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**Optogenetic probing of fast glutamatergic transmission from hypocretin/orexin to
histamine neurons *in situ***

Running title: "Fast transmission in hypocretin/orexin → histamine circuit"

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ABSTRACT

Hypothalamic hypocretin/orexin (hcrt/orx) neurons coordinate sleep-wake cycles, reward-seeking, and body energy balance. Neurochemical data suggest that hcrt/orx cells contain several transmitters, but what hcrt/orx cells release onto their projection targets is unknown. A major pathway by which hcrt/orx neurons are thought to promote arousal is through projections to tuberomammillary histamine (HA) neurons. To study the impact of the electrical activity of hcrt/orx cells at this site, we genetically targeted the light-activated excitatory ion channel ChR2 to the plasma membrane of hcrt/orx cells, and performed patch-clamp recordings from HA cells in acute mouse brain slices. Stimulation of ChR2-containing fibres with millisecond flashes of blue light produced fast post-synaptic currents in HA neurons, with a high connection probability ($\approx 60\%$ of HA cells were connected to $\approx 40\%$ of hcrt/orx cells expressing ChR2). These inputs depended on tetrodotoxin-sensitive action potentials, had kinetics typical of glutamatergic responses mediated by AMPA receptors, were blocked by the AMPA receptor blocker CNQX, and displayed multiple forms of short-term plasticity (depression in $\approx 70\%$ trials, facilitation in $\approx 30\%$ trials, both often in the same cell). Furthermore, stimulation of hcrt/orx axons at physiological frequencies rapidly and reversibly increased action potential firing in HA cells. This stimulation was abolished by blockade of AMPA receptors. These results provide the first functional evidence that hcrt/orx neurons are capable of fast glutamatergic control of their projection targets, and suggest that variations in electrical activity of hcrt/orx axons can induce rapid changes in long-range signals generated by HA neurons.

INTRODUCTION

Hypothalamic hypocretin/orexin (hcrt/orx) neurons project widely throughout the brain and orchestrate sleep-wake cycles, reward-seeking, and body energy balance (de Lecea et al., 2006; Sakurai, 2007). Loss of hcrt/orx cells leads to narcolepsy and obesity (Nishino et al., 2000; Thannickal et al., 2000; Hara et al., 2001), their selective opto- or pharmacogenetic silencing induces slow-wave sleep, while their opto- or pharmacogenetic stimulation of hcrt/orx cells causes awakening (Tsunematsu et al., 2011; Sasaki et al., 2011; Adamantidis et al., 2007). However, how the endogenous firing of hcrt/orx cells regulates their projection targets is unclear due to technical difficulties associated with non-selective electrical stimulation of hcrt/orx cell axons *in situ*. Functional studies of this issue are important because the effect of hcrt/orx cell firing on downstream targets is not clear from current neurochemical data. Indeed, such data suggest that hcrt/orx neurons may contain both excitatory and inhibitory transmitters, such as hcrt/orx, dynorphin (Chou et al., 2001), glutamate (Torrealba et al., 2003; Henny et al., 2010), and GABA (Harthoorn et al., 2005).

To study the neurochemical and electrical impact of electrical activity in endogenous hcrt/axons, we employed recently-developed optogenetic tools (Bernstein et al., 2011; Yizhar et al., 2011) for selective optical activation of hcrt/orx cells in acute mouse brain slices. Through viral gene targeting, we expressed the light-activated excitatory ion channel channelrhodopsin-2 (ChR2) selectively in hcrt/orx cell membrane, which allows fast electrical control of action potential firing with blue light, even in axons severed from cell bodies (Petreanu et al., 2007). We then explored the effects of fast and selective optogenetic stimulation of hcrt/orx cell axons one of their key projection targets, the histamine (HA) neurons in the tuberomammillary hypothalamus. The HA neurons are innervated by hcrt/orx cell axons and express hcrt/orx type-2 receptors, while HA signalling is important for arousal

induced by exogenously applied hcrt/orx (Eriksson et al., 2001; Huang et al., 2001; Yamanaka et al., 2002). However, it is unknown how the physiological output (firing rate) of HA cells is affected by endogenous firing of hcrt/orx cells at physiological frequencies. By combining whole-cell patch-clamp recordings from HA neurons with optogenetic stimulation of hcrt/orx cell axons in acute mouse brain slices, we asked whether hcrt/orx cell terminals can release fast transmitters, and whether changes in electrical activity of hcrt/orx cells can influence the firing rate of HA neurons.

METHODS

Gene transfer

Animal procedures followed UK Home Office regulations. Adenoviruses with ChR2 constructs flanked by a FLEX switch (Figure 1A, see (Atasoy et al., 2008)) were infused into the LH of orexin-cre mice (Matsuki et al., 2009; Sasaki et al., 2011). The mice were bred in het-WT breeding pairs with C57BL/6 mice. 4-6 week-old male and female mice were anesthetized with isoflurane and placed in a stereotaxic frame (David Kopf Instruments). A hole was drilled in the skull and a borosilicate glass pipette with a 20-40 μm tip diameter inserted into the brain. Three 50 nl injections were made at: -1.3 to -1.4 mm from bregma; -0.9 mm from midline; and -5.30 mm, -5.15 mm, and -5.00 mm from skull surface. A precision pump (Harvard instruments) was used to control injection speed at 75 nl/min, and pipette was withdrawn 6 min after the final injection (Adamantidis et al., 2007). The titer of rAAV2/1.CAGGS-FLEX-rev-ChR2tdtomato-SV40 construct (Addgene #18917) was 1.42×10^{13} genomic copies/ml. The titer of rAAV2/1.EF1a-DIO-hChR2(H134R)-EYFP.WPRE.hGH (Addgene #20298) was 1.5×10^{12} genomic copies/ml. Viral vectors were produced by Vector core, University of Pennsylvania.

Electrophysiology and photostimulation

Acute slices containing the LH or ventral tuberomammillary nucleus (TMN) were prepared 4-6 weeks post-injection (i.e. from 8-12 week old mice), as described in our previous studies (Williams et al., 2011). 250 μm thick coronal sections were cut using Leica VT 1200S vibratome while immersed in ice-cold ACSF (see below). After one hour recovery at 35 °C in ACSF, slices were used for recordings. TdTomato or eYFP containing neurons were visualized,

respectively, using an mCherry filter set (Chroma) or customised filter set (excitation 510/10 nm, dichroic 520 nm; emission 542/27 nm; from Laser 2000). To stimulate ChR2, we used a LAMBDA DG-4 fast filter-switcher (Sutter Instruments) with a Xenon lamp and ET470/40 nm band pass filter. Blue light ($\sim 10 \text{ mW/mm}^2$) was delivered onto ChR2-FP axons around the recorded cell via a 40x 0.8 NA objective. Patch pipettes were made from borosilicate glass, and their tip resistances were 4-6 M Ω with K-gluconate solution (see below). Whole-cell recordings were performed at 37 °C using an EPC-10 amplifier and PatchMaster software (HEKA Elektronik, Germany). Only cells with access resistances of <20 M Ω were used for analysis. Current signals were low-pass filtered at 3 kHz and digitized at 10 kHz. Averaged data are presented as mean \pm SEM. Statistical significance was evaluated using Student's t-test except where stated otherwise. Statistical analysis of PSCs was performed using MiniAnalysis (Synaptosoft) as in our previous study (Schone et al., 2011).

Chemicals and solutions

ACSF was gassed with 95% O₂ and 5% CO₂ and contained (in mM): 125 NaCl, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 21 NaHCO₃, 1 d-(+)-glucose, 0.1 Na⁺-pyruvate and 0.4 ascorbic acid. For standard whole-cell recordings, pipettes were filled with intracellular solution containing (in mM): 124 K-gluconate, 14 KCl, 10 HEPES, 1 EGTA, 5 MgATP, 0.3 Na₂GTP, 10 Na₂Phosphocreatine, pH = 7.3 with KOH. All chemicals were from Sigma or Tocris.

Immunostaining

To confirm the histaminergic nature of TMN neurons with electrical signatures of HA cells (Haas and Reiner, 1988; Haas et al., 1989), neurons were filled with biocytin (0.2%, Tocris) as in our previous work (Schone et al., 2011). We used streptavidin linked to Cy2 (1:500, Invitrogen) to detect Biocytin. To detect adenosine deaminase we used rabbit anti adenosine deaminase (Chemicon; 1:250) and goat anti-rabbit Alexa 555 (Invitrogen; 1:1000). Cy2 was

excited with an argon laser at 488 nm, and its fluorescence collected at 570–670 nm using a spectral detector (Olympus). Alexa 555 was excited with a diode-pumped solid-state laser at 559 nm, and its fluorescence collected at 570–670 nm using a spectral detector (Olympus).

To confirm the published specificity of cre-recombinase expression in orexin-cre mice (Matsuki et al., 2009), we performed immunostaining on 50 μ m cryostat sections of the LH as in our previous work (Karnani et al., 2011). Primary antibodies were rabbit anti-orexin A (Phoenix Pharmaceuticals; 1:250) and mouse anti-cre-recombinase (Millipore; 1:500). Secondary antibodies were goat anti-rabbit alexa 647 (1:500) and anti-mouse alexa 488 (1:1000) from Invitrogen. Alexa 488 and 647 signals were collected using BP525/50 nm and BP700/75 nm filters respectively. Orexin-immunoreactivity (ir) was detectable in $94\pm 2\%$ of cre-ir neurons in the LH, while cre-ir was detectable in $58\pm 5\%$ of orexin-ir neurons ($n = 3$ animals, 1200 cells). Following viral transduction, orexin-ir was detectable in $86 \pm 2\%$ of ChR2-FP cells, and ChR2-FP was detectable in $41 \pm 2\%$ of orexin-ir cells ($n = 4$ animals, 1560 cells). This expression pattern is consistent with specific expression of cre in hcrt/orx cells in this mouse line (Matsuki et al., 2009). The selective expression of ChR2-FP was further confirmed by observations of typical hcrt/orx cell electrical signatures in ChR2-FP cells (see Results).

RESULTS

Selective photostimulation of hcrt/orx cell membranes in acute brain slices

In ChR2-containing hcrt/orx cells (identified by fluorescence and verified by immunostaining and electrical fingerprinting, see Methods and Figure 1D,E), flashing blue light robustly produced time-locked whole-cell currents (Figure 1B, current amplitude at -60 mV was 686 ± 150 pA, $n = 9$) and action potentials (Figure 1B, $n = 20$). Flash durations of 1-5 ms and flash frequencies of 1-20 Hz (the physiological firing band for hcrt/orx cells *in vivo*, (Lee et al., 2005; Mileykovskiy et al., 2005), produced time-locked action potentials in ChR2-containing hcrt/orx neurons with 100% reliability (assessed using a 10 sec flash train, $n = 20$, e.g. Figure 1B). LH neurons not containing ChR2 showed no responses to blue light ($n = 5$, data not shown), confirming that responses of ChR2-FP cells were not due to non-specific photodamage. ChR2-expressing cells retained characteristic membrane potential dynamics of wild-type hcrt/orx cells (described in (Williams et al., 2008; Schone et al., 2011)), such as typical postinhibitory rebounds and tonic firing at 4.7 ± 1.4 Hz (Figure 1E, $n = 8/8$ cells). Together, these data confirm that ChR2 enables selective and precise electrical control of hcrt/orx cell membrane without disrupting intrinsic properties of the cells.

Detecting spike-mediated transmitter release from hcrt/orx axons onto HA neurons

Following the expression of fluorescent ChR2 in hcrt/orx neurons, we observed a network of ChR2-containing axons in the TMN (Figure 2A). To test if stimulation of these axons modulates HA cells, we performed whole-cell patch-clamp recordings from TMN HA neurons while optically stimulating ChR2-containing axons. Note that, although some of our TMN

slices contained hcrt/orx neurons, successful stimulation and transmitter release from ChR2-containing axons does not require them to be connected to cell bodies (Petreanu et al., 2007)). As in our previous studies (Williams et al., 2011), we identified TMN HA neurons by: a) large (20-30 μm diameter) multipolar cell body; b) hyperpolarisation-activated depolarizing “sag” indicative of H-current expression; c) prominent post-inhibitory after-hyperpolarisation, and d) tonic firing at resting membrane potential (Figure 2B, see (Haas and Reiner, 1988; Haas et al., 1989)). Immunolabelling confirmed that TMN neurons displaying such characteristics contained the HA cell marker adenosine-deaminase (Figure 2A, n = 3/3).

Optical stimulation of TMN slices containing ChR2-axons produced fast post-synaptic currents (PSCs) in HA cells (Figure 2C, n = 36/65 cells). HA cells from slices that did not contain ChR2 axons did not show any membrane responses to the same light flashes (n = 10, data not shown), confirming that without ChR2, our optical stimulation does not affect synaptic input to HA cells. In ChR2-expressing slices, the delays between flash onset and postsynaptic response (≈ 5 ms, Figures 2C, quantified in Figure 3A,B), were consistent with previous studies using optogenetics to fast transmitter release (Atasoy et al., 2008; Tecuapetla et al., 2010). Tetrodotoxin completely abolished the light-triggered PSCs (Figure 2C, n = 8 cells), suggesting that they were triggered by tetrodotoxin-sensitive Na^+ spikes in the axon, rather than by unphysiological influx of Ca^{2+} through ChR2 channels (Nagel et al., 2003).

Functional properties of fast transmission in hcrt/orx \rightarrow HA circuit

We next studied the kinetics, pharmacology, frequency-dependence, and release probability of the optogenetically-evoked PSCs in HA cells. The PSCs had amplitudes of 26.0 ± 1.8 pA and monoexponential decay time constants of 3.2 ± 0.4 ms (for spread of values, see Figure 3B; $n = 15$ cells), consistent with PSCs mediated by glutamate AMPA receptors (Dingledine et al., 1999). Blockade of AMPA receptors with CNQX completely blocked evoked PSCs (Figure 3A, $n = 12$), indicating that axons originating from hcrt/orx neurons may modulate TMN HA neurons via fast AMPA receptor-mediated synaptic transmission.

Current evidence indicates that hcrt/orx neurons fire < 20 Hz *in vivo*, but can be driven to fire up to 300 Hz *in vitro* (Lee et al., 2005; Mileykovskiy et al., 2005) (Li et al., 2002). However, it is unknown which firing frequencies are optimal for controlling transmitter release. We thus optically stimulated hcrt/orx axons at different frequencies and quantified the total charge input and the success rate of the occurrence of glutamate PSCs in HA cells in a 10 sec long train of flashes (Figure 3C,D). The positive relationship between the effect of glutamate on the postsynaptic cell and the presynaptic stimulation frequency saturated above 5Hz (Figure 3D). This was presumably in part because the success rate for PSC generation fell with stimulation frequency: with 5 ms flashes, increasing flash frequency from 1 to 5 Hz to 20 Hz reduced success from 76 ± 6 % to 53 ± 7 % to 15 ± 5 % respectively (Figure 3C, $n = 8$, $p < 0.05$ for 1 v 5 Hz, and $p < 0.001$ for 1 v 20 Hz using one-way ANOVA). We also quantified paired-pulse ratio (PPR) of PSC amplitudes, a measure of short-term plasticity at central synapses (Dobrunz and Stevens, 1997). We observed both paired-pulse depression and paired-pulse facilitation, often in different trials from the same cell ($n = 11$ cells; Figure 3E). However, on average each cell predominantly showed paired-pulse depression, with mean PPR significantly below 1 (Figure 3E).

Stimulation of hcrt/orx cell axons rapidly alters HA cell firing

Finally, we investigated the impact of hcrt/orx cell stimulation on the physiological output (firing rate) of HA cells. When HA cells were recorded in current-clamp with zero holding current, individual light flashes evoked clear depolarizations (Figure 2A, the amplitude of subthreshold depolarizations was 3.7 ± 0.5 mV, $n = 8$ cells). After recording the natural HA cell membrane potential for 10 seconds without stimuli, we applied 5 ms light stimuli for 10 sec at stimulus frequencies of 1, 5 and 10 Hz, and recorded for another 10 sec to monitor recovery from the stimulus (Figure 4B). We analysed changes in firing rate by first comparing the firing “before”, “during”, and “after” stimulation using two-way ANOVA followed by Bonferroni post-test comparison of the firing “before” and “during” stimulation. In all HA cells functionally connected to hcrt/orx axons (identified by first looking at optically-evoked PSCs as in Figure 2C), this analysis showed significant effects of optical stimulation on firing rate at 5-20 Hz stimulation frequencies (quantification is summarized in Figure 4C, $n = 10$ cells). In all optogenetic stimulation conditions used here, the acceleration of HA cell firing was completely abolished by pharmacological blockade of AMPA receptors (with CNQX, $n = 8$ cells); examples of raw data are shown in Figure 4B and the full dataset is statistically summarized in Figure 4C. These data are consistent with previous voltage-clamp data (Figure 3A) and show that glutamate release is necessary for the electrical activity in hcrt/orx axons to cause significant changes in HA cell firing.

DISCUSSION

The present study is the first direct demonstration that hcrt/orx cells can release glutamate under physiological conditions *in situ*. This suggests that the firing of hcrt/orx cells can be translated into changes in their postsynaptic targets very rapidly (on a millisecond timescale), in addition to the much slower actions of neuropeptides produced by hcrt/orx cells (Li and van den Pol, 2006).

We observed a high glutamatergic connection probability in the hcrt/orx → HA circuit (≈60% of HA cells received connections from ≈40% of hcrt/orx cells expressing ChR2). To the best of our knowledge, there are few studies of functional connectivity between neuronal types in subcortical circuits. Our data suggest that the hcrt/orx → HA circuit is robustly connected, which is similar to recent findings in cortical circuits with connection probabilities approaching 100% (Petreanu et al., 2007). In our analysis, the reduced release probability at increased presynaptic firing rates resulted in functional saturation of the hcrt/orx → HA connection above about ~5 Hz (Figure 3C,D). Interestingly, this is consistent with the saturating relationship between hcrt/orx cell firing and arousal effects observed *in vivo* (Adamantidis et al., 2007). In stimulation paradigms attempted here, blockade of AMPA receptors completely abolished the postsynaptic effect of hcrt/orx axon stimulation, suggesting that release of other transmitters was not sufficient to alter HA cell firing. The role(s) of other transmitter released by orx/hct cells in postsynaptic electrical responses remain to be determined.

The contribution of HA signalling to arousal caused by the activity of hcrt/orx cells has been a matter of recent debate. Arousal stimulated by exogenously applied hcrt/orx peptide

depends on HA signalling (Huang et al., 2001; Yamanaka et al., 2002), but arousal produced by optogenetic stimulation of endogenous hcrt/orx neurons persists in mice lacking histidine decarboxylase, the enzyme that produces HA (Carter et al., 2009). The latter finding does not detract from the potential physiological significance of our data, because 1) apart from HA, HA neurons contain many other transmitters such as GABA, galanin, enkephalin, TRH, and substance P (Haas et al., 2008), which could propagate hcrt/orx cell signals in the absence of HA; and/or 2) the glutamatergic drive to HA cells described in our study may contribute to aspects of brain function distinct from the arousal outputs measured in the above studies, e.g. cognitive processing. It is also possible that in mice lacking HA, other arousal systems stimulated by hcrt/orx cells compensate, for example the noradrenaline, serotonin, and acetylcholine systems (Peyron et al., 1998; Jones, 2003; Saper et al., 2005).

Glutamatergic signalling from hcrt/orx to HA neurons could be important because it offers a novel mechanism for hcrt/orx cell signalling that may contribute sufficiently fast excitatory PSPs for transferring information from transient spike bursts of hcrt/orx observed *in vivo* (Lee et al., 2005; Mileykovskiy et al., 2005). It may also potentially serve to synchronize the firing of HA cells and/or rapidly regulate plasticity in HA cell synapses. Of more general importance, our observations suggest that, in addition to their slow neuromodulatory role, hcrt/orx cell axons may rapidly alter brain function through fast glutamatergic transmission.

FIGURE LEGENDS

Figure 1. *Selective optogenetic control of electrical activity of hcrt/orx cell membrane.*

A: Chr2-containing construct. In the presence of cre, the double-floxed inverted open-reading frame of Chr2(H134R)-EYFP is flipped, and the loxP/lox2272 sites inactivated. ITR: inverted terminal repeat; EF1a: Elongation factor 1alpha promoter; WPRE: WHP-posttranscriptional response element; hGH PA: human growth hormone polyadenylation signal **B:** Light flashes (black bars) evoke whole-cell currents (bottom trace, holding potential = -60 mV) and action potentials (top trace). **C:** Co-localization of Orexin A- and cre-recombinase-like immunoreactivity. Scalebar: 50 μ m. **D:** Co-localization of Chr2-eYFP with orexin-like immunoreactivity. Scalebar: 50 μ m. **E:** Electrical fingerprints of Chr2-expressing cells showing D and H-type “signatures” of hcrt/orx cells.

Figure 2. *Spike-mediated transmitter release from hcrt/orx axons onto HA neurons.*

A: Left: Coronal schematic at Bregma -2.3 mm showing ventral TMN (VTM); f: fornix; 3V third ventricle. Right: z-projections from confocal stacks. Top panels: biocytin (BC)-filled cell, colocalizing with ADA-like immunoreactivity (arrowed). Bottom right: same cell as in top panel (red), overlaid with Chr2-eYFP fibres (green). Scalebar: 20 μ m. Bottom left: higher magnification of same cell showing single confocal planes, with fiber-cell appositions arrowed. **B:** Typical electrical current-clamp fingerprint of an HA cell. **C:** Fast PSCs in an HA neuron upon Chr2 stimulation (light flash shown as black bar), and recorded at -60 mV (individual traces in grey, averages in black or blue), with and without tetrodotoxin (TTX).

Figure 3. *Pharmacological and biophysical properties of fast hcrt/orx \rightarrow HA inputs.*

A: Optogenetically-induced PSCs (light stimulus shown as black bar) with and without CNQX (recordings at -60 mV, individual responses are in grey and averages in black or blue). **B:** Properties of PSCs (black dots are means from individual cells obtained using 0.1 Hz stimulation, population means \pm SEM are in red). **C:** Success rate of optogenetic stimulation to evoke PSCs. **D:** Total charge transfer in hcrt/orx \rightarrow HA PSCs during a 10 sec stimulus train (sum of charges of PSCs); ** $p < 0.01$ by Student's t-test. **E:** Short-term plasticity in hcrt/orx \rightarrow HA PSCs. Examples of paired-pulse depression (PPD, top left trace) and facilitation (PPF, top right trace). Bar graph shows mean proportions of different types of responses in 10 cells (% of trials that failed to produce PSC on 1st or 2nd flash are also shown). **F:** Paired-pulse ratio at different interpulse intervals, * $p < 0.05$; ** $p < 0.01$.

Figure 4. *Optogenetic stimulation of hcrt/orx cell axons rapidly alters HA cell firing.*

A: Example membrane potential responses to a 5 ms flash. **B:** Example changes in HA cells firing induced by optical stimulation in the absence (top trace), and presence (bottom trace) of CNQX. Action potentials are truncated at -15 mV. Bottom graph shows group data for response and recovery at 10 Hz stimulation, expressed at % change in firing, *** $p < 0.001$. **C:** Effects of a range of stimulation frequencies on firing rate with and without CNQX, ** $p < 0.01$; *** $p < 0.001$.

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Figure 1

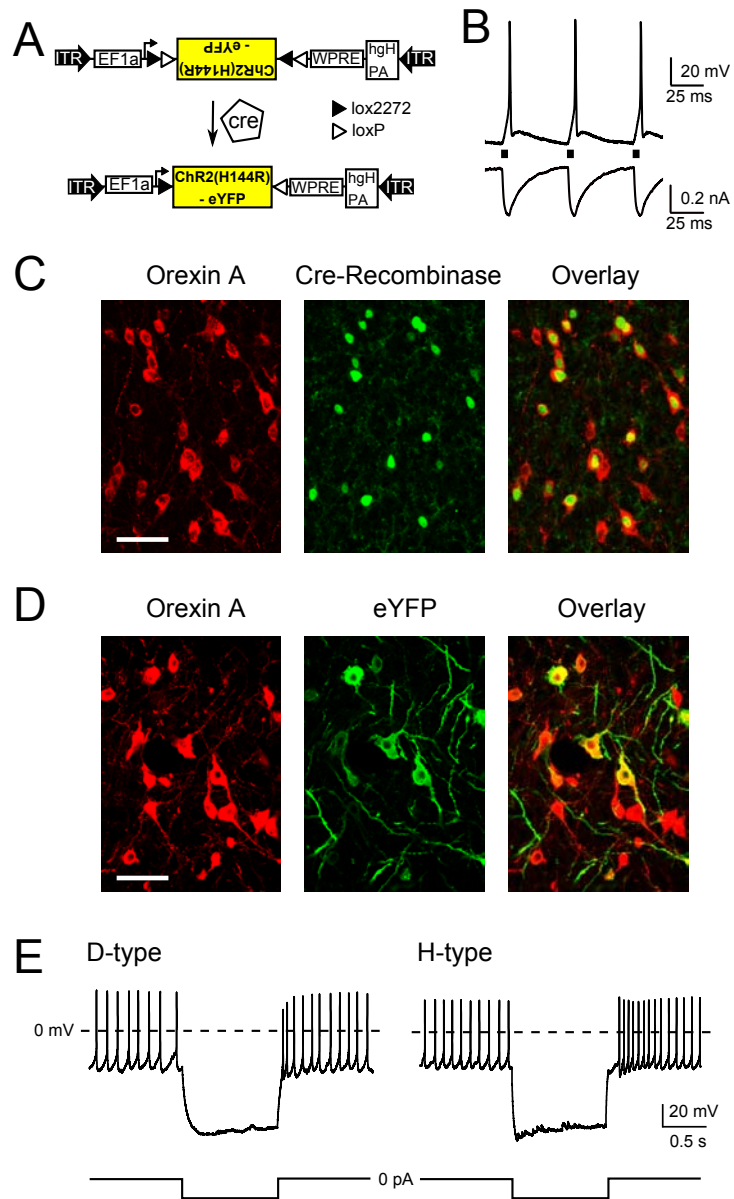


Figure 2

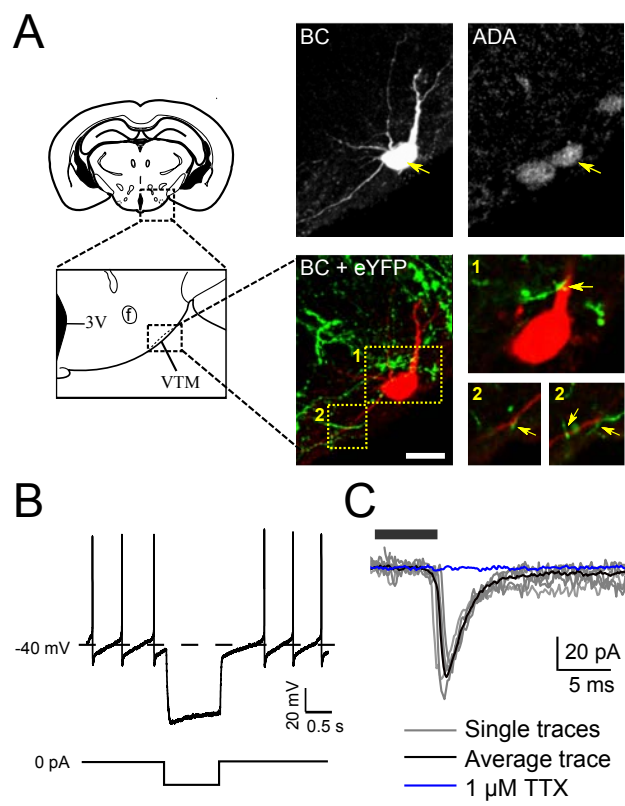


Figure 3

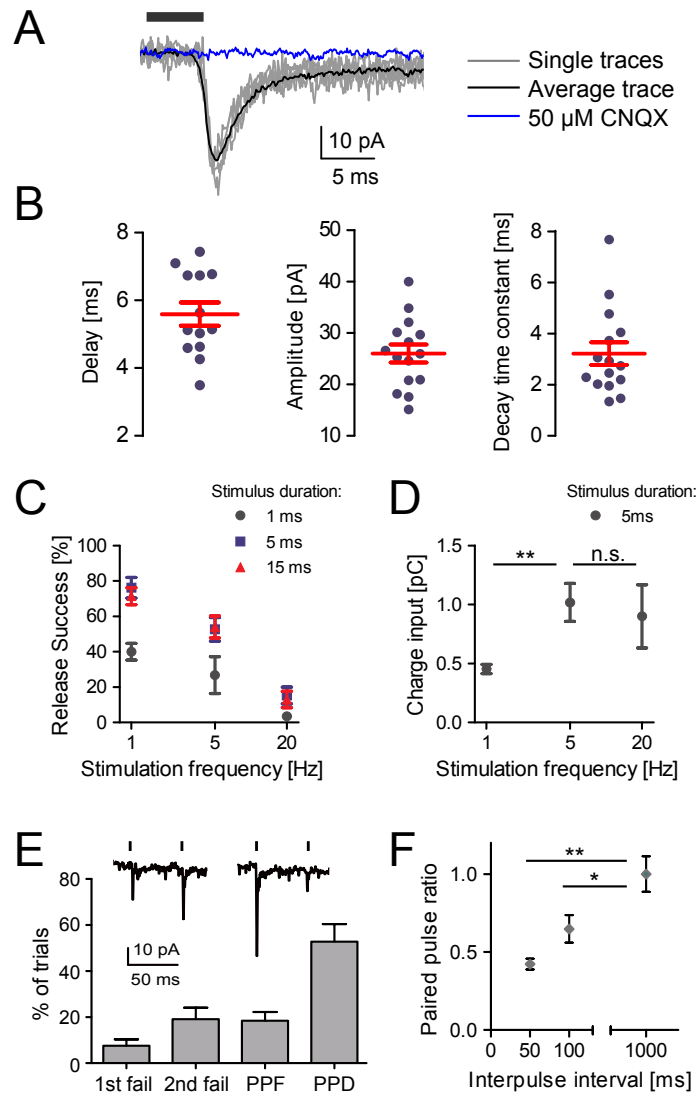


Figure 4

