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Imaging Neural Activity Using Thy1-GCaMP Transgenic Mice

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SUMMARY

The ability to chronically monitor neuronal activity in the living brain is essential for understanding the organization and function of the nervous system. The genetically encoded green fluorescent protein-based calcium sensor GCaMP provides a powerful tool for detecting calcium transients in neuronal somata, processes, and synapses that are triggered by neuronal activities. Here we report the generation and characterization of transgenic mice that express improved GCaMPs in various neuronal subpopulations under the control of the Thy1 promoter. In vitro and in vivo studies show that calcium transients induced by spontaneous and stimulus-evoked neuronal activities can be readily detected at the level of individual cells and synapses in acute brain slices, as well as chronically in awake, behaving animals. These GCaMP transgenic mice allow investigation of activity patterns in defined neuronal populations in the living brain and will greatly facilitate dissecting complex structural and functional relationships of neural networks.

INTRODUCTION

Monitoring neuronal activity is critical for our understanding of both normal brain function and pathological mechanisms of brain disorders. Because neuronal activity is tightly coupled to intracellular calcium dynamics, calcium imaging has proven invaluable for probing the activities of neuronal somata, processes, and synapses both in vitro and in vivo (Andermann et al., 2011; Chen et al., 2011; Kerr and Denk, 2008; Yasuda et al., 2004). Compared to multielectrode recording approaches, calcium imaging has the advantages of detecting activity in large or disperse populations of neurons simultaneously over extended periods of time with little or no mechanical disturbance to brain tissues.

Synthetic calcium dyes have been widely used to monitor intracellular calcium dynamics in cultured neurons, brain slices, as well as in the intact brain (Chen et al., 2011; Dombeck et al., 2007; Kerr and Denk, 2008; Marshal et al., 2011; Rothschild et al., 2010; Yasuda et al., 2004). However, loading calcium dyes into specific neuronal populations is technically challenging. It is difficult, if not impossible, to image activities of the same neuronal populations repeatedly over extended periods of time. Genetically encoded calcium indicators (GECIs) overcome these difficulties, permitting chronic imaging of calcium dynamics within specific cell types. GECIs are composed solely of translated amino acids and do not require the addition of synthetic compounds or cofactors. They can be targeted to specific cell types or subcellular compartments when used in combination with cell type-specific promoters or cellular targeting sequences. In addition, GECIs can be delivered and expressed in brain tissues via viral vectors, in utero electroporation, or through transgenic techniques (Hasan et al., 2004; Mao et al., 2008; Wallace et al., 2008; Yamada et al., 2011). Importantly, recently developed GECIs are capable of detecting calcium dynamics at the sensitivity level close to that of synthetic calcium dyes (Hendel et al., 2008; Pologruto et al., 2004).

At least one class of green fluorescent protein (GFP)-based GECIs, the GCaMP family, has been effective for detecting calcium dynamics induced by neuronal activity in multiple model organisms (Muto et al., 2011; Reiff et al., 2005; Tian et al., 2009; Warp et al., 2012). Recently, a new generation of GCaMPs (e.g., GCaMP3) has been successfully used to monitor neuronal...
We further demonstrate the broad utility of sensitive changes in fluorescence upon neuronal stimulation. Both GCaMP2.2c and GCaMP3 show strong and sensitive changes in fluorescence upon neuronal stimulation. We further demonstrate the broad utility of Thy1-GCaMP2.2c and Thy1-GCaMP3 transgenic mice in reporting neuronal activity in vitro and in vivo.

**RESULTS**

**Generation of Thy1-GCaMP2.2c and Thy1-GCaMP3 Transgenic Mice**

To generate GCaMP transgenic mice, we utilized the previously described GCaMP3 and a further modified GCaMP2.2b (Tian et al., 2009). Previous studies suggested that the N-terminal arginine located immediately after the initiator methionine of GCaMP2.0 destabilizes the protein, and changing the serine at 118 to cysteine could improve brightness and sensor response (Tian et al., 2009). Thus, we changed the second arginine in GCaMP2.0 to valine to increase its stability according to the N-terminal rule of protein degradation (Varshavsky, 2011) and changed the serine at 118 to cysteine as in GCaMP2.2b to create GCaMP2.2c. The domain structure and specific mutations of GCaMP2.2c and GCaMP3 are summarized in Figure S1A, available online.

Two important properties to consider when evaluating GECIs are basal levels of fluorescence and stimulation-induced changes in fluorescence ($\Delta F/\Delta F_0$). To assess these properties for GCaMP2.2c and GCaMP3, we coexpressed GCaMPs and the red fluorescence protein tdTomato in the same construct using the 2A peptide (P2A) sequence (Szymczak et al., 2004) in HEK293 cells. To normalize for transfection efficiency, we used the fluorescence intensity ratio of GCaMPs/tdTomato. We found that GCaMP3 showed significantly higher basal fluorescence compared to GCaMP2.2c and GCaMP2.0, whereas there was no significant difference between the basal fluorescence of GCaMP2.0 and GCaMP2.2c (Figures S1B and S1C). In addition, we found that fluorescence intensity changes elicited by 100 μM ATP are ~1.9-fold (1.9 ± 0.1, n = 56) and ~3.2-fold (3.2 ± 0.3, n = 61) higher in cells expressing GCaMP2.2c and GCaMP3 than in cells expressing GCaMP2.0, respectively (Figure S1D).

The studies above indicate that GCaMP2.2c has a low basal fluorescence with a modest fluorescence change in response to stimulation, whereas GCaMP3 shows higher basal fluorescence and a more robust change in fluorescence after stimulation. Because GCaMP (and any GECI) binds calcium, there is a risk of neuronal toxicity associated with calcium binding and expression level. To increase the chances of finding lines with both strong signal and low toxicity, we generated both GCaMP2.2c and GCaMP3 transgenic lines.

To generate GCaMP transgenic mice, we used the well-characterized Thy1 promoter to express GCaMPs in neurons.

We generated eight founder lines of GCaMP2.2c and six founder lines of GCaMP3. Our previous studies have shown that the Thy1 promoter predominantly drives transgene expression in projection neurons in the CNS. Due to strong transgenic position-effect variegation, a Thy1-driven transgene is often stochastically and differentially expressed in subsets of neurons in different transgenic lines (Feng et al., 2000; Young et al., 2008). Consistent with these findings, we found that all founder lines differed in levels and patterns of expression. For further characterization, we focused on Thy1-GCaMP2.2c line 8 and Thy1-GCaMP3 line 6 because these lines had the highest levels of transgene expression. Both lines of mice are born at the expected Mendelian rate and are healthy with no apparent histological or behavioral abnormality. GCaMP2.2c and GCaMP3 expression in these lines was widespread in the CNS including cortex, hippocampus, thalamus, cerebellum, superior colliculus, amygdala, brain stem, retina, and spinal cord (Figures 1A, 1B, and 2; Figure S2). However, some notable differences in expression...
neuronal activities, we performed cell-attached recording of spontaneous fluorescence changes. The fluorescence changes were well correlated with the spontaneous spiking activities in these neurons (Figure S4).

Next, we measured action potential (AP)-triggered fluorescence responses of GCaMP2.2c and GCaMP3. We made whole-cell recordings from GCaMP-expressing hippocampal dentate granular cells and evoked APs by brief current injections (3-5 nA, 2 ms). Single AP evoked \( \text{Ca}^{2+} \) transients with average \( \Delta F/F \) amplitudes of 21.6% ± 1.4% and 25.8% ± 2.0% (n = 9 cells) in Thy1-GCaMP2.2c and Thy1-GCaMP3 acute slices, respectively. Moreover, the average \( \Delta F/F \) and the number of APs were well correlated. The average \( \Delta F/F \) of GCaMP3 (n = 9 cells) was 65.0% ± 10.5%, 96.6% ± 13.0%, 126.1% ± 15.2%, 146.6% ± 16.8%, 261.4% ± 23.3%, and 308.9% ± 24.2% for 3, 5, 7, 9, 20, and 40 APs, respectively. Similarly, the average \( \Delta F/F \) of GCaMP3 (n = 9 cells) was 69.8% ± 14.5%, 119.5% ± 16.1%, 159.8% ± 19.9%, 200.2% ± 22.1%, 343.5% ± 31.2%, and 396.6% ± 28.2% for 3, 5, 7, 9, 20, and 40 APs, respectively (Figures 3A-3D). The signal-to-noise ratio (SNR) of GCaMP2.2c and GCaMP3 was 7.5 ± 0.4 versus 11.9 ± 0.9, 32.7 ± 6.0 versus 46.9 ± 5.5, and 110.5 ± 15.4 versus 148.1 ± 13.6 for 1, 5, and 40 APs, respectively (Figure 3E). The rise times of fluorescence changes range from 214.1 ms to 374.1 ms for both GCaMP2.2c and GCaMP3. Decay times were between 0.9 s and 1.9 s for GCaMP2.2c and 1.4 s and 2.6 s for GCaMP3 (Figures S3F and S3G). Finally, we tested Thy1-GCaMP2.2c and Thy1-GCaMP3 for the ability to image calcium transients in populations of neuronal somata. For this, we treated acute brain slices from Thy1-GCaMP2.2c and Thy1-GCaMP3 mice with a high-potassium bath solution. We found that depolarization with high potassium (10 mM and 30 mM KCl) induced dramatic fluorescence changes in dentate granular neurons of the hippocampus in both transgenic lines (Movie S3). The average \( \Delta F/F \) of GCaMP3 was 297.2% ± 308.7% versus 469.2% ± 55.6% and 1105.2% ± 15.4% for 3, 5, and 40 APs, respectively (Figures S5A and S5B). Thus, at the single cell or population levels, both GCaMP2.2c and GCaMP3 robustly detect spontaneous and evoked responses in vitro in acute brain slice preparations.

**In Vivo Ca\(^{2+}\) Imaging of Apical Dendrites of Layer V Pyramidal Neurons in the Motor Cortex**

To evaluate GCaMP expression in the intact brain, we performed transcranial two-photon imaging of the motor cortex of adult
Thy1-GCaMP2.2c and Thy1-GCaMP3 mice. Under in vivo imaging conditions in both transgenic lines, GCaMP expression was clearly perimembrane and was never detected in the nucleus (Figures 4A–4F and Movies S4 and S5). The baseline fluorescence intensity of GCaMP was similar in both lines in 5-month-old animals (Figure 4G). In Thy1-GCaMP2.2c mice, densely packed yet resolvable individual apical tuft dendrites were clearly visible in superficial cortical layers (Figures 4A and 4B). In comparison, the density of labeled dendrites was substantially higher in Thy1-GCaMP3 animals, making individual dendritic imaging difficult (Figures 4D and 4E). Consistent with the expression data from fixed brain slices (Figure S2B), Thy1-GCaMP2.2c mice had mainly layer V neuron labeling with very rare layer II/III neuron labeling (Figures 4C and 4H), whereas GCaMP3 was expressed in layer V neurons as well as in the majority of layer II/III neurons (Figures 4F and 4H). Therefore, unlike Thy1-GCaMP3 mice, Thy1-GCaMP2.2c mice offer an opportunity to image the activity of apical dendrites and spines of layer V pyramidal neurons in the cortex.

We next investigated whether Thy1-GCaMP2.2c and Thy1-GCaMP3 mice could report neuronal activity responses in the intact brain. Since individual dendrites are clearly resolvable in Thy1-GCaMP2.2c mice compared to Thy1-GCaMP3 mice, we tested whether calcium transients could be detected in the apical dendrites of layer V neurons of Thy1-GCaMP2.2c mice using two-photon microscopy in the primary motor cortex (M1). In awake, head-fixed animals, we observed numerous dendritic Ca^{2+} transients with large amplitudes (Figures 5A and 5C). These dendritic Ca^{2+} transients typically lasted several hundreds of milliseconds with a ΔF/F ranging from ~50% up to 200% (Figure 5B). The duration and amplitude of these dendritic calcium transients are comparable to dendritic calcium spikes observed in vitro (Larkum et al., 2009). In contrast, we rarely observed such robust Ca^{2+} transients in dendritic branches in anesthetized mice (Figure 5C). Furthermore, in the awake state, large elevations of calcium influx were readily detected not only in the entire dendritic shafts but also in their associated dendritic spines (Figures 5A and 5D and Movie S6). In both anesthetized and awake mice, we were able to detect transient calcium elevations within single dendritic spines over tens of milliseconds (Figure 5E). Thus, Thy1-GCaMP2.2c mice provide a means for investigating calcium transients over time in dendritic spines, as well as dendritic branches in layer V pyramidal neurons.
We also imaged neuronal activity in the motor cortex of awake mice using a fixed-head imaging design (Dombeck et al., 2007, 2009). In awake, behaving animals, we were able to detect activated neurons both in Thy1-GCaMP2.2c and Thy1-GCaMP3 mice (Figures 6A and 6B; Figure S6; Movie S7). In Thy1-GCaMP3 mice, we detected many more activated neurons (34.4 ± 1.7 cells in a 250 × 250 µm imaging window, n = 5 areas from 3 mice) over a 10 min period (Figures 6A and 6B) compared to anesthetized animals (see above). The population activity in the primary motor cortex of both Thy1-GCaMP mice was correlated with locomotor activity (Figure 6C; Figure S6). Repeated imaging of the same brain area at 15 days after the first imaging showed that most of the same neurons were active in both views (Figure 6D).

From these observations, we conclude that both Thy1-GCaMP2.2c and Thy1-GCaMP3 mice can be used to monitor neuronal activity over extended periods of time in the motor cortex of living animals.

**In Vivo Imaging of Sensory Stimulation-Evoked Ca^{2+} Transients in the Somatosensory Cortex**

In recent studies, viral expression of the ratiometric GECIs (YC3.60 and D3cpV) and GCaMP3 in pyramidal neurons of the mouse somatosensory (barrel) cortex allowed the detection of neuronal activity induced by whisker stimulation (Lütcke et al., 2010; Mittmann et al., 2011; O’Connor et al., 2010; Wallace et al., 2008). To determine whether Ca^{2+} transients could be detected in the barrel cortex in response to sensory stimulation in our Thy1-GCaMP mice, we performed a similar test. We used Thy1-GCaMP3 mice because in vivo two-photon imaging in the barrel cortex revealed sparse labeling of layer II/III pyramidal neurons in Thy1-GCaMP2.2c mice and dense labeling in Thy1-GCaMP3 mice (data not shown). To induce sensory stimulation, we deflected multiple mystacial vibrissae ten times using 500 ms air puffs with 10 s interpuff intervals. In Thy1-GCaMP3 transgenic mice, we routinely detected calcium transients associated with whisker stimulation in both cell somata and the adjacent layer II/III neuropil (Figures 7A–7C and Movie S8). To further characterize the kinetics of GCaMP3 signals in vivo, we next recorded changes in reporter fluorescence in 64 × 64 pixel frames at a frame rate of 40 Hz from layer II/III GCaMP3-expressing neurons. Single air puffs induced ΔF/F amplitude changes of 242.9% ± 14.2% (n = 8 from 3 mice), similar to those seen with virally transduced GCaMP3 (O’Connor et al., 2010) and higher than the ratio changes seen from YC 3.60 and D3cpV (Lütcke et al., 2010; Wallace et al., 2008). The rise and decay time of the calcium transients in GCaMP3 were 477.9 ± 17.1 ms and 1,072.5 ± 29.4 ms, respectively (n = 8 from 3 mice; Figures 7D–7F). Thus, Thy1-GCaMP3 mice allow the detection of dynamic changes in neuronal activity in vivo in response to sensory stimulation.

**In Vivo Imaging of Odor Responses in the Olfactory Bulb**

In Thy1-GCaMP3 transgenic mice, GCaMP is expressed in the glomerular layer, the external plexiform layer, and the mitral cell layer, but not within the olfactory nerve layer or the granule cell layer (Figures S7A and S7B). Two-photon imaging showed that GCaMP fluorescence was detected in the olfactory bulb...
in vivo (Figure S7C and Movie S9). Based on the location and soma size, GCaMP3-expressing cells appeared to be mainly mitral cells, in addition to a small subset of periglomerular and external tufted cells. GCaMP fluorescence can be seen throughout the soma and the dendrites.

To characterize activity-induced GCaMP3 responses in the olfactory bulb, we performed in vivo two-photon Ca$^{2+}$ imaging in the dorsal olfactory bulb during odor presentation. For odor stimulation, we chose four odorants, methyl salicylate, amyl acetate, eugenol, and 1-pentanol, because they have different molecular structures and have previously been shown to strongly activate distinct glomeruli in the dorsal olfactory bulb (Lin et al., 2006; Rubin and Katz, 1999; Wachowiak and Cohen, 2001). As shown in Figure S7D, 1% odorants trigger strong calcium responses in the olfactory bulbs of Thy1-GCaMP3 mice. Similar to previous in vivo imaging data using Kv3.1 potassium channel promoter-driven expression of GCaMP2.0 in the olfactory bulb (Fletcher et al., 2009), each odor induced two types of signals within the odor maps. The first response type was relatively weak and diffuse, whereas the second type of response was more focused and formed "hot spots" that corresponded to individual glomeruli (Figure S7D). Consistent with previous studies (Wachowiak and Cohen, 2001; Fried et al., 2002; Bozza et al., 2004), we found that different odorants activated discrete glomeruli in Thy1-GCaMP3 mice (Figure S7D). We also found that initial odor responses were often higher than subsequent stimuli (Figure S7E), a phenomenon we attributed to odor habituation (Holy et al., 2000; Verhagen et al., 2007). Notably, we found that odorant-triggered fluorescence changes with GCaMP3 are in the range of 30%–150%, much greater than in previous reports that used other calcium indicators (De Saint Jan et al., 2009; Fletcher et al., 2009).

Olfactory coding is multidimensional. In addition to response profiles being dictated by the molecular structure of odorants, odorant concentration also influences the receptor repertoire recruited to the stimulus and thus shapes the composite response pattern. With increasing concentrations of odorants, new glomeruli may be recruited into the response pattern, while previously active glomeruli often respond more intensively (Fletcher et al., 2009; Johnson and Leon, 2000). These effects were observed for all odorants tested in Thy1-GCaMP3 mice; the representative odor maps are shown in Figure S7F. Taken together, these data show that Thy1-GCaMP3 mice can detect changes of neuronal activity in mitral cells in response to specific odorants in a population and concentration-dependent manner. Furthermore, unlike previous methods for monitoring Ca$^{2+}$-mediated olfactory responses at presynaptic terminals (Bozza et al., 2004; Fried et al., 2002), the Thy1-GCaMP3 reporter line described here reflects postsynaptic responses.
DISCUSSION

In this study, we generated transgenic mice that stably express improved GCaMPs, GCaMP2.2c and GCaMP3 (Tian et al., 2009), in subsets of CNS neurons under the control of the mouse Thy1 promoter. Our findings indicate that these GCaMP transgenic lines provide an excellent tool for detecting neural activity in acute brain slices as well as the intact brain. First, we show that both spontaneous and evoked calcium transients can be detected in acute brain slices prepared from both transgenic lines. Notably, we were able to detect small calcium transients in neuronal somata triggered by a single action potential. Second, calcium transients were also readily detected in apical dendrites and dendritic spines in the living cortex of Thy1-GCaMP2.2c transgenic mice. Third, large, robust calcium signals can be detected in populations of layer II/III cortical neurons in both GCaMP transgenic lines with natural motor or sensory stimuli. Lastly, odor-evoked calcium transients can be detected at single glomerulus resolution in Thy1-GCaMP3 mice. Together, these results indicate that GCaMP2.2c and GCaMP3 mice provide a sensitive means to detect patterns of neuronal activity at the level of individual neurons and synapses, as well as populations of neurons in vitro and in vivo.

Until recently, calcium imaging with synthetic calcium dyes has been the method of choice to monitor activity in neuronal cultures, acute brain slices, and intact brains. However, routine and reliable loading of Ca^{2+} dyes into targeted neuronal populations in vivo has proven difficult. Bulk loading of calcium indicators indiscriminately labels mixtures of cells, making cell type-specific labeling nearly impossible. Single-cell labeling is technically challenging and allows for only a few cells to be loaded during a given imaging session. Furthermore, imaging with calcium dyes can only last for periods of hours, making chronic recordings of neuronal activity over extended times difficult, if not impossible. Genetically encoded calcium indicators overcome many of these limitations (Hasan et al., 2004; Looger and Griesbeck, 2012; Miyawaki et al., 1997). Incremental rounds of reporter optimization have resulted in new GCaMPs with significantly improved fluorescence characteristics and higher sensitivity to calcium (Muto et al., 2011; Ohkura et al., 2005; Souslova et al., 2007; Tallini et al., 2006; Tian et al., 2009; Zhao et al., 2011b).

A number of methods are available for transgene delivery, including in utero electroporation, biolistic delivery, and viral transduction. Some viral delivery methods have distinct advantages, e.g., the retrograde transsynaptic tracing ability afforded by rabies virus, into which GCaMP3 has recently been incorporated (Osakada et al., 2011). However, they have a number of drawbacks as well: limited payload capacity, inherent tropism, local delivery, incompatibility with early developmental events, and the requirement that each experimental animal be subjected to a survival surgery. Only transgenic incorporation into the genome affords stable expression of a transgene in all target tissues, reliable animal-to-animal comparisons, and the ability to image the embryo and other early developmental states. In this study, we demonstrated the feasibility and functionality of long-term expression of GCaMPs from the Thy-1 promoter for in vitro and in vivo calcium imaging.

As any GECI buffers Ca^{2+} and may interfere with endogenous signaling events, there is an inherent risk of neuronal toxicity with long-term and/or high levels of expression. Indeed, overexpression of GCaMP3 using in utero electroporation or viral infection...
showed that high expression levels can induce neural dysfunction and altered subcellular localization (e.g., nuclear), particularly near the injection site (Dombeck et al., 2010; Tian et al., 2009). In our transgenic animals, GCaMP was widely expressed in many neuronal subtypes throughout the CNS. Analysis of Thy1-GCaMP2.2c and Thy1-GCaMP3 transgenic mice did not reveal any obvious gross or cellular abnormalities. Importantly, distribution of GCaMP was cytosolic and homogeneous, with no signs of aggregation or compartmentalization in the nucleus in vivo. These results suggest that our transgenic mice exhibit stable, long-term expression of GCaMPs in neurons with normal functions and thus allow sensitive detection of calcium transients in vivo.

One key advantage of calcium imaging is that it allows the simultaneous mapping of neuronal activities from numerous cells within complex neuronal networks. Given that GCaMP3 transgenic expression targets most pyramidal neurons (~90%) throughout the cortical layers, this mouse line could allow activity monitoring from large populations of neurons across various cortical layers in behaving animals. The stable expression of GCaMP3 at nontoxic levels in our transgenic mice makes their application ideal for long-term in vivo monitoring of somatic activity. On the other hand, due to the low basal fluorescence and sparser labeling, Thy1-GCaMP2.2c mice provide a suitable means for imaging of Ca2+ transients in the dendrites of layer V neurons in vivo. Large calcium transients could readily be detected in dendrites and dendritic spines of layer V pyramidal neurons in Thy1-GCaMP2.2c mice and in the somata of layer II/III neurons in Thy1-GCaMP3 transgenic mice.

In the CNS, odors are represented as patterns of neural activity encoded by time and space. Previous mapping approaches in the olfactory bulb have used 2-deoxyglucose staining, intrinsic optical signal imaging, and pH-sensitive exocytosis detection to monitor odor-induced changes in neuronal activity. Such functional mapping strategies can provide temporal and spatial resolution of neuronal activity but to date have primarily reported olfactory nerve presynaptic activity, with little (or no) contribution from postsynaptic neurons. On the other hand, odor responses imaged by bulk-loaded voltage-sensitive dyes comprise a mixture of both pre- and postsynaptic components and do not show genetic specificity (Friedrich and Korsching, 1998; Spors and Grinvald, 2002). More recently, GCaMP2.0 transgenic mice, driven by a Kv3.1 potassium channel promoter, allowed detection of postsynaptic odor representation within the glomerular cell layer, but responses were relatively weak and did not span a dynamic range of odor concentration or specificity (Fletcher et al., 2009). In Thy1-GCaMP3 mice, GCaMP3 is expressed strongly in the glomerular and mitral cell layers, and responses to odorants were encoded by distinct sets of glomeruli. Concentration coding involved both graded responses from each activated glomerulus, as well as an increase in the total number of...
Thy1-GCaMP Transgenic Mice

EXPERIMENTAL PROCEDURES

Animal Use
All experiments were conducted according to protocols approved by the Institutional Animal Care and Use and Institutional Biosafety Committees of MIT and the NYU School of Medicine.

DNA Constructs
GCaMP2.0 and GCaMP3 expression constructs were previously reported (Tian et al., 2009). GCaMP2.2c was generated by changing the second arginine to valine and serine at 118 to cysteine of GCaMP2.0. All in vitro expression constructs of GCaMPs were connected with the coding sequence of tdTomato via a 2A peptide (P2A) sequence and subcloned into a modified pBluescript plasmid, which contained the CAG promoter (a combination of the cytomegalovirus early enhancer element and chicken beta-actin promoter). To generate the Thy1-GCaMP transgenic mouse, we subcloned GCaMP2.2c and GCaMP3 coding sequences into a Thy1 transgenic construct (Arenkiel et al., 2007; Feng et al., 2000). All constructs were verified by sequencing.

Fluorescence Measurements in HEK293 Cells
HEK293 cells were cultured in DMEM/F12 containing 10% FBS and GCaMP-P2A-tdTomato plasmid transfection was performed with Lipofectamine 2000. Imaging experiments were performed ∼36–48 hr after the transfection as described previously (Nakai et al., 2001). Imaging was performed using an Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm) using the Olympus Fluoview 1000 confocal microscope equipped with multiline argon laser (457 nm, 488 nm, and 515 nm) and HeNe (G) laser (543 nm). The slices were then crossed to C57BL6/J mice for analysis of expression patterns. 

Transgenic Mice
Thy1-GCaMP2.2c and Thy1-GCaMP3 transgenic mice were generated by injection of gel-purified DNA into fertilized oocytes using standard techniques (Feng et al., 2004; Zhao et al., 2011a). Embryos for injection were obtained by mating (C57BL6/J and CBA) F1 hybrids. Transgenic founders were back-crossed to C57BL6/J mice for analysis of expression patterns. Primers for genotyping were 5′- TCT GAG TGG CAA AGG ACC TTA GG –3′ (forward) 5′- TTA CGA CGT GAT GAG TCG ACC –3′ (reverse). The mouse strains have been deposited at The Jackson Laboratory. The JAX stock number for Thy1-GCaMP2.2c line is 017892 and the JAX stock number for Thy1-GCaMP3 line 6 is 017893.

Immunohistochemistry
GCaMP mice were anesthetized by the inhalation of isoflurane and were intracardially perfused with 20 ml 1× PBS, followed by 20 ml 4% paraformaldehyde (PFA) in PBS. Mouse brains were then postfixed in 4% PFA/PBS overnight at 4°C. We cut 50 μm sagittal sections using a vibratome. Rabbit anti-GFP antibody (Invitrogen, 1:100) was used to enhance the GCaMP fluorescence. Briefly, sections were incubated with blocking buffer (5% normal goat serum, 2% BSA, and 0.2% Triton X-100 in 1× PBS) for 1 hr at room temperature and then incubated with rabbit anti-GFP antibody overnight at 4°C. After incubation with the first antibody, sections were washed with 1× PBS three times for 20 min each, followed by incubation with Alexa 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) for 2–4 hr at room temperature and then washed with 1× PBS. Sections were transferred onto slides, mounted with 0.1% paraformalin/diadenine in 90% glycerol/PBS, and imaged with a microscope (BX61, Olympus).

Slice Preparation and Electrophysiology
Acute slices were prepared according to published procedures (Peça et al., 2011). Briefly, mice were anesthetized with Avertin solution (20 mg/ml, 0.5 mg/g body weight) and perfused through the heart with 20 ml of ice-cold oxygenated (95% O2, 5% CO2) cutting solution containing 105 mM NMDG, 155 mM Cl−, 2.5 mM KCl, 1.2 mM NaH2PO4, 28 mM NaHCO3, 25 mM glucose, 10 mM MgSO4, 0.5 mM CaCl2, 5 mM L-ascorbic acid, 3 mM sodium pyruvate, and 2 mM thioare (pH was 7.4, with osmolarity of 295–305 mosm). The brains were rapidly removed and placed in ice-cold oxygenated cutting solution. Coronal or transverse hippocampal slices (300 μm) were prepared using a slicer (Vibratome 1000 Plus, Leica Microsystems) and then transferred to an incubation chamber (BSK4, Scientific System Design) at 32°C with carbogenated cutting solution, which was gradually replaced with artificial cerebral spinal fluid (ACSF) in 30 min through a peristaltic pump (Dynamax Model RP-1; Rainin Instruments), allowing a precise regulation of fluid flow rates. The slices were then kept in the ACSF that contained 119 mM NaCl, 2.3 mM KCl, 1.0 mM NaH2PO4, 26 mM NaHCO3, 11 mM glucose, 1.3 mM MgSO4, and 2.5 mM CaCl2 (pH was adjusted to 7.4 with HCl, with osmolarity of 295–305 mosm) at room temperature for at least 30 min.

Recordings were performed in oxygenated ACSF. Intracellular solution consisted of 130 mM KMeSO4, 10 mM HEPES, 4 mM MgCl2, 4 mM NaATP, 0.4 mM NaGTP, 10 mM Na-phosphocreatine, and 3 mM Na-L-aspartate; pH was adjusted to 7.3 with KOH. Recordings were performed at room temperature in ACSF. To evoke APs, we held cells in the current-clamp configuration, and we injected 3–5 nA of current for 2 ms through the recording electrode. Cells were selected if their GCaMP fluorescence was homogeneously distributed in the cytoplasm.

Fluorescent signals were imaged by a confocal microscope (Fluoview FV 1000; Olympus) with a 30 mW multiline argon laser, at 5%–10% laser power. The laser with a wavelength of 488 nm was used for excitation, and fluorescence was recorded through a band-pass filter (505–525 nm). The images were acquired using 40× water-immersion objectives (NA = 0.8) with 5 Hz scanning speed. X/Y image galleries were collected and average fluorescence intensity in the soma was measured for the quantification by Fluoview data processing software. We report time series as ∆F/F = (F − Fb) / (Fo − Fb), where F is the raw fluorescence signal, Fb is the background, and Fo is the mean fluorescence signal in a baseline period prior to the action potential stimulus. SNR was calculated as the ratio of ∆F/F to SD of the basal fluorescence, 1 s before the stimulus up to stimulus onset. Rise time was measured as the time between onset of current injection and the maximal response. Decay time was measured as the time between the maximal response and the decay back to baseline.
In Vivo Ca²⁺ Imaging of Layer V Apical Dendrites in Motor Cortex

A head holder composed of two parallel micrometal bars was attached to the animal’s skull to reduce motion-induced artifact during imaging. First, surgical anesthesia was achieved with an intraperitoneal injection (5–6 μg/g) of a mixture of ketamine (20 mg/mL) and xylazine (3 mg/mL). A midline incision of the scalp exposed the peristeum, which was manually removed with a microsurgical blade. A small skull region (~0.2 mm in diameter) was located over the left motor cortex based on stereotactic coordinates (0.5 mm posterior from the bregma and 1.5 mm lateral from the midline) and marked with a pencil. A thin layer of cyanacrylate-based glue was first applied to the top of the entire skull surface and to the metal bars, and the head holder was then further fortified with dental acrylic cement (Lang Dental Manufacturing). The dental cement was applied so that a well was formed leaving the motor cortex with the marked skull region exposed between the two bars. All procedures were performed under a dissection microscope. After the dental cement was completely dry, the head holder was screwed to two metal cubes that were attached to a solid metal base, and a cranial window was created over the previously marked region. The procedures for preparing a thinned skull cranial window for two-photon imaging have been described in detail in previous publications (Yang et al., 2010). Briefly, a high-speed drill was used to carefully reduce the skull thickness by approximately 50% under a dissecting microscope. The skull was immersed in artificial cerebrospinal fluid during drilling. Skull thinning was completed by carefully scraping the cranial surface with a microsurgical blade to ~20 μm in thickness. For anesthetized imaging, animals were immediately imaged under a two-photon microscope tuned to 910 nm with a 40× objective immersed in an artificial cerebrospinal fluid solution and a 3× digital zoom. For awake animal imaging, the completed cranial window was covered with silicon elastomer (World Precision Instruments) and mice were given at least 4 hr to recover from the surgery-related anesthesia. Mice with head mounts were habituated for a few times (10 min for each time) in the imaging apparatus to minimize potential stress effects of head restraining and imaging. To image dendrites in awake mice, we screwed the head holder to two metal cubes attached to a solid metal base, and the silicon elastomer was peeled off to expose the thinned skull region and ACSF was added to the well. The head-restrained animal was then placed on the stage of a two-photon microscope.

These in vivo two-photon imaging experiments were performed using a Bio-Rad Radiance 2000 two-photon system equipped with a Tunami Ti:sapphire laser pumped by a 10 W MilleniaXs laser (Spectra-Physics). The average laser power on the sample was ~20–30 mW. Most experiments were acquired at frame rates of 1 Hz at a resolution of 512 × 512 pixels using a 40× water-immersion objective (Nikon). Image acquisition was performed using Laser Sharp 2000 software and analyzed post hoc using ImageJ software (NIH). ∆F/F was calculated identical to slice imaging experiments. For detecting calcium signals in layer V apical tuft dendritic spines, a line crossing the dendrite and the middle of the spine head was drawn and fluorescence intensity along the line was measured using ImageJ (NIH).

In Vivo Ca²⁺ Imaging of Neuronal Activity in the Motor Cortex, Somatosensory Cortex, and Olfactory Bulb

Imaging experiments were performed on 4- to 5-month-old mice. The surgery was performed as described previously (Dombeck et al., 2009). Briefly, the mice were anesthetized with Avertin solution (20 mg/mL, 0.5 mg/g body weight) and were placed in a stereotactic apparatus with a heating pad underneath to maintain body temperature. A 2 × 2 mm piece of bone was removed above the motor cortex, somatosensory cortex, or olfactory bulb as determined by stereotactic coordinates, and the dura was kept intact and moist with saline. To dampen heartbeat and breathing-induced motion, we filled the cranial window with Kwik-sil (World Precision Instruments) and covered it with an immobilized glass coverslip. A custom-designed head plate was cemented on the cranial window with Meta-bond (Parkell) when the Kwik-sil set. For chronic imaging, two coverslips were joined with ultraviolet curable optical glue (NOR-138, Norland). A smaller insert fit into the craniotomy and a larger piece was attached to the bone. Imaging was performed 7 days post-surgery to allow the window to clear.

During imaging of neuronal activity in motor cortex, the head-fixed animals were placed in water to induce swimming-like behavior. The animals were kept alert by presenting a pole or by mild air puffs to the whisker field. An infrared charge-coupled device camera (CCTV) was used for observing the animal’s behavior during imaging sessions. Sensory stimulations, consisting of puffs of compressed air delivered by a Picospritzer unit (Picospritzer II; General Valve), were applied through a 1-mm-diameter glass pipette placed 15–25 mm rostrolateral from the whiskers. Air puffs (500 ms duration) were given ten times with 10 s intervals to prevent adaptation of whisker-evoked responses.

Odorants were delivered using a custom-built odor delivery system in which the saturated vapor of an odorant was diluted into a main stream of clean air. The clean air stream was fixed at 0.6–0.8 L/min throughout the experiment and the odor vapor stream was adjusted to give the final concentration to the animal. A tube opening was positioned <1 cm from the animal’s nostrils. To avoid cross-contamination, we used separate Teflon tubing in parallel for delivery of different odorants. Odorants were usually presented with pulse duration of 1 s and inter-stimulus interval of 30 s to avoid potential sensory adaptation. A constant suction system was positioned close to the odorant delivery system and used to quickly remove remnant odorants. The odorants used in this study included methyl salicylate, amy1 acetate, eugenol, and 1-menthol (Sigma-Aldrich).

In these experiments, in vivo two-photon imaging was performed at the McGovern Institute two-photon microscopy core facility. Imaging was performed on a custom two-photon laser-scanning microscope (Ultima; Prairie Technologies) coupled with a Mai Tai Deep See laser (Spectra Physics). The laser was operated at 910 nm (~30–40 mW average power on the sample). The emission filter set for imaging GCaMP fluorescence consisted of a 575 nm dichroic mirror and a 525/70 nm band-pass filter. Fluorescence signal was detected using Hamamatsu multialkali PMTs. In most experiments, images were acquired at frame rates of 1.5–2 Hz at a resolution of 512 × 256 pixels using a 20×, 1.0 NA water-immersion objective (Zeiss). For in vivo z stack imaging, images were taken at a resolution of 512 × 512 pixels with 2 μm intervals. Image acquisition was performed using custom Prairie View Software. The images were analyzed post hoc using NIH ImageJ and Image-Pro Plus 5.0 software (Media Cybernetics). ∆F/F was calculated identical to slice imaging experiments.

Statistical Analysis
All statistical analyses were performed using SPSS (IBM) software and graphs were drawn in SigmaPlot 2000 (Systat Software). Values are expressed as mean ± SEM. The data between two groups were compared using unpaired t-test. The data among three groups were compared using one-way ANOVA. Statistical significance was defined as “p < 0.05 or “p < 0.005.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and nine movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2012.07.011.

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