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Cholecystokinin action on layer 6b neurons in somatosensory cortex

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Abstract

Layer 6b in neocortex is a distinct sublamina at the ventral portion of layer 6. Corticothalamic projections arise from 6b neurons, but few studies have examined the functional properties of these cells. In the present study we examined the actions of cholecystokinin (CCK) on layer 6b neocortical neurons using whole-cell patch clamp recording techniques. We found that the general CCK receptor agonist CCK8S (sulfated CCK octapeptide) strongly depolarized the neurons, and this action persisted in the presence of tetrodotoxin, suggesting a postsynaptic site of action. The excitatory actions of CCK8S were mimicked by the selective CCK_B receptor agonist CCK4, and attenuated by the selective CCK_B receptor antagonist L365260, indicating a role for CCK_B receptors. Voltage clamp recordings revealed that CCK8S produced a slow inward current associated with a decreased conductance with a reversal potential near the K^+ equilibrium potential. In addition, intracellular cesium also blocked the inward current, suggesting the involvement of a K^+ conductance, likely K_{leak}. Our data indicate that CCK, acting via CCK_B receptors, produces a long-lasting excitation of layer 6b neocortical neurons, and this action may play a critical role in modulation of corticothalamic circuit activity.

Keywords

corticothalamic; layer 6b; CCK; neuropeptide; rat; electrophysiology

1. INTRODUCTION

Corticothalamic neurons modulate information transfer through the thalamus by influencing firing mode and synchronization of relay neurons as well as modulate thalamocortical rhythms (Sherman and Guillery, 1996;Godwin et al., 1996;Sillito and Jones, 2002;Sillito AM et al., 1994; Steriade, 2001;Blumenfeld and McCormick, 2000;Bal et al., 2000). Corticothalamic innervation arises from deep layer 5 or 6 glutamatergic neocortical neurons (Bourassa et al., 1995;Killackey and Sherman, 2003). These excitatory neurons can activate both ionotropic

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and metabotropic glutamate receptors on thalamic relay neurons leading to both short- and long-term synaptic responses (Sherman and Guillery, 1996;Reichova and Sherman, 2004;Kao and Coulter, 1997;McCormick and von Krosigk, 1992;Turner and Salt, 1998;Alexander and Godwin, 2005).

Layer 6 can be subdivided into two distinct laminae: layers 6a and 6b. Layer 6b has also been referred to as layer VII, subplate, or subgriseal layer and these neurons and these neurons project to thalamus in rodents (Clancy and Cauller 1999; Killackey and Sherman, 2003). The morphology of layer 6b neurons is more diverse than that of layer 6a neurons in that 6b neurons have apical dendrites that extend in horizontal or oblique directions (Killackey and sherman, 2003; Torres-Reveron and Friedlander, 2007), and some 6b neurons lack apical dendrites altogether (Clancy and Cauller, 1999; Andjelic et al., 2009). In addition, certain proteins are predominantly expressed within layer 6b such as orphan nuclear receptor Nurr1 and neurexophilin 3 (Arimatsu et al., 2003; Beglopoulos).

There is limited understanding of the functional significance of layer 6b as a distinct layer (Torres-Reveron and Friedlander, 2007). It is speculated that layer 6b neurons may modulate arousal or wakefulness because the neuropeptide orexin produces strong excitation of layer 6b neurons and little effect on cortical neurons in other layers (Bayer, 2004; Sakurai, 2007). A source of orexin in the brain are hypothalamic neurons, and these cells are strongly excited by an anorexinergic neuropeptide, cholecystokinin (CCK, Tsujino 2005).

CCK was initially found in gut and later widespread within the brain (Crawley and Corwin, 1994). In the thalamocortical circuit, CCK is present in some corticothalamic and thalamocortical projection neurons. CCK receptors are localized in both deep layers of neocortex as well as thalamus (Burgunder and Young, 1990; Mercer et al., 2000; Mercer and Beart, 2004; Schiffmann and Vanderhaeghen, 1991; Zarbin et al., 1983). In thalamus, CCK selectively depolarizes GABA-containing thalamic reticular nucleus neurons, but has no effect on thalamocortical relay neurons (Cox et al., 1995). At the circuit level, CCK significantly alters intrathalamic rhythmic activities (Cox et al., 1997).

In the present study, we have investigated the actions of CCK on layer 6b. Our results indicate that CCK, acting at CCK_B receptors, selectively depolarizes layer 6b projection neurons by suppressing a leak K^+ current. This effect will increase the excitability of these deep layer neurons as well as potentially facilitate excitatory transmission through this circuit, ultimately influencing thalamocortical circuit activity by modulating the corticothalamic feedback.

2. RESULTS

Intracellular recordings in whole cell configuration were obtained from a total 117 layer 6b, 25 layer 6a, and 10 layer 5 projection neurons. Layer 6b could be identified unambiguously before recording (See Experimental procedures; Figure 1A). The resting membrane potential and input resistances of these neurons are shown in Table 1.

2.1 CCK depolarizes corticothalamic neurons via postsynaptic mechanisms

Because we were interested in the possible modulatory role of CCK in the thalamocortical system, we tested whether CCK altered the excitability of corticothalamic projection neurons, which are present in layers 5, 6a and 6b. In control conditions, CCK (1.25 μM, 60 s) produced a robust depolarization in all layer 6b neurons tested (n=6, Figure 1B), which led to action potential discharge in three of these cells. In the remaining 3 cells, the depolarization averaged 8.6 ± 1.8 mV. In layer 5 and 6a neurons, the overall CCK effect was weaker. In layer 5, CCK evoked depolarization in 5 of 10 cells that was not sufficient to produce action potential discharge. The peak depolarizations averaged 3.8 ± 1.6 mV (125 nM, n=5) and 2.8 ± 2.7 mV

(1.25 μM, n=5). In layer 6a neurons, CCK produced a depolarization in 20 of 25 cells, and this response was sufficient to evoke action potential discharge in four cells. The peak depolarization produced by CCK in layer 6a neurons averaged 1.1 ± 0.7 mV (12.5 nM, n=3), 5.6 ± 1.7 mV (125 nM, n=12), and 2.5 ± 1.2 (1.25 μM, n=6). Because layer 6b neurons showed the most robust response, we focused on this layer for all subsequent experiments.

The CCK8S-mediated depolarization in layer 6b neurons persisted in the presence of the voltage-dependent sodium channel blocker, tetrodotoxin (TTX, 0.5, 1 μM), suggesting that CCK8S acts on postsynaptic CCK receptors (Figure 2A). Across the concentration range tested (0.125–2.5 μM), CCK8S produced a significant concentration-dependent depolarization in layer 6b neurons, averaging 1.9 ± 0.3 (n=5, 12.5 nM), 5.0 ± 0.6 (n=38, 125 nM), 6.7 ± 1.3 (n=7, 1.25 μM), and 6.3 ± 0.9 mV (n=4, 2.5 μM) (Figure 2B). During the depolarization, the apparent input resistance was significantly increased by 28±4% (n=12) in 1.25 μM CCK8S.

2.2 Cholecystokinin excites cortical neurons via CCK_B (CCK_2) receptors

Two subtypes of CCK receptors have been characterized: CCK_A (CCK_1) and CCK_B (CCK_2) (Wank, 1995; Noboe et al., 1999). In the central nervous system, the CCK_B receptor subtype generally predominates while the CCK_A subtype is present in restricted regions. Similarly, in the neocortex CCK_B receptors are most abundant (Carlberg et al., 1992; Noble et al., 1999); however, CCK_A receptors are also present (Mercer and Beart, 2004). In thalamic reticular neurons, the robust depolarization by CCK is mediated via CCK_A receptors (Cox et al., 2005) thereby suggesting that both CCK_A and CCK_B receptors may play an important role in the brain. In order to determine the receptor subtype underlying CCK8S-mediated depolarization, we tested receptor specific agonists and antagonists on layer 6b cells. Repeated exposure to CCK8S (125 nM - 2.5 μM) showed partial desensitization even at intervals up to 30 minutes. Using 30 minute intervals between applications (125 nM, 60 s), the second CCK8S-mediated response averaged 51 ± 8% (n=9) of the initial response (Figure 3A). In the presence of the CCK_A receptor antagonist L364718 (1 μM), the second CCK8S-mediated response was not significantly altered compared with the second CCK8S in the absence of an antagonist (Figure 3B, 53 ± 5% of initial response, n=5). In contrast, in the presence of selective CCK_B receptor antagonist L365260 (1 μM), the second CCK8S-mediated depolarization was strongly attenuated (Figure 3C, 22 ± 5% of initial CCK8S control response, n=6). The CCK8S-mediated depolarization in the presence of L365260 was significantly smaller than those found in L364718 or control conditions. Considering the range of initial CCK8S responses, we also plotted the amplitude of the responses of the repeated CCK8S applications (Figure 3D). From these plots we calculated regression lines for the three different conditions: control, L364718, and L365260. The greater slope of the regression line indicates less reduction in the second CCK8S response. The slopes of the regression lines were 0.62, 0.55, and −0.03 for control, L364718, and L365260, respectively (Figure 3D). The slopes of control and L364718 did not significantly differ, but the slope of L365260 was significantly less than that of control or L364718. These data suggest that the CCK8S depolarization is mediated through activation of CCK_B receptors.

To further test the role of CCK_B receptors, we next tested the action of the selective CCK_B receptor agonist CCK4 on layer 6b neurons. CCK4 (125 nM - 1.25 μM) produced a depolarization, mimicking the actions of CCK8S (Figure 4A). Lower CCK4 concentrations (125 nM) produced a depolarization in 9 of 10 neurons that averaged 3.5 ± 1.0 mV (n=10), and at a higher CCK4 concentrations (1.25 μM) the depolarization averaged 5.7 ± 0.7 mV (n=21). CCK4 receptor-mediated depolarization was also associated with a significant increase in input resistance (23 ± 4%, n=6). As with CCK8S, repeated CCK4 applications resulted in smaller responses with a second application after 30 minute intervals. The amplitude of the second CCK4 response averaged 78 ± 11% (n=10) of the first response (Figure 4A). The CCK4

mediated depolarization was persistent in a specific CCK_A receptor antagonist L364718 (1 μM, Figure 4B) (74 ± 6%, n=5). However, the CCK_B receptor specific antagonist L365260 (1 μM), significantly attenuated the actions of CCK4 (17 ± 10%, n=5, Figure 4C). The amplitude ratio for L365260 was significantly smaller than control or L364718. Considering the limitations of these amplitude ratios, we also calculated the slope of regression lines from the plots of the first CCK4 response versus second CCK4 response (Figure 4D). The slopes of the regression lines were 0.38 in absence of antagonists and 0.66 in L364718, and these did not differ significantly. In contrast, in L365260, the slope was −0.02, which was significantly smaller than control conditions and L364718.

2.3 CCK excitation of layer 6b neurons is mediated via suppression of a K^+ current

In the present study, the CCK-mediated depolarization in cortical neurons was associated with an increased input resistance. We next used voltage clamp recordings to determine the ionic mechanisms underlying the membrane depolarization produced by CCK8S. At a holding potential of −60 mV, CCK8S produced an inward current in all cells tested (125 nM, n=2; 1.25 μM, n=13). The peak amplitude of the inward current in response to 1.25 μM CCK8S averaged 23.7 ± 3.0 pA (n=13). To examine the voltage dependence of the CCK-mediated current, voltage command ramps (−60 to −110 mV, 3 s duration, 0.1 Hz) were repeatedly applied. CCK8S produced an inward current associated with a decreased slope in response to the ramped voltage command, indicating decreased membrane conductance (Figure 5Aii). The difference in the membrane responses to the ramped voltage commands in control and CCK8S represents the CCK-sensitive current (I_{diff}, Figure 5Aiii). This current (I_{diff}) was linear over the voltage range tested (−60 to −110 mV) with an average conductance of 0.49 ± 0.05 nS (n=13). In 6 of 13 neurons, I_{diff} reversed within the voltage range tested (−60 to −110 mV) and averaged −86.9 ± 3.2 mV (n= 6), close to the estimated reversal potential for K^+ ions based on our solutions (−93 mV). Our voltage ramp data indicate that activation of CCK receptors suppress a resting, linear conductance mediated by K^+. To confirm the role of a K^+ current, we next substituted Cs^+ for K^+ within the recording pipette. With the Cs^+-containing electrodes and a holding potential of either −60 mV (n=3) or −30 mV (n=8), CCK8S (1.25 μM) did not alter the holding current (Figure 5B). This lack of effect contrasts with our K^+-containing pipette solution. These data support the notion that activation of CCK receptors reduces a voltage-independent K^+ conductance such as K_{leak}.

3. DISCUSSION

The present data indicate that CCK can produce long-lasting excitatory responses in layer 6b cortical neurons by suppressing a K^+ conductance. The excitatory actions of CCK are mediated by CCK_B receptors because of their sensitivity to the CCK_B antagonist, L365260, and insensitivity to the CCK_A antagonist, L364718. This is consistent with the anatomical distribution of CCK_B receptors throughout the brain (Durieux et al., 1988). In addition, CCK4, a selective agonist for CCK_B receptors (Innis and Snyder, 1980) produced a depolarization similar to CCK8S that was also attenuated by CCK_B receptor antagonist, L365260. CCK mediated depolarization by CCK_B receptors in layer 6b cortical neurons contrasts with the depolarization mediated by CCK_A receptors in thalamocortical reticular neurons (Cox et al., 1995). Hence, within the thalamocortical circuit CCK may act on two different types of receptors.

CCK has been found to produce a variety of actions in different regions of the central nervous system including direct postsynaptic actions (Cox et al., 1995; Dodd and Kelly, 1981; Miller et al., 1997; Shinohara and Kawasaki, 1997), increasing neurotransmitter release (Cox et al., 1997; Etou et al., 1998; Ferraro et al., 1999), and modulating synaptic activity (Delfs and Dichter, 1985; Chung and Moore, 2007). Our results indicate that CCK produced a postsynaptic

depolarization that persisted in TTX. In other brain regions, CCK-mediated depolarizations result from decreased K^+ current (Cox et al., 1995; Miller et al., 1997; Shinohara and Kawasaki, 1997), increased mixed cation current (Dodd and Kelly, 1981; Jarvis et al., 1992; Wu and Wang, 1996; Meis et al., 2007), or increased Ca^{2+} current (Shinohara and Kawasaki, 1997). In layer 6b neurons, we found CCK evokes a long lasting depolarization that appears to be mediated by a decreased voltage independent K^+ current, consistent with K_{leak}.

3.1 Functional significance of CCK-mediated actions

Different neuropeptides and their receptors are present in the neocortex, including but not limited to: CCK (Delfs and Dichter, 1985; Harro et al., 1993), vasoactive intestinal peptide (Murphy et al., 1993; Pawelzik et al., 1992), neuropeptide Y (Bacci et al., 2002), somatostatin (Delfs and Dichter, 1983; Vidal and Zieglgansberger, 1989; Wang et al., 1989), and orexin (Bayer et al., 2004; Lambe et al., 2005). A potentially interesting aspect of these neuropeptides is that they tend to produce long-lasting changes in neuronal excitability. For example, orexin depolarizes neurons specifically in layer 6b in cortex (Bayer et al., 2004). On the other hand, CCK excites neurons in hypothalamus that release orexin (Tsujino et al., 2005). This finding suggests a potentially interesting interaction, considering CCK has been found to inhibit food intake whereas orexin mildly stimulates it (Noble et al., 1999; Sakurai, 2007). The activation of layer 6b neurons by CCK and orexin may indicate cooperatively in physiological functions other than food intake (Harris and Aston-Jones, 2006).

Layer 6b neurons gives rise to both corticothalamic and corticocortical projections (Clancy and Cauller, 1999; Prieto and Winer, 1999; Killackey and Sherman 2003). Because CCK8S depolarized all layer 6b neurons, both circuits are likely affected. In the corticothalamic circuit, we speculate that the CCK-mediated depolarizations in layer 6b neurons could serve to modulate thalamic rhythmic activity. Thalamic neurons produce action potential discharge in two basic modes: burst and tonic firing (Jahnsen and Llinás, 1984; Steriade and Llinás, 1988). The burst discharge of thalamic neurons is required to produce certain types of intrathalamic rhythmic activities that are associated with levels of arousal as well as pathophysiological conditions such as absence epilepsy (McCormick and Bal, 1997; McCormick and Contreras, 2001). The long lasting depolarization by CCK in layer 6b may lead to prolonged discharge of these corticothalamic glutamatergic neurons that may activate metabotropic glutamate receptors leading to a slow depolarization of thalamocortical neurons, shifting the firing mode from burst- to tonic-firing mode (Kao and Coulter, 1997; McCormick and von Krosigk, 1992; Turner and Salt, 1999).

In addition to the possible anti-oscillatory actions of corticothalamic feedback, this input may also play an important role in sensory information processing (Sillito and Jones, 2002). The layer 6 inputs have been referred to as modulatory in that these inputs may play an important role in setting the gain, or sensitivity, of neurons to afferent inputs (Guillery and Sherman, 2002; Sherman and Guillery, 1996). Corticothalamic input from layer 6 is hypothesized to provide a modulatory input to first- and higher-order thalamic nuclei while higher order thalamic nuclei receive driver input from layer 5 corticothalamic neurons. Functionally, such an arrangement could serve as a means for interconnecting different cortical areas. In the somatosensory system, anatomical data indicate that layer 6b neurons provide innervation of a higher order thalamic nucleus, the posterior medial nucleus (Killackey and Sherman, 2003). Activity changes in layer 6b cells induced by neuromodulators can affect other cortical areas through this pathway.

Cortical projections of layer 6b neurons innervate different layers (e.g., layer 1) and adjacent ipsilateral cortical areas (Clancy and Cauller 1999; Prieto and Winder 1999; Arimatsu et al., 2003). Considering layer 6b neurons are glutamatergic, the prolonged excitatory output of these cells by CCK could modulate excitatory input to other cortical neurons (Andjelic et al.,

2009); however, the physiological function of these neurons in the cortical circuit is poorly understood (Reveron and Friedlander 2007). As for a specific function of layer 6b, a transgenic mouse model lacking the protein neurexophilin 3 suggests a role for layer 6b neurons (Beglopoulos et al., 2005). Neurexophilin 3 is highly localized in layer 6b, though selective deletion of this protein does not kill the neurons. However these animals show defects in sensory motor gating including increased startle response and reduction of prepulse inhibition. In our study, we did not distinguish corticothalamic or corticocortical neurons because CCK depolarized all layer 6b neurons. However, after identification of the functional cell types (corticothalamic vs corticocortical) (Brumberg et al., 2003), CCK effects on each type could be distinguished. In the future, we plan to study CCK effects on different cortical layers and diverse cell types in each layer, including inhibitory interneurons.

4. EXPERIMENTAL PROCEDURE

4.1 Slice preparation

All experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Animal Care and Use Committee. Young Sprague-Dawley rats (postnatal age: 8–14 days) were deeply anesthetized with pentobarbital sodium (50 mg/kg) and decapitated. The brains was quickly removed and placed into cold, oxygenated slicing medium containing (in mM) 2.5 KCl, 1.25 NaH_2PO_4, 10.0 $MgCl_2$, 0.5 $CaCl_2$, 26.0 $NaHCO_3$, 11.0 glucose, and 234.0 sucrose. Tissue slices (300 μm) were cut in the coronal plane at the level of somatosensory cortex using a Vibratome. The slices were transferred to a physiological solution containing (in mM): 126.0 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 2.0 $CaCl_2$, 1.25 NaH_2PO4, 26.0 $NaHCO_3$ and 10.0 glucose. The slices were incubated in the physiological solution at 30°C, gassed with 95% O_2/5% CO_2 for at least 1 hour before recording. Individual slices were transferred to a recording chamber and continuously superfused with warm (30 °C) oxygenated physiological solution.

4.2 Recording procedures

We obtained whole cell recordings with the visual aid of a Axioskop 2FS equipped with infrared differential interference contrast (DIC) optics (Zeiss Instruments, Thornwood, NY) as described previously (Govindaiah and Cox, 2004). A low power objective was used to initially identify neocortical layers and a high-power water immersion objective was used to visualize individual neurons. Layer 6b was identified as a distinct layer under DIC (Figure 1A), appearing above the white matter and below a thin layer of axons at the bottom of layer 6a. Below this axon, the soma sizes are larger and the apical dendrite is not clearly aligned toward the pia as seen in the cells above the thin layer of axons (layer 6a). In contrast, layer 6a cell somata are smaller than those of layer 6b and most cells show an apical dendrite oriented toward the pia. They are also aligned in a more regular fashion. We could easily tell the difference clearly enough to distinguish the 6a and 6b layers. Similar criteria were used in previous studies (Bayer et al., 2004; Torres-Reveron and Friedlander, 2007). Recording pipettes had tip resistances of 2–5 MΩ when filled with an intracellular solution containing (in mM): 117 K-gluconate, 13 KCl, 1.0 $MgCl_2$, 0.07 $CaCl_2$, 0.1 EGTA, 10.0 HEPES, 2.0 Na_2-ATP, 0.4 Na-GTP, and 0.3% biocytin. The pH and osmolarity were adjusted to 7.3 and 290 mosm, respectively. In some experiments a Cs^+-based recording pipette solution was used in which cesium gluconate and CsCl were substituted for potassium gluconate and KCl. The access resistance typically ranged from 10 to 25 MΩ and remained stable during recordings included for analyses in this study.

Recordings were obtained using an Axoclamp 2B amplifier (Molecular Devices, Foster City, CA) in bridge mode for voltage recordings (current clamp) or switching single-electrode voltage clamp mode for current recordings. Voltage and current protocols were generated using

pClamp software (Molecular Devices), and data were stored on computer for off-line analyses. For current-clamp recordings, an active bridge circuit was continuously adjusted to balance the drop in potential produced by passing current through the recording electrode. The apparent input resistance was calculated from the linear slope of the voltage-current relationship obtained by applying constant current pulses ranging from −100 to +40 pA (800 ms duration). Changes in input resistance following agonist application were determined by membrane responses to single-intensity constant current hyperpolarizing pulses (−10 to −30 pA, 500 ms, 0.2 Hz).

For voltage-clamp recordings, the amplifier was used in discontinuous mode. In these recordings, the switching frequency ranged from 2.7 – 3.5 kHz and the headstage was continually monitored to ensure that the current transients completely decayed before voltage measurements. These recordings were limited to neurons that had stable access resistances less than 20 MΩ. To quantify CCK-mediated changes in membrane conductance, slow ramped voltage commands (−60 to −100/110 mV, 4 s duration, 0.1 Hz) were applied to the neuron (Cox et al., 1995). Five subsequent current traces were averaged prior to agonist application and during the peak changes produced by the agonists.

4.3 Pharmacological agents

Pharmacological agents were dissolved in appropriate solvents and stored as recommended by the manufacturer. Concentrated stock solutions of CCK agonists (100 μM) were prepared in distilled water and diluted in physiological solution to a final concentration of 0.05 – 10 μM. Stock solutions of CCK antagonists were prepared in 1:1 dimethyl sufoxide:dH_2O and were bath applied in the final concentration. The agonists were applied by injecting a bolus into the chamber input line using a motorized syringe pump (Cox and Sherman, 2000). Based on the rates of agent injection and of chamber perfusion, the final bath concentration of the agonists was estimated to be one-fourth of the concentration introduced in the flow line. All agents were purchased from Tocris (Ellisville, MO) or Sigma (St. Louis, MO).

4.4 Histology and statistics

To recover the morphology of recorded neurons, biocytin (0.3%) was included in the intracellular solution. After recording, slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Slices were then reacted with avidin-biotin-peroxidase complex (ABC Elite, Vector Labs, CA) using established procedures and mounted with permount (Govindaiah and Cox, 2004). The neurons were examined under a light microscope and digitally photographed. Data are expressed as mean ± standard error of mean. Regression lines were plotted using the method of least squares. The difference between means was evaluated using the Student's *t*-test for two groups or one-way ANOVA with Tukey-Kramer test for post-hoc mean comparisons. Significance level was set at $p < 0.05$.

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Abbreviations

CCK cholecystokinin

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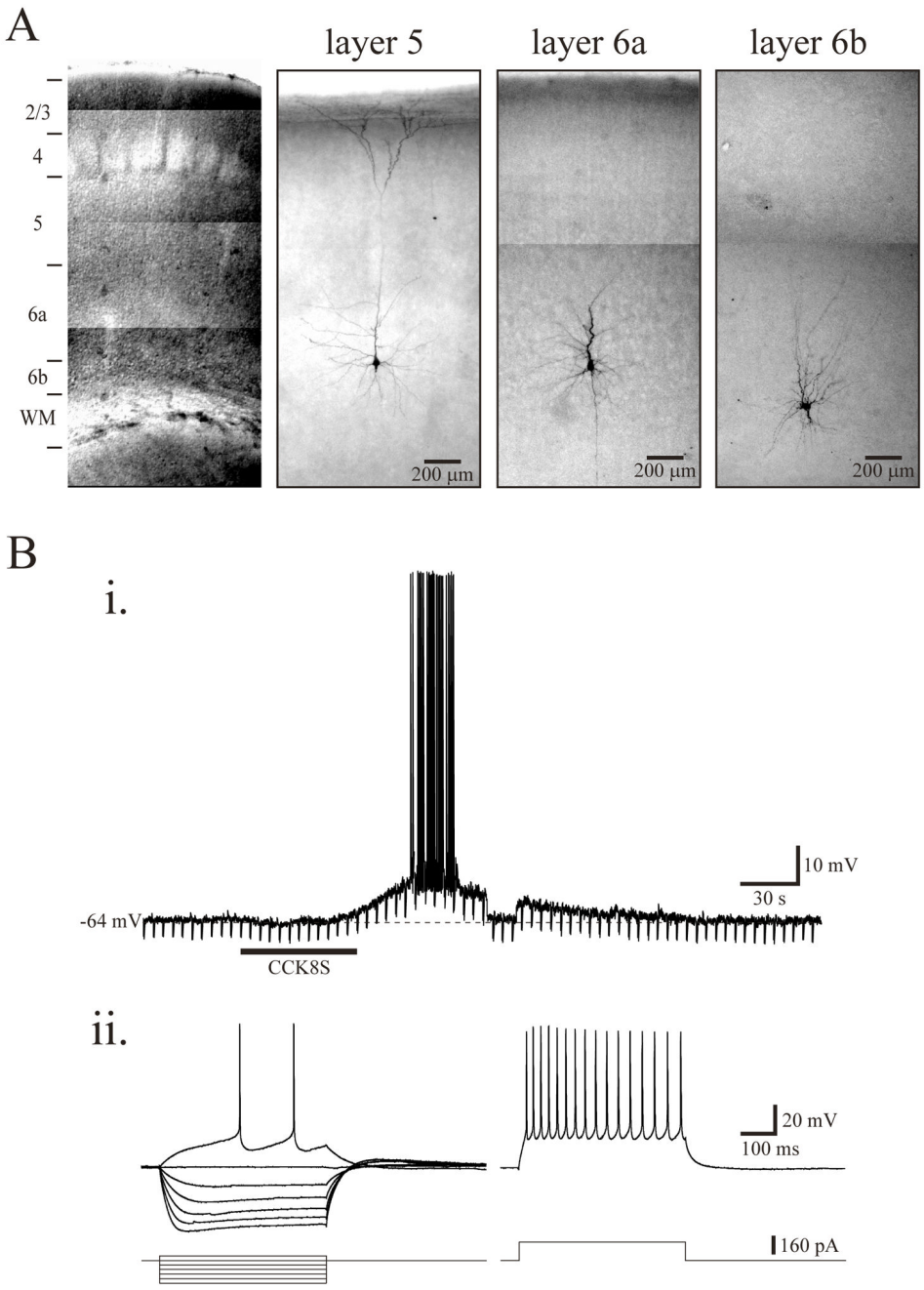

Figure 1.

CCK depolarizes layer 6b neurons. **A:** *Left*: Low power image of somatosensory cortex in coronal slice. Layer 6b can be identified from the image above white matter. *Right*: Biocytin-filled morphologies representative of layer 5, 6a and 6b neurons. **B: i.** In a current clamp recording from a layer 6b neuron in control conditions, CCK8S (1.25 μM, 60 seconds) depolarizes the membrane leading to action potential discharge. The short downward deflections are voltage responses to short hyperpolarizing current pulses (−15 pA, 500 ms). The prolonged hyperpolarization to baseline levels is produced by adding hyperpolarizing current and serves to determine voltage-independent alterations in the transient hyperpolarizing

steps. **ii.** Voltage responses and firing pattern to the hyperpolarizing and depolarizing DC current steps to the neuron.

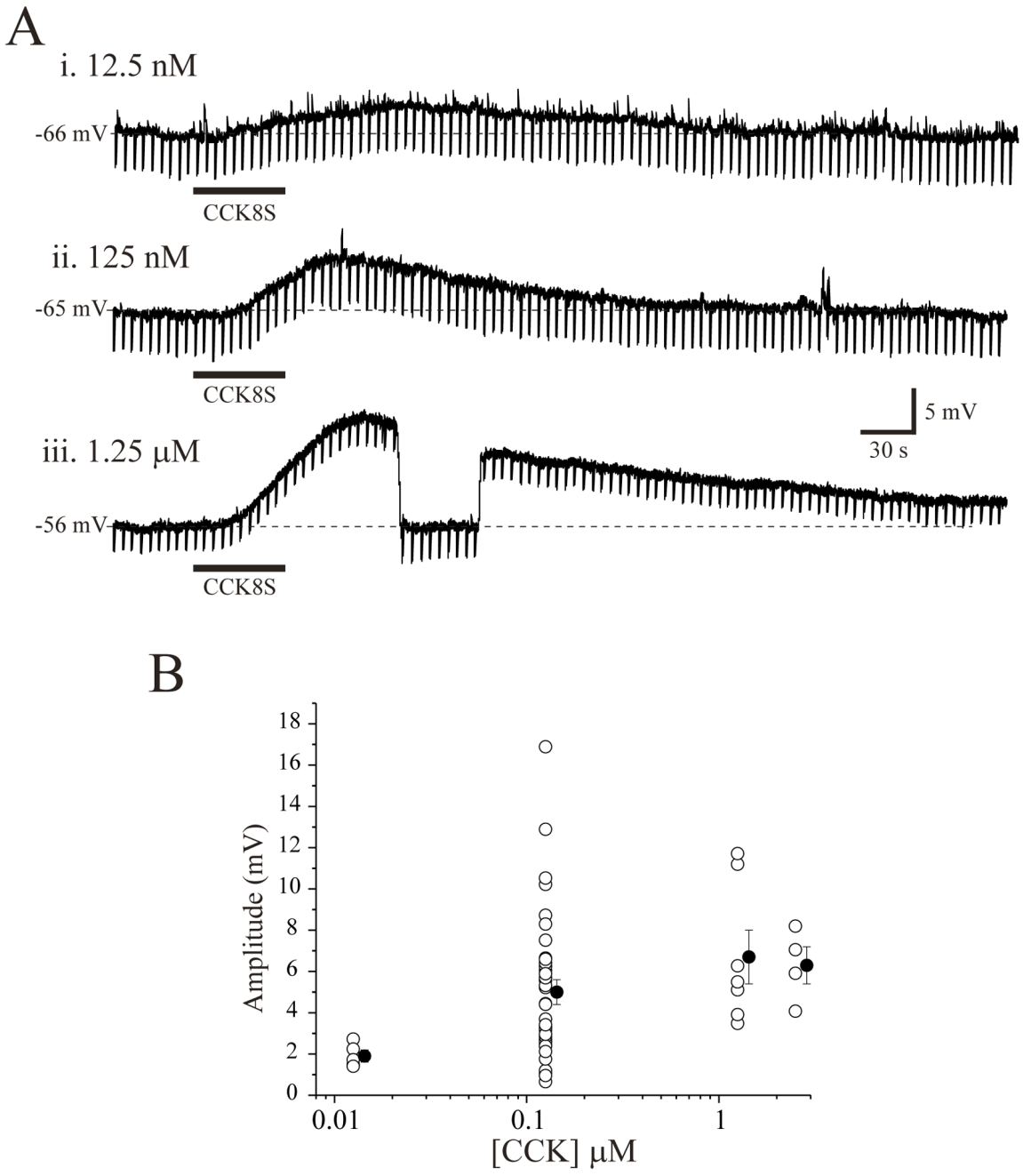

Figure 2.

In the presence of TTX (1 μM), CCK8S depolarizes layer 6b cortical neurons in a concentration-dependent manner. A: Examples of CCK8S effect at 3 concentrations (12.5 nM, 125 nM, and 1.25 μM) from different neurons. B: Population data reveal the concentration dependence of the CCK8S-mediated membrane depolarization. Open circles are from individual cells. Filled circles and error bars represent mean and standard error.

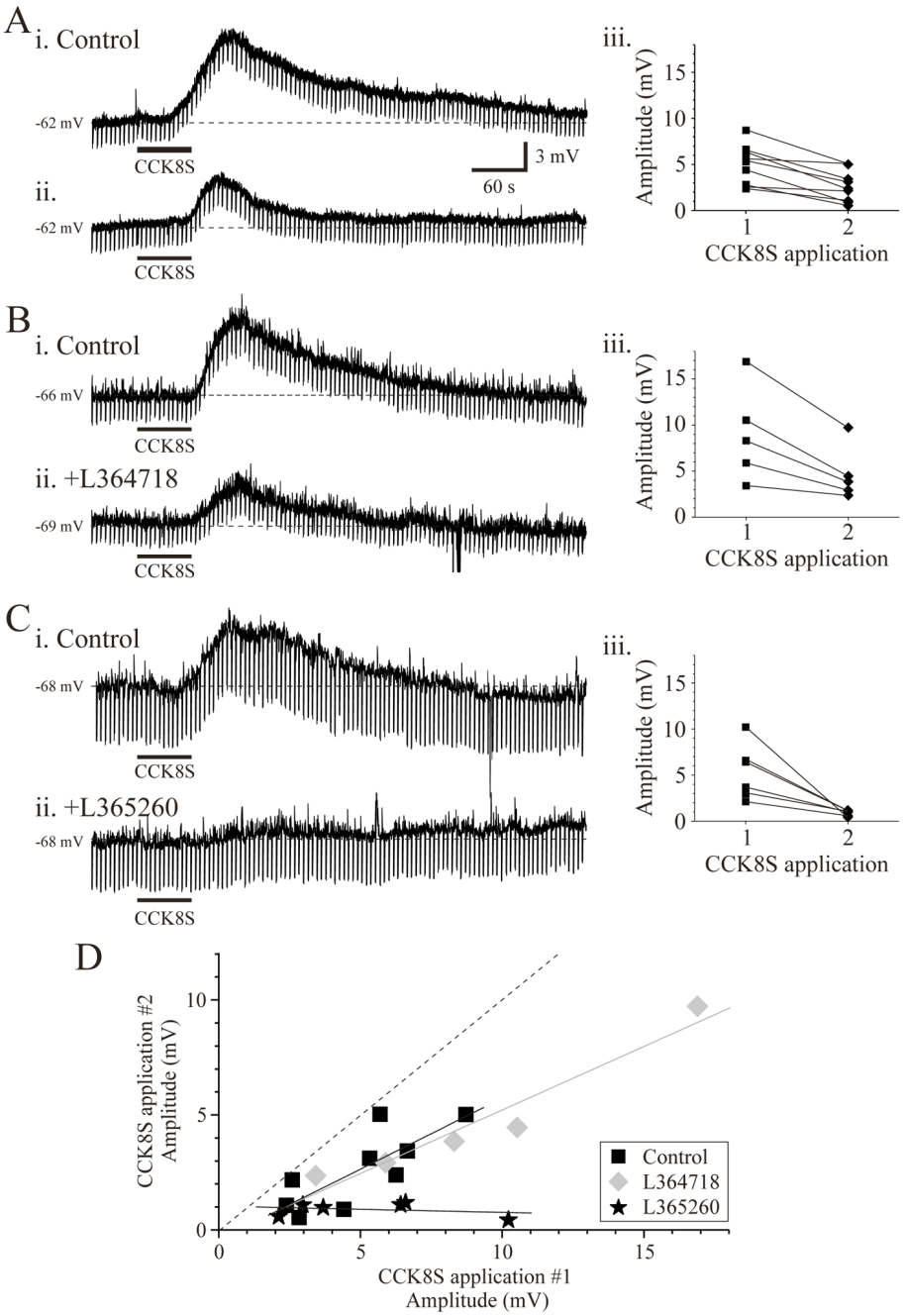

Figure 3.

The depolarizing actions of CCK on layer 6b neurons are mediated through CCK_B receptors. **A:** In a representative neuron, CCK8S depolarizes the membrane in a reversible manner (Ai). Subsequent application of CCK8S at a 30 minute interval results in a smaller depolarization (Aii). The reduction in the amplitude of the second response was consistent across neurons. The average ratio of the second to the first amplitude was 51 ± 8% (n=9; Aiii). **B:** The depolarizing actions of CCK8S were not blocked in the presence of CCK_A antagonist (L364718, 1 μM). Following the first CCK8S application, L364718 was then bath applied. Following 30 minute interval, and in the presence of L364718, CCK8S was then reapplied and the response to second application was 53 ± 5% (n=5) of the initial response. **C:** In a different

neuron, the depolarizing actions of CCK8S were completely attenuated in the presence of CCK_B antagonist L365260 (1 μM). The ratio between first and second response was significantly attenuated. The average ratio of the second to the first amplitude was 22 ± 5% (n=6). **D:** The regression lines of the second response amplitude relative to the first response amplitude show the antagonist effects clearly. The slopes of control, CCK_A antagonist, and CCK_B antagonist were 0.62, 0.55, and −0.03. The slope of CCK_B antagonist was significantly smaller than those of control and CCK_A antagonist. The dashed line indicates the slope of 1. All experiments were done in TTX.

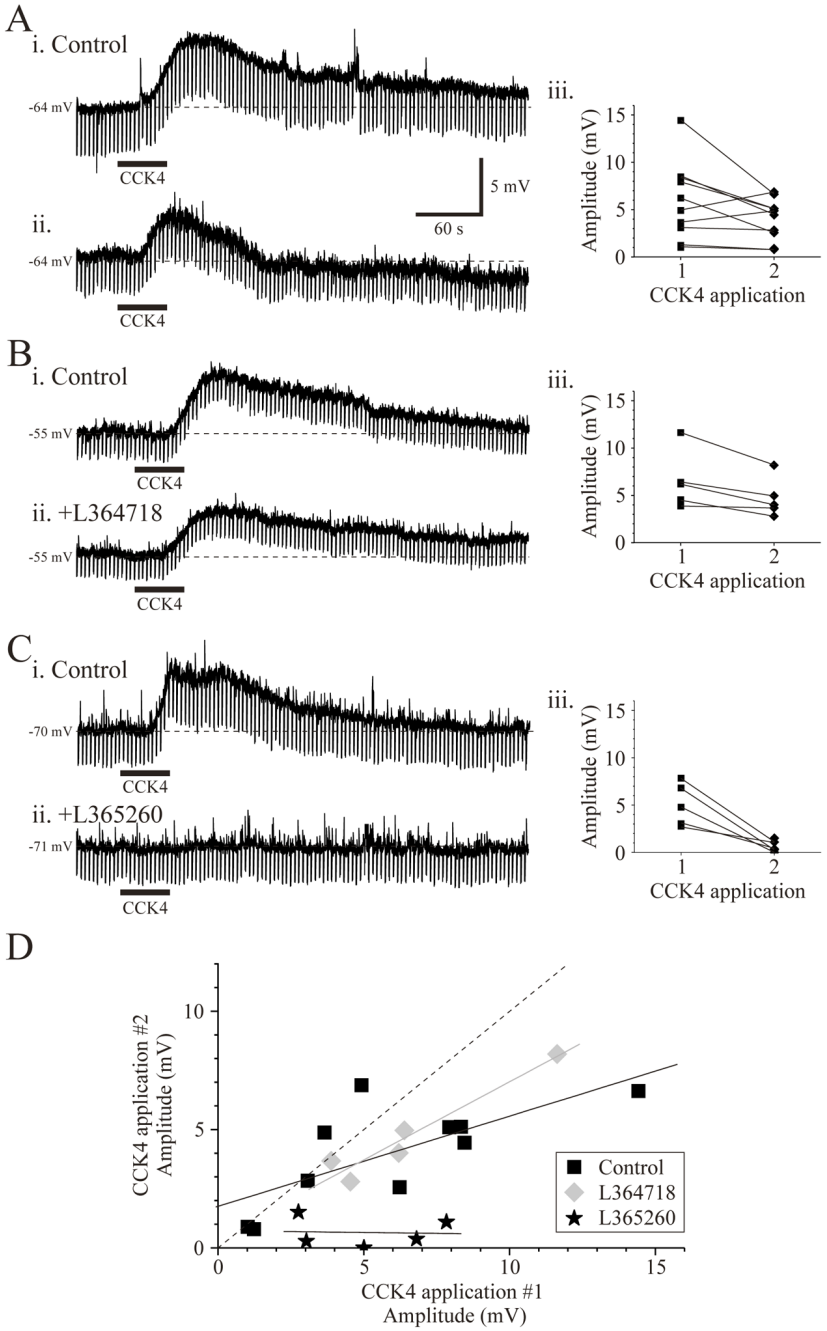

Figure 4.

Selective CCK_B agonist depolarizes layer 6b neurons. **A:** CCK4 (1.25 μM) was applied two times with a 30 min interval. The second response was smaller than the first response (**Ai** & **Aii**). **Aiii:** The average ratio of the second response amplitude to the first response amplitude was 78 ± 11% (n=10). **B:** The effects of CCK4 were not blocked in the presence of CCK_A antagonist L364718 (1 μM). The average ratio of the second response amplitude to the first response amplitude was 74 ± 6% (n=5). **C:** In contrast, CCK_B antagonist L365260 (1 μM) completely attenuated the actions of CCK4. The average ratio of the second response amplitude to the first response amplitude was 17 ± 10% (n=5). **D:** The slopes of control, CCK_A antagonist, and CCK_B antagonist were 0.38, 0.66, and −0.02. The slope of CCK_B antagonist differed

significantly from the control and CCK_A antagonist regressions. The dashed line indicates the slope of 1. All experiments were done in TTX.

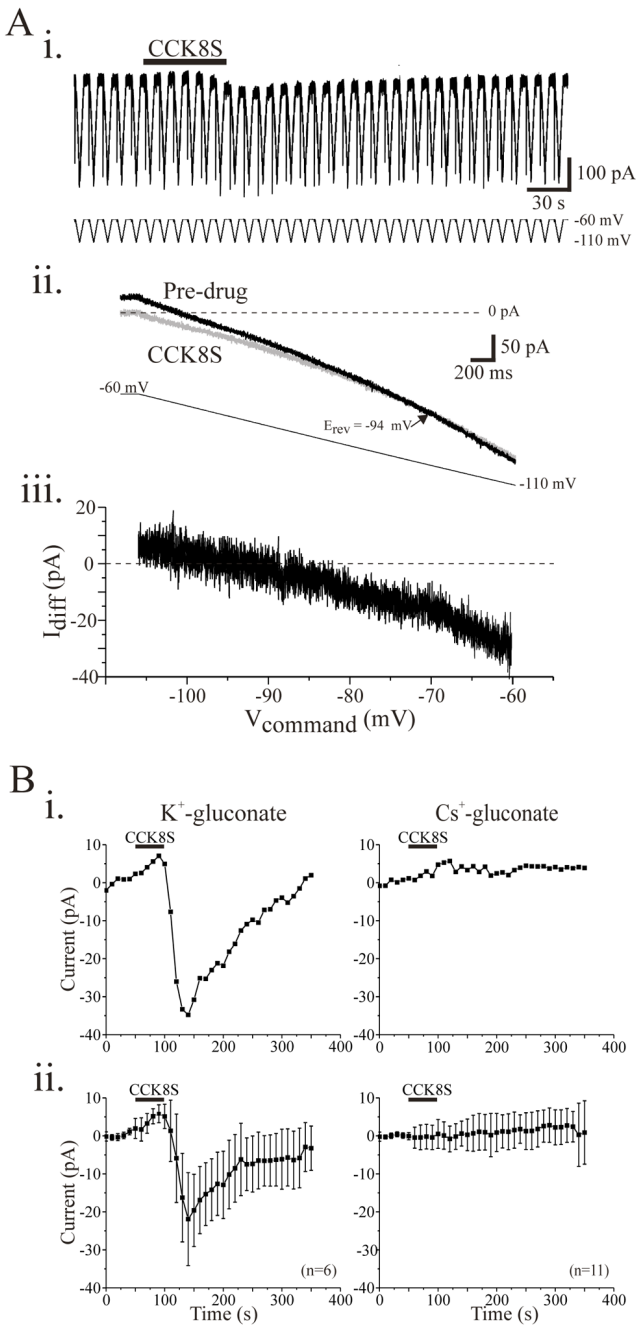

Figure 5.

The CCK depolarization is mediated through suppression of K_{leak} current. Representative current trace revealing the application of CCK8S elicits an inward current associated with a voltage-independent decrease in conductance. **A: i.** CCK8S (1.25 μM, 60 s) evokes inward current. The downward deflections are current responses to voltage ramps (−60 to −110 mV, 3 s in duration). **ii.** Expanded time base of responses to hyperpolarizing voltage ramps before CCK8S application (Pre-drug) and during the peak inward current (CCK8S) are shown. Each trace is an average of 5 sequential responses. After CCK8S application there is an inward shift of the holding current at V_{hold} = −60 mV, and a decrease in the slope of ramp response. The current responses intersect at approximately −94 mV. **iii.** CCK8S reduces a voltage-

independent conductance that reverses near the potassium equilibrium potential. I_{diff} is the difference between the Pre-drug and CCK8S current responses in Aii plotted current vs. voltage. I_{diff}, which is attributable to the actions of CCK8S, has a linear slope, indicating a reduction in conductance. **B:** Intracellular cesium (Cs^+) blocks the inward current evoked by CCK8S (1.25 μM, 60 s). **i.** *Left*, CCK8S evokes slow inward current in recording with K-gluconate electrode solution at holding potential of −60 mV. *Right*, Using cesium Cs^+-gluconate pipette solution, CCK8S did not evoke inward current. **ii.** Population data illustrating the absence of the CCK8S-mediated inward current using the Cs^+-containing pipette solution. All experiments were done in TTX.

Table 1

Basic intrinsic characteristics of different neocortical neurons.

	Layer 6b (n=106)	**Layer 6a (n=25)**	**Layer 5 (n=10)**
Resting membrane potential (mV)	−66.7 ± 0.4	−66.3 ± 0.8	−60.5 ± 1.2
Input resistance (MΩ)	367 ± 15	244 ± 49	257 ± 51