Enhanced dendritic activity in awake rats

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Almost nothing is known about dendritic activity in awake animals and even less about its relationship to behavior. The tuft dendrites of layer 5 (L5) pyramidal neurons lie in layer 1, where long-range axons from secondary thalamic nuclei and higher cortical areas arrive. This class of input is very dependent on active thalamo-cortical loops and activity in higher brain areas and so is likely to be heavily influenced by the conscious state of the animal. If, as has been suggested, the dendrites of pyramidal neurons actively participate in this process, dendritic activity should greatly increase in the awake state. Here, we measured calcium activity in L5 pyramidal neuron dendrites using the ''periscope'' fiberoptic system. Recordings were made in the sensorimotor cortex of awake and anesthetized rats following sensory stimulation of the hindlimb. Bi-phasic dendritic responses evoked by hindlimb stimulation were extremely dependent on brain state. In the awake state, there was a prominent slow, delayed response whose integral was on average 14-fold larger than in the anesthetized state. Moreover, the dramatic increases in dendritic activity closely correlated to the strength of subsequent hindlimb movement. These changes were confined to L5 pyramidal dendrites and were not reflected in the response of layer 2/3 (L2/3) neurons to air-puff stimuli in general (which actually decreased in the awake state). The results demonstrate that the activity of L5 pyramidal dendrites is a neural correlate of awake behavior.

calcium imaging | calcium spike | fiberoptics | layer 5 pyramidal neuron | neocortex

The changes that occur at the neuronal level from the anesthetized to the awake brain states is one of the most fundamental questions remaining in neuroscience (1–3). One leading hypothesis is that during awake sensory processing, thalamo-cortical interactions involving deep-layer pyramidal neurons result in non-specific thalamic nuclei projecting feedback input to the tuft dendrites of L5 pyramidal neurons (4). It is also hypothesized that higher cortical areas (more active during conscious processing) project to the same tuft dendrites in L1 in primary sensory areas (5, 6). Both these hypotheses predict specific input to pyramidal cell dendrites during the awake state. Given the intrinsic excitability of pyramidal tuft dendrites with their ability to sustain regenerative Ca^{2+} activity (7, 8), these hypotheses also predict that dendritic activity in these neurons should increase in the awake state.

So far, it has only been possible to test the effectiveness of feedback input to L1 in vitro (9, 10). In vivo recordings of dendritic calcium activity has been restricted to anesthetized animals. In these studies, there is evidence for sensory stimulus-evoked dendritic Ca^{2+} spikes in the apical dendrites (11, 12), which is, however, suppressed by powerful dendritic inhibitory mechanisms (13) that are more active during anesthesia (14). These studies also predict that dendritic activity should increase in the awake preparation; however, until now, dendritic recordings have not been feasible in awake animals.

We recently developed a fiberoptic system (the ''periscope'') for recording dendritic Ca^{2+} signals in freely moving rats (15, 16). Using this method, we recorded sensory stimulus-evoked dendritic Ca^{2+} activity of L5 pyramidal cells in primary sensory cortex under anesthesia and in the awake state. The method records from populations of apical dendrites of L5 pyramidal cells, ensuring that these structures are the only fluorescent elements in the upper layers and then uses a prism attached to an optical fiber to image the upper cortical layers horizontally.

Results

Fiberoptic Imaging of Dendritic Activity in Somatosensory Cortex. We performed fiberoptic calcium imaging (Fig. 1*A*) of the top 700-μm of somatosensory cortex, in which we could specifically monitor that activity of a local population of L5 pyramidal cell dendrites. The method has been shown to be equally effective for recording signals in awake, freely moving animals using a head-mount (15, 16) for fixing the fiberoptic cable (Fig. 1*A*). We imaged dendritic Ca^{2+} changes in the dendrites of L5 pyramidal neurons in the hindlimb primary somatosensory area (HLA) of the cortex (determined beforehand with intrinsic imaging) in awake and anesthetized rats. Stimuli consisted of air-puffs to the contralateral hindlimb (50-ms duration, Fig. 1*A*), whilst simultaneously measuring the movement of the stimulated limb. Using this method, we addressed whether (*i*) the level of dendritic activity in the awake state differs from the anesthetized state and (*ii*) dendritic activity correlates to the behavior of the animal.

Dendritic Responses to Air-Puff Stimuli in Awake Versus Anesthetized States. Dendritic activity increased dramatically in the awake state relative to anesthesia (Fig. 1*B*). Moreover, the dendritic signals appeared to increase in amplitude and duration in a behaviorally relevant way (Fig. 1*B*). In the awake state, stimulation sometimes triggered delayed hindlimb movements which could be easily visually observed in all rats but only 42.9 ± 3.5% of trials ($n = 6$ rats) whereas the same air-puff stimulation never caused hindlimb movement during anesthesia ($n = 47$ rats). We therefore separated the awake results into two categories (with and without movement) to compare both these conditions to the anesthetized data. The amplitude of the fast component of the biphasic signal increased approximately 2.5-fold compared to the anesthetized response ($n = 6$ rats; $P < 0.05$) regardless of the hindlimb movement (239.4 ± 25.3% without movement vs. 227.2 ± 21.3% with movement; $n = 6$ rats) (Fig. 1*C*). On the other hand, the slow component differed dramatically across all three cases. Here, dendritic activity, measured as the area of the slow component, increased approximately 4-fold in the awake state without hindlimb movement and 14-fold with movement (378.4 ± 99.4% and 1,434.9 ± 282.6%, respectively) (Fig. 1*C*). Note, the area reflects the combined increases in peak and duration which are given separately in Fig. S1. We could not record the duration of the fast component because it was obscured by the onset of the slow component. The correspondence between the integral of the slow component and hindlimb movement could be recorded in the electromyogram (EMG) and tension of the hindlimb (Fig. 1*D*).

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The authors declare no conflict of interest.

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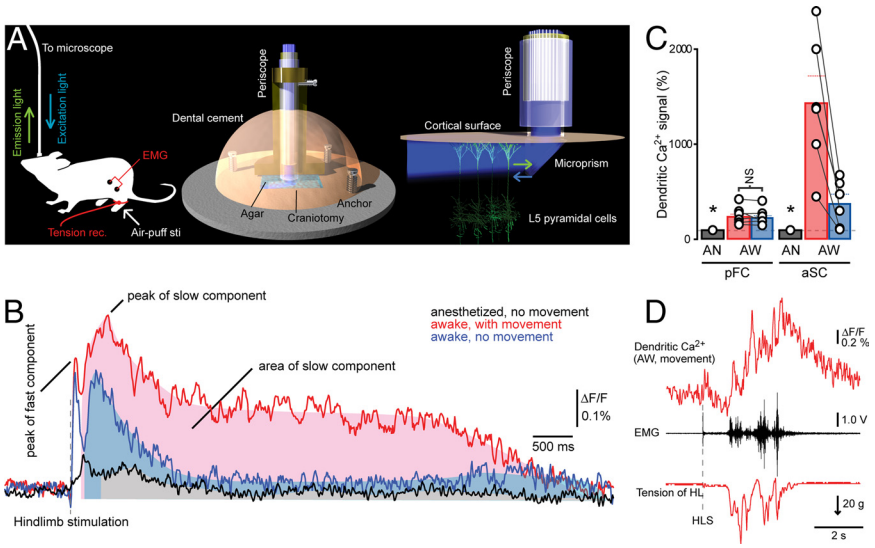

Fig. 1. Dramatic increase in dendritic calcium in awake rats. (*A*) Schematic diagram of experimental design. Left, ''periscope'' fiberoptic imaging device attached to rat cortex. Tension and EMG of upper hindlimb muscles were measured in response to an air-puff to the hindlimb. Middle, head mount allowing application of drugs with simultaneous imaging of the somatosensory cortex. Right, schematic diagram showing the light path for collecting fluorescence changes from populations of L5 pyramidal neuron dendrites (blue arrow, excitation at 475 nm; green arrow, emission at 520 nm). (*B*) Dendritic Ca^{2+} fluorescence changes recorded under anesthesia (AN, black), in awake state (AW) with hindlimb movement (red) and without movement (blue). Signals were evoked by air-puff stimulation (50-ms, dashed line) to contralateral hindlimb. The data are shown as averages (20 trials for AN, 11 trials for AW with movement, 16 trials for AW without movement). The areas of the slow components are highlighted. (*C*) Summary of dendritic Ca^{2+} signals in anesthetized (AN) and awake (AW) states (n = 6 rats). pFC, peak amplitude of fast component and aSC, area of slow component (see also Fig. S1 for pSC, peak amplitude of slow component; dSC, duration of slow component). All statistics show significant differences ($P < 0.05$), except pFC between movement and no movement in the awake state (NS, no significant difference). Dashed lines show SEM. (*D*) Example of single, simultaneous recording of dendritic Ca^{2+} signals, electromyogram (EMG) and tension of hindlimb in awake state.

Response of Upper-Layer Neurons to Air-Puff Stimuli. The increase in activity was specifically dendritic and was not reflected in the general activity of other cells in L2/3. We could show this by injecting the bolus of calcium indicator (OGB-1 AM) directly into L2/3 of the same cortical area (Fig. 2*A*). Here, the change in fluorescence in response to air-puff stimulus of the hindlimb actually decreased in the awake versus the anesthetized state and there was no difference between responses with and without movement (Fig. 2 *B* and *C* and Fig. S2). The probability of hindlimb movement by the rat was not significantly different for these experiments (47.4 ± 4.9% for L2/3 imaging versus 42.9 ± 3.5% for dendritic imaging, $P = 0.24$, $n = 6$ rats, respectively). The baseline fluorescence level in L2/3 increased slightly in the awake state (113.1 ± 2.4%, $P < 0.05$, $n = 6$ rats), which corresponds to the changes in spiking activity of L2/3 neurons reported for recordings from cell bodies in awake versus anesthetized rodents (17–20). For example, Greenberg et al. found that L2/3 neurons fire at approximately 0.32 Hz in anesthetized rats versus 0.44 Hz in awake rats (17). Taken together, the data show a specific relationship between the slow component of dendritic activity and cortical output in the awake state.

Dendritic Activity and Behavior. We have shown previously that sensory inputs are encoded by population dendritic activity (13), where the peak of the fast component is linearly correlated with the strength of sensory (bottom-up) input in both anesthetized and awake states. Here we examined the responses to hindlimb stimulation and subsequent tension of the hindlimb movement on a trial by trial basis (Fig. 3*A*). The fast component of dendritic activity was not related to the intensity of the movement (r = 0.12, 85 data points, $n = 6$ rats) (Fig. 3*B*). In contrast, the area of the slow component correlated strongly with the intensity of movements ($P < 0.001$, $r = 0.63$, 85 data points, $n = 6$ rats) (Fig. 3 *A* and *C*). The first and second components were also not correlated to each other (r = 0.23, 85 data points, $n = 6$ rats) (Fig. S3). This suggests that the slow, long-lasting dendritic

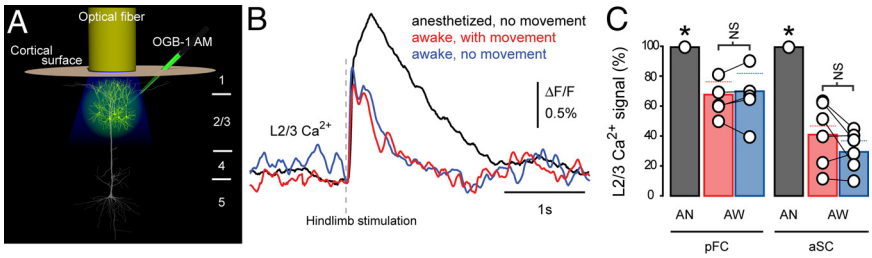

Fig. 2. No increase in L2/3 evoked response in awake rats. (*A*) Schematic diagram showing the experimental setup with bolus loading of L2/3 and imaging vertically from the cortical surface with an optical fiber. (*B*) Average L2/3 Ca^{2+} fluorescence changes recorded under anesthesia (black), in awake state with hindlimb movement (red) and without movement (blue, $n = 6$ rats). Signals were evoked by air-puff stimulation to contralateral hindlimb. (*C*) Summary of L2/3 Ca^{2+} activity in anesthetized (AN) and awake (AW) states ($n = 6$ rats). pFC, peak amplitude of fast component and aSC, area of slow component (see also Fig. S2 for pSC, peak amplitude of slow component; dSC, duration of slow component). Dashed lines show SEM.

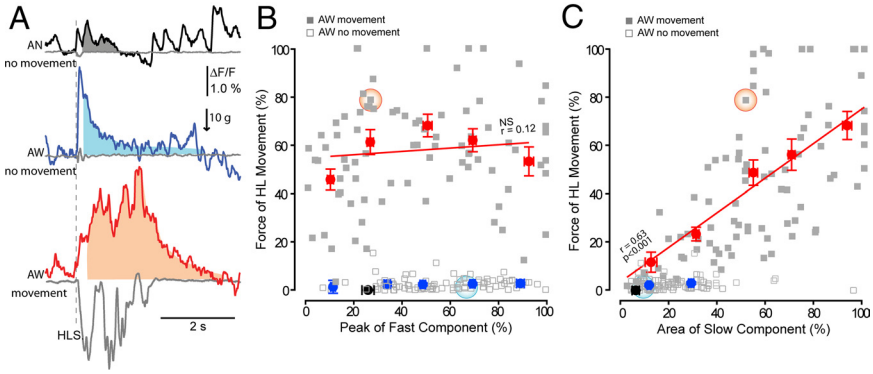

Fig. 3. Dendritic slow Ca^{2+} activity correlates with the strength of hindlimb movement. (*A*) Single trials of simultaneous recordings of dendritic Ca^{2+} signals (colored) and tension of movement (gray) in the same rat. Area of slow component is highlighted. AN, anesthetized; AW, awake; HLS, hindlimb stimulus. (*B* and *C*) Strength of movement versus peak amplitude of fast component (*B*) and area of slow component (*C*) evoked by hindlimb stimulation ($n = 6$ rats). Blue and orange circles indicate the data corresponding to the traces shown in *A*. Filled squares (gray) show trials with movement. Open squares show those without movement. The linear fits (red) were applied to all movement data points (85 trials in 6 rats). Statistics of the Pearson's *r* correlation were performed from the 85 points. Black, blue and red points show binned averages (bin size, 20%) of awake data. Anesthetized data are averaged as a single value (i.e., no movement).

activity across the population of layer 5 pyramidal cells decodes the intensity of motor output. We conclude that processes intrinsic to the awake state (e.g., increased feedback to the dendrites) have an enormous impact on dendritic activity.

Discussion

The conclusion that dendritic activity is greatly enhanced during the awake state is contingent on the recordings representing physiologically relevant activity in L5 pyramidal cells and that the increases that occur are dendrite specific.

In principle, the physiological relevance of the data may have been compromised by tissue damage caused by the insertion of the 500 × 500-μm prism into the cortex. However, the dendritic Ca^{2+} change was correlated to behavior and the behavior was not dependent on insertion of the prism (Figs. 1 and 2). Furthermore, we have previously tested the effect of the periscope on signals by comparing the inserted prism approach with a completely non-invasive version (15). In these experiments, no difference could be detected in the fluorescence signal. In the same experiments, investigation of the damaged tissue afterward (in ex vivo slices) revealed that pyramidal neuron dendrites near the tissue damage displayed normal Ca^{2+} activity. In general, it appears that cortical neurons are extremely resilient to tissue damage which is reflected in the great body of literature in sliced brain tissue. The biggest effect of the periscope insertion is likely to be the cutting of some horizontal fibers which should have only reduced the synaptic drive for dendritic activity (and the 14-fold increase even more significant).

The dendritic origin of the signals is also implied by many factors. We showed previously in brain slices that bolus loading of L5 (either in vivo or in vitro) filled the dendrites of L5 specifically and sufficiently to give dendritic signals while the nearby neuropil in the upper layers gave no signal (15). In the same experiments, we found that EPSPs had a negligible effect on intracellular Ca^{2+} as have others (21–23). We examined the effect of back-propagating action potentials (BPAPs) on dendritic Ca^{2+} by applying TTX to block Na^+ channels first to the cortical surface and then injected to L5. TTX to L5, but not to the cortical surface, abolished the small spontaneous fluctuations normally observed implying that these small signals are due to BPAPs. On the other hand, TTX to L5 tripled the size of hindlimb stimulus-evoked dendritic responses (13). The evoked signals with TTX in L5 (which could not have been due to BPAPs) were an order of magnitude greater than the spontaneous fluctuations blocked by TTX in the upper layers. This argues powerfully for the dominance of local dendritic activity in causing dendritic Ca^{2+} influx in the tuft compared to BPAPs. In the same study we showed, both in vivo and in vitro, that a dendritic inhibitory circuit can block dendritic spikes specifically without influencing the nearby L2/3 activity. Taken together, we conclude that large signals recorded by the periscope in the anesthetized rat cortex are the result of local Ca^{2+} electrogenesis in the apical dendrites of L5 pyramidal neurons.

The increases in dendritic activity in the awake state observed in this study were of particularly long duration (up to 6 s). This raises interesting questions about the underlying mechanisms involved since dendritic Ca^{2+} spikes observed so far in vitro typically only last approximately 20–50 ms and have a relative refractive period of approximately 250 ms (12). One possible explanation for our data is that the periscope method sees only the population response of the pyramidal neuron dendrites averaged over time and space. On the other hand, longer dendritic plateau potentials can be seen in vitro under conditions where inhibition is pharmacologically suppressed (24), and it is possible that there exists some as yet unknown disinhibitory mechanisms present in the awake state to prevent the normally powerful dendritic inhibitory mechanisms (13, 25). Lastly, it is possible that invasion of the distal apical dendrites by BPAPs is enhanced in the awake state so that the AP activity of the pyramidal neurons themselves can contribute to dendritic Ca^{2+} responses. For instance, BPAPs cause enhance dendritic Ca^{2+} influx into the tufts of L2/3 neurons during upstates induced by urethane anesthesia (26).

Since the dendritic activity we recorded was correlated to movement of the hindlimb in the awake state, it is not unlikely that the AP output of the pyramidal neurons was also correlated to behavior. However, in the awake state we could not distinguish between the possibility that dendritic electrogenesis drove the output AP activity or AP activity caused dendritic electrogenesis. Under anesthesia, as noted above, we could show that dendritic Ca^{2+} electrogenesis was not dependent on BPAPs using local injection of TTX to L5 (13). Importantly, however, regardless of the initial direction of causality, the presence of dendritic Ca^{2+} activity would interact with AP activity from the soma and increase the proportion of bursts of Na^+ APs in pyramidal neuron output (10, 27). This would both increase overall firing rates and provide a mechanism for downstream "read out" in the form of prolonged bursts of Na^+ spikes. Thus, in both cases, we would expect dendritic activity to influence spiking behavior (and vice versa) which therefore predicts an inter-causal relationship between these two phenomena. On the other hand, our data provides no conclusive evidence about the

causal relationship between dendritic activity (or APs) and movement of the hindlimb. Indeed, more likely explanations would seem to be that activity in the somatosensory area represents an "efferent copy" of the output signal for feedback control of movement (28) or a proprioreceptive response.

We observed increases in baseline Ca^{2+}-fluorescence in the awake state with bolus loading of the upper layers which is consistent with relatively small changes in activity of L2/3 neurons reported in other awake versus anesthetized studies in rodents (17–19, 29, 30). The decrease in evoked activity of L2/3 neurons in our study may be due in part to the increase in spontaneous activity which increases the fluorescence baseline (and decreases relative changes) and possibly also decreases the driving force for synaptic input. Both these factors also applied to the dendritic signal so that the discrepancy between the two signals therefore indicates a specific dendritic mechanism is involved. This result implies that a subpopulation of neurons within the column (i.e., L5 neurons) is "selected" to participate during certain behavior. One possibility is that this arises from specific wiring of microcircuits within the column (31). Another suggestion is that L5 pyramidal neurons are specifically sensitive to coincident input to the upper and lower layers of the cortex (9, 32). This second possibility would render the cortex particularly sensitive to enhanced or synchronous activity thought to be evoked by thalamocortical interactions in the awake state (33). The selection of particular pyramidal neurons through the interplay of thalamocortical loops projecting to different cortical lamina has been proposed as a solution to the binding problem (34), and if so, would imply that the dendritic activity seen here is intrinsically involved in this process (4, 9, 33).

The neuronal basis of general anesthesia remains mysterious particularly since different anesthetics target diverse mechanisms. However, common to all anesthesia appears to be a disruption of coherent thalamocortical activity and other top-down influences on primary cortical regions (35). The central role of pyramidal neurons in deep cortical layers in these processes is beyond doubt because they are the only elements projecting to the thalamus, reticular nucleus and they also contribute to long range cortico-cortical connections. The recurrent projection from the thalamus to the upper cortical layers is highly likely to influence the tuft dendrites of pyramidal neurons since these neurons provide the bulk of the total synapses in L1. Without the presence of dendritic spiking mechanisms that could transmit this input to the axonal AP initiation region (36), this architecture would be highly enigmatic (37). On the other hand, disruption of this mechanism could serve as an explanation for the anesthetic effects (14). The dramatic increase in Ca^{2+} electrogenesis after release from anesthetic block therefore represents crucial supporting evidence that dendritic activity is involved in fundamental cortical interactions during awake behavior.

Materials and Methods

Animals and Surgery. Female Wistar rats (P30–P45) were used in these experiments. Isoflurane (1.5–3%, Baxter) and a local anesthetic (lidocaine; Sigma–Aldrich) was used for for anesthesia before surgery, according to the guidelines of the Federal Veterinary Office of Switzerland. Initially, the rat was prepared under anesthesia for later surgery and recording by exposing the skull over the region intended for recording. The rat was then allowed to recover over 2–3 days, during which time an analgesic was administered (buprenorphine, twice per day; Essex Chemie) and local anesthetic applied to the scalp. On the day of the experiment, the animal was anesthetized again with isofluroane and the head was fixed in a stereotaxic instrument (Narishige) and body temperature maintained at 36 to 37 °C. A craniotomy was performed above primary somatosensory cortex (3 × 4.4 mm square), centered at 1.5 mm posterior to bregma and 2.2 mm from midline in the right hemisphere. Intrinsic imaging during hindlimb stimulus was performed to identify the hindlimb area on the sensory cortex. The dura mater was surgically removed immediately before attachment of the fiberoptic cable (see below). For the awake behavior part of the experiments, the animals were allowed to recover from anesthesia for at least 2 h and then restrained in a harness (Kent Scientific Corp.) which allowed free movement of the head and limbs whilst preventing the animal from moving location.

In Vivo Loading of Ca^{2+}-Sensitive Dye. Oregon Green 488 BAPTA-1 (OGB-1) AM (50 μg; Molecular Probes) was mixed with 5 μL pluronic acid (Pluronic F-127, 20% solution in DMSO, Molecular Probes) for 15 min. The solution was then diluted in 28 μL of HEPES-buffered solution (125 mM NaCl, 2.5 mM KCl, and 10 mM HEPES) and mixed for a further 15 min. The OGB-1 AM solution was loaded into a glass pipette (tip diameter: 5–20 μm) and pressure-injected into L5 (pressure: 10–25 kPa) for 1 min. The pipette was withdrawn and the area of the craniotomy was then submerged with rat ringer (135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES) for 2 h.

In Vivo Ca^{2+} Imaging. A blue light-emitting-diode (LED, IBF+LS30W-3W-SlimRX, Imac Co., Ltd.) was used as a light source. An excitation filter, a dichroic mirror, and an emission filter (as a filter set 31001, Chroma Technology) were used for epifluorescence Ca^{2+} recordings. A 10× objective (Edmund Optics GmbH) was used for illuminating and imaging a fiber bundle (see below). A cooled charge-coupled device (CCD) camera (MicroMax, Roper Scientific) was used for collecting fluorescence.

A fiber bundle (IGN-06/17, Sumitomo Electric Industries) consisting of 17,000 fiber elements was used for a combined illuminating-recording fiber. The end face of the bundle was fitted with a prism-lens assembly which consisted of a right-angle prism (dimension of 0.5 × 0.5 × 0.5 mm, GrinTech) attached to a GRIN lens (a diameter of 0.5 mm and a NA of 0.5, GrinTech). The working distance was nominally 100 μm and the magnification ×0.73, resulting in a field of view of 685-μm diameter. A single core fiber (470-μm diameter, Edmund Optics) was used for recording L2/3 Ca^{2+} fluorescence changes. Sensory responses were evoked by a brief air-puff (50-ms duration) delivered to the contralateral hindlimb. Fluorescence changes were sampled at 100 Hz. Data were acquired on a PC using WinView software (Roper Scientific). Regions of interest (ROIs) were chosen offline for measuring fluorescence changes (see *Data Analysis*).

Electromyogram, Tension Recording, and Motion Recording. Surface electrodes were used for electromyogram recordings of muscle electrical activity of contralateral hindlimb. The signals were amplified with a differential amplifier (Tektronix, Switzerland) and digitized at 5 kHz with an A/D converter (Digidata 1322A, Axon Instruments). Force of movement was recorded with a modified TF3V3 level transducer (Hugo Sachs Elektronik). The tension applied to the transducer was digitized with the Digidata A/D converter. These recordings were acquired on a PC using Axoscope software (Axon Instruments). Hindlimb movement was visually observed by using a CCD camera (Qcam for notebooks pro, Logitech). The video recording was acquired and stored to disk using QuickCam software (Logitech).

Data Analysis. The fluorescence signals in vivo were quantified by measuring the mean pixel value of a manually selected (offline) ROI for each frame of the image stack using Igor software. Ca^{2+} changes were expressed as $\Delta F/F = F_t/F_0$, where F_t was the average fluorescence intensity within the ROI at time t during the imaging experiment and F_0 was the mean value of fluorescence intensity before stimulation. All numbers are indicated as mean ± SEM. Significance was determined using paired t tests (* denotes $P < 0.05$).

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