed and plotted using Origin 8.0 software (Northampton, MA, USA). Current-voltage relationships were analyzed using built in software in pClamp, taking each respective voltage to an inquired current. The I-V relationship was fit with the Boltzman I-V equation:

$$I = \frac{(V_m - V_{rev}) g_{max}}{1 + e^{\frac{V_m - V_{\frac{1}{2}}}{k}}} \quad (1)$$

Activation and deactivation kinetics were determined with mono- or bi-exponential fits of test pulses after the initial lag period, as shown. Steady-state activation curves were fit with the Boltzmann equation:

$$G/G_{Max} = \frac{1}{1+ e^{\frac{V_m - V_{\frac{1}{2}}}{k}}} \quad (2)$$

where V_m corresponds to the test pulse, $V_{1/2}$ is the midpoint of activation and k is the slope factor. Concentration dependences of the drug-induced shift in $V_{1/2}$ ($\Delta V_{1/2}$) were fit with the Hill equation:

$$\Delta V_{\frac{1}{2}} \Big/ \Delta V_{\frac{1}{2}max} = \frac{1}{1+\left(\frac{IC_{50}}{[drug]}\right)^{nH}} \quad (3)$$

where nH is the Hill co-efficient. Data are presented as means (±) standard error of the total number of cells (*N*). Statistical significance for I-V curves were determined measured using two-way ANOVA with Tukey HSD post-hoc analysis. $V_{1/2}$'s of steady-state dependencies were determined for each recording and pooled for a given treatment then analyzed by pairwise student t-test. A P value < 0.05 was considered as statistically significant.

Results

Inhibition of HCN1 channel by norquetiapine but not quetiapine

Since 30 μM quetiapine (QTP) induces ~80% inhibition of hERG channels (Lee et al., 2018) and 50% inhibition of Nav1.5 channels (D. H. Kim et al., 2020), we examined the effects of 30 μM QTP on HCN1 channels expressed in *Xenopus laevis* oocytes using TEVC. At this concentration, in paired experiments, we observed no significant changes in the current-voltage relationship, steady-state voltage-dependence, nor gating kinetics of HCN1 (Fig. 1). No effect of QTP on HCN1 is observable, even if the incubation period is extended from 15 mins to 30 mins (Fig. 1).

Since NQTP also exhibits antidepressant activity (Jensen et al., 2008; Lopez-Munoz & Alamo, 2013) and a pharmacological activity that differs from QTP (Bakken et al., 2012; DeVane & Nemeroff, 2001), we also examined the effects of 30 μM NQTP on HCN1 channels. NQTP reduces HCN1 currents (Fig. 2A), with a -11.8 ± 0.5 mV hyperpolarizing shift in the voltage-dependence of activation (Fig. 2B) and slowing the kinetics of activation (Fig. 2C). Deactivation kinetics are unchanged with NQTP treatment (Fig. 2D). Examination of the concentration dependence of NQTP on the shift in voltage-dependence of activation ($\Delta V_{1/2}$) indicates an IC_{50} of 13.9 ± 0.8 μM with a maximum $\Delta V_{1/2}$ of -15.4 ± 1.2 mV and a Hill co-efficient of 4.2 ± 0.1 (Fig. 2E). Contrary to the effects of NQTP on HCN1 function, 7-OH QTP had no observable effects on HCN1 current, voltage-dependence, or gating kinetics (Fig. 3).

Inhibition of HCN1 and HCN2 channels by norquetiapine

To determine if NQTP inhibition is specific to HCN1 channels, we assessed if NQTP can also inhibit HCN2 channels. No significant changes in HCN2 function (I-V relationship, voltage-dependence of activation, or gating kinetics) are observed following incubation with NQTP at concentrations between 10 μM to 25 μM (Fig. 4). To ensure the lack of an effect was due to equilibration within the membrane, we extended the period of incubation from 7.5 mins to 15 mins, however, still no effect is observable. Thus, we conclude that NQTP inhibition is specific to HCN1 at the concentrations tested.

Mechanistic characterization of norquetiapine modulation of HCN1 channels

To assess if NQTP inhibits HCN function from the open state we applied a prolonged activation step to -130 mV to fully activate the channels, and applied 30 μM NQTP at steady-state (Fig. 5A). We observe a current ratio ($I_{NQTP}/I_{control}$) of 0.95 ± 0.01 following this protocol, which is similar to the decrease in the I-V relationship observed in our previous pair-wise experiments (Fig. 5A). Thus, NQTP has minimal effects on HCN channels in the open state.

We also examined if NQTP inhibition involves interactions in the closed state. We performed a repetitive pulse protocol in which channels were opened at -130 mV for 2 seconds then closed at +30

mV every 30 seconds. 30 µM NQTP was applied after the stabilization of HCN1 currents, and resulted in a decrease of current by 11.5 ± 0.7% (Fig. 5A). This is similar to the amount of inhibition that is induced is 15.5 ± 1.3% when the repetitive protocol is interrupted and cells are held at V_H = -10 mV for 7.5 mins during the application of NQTP (Fig. 5B). Under this condition, cells are predominately closed. Therefore, HCN1 channels can also be inhibited by NQTP from the closed state.

To determine if NQTP acts on HCN1 channels through interactions with the CNBD, we examined the effects on HCN1 channels lacking this domain (HCN1ΔCNBD). Similarly to full-length HCN1 channels, 30 µM NQTP induces an approximate 10% reduction in the I-V relationship of HCN1ΔCNBD compared to control (Fig. 6A). Additionally, 30 µM NQTP shifts the $V_{1/2}$ of activation to more hyperpolarized potentials by -11.8 ± 1.2 mV (Fig. 6B). Thus, inhibition of HCN1 channels by NQTP is not dependent on the CNBD.

If NQTP inhibits HCN channels by binding in the pore region, similarly to ivabradine, ZD7288, clonidine, lidocaine, and other inhibitors (Cheng, Kinard, Rajamani, & Sanguinetti, 2007; Tanguay, Callahan, & D'Avanzo, 2019), we would anticipate that NQTP inhibition would depend on the extracellular K^+ concentration ($[K^+]_o$). Specifically, we would anticipate that increasing $[K^+]_o$ would reduce the effect of NQTP on HCN1. Instead, we observe that increasing $[K^+]_o$ from 5 mM to 30 mM (by replacing the equivalent amount of extracellular Na^+) had no effect on NQTP inhibition of HCN1 currents. 30 µM NQTP continues to reduce the I-V relationship (Fig. 7A), and induces a hyperpolarizing shift in the $V_{1/2}$ of activation by -9.5 ± 0.4 mV (Fig. 7B) and slowed activation of HCN1 in 30 mM $[K^+]_o$. Thus, these data suggest that NQTP inhibition occurs via a different mechanism than many other known inhibitors of HCN channels which block the pore-domain and are sensitive to $[K^+]_o$.

DISCUSSION

Antipsychotics are a class of psychotropic medication primarily used to manage psychosis (including delusions, hallucinations, paranoia or disordered thought), principally in schizophrenia and bipolar disorder. All antipsychotics block dopamine D2 receptors, however, “atypical” antipsychotics (AAPs) bind less avidly to D2 receptors, leading to fewer extrapyramidal side effects at appropriate doses. AAPs also antagonize serotonin receptors, mainly 5HT2A. Consequently, AAPs are being used more often in recent years to treat anxiety, MDD, PTSD and other disorders. Quetiapine (QTP), a second-generation AAP commonly prescribed for the treatment of schizophrenia (Dev & Raniwalla, 2000; Small et al., 1997) and acute bipolar mania (Janicak & Rado, 2012), is now also used to treat insomnia (Lin et al., 2023), MDD (Ravindran et al., 2022), anxiety (Bandelow et al., 2010; Crapanzano et al., 2021; Ravindran et al., 2022), and PTSD (Crapanzano et al., 2023). Like other AAPs, QTP is structurally similar to clozapine and acts as an antagonist to serotonin, dopamine, histamine, and adrenergic receptors (Burns, 2001; Saller & Salama, 1993). QTP is primarily metabolized by hepatic cytochrome P450 3A4 (Dev & Raniwalla, 2000), with norquetiapine (NQTP) as its major active metabolite. NQTP exhibits pharmacological activity that differs from QTP (Bakken et al., 2012; DeVane & Nemeroff, 2001) and also exhibits antidepressant activity (Jensen et al., 2008; Lopez-Munoz & Alamo, 2013). In fact, NQTP shares structural similarities with several antidepressants including amoxapine and desipramine, and its physicochemical properties confer greater potential for its use as an antidepressant agent (D. W. Kim et al., 2016; Lopez-Munoz & Alamo, 2013). Indeed, the effect of QTP in major depressive disorder is probably mediated, at least in part, by NQTP, which selectively inhibits norepinephrine transporter reuptake (Bandelow et al., 2010; Lopez-Munoz & Alamo, 2013). Given the role of HCN channels in MDD, anxiety, and schizophrenia, we hypothesize that QTP and its major metabolites NQTP and 7-OH may work effectively in part via inhibition of HCN function.

Here we demonstrate that norquetiapine, but not quetiapine, inhibits HCN channels by shifting the voltage-dependence to hyperpolarized potentials and slowing channel opening. NQTP inhibited HCN1 channels with an IC_{50} of 13.9 ± 0.8 μM. This is similar to the ranges in which QTP and NQTP were found to inhibit other ion channels. Specifically, QTP and NQTP were found to block hERG current with a half-maximal inhibitory concentration of 8.3 and 10.8 μM, respectively (Lee et al., 2018). Nav1.5 currents were also shown to be inhibited by QTP and NQTP with IC_{50} of 30 and 6 μM respectively (Kim et al, 2020). It is speculated that the inhibition of cardiac sodium channels by these drugs can reduce the risk of cardiotoxicity induced by the inhibition of hERG current. By comparison, inhibition of HCN channels by other molecules including ivabradine, ZD7288, clonidine, lidocaine, ketamine, and carvedilol also act within a similar concentration range (BoSmith, Briggs, & Sturgess, 1993; Bucchi, Tognati, Milanesi, Baruscotti, & DiFrancesco, 2006; Cao et al., 2018; Cheng et al., 2007; Putrenko, Yip, Schwarz, & Accili, 2017; Xing et al., 2017). Therefore, inhibition of HCN channels by NQTP occurs within the physiological range, and likely contributes to its therapeutic role.

Inhibition of HCN1 channels has been suggested as a key therapeutic target for depression. $HCN1^{-/-}$ mice show impaired motor learning but enhanced spatial learning and memory (Nolan et al., 2004; Nolan et al., 2003) and enhanced resistance to depression (Huang et al., 2009; Lewis et al., 2011). Targeted knockdown of HCN1 by shRNA in the CA1 hippocampal region enhances mobility in the Porsolt swim test, a behavioural model for anti-depressant effects (C. S. Kim et al., 2012). Furthermore, HCN1 expression increases in the CA1 region of the dorsal hippocampus of chronic unpredictable stress rats, while stress responses are reduced upon HCN1 shRNA knockdown (C. S. Kim et al., 2018). Additionally, *Trip8b* knockout mice are also more resistant to depression (Lewis et al., 2011). Recently, the benzisoxazole derivative Org 34167, which has been patented for the treatment of depression and progressed to Phase I trials, was shown to be a broad-spectrum brain penetrant inhibitor of HCN channels, and resulted in reduced marble burying and increased the time spent mobile in the Porsolt swim and tail suspension tests in both male and female mice, suggesting reduced depressive-like behaviour (Pinares-Garcia et al., 2023). Thus, the inhibition of HCN1 channels by norquetiapine could provide a therapeutic benefit and contribute to the molecular mechanism of its anti-depressant action. Selectivity between QTP and NQTP may also provide a platform from which to develop even more specific negative gating modulators of HCN channels.

NQTP selectivity for HCN1 over HCN2 may also be an important factor in its therapeutic role. Intriguingly, the role of HCN2 in major depressive disorder is less straightforward than HCN1. $HCN2^{-/-}$ mice spend more time mobile in the tail suspension test (Lewis et al., 2011), suggesting a reduction in HCN2 has antidepressant effects. However, the expression of HCN2 is reduced in cholinergic interneurons in the nucleus accumbens of mice subjected to chronic stress, while HCN2 overexpression rescues the depressive phenotypes (Cheng et al., 2019). Similarly, HCN2 overexpression in dopamine neurons of the ventral tegmental area is effective in reversing the depressive phenotypes caused by chronic mild unpredictable stress in mice (Zhong et al., 2018). These data suggest that unlike what was observed for HCN1, stimulation of HCN2 is more favourable for the treatment of depression than HCN2 inhibition. Thus, further supporting a role for NQTP inhibition of HCN1 as part of the therapeutic mechanism of action of NQTP.

HCN channels are blocked by a number of inhibitors, with ivabradine arguably the best characterized. Ivabradine does not demonstrate isoform specificity between HCN1-4 channels (Bucchi et al., 2006; Stieber, Wieland, Stockl, Ludwig, & Hofmann, 2006). Ivabradine blocks the open state of HCN4 when the channels are opened by hyperpolarization, with enhanced binding upon frequent changes in the direction of ion flow (Bois, Bescond, Renaudon, & Lenfant, 1996; Bucchi, Baruscotti, & DiFrancesco, 2002; Bucchi et al., 2006) However, HCN1 channels can also be inhibited from the closed state (Bucchi et al., 2006). ZD7288 is also an open-state blocker of HCN channels (Benetos, Rudnichi, Thomas, Safar, & Guize, 1999; Cheng et al., 2007; Shin, Rothberg, & Yellen, 2001; Wu et

al., 2012) that induces at -15 mV shift in voltage-dependent I_h activation and reduces maximal activity by more than 50% (BoSmith et al., 1993). Lidocaine, bupivacaine and mepivacaine blockade of HCN channels also occurs from the inside of the cell (Putrenko et al., 2017). These inhibitors may bind in the open pore interacting with residues C358, A383, Y386, A387, V390 (HCN1 numbering) with residues C358, Y386, and A387 lining a hydrophobic groove within the pore cavity that may conformationally restrict the smaller ligands (Tanguay et al., 2019). Notably, these inhibitors are sensitive to extracellular potassium concentrations. Thus, it does not appear that NQTP inhibition of HCN1 follows the same pore-binding mechanism as these inhibitors.

On the other hand, other HCN inhibitors identified appear to act primarily as negative gating modulators of HCNs, rather than blockers of the ion conduction pathway. Niflumic acid may interact with the outer voltage-sensor domain (Cheng & Sanguinetti, 2009) While the binding site of carvedilol is not yet resolved, it is a closed-state inhibitor of HCN channels that induces a hyperpolarizing shift in the voltage-dependence of activation, but interacts with the channels at a site distinct from the pore-binding site of ivabradine or ZD7288 (Cao et al., 2018). There is also no evidence that ketamine, which inhibits HCN1 channels with a -20 mV shift in voltage-dependence and a reduction in activation kinetics at 25 μM, but not HCN2 and HCN4 at that concentration, could act as a pore inhibitor (Xing et al., 2017). Our data indicates that NQTP acts to inhibit HCN1 more like a negative gating modulator, rather than a pore blocker, since it modulates the voltage-dependence and gating kinetics of activation, is a closed-state blocker, and insensitive to external potassium concentration.

Our findings that NQTP is a selective inhibitor of HCN1 channels contributes to the understanding of the mode of action of quetiapine for the treatment of neuropsychiatric disorders such as anxiety, major mood disorder, and others. Our results may assist in the development of improved therapeutics based on this molecular scaffold.

FIGURES AND LEGENDS

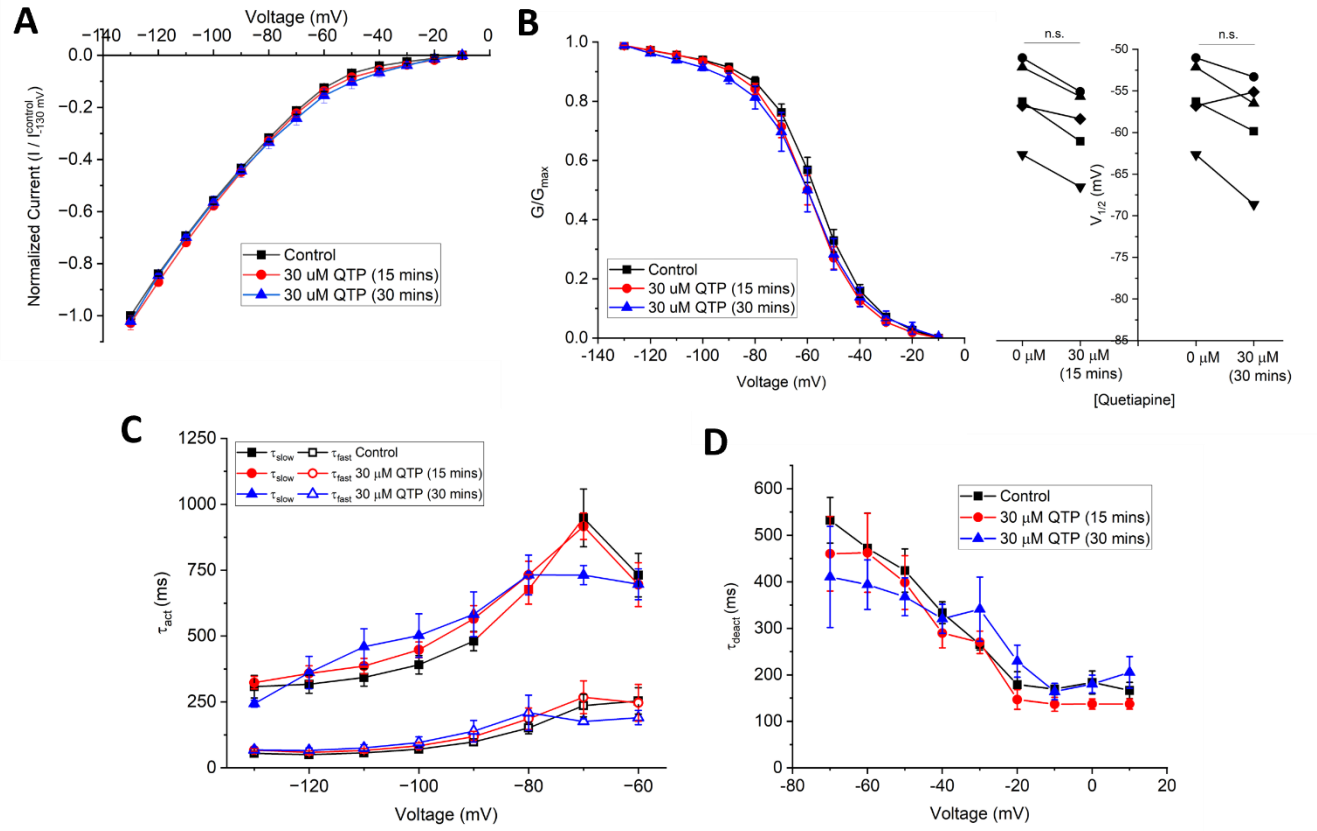

Figure 1. Quetiapine (QTP) does not regulate HCN1 channels. 30 µM QTP does not affect the current-voltage (I-V) relationship (n = 5, P = 0.35) **(A)**, voltage-dependence of activation ($V_{1/2}$ of each cell is shown on the right) (n = 5, P = 0.11) **(B)**, activation kinetics (n = 5, P = 0.56) **(C)**, or deactivation kinetics **(D)** (n = 5, P = 0.72) of HCN1 channels**.**

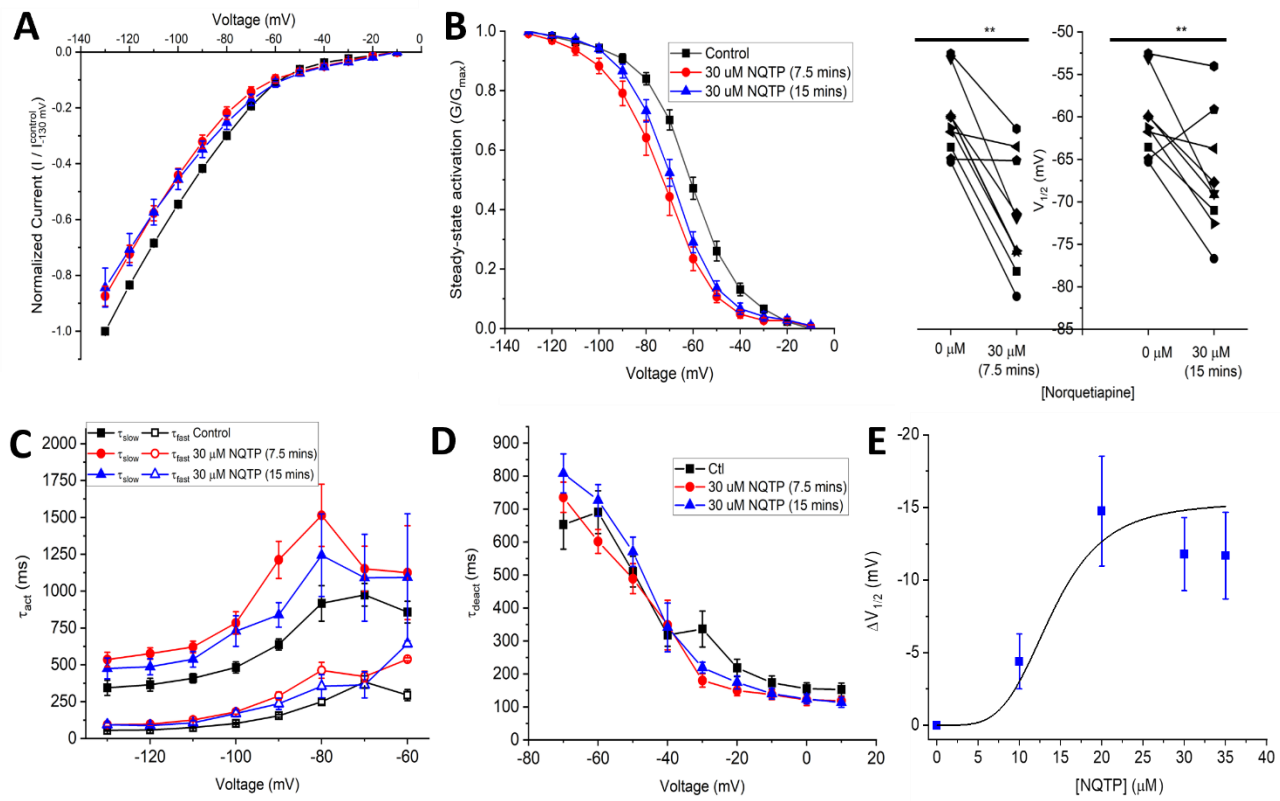

Figure 2. Norquetiapine (NQTP) inhibits HCN1 channels. (A) Representative traces from a paired experiment following the addition of 30 µM NQTP to oocytes expressing full-length HCN1. **(B)** Current-voltage (I-V) relationship in presence of NQTP normalized to maximal current ($I_{Control\ (-130\ mV)}$). (n = X; P < 0.05 for 7.5 and 30 mins compared to control). **(C)** NQTP induces a hyperpolarizing shift in the steady-state voltage-dependence of activation (P < 0.05 for $V_{1/2}$). **(D)** Activation time constants (τ_{fast} and τ_{slow}) are greater in the presence of NQTP (P < 0.05). **(E)** Deactivation time constant (τ) kinetics is unchanged in presence of NQTP. (n = 6; P = 0.23). **(F)** Concentration dependence of $\Delta V_{1/2}$ fit with a Hill equation (3) indicates NQTP inhibits HCN1 channels with an IC_{50} of 13.9 ± 0.8 µM, a maximum $\Delta V_{1/2}$ of -15.4 ± 1.2 mV and a Hill co-efficient of 4.2 ± 0.1.

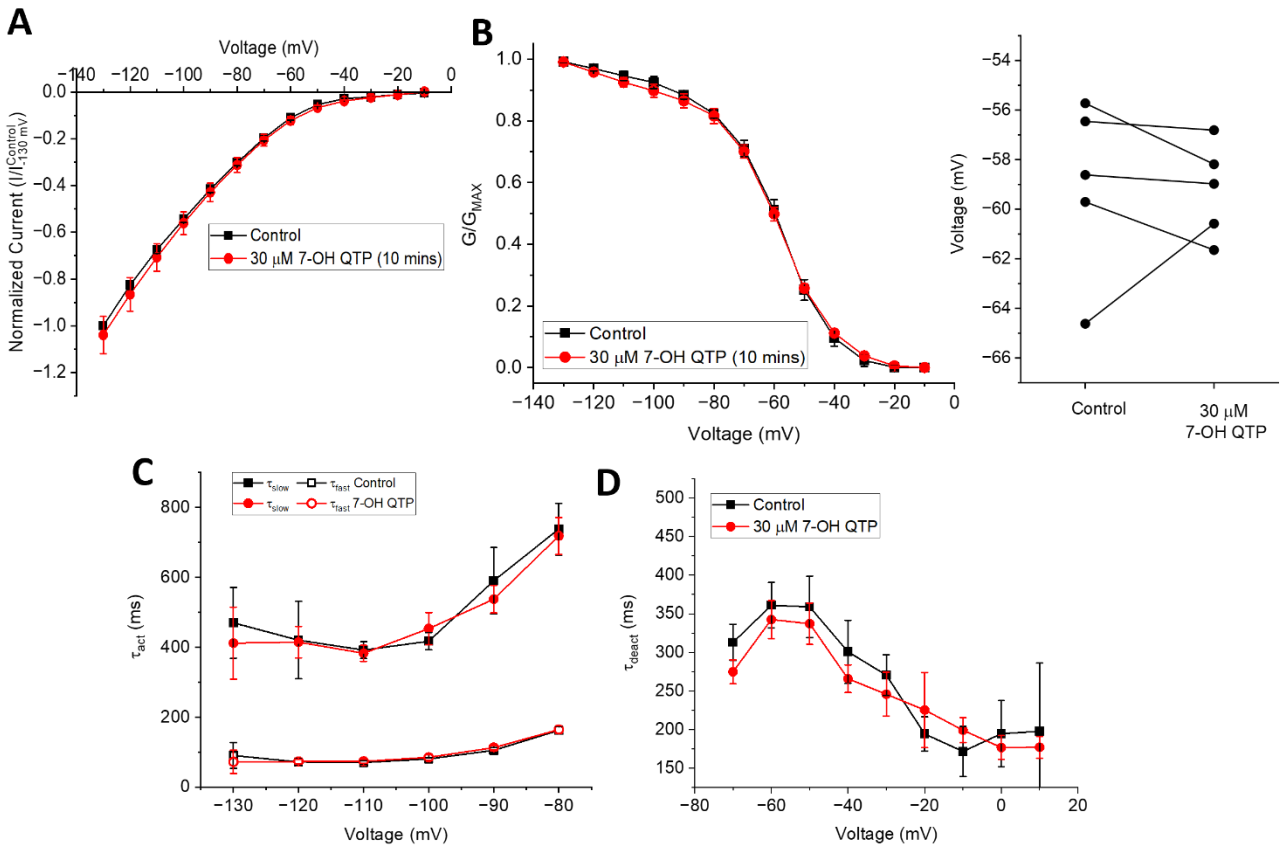

Figure 3. 7-hydroxyquetiapine (7-OH QTP) does not regulate HCN1 channels. 30 μM 7-OH QTP does not affect the current-voltage (I-V) relationship (n = 5, P = 0.47) **(A)**, voltage-dependence of activation ($V_{1/2}$ of each cell is shown on the right) (n = 5, P = 0.64) **(B)**, activation kinetics (n = 5, P = 0.88) **(C)**, or deactivation kinetics **(D)** (n = 6, P = 0.35) of HCN1 channels**.**

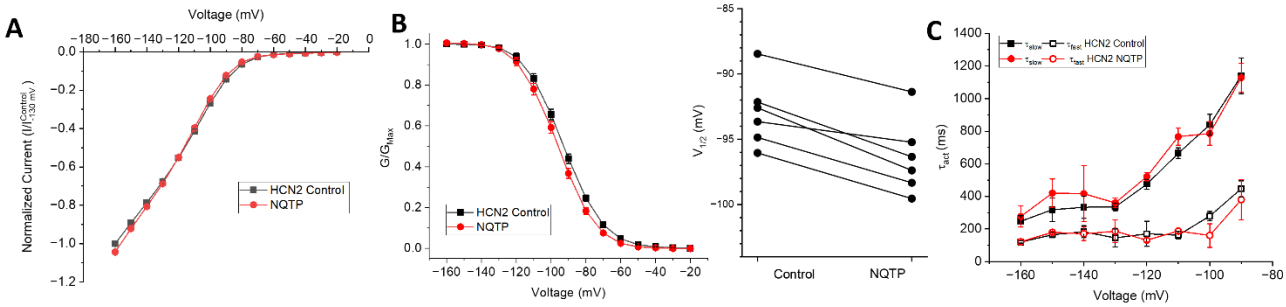

Figure 4. Norquetiapine (NQTP) does not inhibit HCN2 channels. Unlike what we observe for HCN1 channels, 30 μM NQTP does not alter the current-voltage (I-V) relationship (n=6, P=0.79) **(A)**, the voltage-dependence of activation ($V_{1/2}$ of each cell is shown on the right) (n = 6, P = 0.09) **(B)**, nor the activation kinetics (n = 6, P = 0.65) **(C)**. Therefore, NQTP appears to be a selective inhibitor for HCN1 currents.

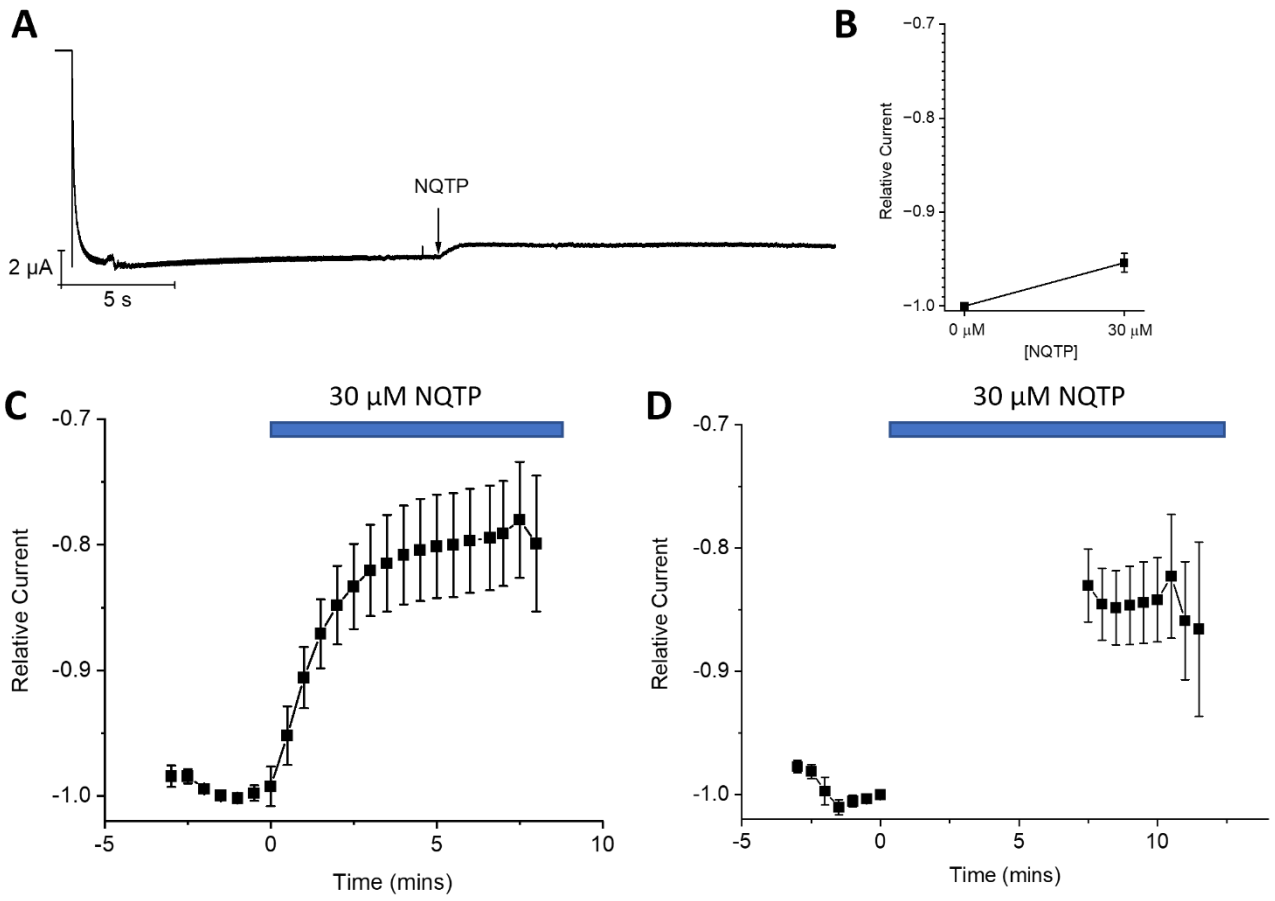

Figure 5. State-dependence of norquetiapine (NQTP) inhibition of HCN1 channels. (A) Open-state block was assessed by a prolonged activation step to -130 mV and applying 30 µM NQTP at steady-state. **(B)** Relative current ($I_{NQTP}/I_{control}$) to be 0.95 ± 0.01 following NQTP treatment (n = 5; P < 0.05). **(C)** Closed-state block was assessed using a repetitive -130 mV/+30mV pulse protocol every 30 seconds. 30 µM NQTP was applied after the stabilization of HCN1 currents, and resulted in a decrease of current by 11.5 ± 0.7 % (n = 7). **(D)** When the repetitive protocol is interrupted and cells are held at V_H = -10 mV for 7.5 mins during the application of NQTP the amount of inhibition that is induced is 15.5 ± 1.3 % (n = 9) indicating NQTP is a closed state blocker of HCN1.

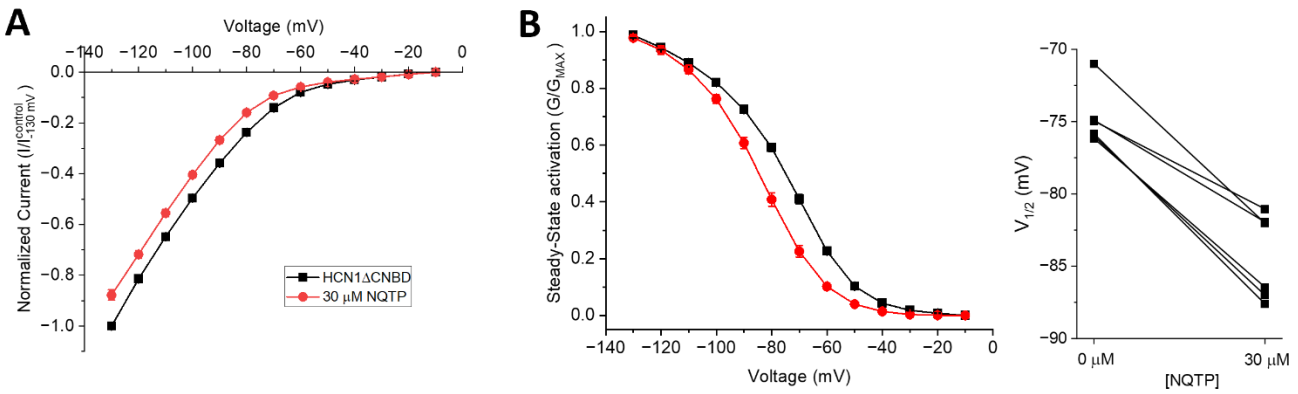

Figure 6. Norquetiapine (NQTP) inhibition of HCN1 channels does not depend on the CNBD. (A) Current-voltage (I-V) relationship in presence of NQTP normalized to maximal current ($I_{control\ (-130\ mV)}$). (n = 6; P < 0.05). **(B)** NQTP induces a hyperpolarizing shift in the steady-state voltage-dependence of activation (P < 0.05).

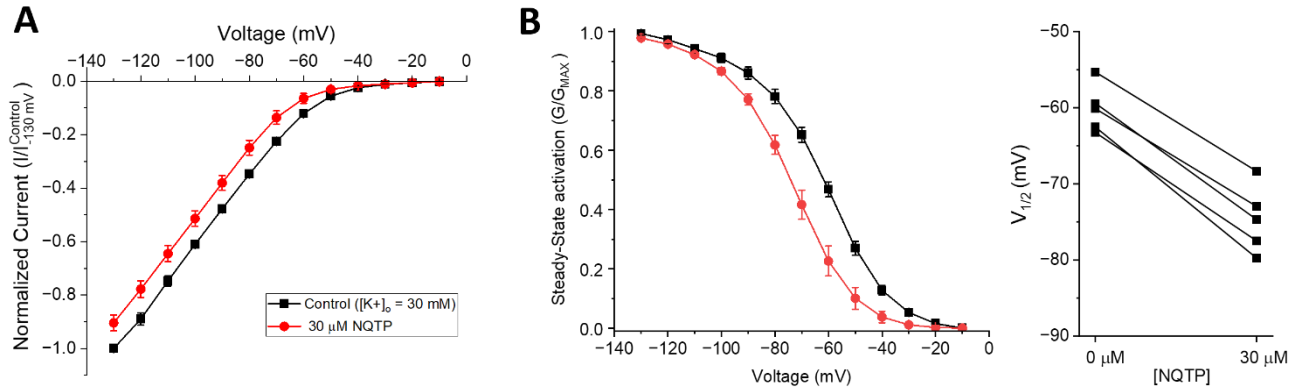

Figure 7. Norquetiapine (NQTP) inhibition of HCN1 channels is not affected by increasing $[K^+]_o$. (A) Current-voltage (I-V) relationship in presence of NQTP normalized to maximal current ($I_{control\ (-130\ mV)}$). (n = 6; P < 0.05). **(B)** NQTP induces a hyperpolarizing shift in the steady-state voltage-dependence of activation (P < 0.05 for $V_{1/2}$).

Table 1. Voltage-dependence of activation in the presence and absence of QTP or NQTP

Pair-wise Conditions	V ½ (mV)	k	n	P
HCN1 Control	-56.4 ± 4.4	10.3 ± 0.8	5	
HCN1 30µM QTP	-59.3 ± 2.1	10.3 ± 0.3	5	0.11
HCN1 Control	-60.2 ± 1.4	10.6 ± 1.2	10	
HCN1 30µM NQTP	-72.0 ± 2.1	10.9 ± 1.8	10	< 0.001
HCN1 Control	-59.0 ± 1.6	10.4 ± 0.5	5	
HCN1 7-OH QTP	-59.2 ± 0.9	11.1 ± 0.7	5	0.86
HCN2 Control	-93.0 ± 1.1	11.1 ± 0.5	5	
HCN2 30 µM NQTP	-96.3 ± 1.2	10.6 ± 0.7	5	0.09
HCN1 30 mM $[K^+]_o$ Control	-60.9 ± 3.4	13.3 ± 2.3	6	
HCN1 30 mM $[K^+]_o$ 30µM NQTP	-72.7 ± 6.2	11.7 ± 2.4	6	< 0.01
CN1ΔCNBD Control	-74.8 ± 2.0	13.7 ± 0.5	6	
HCN1ΔCNBD 30µM NQTP	-84.3 ± 3.0	12.2 ± 0.9	6	< 0.01

REFERENCES

Arnsten, A. F. (2011). Prefrontal cortical network connections: key site of vulnerability in stress and schizophrenia. *Int J Dev Neurosci, 29*(3), 215-223. doi:10.1016/j.ijdevneu.2011.02.006

Bakken, G. V., Molden, E., Knutsen, K., Lunder, N., & Hermann, M. (2012). Metabolism of the active metabolite of quetiapine, N-desalkylquetiapine in vitro. *Drug Metab Dispos, 40*(9), 1778-1784. doi:10.1124/dmd.112.045237

Bandelow, B., Chouinard, G., Bobes, J., Ahokas, A., Eggens, I., Liu, S., & Eriksson, H. (2010). Extended-release quetiapine fumarate (quetiapine XR): a once-daily monotherapy effective in generalized anxiety disorder. Data from a randomized, double-blind, placebo- and active-controlled study. *Int J Neuropsychopharmacol, 13*(3), 305-320. doi:10.1017/S1461145709990423

Benetos, A., Rudnichi, A., Thomas, F., Safar, M., & Guize, L. (1999). Influence of heart rate on mortality in a French population: role of age, gender, and blood pressure. *Hypertension, 33*(1), 44-52. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9931080

Bois, P., Bescond, J., Renaudon, B., & Lenfant, J. (1996). Mode of action of bradycardic agent, S 16257, on ionic currents of rabbit sinoatrial node cells. *Br J Pharmacol, 118*(4), 1051-1057. doi:10.1111/j.1476-5381.1996.tb15505.x

BoSmith, R. E., Briggs, I., & Sturgess, N. C. (1993). Inhibitory actions of ZENECA ZD7288 on whole-cell hyperpolarization activated inward current (If) in guinea-pig dissociated sinoatrial node cells. *Br J Pharmacol, 110*(1), 343-349. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7693281

Bucchi, A., Baruscotti, M., & DiFrancesco, D. (2002). Current-dependent block of rabbit sino-atrial node I(f) channels by ivabradine. *J Gen Physiol, 120*(1), 1-13. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12084770

Bucchi, A., Tognati, A., Milanesi, R., Baruscotti, M., & DiFrancesco, D. (2006). Properties of ivabradine-induced block of HCN1 and HCN4 pacemaker channels. *J Physiol, 572*(Pt 2), 335-346. doi:10.1113/jphysiol.2005.100776

Burns, M. J. (2001). The pharmacology and toxicology of atypical antipsychotic agents. *J Toxicol Clin Toxicol, 39*(1), 1-14. doi:10.1081/clt-100102873

Cao, Y., Chen, S., Liang, Y., Wu, T., Pang, J., Liu, S., & Zhou, P. (2018). Inhibition of hyperpolarization-activated cyclic nucleotide-gated channels by beta-blocker carvedilol. *Br J Pharmacol, 175*(20), 3963-3975. doi:10.1111/bph.14469

Chaplan, S. R., Guo, H. Q., Lee, D. H., Luo, L., Liu, C., Kuei, C., … Dubin, A. E. (2003). Neuronal hyperpolarization-activated pacemaker channels drive neuropathic pain. *J Neurosci, 23*(4), 1169-1178. doi:10.1523/JNEUROSCI.23-04-01169.2003

Cheng, L., Kinard, K., Rajamani, R., & Sanguinetti, M. C. (2007). Molecular mapping of the binding site for a blocker of hyperpolarization-activated, cyclic nucleotide-modulated pacemaker channels. *J Pharmacol Exp Ther, 322*(3), 931-939. doi:10.1124/jpet.107.121467

Cheng, L., & Sanguinetti, M. C. (2009). Niflumic acid alters gating of HCN2 pacemaker channels by interaction with the outer region of S4 voltage sensing domains. *Mol Pharmacol, 75*(5), 1210-1221. doi:10.1124/mol.108.054437

Crapanzano, C., Damiani, S., Casolaro, I., & Amendola, C. (2023). Quetiapine Treatment for Post-traumatic Stress Disorder: A Systematic Review of the Literature. *Clin Psychopharmacol Neurosci, 21*(1), 49-56. doi:10.9758/cpn.2023.21.1.49

Crapanzano, C., Damiani, S., & Guiot, C. (2021). Quetiapine in the Anxiety Dimension of Mood Disorders: A Systematic Review of the Literature to Support Clinical Practice. *J Clin Psychopharmacol, 41*(4), 436-449. doi:10.1097/JCP.0000000000001420

Dev, V., & Raniwalla, J. (2000). Quetiapine: a review of its safety in the management of schizophrenia. *Drug Saf, 23*(4), 295-307. doi:10.2165/00002018-200023040-00003

DeVane, C. L., & Nemeroff, C. B. (2001). Clinical pharmacokinetics of quetiapine: an atypical antipsychotic. *Clin Pharmacokinet, 40*(7), 509-522. doi:10.2165/00003088-200140070-00003

Gamo, N. J., Lur, G., Higley, M. J., Wang, M., Paspalas, C. D., Vijayraghavan, S., … Arnsten, A. F. (2015). Stress Impairs Prefrontal Cortical Function via D1 Dopamine Receptor Interactions With Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels. *Biol Psychiatry, 78*(12), 860-870. doi:10.1016/j.biopsych.2015.01.009

Gauthier, J., Champagne, N., Lafreniere, R. G., Xiong, L., Spiegelman, D., Brustein, E., … Team, S. D. (2010). De novo mutations in the gene encoding the synaptic scaffolding protein SHANK3 in patients ascertained for schizophrenia. *Proc Natl Acad Sci U S A, 107*(17), 7863-7868. doi:10.1073/pnas.0906232107

Guilmatre, A., Huguet, G., Delorme, R., & Bourgeron, T. (2014). The emerging role of SHANK genes in neuropsychiatric disorders. *Dev Neurobiol, 74*(2), 113-122. doi:10.1002/dneu.22128

Huang, Z., Walker, M. C., & Shah, M. M. (2009). Loss of dendritic HCN1 subunits enhances cortical excitability and epileptogenesis. *J Neurosci, 29*(35), 10979-10988. doi:10.1523/JNEUROSCI.1531-09.2009

Janicak, P. G., & Rado, J. T. (2012). Quetiapine for the treatment of acute bipolar mania, mixed episodes and maintenance therapy. *Expert Opin Pharmacother, 13*(11), 1645-1652. doi:10.1517/14656566.2012.681377

Jensen, N. H., Rodriguiz, R. M., Caron, M. G., Wetsel, W. C., Rothman, R. B., & Roth, B. L. (2008). N-desalkylquetiapine, a potent norepinephrine reuptake inhibitor and partial 5-HT1A agonist, as a putative mediator of quetiapine's antidepressant activity. *Neuropsychopharmacology, 33*(10), 2303-2312. doi:10.1038/sj.npp.1301646

Kim, C. S., Brager, D. H., & Johnston, D. (2018). Perisomatic changes in h-channels regulate depressive behaviors following chronic unpredictable stress. *Mol Psychiatry, 23*(4), 892-903. doi:10.1038/mp.2017.28

Kim, C. S., Chang, P. Y., & Johnston, D. (2012). Enhancement of dorsal hippocampal activity by knockdown of HCN1 channels leads to anxiolytic- and antidepressant-like behaviors. *Neuron, 75*(3), 503-516. doi:10.1016/j.neuron.2012.05.027

Kim, D. H., Park, K. S., Park, S. H., Hahn, S. J., & Choi, J. S. (2020). Norquetiapine blocks the human cardiac sodium channel Na(v)1.5 in a state-dependent manner. *Eur J Pharmacol, 885*, 173532. doi:10.1016/j.ejphar.2020.173532

Kim, D. W., Weon, K. Y., Hong, E. P., Chung, E. K., & Lee, K. T. (2016). Comparative Physicochemical and Pharmacokinetic Properties of Quetiapine and Its Active Metabolite Norquetiapine. *Chem Pharm Bull (Tokyo), 64*(11), 1546-1554. doi:10.1248/cpb.c16-00223

Knop, G. C., Seeliger, M. W., Thiel, F., Mataruga, A., Kaupp, U. B., Friedburg, C., … Muller, F. (2008). Light responses in the mouse retina are prolonged upon targeted deletion of the HCN1 channel gene. *Eur J Neurosci, 28*(11), 2221-2230. doi:10.1111/j.1460-9568.2008.06512.x

Kongsamut, S., Kang, J., Chen, X. L., Roehr, J., & Rampe, D. (2002). A comparison of the receptor binding and HERG channel affinities for a series of antipsychotic drugs. *Eur J Pharmacol, 450*(1), 37-41. doi:10.1016/s0014-2999(02)02074-5

Lee, H. J., Choi, J. S., Choi, B. H., & Hahn, S. J. (2018). Effects of norquetiapine, the active metabolite of quetiapine, on cloned hERG potassium channels. *Neurosci Lett, 664*, 66-73. doi:10.1016/j.neulet.2017.11.029

Lewis, A. S., Vaidya, S. P., Blaiss, C. A., Liu, Z., Stoub, T. R., Brager, D. H., … Chetkovich, D. M. (2011). Deletion of the hyperpolarization-activated cyclic nucleotide-gated channel auxiliary subunit TRIP8b impairs hippocampal Ih localization and function and promotes antidepressant behavior in mice. *J Neurosci, 31*(20), 7424-7440. doi:10.1523/JNEUROSCI.0936-11.2011

Lin, C. Y., Chiang, C. H., Tseng, M. M., Tam, K. W., & Loh, E. W. (2023). Effects of quetiapine on sleep: A systematic review and meta-analysis of clinical trials. *Eur Neuropsychopharmacol, 67*, 22-36. doi:10.1016/j.euroneuro.2022.11.008

Lopez-Munoz, F., & Alamo, C. (2013). Active metabolites as antidepressant drugs: the role of norquetiapine in the mechanism of action of quetiapine in the treatment of mood disorders. *Front Psychiatry, 4*, 102. doi:10.3389/fpsyt.2013.00102

Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F., & Biel, M. (1998). A family of hyperpolarization-activated mammalian cation channels. *Nature, 393*(6685), 587-591. doi:10.1038/31255

Moosmang, S., Stieber, J., Zong, X., Biel, M., Hofmann, F., & Ludwig, A. (2001). Cellular expression and functional characterization of four hyperpolarization-activated pacemaker channels in cardiac and neuronal tissues. *Eur J Biochem, 268*(6), 1646-1652. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11248683

Nolan, M. F., Malleret, G., Dudman, J. T., Buhl, D. L., Santoro, B., Gibbs, E., … Morozov, A. (2004). A behavioral role for dendritic integration: HCN1 channels constrain spatial memory and plasticity at inputs to distal dendrites of CA1 pyramidal neurons. *Cell, 119*(5), 719-732. doi:10.1016/j.cell.2004.11.020

Nolan, M. F., Malleret, G., Lee, K. H., Gibbs, E., Dudman, J. T., Santoro, B., … Morozov, A. (2003). The hyperpolarization-activated HCN1 channel is important for motor learning and neuronal integration by cerebellar Purkinje cells. *Cell, 115*(5), 551-564. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14651847

Pape, H. C. (1996). Queer current and pacemaker: the hyperpolarization-activated cation current in neurons. *Annu Rev Physiol, 58*, 299-327. doi:10.1146/annurev.ph.58.030196.001503

Paspalas, C. D., Wang, M., & Arnsten, A. F. (2013). Constellation of HCN channels and cAMP regulating proteins in dendritic spines of the primate prefrontal cortex: potential substrate for working memory deficits in schizophrenia. *Cereb Cortex, 23*(7), 1643-1654. doi:10.1093/cercor/bhs152

Pinares-Garcia, P., Spyrou, J., McKenzie, C. E., Forster, I. C., Soh, M. S., Mohamed Syazwan, E., … Reid, C. A. (2023). Antidepressant-like activity of a brain penetrant HCN channel inhibitor in mice. *Front Pharmacol, 14*, 1159527. doi:10.3389/fphar.2023.1159527

Putrenko, I., Yip, R., Schwarz, S. K. W., & Accili, E. A. (2017). Cation and voltage dependence of lidocaine inhibition of the hyperpolarization-activated cyclic nucleotide-gated HCN1 channel. *Sci Rep, 7*(1), 1281. doi:10.1038/s41598-017-01253-x

Ravindran, N., McKay, M., Paric, A., Johnson, S., Chandrasena, R., Abraham, G., & Ravindran, A. V. (2022). Randomized, Placebo-Controlled Effectiveness Study of Quetiapine XR in Comorbid Depressive and Anxiety Disorders. *J Clin Psychiatry, 83*(3)doi:10.4088/JCP.21m14096

Saller, C. F., & Salama, A. I. (1993). Seroquel: biochemical profile of a potential atypical antipsychotic. *Psychopharmacology (Berl), 112*(2-3), 285-292. doi:10.1007/BF02244923

Santoro, B., Grant, S. G., Bartsch, D., & Kandel, E. R. (1997). Interactive cloning with the SH3 domain of N-src identifies a new brain specific ion channel protein, with homology to eag and cyclic nucleotide-gated channels. *Proc Natl Acad Sci U S A, 94*(26), 14815-14820. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9405696

Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A., & Tibbs, G. R. (1998). Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. *Cell, 93*(5), 717-729. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9630217

Shin, K. S., Rothberg, B. S., & Yellen, G. (2001). Blocker state dependence and trapping in hyperpolarization-activated cation channels: evidence for an intracellular activation gate. *J Gen Physiol, 117*(2), 91-101. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11158163

Small, J. G., Hirsch, S. R., Arvanitis, L. A., Miller, B. G., & Link, C. G. (1997). Quetiapine in patients with schizophrenia. A high- and low-dose double-blind comparison with placebo. Seroquel Study Group. *Arch Gen Psychiatry, 54*(6), 549-557. doi:10.1001/archpsyc.1997.01830180067009

Stevens, D. R., Seifert, R., Bufe, B., Muller, F., Kremmer, E., Gauss, R., … Lindemann, B. (2001). Hyperpolarization-activated channels HCN1 and HCN4 mediate responses to sour stimuli. *Nature, 413*(6856), 631-635. doi:10.1038/35098087

Stieber, J., Wieland, K., Stockl, G., Ludwig, A., & Hofmann, F. (2006). Bradycardic and proarrhythmic properties of sinus node inhibitors. *Mol Pharmacol, 69*(4), 1328-1337. doi:10.1124/mol.105.020701

Szabo, Z., Bacskai, T., Deak, A., Matesz, K., Veress, G., & Sziklai, I. (2011). Dendrodendritic connections between the cochlear efferent neurons in guinea pig. *Neurosci Lett, 504*(3), 195-198. doi:10.1016/j.neulet.2011.09.012

Tanguay, J., Callahan, K. M., & D'Avanzo, N. (2019). Characterization of drug binding within the HCN1 channel pore. *Sci Rep, 9*(1), 465. doi:10.1038/s41598-018-37116-2

Wu, S., Gao, W., Xie, C., Xu, X., Vorvis, C., Marni, F., … Zhou, L. (2012). Inner activation gate in S6 contributes to the state-dependent binding of cAMP in full-length HCN2 channel. *J Gen Physiol, 140*(1), 29-39. doi:10.1085/jgp.201110749

Xing, J., Zhang, C., Jiang, W., Hao, J., Liu, Z., Luo, A., … Ma, J. (2017). The Inhibitory Effects of Ketamine on Human Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels and Action Potential in Rabbit Sinoatrial Node. *Pharmacology, 99*(5-6), 226-235. doi:10.1159/000452975

Yi, F., Danko, T., Botelho, S. C., Patzke, C., Pak, C., Wernig, M., & Sudhof, T. C. (2016). Autism-associated SHANK3 haploinsufficiency causes Ih channelopathy in human neurons. *Science, 352*(6286), aaf2669. doi:10.1126/science.aaf2669