NMDA receptors are the basis for persistent network activity in neocortex slices

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During behavioral quiescence the neocortex generates spontaneous slow oscillations that consist of Up and Down states. Up states are short epochs of persistent activity but their underlying source is unclear. In neocortex slices of adult mice, we monitored several cellular and network variables during the transition between a traditional buffer, that does not cause Up states, and a lower divalent cation buffer, that leads to the generation of Up states. We found that the resting Vm and input resistance of cortical cells did not change with the development of Up states. The synaptic efficacy of excitatory postsynaptic potentials mediated by non-NMDA receptors was slightly reduced but this is unlikely to facilitate the generation of Up states. On the other hand, we identified two variables that are associated with the generation of Up states; an enhancement of the intrinsic firing excitability of cortical cells and an enhancement of NMDA mediated responses evoked by electrical or optogenetic stimulation. The fact that blocking NMDA receptors abolishes Up states indicates that the enhancement in intrinsic firing excitability alone is insufficient to generate Up states. NMDA receptors have a crucial role in the generation of Up states in neocortex slices.
Neocortex slow oscillations, characteristic of slow wave sleep and anesthesia in vivo (Steriade et al. 1993), can be generated in slices maintained in vitro (Castro-Alamancos 2009; Crunelli and Hughes 2010; McCormick et al. 2003). Slow oscillations are characterized by rhythmic cycles of synaptically mediated depolarization and action potentials (Up states), followed by decrease of synaptic inputs, leading to membrane hyperpolarization and cessation of firing (Down states). Slow oscillations consisting of Up and Down states occur spontaneously in isolated cortical slices of adult ferrets (Sanchez-Vives and McCormick 2000; Shu et al. 2003b), developing [postnatal day 14 (P14) to P21] (Wester and Contreras 2013) or adult rats (Cunningham et al. 2006), and adult mice (Castro-Alamancos and Rigas 2002; Rigas and Castro-Alamancos 2007) bathed in an ACSF that contains a concentration of divalent cations that resembles the “natural” CSF (i.e., ~1 mM $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$). This concentration is lower than the traditional ACSF (i.e., 2 mM or higher) typically used in slices.

Slice studies have used this method to determine the balance of excitatory and inhibitory conductances during slow oscillations (Shu et al. 2003b), the impact of slow oscillations on afferent inputs (Rigas and Castro-Alamancos 2009; Shu et al. 2003a), the role of thalamocortical and intracortical afferents in controlling slow oscillations (Favero and Castro-Alamancos 2013; Rigas and Castro-Alamancos 2007), and the effects of neuromodulators on slow oscillations (Favero et al. 2012; Wester and Contreras 2013), among other studies.

For the most part, previous studies have not focused on the cellular and synaptic variables that change in cortical networks, as the ACSF is adjusted, to allow the generation of slow oscillations. This requires holding intracellular and extracellular recordings during the transition between before and after the generation of slow oscillations. A previous study mentioned that the amplitude of postsynaptic potentials evoked by electrical stimulation did not change significantly
in five cells held during this transition (see Methods in (Reig et al. 2006). Unpaired comparisons between different cells in different ACSF conditions found that short-term depression was reduced by the ACSF that produces slow oscillations (Reig et al. 2006), which is expected based on the known effects of reducing \([\text{Ca}^{++}]_o\) on short-term depression in neocortex (Castro-Alamancos and Connors 1997). Here we monitored cellular and synaptic properties of cortical networks during this transition in the same cells (paired comparisons). The results show that a main change in cortical networks associated with the development of slow oscillations in slices is the enhancement of NMDA synaptic responses. Together with the fact that slow oscillations are abolished by NMDA receptor antagonists highlights a critical role of these receptors in the generation of neocortex slow oscillations in vitro.

**METHODS**

All procedures were reviewed and approved by the Animal Care Committee of Drexel University. Slices were prepared as previously described (Favero and Castro-Alamancos 2013; Favero et al. 2012; Rigas and Castro-Alamancos 2009; 2007) from adult (>8 weeks) CD-1 or Thy1-COP4/EYFP (line18) mice. Some CD-1 mice were anesthetized with ketamine-xylazine (100-5 mg/kg) and injected with AAV2/9-hSyn-ChR2(H134R)-eYFP (UPenn Vector core) into the somatosensory thalamus (0.2-0.4 µl) using the following coordinates (from bregma):

- posterior: 1.5, lateral: 2.0, and ventral: 3.0 mm. Slices were prepared from these animals 1-2 weeks after the injection.

For slice preparation, mice were deeply anesthetized with an overdose of ketamine. Upon losing all responsiveness to a strong tail pinch, the animal was decapitated and the brain was rapidly extracted. Slices (400 µm thick) were cut in the thalamocortical plane (Agmon and
Connors 1991) using a vibratome. Slices were transferred to an interface chamber where they were bathed constantly (1-1.5 ml/min) with artificial cerebrospinal fluid (ACSF) at 32.5°C. We used two ACSFs; control and low buffer. The *control* ACSF contained (in mM): NaCl (126), KCl (3.5), NaH2PO4 (1.25), NaHCO3 (26), Dextrose (10), MgSO4 7H2O (2), CaCl2 2H2O (2). The *low buffer* ACSF was identical to the control ACSF except for MgSO4 7H2O (1), and CaCl2 2H2O (1), which were lowered to 1 mM. Field potential (FP) recordings were made using low impedance (~0.5 MΩ) glass pipettes filled with ACSF. Blind whole-cell recordings were obtained from layers V, IV, and III cells of somatosensory cortex using patch electrodes of 4-12 MΩ impedance. For current clamp recordings, the electrodes were filled with internal solution containing (in mM): 135 K-gluconate, 4 KCl, 2 NaCl, 0.2 EGTA, 10 Tris-Phosphocreatine, 0.3 trisGTP, 10 HEPES, 4 MgATP (290 mOsm). Under our conditions, the Nernst equilibrium potential for Cl⁻ is -81 mV and for K⁺ is -96.7 mV. In most cases, the internal solution contained neurobiotin (0.2%) to label the recorded cells. The results from cells located in different layers (V-III) were pooled together because we found no significant differences in the effects of the experimental manipulations.

After each experiment, the slices were fixed in 4% paraformaldehyde with 1% glutaraldehyde, later cryoprotected with sucrose (30%) and re-sectioned on a cryostat (80 µm). Non-fluorescent sections incubate in 3% hydrogen peroxide, followed by 0.2% Triton X-100 and by incubation in 2% goat serum. Incubation with ABC reagent (Vectorlabs, CA) occurs overnight. The following day, diaminobenzidine is applied to the sections. After color development, sections are mounted and cleared in xylene. Fluorescent sections (from animals injected with AAV or line18) are mounted, cover-slipped with DAPI mounting media, and photographed using a fluorescent microscope. Fluorescent sections that contain neurobiotin-
filled cells are incubated in 0.2% Triton X-100 and 2% goat serum followed by Dylight594-Streptavidin. Sections are then mounted, cover-slipped with DAPI mounting media, and photographed using a fluorescent microscope. The eYFP from the ChR2 expression appears greenish, the neurobiotin filled cell appears redish, and DAPI appears blueish. Examples of these fluorescent sections and of reconstructed cells recorded in similar experiments can be found in previous publications (Favero and Castro-Alamancos 2013; Favero et al. 2012; Rigas and Castro-Alamancos 2007).

A concentric bipolar stimulating electrode (125-µm diameter) were used to electrically stimulate the cortex (intracortical). The intracortical electrical stimulating electrode was placed lateral to the recording electrodes (~400 µm) in layers III-II. To derive an input-output curve, 5 stimulation intensities, typically between 10-50 µA, were used. A 200-µm core diameter multimode optic fiber was used to apply pulses of blue light in the cortex (around the recording site). The light source was an LED (~473 nm) driven by pulses of 0.2-5 ms duration (or longer to test if the cell responds directly to the light). The blue light intensity was controlled by adjusting the output range of the light source or the duration of the pulse, and could be easily monitored by recording the evoked short-latency FP response. In addition, the intensity (irradiance) of the light beam exiting the optic fiber was measured by flashing a photodiode power sensor placed in the location of the slice. The light intensity range was 0-8 mW/mm², which correspond to relative values of 0-5. The FP electrode was first used to identify the cortical region with a strong and short latency response evoked by electrical stimulation and light. Intracellular recordings were then obtained adjacent to the FP electrode. Drugs were dissolved in the ACSF at the indicated concentrations.
Electrical or blue light stimulation were applied at a minimum 5 sec apart between each
other and recurred at an interval of 5 sec or higher. During each stimulus the membrane potential
(Vm) could be set at different potentials by applying negative and positive current pulses (before
the synaptic stimulus onset), up to the level which produced spontaneous firing. This allowed
deriving the reversal potential for each point of the synaptic response and estimating the
excitatory and inhibitory synaptic conductance (Gsyn_{exc} and Gsyn_{inh}, 0 mV and -75 mV reversal
potential, respectively) before and during a drug (see (Favero and Castro-Alamancos 2013;
Favero et al. 2012) for details).

Up states were detected offline by using a threshold detector in the FP recording (see
(Rigas and Castro-Alamancos 2009; 2007). Relatively rare transient FP events that are not Up
states are easily rejected by setting the detection algorithm to reject short duration events (<50
ms). In addition, all detected events are sent to a sorting algorithm (similar to those used to sort
spikes) and this allows classifying all detected Up states based on several projections (e.g.
principal components, etc). Finally, all detected events (selected or unselected as Up states) are
inspected by eye to assure that the procedure was adequate.

For statistical analyses, data were first tested for normality using the Shapiro–Wilk test. If
the data was considered normally distributed, parametric statistics were applied (ANOVA
repeated measures or t-test paired). Otherwise, we applied non-parametric statistics (Wilcoxon
signed ranks for paired comparisons, Mann-Whitney for non-paired comparisons, Kruskal-
Wallis for multiple groups). To compare input-output curves or firing excitability between two
conditions we used a two factor repeated measures ANOVA. The buffer factor had two levels
(control and low buffer) and the intensity factor had as many levels as the intensities tested.
RESULTS

Low Buffer leads to the generation of Up states that depend on NMDA receptors

Traditionally, cortical slices of adult mice maintained in vitro are studied using ACSF buffer in which the concentration of divalent cations (Ca$^{2+}$ and Mg$^{2+}$) is 2 mM or higher (termed here *control buffer*). Under these conditions, adult cortical slices show nil spontaneous network activity. As described above, lowering the divalent cation concentration to ~1 mM (termed here *low buffer*) in cortical slices of adult mice leads to the generation of spontaneous and evoked network activity that resembles cortical slow oscillations observed in vivo. Figure 1 shows the appearance of slow oscillations consisting of Up and Down states upon changing between control buffer and low buffer. During control buffer there was little or nil spontaneous network activity reflected in both the Vm of the recorded cell and the population FP. In contrast, after switching to low buffer, network activity consisting of Up and Down states readily emerged. This activity was rapidly and completely abolished by blocking NMDA receptors (Fig. 1C; D-AP5; 50 µM; n=12), as previously described (Favero and Castro-Alamancos 2013). Note that Up states are not abolished by blocking AMPA receptors; instead Up states become less frequent and shorter in duration but are readily evoked by electrical or optogenetic stimulation of cortical pathways (Favero and Castro-Alamancos 2013).

Similar to the effects on spontaneous Up states, low buffer also unmaskes evoked Up states that depend on NMDA receptors. FP and intracellular responses were evoked by a stimulating electrode placed in layers III-II during control and low buffers. Figure 2A,B shows a typical experiment in which the buffer was changed from control to low buffer. Each trace is an average of 5-10 FP and intracellular responses evoked by 5 different intensities (10-50 µA) to produce an input-output curve during each condition. Subsequent addition of AP5 (50 µM),
completely abolished the evoked Up states leaving only the non-NMDA mediated response (termed AMPA response, for simplicity; note that GABA receptors are not blocked). As previously, we measured the amplitude of short-latency evoked FP responses (<15 ms), and the amplitude of the longer latency evoked Up states (Favero and Castro-Alamancos 2013; Rigas and Castro-Alamancos 2007). Low buffer had negligible effects on the amplitude of the short-latency responses (see below), but led to the development of evoked Up states (Fig. 2C; n=6 p<0.001). Note also that evoked Up states are suppressed by higher intensities (due to stronger feed-forward inhibition driven by cooperativity) (Favero and Castro-Alamancos 2013). This is reflected in the population data (Fig. 2C) because different slices have different thresholds for Up state induction and suppression.

**Low Buffer enhances NMDA mediated responses**

The dependence of Up states on NMDA receptors led us to test the hypothesis that low buffer causes Up states by enhancing NMDA synaptic responses as a result of lowering the divalent cation concentration in the ACSF. At resting Vm, the NMDA receptor channel is blocked by Mg$^{2+}$ depending on the [Mg$^{2+}$]$_o$ in the ACSF; at lower [Mg$^{2+}$]$_o$, NMDA responses are potentiated (Mayer et al. 1984; Nowak et al. 1984). Cortical slices bathed in 1-2 mM [Mg$^{2+}$]$_o$ are known to show evoked NMDA responses at resting Vm (Castro-Alamancos and Connors 1996; Espinosa and Kavalali 2009; Kanter et al. 1996). In contrast to the potentiating effects of lowering [Mg$^{2+}$]$_o$ on NMDA synaptic responses, lowering [Ca$^{2+}$]$_o$ is well known to suppress synaptic responses by reducing release probability (Katz 1969), including in neocortex (Castro-Alamancos and Connors 1997; Rozov et al. 2001). To decipher these contrasting effects on synaptic responses, we tested the effect of low buffer on isolated AMPA and NMDA mediated
responses. We focused on evoked responses because spontaneous events (such as mini
frequencies) are difficult to compare between control and low buffer conditions due to the
absence of any spontaneous network activity during the former and the development of Up states
during the latter.

AMPA responses, measured in the presence of AP5 (50 µM), consist of sharp short
latency FP responses (< 20 ms) coincident with intracellular PSPs. Low buffer had no significant
effect on the peak amplitude of short-latency FP AMPA responses (n=19 slices; p=0.8; Fig. 2D,
left panel). Also, low buffer did not affect FP AMPA responses >20 ms, and thus did not
generate evoked Up states (n=19 slices; p=0.4). However, low buffer had subtle but consistent
effects on the short-latency AMPA response evoked by electrical stimulation. Figure 3 shows
these effects in two example cells. First, low buffer slightly but significantly (n=19 slices;
p=0.003) delayed the time-to-peak of the evoked FP response (Fig. 2D, right panel and Fig. 3A).
This change in the FP response was associated with a significant reduction in the rising slope of
the evoked EPSPs in simultaneously recorded cells (n=5 cells; p=0.01; Fig. 3). Second, the short
latency FP response was slightly broadened so that the falling phase of this response occurred
slightly later. The FP broadening was associated with an increase in spike probability in the
evoked intracellular response on top of the slower rising EPSP slope (Fig. 3C). We will return to
this increase in firing excitability later.

NMDA responses, measured in the presence of CNQX (10 µM), consist of slower rising
and longer lasting FP and PSPs than AMPA responses (Fig. 2E and Fig. 4). Low buffer had a
robust enhancing effect on both FP and intracellular NMDA responses, which were abolished by
subsequent AP5. Figure 4 shows these effects in three example experiments; one FP (Fig. 4A)
and two intracellular (Fig. 4B) recordings. The second cell example in figure 4B (lower panel)
showed an evoked EPSP followed by an IPSP at higher intensities; both were abolished by subsequent application of AP5, confirming that they were driven by NMDA receptors. Population measurements revealed that low buffer significantly increased both the peak amplitude (n=21 slices; p<0.001; Fig. 2E, left panel) and the area of the FP NMDA response (p<0.001; Fig. 2E, right panel) measured between 3-50 ms poststimulus. The peak amplitude of intracellular EPSPs, excluding spikes, was also significantly enhanced by low buffer (n=8 cells; p=0.001).

These results indicate that low buffer enhances NMDA mediated responses, and slightly suppresses the synaptic efficacy of AMPA responses. In addition, low buffer seems to enhance the firing excitability of cortical cells, which will be addressed below.

Low buffer also enhances optogenetically evoked NMDA responses

Electrical stimulation within neocortex is fairly unselective; it directly excites the membranes of all excitatory and inhibitory cells surrounding the stimulating electrode. To address this confound, we used slices from Thy1(line18) mice, which primarily express ChR2 in excitatory cortical cells (Wang et al. 2007). In these mice, layer V pyramidal cells of the somatosensory cortex robustly express ChR2 (Favero and Castro-Alamancos 2013). We also used a few CD-1 (n=4) mice that express ChR2 in thalamocortical fibers. This was accomplished by infusing an AAV into the somatosensory thalamus of CD-1 mice to express ChR2 in thalamocortical fibers (Cruikshank et al. 2010; Favero and Castro-Alamancos 2013; Zhang et al. 2006; Zhang et al. 2007). Note that FP and intracellular responses evoked by optogenetic stimulation (blue light) have been characterized previously in these mice (Favero and Castro-Alamancos 2013). In Thy1 slices, many cortical cells respond directly to blue light, and FP
responses have a short-latency response component driven by direct activation of responding
cells, which is not abolished by glutamate receptor antagonists or TTX. In CD-1 slices that
express ChR2 in thalamocortical fibers, cortical cells do not respond directly to blue light, but
the FP has a short-latency component that reflects the direct activation of thalamocortical fibers,
which is also not abolished by the mentioned blockers. Here the effect of low buffer was tested
on isolated AMPA and NMDA responses evoked by a spot of blue light delivered by an optic
fiber centered in layer IV.

Figure 5 shows examples obtained from three experiments. In the first case (Fig. 5A),
AMPA responses (during AP5) were evoked by five different intensities of blue light in a Thy1
slice. As per electrical stimulation, the spike probability increased during low buffer compared to
control, and there was also a corresponding broadening of the evoked FP response. Similar
effects were obtained in four other cells. Population measurements of FP responses revealed that
low buffer did not significantly affect the peak amplitude of short-latency AMPA responses in
Thy1 mice (n=18; p=0.4). Also, low buffer did not unmask long latency responses (>20 ms).
Measurement of the rising slope of EPSPs evoked in intracellular recordings by blue light did not
reveal a significant change (n= 5 cells; p=0.3). This was different compared to electrically-
evoked responses, which decreased, and may be due to the fact that ChR2 channels provide an
additional Ca\(^{2+}\) source (Zhang et al. 2006), which may counteract the effect of lowering [Ca\(^{2+}\)]\(_o\)
on synaptic transmission.

Figure 5B shows NMDA responses (during CNQX) evoked by five different intensities
of blue light in a Thy1 slice in a cell that expresses ChR2. As per electrical stimulation, low
buffer caused a significant enhancement of the NMDA response evoked by blue light, which
appeared as a long-latency depolarization. In this cell, the unmasked NMDA response was fairly
long latency and subsequent application of AP5 completely abolished it. AP5 left intact the
short-latency response indicating that this cell expressed ChR2; responding directly to blue light
in the presence of glutamate receptor antagonists. This was confirmed by using long pulses of
blue light which directly depolarize the cell for the duration of the pulse, as previously shown
(Favero and Castro-Alamancos 2013). Similar effects in cells expressing or not expressing ChR2
(see Fig. 5C) were obtained in six other cells. Population measurements of FP responses revealed
that low buffer significantly increased the peak amplitude and the area of the evoked NMDA
response (3-50 ms) in Thy1 mice (n=26; p<0.001).

Figure 5C shows NMDA responses (during CNQX) evoked by five different intensities
of blue light in a slice from an animal expressing ChR2 in thalamocortical fibers. This cell
showed NMDA responses at the lowest intensities, but these were inhibited at higher intensities
(see (Favero and Castro-Alamancos 2013)). Low buffer caused the NMDA evoked responses to
become much larger and also revealed IPSPs driven by the thalamocortical stimulation,
indicating that the enhancement of the NMDA response produced a stronger thalamocortical
response that more effectively drove inhibitory interneurons (Cruikshank et al. 2010; Sun et al.
2006). As expected, the intracellular NMDA evoked response was completely abolished by AP5.
The postsynaptic FP response was also abolished, except for the thalamocortical fiber volley that
is driven by ChR2 ion channels (Favero and Castro-Alamancos 2013). Similar results were
obtained in three cells. Population measurements of FP responses revealed that low buffer
significantly increased the area of the evoked NMDA FP response (3-50 ms) in slices expressing
ChR2 in thalamocortical fibers (n=5; p<0.001). Thus, low buffer appears to also potentiate
NMDA responses driven by thalamocortical fibers, which may explain the high effectiveness of
thalamocortical stimulation in triggering Up states (Favero and Castro-Alamancos 2013; Rigas and Castro-Alamancos 2007).

Effect of low Buffer on isolated IPSPs

We next determined the effect of low buffer on isolated IPSPs evoked in the presence of glutamate receptor antagonists (CNQX and AP5, 10 and 50 µM, respectively) by a stimulating electrode located adjacent (200-400 µm lateral) to the recorded cells within layers V-III. We estimated the inhibitory synaptic conductance from synaptic potentials evoked at different Vm set by variable current pulses (see (Favero and Castro-Alamancos 2013; Favero et al. 2012)). Figure 6A shows the effect of low buffer on two different cells. Both of these cells show a relatively small suppression of the evoked inhibitory conductance. However, this effect was statistically significant when considering the population of cells (n= 8; p=0.002; Fig. 6B). Thus, low buffer produces a very small but significant suppression of the isolated inhibitory conductance.

Low buffer increases firing excitability

From the population of cells that were recorded during control and low buffer, we measured resting Vm, input resistance, and intrinsic firing. Vm did not change significantly as a consequence of low buffer for the whole population of cells measured (Fig. 7A; n=39 cells; p=0.87). To measure input resistance we applied negative current pulses of different amplitudes (-0.1 to -0.4 nA). Input resistance was estimated in two different ways. First, we measured the voltage drop caused by each of the negative current pulses (-0.4 to -0.1 nA; Fig. 7B). Second, we measured the decay time constant of the voltage after the offset of the -0.2 nA current pulse (Fig.
7C), which is less sensitive to changes in access resistance that may confound changes in input resistance. We found that low buffer did not cause a significant change in input resistance measured with any of these two methods (n=32 cells; p>0.5).

The preceding results that tested evoked excitatory responses suggest that low buffer increases firing excitability. To measure intrinsic firing we applied positive current pulses (500 ms) of different amplitudes. The amplitude of the current pulses was the same for each cell before and after low buffer but varied slightly between cells as a function of input resistance (the mean currents applied ranged between: 0.05 to 0.2 nA). We found that low buffer significantly increased the number of spikes evoked by the same current pulse (n=32 cells; p<0.001; Fig. 7D,E). Thus, low buffer increases intrinsic firing excitability.

**DISCUSSION**

*The effects of low buffer*

The low buffer that leads to the generation of Up states in neocortex slices consists of lowering [Mg$^{2+}$]$_o$ and [Ca$^{2+}$]$_o$ from the traditional 2 mM to 1 mM. Here we show that low buffer is associated with a potentiation of NMDA mediated responses, and NMDA receptors are required for Up states to occur. Thus, NMDA receptors seem to be at the basis of Up states in neocortex. Apart from enhancing NMDA receptor responses, we also found that low buffer produces two other major effects.

First, synaptic efficacy is slightly reduced. This is apparent in both the rising slope of the non-NMDA EPSPs and the amplitude of isolated IPSPs evoked by electrical stimulation, and is a direct consequence of lowering [Ca$^{2+}$]$_o$ on synaptic transmission (Katz and Miledi 1968). Importantly, even though lowering [Ca$^{2+}$]$_o$ alone reduces synaptic efficacy in neocortex slices, it
does not lead to the development of Up states (Castro-Alamancos and Connors 1997). Thus, a reduction in synaptic efficacy caused by lower $[Ca^{2+}]_o$ does not underlie Up state generation. It is also worth mentioning that a reduction in release probability in neocortex caused by lowering $[Ca^{2+}]_o$ causes a significant increase in synaptic facilitation of subsequent inputs (Castro-Alamancos and Connors 1997; Katz and Miledi 1968), which could be somewhat supportive of ongoing Up states. Another important consideration is that low buffer involves lowering both divalent cations. So the suppressive effect of lowering $[Ca^{2+}]_o$ on synaptic transmission is countered by the simultaneous lowering of $[Mg^{2+}]_o$, which is known to enhance synaptic transmission (Katz 1969). The reduction in synaptic efficacy caused by low buffer will be less than if only $[Ca^{2+}]_o$ was lowered. In conclusion, the relatively small reduction in synaptic efficacy caused by low buffer is unlikely to be a major factor in the generation of Up states.

Interestingly, the reduction in synaptic efficacy produced by low buffer was observed for synaptic responses evoked by electrical stimulation but not for responses evoked by blue light. This difference can be explained by the fact that ChR2 channels allow $Ca^{2+}$ to flow through them providing an additional $Ca^{2+}$ source (Zhang et al. 2006) that can support synaptic transmission in different ways than electrical stimulation.

Second, low buffer consistently enhanced cell firing excitability. This is caused by the well-known actions of lowering the overall divalent cation concentration on ion channel gating. Lowering the cation concentration lowers the activation threshold of many channels, including $Na^+$ channels (Frankenhaeuser and Hodgkin 1957; Hille 2001; Wang et al. 2004). It is also worth noting that lowering $[Ca^{2+}]_o$ can lead to less efficient activation of calcium-activated potassium channels, which can facilitate excitability during sustained firing (Storm 1990). The enhanced
firing excitability associated with low buffer can boost the network activity associated with Up states.

**NMDA receptors and Up states**

NMDA receptors have been proposed to contribute to the ability of prefrontal cortical neurons to exhibit persistent activity related to short-term memory (Lisman et al. 1998). Several models have emphasized a critical role for NMDA-receptor dendritic spikes (Schiller et al. 2000) in the generation of Up states (Antic et al. 2010; Kepecs and Raghavachari 2007; Milojkovic et al. 2005). NMDA receptors are thought to facilitate sustained recurrent excitation because of their slow kinetics and voltage-dependency, and they can ease the propagation of bursting in cortical networks (Polsky et al. 2009).

Although block of non-NMDA and NMDA receptors have been reported to suppress spontaneous Up states (McCormick et al. 2003; Sanchez-Vives and McCormick 2000), the relative importance of NMDA compared to AMPA receptors in the generation of Up states was recognized recently (Favero and Castro-Alamancos 2013). In adult mice, we found that blocking NMDA receptors completely abolishes Up states, while blocking AMPA receptors does not; the frequency and duration of spontaneous Up states is reduced by AMPA antagonists, but these are still evoked reliably by afferent stimulation. The fact that Up states are abolished by blocking NMDA receptors, together with the present findings, showing that NMDA responses are robustly enhanced by the low buffer that triggers Up states, points toward a prominent role of NMDA receptors in the generation of Up states in slices.

Interestingly, spontaneous recurrent network activity, consisting of Up and Down states, have also been shown to arise in slices of developing mice [P14-P18] bathed in a “traditional”
slice buffer (i.e., ~2 mM \([\text{Ca}^{2+}]_o\) and \([\text{Mg}^{2+}]_o\)) (MacLean et al. 2005; Mao et al. 2001); although other studies using rats of the same age appear to have required low buffer (Wester and Contreras 2013). In any case, NMDA receptor responses of developing neocortex slices around this age have very different kinetics (i.e. much longer decay time constants) than adult slices (Carmignoto and Vicini 1992; Crair and Malenka 1995); which is also the case in other brain regions (Hestrin 1992; Lester et al. 1990). The longer decays of NMDA responses could facilitate to the generation of Up states in developing slices bathed in 2 mM buffer.

NMDA receptors are well known to be located postsynaptically, where their properties have been extensively characterized (Dingledine et al. 1999). However, there is also growing evidence that NMDA receptors are located presynaptically at some synaptic connections in various cortical regions, including enthorhinal cortex (Beretta and Jones 1996), visual cortex (Buchanan et al. 2012), and somatosensory cortex (Brasier and Feldman 2008), where they enhance neurotransmitter release (Corlew et al. 2008; Kunz et al. 2013). Thus, it is possible that presynaptic NMDA receptors facilitate the generation of Up states by potentiating neurotransmitter release at specific synapses that are involved in the generation of Up states.

Alas, testing the pre- versus postsynaptic contribution of NMDA receptors to the generation of Up states will be tricky because each cell serves both pre- and postsynaptic roles in highly interconnected and recurrent neocortex circuits. In addition, Up states are network events and so blocking NMDA receptors in single or a few cells (e.g. through the patch internal solution) is unlikely to have a major impact on the network event.

An important question relates to the role of NMDA receptors in the generation of slow oscillations in vivo. NMDA receptors have been shown to mediate persistent visual-evoked responses in neocortex (Miller et al. 1989), and application of AP5 locally into the barrel cortex
or thalamus attenuates spontaneous activity (Hirata and Castro-Alamancos 2006). In principle, it is possible that NMDA receptors have a critical role in the generation of Up states in vivo, but future work needs to address this question directly in vivo. Alas, this is not a trivial experiment to conduct in vivo because of various technical difficulties, such as the difficulty in blocking NMDA receptors in all cortical regions to avoid slow oscillations propagating from them to the test region, or the confounds from the impact of cortical activity changes on thalamic activity that will feed back to the cortex, to name a few.

Conclusion

Here we monitored several cellular and network variables during the transition between a traditional buffer and the low buffer that leads to the generation of Up states in slices of adult rodents. We found variables that did not change during this transition, variables that changed but are unlikely to cause the development of Up states, and variables that changed and are associated with the development of Up states. Among the variables that did not change significantly were the resting Vm and input resistance of cortical cells. Among the variables that changed but are unlikely to facilitate the development of Up states was a reduction in synaptic efficacy associated with lowering $[\text{Ca}^{2+}]_o$. We identified two other variables that can be associated with the development of Up states. First, an enhancement of the firing excitability of cortical cells caused by lowering the overall concentration of divalent cations. Second, an enhancement of NMDA mediated responses caused by lowering $[\text{Mg}^{2+}]_o$. The fact that blocking NMDA receptors alone is sufficient to completely abolish Up states implies that NMDA receptors are
critically important for the generation of Up states in cortical slices, and that the change in firing excitability has only a supportive role.
REFERENCES


FIGURE LEGENDS

Figure 1. Low buffer generates spontaneous slow oscillations consisting of Up and Down states in neocortex slices. **A**, Whole-cell intracellular (Vm) and field potential (FP) recordings from a cell in somatosensory cortex before and during low buffer. **B**, Close-ups of Up states shown in A marked by the asterisks. **C**, Population data showing the abolishment of spontaneous Up states by application of D-AP5 (50 µM).

Figure 2. Effect of low buffer on input-output curves of electrically evoked synaptic responses. **A**, Example showing averaged synaptic responses (Vm and FP) evoked at different intensities by a stimulating electrode placed in layers III-II during control buffer, low buffer and subsequent addition of AP5. **B**, Overlaid responses evoked by one intensity (20 µA) for the data shown in A. Arrows point to short- and long-latency responses. **C**, Population data showing the effects of low buffer on the amplitude of short-latency (2-15 ms) and long-latency (15-50 ms; reflecting the Up states) FP responses under normal conditions (no glutamate receptor antagonists). **D**, Population data showing the effects of low buffer on AMPA FP responses (in the presence of AP5). The left panel measures the peak amplitude and the right panel measures the time-to-peak. **E**, Population data showing the effects of low buffer on NMDA FP responses (in the presence of CNQX). The left panel measures the peak amplitude and the right panel measures the area of the FP response (3-50 ms). Note that low buffer enhances NMDA responses. * p<0.01

Figure 3. Examples depicting the effect of low buffer on AMPA responses. **A**, One experiment showing simultaneous FP and intracellular recordings of AMPA responses evoked by electrical stimulation at different intensities during control and low buffer. The right panel overlays the
responses evoked by two intensities for comparison. The arrows in the FP panel point to a shift in the peak and a broadening of the FP response. **B,** Another experiment showing intracellular responses as described in A. **C,** Individual traces used to derive the average traces shown in B for the 50 µA stimulus intensity (black is control and red is low buffer). Note the reduction in the slope of the EPSP and the enhanced spiking during low buffer.

**Figure 4.** Examples depicting the effect of low buffer on NMDA responses. **A,** One FP experiment is shown. NMDA FP responses are evoked by electrical stimulation at different intensities during control and low buffer. The right panel overlays the responses evoked by one intensity for comparison, and also shows (gray trace) the normal response evoked before addition of CNQX to isolate NMDA responses. **B,** Two additional experiments are shown in the upper and lower panels. Intracellular NMDA responses are evoked by electrical stimulation at different intensities during control and low buffer. The upper right panel overlays the responses evoked by two of the intensities for comparison. The lower right panel shows the responses evoked after AP5.

**Figure 5.** Effect of low buffer on optogenetically evoked responses. **A,** One experiment showing simultaneous FP and intracellular AMPA responses evoked by blue light pulses at different intensities during control and low buffer in a Thy1 slice. The right panel overlays the responses evoked by two intensities for comparison. **B,** Another experiment showing simultaneous FP and intracellular NMDA responses evoked by blue light pulses at different intensities during control and low buffer in a Thy1 slice. The third panel overlays the responses evoked by two intensities for comparison. The fourth panel shows the effect of AP5 on the responses evoked by the five
intensities; it also overlays one of the traces before AP5 (in gray) for comparison. This cell responded directly to blue light (in the presence of glutamate receptor antagonists) indicating that it expressed ChR2. C, A third experiment showing simultaneous FP and intracellular NMDA responses evoked by blue light pulses at different intensities during control and low buffer in a slice expressing ChR2 in thalamocortical fibers. The third panel overlays the responses evoked by two intensities for comparison. The fourth panel shows the effect of AP5; it overlays one of the traces before AP5 (in gray) for comparison. AP5 abolished the intracellular responses and most of the FP response except for the short-latency fiber volley reflecting the direct activation of thalamocortical fibers.

Figure 6. Low buffer slightly suppresses the isolated inhibitory synaptic conductance evoked by intracortical electrical stimulation. A, Two examples showing the effect of low buffer on the inhibitory synaptic conductance derived from isolated inhibitory postsynaptic potentials evoked at different Vm’s. B, Population data showing the effects of low buffer on the peak amplitude of the isolated inhibitory synaptic conductance evoked by electrical stimulation. (* p<0.05)

Figure 7. Low buffer increases firing excitability. A, Effect of low buffer on resting Vm. B, Effect of low buffer on input resistance estimated by measuring the voltage drop caused by negative current pulses of different amplitudes. C, Effect of low buffer on input resistance estimated by measuring the decay time constant after pulse offset. D, Effect of low buffer on firing excitability estimated by measuring the number of spikes evoked by 500 ms positive current pulses of different amplitudes. (*p<0.05) E, The same data shown in D but plotted as perievent time histograms with 100 ms bins.
A

Control  Low Buffer  +AP5

V_m

-60 -65 -70 -75

0 50 100 150 0 50 100 150 ms

0.5 mV

B

20 μA

V_m

-60 -65 -70 -75

0 50 100 150 ms

0.5 mV

C

Short latency

FP Amplitude (mV)

0.0 0.5 1.0 1.5 2.0 2.5

10 20 30 40 50 Intensity (μA)

n.s.

D

AMP A response

FP Amplitude (mV)

0.0 0.5 1.0 1.5

10 20 30 40 50 Intensity (μA)

n.s.

E

NMDA response

FP Amplitude (mV)

0.0 0.2 0.4 0.6

10 20 30 40 50 Intensity (μA)

FP Area

0 4 8 12

10 20 30 40 50 Intensity (μA)