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Monosynaptic GABAergic projections from a subset of suprachiasmatic nucleus neuromedin S neurons suppress preoptic area kisspeptin neuron firing in female mice.

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Abstract

Neural control of ovulation is critical for reproductive success. In spontaneous ovulators, this is achieved through hypothalamic neural circuits relaying ovarian follicle maturity cues to gonadotropin-releasing hormone (GnRH)-secreting neurons to drive the preovulatory luteinizing hormone (LH) surge. Within these circuits, kisspeptin-expressing neurons in the rostral periventricular region of the third ventricle (RP3V^{KISS1} neurons) are thought to stimulate GnRH neurons for the LH surge. In female rodents, the central circadian clock in the suprachiasmatic nucleus (SCN) regulates the timing of RP3V^{KISS1} and GnRH neuron activation, coordinating neuroendocrine control of ovulation and behavior. However, the mechanisms thereof are not fully understood. Here, we examined the potential regulation of RP3V^{KISS1} neuron activity by neuromedin S (NMS)-expressing SCN neurons – a neuronal population essential for circadian rhythms – using anatomical tract-tracing, optogenetics and electrophysiology in female mice. Our observations indicate that a subset of SCN^{NMS} neurons, likely distinct from the canonical arginine vasopressin and vasoactive intestinal peptide SCN populations, projects to RP3V^{KISS1} neurons. Optogenetic activation of these projections evokes monosynaptic release of GABA onto $\approx 75\%$ of RP3V^{KISS1} neurons. Whereas exogenous NMS only moderately affected RP3V^{KISS1} neuron activity, with no effect on firing and altered intracellular calcium concentration in subsets of cells, GABA release from SCN^{NMS} fibers in the RP3V potently suppressed KISS1 neuron action potential firing, through activation of GABA_A receptors. Together, these findings reveal additional complexity in the regulation of the GnRH neuronal network by the central circadian clock. Potential implications of these observations for the timing of the LH surge are discussed.

Significance statement

In females of multiple species, neuroendocrine circuits in the hypothalamus, which include preoptic area kisspeptin neurons and converge on the gonadotropin-releasing hormone (GnRH) neurons, integrate information about gonadal state with circadian cues to triggers ovulation. The mechanisms through which the central circadian clock in the suprachiasmatic nucleus (SCN) relays timing cues to the GnRH neuronal network are, however, incompletely understood. Using anatomical and functional approaches, we found that a previously unsuspected subpopulation of SCN neurons projects to preoptic area kisspeptin neurons and, unexpectedly, suppresses these cells' activity through the release of the inhibitory neurotransmitter GABA. Our observations suggest that control of activity within the GnRH neuronal network for ovulation might extend beyond modulation by canonical SCN neuropeptides.

Introduction

Most aspects of physiology and behavior exhibit endogenously generated circadian rhythms, enabling organisms to anticipate predictable changes in the environment like the light and dark cycle. The central circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus is a key regulator of circadian rhythms, including rhythms in hormone secretion (Buijs et al., 2003; Pevet and Challet, 2011; Hastings et al., 2018; Focke and Iremonger, 2020; Piet, 2023). However, despite a growing understanding of the molecular clockwork enabling timekeeping (Takahashi, 2017), how the SCN clock relays timing signals to downstream effectors remains underexplored.

The timing of the female rodent surge in luteinizing hormone (LH) secretion that triggers ovulation is an example of the SCN's influence on neuroendocrine circuits. The LH surge and ovulation both require a daily neural signal that occurs within a critical period at mid-day (Everett and Sawyer, 1950; Legan and Karsch, 1975) and likely originates in the SCN (Wiegand and Terasawa, 1982; Meyer-Bernstein et al., 1999). The SCN is thought to relay timing signals to the circuits responsible for the LH surge through axonal projections to the gonadotropin-releasing hormone (GnRH) neurons and their afferent partners (van der Beek et al., 1993; Meyer-Bernstein et al., 1999; de la Iglesia et al., 2003; Vida et al., 2010; Russo et al., 2015).

Among the afferent neuronal populations controlling GnRH neuron activity, hypothalamic kisspeptin (KISS1) neurons have emerged as key regulators of fertility (de Roux et al., 2003; Seminara et al., 2003). KISS1 neurons located in the arcuate nucleus are likely the GnRH/LH pulse generator (Clarkson et al., 2017; Nagae et al., 2021; Moore et al., 2022; Han et al., 2023), whereas KISS1 cells located in the anteroventral periventricular and preoptic periventricular nuclei, collectively referred to as the rostral periventricular area of the third ventricle (RP3V), are thought to contribute to generating the LH surge (Smith et al., 2006; Adachi et al., 2007; Clarkson et al., 2008; Yip et al., 2015; Piet et al., 2018; Wang and Moenter, 2020; Clarkson et al., 2023;

Zhou et al., 2026). Activation of RP3V^{KISS1} neurons and RP3V *Kiss1* gene expression for the LH surge are under circadian control (Robertson et al., 2009). SCN neurons that express arginine vasopressin (AVP) project to RP3V^{KISS1} neurons and release AVP onto these cells, thereby increasing their action potential firing (Jamieson et al., 2021), providing a potential pathway through which the SCN relays timing signals to the GnRH neuronal network. However, tract-tracing studies suggest that AVP-containing fibers may only be a subset of SCN projections to RP3V^{KISS1} neurons (Vida et al., 2010). The identity of SCN neurons, beyond AVP cells, that contribute projections to KISS1 neurons is unknown.

Among possible SCN neuronal subpopulations, the neuromedin S (NMS) neurons, which comprise most of the AVP- and vasoactive intestinal peptide (VIP)-expressing cells and play a central role in generating circadian rhythms (Lee et al., 2015), are good candidates for regulating the GnRH neuronal network for the LH surge. Because they overlap with SCN^{AVP} neurons, at least some SCN^{NMS} neurons may project to RP3V^{KISS1} neurons. In addition, *Nms* gene expression in the SCN peaks on the day the preovulatory LH surge is generated, and central NMS administration stimulates LH secretion (Vigo et al., 2007). Lastly, RP3V^{KISS1} neurons express the *Nmur2* gene (Stephens and Kauffman, 2021) that encodes a G protein-coupled receptor that binds NMS. We, therefore, hypothesized that SCN^{NMS} neurons project to RP3V^{KISS1} neurons and regulate their electrical activity. Using anatomical tract-tracing, optogenetics, patch-clamp electrophysiology and calcium imaging, we found that a subset of SCN^{NMS} neurons, likely distinct from AVP- or VIP-expressing cells, establishes monosynaptic GABAergic inputs onto RP3V^{KISS1} neurons that suppress action potential firing in these cells. Together with our previous findings (Jamieson et al., 2021), these observations reveal that the SCN exerts opposing influences on RP3V^{KISS1} neuron electrical activity. This might be important for gating activation of these neurons for the preovulatory surge.

Materials and Methods

Animals

Multiple genetically modified mouse strains were used in these studies. Mice expressing the DNA-recombining enzyme Cre recombinase in NMS-expressing cells (Lee et al., 2015) (*Nms*-Cre; Jackson Laboratory; stock #027205), in VIP-expressing cells (*Vip*-Cre; Jackson Laboratory; stock #031628) or in KISS1-expressing cells (Cravo et al., 2011) (*Kiss1*-Cre; Jackson Laboratory; stock # 023426) were used to drive conditional expression of transgenes in NMS, VIP or KISS1 neurons, respectively. The *Nms*-Cre and *Kiss1*-Cre strains have been found to report faithfully (>90% specificity) driver gene activity (Cravo et al., 2011; Lee et al., 2015). Mice that express the humanized *renilla* green fluorescent protein in KISS1 cells (Cravo et al., 2013) (*Kiss1*-hrGFP; Jackson Laboratory; stock #023425) were used to target KISS1 neurons for brain slice electrophysiology. Our assessment of the specificity of this strain reveals that approximately 70% of hrGFP cells are KISS1-immunoreactive (ir) in the RP3V (Braine and Piet, unpublished data), suggesting that some hrGFP targeted for electrophysiological recordings in our studies might not be *bona fide* kisspeptin neurons. For optogenetic stimulation of SCN neuronal projection fibers in brain slices, *Nms*-Cre mice or *Vip*-Cre mice were crossed with *Kiss1*-hrGFP mice and the female offspring carrying both the *Nms/Vip*-Cre and *Kiss1*-hrGFP alleles were used in experiments. For calcium imaging, mice expressing the genetically-encoded fluorescent calcium indicator GCaMP6f (Chen et al., 2013) in KISS1 neurons, obtained by crossing *Kiss1*-Cre mice with mice that carry Cre-dependent GCaMP6f at the ROSA26 locus (Madisen et al., 2015) (Jackson laboratory strain #028865), were used (Buo et al., 2023; Bearss et al., 2025). Estrous cycle stage was determined by vaginal lavage (3.5 μ L H₂O; taken *antemortem* at zeitgeber [ZT] 1-3 or *postmortem*) smeared on slides, stained with methylene blue and examined by light microscopy (Quignon, 2023). All experimental mice were adult (≥ 8 weeks old) group-housed with littermates under controlled temperature ($23 \pm 2^\circ\text{C}$) and lighting conditions (12-hour light/dark

cycles) with *ad libitum* access to food and water. All experiments were approved by the University of Otago Animal Ethics Committee and the Kent State University Institutional Animal Care and Use Committee.

Stereotaxic surgery

Mice, anesthetized with isoflurane, were placed in a stereotaxic apparatus and uni- or bilaterally injected with 200-400 nL/side of AAV_{DJ}-EF1-DIO-mCherry (AAV-DIO-mCherry, 5.05×10^{13} GC/mL) or AAV_{DJ}-EF1-DIO-hChR2^(E123T/T159C)-p2a-mCherry-WPRE (Berndt et al., 2011; Mattis et al., 2011) (AAV-DIO-ChR2^(ET/TC), 1.60×10^{13} GC/mL; AAV stocks were purchased from the Stanford University Gene Vector and Virus Core) in the SCN at 100 nL/min using a 1 μ L Hamilton syringe. Coordinates were (from bregma) 0.5 mm anterior, $\pm$ 0.2 mm lateral and 5.8 mm deep from the top of the skull for bilateral injections, or 0.4-0.5 mm anterior, 0.81 mm lateral with a 6-degree angle and 5.6-5.7 mm deep from dura for unilateral injections. The needle was left *in situ* for 3-5 minutes before, and 10 minutes after virus injection. Injected mice were used in experiments $\geq$ 2 weeks after surgery.

Immunohistochemistry

Mice injected with AAV-DIO-mCherry were deeply anesthetized by pentobarbital overdose (100 μ L i.p. 150 mg/kg) and transcardially perfused with 4% paraformaldehyde at ZT4-6. Three sets of 40 μ m thick coronal brain sections were cut through the hypothalamus and processed for free-floating immunohistochemistry to enhance endogenous mCherry and visualize RP3V^{KISS1} neurons. Antibodies used were rabbit anti-mCherry (1:5000; AbCam, catalog #ab167453), which was visualized with donkey anti-rabbit AlexaFluor-568 (1:200; ThermoFisher Scientific, catalog #A10042); and sheep anti-KISS1 (1:1000; Gift from Prof. Alain Caraty, AC053) followed with

biotinylated donkey anti-sheep (1:1000; ThermoFisher Scientific, catalog #A16045), which was visualized with streptavidin AlexaFluor-488 (1:1000; ThermoFisher Scientific, catalog #S32354).

Confocal imaging

Brain sections were imaged using an inverted Nikon A1R confocal microscope (Nikon Instruments Inc., Tokyo, Japan), with lasers of 488 and 543 nm wavelengths. Z-stack images were taken through the SCN and RP3V using 10x (1.0 to 2.0 μm Z-step) or 40x (0.5 μm Z-step) objectives. Images were analyzed offline using FIJI (Schindelin et al., 2012).

mCherry-ir projection fiber density was measured on 10x Z-stack images of sections containing the RP3V. Maximum projection images were binarized and the percentage of area covered by mCherry-ir fluorescent pixels was measured in regions of interest (ROI) including the RP3V. To estimate the extent of SCN transfection, the percentage of area covered by mCherry-ir fluorescence was measured in ROIs delineating the SCN. Injections were considered successful (SCN hit) if mCherry-ir covering a substantial part of the SCN, with numerous mCherry-ir somata, was seen on at least one side of the brain. Injections were considered to have missed the SCN if the bilateral SCN contained only a few (< 10) or no mCherry-ir somata.

RP3V KISS1-ir neurons were imaged with a 40x objective. KISS1-ir somata, digitally magnified by 4x, were inspected in individual confocal planes of the Z-stack to determine the presence of apposition by mCherry-ir fibers. An apposition between an mCherry-ir fiber and a KISS1-ir soma was counted if there were no black pixels between the red and green signals in a single confocal plane, as described previously (Jamieson et al., 2021; Jamieson et al., 2022).

In each mouse, one section containing the mid-level SCN and two sections containing the RP3V were analyzed. However, in three animals (one diestrus and two proestrus, including one miss), only one RP3V section could be recovered. Analyses in the RP3V were carried out on the

side ipsilateral to the most transfected SCN and averaged across the two sections. mCherry-ir is displayed in magenta in figures to enhance contrast with the dark background.

Brain slice preparation, electrophysiology and optogenetic stimulation

Mice for electrophysiological studies were decapitated between ZT1.5 and ZT7.5 (ZT4.7 $\pm$ 0.2, N = 53 *Nms-Cre;Kiss1-hrGFP* mice; ZT4.1 $\pm$ 0.2, N = 14 *Kiss1-hrGFP* mice) after either cervical dislocation or anesthesia with isoflurane, and the brains quickly removed. Coronal brain slices (200 μ m thick) containing the RP3V or the SCN were cut using a vibrating blade microtome (VT1000 S or VT1200S, Leica) in an ice-cold slicing solution containing (in mM): 87 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 0.5 CaCl₂, 6 MgCl₂, 25 glucose and 75 sucrose as described previously (Buo et al., 2023; Bearss et al., 2025). Brain slices were left to incubate at $\approx$ 30-34°C for $\geq$ 1 hour in artificial cerebrospinal fluid (aCSF) containing (in mM): 120 NaCl, 3 KCl, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂ and 10 glucose. All solutions were equilibrated to pH 7.4 with a mixture of 95% O₂/5% CO₂.

Individual slices were then placed under an upright epifluorescence microscope (Scientifica or Prior Scientific) and constantly perfused (1.5 mL/min) with warm ($\approx$ 30°C) aCSF. In AAV-DIO-ChR2^(ET/TC)-injected mice, we first confirmed that the SCN was sufficiently transfected by visualizing mCherry fluorescence using light-emitting diode (LED; CoolLED) illumination (excitation 563-588 nm; emission 604-800 nm; Semrock). In slices containing the RP3V, GFP-expressing KISS1 neurons were visualized using brief LED illumination (excitation 460-487 nm; emission 500-546 nm; Semrock) and subsequently approached with recording electrodes under infrared differential interference contrast illumination. Because the ChR2^(ET/TC) excitation spectrum overlaps that of GFP, low intensity LED illumination ($\approx$ 0.8 mW) was used and a waiting period $\geq$

10 min following identification of a GFP-expressing neuron was allowed before recordings started (Piet et al., 2018; Jamieson et al., 2021; Jamieson et al., 2022).

Electrophysiological recordings were made using glass microelectrodes pulled from borosilicate glass (Sutter Instrument; catalog # BF-150-110-10) using a model P-1000 puller (Sutter Instrument). Synaptic transmission was recorded in the whole cell voltage clamp (WCVC) configuration (-60 mV holding potential), using glass microelectrodes (1-3 M Ω) filled with a chloride-based internal solution containing (in mM): 130 KCl, 10 HEPES, 0.2 Na₂GTP, 2 MgATP and 1 QX-314-chloride (adjusted to pH 7.2 with KOH and to $\approx$ 290 mosm with sucrose). Action potential firing was recorded in the “loose patch” cell-attached configuration (12-20 M Ω seal resistance) in voltage-clamp mode (no holding potential applied). Glass microelectrodes (1-3 M Ω) were filled with aCSF and the recording configuration was achieved by applying the lowest amount of suction required to detect spikes. Electrophysiological signals were acquired using either a double integrated patch-clamp amplifier[®] and the Sutterpatch software (Sutter Instrument) or a Multiclamp 700B amplifier and 1440a digitizer along with the pClamp10 software (Molecular Devices). Acquisition settings were 10-20 kHz sampling rate and 2 kHz filter for synaptic transmission or 10 kHz sampling rate and 2 kHz filter for action potential firing.

Optogenetic stimulation was achieved by delivering brief pulses (2-5 ms duration) of blue LED light (460-487 nm) to the slice through a 40x immersion objective (0.8 NA, Olympus). In WCVC recordings, optogenetically-evoked post-synaptic currents (oPSCs) were stimulated by pairs of blue LED light pulses (200 ms intervals) occurring every 10-20 seconds. In loose patch recordings, the effect of optogenetic stimulation on action potential firing was assessed by delivering trains of blue LED light pulses. In mCherry-expressing SCN neurons, we tested the efficacy of blue light pulses at evoking firing (spike fidelity) by delivering 10 pulses at 2, 5, 10, 20 or 50 Hz. 10-pulse trains of various frequencies, each repeated five times, occurred in random order every 10 seconds. In GFP-expressing RP3V neurons, the impact of ChR2-expressing axon

blue light stimulation on firing was tested with 600 pulses at 10 Hz or, in some cases, 300 pulses at 5 Hz. Stimulation trains, when repeated, were separated by ≥ 5 minutes.

Analysis of oPSCs and action potential firing was carried out using Sutterpatch and Clampfit (Molecular Devices). In WCVC recordings, we considered that optogenetic stimulation successfully evoked oPSCs when synaptic events initiating with similar latency within 10 milliseconds of the blue light pulse onset were observed in multiple overlaid sweeps (see for example, Figure 2B and Figure 3C). In each cell, including those in which optogenetic stimulation failed to evoke oPSCs, peak PSC amplitude was measured in 10-to-20 sweep averages during a time window between 5 and 20 ms after the onset of the stimulus. In 10 RP3V^{KISS1} neurons with oPSCs, we estimated the relationship between stimulus intensity and oPSC amplitude (input-output) by varying the light intensity in steps (1, 2, 5, 10, 20, 50 and 100% of maximum LED intensity; ranging from 0.1 to > 10 mW). In these cells, synaptic latency was estimated in the 10 sweeps in which oPSCs were evoked by 50% of maximum LED intensity, the stimulus intensity that evoked the largest PSCs on average (see Results). This was done by first differentiating the current traces (dl/dt) and, then, determining the delay between the onset of the stimulus and the time at which the derivative trace crossed a threshold set to 1.5x the baseline noise. Synaptic jitter was defined as the standard deviation of synaptic latencies in those sweeps (Doyle and Andresen, 2001).

In loose patch recordings, spikes – corresponding to single action potentials (Perkins, 2006) – were detected using the threshold crossing method. In SCN neurons, spike fidelity was defined as the percentage of stimuli that evoked spikes. In RP3V^{KISS1} neurons, the effect of stimulation trains was assessed by comparing action potential firing rates during the train to those during a one-minute baseline period immediately preceding the train. The change in firing was calculated as the difference in mean firing rate between the baseline and stimulation train periods. We defined a response as mean action potential firing rate changing by $\geq 50\%$; because the effect

of blue light stimulation on RP3V^{KISS1} firing was sometimes transient (see Figures 7B and 8B), only the first 15 seconds following the stimulus train onset were considered for classifying a neuron as responding or not. The effect of local applications of peptides or aCSF on RP3V^{KISS1} neuron firing was assessed by comparing action potential firing rates in response to the applications to those during a 10-second baseline period immediately preceding the applications. A response to local application of a peptide was defined as the firing rate changing by $\geq 50\%$ during a floating 10-second period comprising the largest variation from baseline (peak effect) within 40 seconds from the “puff” onset. The change in firing rate was calculated as $mean\ firing\ rate(baseline) - mean\ firing\ rate(peak\ effect)$.

All electrophysiological recordings were carried out between ZT5 and ZT15 ($ZT8.8 \pm 0.1$, $n = 134$; four cells were recorded after ZT12) in *Nms-Cre;Kiss1-hrGFP* mice and between ZT5.5 and ZT10.5 ($ZT7.9 \pm 0.2$, $n = 37$) in *Kiss1-hrGFP* mice.

Calcium imaging

Variations in $[Ca^{2+}]_i$ in RP3V^{KISS1} neurons were estimated by measuring fluorescence changes in individual GCaMP6f-expressing neurons in brain slices (prepared as above) from *Kiss1-Cre;GCaMP6f* mice killed between ZT2 and ZT5.5 ($ZT3.8 \pm 0.2$, $N = 9$ *Kiss1-Cre;GCaMP6f* mice). Slices were illuminated through the 40x immersion objective, using the microscope LED light source filtered for blue light excitation (460-487 nm). Epifluorescence (emission 500-546 nm) was collected using an ORCA-FLASH 4.0 LT+ CMOS camera (Hamamatsu). LED and camera were controlled and synchronized with the μ -manager 1.4 software (Edelstein et al., 2010). After a 10- to 30-minute stabilization period in the recording chamber, a focal plane including several fluorescent cell bodies was chosen and acquisitions (100 ms light exposure at 2 Hz for 10-15 minutes) started. Low-intensity LED illumination was used (~ 0.1 to 0.8 mW) to minimize GCaMP6f

photobleaching. Cell viability was tested at the end of the recording by bath application of (S)- α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 10 μ M). All $[Ca^{2+}]_i$ recordings were carried out between ZT5.5 and ZT9.5 ($ZT7.3 \pm 0.3$, $n = 17$ slices).

Analysis of fluorescence image time-series was carried out in FIJI software (Schindelin et al., 2012). Regions of interest (ROIs) were drawn around individual, in-focus fluorescent somata (10.3 ± 0.7 cells per slice, ranging 5 to 16). Average fluorescence intensity within each ROI was measured in each frame. Fluorescence intensity data were analyzed using scripts written in R (<http://www.r-project.org/>). For each ROI, normalized fluorescence was calculated as $\frac{F_t}{F} * 100$, where F is the baseline fluorescence intensity calculated as the mean fluorescence intensity over a one-minute period preceding a drug application and F_t is the fluorescence measured at any time point. Time-dependent bleaching was manually subtracted from normalized fluorescence traces using Clampfit. ROIs in which the corrected normalized fluorescence changed by more than two standard deviations of the baseline over a one-minute period within five minutes of the drug application onset were recorded as having been affected by the application. Effects are reported as percentage of ROIs stimulated or inhibited per slice.

Drugs

4-amino pyridine (4-AP; catalog #275875) was obtained from Sigma. AMPA (catalog #HB0052), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5; catalog #HB0225), 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX; catalog #HB0443) and SR 95531 hydrobromide (gabazine; catalog #HB0901) were purchased from HelloBio. Tetrodotoxin citrate (TTX) was obtained from Alomone Labs (catalog #T-550) or from HelloBio (catalog #HB1034). Arginine vasopressin (AVP) was purchased from Tocris (catalog #2935). Mouse NMS (LPRLRLDSRMATVDFPKKDPTTSLGRPFLLFRPN) was custom synthesized by

ThermoScientific. All drugs and peptides, except for NMS, were dissolved in deionized H₂O to a stock concentration, aliquoted and stored at -20°C. NMS was dissolved in deionized H₂O and used fresh. All drugs were bath applied, except for AVP and NMS that were also pressure ejected for local applications. This was carried out *via* a glass micropipette connected to the valve of a picospritzer (NPI electronic GmbH). The pressure micropipette was filled with peptide-containing aCSF and was placed in the tissue 20 to 30 mm from the recorded cell. Pressure applications (2-5 psi) occurred for ten seconds after a twenty-second baseline. Because pressure applications typically caused tissue displacement around recorded cells, control experiments in which aCSF was locally applied were carried out to confirm the specificity of the reported effects (see Results). In recordings involving receptor antagonists or ion channel blockers, compounds were bath applied for ≥ 5 minutes.

Statistics

Statistical analyses were performed using Prism 10.0 (GraphPad). Data are reported in the text and figures as mean ± SEM. Normality of data sets was tested using the Shapiro-Wilk test. Non-parametric statistical tests were used when at least one comparison group did not pass this test. Comparisons between two paired groups were made using the paired t-test or the Wilcoxon signed rank test, while comparisons between two independent groups were made using the unpaired t-test with Welch's correction or the Mann-Whitney test, as appropriate. Comparisons between multiple groups with repeated measures were made with the repeated measure one-way ANOVA (and Tukey's post-test), mixed effect model (in case of missing values) or Friedman (and Dunn's post-test) tests, as appropriate. Comparisons between multiple groups without repeated measures were made with the one-way ANOVA test followed by Tukey's post-tests. Comparisons between multiple groups where two factors were considered were carried out with the two-way ANOVA test and Fisher's LSD tests. Comparisons of proportions were made using

326 Fisher's exact tests. Correlation analyses were performed using linear regressions. Differences
327 were considered statistically significant for $p < 0.05$.

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Results

SCN^{NMS} neurons project to RP3V^{KISS1} neurons in female mice

We first sought to determine whether SCN^{NMS} neurons project to the RP3V, using AAV-mediated anterograde tract-tracing in *Nms*-Cre females. AAV injections resulted in SCN mCherry-ir in 8 mice ("SCN hit" [see methods]; 4 diestrus and 4 proestrus; Figure 1A and E) and in little or no SCN mCherry-ir in 4 ("SCN miss" [see methods]; 1 diestrus and 3 proestrus; Figure 1C and E). Consistent with previous observations of Cre activity outside the SCN in *Nms*-Cre mice (Lee et al., 2015), transfection was seen in hypothalamic areas outside the SCN in all animals following AAV-DIO-mCherry injection directed to the SCN (see, for example, Figure 1A and C). In "SCN hit" mice, we observed extensive mCherry-ir innervation of the RP3V and of KISS1-ir neurons therein (Figure 1B and F). However, in "SCN miss" mice, RP3V innervation was significantly lower (4.43 ± 1.96 vs. $34.23 \pm 3.68\%$ of RP3V area covered by mCherry-ir fibers in miss vs. hit mice, $N = 4$ and 8 mice, respectively; $p < 0.001$, unpaired t-test with Welch's correction; Figure 1D and F). Together, these observations indicate that NMS neurons extend projections to the RP3V and that this innervation originates, for the most part, in the SCN.

Analysis of KISS1-ir somata in 40x confocal image stacks obtained in "SCN-hit" mice revealed that $89.82 \pm 4.03\%$ of KISS1-ir cells (16.3 ± 2.1 cells analyzed per section) received 2.83 ± 0.31 close appositions by mCherry-ir fibers (Figure 1B, G and H). In "SCN-miss" mice, the percentage of KISS1-ir somata with close appositions ($19.26 \pm 8.27\%$; 23.6 ± 3.9 cells analyzed per section) and the number of close appositions per neuron (1.07 ± 0.06) were both significantly lower ($p = 0.001$ and $p < 0.001$, respectively, unpaired t-test with Welch's correction; Figure 1D, G and H). In "SCN hit" animals, proestrous mice tended to have denser mCherry-ir innervation in the RP3V (40.37 ± 4.01 vs. $28.09 \pm 4.69\%$; $p = 0.095$, unpaired t-test with Welch's correction) and a higher percentage of RP3V KISS1-ir neurons with close appositions by mCherry-ir fibers than diestrus mice (97.40 ± 1.50 vs. $82.24 \pm 5.92\%$, respectively; $p = 0.029$, Mann-Whitney test; not

shown). This observation, however, is difficult to interpret due to the small sample sizes (N = 4 each for diestrus and proestrus) and mouse-to-mouse variability in SCN transfection ($50.62 \pm 13.60\%$ in diestrus vs. $39.94 \pm 5.82\%$ in proestrus, ranging 19.43 to 75.76% and 23.09 to 48.95%, respectively; $p = 0.509$, unpaired t-test with Welch's correction).

Taken together, the results of this anterograde study indicate that SCN^{NMS} neurons send projections to the RP3V that come in close apposition with the majority of RP3V^{KISS1} neuron somata.

Optogenetic activation of SCN^{NMS} neuron fibers in the RP3V evokes synaptic transmission in KISS1 neuron

We next examined whether SCN^{NMS} neurons establish functional synaptic inputs onto RP3V^{KISS1} neurons using ChR2-assisted circuit mapping (CRACM) (Petreanu et al., 2007). Injection of AAV-DIO-ChR2^(ET/TC) in the SCN of *Nms-Cre* females resulted in functional expression of ChR2^(ET/TC) in SCN neurons. SCN mCherry (ChR2^(ET/TC))-expressing neurons responded to brief 10-pulse blue light stimulation trains with high-fidelity action potential firing at various frequencies (2-50 Hz). Fidelity varied in SCN mCherry-expressing neurons with stimulation frequency ($p = 0.003$, Friedman test) and tended to be lower at 50 Hz, although not significantly so compared with fidelity at 2, 5, 10 and 20 Hz ($p > 0.282$, Dunn's multiple comparisons tests, $n = 12$ cells in 2 diestrus and 1 estrous mice; Figure 2A). In brain slices from *Nms-Cre; Kiss1-hrGFP* females that received AAV-DIO-ChR2^(ET/TC) in the SCN, optogenetic activation of ChR2^(ET/TC)-expressing fibers evoked large inward PSCs (oPSCs) in RP3V GFP-expressing (i.e. KISS1) neurons recorded in WVCV (Figure 2B). This was seen in 28 out of 29 mice in which there was mCherry expression in the SCN, and in 58 out of 74 KISS1 neurons recorded in the RP3V (Figure 2C; see Table 1 for cycle stage breakdown). In mice in which injection of AAV-DIO-ChR2^(ET/TC) missed the SCN,

however, blue light stimulation failed to evoke synaptic transmission in RP3V^{KISS1} neurons (n = 20 cells tested in 4 diestrous, 2 proestrous and 2 estrous mice; $p < 0.001$ vs. SCN hit for cells and for mice, Fisher's exact tests; Figure 2B and C). Interestingly, the proportion of cells in which oPSCs could be evoked was smaller in diestrous than in proestrous mice, while the amplitude of oPSCs was similar across cycle stages (Table 1). However, similar to the tract-tracing, the interpretative value of these observations is limited due to between-mouse variability in SCN transfection rates. The percentage of cells displaying oPSCs did not correlate with time of death (ZT_{death}). Likewise, oPSC amplitude in those RP3V^{KISS1} neurons in which optogenetic stimulation evoked synaptic transmission did not correlate with ZT_{death} or time of recording (ZT_{recording}; Table 2).

These observations show that SCN^{NMS} neurons establish functional synaptic inputs onto the majority (> 75%) of RP3V^{KISS1} neurons. This contrasts with our previous findings in *Avp-Cre; Kiss1-hrGFP* mice, in which optogenetic stimulation evoked synaptic transmission in only ~5% of KISS1 neurons under similar recording conditions (Jamieson et al., 2021), suggesting that the SCN^{NMS}-derived synaptic inputs identified here may not originate from AVP-expressing neurons. Although previous studies indicate that SCN VIP-expressing (SCN^{VIP}) neurons may not project extensively to RP3V^{KISS1} neurons (Vida et al., 2010), we also tested whether SCN^{VIP} cells provide synaptic inputs to KISS1 neurons. In *Vip-Cre; Kiss1-hrGFP* mice that were transduced with AAV-DIO-ChR2^(ET/TC) in the SCN, 10-pulse blue light stimulation trains (2-50 Hz) evoked high fidelity action potential firing in a frequency-dependent manner in mCherry-expressing SCN cells ($p = 0.001$, Friedman test; $p > 0.109$ in Dunn's multiple comparison tests between frequencies; n = 10 in 3 mice; Figure 2D). However, CRACM experiments revealed that none of the 14 KISS1 neurons we recorded in 3 mice (1 diestrus, 1 proestrus and 1 estrus) with SCN transfection exhibited oPSCs upon blue light stimulation (Figure 2E and F). Taken together, these findings

indicate that an SCN^{NMS} neuronal subpopulation, likely distinct from the AVP and VIP subpopulations, provides functional fast synaptic inputs to RP3V^{KISS1} neurons.

SCN^{NMS}→RP3V^{KISS1} neuronal circuit is monosynaptic

Increasing blue light stimulation intensity resulted in RP3V^{KISS1} neurons in graded oPSC amplitude (Figure 3A) that appeared to plateau, on average, in response to stimuli > 10% of maximum intensity (n = 10 cells in 2 proestrous and 3 estrous mice; Figure 3B), suggesting recruitment of multiple ChR2^(ET/TC)-expressing fibers by light stimuli. Analysis of synaptic latencies in these cells (see methods) revealed that light pulses evoked oPSCs with short latencies (3.64 ± 0.20 ms) and submillisecond synaptic jitter (0.21 ± 0.03 ms; Figure 3C and D), suggesting a monosynaptic circuit (Doyle and Andresen, 2001).

We investigated this further using a pharmacological approach (Petreanu et al., 2009). We first blocked action potential generation with the voltage-gated sodium channel blocker tetrodotoxin (TTX, 0.5 mM), which abolished oPSCs (normalized amplitude 0.02 ± 0.01 in TTX vs. 1.00 ± 0.22 in control; p < 0.001 repeated measure ANOVA, p = 0.006 Tukey's multiple comparisons test; Figure 3E and F). In the presence of TTX, blocking voltage-gated potassium channels with 4-amino pyridine (4-AP; 100 mM) – used to boost blue-light evoked depolarization of ChR2^(ET/TC)-expressing terminals – restored oPSCs (normalized amplitude 0.66 ± 0.16; p < 0.01 vs. TTX and p = 0.21 vs. control, Tukey's multiple comparisons test, n = 9 cells in 3 diestrous, 2 proestrous and 1 estrous mice; Figure 3E and F). Recovery of oPSCs in the presence of TTX indicates that these events do not require activation of intermediate neuron(s).

Taken together, these observations are consistent with oPSCs resulting from activation of a monosynaptic circuit. This provides functional evidence that SCN^{NMS} neurons project directly to RP3V^{KISS1} neurons, corroborating our anatomical results.

SCN^{NMS} neurons release GABA onto RP3V^{KISS1} neurons

We then examined the pharmacology of oPSCs. Blockade of ionotropic glutamate AMPA and NMDA receptors with NBQX (20 mM) and D-AP5 (40 mM), respectively, had no effect on oPSC amplitude (normalized amplitude 0.99 ± 0.17 ; $p = 0.65$ mixed effect model, $n = 8$ cells in 1 undetermined, 2 diestrous and 1 proestrous mice; Figure 4A and B). However, the ionotropic GABA_A receptor antagonist gabazine (5 mM) reversibly reduced oPSC amplitude by > 95% (normalized amplitude: control 1.00 ± 0.18 , gabazine 0.02 ± 0.01 , washout 0.57 ± 0.14 ; $p < 0.001$ Friedman test; control vs. gabazine $p < 0.001$, gabazine vs. washout $p = 0.042$, control vs. washout $p = 0.221$, Dunn's multiple comparisons test; $n = 10$ in 2 diestrous, 2 proestrous and 4 estrous mice; Figure 4C and D).

Optogenetic activation of ChR2^(ET/TC)-expressing SCN^{NMS} fibers, therefore, results in synaptic release of GABA, but not glutamate, and activation of GABA_A receptors on RP3V^{KISS1} neurons. That stimulated SCN^{NMS} fibers release GABA is consistent with previous observations that most SCN neurons are GABAergic (Moore and Speh, 1993).

SCN^{NMS} neuron projections may establish functional fast synaptic inputs primarily onto KISS1 neurons in the RP3V

We next asked whether SCN^{NMS} neuron projections communicate with non-KISS1 neurons within the RP3V using fast synaptic transmission. To answer this question, we successively recorded RP3V GFP positive (GFP^{pos}, i.e. KISS1) and GFP negative (GFP^{neg}, i.e. non-KISS1) neurons and optogenetically activated ChR2^(ET/TC)-expressing SCN fibers (Figure 5A). Light pulses evoked oPSCs in 11 out of 12 RP3V^{KISS1} neurons and in 4 out of 12 GFP^{neg} RP3V neurons ($p = 0.009$, Fisher's exact test; 3 diestrous and 2 estrous mice; Figure 5B-D).

oPSCs had larger amplitudes in KISS1 neurons (ranging -139.82 to -1,469.97 pA, 11 cells) than those seen in GFP^{neg} neurons (ranging -5.43 to -35.94 pA, 4 cells; Figure 5B and C) and, overall, oPSC amplitude was significantly larger in KISS1 neurons (normalized amplitude 1.00 ± 0.23 vs. 0.01 ± 0.01 ; $p < 0.001$ Mann-Whitney test; $n = 12$ GFP^{pos} and 12 GFP^{neg} cells; Figure 5E). In addition, stimulation intensities used to probe synaptic transmission were significantly larger in GFP^{neg} neurons than in KISS1 neurons (78.17 ± 10.17 vs. $7.42 \pm 1.87\%$; $p < 0.001$, Mann-Whitney test; not shown).

Together, these observations suggest that SCN^{NMS} neurons may establish functional fast synaptic inputs primarily, although not exclusively, onto KISS1 neurons within the RP3V.

Exogenous NMS does not affect RP3V^{KISS1} neuron firing

We next examined the effects of NMS on RP3V^{KISS1} neuron action potential firing. As NMS was previously reported to stimulate periventricular nucleus A14 dopamine neuron activity (Romanov et al., 2017), we hypothesized that it might excite RP3V^{KISS1} neuron activity, for a potential effect on the preovulatory surge. These experiments were, therefore, carried out in proestrous mice. We recorded KISS1 neurons in the loose patch configuration and applied NMS (1 mM; 10 s) locally, using a pressure ejection system (2-5 psi) (Piet et al., 2015; Bearss et al., 2025). In separate sets of recordings, AVP (1 mM; 10 s), previously reported to excite KISS1 neurons (Piet et al., 2015; Jamieson et al., 2021; Jamieson et al., 2022), or aCSF were also locally applied as positive and negative controls, respectively.

Pressure applications of aCSF either had no detectable effect (defined as a change in firing by $\geq 50\%$; see methods) or increased action potential firing (6 out 11 cells, 6 mice; Figure 6A and D) in RP3V^{KISS1} neurons. Pressure applications of NMS, likewise, either had no detectable effect or increased action potential firing (6 out 12 cells, 4 mice; Figure 6B and D). Locally applied

AVP (1 mM; 10 s) increased firing in 10 out of 14 cells (4 mice; Figure 6C and D). The proportions of cells excited by aCSF, NMS or AVP did not significantly differ ($p = 0.494$, Fisher's exact test; not shown). A two-way ANOVA analysis revealed, however, a main effect of pressure applications ($p < 0.001$), no main effect of treatment (aCSF vs. NMS vs. AVP; $p = 0.2539$) and a significant interaction between these two factors ($p = 0.014$). Post-hoc tests showed no significant differences in baseline firing rates between datasets (aCSF vs. NMS: $p = 0.950$; aCSF vs. AVP: $p = 0.786$; NMS vs. AVP: $p = 0.731$), significant differences between treatments in firing rates recorded after pressure applications (aCSF vs. NMS: $p = 0.944$; aCSF vs. AVP: $p = 0.026$; NMS vs. AVP: $p = 0.019$) and a significant baseline vs. after pressure application difference for AVP ($p < 0.001$) but not for aCSF ($p = 0.063$) or for NMS ($p = 0.054$; Fisher's LSD post-tests; Figure 6D and Table 3). Consistent with this, AVP-induced changes in firing were significantly larger than those induced by aCSF or NMS ($p = 0.014$, one-way ANOVA; AVP vs. NMS $p = 0.028$, AVP vs. aCSF $p = 0.033$, NMS vs. aCSF $p > 0.99$; Table 3). Effects of NMS, AVP or aCSF did not correlate with ZT or with baseline firing rates (Table 2).

Together, these observations indicate that, unlike AVP, exogenous NMS does not measurably affect RP3V^{KISS1} neuron action potential firing and, therefore, suggest that NMS is not a primary driver of these cells' electrical activity.

Exogenous NMS increases $[Ca^{2+}]_i$ in a subset of RP3V^{KISS1} neurons

We also examined the effects of NMS on populations of RP3V^{KISS1} neurons using $[Ca^{2+}]_i$ imaging in brain slices from proestrous *Kiss1-Cre;GCaMP6f* mice (Figure 6E). Bath applications of NMS (1 mM; 2 minutes) elicited increases and decreases in $[Ca^{2+}]_i$ or had no discernable effect in RP3V^{KISS1} neurons (Figure 6F). On average, NMS excited $30.37 \pm 7.57\%$ and inhibited $6.59 \pm 3.24\%$ of RP3V^{KISS1} neurons (102 cells total; $n = 10$ slices from 5 mice; Figure 6G), leaving the

rest ($\approx 65\%$) unaffected. To determine whether these effects were direct, we repeated this experiment in slices obtained from a different set of proestrous mice and in the presence of TTX (0.5 mM), to block action potential generation, and NBQX (10 mM), D-AP5 (40 mM) and gabazine (5 mM), to block excitatory and inhibitory synaptic transmission. In the presence of this drug cocktail, the percentage of RP3V^{KISS1} neurons (73 cells total; $n = 7$ slices from 4 mice) exhibiting excitations in response to NMS ($11.06 \pm 3.92\%$) was significantly lower than under control conditions ($p = 0.041$, unpaired t test with Welch's correction). The percentage of RP3V^{KISS1} neurons inhibited by NMS in the presence of the cocktail ($1.43 \pm 1.43\%$) was, however, statistically comparable to that under control conditions ($p = 0.247$, Mann-Whitney test; Figure 6G). Linear regression analyses revealed that the percentages of neurons excited or inhibited by NMS did not correlate with ZT_{death} or $ZT_{\text{recording}}$ (Table 2).

Together, these data indicate that NMS increases $[Ca^{2+}]_i$ in approximately 30% of RP3V^{KISS1} neurons while suppressing activity in a small subset ($< 10\%$) of RP3V^{KISS1} neurons. In addition, NMS appears to be producing these effects, for the most part, through indirect means.

Activation of SCN^{NMS} neuron projections suppresses RP3V^{KISS1} neuron action potential firing through GABA release and activation of GABA_A receptors

Lastly, we tested the impact of SCN^{NMS} neuron activity on RP3V^{KISS1} neuron action potential firing. Because exogenous NMS had no impact on RP3V^{KISS1} neuron firing, we focused on the potential effect of synaptic transmission between SCN^{NMS} and RP3V^{KISS1} neurons.

Sustained optogenetic activation of SCN^{NMS} neuron projection fibers (600 pulses of blue light delivered at 10 Hz) caused an immediate and reversible suppression of spontaneous action potential firing in a majority of diestrous RP3V^{KISS1} neurons (14 out of 19 cells in 7 out of 7 mice with SCN transfection) or had no effect (Figure 7A and C). On proestrus, 600 pulse 10 Hz trains

suppressed firing in 10 out of 21 RP3V^{KISS1} neurons (6 out of 9 mice with SCN transfection; $p = 0.213$ for proportions of mice and $p = 0.117$ for proportions of cells, Fisher's exact tests; Figure 7B and C). Optogenetic stimulation-induced suppressions of firing did not statistically differ between diestrus and proestrus whether we compared all cells (diestrus $50.66 \pm 10.34\%$ vs. proestrus $32.16 \pm 10.61\%$; $p = 0.219$, unpaired t-test with Welch's correction; Figure 7D) or only those that met the criterion for a suppression of firing (diestrus $70.57 \pm 7.67\%$ vs. proestrus $68.84 \pm 9.31\%$; $p = 0.666$, Mann Whitney test; Figure 7E). The percentage of RP3V^{KISS1} cells in which optogenetic stimulation suppressed firing did not correlate with ZT_{death}. Likewise, the impact of optogenetic stimulation did not correlate with ZT_{death} and ZT_{recording} or with baseline firing rates (table 3). Suppressions of firing could also be seen in response to moderate stimuli (300 pulses at 5 Hz; $69.3 \pm 11.0\%$ suppression; $n = 7$ in 2 diestrous mice; not shown). By contrast, trains of light pulses (600 at 10Hz) failed to affect firing in RP3V^{KISS1} neurons from mice with mistargeted injections or from *Kiss1*-hrGFP mice ($n = 12$ cells in 6 mice; not shown), ruling out off-target light-induced effects.

We next investigated the role of GABA_A receptor activation in these responses. As illustrated by the example shown in figure 8A, in RP3V^{KISS1} neurons that showed a decrease in firing upon optogenetic stimulation (600 pulses at 10 Hz), blockade of GABA_A receptors with gabazine almost completely prevented the suppression of firing upon a second round of stimulation. The average time-course (Figure 8B) shows that suppression of firing in response to optogenetic activation of SCN^{NMS} neuronal projections was not seen in the presence of gabazine (5-10 mM). In fact, the light-induced suppression of RP3V^{KISS1} neuron firing ($73.15 \pm 11.99\%$) measured during the stimulus train was significantly smaller in the presence of gabazine ($-17.15 \pm 10.90\%$; $n = 7$ in 6 mice; $p = 0.002$, paired t-test; Figure 8C). This effect reflects blockade of GABA_A receptors rather than fatigue of ChR2^(ET/TC)-expressing fibers upon repeated stimuli. Indeed, figure 8D shows that blue light-induced (600 pulses at 10 Hz) suppressions could be

readily evoked by a second round of stimulation in the absence of gabazine (filled circles) or by a third round of stimulation after gabazine washout (filled diamonds). When pooled together, normalized suppressions upon the second or third stimulus ($86.40 \pm 8.71\%$) were similar in magnitude to those upon the first stimulus ($91.20 \pm 6.12\%$; $n = 6$ in 5 mice [4 diestrus and 1 estrus]; $p = 0.188$, Wilcoxon test).

Taken together, these findings indicate that synaptic release of GABA from SCN^{NMS} neuron projections suppresses RP3V^{KISS1} neuron action potential firing through activation of GABA_A receptors in slices obtained from diestrous and proestrous mice.

Discussion

We report here that NMS-expressing neurons in the SCN project to the RP3V region of the hypothalamus, establishing close appositions onto the vast majority of KISS1-ir somata. Furthermore, our ChR2-assisted circuit mapping (Petreanu et al., 2007) approach in brain slices revealed that a subset of SCN^{NMS} neurons provides GABAergic monosynaptic inputs to > 75% of RP3V^{KISS1} cells. Lastly, while we observed that exogenous NMS had no effect on firing and affected $[Ca^{2+}]_i$ in subsets of cells, GABA release from SCN^{NMS} neuronal projections suppressed RP3V^{KISS1} neuron action potential firing on diestrus and on proestrus.

SCN^{NMS} neurons projecting to RP3V^{KISS1} neurons

Our anterograde tract-tracing studies revealed that the great majority (80-95% on average) of RP3V^{KISS1} neurons received at least one close apposition by mCherry-ir fibers. Assessment of such appositions in animals in which the viral vector injection only gave rise to very sparse transfection (< 10 transfected SCN somata) indicates that most of this innervation originates in the SCN. As the SCN^{NMS} neuronal population includes most SCN AVP-ir neurons, and because SCN^{AVP} neurons project to RP3V^{KISS1} neurons (Vida et al., 2010; Jamieson et al., 2021), at least part of the KISS1 neuron innervation seen in *Nms*-Cre mice may be contributed by SCN^{AVP} cell projections. However, our observations suggest that an additional NMS-expressing SCN cell population, distinct from AVP neurons, might be providing inputs to KISS1 neurons. First, both the proportion of innervated cells and the number of close appositions per neuron were larger in the present tract-tracing experiment than in our previous report of projections of SCN^{AVP} neurons (Jamieson et al., 2021). Second, our functional assessment revealed a strikingly different pattern of RP3V^{KISS1} neuron synaptic transmission in *Nms*-Cre mice than in *Avp*-Cre mice in our previous studies (Jamieson et al., 2021). While in the latter, SCN^{AVP} cells rarely established functional synaptic inputs onto RP3V^{KISS1} neurons ($\approx$ 5% of KISS1

neurons), > 75% of KISS1 neurons exhibited synaptic currents in response to optogenetic stimulation of SCN^{NMS} neuronal fibers. Together these observations indicate that, in addition to AVP neurons, a subpopulation of SCN^{NMS} neurons that do not co-express AVP provides synaptic inputs to RP3V^{KISS1} neurons. It is unlikely that this subpopulation comprises SCN vasoactive intestinal peptide (VIP)-expressing cells, reported to overlap with the NMS population (Lee et al., 2015). Indeed, it was previously reported that VIP neurons do not project extensively to the RP3V (Abrahamson and Moore, 2001; Vida et al., 2010) and our findings indicate that SCN VIP-expressing cells do not communicate with RP3V^{KISS1} neurons *via* fast synaptic transmission. Therefore, SCN^{NMS} neurons that establish GABAergic synaptic inputs onto RP3V^{KISS1} neurons are likely neither AVP- nor VIP-expressing.

Interestingly, we observed that while KISS1 neurons consistently received synaptic inputs from SCN^{NMS} neurons, this might not be the case of other RP3V neurons. Indeed, in our relatively small sample, we found evidence of oPSCs in a significantly smaller proportion of GFP^{neg} neurons compared with GFP^{pos} cells. In addition, oPSCs in the former were substantially smaller and required higher stimulation intensity than in the latter. These observations suggest that SCN^{NMS} neuron synaptic innervation might be, to a certain degree, specific to RP3V^{KISS1} neurons. Given the role that these cells are thought to play in the LH surge (Piet, 2023), we speculate that their preferential synaptic innervation by SCN^{NMS} neurons might be involved as well.

Impact of SCN^{NMS} neuron projections on RP3V^{KISS1} neuron firing

Using electrophysiology and GCaMP-based [Ca²⁺]_i imaging, we observed that exogenous NMS had no effect on action potential firing but increased [Ca²⁺]_i in a subset of RP3V^{KISS1} cells. The latter effect is mostly mediated indirectly, although some NMS-induced stimulation remained in the presence of TTX and synaptic receptor antagonists, consistent with the low expression

levels of the *Nmur2* gene, which encodes a G-protein coupled receptor that binds NMS, in RP3V^{KISS1} neurons (Stephens and Kauffman, 2021; Tonsfeldt et al., 2022). This suggests that NMS, potentially released by SCN^{NMS} neurons, might not be a primary driver of RP3V^{KISS1} neuron electrical activity, although intracellular effects cannot be discounted. Additional experiments will be required to test this further.

Release of GABA upon optogenetic stimulation, on the other hand, had a distinct effect on KISS1 neuron activity. Activating SCN^{NMS} projections fibers in the RP3V indeed resulted in GABA_A receptor-mediated suppression of firing in many KISS1 cells (~48-74%) in slices from diestrous and proestrous mice. Similar GABA-dependent inhibitory responses were previously observed in RP3V^{KISS1} neurons in response to optogenetic activation of SCN^{AVP} projection fibers in slices from estrous females. These responses, however, were not associated with synaptic communication between SCN^{AVP} and RP3V^{KISS1} neurons and were rarely seen in diestrous and proestrous KISS1 neurons (Jamieson et al., 2021), providing further evidence that the GABAergic SCN^{NMS} → RP3V^{KISS1} neuronal input likely originates from NMS neurons that do not express AVP.

Potential role of SCN^{NMS} → RP3V^{KISS1} projections in the preovulatory surge

Previous studies have revealed a prominent role of neuropeptides in the circadian control of the GnRH/LH surge, with evidence that AVP can restore the surge in circadian clock-deficient rodents (Palm et al., 1999; Miller et al., 2006), and that AVP signaling contributes to surge generation (Funabashi et al., 1999) – although this has not been consistently seen (Palm et al., 2001; Miller et al., 2006). In addition, prokineticin 2, a neuropeptide important for circadian rhythms (Cheng et al., 2002), and VIP signaling may also play a role in the surge (Harney et al., 1996; van der Beek et al., 1999; Xiao et al., 2014). These in vivo observations are corroborated by reports that exogenous AVP and VIP stimulate action potential firing in RP3V^{KISS1} and GnRH

neurons, respectively, ex vivo (Christian and Moenter, 2008; Piet et al., 2015; Piet et al., 2016; Constantin et al., 2024).

By contrast, the data presented here show that a subpopulation of SCN^{NMS} neurons suppresses RP3V^{KISS1} neuron activity *via* synaptic GABA release, an effect that does not substantially vary from diestrus to proestrus, at least under our recording conditions. Together with our previous observations that SCN^{AVP} neurons release AVP onto RP3V^{KISS1} neurons and drive their action potential firing on proestrus (Jamieson et al., 2021), this indicates that distinct SCN^{NMS} neuronal subpopulations may exert opposing influences over RP3V^{KISS1} neuron firing, exciting these cells through the release of AVP and inhibiting them through the release of GABA. We, thus, speculate that GABA-mediated suppression of RP3V^{KISS1} neurons – along with plasticity in their AVP-dependent activation (Jamieson et al., 2021) – might contribute to preventing inappropriate activation of these cells by daily SCN signals (Piet, 2023) on days of the cycle other than proestrus, whereas AVP-mediated excitation would drive their stimulation on proestrus (Jamieson et al., 2021). In addition, because amino acid neurotransmitter release and neuropeptide release require different rates of presynaptic activity (Liu et al., 2011; Schone et al., 2014; Piet and Herbison, 2018), it is possible that low frequency SCN^{NMS} neuron action potential firing would preferentially release GABA, thereby suppressing RP3V^{KISS1} neuron activity, whereas higher frequency SCN^{NMS} neuron activity would cause the release of both GABA and AVP, potentially driving KISS1 neuron activity. Although the firing patterns of SCN^{NMS} neurons in vivo are unknown, it is worth noting that unidentified SCN neurons fire faster during the (subjective) day than during the (subjective) night in vivo and *in vitro* [see, for example, (Nakamura et al., 2008; Hermansteyne et al., 2023)] and that SCN^{AVP} neurons fire at rates up to 10 Hz during the subjective day in brain slices (Thirouin et al., 2023) in rodents. We speculate, therefore, that activity-dependent release of GABA vs. AVP contributes to timing activation of RP3V^{KISS1} neurons

for the preovulatory LH surge, occurring in the hours that precede darkness. Further work will be needed to directly test these hypotheses.

Limitations

Our observations should be interpreted in light of the limitations associated with the transgenic mouse strains we used (see methods). Indeed, in *Kiss1*-hrGFP mice, not all RP3V hrGFP cells are KISS1-ir. Our electrophysiological recordings, thus, may include RP3V neurons that are not kisspeptin-expressing cells. We note, however, that the percentage of hrGFP neurons that receive synaptic transmission from SCN^{NMS} neurons in electrophysiological studies is comparable to that of KISS1-ir neurons receiving close apposition by SCN^{NMS} neuron fibers in tract-tracing experiments. This suggests that what we observed using hrGFP to target kisspeptin neurons is, for the most part, a good reflection of the situation in *bona fide* kisspeptin neurons.

Conclusion

Despite the limitations listed above, the findings reported here indicate that control of the GnRH neuronal network by the central circadian clock for the preovulatory surge might go beyond the classical AVP and VIP neuropeptides. Indeed, our studies reveal that a subpopulation of NMS neurons, which does not appear to overlap with neuronal populations that produce AVP or VIP, establishes synaptic inputs onto RP3V^{KISS1} neurons and suppresses their firing through synaptic GABA release. Although it is unknown whether those RP3V^{KISS1} neurons that receive these inputs also project to GnRH neurons, our observations suggest a previously unsuspected pathway through which the central circadian clock might regulate the activity of the GnRH neuronal network and, potentially, the preovulatory LH surge.

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Figure legends

Figure 1: SCN^{NMS} neurons extend projections to RP3V^{KISS1} neurons.

A: maximum projection confocal image (10x objective) showing mCherry-ir (magenta) in the SCN of an *Nms*-Cre female injected bilaterally with the AAV-DIO-mCherry vector. 3V = third ventricle; OC = optic chiasm. **B:** mCherry-ir fibers innervate the RP3V and are seen in the vicinity of KISS1-ir (green) somata (maximum projection image; 20x objective). Insets (i, ii, iii) are single confocal sections (40x objective) showing KISS1-ir somata receiving close appositions (arrows) by mCherry-ir fibers. **C-D:** Example of a female in which the AAV-DIO-mCherry injection was mistargeted (**C**). Few mCherry-ir fibers are seen in the RP3V and in the vicinity of KISS1-ir somata (**D**). Single confocal sections show the relative absence of mCherry-ir fibers near KISS1-ir cells, with rare close appositions (arrow). **E-H:** Quantifications of SCN transfection (**E**), RP3V innervation (**F**), percentage of KISS1-ir somata with close appositions (**G**) and number of appositions per KISS1-ir soma (**H**) in mice in which stereotaxic injections hit (orange, n = 8) or missed (blue, n = 4) the SCN (see methods). *** p ≤ 0.001, unpaired t-test with Welch's correction. mCherry-ir is shown in magenta to enhance contrast with the dark background.

Figure 2: Optogenetic activation of SCN^{NMS} neuron projection fibers evokes synaptic transmission in RP3V^{KISS1} neurons.

A: Action potential firing (spike fidelity) of ChR2^(ET/TC)-expressing SCN^{NMS} neurons in response to blue light pulses delivered at various frequencies. Inset shows an example response to a 10 Hz train. **B:** Sample traces illustrating that blue light evoked PSCs in RP3V^{KISS1} neurons in slices from mice in which AAV-DIO-ChR2^(ET/TC) was successfully injected in the SCN (SCN “hit”; *top*) but not in slices from mice in which the injection missed (SCN “miss”; *bottom*). Traces are 10 individual sweeps (gray) and their average (red) overlaid. **C:** oPSCs were detected in at least one RP3V^{KISS1} neuron in the great majority of mice with transfection in the SCN but never in those without SCN transfection (*left*). Blue light stimulation evoked oPSCs in the majority (> 75%) of RP3V^{KISS1} neurons recorded in slices from mice with SCN transfection. This was not seen in RP3V^{KISS1} neurons recorded in slices from mice without SCN transfection (*right*). *** $p < 0.001$ Fisher’s exact test. **D:** Spike fidelity of ChR2^(ET/TC)-expressing SCN^{VIP} neurons in response to blue light stimuli at various frequencies. Inset shows an example response to a 10 Hz train. **E:** Sample traces illustrating that blue light did not evoke PSCs in RP3V^{KISS1} neurons in slices from mice that were transduced with AAV-DIO-ChR2^(ET/TC) in the SCN. Traces are 10 individual sweeps (gray) and their average (red) overlaid. Blue rectangles indicate the timing of the stimuli. **F:** None of the 14 RP3V^{KISS1} neurons recorded in 3 mice exhibited oPSCs in response to blue light stimuli.

Figure 3: SCN^{NMS} → RP3V^{KISS1} synaptic transmission is monosynaptic.

A: Sample traces illustrating graded synaptic responses evoked by increasing stimulus intensities in an RP3V^{KISS1} neuron recorded in a brain slice obtained from an *Nms-Cre; Kiss1-hrGFP* mouse transduced AAV-DIO-ChR2^(ET/TC) in the SCN. Traces are averages of 10 sweeps.

B: Graph summarizing data obtained from 10 different cells in which oPSCs were evoked by increasing blue light stimulation intensities. oPSC amplitude increased as a function of light intensity and plateaued at intensities ≥ 10% of maximum light intensity. **C:** Overlaid successive sweeps (oPSCs evoked with 50% light intensity) showing the latency and jitter of oPSC onset.

D: Quantifications in the 10 cells used in *B* revealed short synaptic latencies and submillisecond jitter, suggesting activation of a monosynaptic input. **E:** Average oPSCs recorded in an RP3V^{KISS1} neuron under control conditions, in the presence of TTX (0.5 mM) and in the presence of TTX and 4-AP (100 mM). **F:** Summary graph of the effect of TTX and TTX+4-AP in 9 different cells. *** $p < 0.001$ repeated measure one-way ANOVA; groups with different letters were significantly different ($p < 0.01$) in Tukey's multiple comparisons test. Blue rectangles in *A*, *C* and *E* indicate the timing of light stimulation.

Figure 4: SCN^{NMS} → RP3V^{KISS1} synaptic transmission involves the release of GABA.

A: Average oPSCs recorded in an RP3V^{KISS1} neuron under control conditions, in the presence of ionotropic glutamate receptor antagonists NBQX (20 mM) and D-AP5 (40 mM), and after washout. **B:** Quantification of the effect of NBQX and D-AP5 on oPSC amplitude. Mixed-effect model analysis revealed that amplitude did not significantly change across treatments (n = 8). **C:** Average oPSCs recorded in another RP3V^{KISS1} neuron under control conditions, in the presence of the GABA_A receptor antagonist gabazine (5 mM), and after washout. **D:** Summary graph of the reversible gabazine-induced suppression of oPSC amplitude in 10 different cells. *** p < 0.001 Friedman test; groups with different letters were significantly different (p < 0.05) in Dunn's multiple comparisons test. Blue rectangles in A and C indicate the timing of light stimulation.

Figure 5: KISS1 neurons are preferential synaptic targets of SCN^{NMS} neuron projections in the RP3V.

A: Cartoons illustrating the recording protocol. GFP^{pos} (KISS1; *left*) and GFP^{neg} (non-KISS1; *right*) were recorded in WVCV while ChR2^(ET/TC)-expressing fibers were stimulated with blue light in the RP3V. GFP^{pos} and GFP^{neg} neurons were recorded in succession and were in the vicinity of one another. **B,C:** Average traces showing synaptic responses (or lack thereof) to blue light stimulation in RP3V cells recorded in slices from two different mice. Note the small amplitude oPSC evoked in a GFP^{neg} neuron in C. Blue rectangles indicate the timing of light stimuli. **D:** Most ($\approx 90\%$) RP3V^{KISS1} neurons, but only a third of unidentified RP3V cells, received SCN^{NMS} synaptic innervation. ** $p = 0.009$ Fisher's exact test, $n = 12$ in each group. **E:** oPSC amplitude was significantly larger in KISS1 neurons than in unidentified neurons. *** $p < 0.001$ Mann Whitney test.

Figure 6: NMS does not change action potential firing but alters $[Ca^{2+}]_i$ in subsets of RP3V^{KISS1} cells.

A: Example traces and corresponding rate meters (1 second bins) of RP3V^{KISS1} neuron firing in response to aCSF local applications (10 seconds). Cells were either unaffected (*top*) or showed increased firing (*bottom*). **B:** Example traces and rate meters illustrating the effects of NMS local applications (1 mM; 10 seconds). NMS had no discernable effect (*top*) or increased action potential firing (*bottom*) in RP3V^{KISS1} neurons. **C:** Example traces showing the effect of AVP local applications (1 mM; 10 seconds). AVP-induced increases in firing were consistently observed. **D:** Two-way ANOVA analysis showed a significant main effect of pressure applications ($p < 0.001$) but no difference across treatments (aCSF vs. NMS vs. AVP; $p = 0.254$). There was, however, a significant interaction between these two factors ($p = 0.014$). Post-hoc analyses revealed a significant baseline vs. local application difference for AVP ($** p < 0.001$) but not for aCSF ($p = 0.063$) or for NMS ($p = 0.054$; Fisher's LSD tests). There were no differences in baseline firing ($^a p = 0.731-0.950$) but significant differences after local pressure applications ($^b p = 0.944$ aCSF vs. NMS; $^c p = 0.026$ aCSF vs. AVP and $p = 0.019$ NMS vs. AVP). **E:** Mean intensity projection image ($z = 30$ seconds) showing GCaMP6f expression in the RP3V of a female *Kiss1-Cre*;GCaMP6f mouse. **F:** Normalized GCaMP6f fluorescence traces for the five cells circled in *E*. While NMS bath application (1 mM; 2 minutes) did not affect GCaMP6f fluorescence in some cells (1, 2), it increased (3, 4) or decreased (5) it in others. **G:** Graph summarizing the percentage of KISS1 cells excited or inhibited by NMS ($n = 10$ slices). In the presence of a cocktail of TTX (0.5 mM) and synaptic receptor antagonists (NBQX 10 mM, D-AP5 40 mM and gabazine 5 mM), NMS increased $[Ca^{2+}]_i$ in a significantly smaller percentage of RP3V^{KISS1} neurons ($n = 7$ slices; $p < 0.05$ unpaired t-test with Welch's correction) than under control conditions. Inhibition percentages were compared with a Mann-Whitney test.

Figure 7: Optogenetic activation of SCN^{NMS} neuron projection fibers suppresses RP3V^{KISS1} neuron action potential firing.

A,B: Example traces and corresponding rate meters (5 second bins) illustrating that optogenetic activation of SCN^{NMS} neuronal fibers (600 pulses delivered at 10 Hz) in the RP3V suppresses (Left) or does not affect (Right) diestrous (**A**) and proestrous (**B**) KISS1 neuron action potential firing. **C:** Summary graph of the percentages of diestrous and proestrous *Nms-Cre;Kiss1-hrGFP* mice (Left) and RP3V^{KISS1} neurons (Right) in which optogenetic stimulation suppressed firing. Proportions were compared using Fisher's exact tests. **D,E:** Quantifications of action potential firing suppression in all cells (**D**; unpaired t test with Welch's correction) or in those cells showing suppressed firing (**E**; Mann Whitney test) revealed no difference between diestrous and proestrous RP3V^{KISS1} neurons.

Figure 8: GABA release from SCN^{NMS} neuron projection fibers suppresses RP3V^{KISS1} neuron activity.

A: Example RP3V^{KISS1} neuron in which blue light stimulation suppressed action potential firing under control conditions (*left*) but not in the presence of gabazine (5 mM; *right*). Rate meters (5 second bins) are displayed below traces. **B:** Superimposed time courses of RP3V^{KISS1} neuron action potential firing in the absence (filled circles) and in the presence of gabazine (5-10 mM; filled squares) in 7 different neurons. Note the absence of light-induced suppression in the presence of gabazine. **C:** Summary graph of light-induced suppression of RP3V^{KISS1} neuron firing in the absence and in the presence of gabazine. ** $p = 0.002$ paired t-test. **D:** Light-induced suppression of firing in response to consecutive stimuli in the absence of gabazine. Filled circles are responses to first and second stimuli and filled diamonds are responses to first and third stimuli, after gabazine washout. Suppression of firing was repeatable and consistent upon multiple stimuli (Wilcoxon matched-pairs signed rank test).

	Diestrus	Proestrus	Estrus
# mice with oPSCs / total # mice ^a	12/13	6/6	9/9
# cells with oPSCs / total # cells ^b	25/38	14/14	17/20
oPSC amplitude (-pA) ^c	522.77 ± 69.95	669.48 ± 100.60	484.30 ± 110.22

Table 1: SCN^{NMS} neuron to RP3V^{KISS1} neuron synaptic transmission across the estrous cycle.

^a p > 0.999 diestrus vs. proestrus, diestrus vs. estrus, proestrus vs. estrus; Fisher's exact test.

^b p = 0.011 diestrus vs. proestrus, p = 0.215 diestrus vs. estrus, p = 0.251 proestrus vs. estrus; Fisher's exact test.

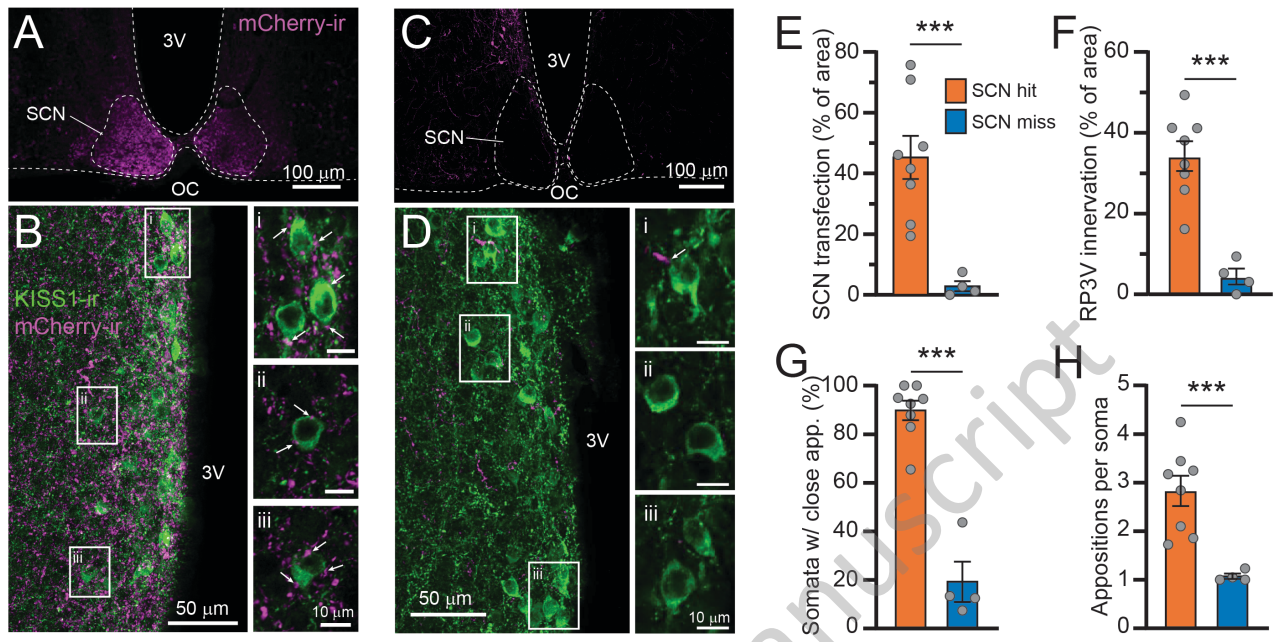
^c p = 0.210 Kruskal-Wallis test.

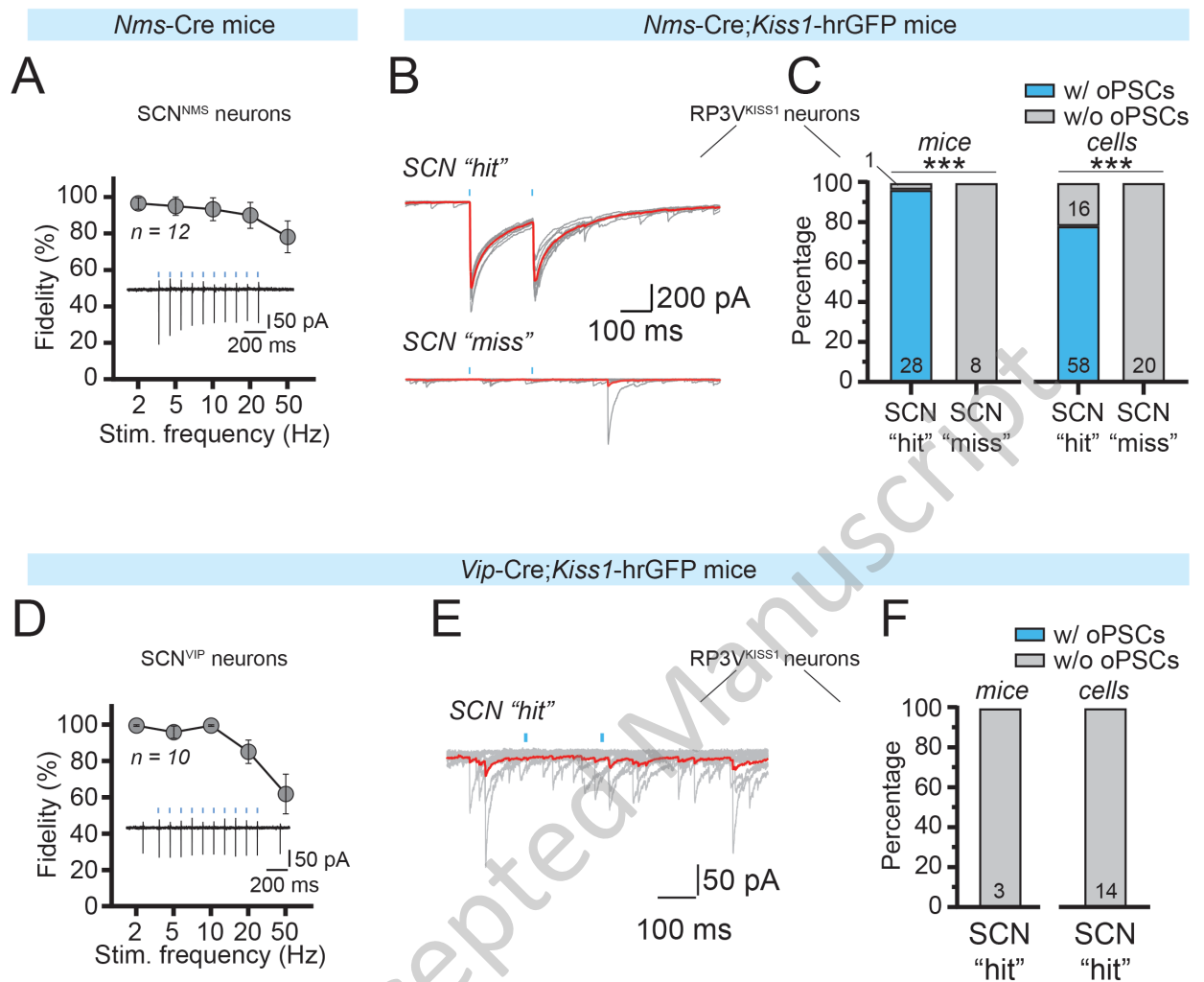
	ZT _{death}	ZT _{recording}	Baseline firing
Percentage of cells with oPSCs	R ² = 0.0248 p = 0.424	/	/
oPSC amplitude	R ² = 0.0011; p = 0.801	R ² = 0.0153; p = 0.354	/
aCSF local applications	R ² = 0.1435; p = 0.251	R ² = 0.0247; p = 0.627	R ² = 0.1781; p = 0.196
NMS local applications	R ² = 0.0053; p = 0.821	R ² = 0.0174; p = 0.682	R ² = 0.1359; p = 0.238
AVP local applications	R ² = 0.1210; p = 0.223	R ² = 0.1911; p = 0.118	R ² = 0.0692; p = 0.364
Percentage of cells with NMS-induced increases in [Ca ²⁺] _i	R ² = 0.0853; p = 0.255	R ² = 0.0129; p = 0.664	/
Percentage of cells with NMS-induced decreases in [Ca ²⁺] _i	R ² = 0.1732; p = 0.097	R ² = 0.1008; p = 0.214	/
Percentage of cells with suppression of firing	R ² = 0.1711; p = 0.111	/	/
Blue light train-induced suppression of firing	R ² = 0.0550 p = 0.145	R ² = 0.0352 p = 0.246	R ² = 0.0013 p = 0.823

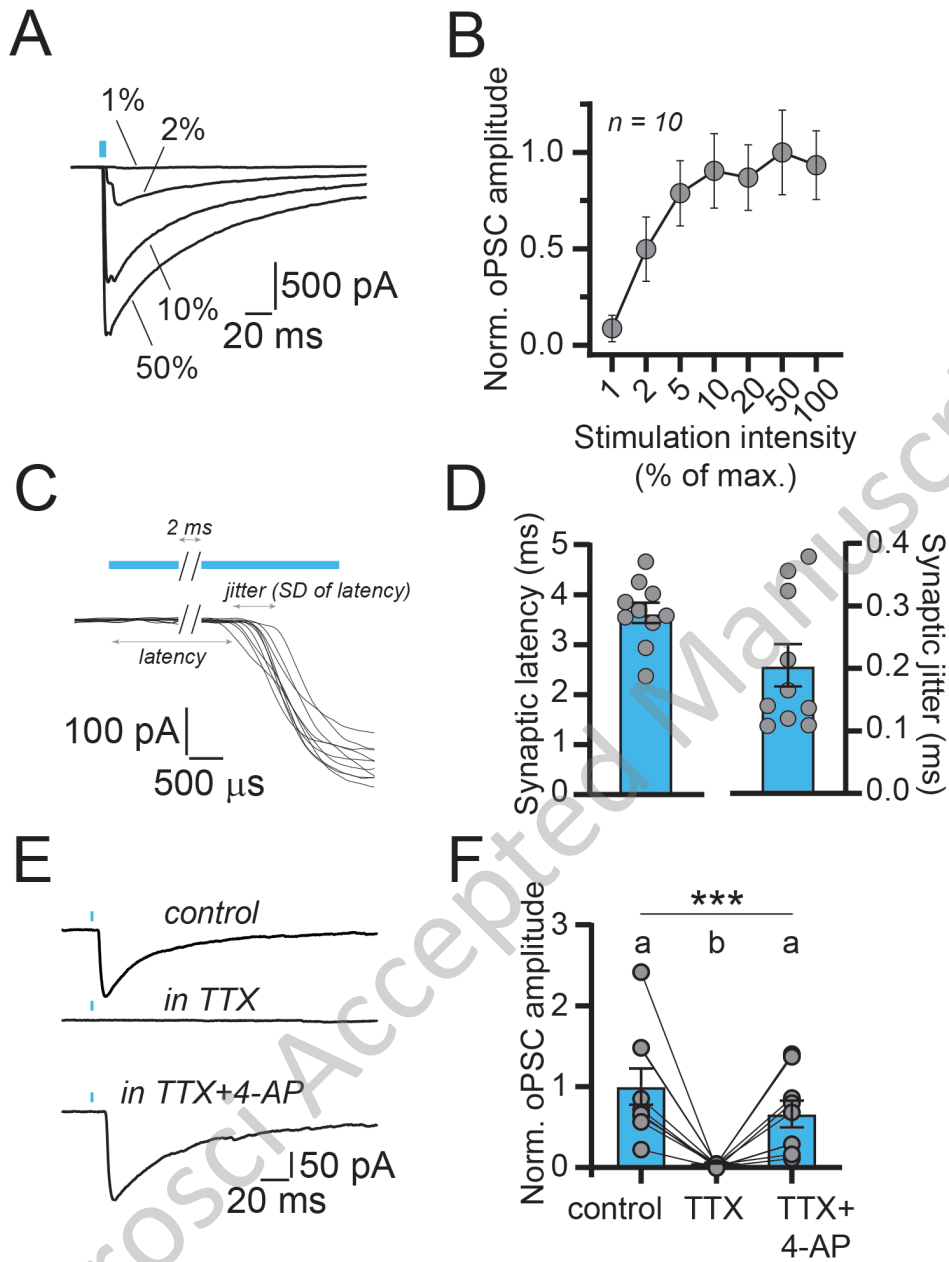
Table 2: Linear regression analyses of the impact of ZT or baseline firing rate on SCN^{NMS} → RP3V^{KISS1} neurotransmission, the effect of local pressure applications on RP3V^{KISS1} neuron firing and the effect of NMS on RP3V^{KISS1} neuron [Ca²⁺]_i, as applicable.

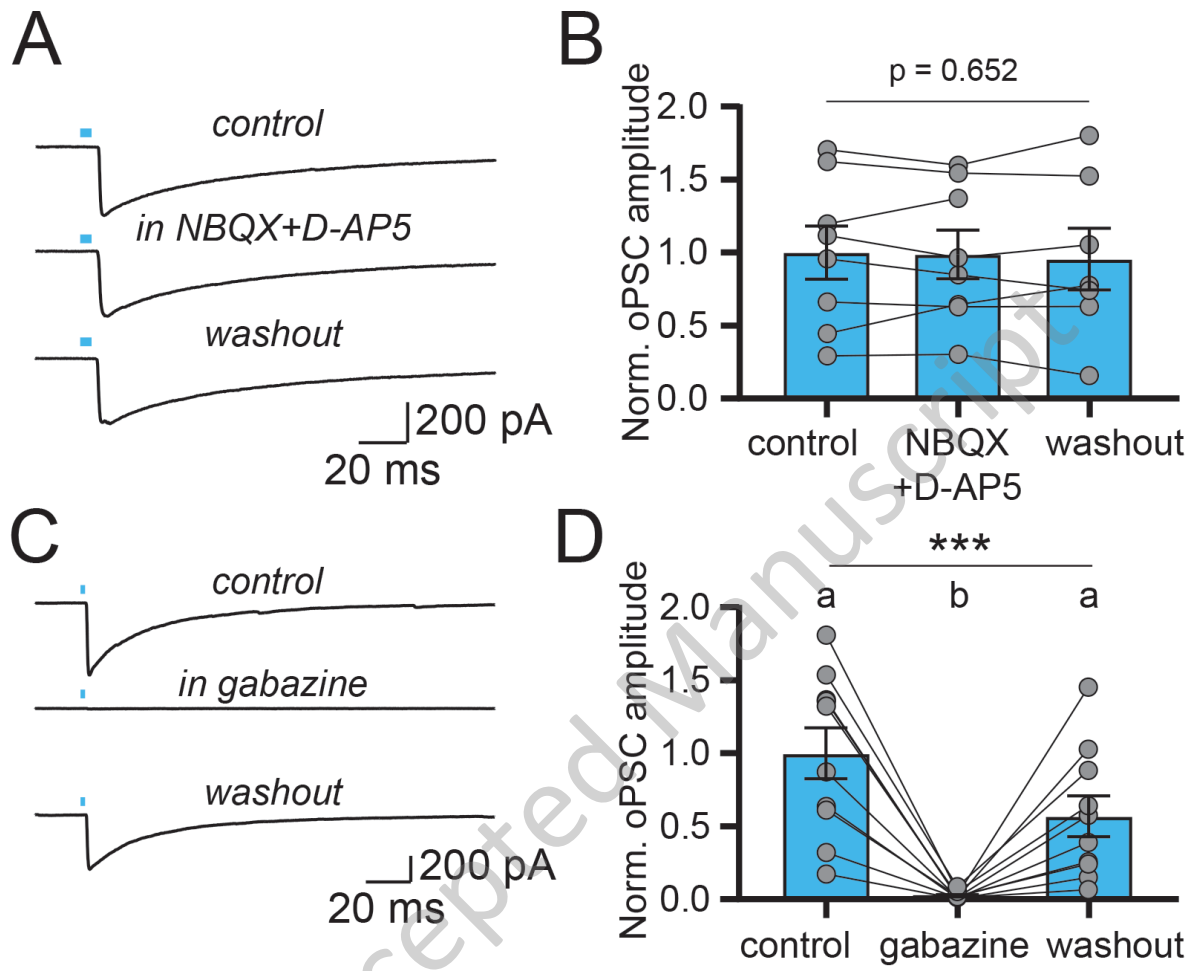
	Baseline firing (Hz)	Firing after pressure application (Hz)	Change in firing (Hz)
aCSF	2.37 ± 0.62	3.46 ± 0.77	1.09 ± 0.26
NMS	2.31 ± 0.58	3.39 ± 0.86	1.08 ± 0.47
AVP	2.64 ± 0.38	5.73 ± 0.87	3.09 ± 0.68

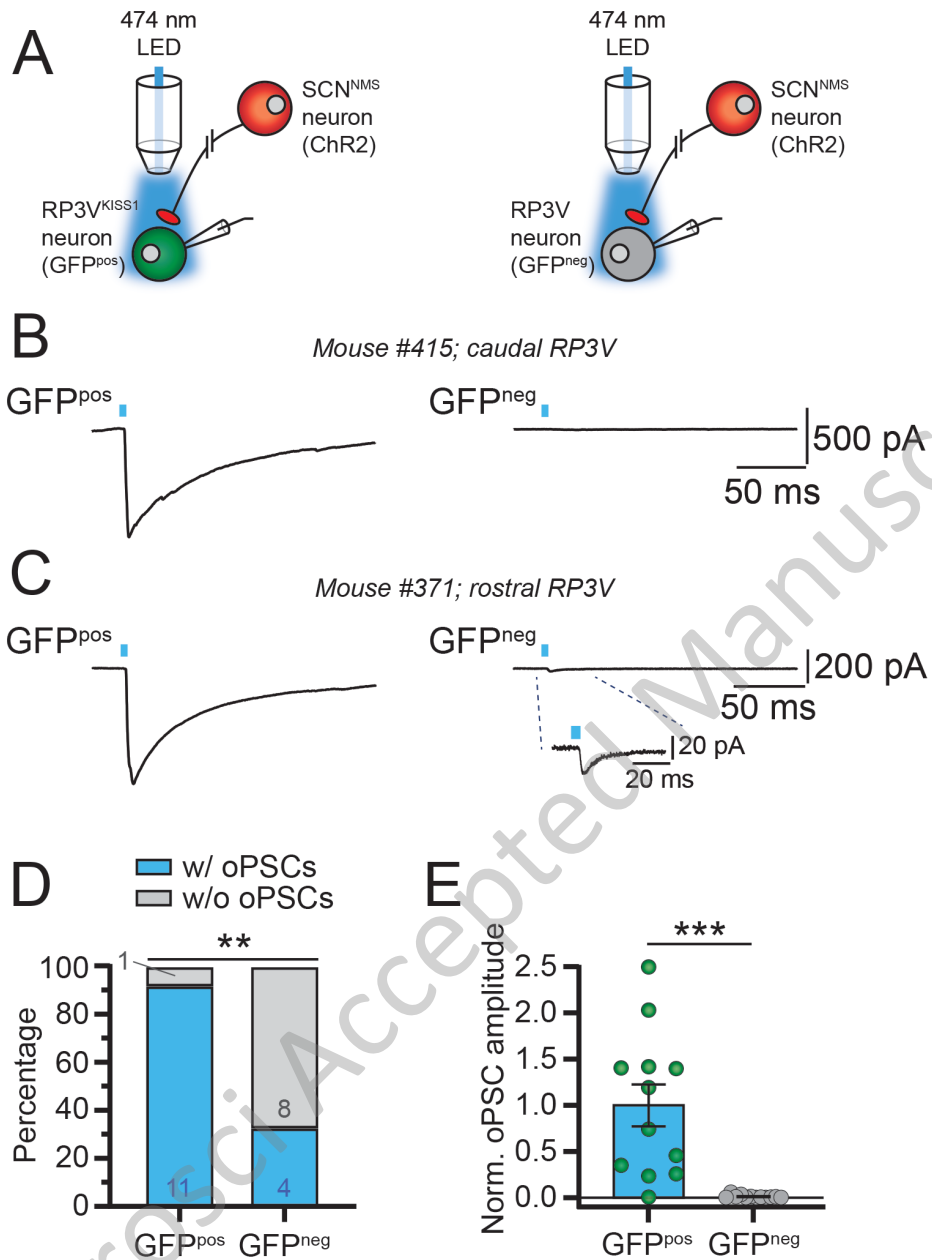
Table 3: RP3V^{KISS1} neuron firing in local pressure application experiments. Bold characters indicate statistically significant differences (see text for details).



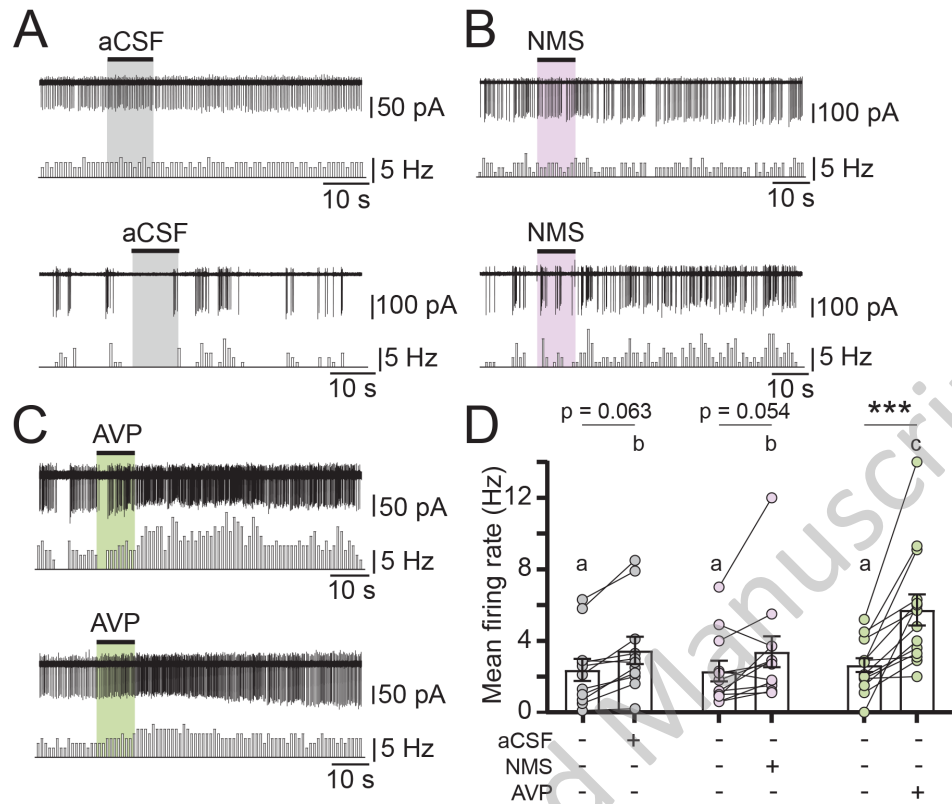




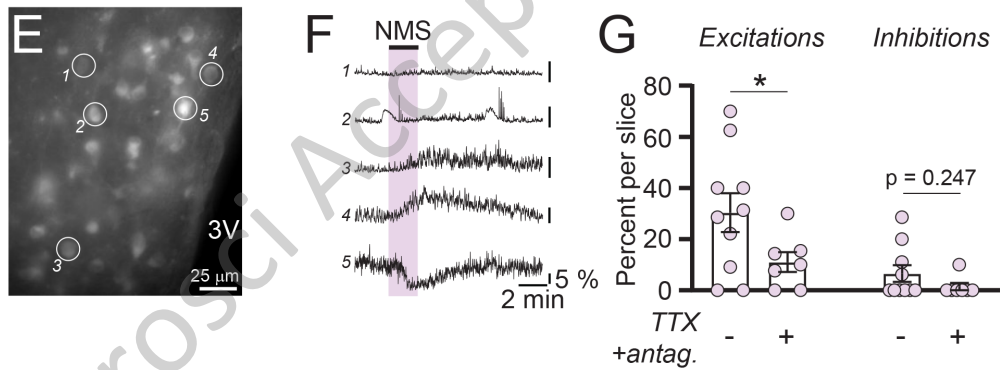


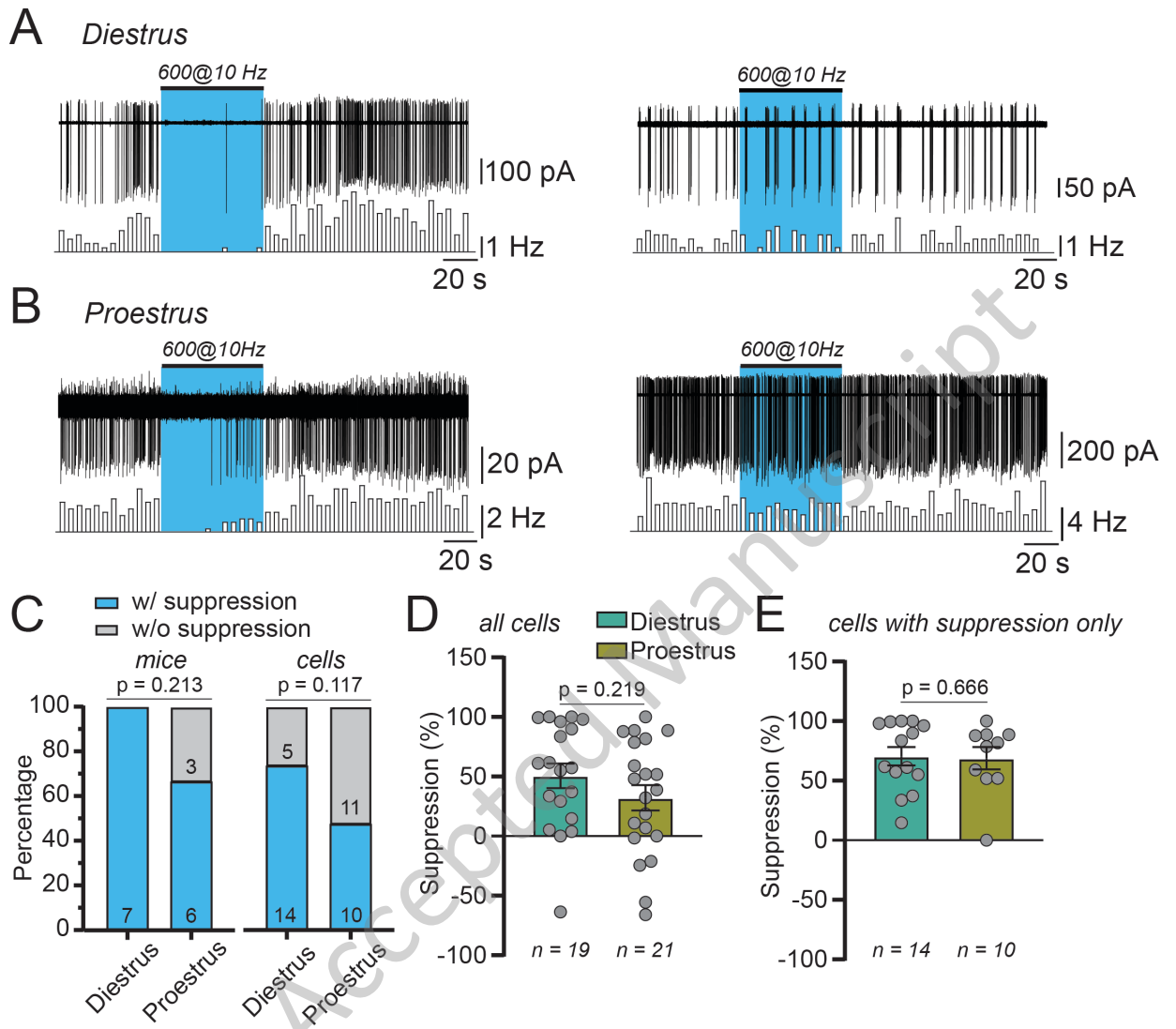


Kiss1-hrGFP mice

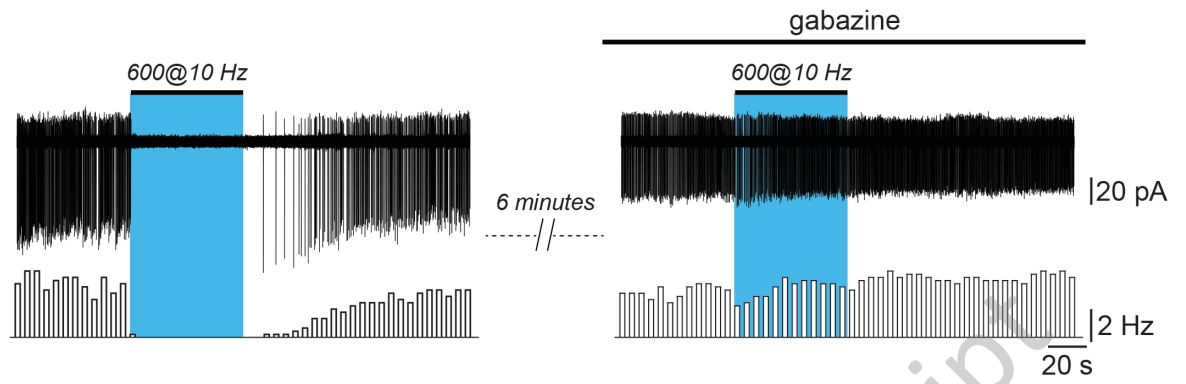


Kiss1-Cre;GCaMP6f mice

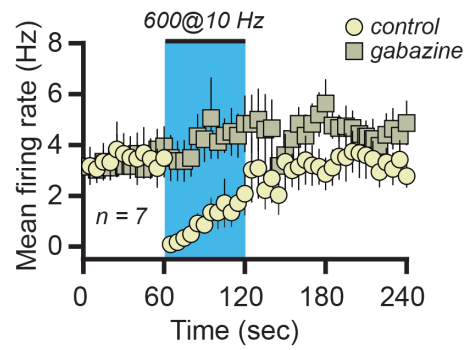




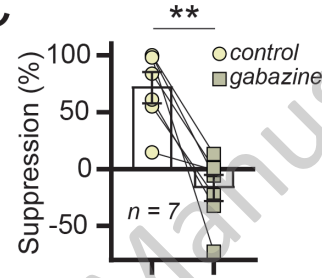
A



B



C



D

