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Title Page

A novel peptide restricts ethanol modulation of the BK channel *in vitro* and *in vivo*

Luisa L. Scott, Sangeetha Iyer, Ashley E. Philpo, Melva N. Avalos, Natalie S. Wu, Ted Shi, Brooke A. Prakash, Thanh-Tu Nguyen, S. John Mihic, Richard W. Aldrich, Jonathan T. Pierce

Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, Austin, TX, 78712 (LLS, SI, AEP, MNA, NSW, TS, BAP, T-TN, SJM, RWA, JTP)

Department of Neuroscience, The University of Texas at Austin, Austin, TX 78712 (SJM, RWA, JTP)

Center for Learning and Memory, The University of Texas at Austin, Austin, TX 78712 (RWA, JTP)

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Running Title Page

Running title: Peptide restricts BK channel modulation by ethanol

Corresponding author:
Jonathan T. Pierce, Ph.D.
2506 Speedway, C7350
Department of Neuroscience
The University of Texas at Austin
Austin, TX 78712
Tel. (512) 232-4137
jonps@austin.utexas.edu

Co-corresponding author:
Luisa L. Scott, Ph.D.
2506 Speedway, C7350
Department of Neuroscience
The University of Texas at Austin
Austin, TX 78712
Tel. (512) 507-0970
luisa_scott@utexas.edu

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List of abbreviations

BK channel = large-conductance, calcium- and voltage-activated potassium channel
hSLO= human BK channel
SLO-1 = worm BK channel
CNS = central nervous system
hGlyRα1 = human glycine receptor α1
rSK2 = rat small conductance calcium-activated channel 2
NGM = nematode growth media
wild-type = WT

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Abstract

Alcohol is a widely used and abused substance. A major unresolved issue in the alcohol research field is determining which of the many alcohol target proteins identified to date is responsible for shaping each specific alcohol-related behavior. The large-conductance, calcium- and voltage-activated potassium channel, or BK channel, is a conserved target of ethanol. Genetic manipulation of the highly conserved BKα channel influences alcohol-related behaviors across phylogenetically diverse species that include worm, fly, mouse, and man. A pharmacological tool that prevents alcohol's action at a single target, like the BK channel, would complement genetic approaches in the quest to define the behavioral consequences of alcohol at each target. To identify agents that specifically modulate the action of ethanol at the BK channel, we executed a high-throughput phagemid-display screen in combination with a *C. elegans* behavioral genetics assay. This screen selected a novel nonapeptide, LS10, that moderated acute ethanol intoxication in a BK channel-humanized *C. elegans* strain without altering basal behavior. LS10's action *in vivo* was dependent upon BK channel functional activity. Single-channel electrophysiological recordings *in vitro* showed that pre-incubation with a sub-micromolar concentration of LS10 restricted ethanol-induced changes in human BKα channel gating. In contrast, no substantial changes in basal human BKα channel function were observed after LS10 application. The LS10 peptide provides a proof of concept that a combined phagemid-display/behavioral genetics screening approach can provide novel tools for understanding the action of alcohol at the BK channel and how this, in turn, exerts influence over CNS function.

Introduction

The large-conductance, calcium- and voltage-activated potassium (BK) channel is widely expressed in excitable cells, where it regulates muscle tone and neuronal signaling (Hoshi et al., 2013). The BK channel is a well-conserved target of ethanol in species as diverse as worm, fly, mouse and man (Mulholland et al., 2009; Treistman and Martin, 2009; Bettinger and Davies, 2014). Pharmacologically-relevant concentrations (10-100 mM) of ethanol are sufficient to alter BK channel gating across this phylogenetic spectrum (Chu and Treistman, 1997; Jakab et al., 1997; Dopico et al., 1998; Brodie and Appel, 2000; Walters et al., 2000; Dopico, 2003). The constitutive pore-forming α subunit is the minimal unit required for ethanol modulation of the BK channel (Brodie et al., 2007). The channel's probability of opening changes within minutes after ethanol exposure. However, whether the P_o goes up or down, either transiently or more persistently, depends upon regulatory subunit expression, post-translational modifications, and the channel's microenvironment (Dopico et al., 2016).

Expressed in multiple neuronal compartments, the BK channel is well-positioned to broadly mediate the effects of ethanol on central nervous system (CNS) function (Wang et al., 2001b; Dopico et al., 2014a; Alqadah et al., 2016). In *Caenorhabditis elegans* and *Drosophila*, genetic manipulation of the highly conserved BKα channel influences alcohol-related behaviors; null mutations reduce acute ethanol intoxication and tolerance (Davies et al., 2003; Ghezzi and Atkinson, 2011), and overexpression reduces ethanol withdrawal severity (Scott et al., 2017b). Similarly, genetic manipulations that alter BK channel function in rodents influence ethanol tolerance and consumption (Martin et al., 2008; Kreifeldt et al., 2013), and a gain-of-function mutation increases acute responses to alcohol in humans (Du et al., 2005).

Genetic approaches to test the *in vivo* importance of this molecular target of ethanol have proved informative. Behavioral genetic and electrophysiological screening of BKα channel mutants have yielded several mutations that restrict ethanol modulation of BKα channel gating

(Bukiya et al., 2014; Davis et al., 2014). We recently identified a BK channel mutant that exhibited limited ethanol modulation *in vitro*, and prevented BK channel-mediated acute ethanol intoxication *in vivo*. Restricted ethanol responses occurred without gross changes to baseline gating or other BK channel-dependent behaviors (Davis et al., 2014). Nevertheless, genetic approaches may be limited by changes in nervous system function caused directly by life-long alteration of a molecule and/or indirectly via mechanisms compensating for the molecular alteration. BK channel deletion in mice results in obvious CNS and neuromuscular abnormalities (Meredith et al., 2004; Rüttiger et al., 2004), which can obscure the interpretation of alcohol-related behavioral effects. Even without widespread defects in basal physiology, one cannot disentangle the direct effect of manipulating the genetic target from the influence of potential compensatory changes. For these reasons, pharmacological approaches complement genetic approaches to address the *in vivo* importance of putative ethanol targets. Pharmacological agents can be administered during a circumscribed period, and are particularly powerful if they selectively occlude the functional impact of ethanol on the target molecule. Agents with this pharmacological action are lacking for most ethanol targets, including the BK channel.

To expand our toolset for understanding the action of ethanol at the BK channel, we developed a screen to search for pharmacological agents that restricted the BK channel's physiological response to ethanol with little impact on basal channel function. Phage display for ion channels, pioneered by Tipps et al. (2010), allows a high-throughput screen for target binding. Though not as high-throughput, phenotypic screens can select agents with functional activity at a specific molecular target (Kwok et al., 2006) or a specific action (e.gs., neuroprotection (Mondal et al., 2018) or antagonizing fetal alcohol syndrome (Wilkemeyer et al., 2003)). Previously, we combined phage display and a secondary phenotypic screen in worm to identify a high-affinity modulator of BK channel function (Scott et al., 2017a). In the present study, our challenge was to select a modulator of the effects of another pharmacological agent at a specific target. Our high-

throughput peptide-identification screen and a worm assay for rapid functional screening identified novel peptides that antagonized ethanol effects at the BK channel. One novel 9-amino acid peptide, LS10, acted specifically at the BK channel to reduce acute ethanol intoxication in wild-type and BK channel-humanized *C. elegans* strains. LS10 also restricted ethanol-induced changes in human BKα channel gating *in vitro* with limited impact on basal function. These findings provide proof of concept that a combined phagemid-display/behavioral genetics screening approach provides novel tools like LS10 to further our understanding of how alcohol acts at the BK channel to exert influence over CNS function.

Methods

Peptide selection and synthesis

A monovalent phagemid display library (library C, Mobitec) was panned against three sets of HEK293 cells expressing one of three ion channels. Two sets were for negative selection. For one, cells were transfected (Lipofectamine 2000, Invitrogen) with the human glycine receptor α1 (hGlyRα1, X52009) and used 48 hours later. Another line stably expressed the rat small conductance calcium-activated channel 2 (rSK2, U69882.1). For positive selection, cells stably expressed the human BKα channel ZERO isoform (NM_002238). Phagemid particles obtained by superinfection were purified and diluted to a titer of 10^{11} – 10^{12} per mL using standard procedures (Röttgen and Collins, 1995). 1 mL of this solution was serially incubated with each set of HEK293 cells for 45 minutes. For the negative selection plates, the supernatant was collected and transferred to the next plate. After incubation with the positive selection plate, the cells were rinsed 3X and the phage were eluted at a pH of 2.2. The progress of the phagemid selection process was monitored after each of 5 panning rounds by titering and sequencing the phagemid DNA. The selected peptide sequences were then synthesized as trifluoroacetic acid salts at 98–99% purity verified by HPLC and MS analysis (Genscript, Piscataway, NJ). Stocks were dissolved

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in water at 10 mM and aliquots were lyophilized and stored at −80 °C. The concentration of these aliquots was verified by disulfide bond UV absorption at 280 nM (Beckman spec). As a secondary determination of identity and purity of LS10, in-house LC/MS was performed on a single quadrupole Mass Spectrophotometer (Agilent 6130) interfaced with a HPLC with a diode-array (UV-vis) detector (Agilent 1200).

C. elegans strains

Worms were maintained at 20° C on standard 6-cm diameter Petri dishes filled with 12-mL Nematode Growth Media (NGM)-agar and seeded with OP50 bacteria as described previously (Brenner, 1974). Worms cultured on plates contaminated with fungi or other bacteria were excluded from this study. The reference wild-type (WT) strain was N2 Bristol. The *slo-1* null strain was NM1968, harboring the previously characterized null allele, *js379* (Wang et al., 2001a). The transgenic human BKα strain (*hSLO(+)*) was JPS340, which expressed cDNA of the human BKα channel ZERO isoform under the endogenous *pslo-1* promoter on a *slo-1(js379)* background (Davis et al., 2014).

C. elegans behavioral assays

Ethanol plates (400 mM) were prepared at the start of the experiment by adding 280 µL of 200-proof ethanol (Sigma Aldrich) beneath the agar of a standard unseeded plate. The plates were sealed with Parafilm. Age-matched day one adults were cleaned of bacteria by crawling on an unseeded plate. The worms were then incubated in a puddle of vehicle (NGM) or 750 µM peptide on an unseeded plate. The puddle was refreshed 1-2 times as needed, but let to fully absorb into the agar by 30 minutes. Pharmaco-behavioral analyses in *C. elegans* requires high doses because of the difficulty in getting pharmacological agents across the worm cuticle; further, the brief duration (30 minutes) and mode of treatment (swimming in liquid) allows for minimal ingestion (Bull et al., 2007). Crawl behavior was then videoed (Flea2 camera, Point Grey

Research, Canada; StreamPix 3, NorPix, Canada). Copper rings were used to restrict movement to a proscribed area for the crawl videos. The worms were moved to ethanol plates, and, after 20 minutes, crawl behavior was recorded again. The worms were tracked offline using semi-automated custom macros (Image-Pro, MediaCybernetics, Rockville, MD) for 1 minute to obtain crawl speed (cm/min) by an impartial observer blind to genotype and peptide treatment. Crawl speeds were normalized to the mean speed of vehicle-treated yoked controls run concurrently with peptide-treated worms in each condition (baseline or ethanol-exposed). This controlled for drifts in behavior and/or conditions over the several year course of data collection. However, similar results were found with non-normalized data (Figure 2 vs. Supplemental Table 1). Normalized group means±SEM for the peptide-treated and NGM-control groups run in tandem were compared with Student's *t*-tests. Selection criteria for each step of the screen is described in the Results.

HEK293 cell maintenance and transfection

HEK293 cells (ATCC, Manassas, VA) were grown according to standard procedures. Cells were cultured at 37° C in a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium with l-glutamine, sodium pyruvate and 10% fetal bovine serum (Invitrogen). Cell lines were split with trypsin/EDTA in Hanks' balanced salt solution (Invitrogen) up to 25-30 cycles. For electrophysiological recordings, cells were transfected (Polyfect, Qiagen) with the human BKα (hSLO) ZERO or STREX isoform. Enhanced green fluorescent protein was co-transfected as a marker. Electrophysiological recordings were made 16–72 h after transfection.

Xenopus oocyte expression

SLO-1 was expressed heterologously in oocytes because the channel did not express at detectable levels in HEK293 cells. To obtain *Xenopus laevis* oocytes, frogs (Nasco, Fort Atkinson, WI) were anesthetized with tricaine, and portions of their ovaries were removed surgically in

accordance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations. A plasmid containing the coding sequence for the *slo-1a* variant, mg180 (Addgene plasmid #34660), was a gift from Miriam Goodman (Johnson et al., 2011a). The *slo-1a* variant was chosen because it is the most abundantly expressed in worm (Glauser et al., 2011), and expression of this variant is sufficient to rescue acute behavioral intoxication to ethanol in *slo-1* null worms (Davis et al., 2014). This plasmid was linearized with *Xba*I and transcribed *in vitro* (mMessage mMachine kit, Life Technologies). Oocytes were injected with 5 ng of cRNA and then stored individually at room temperature in modified Barth's solution.

Patch-clamp recordings

Voltage-clamp recordings were performed at room temperature (22–24 °C) using an inside-out configuration. For all recordings the extracellular solution contained the following (in mM): 2 KCl, 136 KOH, 20 Hepes, 2 $MgCl_2$, adjusted to pH 7.2 with $MeSO_3H$. In order to apply peptide to the extracellular surface, patch electrodes (7–20 MΩ in resistance) were tip filled with normal extracellular solution and backfilled with extracellular solution containing 500 nM LS10. Enough normal extracellular solution was included to provide about 10 minutes of peptide-free recording (Scott et al., 2017b). The intracellular solution contained the following (in mM): 6 KCl, 132 KOH, 20 Hepes, adjusted to pH 7.2 with $MeSO_3H$. For hSLO recordings without ethanol, ~750 nM free Ca^{2+} was achieved with 4.17 mM $CaCl_2$ and 5 mM EGTA, a ratio verified by measurement with a Ca^{2+}-sensitive electrode. For hSLO recordings with ethanol, 2 mM $MgCl_2$, 3.85 mM $CaCl_2$ and 5 mM EGTA were added for an estimated 638 nM free Ca^{2+} (MaxChelator). For all SLO-1 recordings, the internal solution contained ~5 μM free Ca^{2+} achieved with 2.644 mM $CaCl_2$ and 5 mM HEDTA, a ratio verified by measurement with a Ca^{2+}-sensitive electrode. For recordings with ethanol treatment, 50 mM ethanol was added to the bath for intracellular application.

Voltage-clamp recordings were made with an Axopatch 200A amplifier (Molecular Devices, 10 kHz filtering) at 50 kHz sampling using custom macros in IgorPro (Wavemetrics) or

Patchmaster (HEKA). The measurement of P_o for each experiment is detailed in Figure 5 and Supplemental Figures 2-3. Group comparisons for means±SEM were made using planned paired or unpaired Student's *t*-tests and two-way repeated measures ANOVA with Holm-Sidak post-hoc correction as stated in the figure legends.

Results

Phagemid display screen for BK channel-binding peptides selects unique sequence motifs

To screen for small peptides that modulate the BK channel's physiological response to ethanol, we first performed a high-throughput phagemid-display screen designed to enrich BK channel-binding peptides. The phagemid library was comprised of approximately $3x10^7$ peptide sequences generated on a cyclic, 9-amino acid scaffold with 6 randomized positions. For positive selection, the phagemid library was panned against HEK293 cells expressing the ZERO isoform of the human BKα channel (hSLO). The ZERO isoform was chosen because it is widely expressed in the mammalian nervous system and is sensitive to ethanol (Pietrzykowski et al., 2008b; Dopico et al., 2016). For negative selection, the library was screened against another ethanol target protein, the glycine receptor (hGlyRα1), as well as another calcium-sensitive potassium channel (rSK2) (Figure 1A). Positively charged residues were enriched in identified peptide sequences, particularly arginine, as were the small amino acids glycine and alanine (Figure 1B). The sequences were further analyzed to determine whether the panning procedure enriched certain sequence motifs. A sequence motif was defined as two or more amino acids in a fixed position, or three or more amino acids in a sliding position. Among the twenty peptides derived from our screen (Table 1), thirteen sequence motifs were enriched 150- to ~60,000-fold over their expected frequencies in the original library (Figure 1C). A previous study from our laboratory characterized the ethanol-independent effects of the single peptide LS3 (26). Here we present a detailed analysis of all twenty peptides LS1-20 in the presence and absence of ethanol.

C. elegans behavioral screen selects peptides with BK channel- and ethanol-dependent activity in vivo

We synthesized the twenty peptides and screened them for functional activity at the BK channel using a *C. elegans* phenotypic assay. *C. elegans* expresses a highly conserved BK channel ortholog, SLO-1 (Sonnhammer and Durbin, 1997; Lai et al., 2000). SLO-1 is one of the many gene products that modulate the *C. elegans* neuromuscular circuitry to control crawling locomotion (Wang et al., 2001b). Moreover, SLO-1 plays a key role in mediating the locomotor effects of acute ethanol intoxication in worms (Davies et al., 2003). To probe for peptide-induced changes in locomotion, age-matched, day 1 adult wild-type (WT) worms were pre-incubated with either peptide or vehicle for 30 minutes. Crawl speed was then measured before and after a 20-minute exposure to ethanol. This acute ethanol exposure leads to an internal concentration of ~40 mM ethanol (Scott et al., 2017b), a pharmacologically-relevant concentration for ethanol intoxication (Dopico et al., 2014a). Intoxication was observed as a ~45% reduction in crawl speed for vehicle-treated WT worms (crawl speed before and after ethanol exposure, respectively: 0.92 $\pm$ 0.011 and 0.51 $\pm$ 0.011 cm/min; [$t(1953)=26.05$, $p < 0.001$]). To ascertain whether peptide treatment altered crawl speed at baseline or in response to acute ethanol exposure, the crawl speeds of peptide-treated worms were compared to the speeds of vehicle-treated yoked controls tested concurrently. Half of the peptides caused a significant (set at $p < 0.001$) change in the crawl speed of WT worms at baseline (Figure 2A) and/or after ethanol exposure (Figure 2B). Five more peptides showed a trend towards altering the crawl speed of WT worms in either condition (set at $p < 0.05$). Finally, five peptides, LS15-18 and LS20, showed no effects on WT crawl speed in either condition, and were eliminated from our screen (Figure 2C and 2D, hatched boxes).

Next, we used a strain lacking the BK channel (*slo-1* null) to determine whether the ethanol- and ethanol-independent locomotor effects of the 15 peptides remaining in the screen were

mediated by changes in BK channel function. As for WT worms, *slo-1* null worms were pre-incubated with either peptide or vehicle for 30 minutes and then crawl speed was measured before and after a 20-minute exposure to ethanol. Consistent with previous findings (Davies et al., 2003), vehicle-treated worms carrying the *slo-1* null allele, *js379*, were largely resistant to the intoxicating effects of ethanol, showing only a ~10% reduction in crawl speed (0.52 ± 0.011 and 0.45 ± 0.0090 cm/min before and after ethanol exposure, respectively; [$t(1546)=4.46$, $p < 0.001$]). The crawl speeds of peptide-treated worms were compared to the speeds of vehicle-treated yoked controls tested concurrently. Of the 15 peptides that showed strongly significant effects on WT crawl speed, only five peptides showed no effect (set at $p > 0.05$) on *slo-1* null mutant crawl speed either before or after ethanol exposure (Figure 2C,D). These five peptides, LS3, LS10, LS11, LS13 and LS19 (Figure 2, turquoise bars; Supplemental Table 1), were designated as having BK channel-dependent physiological activity; i.e., they likely altered *C. elegans* locomotor behavior by specific modulation of worm BK channel function. The other ten peptides that modified crawl speed in *slo-1* null mutant worms may act on additional non-specific targets and were thus eliminated from further analysis (Figure 2C,D, grey bars).

We hypothesized that the five selected peptides would also alter the physiological activity of the human BK channel due to i) conservation of the channel and ii) the use of the human channel during positive selection in our phagemid display screen. To test this hypothesis we used a BK channel-humanized worm strain (*hSLO(+)*) expressing the same human BK channel isoform as for phagemid display (ZERO) in the *slo-1* null mutant background. Similar to WT worms, acute ethanol intoxication caused a ~40% reduction in crawl speed for vehicle-treated humanized worms (crawl speed before and after ethanol exposure: 0.60 ± 0.019 and 0.38 ± 0.012 cm/min, respectively; [$t(1066)=8.30$, $p < 0.001$]). Thus, as we previously reported (Davis et al., 2014), the human BK channel can functionally substitute for the endogenous SLO-1 channel for ethanol intoxication.

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Four out of five of the peptides that affected worm BK channel function in WT worms also caused a significant change in crawl speed before and/or after ethanol exposure for the humanized *hSLO(+)* strain (Figure 3A,B, turquoise bars; Supplemental Table 1). Although they do not share any potential amino acid sequence motifs (Figure 3C, Table 1), two peptides, LS3 and LS10, restricted the sedative effects of acute ethanol exposure (crawl speed before and after ethanol exposure: for LS3-treated, 0.34 ± 0.021 and 0.45 ± 0.027 cm/min, [$t(173)=3.2$, $p < 0.01$]; for LS10-treated, 0.62 ± 0.040 and 0.54 ± 0.053 cm/min, [$t(174)=1.1$, *n.s.*]). LS3 slowed baseline crawl speed for both WT and humanized worms (Scott et al., 2017a). Thus, we focused on investigating LS10 because the peptide limited the physiological effects of ethanol without significantly altering basal BK channel function in the humanized strain.

Peptide LS10 restricts ethanol-induced changes in gating for heterologous BKα channels

To observe the action of peptide LS10 on worm and human BK channel gating *in vitro*, we made electrophysiological recordings of BKα channels expressed heterologously. The ZERO and STREX isoforms of the human BKα channel (hSLO-ZERO and hSLO-STREX, respectively) were expressed in HEK293 cells, while the worm BK channel (SLO-1) was expressed in oocytes. Both were recorded in inside-out patches at ~750 nM free internal calcium for hSLO and ~5 μM for SLO-1. Higher internal calcium concentrations were used for SLO-1 because invertebrate channels show less activation at low calcium concentrations (Johnson et al., 2011b). LS10 was applied via diffusion to the extracellular side of the patch. Neither hSLO isoform showed a net change in P_o across patches with application of 500-nM LS10 (Figure 4B, Supplemental Figure 2A,B). By comparison, we found that the P_o of SLO-1 showed a consistent decrease by over half in the presence of 500-nM LS10 (Figure 4C, Supplemental Figure 2C). Although measured outside of the physiological voltage range, the differential effects we observed of LS10 on the P_o of SLO-1 and hSLO parallels the presence and absence of baseline behavioral effects in WT and humanized worms, respectively (Figure 2A and Figure 3A).

Because peptide LS10 suppressed the BK channel-mediated effects of acute intoxication *in vivo*, we predicted that LS10 would also alter the effects of ethanol on BK channel gating *in vitro*. To test this idea, we recorded hSLO-ZERO in inside-out patches with a combination of high intracellular [Mg^{2+}] and low intracellular [Ca^{2+}]. In control experiments, hSLO-ZERO displayed a highly reliable decrease in P_o several minutes after the intracellular application of 50-mM ethanol (Figure 5A-C, Supplemental Figure 3B). Similar to our findings in the absence of high intracellular [Mg^{2+}], pre-incubation of the patch (~15-minutes) with 500-nM LS10 did not consistently alter the baseline P_o at 20-60 mV (Supplemental Figure 3A). In contrast, pre-incubation with LS10 abolished the ethanol-induced decrease in P_o (Figure 5A-C, Supplemental Figure 3B). In a small set of inside-out patches, we also tested the effect of LS10 on worm BK channels. SLO-1 expressed in oocytes was recorded under the same conditions as used above. Intracellular application of 50-mM ethanol caused a transient increase in SLO-1 P_o, which was limited by pre-incubation with 500-nM LS10 (Figure 5D-F). Together, these findings demonstrate that peptide LS10 restricts changes in BKα channel gating in response to acute ethanol exposure.

Discussion

From worms to humans, the BK channel appears to be a conserved target of alcohol. Tools for understanding the behavioral consequence of alcohol's action at a single molecular target, like the BK channel, would ideally modulate the target's response to ethanol without altering baseline function. In the present study we developed a screening paradigm to search for pharmacological agents that restrict the BK channel's response to ethanol with limited impact on basal channel function. Using this screening paradigm, we report the identification and characterization of a small peptide, LS10, that restricts the effects of ethanol at the BKα channel *in vitro* and *in vivo*. Electrophysiological recordings showed that LS10 suppressed ethanol modulation of human BKα channel gating in the sub-micromolar concentration range, with limited effects on basal channel

function. In both wild-type and BK channel-humanized *C. elegans*, LS10 reduced acute ethanol intoxication via a BK channel-dependent mechanism. Notably, LS10 did not substantially affect baseline behavior of the humanized strain. Together, our findings indicate that our screening paradigm will be useful for developing tools like LS10 to study BK channel modulation by ethanol and how this contributes to alcohol-related behaviors.

To select a peptide that limited ethanol modulation without substantial effects on basal BKα channel function, we harnessed the power of a high-throughput phagemid-display assay to screen millions of peptide sequences against the human BKα channel. We then secondarily screened for BK channel- and ethanol-dependent physiological activity, capitalizing on the importance of the worm BK channel for both acute ethanol intoxication and baseline locomotion (Davies et al., 2003). This screen succeeded in selecting a peptide that alters ethanol modification of BKα channel gating. We found that LS10 restricted acute intoxication for WT and humanized worms and limited ethanol-dependent changes in gating for both human and worm BKα channels *in vitro*. Moreover, eliminating peptides that caused locomotor slowing in ethanol naïve worms successfully eliminated peptides that substantially altered baseline BKα channel function *in vitro*. Gain- and loss-of-function BK channel mutations both slow baseline locomotion in worm (Davies et al., 2003), and several peptides screened here caused BK channel-dependent reductions in baseline locomotion for WT and BK channel humanized worms. We previously reported that one of these, LS3, altered the basal probability of opening of the human BKα channel *in vitro* (Scott et al., 2017a). Here we report that LS10 changed baseline behavior for WT but not humanized worms, corresponding with a reduction in basal probability of opening for the worm but not the human BKα channel. Together these findings support the ability of BK channel-dependent behavioral effects in worm to predict the effect of peptide treatment on BK channel gating *in vitro*. Moreover, the differential effects of LS10 on worm and human BK channel gating are not unexpected. Without splice inserts, the percent identity for these channels is over 60% from S1

through the calcium bowl, with even greater identity in key functional regions like the transmembrane and pore domains, RCK1, RCK2 and the calcium bowl. Nonetheless, there are striking differences in gating kinetics and activation by intracellular calcium for worm and human BK channels (e.g. see Figure 3) that are likely to contribute to different responses to a modulator, even if it binds to an identical or highly similar pocket.

This screening technique could provide powerful tools, like LS10, for understanding ethanol's action at the BK channel. The putative ethanol-binding pocket identified for the mammalian BK channel (Bukiya et al., 2014), and another well-conserved ethanol-sensitive residue found in both the worm and human BK channel (Davis et al., 2014) reside on the channel's long intracellular cytoplasmic domain. LS10, applied extracellularly, may restrict ethanol modulation of BK channel gating via long-range allosteric interactions with the intracellular ethanol-binding site. Binding of ethanol to the BK channel potentiates or inhibits channel gating depending upon channel and cellular conditions, including ionic concentrations (Dopico et al., 2014b). The recording conditions used herein for the human BKα channel obtained a very consistent, stable response to ethanol, which was especially important given the greater fluctuation in the probability of opening for LS10- and ethanol-treated patches (Supplemental Figure 3B). Together with the worm BK channel recordings, our data suggest that LS10 may inhibit ethanol binding or signal transduction of the event. As such, fully probing the biophysical mechanism through which LS10 influences ethanol modulation could provide support for the location of the ethanol binding pocket or elucidate the signal transduction pathway from ethanol binding to channel gating.

Peptides hold a prominent position in ion channel pharmacology. For the BK channel, peptide toxins, predominantly derived from scorpion venom, block the pore with high affinity and specificity but have relatively complex structures that impede large-scale synthesis and efficient blood-brain barrier permeability (Yu et al., 2016). Small, endogenous peptides or peptide

fragments modulate BK channel function, but mainly act with lower affinity (White et al., 1991; Wallner et al., 1999; Xia et al., 2003; Zhang et al., 2014). Recently we have introduced this new class of BK channel-modulating peptides, small 9 amino acid peptides not known to be found in nature (Scott et al., 2017a). The shared scaffold of these peptides, chosen for scalable synthesis and resistance to proteolytic degradation (Gudmundsson et al., 1999; Andersson et al., 2000), allows them to be developed for large-scale applications. We previously reported that one such peptide, LS3, acts with nanomolar efficacy and high specificity at the BK channel, modulating sound-evoked neural activity in the auditory midbrain (Scott et al., 2017a). LS10 shares a similar structure, with an N- to C-terminal disulfide bridge and the presence of arginine, as well as sub-micromolar efficacy at the BKα channel *in vitro*. Thus, like LS3, LS10 may exhibit CNS bioavailability *in vivo* without further modifications (Scott et al., 2017a).

Once in the brain, peptides like LS10 could be used to explore how ethanol modulation of BK channel function mediates alcohol-related phenotypes, such as acute intoxication. Knock-out and knock-in mutations, most of which rely on alterations to basal protein function, have made substantial contributions to our understanding of the functional impact of ethanol's action at individual proteins (Blednov et al., 2012; Borghese et al., 2012; Liu et al., 2013; Howard et al., 2014). A pharmacological tool has the advantage of manipulating the impact of acute ethanol administration at a target protein while eliminating the influence of even moderate compensatory mechanisms provoked by genetic mutations. LS10 limits the acute effects of ethanol on the ZERO isoform of the human BK channel function *in vivo* and *in vitro*. The ZERO isoform of the BK channel is nearly identical in rodents and humans and is the most commonly expressed isoform in brain (Chen et al., 2005). Moreover, the specificity of action at the BK channel in worm probed by our selection techniques may carry over to mammals since *C. elegans* homologues have been identified for many human genes (Consortium, 1998). While restricting acute ethanol modulation of the BK channel may not fully eliminate all aspects of acute ethanol intoxication due to the multi-

target nature of ethanol, we predict that treatment with an LS10-like peptide will reduce at least some of the consequences of ethanol intake, particularly locomotor sedation. Interestingly, a structurally unrelated 9 a.a. peptide restricts ethanol-induced developmental toxicity in mouse (Wilkemeyer et al., 2003). Together, these findings suggest that peptides like LS10, and our recently identified BK channel mutation that restricts ethanol modulation (Davis et al., 2014), may play complementary roles in supporting our understanding how BK channel modulation by ethanol contributes to alcohol-related behaviors.

The screening paradigm used to select LS10 may be able to further expand the pharmacological toolbox for dissecting the influence of BK channel subsets in acute intoxication. Modulation of BK channel function by acute ethanol application is influenced by BKα splice variation, auxiliary subunit expression and phosphorylation state (Dopico et al., 2014a). For example, STREX isoforms show little functional modulation by ethanol while ZERO and ALCOREX-containing isoforms show increasing levels of modulation, respectively (Pietrzykowski et al., 2008a). Future uses of this platform could strive to select peptides that differentially restrict ethanol action dependent upon channel composition or post-translational modifications. Phage display alone has had moderate success in selecting peptides that show composition-dependent modulation of glycine receptor function (Cornelison et al., 2016). Further, worms used in our secondary behavioral screen, could be humanized to express different BKα isoforms or phosphomutants. Combined BKα and auxiliary subunit expression may even be possible; *C. elegans* do not express mammalian-like auxiliary subunits that would require null backgrounds, though it has not yet been shown whether BK channel auxiliary subunits traffic to the plasma membrane in worm. LS10 serves as a proof of concept that this screening platform could provide novel tools to further our understanding of how alcohol acts at the BK channel to exert influence over CNS function.

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Author contributions

Participated in research design: LLS, SJM, RWA and JTP

Conducted experiments: LLS, SI, AEP, MNA, NSW, TS, BAP, T-TN

Performed data analysis: LLS, SI, AEP, MNA, NSW, TS, BAP, T-TN

Wrote or contributed to the writing of the manuscript: LLS, SJM, RWA and JTP

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Footnotes

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The authors declare that they have no conflicts of interest with the contents of this article.

Legends for Figures

Figure 1. **Peptide motifs enriched by phagemid-display screen targeting the BK channel**. ***A***, Phagemid displaying approximately 30 million unique peptide sequences were screened for their putative abilities to bind to the human BKα channel (hSLO). Phagemid were serially incubated with HEK293 cells expressing rat SK2 and human GlyRα1, respectively, to remove non-specific phagemid prior to incubation with HEK293 cells expressing hSLO. Phagemid were sequenced after round 3 through 5 of panning to reveal 26 remaining peptide sequences. ***B***, Sequence logo showing the relative likelihood of amino acid expression for all twenty candidate peptides selected by the phagemid display screen (see Table 1). Only the residues that were randomized in the phagemid display library (a.a. 2-7) are shown. The larger the letter denoting the amino acid, the more prevalent that amino acid was in the identified peptides. Charged amino acids, particularly arginine (R), were highly enriched at all positions followed by small amino acids like glycine (G) and alanine (A). ***C***, Two or more amino acids in a fixed position or three or more amino acids in a sliding position (*) were considered motifs. Motifs are shaded according to fold enrichment over theoretical frequency in the original phagemid display library.

Figure 2**. Subset of peptides selectively altered behavior of *C. elegans* in BK channel-dependent manner**. Worms were pre-incubated with peptide (LS1-LS20) or vehicle, and then crawl speed was measured both before (no ethanol, left panels) and after (ethanol, right panels) acute exposure to ethanol. Crawl speeds were normalized to the mean speed of vehicle-treated yoked controls. Means ± SEM are shown as bars for the peptide-treated groups and as lines (maroon) for the vehicle-treated groups. ***A,*** For WT worms, a subset of peptides decreased baseline crawl speed (normalized crawl speed < 1). ***B,*** For ethanol-exposed WT worms, a subset of peptides either enhanced (normalized crawl speed < 1) or reduced (normalized crawl speed > 1) ethanol-induced locomotor sedation. In all, ten peptides significantly (set at p<0.001)

altered WT crawl speed in the presence or absence of ethanol and were considered physiologically active. Five more were potentially physiologically active, showing a trend towards altered crawl speeds (p<0.05). Five peptides showed no effect on WT with or without ethanol and were eliminated from the screen. ***C,D,*** Of the remaining fifteen peptides, only five showed no effect (significance set at p<0.05) on baseline or ethanol-exposed crawl speeds for a strain lacking a functional BK channel (*slo-1* null). These peptides with BK channel-dependent physiological activity are indicated by turquoise bars. Student's *t*-tests compared the peptide-treated vs. vehicle-treated worms run in tandem, ***p<0.001, **p<0.01, *p<0.05.

Figure 3. **Peptides altered behavior of BK channel-humanized worms.** ***A***,***B***, Day 1 adult worms expressing the human BKα channel (*hSLO(+)*) in a *slo-1*-null background were pre-incubated with peptide or vehicle. Crawl speed was then assessed before (***A***) and after acute exposure to ethanol (***B***). Four peptides showed significant (p<0.05) effects (turquoise). Crawl speeds were normalized to the vehicle-treated yoked controls where 1.0 signifies basal speed (***A***) and intoxicated speed (***B***), respectively. Means ± SEM are shown as bars for the peptide-treated groups and as lines (maroon) for the vehicle-treated groups. Student's *t*-tests compared peptide-treated vs. vehicle-treated worms run in tandem, ***p<0.001, *p<0.05, N > 60. ***C***, Schematic representing the enriched amino acid motifs in the four selected peptides. Each motif is represented by a unique color. Dual coloring indicates the residue is shared between motifs. LS19 and LS11 peptides share a motif.

Figure 4. **Peptide LS10 substantially reduces the probability of opening of the worm but not the human BK channel**. ***A***, Structure of LS10. ***B***, Representative single-channel recording (1 sec) at 100 mV before and after LS10 (500 nM) was applied by diffusion to the extracellular face of ZERO (upper traces) or STREX (lower traces) isoforms of the human BKα channel

(hSLO). ***C***, There was no significant change in P_o in response to LS10 application for the ZERO (open bars) or STREX (shaded bars) isoforms. P_o was measured using ≥ five 3-second traces recorded over a duration of ~5 min. Post-peptide P_o plotted relative to baseline (pre). Pre vs. post, paired Student's *t*-tests, n.s.; *N* = 6-10. ***D***, Representative single channel recordings (1 sec) at 100 mV before and after LS10 (500 nM) was applied by diffusion to the extracellular face of the worm BK channel (SLO-1). ***E***, The P_o decreased in response to LS10 for SLO-1. Pre vs. post, paired Student's *t*-tests, ***$p<0.005$, *N* = 8.

Figure 5. **Peptide LS10 limits changes in BK channel gating by acute ethanol exposure**. ***A***, Representative single-channel recordings (3 sec) of hSLO-ZERO expressed in HEK293 cells at 40 mV before (upper traces) and after (lower traces) bath application of 50-mM ethanol. LS10 (500 nM) was applied by diffusion to the extracellular face. ***B***, Control (open) recordings at 40 mV showed a decrease in P_o relative to baseline after the application of ethanol (time = 0) that was blocked by pre-incubation with 500-nM LS10 (shaded). P_o measured each minute using three 3-second traces and plotted relative to baseline as mean ± SEM. Control vs. peptide, two-way repeated measures ANOVA, post-hoc $p<0.05$, *N* = 7-10. ***C***, The P_o was significantly lower relative to baseline ~10 min after ethanol application for control recordings (open bars) than in the presence of LS10 (shaded bars). P_o was measured using ten 3-second traces recorded over a duration of several minutes. P_o after ethanol plotted relative to baseline. Ethanol-induced change in P_o for control vs. peptide-treated, Student's *t*-tests, ***$p<0.005$, *N* = 6-10. ***D***, Representative single-channel recordings (3 sec) of SLO-1 expressed in oocytes at 100 mV. 50-mM ethanol potentiated the probability of opening in control recordings, which was blocked in the presence of 500-nM LS10. ***E***, P_o measured each minute using two 3-second traces plotted relative to baseline as mean ± SEM. ***F***, Largest potentiation in P_o after ethanol application plotted relative to baseline for control and LS10-preincubated patches. Peak P_o measured over

a 1.5-minute interval (~three 3-sec traces) was compared to baseline (≥five 3-sec traces) via paired Student's *t*-tests, **p*<0.05, *N* = 4-5.

Tables

name	sequence	enriched motifs
LS1	CARGVYRVC	XRXXYR, XRGXXX
LS2	CRVAHRAVC	RXXXXA
LS3	CRRGLVQVC	XRGXXX, RRXXXX
LS4	CPPGRGAVC	XXGRXA
LS5	CGMTKRPVC	GMXXXX, XXXKRX
LS6	CDEMNWWVC	DXMXXX
LS7	CERRMYRVC	XRXXYR
LS8	CRRAYEMVC	RRXXXX
LS9	CRRKRHAVC	RRXXXX, RXXXXA
LS10	CAVGRLAVC	XXGRXA, XXGXLX
LS11	CLQEQRGVC	EQR, XQXXXG
LS12	CRKQGRRVC	GRR
LS13	CEGRRARVC	GRR
LS14	CLDGKLDVC	XXGXLX
LS15	CGGGGSRVC	GGGG
LS16	CFTGGGGVC	GGGG
LS17	CVWVKRNVC	XXXKRX
LS18	CGMASSFVC	GMXXXX
LS19	CDTMEQRVC	EQR, DXMXXX
LS20	CGQQSPGVC	XQXXXG

Table 1. Peptide sequences selected by phagemid display

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Figures

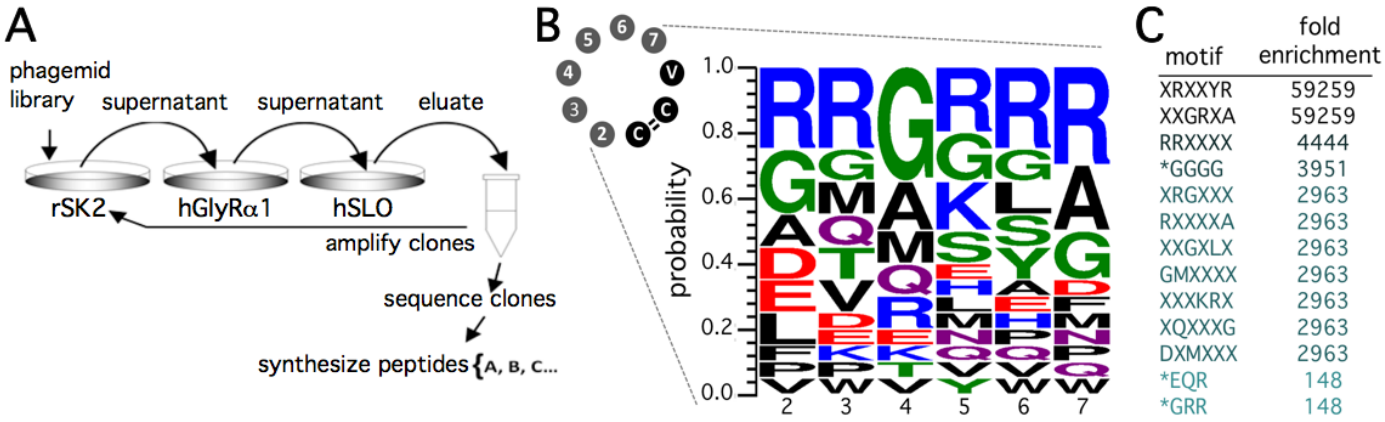

Figure 1

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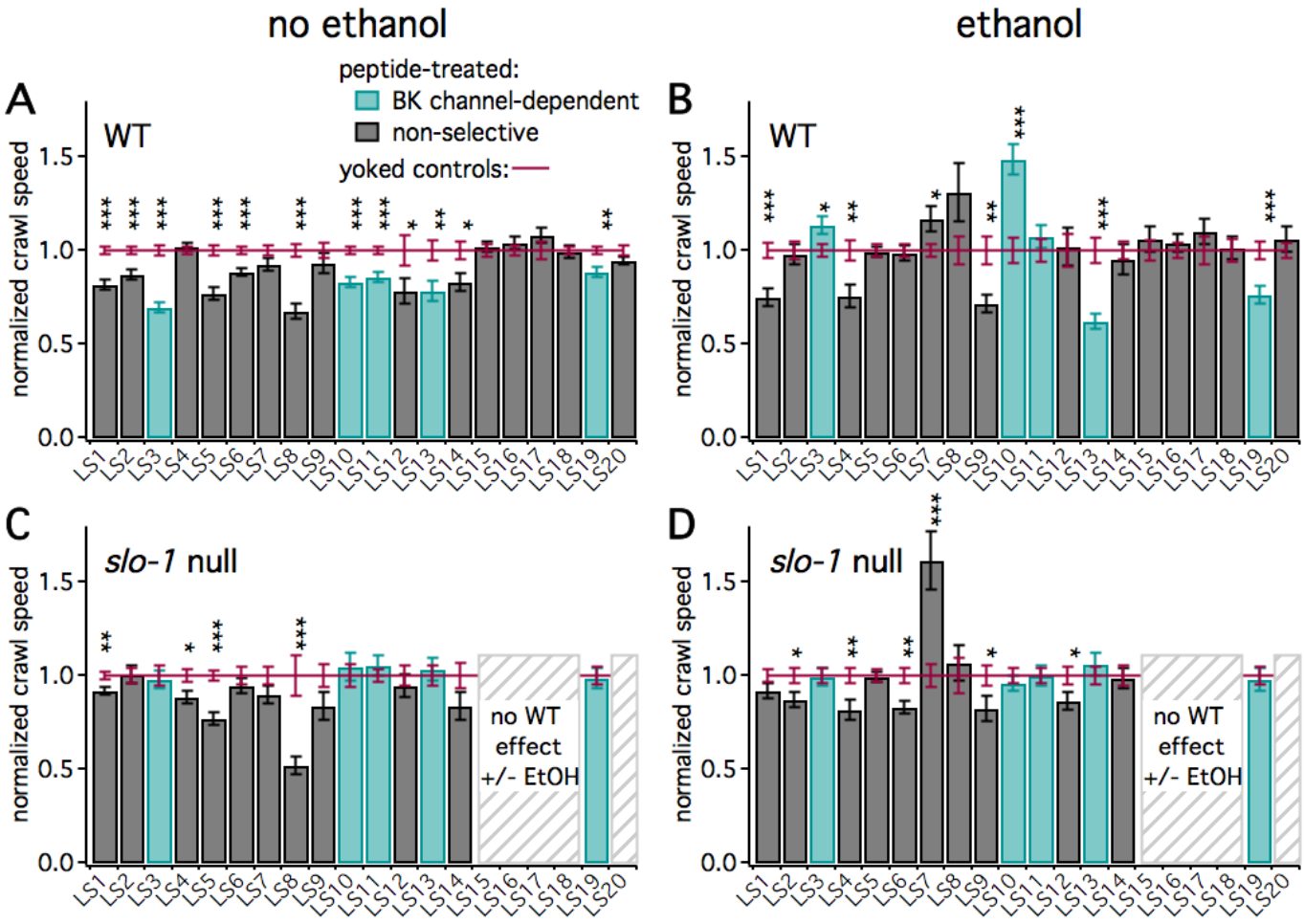

Figure 2

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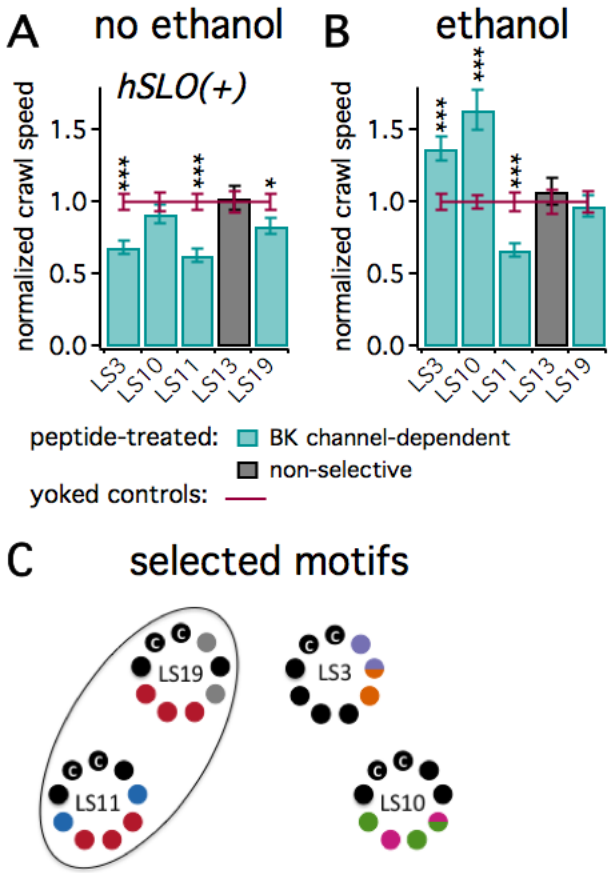

Figure 3

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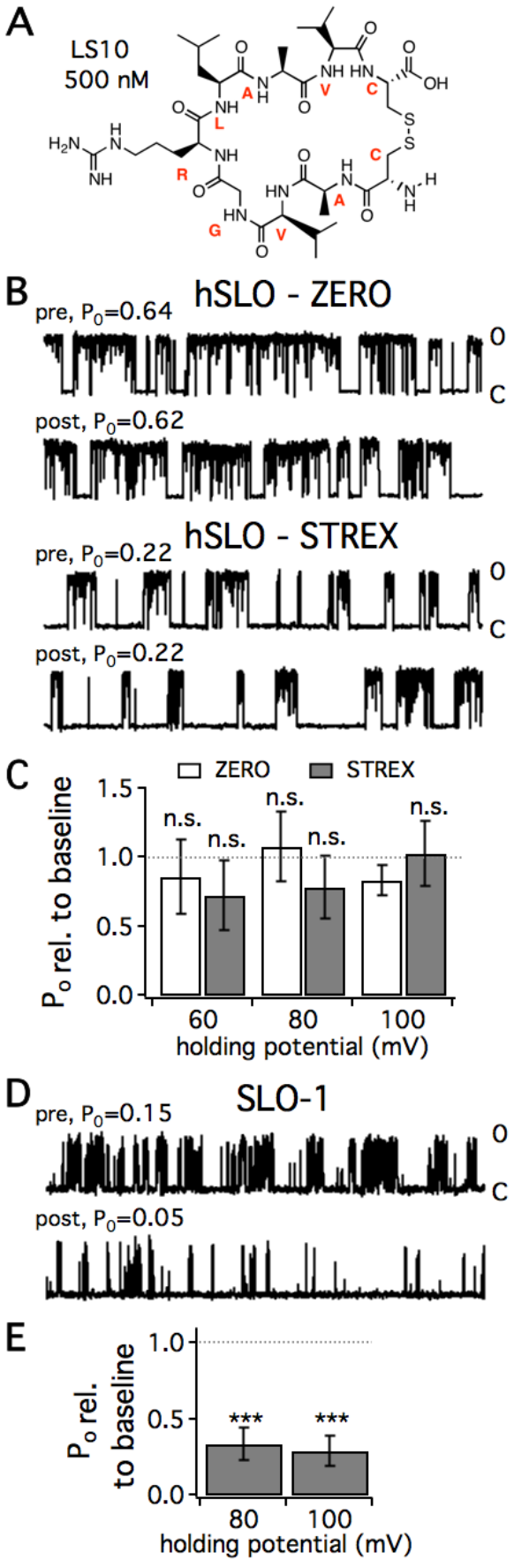

Figure 4

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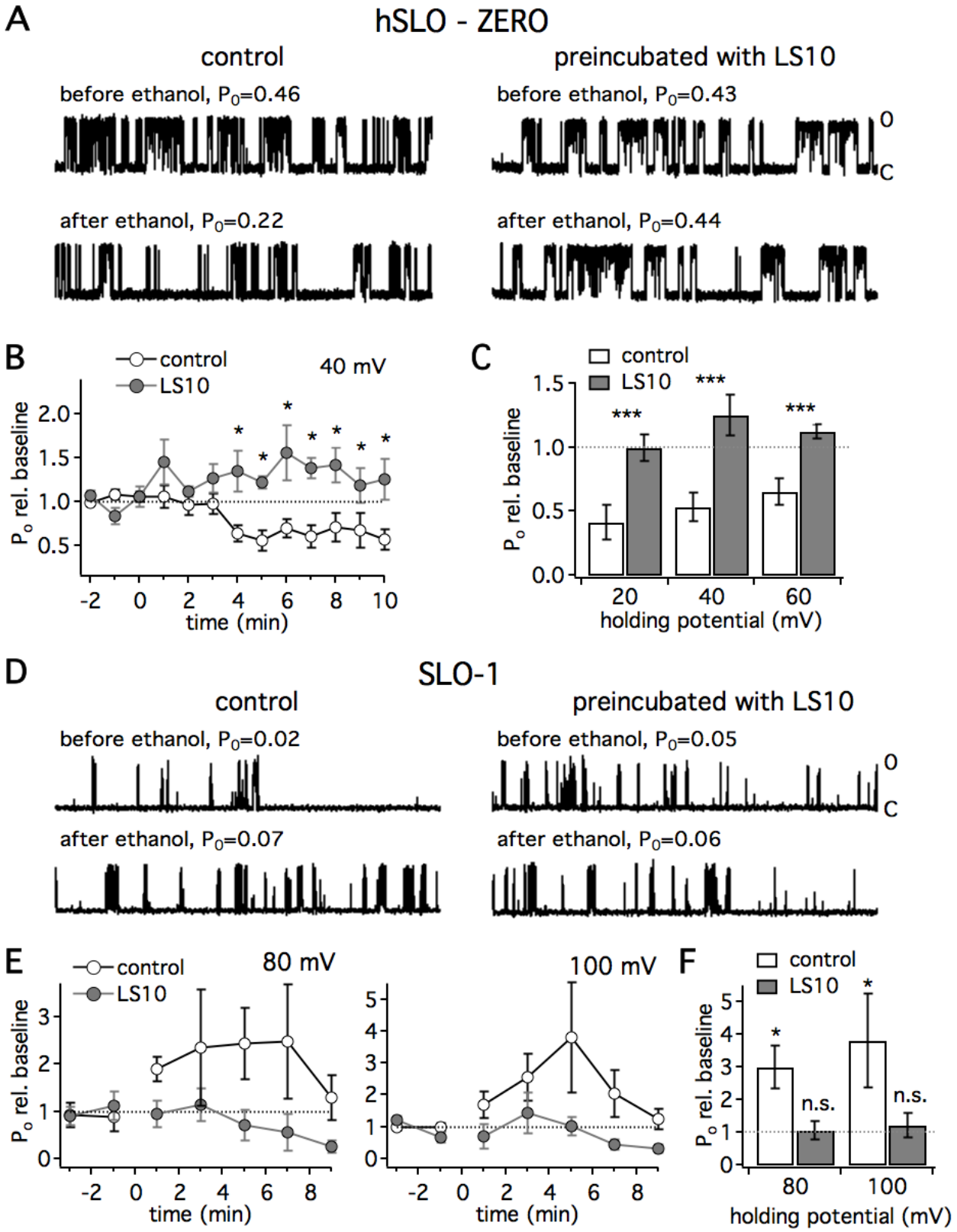

Figure 5