Inhibition dominates the early phase of up-states in the basolateral amygdala

Francois Windels*, James W. Crane*¹ and Pankaj Sah

Queensland Brain Institute, The University of Queensland, Australia. ¹School of Biomedical Sciences, Charles Sturt University, NSW Australia

Running title: Early strong inhibition in the up-state

*equal first authors

Correspondence to:
Pankaj Sah
Queensland Brain Institute
The University of Queensland
Brisbane, QLD 4072
Phone: + 61 7 334 66376
Fax: + 61 7 3346 6301
e-mail: pankaj.sah@uq.edu.au
Summary

Slow oscillations (<1 Hz) in neural activity occur during sleep and quiet wakefulness in both animals and humans. Single-cell recordings in cortical neurons have shown that these oscillations are driven by a combination of excitatory and inhibitory synaptic inputs. During up-states, while the ratio between them varies between cells, excitation and inhibition follow similar time courses. Neurons in the basolateral amygdala (BLA) also display slow oscillations between the resting membrane potential (down-state) and depolarized potentials (up-states). Delivery of footshock during the down-state fully reproduces up-states in these cells. Here we report that up-states in BLA principal neurons up-states begin with an excitatory drive that is rapidly (within ~50ms) overwhelmed by inhibitory input. This excess of inhibitory drive is short lasting (300-400ms) following which up-states are maintained by a tight balance between excitation and inhibition. This initial large inhibitory input restricts action potential generation and reduces the firing frequency of these cells. These results indicate that, in contrast to cortical neurons, up-states in BLA neurons show an initial period of strong cortically driven feed-forward inhibition. For the remainder of the up-state, feedback inhibition then acts to balance excitatory input.

Introduction

Spontaneous oscillations in neural activity over a wide range of frequencies are a common feature of the mammalian brain (Buzsaki 2006; Steriade 1997). These oscillations have been linked to memory formation and consolidation, spatial navigation, and binding of disparate sensory information (Singer and Gray 1995). Of these, the low frequency (< 1 Hz), slow oscillation (Steriade et al. 1993b,c) is seen most prominently during slow-wave sleep and certain forms of anesthesia (Petersen et al. 2003; Steriade et al. 1993b) as well as in acute brain slices maintained in vitro (MacLean et al. 2005; Sanchez-Vives and McCormick 2000; Watson et al. 2008). At the single-cell level, these slow oscillations are seen as spontaneous fluctuations in membrane potential characterized by two phases: down-states, during which the membrane potential is near the resting membrane potential and up-states during which neurons depolarize to near spike threshold and may be accompanied by action

In the basolateral amygdala (BLA), extracellular field recordings have demonstrated a variety of oscillatory states (Pare et al. 2002): slow oscillations are characteristically seen during slow-wave sleep and anesthesia (Collins et al. 2001; Pare and Gaudreau 1996) whereas higher frequency oscillations in the theta range are present during paradoxical sleep and emotional arousal (Pape et al. 2005; Paré and Collins 2000; Seidenbecher et al. 2003). Amygdala-dependent learning leads to synchronization of oscillatory activity within the amygdala (Pare et al. 2002; Seidenbecher et al. 2003). Single cells recordings have shown that principal neurons of the BLA display a slow oscillation in their membrane potential, moving from periods near the resting membrane potential (down-states) to periods at depolarized membrane potentials (up-states) (Crane et al. 2009).

In cortical neurons, up-states are synaptically driven where both excitatory and inhibitory inputs activate and inactivate with a similar trajectory, maintaining a balanced ratio during the up-state (Sanchez-Vives and McCormick 2000; Shu et al. 2003). In BLA neurons, oscillatory activity is also synaptically driven (Crane et al. 2009); however, the excitatory and inhibitory components of up-states remain to be determined. Here we demonstrate that excitation and inhibition in up-states of BLA principal neurons in vivo are not as tightly balanced as in the cortex, being characterized by a dominant inhibitory drive early in the up-state.

Materials and Methods

Data were obtained from Wistar rats (P16-P23; 45±10 g). Animals were housed under standard laboratory conditions with a 12 h light/dark cycle (lights on at 6 AM) and food and water available ad libitum. In all procedures the care and experimental use of animals was in accordance with protocols approved by the University of Queensland Animal Ethics Committee.

Surgery

Animals were anesthetized with urethane (2 g/kg; i.p.). Once a sufficient level of anesthesia was obtained, animals were mounted in a stereotaxic frame and their body temperature maintained at 37°C. Following the exposure of the skull surface, a hole was drilled unilaterally at co-ordinates corresponding to the location of the left
BLA (2.5-2.6 mm posterior and 4.3 mm lateral to midline), and the dura carefully retracted. Another hole was drilled on the contralateral side of the skull to allow a reference electrode to be placed on the cortical surface. Footshocks (5-8 mA, 1 ms) were delivered via two 25 G needles connected to an isolated current generator (Digitimer) and inserted into the footpad contralateral to the side of the BLA recording (i.e. the right footpad).

Electrophysiology

Recording pipettes (shank length of 7.5 mm) were fabricated from borosilicate glass using a Sutter P-87, 3 stage pipette puller. For voltage-clamp recordings the internal solution used was (in mM): 120 CsMeSO4, 20 TEACl, 10 HEPES, 2 Mg2ATP, 0.3 Na3GTP, 0.1 spermine, 10 phosphocreatine, 5 EGTA, 2 CsBAPTA, 5 QX314, and 0.3 % neurobiotin; pH 7.3; Osmolarity 310 mOsm. For control current-clamp recordings pipettes were filled with the following internal solution (in mM):

- 135 KMeSO4, 7 NaCl, 10 HEPES, 2 Mg2ATP, 0.3 Na3GTP, 0.3 EGTA and 0.3 % neurobiotin (pH 7.3; osmolarity 290-300 mOsm). For high-chloride current-clamp recordings pipettes were filled with (in mM): 143 KCl, 7 NaCl, 10 HEPES, 2 Mg2ATP, 0.3 Na3GTP, 0.3 EGTA and 0.3 % neurobiotin (pH 7.3; Osmolarity 290-300 mOsm). Pipettes had a series resistance of 5 – 10 MΩ on fabrication and varied between 20 and 50 MΩ during recordings. Recordings with series resistances higher than this were discarded from the final analysis. Signals were amplified using either a Multiclamp 700B or Axopatch 1D (Molecular Devices), filtered at 5 kHz and digitized at 10 kHz using an Instrutech ITC-18 board. Data acquisition and subsequent analysis were performed using Axograph X (V1.0.8, Axograph scientific).

After completion of the recording, animals were trans-cardially perfused with 2% sodium nitrite solution (in 0.1 M phosphate-buffer, pH 7.4) followed by 50 ml of 4% formaldehyde (in 0.1 M phosphate-buffer, pH 7.4). Brains were then removed and postfixed overnight in 4% formaldehyde at 4°C. Serial, coronal forebrain (100 μm) sections were cut using a freezing microtome and processed using DAB based immunohistochemistry to recover the recorded cell. All cell were confirmed to be pyramidal neurons in the basolateral amygdala (Crane et al. 2009).

Data Analysis

Average traces (n=8-10) of footshock-induced currents occurring at -50 mV (excitatory current) and at +20 mV (inhibitory current) were converted into plots of
conductance. Based on the components of our internal solution and accepted values of the concentration of ions within the extracellular fluid, the estimated driving forces for excitatory-currents was ~ 50 mV, and the estimated driving force for inhibitory-currents was ~ 70 mV. Values for excitatory (G_e) and inhibitory (G_i) conductances, calculated from \( G_{e/i} = I_{e/i}(V_m - E_o) \), where \( G \) is the conductance, \( I_{e/i} \) the current and \( E_o \) the reversal potentials. Conductance was then plotted with respect to time. The latency to the up-state onset was measured as the time from the stimulus artifact to when the inward current attained 5% of the initial peak. All statistical comparisons were made using Students t test and results are presented as the mean ± SEM.

**Results**

In anesthetized animals, neurons in the BLA show sustained slow oscillations in membrane potential with delivery of a single footshock during the down-state evoking an all-or-none transition into an up-state (Fig 1A) and entraining the oscillatory phase (Crane et al. 2009). These oscillations are thought to be driven by extra-amygdalar, presumed cortical, synaptic inputs (Crane et al. 2009). To determine the contribution of excitatory and inhibitory conductances to the up-state, neurons were voltage clamped with a cesium-based internal solution containing QX314 to block intrinsic sodium, potassium and some other voltage dependent channels (see Methods). Under these conditions, at a holding potential of -50 mV (near the estimated chloride equilibrium potential), up-states were detected as periodic bursts of inward-current (Fig 1B). Depolarization of the neuron revealed short bursts of outward currents that appeared to be predominantly located at the start of spontaneous up-states (Fig 1B). At depolarized membrane potentials, the exact onset time of spontaneous up-states, and thus the time course of synaptic inputs driving the up-state, could not be unequivocally established. However, in BLA neurons, footshock-evoked up-states fully reproduce spontaneous up-states (Fig 1A, B) (Crane et al. 2009). For example, at a holding-potential of -50 mV, footshock-evoked up-states were similar in duration to those occurring spontaneously, with a mean duration of 1.12 ± 0.02 s (n=7) while spontaneous up-states had a mean duration of 1.11 ± 0.03 s (n=6, P = 0.07). We therefore assume that foot-shock evoked up-states are equivalent to spontaneous up-states and have examined the time course of up-states evoked by footshocks.
Footshock-evoked up-states recorded over a range of membrane potentials are shown in Fig 1C. It can be seen that at depolarized membrane potentials, as with spontaneous up-states (Fig 1B), there is an early outward current apparent at the start of the up-state that reverses at ~50 mV. The mean reversal potential for this early component was -43 ± 3 mV (n = 5). The cerebrospinal fluid chloride concentration has been measured to be ~150 mM (Hammond and Tritsch 1990) and our internal solution had a chloride concentration of 30 mM, giving a reversal potential of -43 mV for Cl ions. Thus, this early current reverses near the predicted chloride equilibrium potential (Fig 1D), revealing the contribution of GABAergic inhibitory synaptic activity to the up-state. The more long-lasting inward current seen is the summed activity of excitatory glutamatergic inputs. At +20 mV, near the reversal potential for ionotropic glutamate receptor currents, the outward current has a large early component that then decays towards the baseline, showing that that there is an initial large transient component of inhibition during the up-state.

To quantify the time course of excitation and inhibition, inward and outward currents (as measured at -50 mV and +20 mV respectively) were converted to conductance using their predicted reversal potentials (Fig 2A, B). Footshock-evoked up-states began with an excitatory component with a latency of 68.5 ± 6.4 ms (n=7) that was rapidly replaced by inhibition (latency = 101 ± 6 ms; n=7). Inhibitory conductance peaked at 148 ± 12 ms (n=7), and was followed by a period during which inhibition and excitation were evenly matched. At its peak, the mean inhibitory conductance was 2876 ± 1099 pS and the mean excitatory conductance was 1478 ± 433 pS (n=7). To examine the relationship between these two synaptic drives we calculated the ratio of inhibitory conductance to excitatory conductance during up-states. As can be seen (Fig 2C) the up-state begins with an excitatory input but is replaced by a dominant inhibitory drive during its early phase. However, this inhibition is short lasting and excitation and inhibition are tightly balanced during the remainder of the up-state.

To test the functional role of this early dominant inhibition, current-clamp recordings were made with a pipette solution containing high intracellular chloride (143 mM). This internal solution shifts the chloride reversal potential to near 0 mV, such that opening of chloride channels at resting membrane potentials depolarizes the neuron. BLA principal neurons in vivo have a low spontaneous firing rate and, consistent with this, up-states were rarely accompanied by action potentials (Fig 1, 3).
giving a mean firing rate of 0.5 Hz (Crane et al. 2009). In contrast, when loaded with
high intracellular chloride, up-states were accompanied by a burst of action potentials
(Fig 3B) with a mean of 6.2 ± 2.7 action potentials per up-state and an overall spike
frequency of 2.7 ± 1.2 Hz (n=3). This increase in action potentials was confined to
the early part of the up-state with the average spike frequency during the first 150 ms
of the up-state being 5.9 ± 0.8 Hz (n=3) with in chloride loaded neurons, significantly
higher (p < 0.01) than seen in control conditions (0.93 ± 0.2 Hz, n=6). For
comparison, the spike frequency seen 450-600 ms after the onset of the up-state was
5.7 ± 1.6 Hz (n=3), not significantly different from that in recordings performed with
normal chloride internals (4.1 ± 0.7; n=6; p = 0.21). These results are consistent with
peak inhibition occurring within the first 150 ms of the up-state and show that
inhibition acts early in the up-state as a brake on action potential initiation.

Discussion

Rhythmic slow oscillations in membrane potential are seen in a number of
brain regions during some types of anesthesia, as well as slow-wave sleep and periods
of quiet wakefulness (Buzsaki and Draguhn 2004; Petersen et al. 2003; Steriade 2006;
1997). In the BLA, oscillatory activity is seen in extracellular field recordings during
anesthesia, sleep and also during periods of wakefulness (Collins et al. 2001; Paré and
Collins 2000; Pare and Gaudreau 1996; Seidenbecher et al. 2003). Recordings from
BLA neurons in anesthetized animals show a slow oscillation in their membrane
potential, moving from periods near the resting membrane potential (down-state) to
periods at depolarized membrane potentials (up-state) (Crane et al. 2009). In this
study we have determined the properties of synaptic inputs that underlie these
oscillations. Using whole-cell voltage-clamp recordings from principal cells in vivo
we have shown that up-states are driven by synaptic input that begins with an
excitatory input but is overwhelmed by inhibition after about 50ms. Inhibition is the
dominant synaptic drive in the early part of the up-state, but is short lived (~400ms),
being replaced with balanced inhibition and excitation. This dominant inhibition
early in the up-state delays action potential firing, thus limiting the overall firing rate
of these neurons.

Persistent, spontaneous activity is seen in networks of neurons in cortical and
thalamic regions (Steriade 1997; Wilson 2007) with both excitatory and inhibitory
neurons displaying similar oscillatory activity (Wilson 2007). Consistent with this,
recordings from single neurons show tightly balanced excitatory and inhibitory synaptic input such that inhibitory and excitatory activity covaries during up-states both in vitro (Sanchez-Vives and McCormick 2000; Shu et al. 2003) and in vivo (Haider et al. 2006; Wilson 2007) (Rudolph et al. 2007). It is notable that there are clear differences in the absolute ratio of excitation to inhibition with reports where the two conductances are equal during the entire up-state (Shu et al., 2003) whereas in others inhibition is dominant (Rudolph et al., 2007). The reasons for the difference between these two situations is not known. Using a combination of modeling and single-electrode voltage-clamp, this analysis has led to the conclusion that the transition into, and exit from up-states results from changes in excitatory synaptic input, and the tight balance between excitation and inhibition during the up-state results from local feedback inhibition (Haider et al. 2006; Sanchez-Vives and McCormick 2000; Shu et al. 2003). In our study, whole-cell voltage clamping with relatively low-resistance electrodes has allowed us to voltage clamp neurons over a range of membrane potentials to directly assess the contribution of synaptic inputs to the generation of up-states. Although voltage control is not complete, it clearly shows that while oscillations in BLA neurons share some features with those in cortical neurons, there are also clear differences. In cortical neurons, entry into an up-state results from an increase in excitatory drive and, inhibitory input tracks the change in excitation (Haider et al. 2006). In contrast, in the BLA, while entry into the up-state is initiated by a rise in excitatory input, it is rapidly overwhelmed by a large, but transient, inhibitory drive. This inhibition then decreases and, as with cortical neurons (Sanchez-Vives and McCormick 2000; Shu et al. 2003) (Rudolph et al. 2007), excitatory and inhibitory drive covary during the remainder of the up-state. Moreover, in cortical neurons, entry into the up-state is accompanied by action potentials (Haider et al. 2006) whereas in BLA neurons the presence of strong inhibition early in the up-state limits action potential generation resulting in the low action potential discharge during the up-state.

The BLA is a cortical-like structure of which ~80% of the neuronal population are pyramidal-like glutamatergic neurons. The remaining population comprises interneurons with local connections providing both feed-forward and feed-back inhibition that tightly controls principal neuron activity (Ehrlich et al. 2009; Lang and Pare 1997; Woodruff and Sah 2007). Local inhibition is potent and has a strong inhibitory effect on principal cell firing (Woodruff and Sah 2007). The early onset of
strong inhibition, during which time principal neurons show little activity, suggests
that this early inhibition is driven by feed-forward local interneurons. Excitatory and
inhibitory inputs are balanced during the remainder of the up-state, including the
return to the down-state. As principal neurons are active throughout this later period,
the maintained inhibitory activity is likely to result from the activity of local feed-
back interneurons.

The BLA receives extensive projections from a host of cortical regions
(McDonald 1998). As cortical projections drive up-states in thalamic and striatal
neurons (Steriade et al. 1993a; Wilson 1993), it is likely that up-states in BLA
neurons are also driven by projections from cortical networks. The similarity of
footshock-evoked up-states and spontaneous up-states suggest that footshocks trigger
the same cortical networks that are active during spontaneous up-states. Consistent
with this is our finding that footshock reset the phase of spontaneous oscillations and
BLA neurons are unresponsive to footshock when delivered during spontaneous up-
states (Crane et al. 2009). Our results indicate that, as well as providing the initial
excitatory drive to principal neurons of the BLA, cortical inputs also trigger
feedforward inhibition via the activation of local interneurons.

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Figure legends

Figure 1. Up-states in basolateral amygdala principal neurons are characterized by an early period of strong inhibition. A. Current-clamp recording from a principal neuron in vivo shows sustained slow oscillations. Delivery of a footshock (arrow) in the down-state causes an all-or-none transition into an up-state similar to spontaneously occurring up-states. B. Voltage-clamp recordings using a cesium-based internal solution, of spontaneous up-states in a principal neuron voltage clamped at the indicated membrane potentials. Depolarisation shows an early, short lasting outward current. C. Average traces of footshock-induced (arrow) up-states (n=10-15 traces) recorded over a range of membrane potentials. With membrane depolarization, an early short lasting current is apparent, as seen in spontaneous up-states. D, Current-voltage relationship of the early component for the cell shown in C, with peak currents measured at the dotted line in C. The dotted line is the linear regression fit to the data between -60 and 0 mV, and gives a reversal potential of -47 mV.

Figure 2. Inhibition is dominant in the early part of the up-state in the basolateral amygdala. (A) Mean (n=7 cells) excitatory (Ge) and inhibitory (Gi) drive calculated as conductance change from recordings at -50 mV (Ge) and +20mV (Gi) (n=7) during footshock-induced up-states. The grey regions plot ± SD (B) Expanded view of the first 150 ms of plot in A showing that entry into the up-state begins with excitation that is then dominated by inhibition. C. Mean Gi/Ge ratio during footshock-induced up-states. Inhibition dominates the early phase, but is tightly balances with excitation during the later phase of the up-state.

Figure 3. Early inhibition blocks action potential firing during the early part of the up-state. A, Spontaneous oscillations recorded using control internal solution (left) and an internal solution with high chloride (right). Action potentials are more prominent in high chloride internal solutions. B, Individual up-states recorded under
control conditions (upper trace) and with chloride loading (lower trace). C, Average firing frequency of action potentials during the initial part of the up-state (0-150 ms) and later in the up-state (450-600 ms) measured with normal chloride solution (black bars) and high chloride internals (shaded bars). Significantly higher frequency of action potentials are seen during the early part of the up-state in high-chloride loaded neurons. ** indicates $P < 0.01$. 

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