NOVEL METHODOLOGY TO MAP THE MOTOR CORTEX

by

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Abstract

It is very well established that the motor cortex has a distinct cortical location that can be mapped in a variety of species from mice to humans. Traditionally, mapping the motor cortex requires electrodes to stimulate the brain and define motor output pathways. Although effective, electrode-based methods are labor-intensive, potentially damaging to the cortex, can have off-target effects, and are not well suited to long-term application in the same brain due to its invasive nature. As an alternative method to traditional motor mapping, transgenic mice expressing the light-sensitive ion channel channelrhodopsin-2 in predominantly layer-5 output cortical neurons were photostimulated. Implanted electromyogram electrodes or a noninvasive motion sensor were used as a readout of motor cortex output. In addition, electroencephalogram electrodes were used to directly monitor the activity of the motor cortex during periods of optical stimulation. Optical stimulation with a 473 nm laser was delivered to hundreds of cortical locations, in vivo, using a stage scanning laser system. Electrophysiological signals from the muscles and the cortex were used to create highly reproducible automated maps of the mouse forelimb and hindlimb motor cortex much faster than with previous methods. This method was well suited to mapping the same brain over a period of weeks using an implanted cranial window. It is anticipated that this novel method will facilitate the study of changes in the location and properties of motor maps after skilled training or damage to the nervous system.
Preface

This thesis presents work that has been previously published. Ayling, O.G., Harrison, T.C., Boyd, J.D., Goroshkov, A., Murphy, T.H. Automated light-based mapping of motor cortex by photoactivation of channelrhodopsin-2 transgenic mice. *Nature Methods* 6, 219–224 (2009). Dr. Timothy Murphy supervised the project, provided financial support, and aided in writing the manuscript. Alexander Goroshkov provided assistance with optics and fabrication of the hardware. Dr. Jamie Boyd developed the software necessary to control the hardware. Thomas Harrison assisted in the collection of data, analysis, and writing the manuscript. For this paper I conducted the majority of the experiments including light-based motor mapping, ICMS, and pharmacology experiments, analysed data, and wrote the manuscript.

Ethical Approval was obtained from the University of British Columbia animal care committee, the certificate number is A10-0140.
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Introduction

Traditional motor mapping methodology

Motor mapping technologies have been greatly refined since the days of Hitzig and Fritsch (Fritsch & Hitzig, 1870) who used wires and batteries to stimulate the cortex. Wilder Penfield developed handheld electrical probes to stimulate the cortical surface during surgery in epileptic patients that he used to define the cortical location of the motor cortex (Penfield & Boldrey, 1937). Several decades later, Asanuma and colleagues (Asanuma, Arnold, & Zarzecki, 1976) developed intra-cortical micro-stimulation (ICMS) which is now considered the gold standard for motor mapping studies (Donoghue & Sanes, 1987). ICMS involves lowering electrodes into the cortex and passing current in order to drive cells in a region of interest. Subsequently, surface stimulation with electrode arrays was developed for use in rodents (Hosp et al., 2008). The advent of transcranial magnetic stimulation (TMS) has made non-invasive motor mapping feasible in humans (Siebner & Rothwell, 2003). Each of these techniques has a unique set of advantages and limitations. TMS is non-invasive, but has poor spatial resolution. Electrode-based brain stimulation methods have common disadvantages: the inability to selectively recruit neuronal sub-types, indiscriminate activation of axons of passage, and some degree of damage where impalements are made.

Genetically targeted, optical control of neuronal cells

Recently, it has become possible to stimulate neurons using light energy, either by uncaging neurotransmitters (Shepherd et al., 2003; Luo et al., 2008) or directly
activating genetically targeted light-sensitive channels (Huber et al., 2008; Zhang et al., 2007), termed optogenetics. Channelrhodopsin-2 (ChR2) is a light-activated non-selective, 7 transmembrane, cation channel isolated from the green algae Chlamydomonas reinhardtii (Nagel et al., 2005) which when expressed in neurons can transduce light energy into neural activity. The excitation spectrum of ChR2 is consistent with other rhodopsin variants and is slightly blue shifted so that maximum photo currents are evoked at ~470 nm (blue light) (Nagel et al., 2005). When ChR2 is expressed in neuronal cells one has the ability to selectively activate only the cells that are expressing ChR2, using light, with millisecond temporal resolution, and to have reliable action potential generation at up to ~30 Hz (Boyden et al., 2005). Optical stimulation of cells expressing ChR2 initially causes direct activation of these cells but also can activate cells downstream via synaptic transmission (Lee et al., 2010). ChR2 has been expressed in discrete brain locations, such as the olfactory bulbs, subthalamic nucleus, and cortex while being stimulated in vivo with an implanted fiber optic cable that remains fixed in one location (Arenkiel et al., 2007; Gradinaru et al. 2009). A fiber optic cable fixed in one location is very useful when studying awake behaving animals but this method it makes it virtually impossible to stimulate different sites of interest in the same animals or to combine ChR2 and imaging methods. ChR2 has been expressed, using viruses, in various cortical locations and the intrinsic circuitry of the cortex has begun to be worked out by stimulating ChR2 in brain slices while conducting whole cell recordings in areas of interest (Petreanu et al., 2007; Wang et al., 2007; Petreanu et al., 2009). The work in this manuscript aims to extend the in vitro mapping studies and also attempts to circumvent some of the limitations related with ICMS motor
mapping by using transgenic mice expressing ChR2 (Arenkiel et al., 2007) to map the motor cortex in vivo.

**Motor map plasticity**

The primary motor cortex has been noted to exhibit remarkable plasticity, especially after damage to the nervous system but also due to skilled learning (Nudo et al., 1996; Kleim et al. 1998). Damage to either the sensory or motor cortex or peripheral input to the cortex, in the form of experimental lesions (Merzenich et al., 1984, Jones et al., 1999; Ghosh et al., 2010) or strokes in humans (Liepert et al., 2000) leads to impairments of function but there is also incredible cortical plasticity that can lead to restoration of function over time. The sensory cortex, much like the motor cortex, has a clear topology which can be readily mapped. Damage to the sensory cortex, while not only impairing sensation, may also lead to changes in the motor cortex. Prior work in the Murphy lab has demonstrated that strokes targeted to the forelimb sensory cortex of mice cause the sensory map to shift, over a period of weeks to months, into the neighbouring motor cortex (Winship & Murphy, 2008; Brown et al., 2009). It is these previous results that provided the inspiration for a suitable method to monitor any changes in the motor cortex maps that may take place as the sensory maps reorganise after stroke. Prior to this current work, the most suitable method available was ICMS. However, ICMS is very time consuming, and can damage the cortex with electrode penetration (hundreds are required to map the mouse motor cortex) which can potentially lead to confounding effects when studying recovery from nervous system trauma such as stroke. Additionally, due to its invasive nature ICMS is not suited to
longitudinal studies. This thesis presents the work that led to the development of a novel method to map the motor cortex using light rather than stimulating electrodes.

**The history of the motor cortex**

The motor cortex was the first region of the brain to be mapped and to have an overt function attributed to it (Penfield & Boldrey, 1937). Prior to this work a debate raged in the scientific community as to whether the cerebral cortex could be divided into discrete locations that represented individual functions or whether various functions were represented throughout the cortex in an equal and homogeneous manner (Morgan, 1982). For the first half of the 1800’s the dominant belief among the scientific community was that the cerebral cortex was a homogeneous structure and that specific functions, such as movements, were controlled by sub-cortical structures (Morgan, 1982). People were no doubt looking for evidence to counter that of the phrenologists.

As the 20th century drew closer evidence began to emerge that functions were, in fact, discretely localised in the cortex. Evidence in favour of the cortical localisation view-point initially came from a couple of neurologists. First, Paul Broca described that damage to a specific cortical region led to deficits in speech production (Broca, 1861). Next, the English neurologist, John Hughlings Jackson, using astute clinical observations, noted that motor seizures would initiate and travel up a patient’s arm. This led him to conclude, before the advent of technology to directly investigate his conclusion, “the convolutions of the brain must contain nervous arrangements representing movements” (Foerster, 1936). While clinical observations were paramount in initiating the shift from the view that cortical functions are equally distributed, to a view
that functions are localised, there was not any hard experimental evidence to truly convince scientists.

In 1870 Fritsch and Hitzig essentially changed the field by providing definitive evidence that individual functions are indeed localised in the cortex. Applying electrical current to the cortex of dogs Fritsch and Hitzig noted that limbs on the opposite side of the body would contract. Importantly, not every cortical site of stimulation led to the initiation of movements (Fritsch & Hitzig, 1870). Many decades later, Wilder Penfield, began to systematically map out the cortex in epileptic patients and defined the motor cortex in humans, now referred to as the homunculus (Schott, 1993). More than a hundred years after the pioneering work by Fritsch and Hitzig the overwhelming majority of the scientific community subscribes to the notion that the cortex is divided into localised functions (Monfils, Plautz, & Kleim, 2005).

**Organisation of the motor cortex**

**Motor cortex somatotopy**

The main role of the motor cortex is the planning and execution of movements (Sanes & Donoghue, 2000). The landmark work from Wilder Penfield clearly established that the motor cortex occupied a distinct cortical location in humans and furthermore that the motor cortex could be divided into regions based upon movements of different limbs, a somatotopy (Penfield & Boldrey, 1937; Schott, 1993). More recent work has also established that non-human primates, cats, rats, and mice all exhibit a definable motor cortex somatotopy based on body parts or movement repertoires of those limbs (Graziano, Taylor, & Moore, 2004; Nieuwullon & Rispal-Padel, 1976; Neafsey et al., 1986;
Pronichev & Lenkov, 1998; Tennant et al., 2010; Ayling et al., 2009). Maps are by no means unique to the motor cortex, as the somatosensory and visual cortices also are amenable to mapping (Hubel & Wiesel, 1962; Kaas et al., 1979; Grinvald et al., 1986; Ohki et al., 2005). However, maps based on somatotopy are arguably the distinguishing feature of the gross structure of motor cortex.

**Motor cortex structure**

While the motor cortex can be relatively clearly defined by functional parameters (i.e. limb movements) it has been slightly more difficult to determine the fine structural organisation of the motor cortex (Shepherd, 2009). It is likely that the fine structure of the motor cortex is what defines it and separates it from other cortical areas. One attempt to determine the fine structure of the motor cortex has been based primarily on cytoarchitectonics. Cytoarchitectonics is the study of how cell types are arranged in tissue. Cytoarchitectonic work has revealed, not surprisingly, that the motor cortex is divided into six layers, as are the primary sensory cortices, including visual and somatosensory (Donoghue & Wise, 1982). Somatosensory and visual cortices have some very well defined cortical layers that are easily identifiable due to sharply demarcated borders. In particular, the hallmark feature of sensory cortex is the granular layer 4 where the majority of thalamic inputs arrive (Staiger et al., 2004). The term granular refers to the appearance of stellate cells which give a granular appearance when Nissl staining has been employed to visualise cortical layers (Kreutzberg, 1984). Although the motor cortex does have identifiable cortical layers the laminar pattern is not the same as sensory cortex.
Based on cytoarchitectonics there are two notable fine-structural features commonly used to define the motor cortex. The first is that the motor cortex lacks a clear cortical layer 4, in other words, it is agranular (Keller, 1993; Shepherd, 2009). The less prominent layer 4 seen in the motor cortex of gives way to its’ second defining feature, a prominent layer 5. Layer 5 of motor cortex houses large pyramidal cells that form the two main output pathways of the motor cortex: the corticospinal and corticostriatal pathways (Anderson et al., 2010). These corticofugal projections are divided into two sublayers of layer 5. In the superficial layer 5A, cells preferentially project to the striatum. This corticostriatal pathway is important for regulation of movements and goal directed behaviour (Alexander et al., 1986). The lower layer 5B houses the corticospinal projection neurons, which are important for fine digit manipulation (Heffner & Masterton, 1983). Residing in Layer 5B are the Betz cells (Betz, 1874; Walshe, 1942). The Betz cells are among the largest in the brain and send their axons to synapse directly at the spinal cord, defining what is known as the pyramidal tract or corticospinal output pathways (Nathan & Smith, 1955; Brown, 1971).

**What does the motor cortex encode?**

Apart from cytoarchitectonic or histological evidence, physiological studies have shed light on how individual cells encode various parameters related to movements. Most commonly, individual motor cortex neurons are recorded from while primates perform various behavioural tasks (Hatsopoulos, 2005). In 1968, Evarts asked whether motor cortex pyramidal cell firing patterns were related to the force of a movement or whether they encoded the distance of a movement (Evarts, 1968). In attempt to answer
this question Evarts trained monkeys to move a lever back and forth for a defined distance and in a given time window. Attached to the lever, over a series of pulleys was a weight and this weight could be adjusted to oppose either flexion or extension of the wrist. After the monkeys were trained in the behavioural paradigm single units were recorded from the motor cortex. Evarts recorded from individual cells and noticed that many modulated their activity in response to force rather than displacement. Based on this observation, Evarts concluded that the pyramidal tract neurons of the motor cortex encoded the force during a movement. He went on to hypothesise that cells could encode displacement but that it was likely a more complex process than encoding for force. Evarts also noticed that many neurons responded to events other than force, such as the movement of a particular joint. Furthermore, it was also noted that motor cortex neurons preferentially fired in response to a single event (i.e. a cell would fire strongly for digit manipulation but only weakly for shoulder movements). Finally, Evarts observed that cells along the same vertical axis, relative to the cortical surface, fired in response to the same event. The pioneering study of Evarts opened the door for the current motor cortex research by presenting many avenues to follow up on.

Several years later Rosén & Asanuma (1972), based on microelectrode stimulation studies in primates, put forth the theory that the motor cortex was organised around vertical columns that represented different muscles or joints. Asanuma and Rosen began to view the motor cortex as a mosaic structure where individual parameters encoded in the motor cortex slightly overlapped. The relatively early studies, by Evarts, Asanuma, and Rosén, investigating the functional organisation of the motor cortex were perhaps overly influenced by work done on the sensory cortices that
promoted the idea of cortical columnar organisation (Mountcastle, 1957; Hubel and Wiesel, 1962; Abeles & Goldstein, 1970). In subsequent years much more work has been conducted to understand how the motor cortex is organised and the idea of discrete columns in the motor cortex has been losing favour ever since.

The notion that the motor cortex could be organised into discrete columns, that each control a very specific parameter, is attractive because one could imagine various columns working synergistically in a virtually unlimited number of combinations to make various complex movements. However, more recent work has established that motor cortex cells fire in graded responses to events that are more complex than simply encoding force or a particular joint movement. In 1982, Georgopolous et al., (Georgopoulos et al., 1982) demonstrated that individual motor cortex neurons modulate their activity in response to varying movement directions. This initial finding by Georgopolous et al., has also been seen by other labs, suggesting that cellular encoding of movement direction is a fundamental feature of the motor cortex (Georgopoulos et al., 1986; Moran & Schwartz, 1999; Paninski et al., 2004). In these studies, primates were trained to move their hand from a center position to various endpoints at the edge of a circle. The interesting finding from these studies is that individual motor cortex neurons do not fire in response to only one movement direction. Instead, they have an orderly frequency of discharge so that when a monkey moves its’ arm to different endpoints around the circle the neuron will usually fire at different rates for each movement trajectory. When the firing rate versus the movement direction is plotted, a bell shaped curve is produced where the peak will be that particular cell’s preferred movement direction. Studies from behaving primates have put forth the notion
that the motor cortex encodes many different parameters but how these parameters function in unison has been difficult to pin down.

The functional connectivity of motor cortex

Single unit recordings have been paramount in understanding how the motor cortex is functionally organised but as new methodology has emerged, so has our view of the motor cortex’s functional organisation. Single unit recordings, in isolation, are limited as information regarding how a network functions will go unnoticed. Recent advances in cellular imaging, such as multiphoton calcium imaging, are now being applied to the motor cortex (Stosiek et al., 2003). In vivo calcium imaging is able to provide a simultaneous readout from hundreds of different cells in the imaging field and does not suffer from the sampling bias of single unit recording where only cells that respond to activity are recorded from (O’Connor et al., 2010). One major limitation of in vivo calcium imaging is the depth in the cortex that cells can be imaged from due to the scattering of light as deeper tissue needs to be accessed (Svoboda & Yasuda, 2006). The driving cells of the motor cortex are located in layer 5, and even in a mouse they are often too deep to be imaged in vivo using 2-photon microscopy. Nonetheless, important data is being obtained by imaging in layer 2/3 of motor cortex. Using in vivo calcium imaging, Dombeck, Graziano, & Tank (2009), asked how hundreds of simultaneously imaged cells would be functionally organised when mice were either running on a ball or grooming their vibrissae. The authors were able to correlate the activity of every neuron imaged during behaviours and found that motor cortex cells cluster to form functional units that were associated with a specific behaviour. It was not
necessary for the cells in a functional cluster to be next each other, but at the same time
the distances separating cells were not immense (<200 micrometers). The Dombeck et
al., (2009) study is important because it, in a way, filled in the missing cells when single
unit recordings were made. The study did fall short, however, because it was not able to
control specific phases of a behaviour. For example, the mouse was monitored while it
ran but no considerations were made for the movement velocity or phase of the limb
during the motions.

Komiyama et al., (2010) were able to extend the initial findings of Dombeck et al.,
(2009) by also employing in vivo calcium imaging in awake behaving mice. First,
Komiyama et al. (2010) imaged mice that were learning a lick/ no-lick task where the
mice had to lick for a water reward in response to one odor and withhold licking in
response to a separate odor. Mice were either imaged on the first learning session or
were imaged at the fifth/sixth learning session. Regardless, if the imaging was
conducted earlier or later during learning there was a functional clustering of neurons in
the motor cortex. Cells clustered based on the correlation of detectable calcium
transients. The strength of the correlation was a product two factors: distance and
response type. An inverse relationship between distance and correlating pairs was
noted; so that as the distance between pairs of cells increased over a distance of ~150
micrometers the correlation exponentially decayed. Interestingly, ~150 microns is
approximately the size of dendritic and local axonal arbours of pyramidal neurons and
the correlations between cells likely reflects their microcircuitry (Shepherd et al., 2005).
Pairs of neurons were also more strongly correlated if they were found to respond to the
same behavioural response of the mouse (i.e. a correct rejection or a correct lick).
Komiyama et al. (2010) further extended the field by studying how the animals learned the task over the course of a session. Whether it was the first or fifth learning session, as the trial number increased per session so did the correlation between cells that responded to similar aspects of the task (e.g. a correct lick). These correlations were even stronger by the fifth or sixth training session. Importantly, these increasing correlations were not due to increasing activity of the individual cells, suggesting that increased coupling between neurons is an important feature of learning in the motor cortex. Interestingly, when imaging was performed at the fifth/sixth day of training on the licking task fewer neurons were involved in the behaviour but the correlations between cells that encoded similar phases of the task were stronger. Together, the recent studies by Dombeck et al. (2009) and Komiyama et al. (2010) suggest that the motor cortex, at least in layer 2/3, works by encoding functional parameters (e.g. force, direction, or action selection) of movements in small, but overlapping, ensembles of neurons.

The previously discussed electrophysiology and calcium imaging studies raise the question of how these motor cortex ensembles are functionally connected. One could hypothesise that ensembles are connected in a point-to-point manner or that they are all broadly connected in an integrated manner. In an attempt to answer this question Capaday et al., (2009) first mapped out the motor cortex of cats using microstimulation electrodes and recording muscle activity from many proximal and distal forearm muscles. The retrograde tracer biocytin was injected at various depths in a single vertical tract spanning most of the cortex. Several key observations came from this study. The first was that different muscle representations, whether proximal or distal,
were overlapping in the cortex, as determined by EMG recordings and ICMS. The second was that axons projected extensively throughout most of the motor cortex for quite long distances (up to 7 mm). The third was that axons were studded with varicosities along their entire processes. Using electron microscopy it was demonstrated that the majority of these varicosities formed synapses and were thus referred to as axonal boutons. These axonal boutons were primarily excitatory in nature (75% excitatory vs. 25% inhibitory synapses). The density of axons and boutons decreased monotonically with distance from the injection site. In other words, axons of motor cortex do not project in patches but in smooth distributions, rather than in a patchy distribution that had previously been reported (Huntley & Jones, 1991; Keller, 1993). Lastly, when motor maps of various muscles were overlaid with bouton density and axonal projection maps it was evident that most points of the motor cortex were connected with each other. Together, the data from Capaday et al. (2009) strongly suggest that the motor cortex has its’ connections organised in an integrative manner rather than in a patchy point-to-point fashion. Interestingly, this pattern of connectivity is quite different than what has been observed in the visual cortex where patchy distributions of axons have been observed and are thought to connect similar orientation columns (Rockland and Lund, 1982; Gilbert and Wiesel, 1983; Gilbert and Wiesel, 1989; Buzás et al., 2006).

**Motor cortex intracortical circuitry**

It seems as if recent work in the motor cortex has resulted in a confusing view of its functional connectivity. If many cells are active during a movement and most of these
cells seem to be connected then how does the motor cortex establish any specificity? *In vitro* circuit mapping studies have been able to identify specific ascending and descending pathways in the motor cortex that may be able to, in part, explain how the motor cortex can establish specificity. Weiler et al., (2008) used the technique of glutamate uncaging to map the presynaptic inputs from all cortical layers to individual post synaptic pyramidal cells located in layers 2 to 6 in motor cortex. It was found that upper cortical layers (layer 2/3) strongly project to lower cortical layers (layer 5A/B border). There were also strong horizontal connections in upper and lower cortical layers (i.e. layer 2/3 connects with layer 2/3 and layer 5 connects with layer 5). The study by Weiler et al. (2008) suggests that local intracortical circuit interactions in upper cortical layers may determine how and when the motor output cells of layer 5 are engaged to produce the final movement.

**Basic principles and future directions for motor cortex research**

Clearly much work remains to be conducted in the motor cortex. However, several features of the motor cortex seem to be emerging. First, individual muscles are represented in overlapping cortical domains but individual movement repertoires appear to be less overlapping (Graziano et al., 2002; Ayling et al., 2009; Capaday et al., 2009). Second, individual movement repertoires are organised, on a microscopic scale, into neuronal clusters or ensembles that interact over hundreds of microns (Dombeck et al., 2009; Komiyama et al., 2010). Third, motor cortex functions in an integrative manner; the functional ensembles are all connected (Keller, 1993; Capaday et al., 2009). Lastly, motor cortex output depends not simply on corticofugal projections but rather depends
on interactions between active local networks that function in a very specific manner (Weiler et al., 2008; Shepherd, 2009; Anderson et al., 2010).

The motor cortex presents a difficult dichotomy to understand because on one hand it seems that everything in the motor cortex is connected but on the other hand the motor cortex is responsible for exquisite dexterity associated with movements. Future work in the motor cortex will have to establish how functionally clustered cells in the upper layers of the motor cortex are able to effectively engage the motor output cells located in deeper layers. If the motor cortex is truly functioning in an integrative manner then inhibition will play a key role in shaping how motor cortex circuits achieve such remarkable specificity. The role of inhibition in the motor cortex has not been explored in detail. Finally, the cells of the motor cortex are arranged in a very specific manner, as they are in any cortical region, but the role that specific cell types play in creating motor specificity also remains to be understood. Given this, it will be important to control specific cell types using optogenetics to understand their role in determining the organisation of motor maps.
Objective and Hypothesis

The objective of this thesis was to create a novel method, employing transgenic mice expressing ChR2, to map the motor cortex using light rather than stimulating electrodes. The working hypothesis was that mice expressing ChR2 in layer 5 cortical pyramidal cells would be amenable to optical motor mapping.
Methods

Animal protocols were approved by the University of British Columbia Animal Care Committee. Channelrhodopsin-2 transgenic mice were purchased from the Jackson labs (Line 18, Stock # 007612, Strain B6.Cg-Tg(Thy1-COP4/EYFP)18Gfng/J). After craniectomy, the animal was fixed to the scanning stage and the locations of its somatosensory forelimb and hindlimb representations were visualized using IOS imaging (Winship & Murphy, 2008). During craniectomy surgery and IOS imaging the animal was anesthetized with isoflurane (1.5% in air). Ketamine/xylazine (100 mg kg\(^{-1}\) ketamine, 10 mg kg\(^{-1}\) xylazine) anesthetic was used during motor mapping. We generally collected several cortical EEG-based maps at the beginning of each experiment using low laser power (40 mW mm\(^{-2}\)) and short activation duration (1 ms). In some cases when responses were weak (usually when craniectomies were imperfect), we increased laser power (up to 200 mW mm\(^{-2}\)) and/or duration (up to 5 ms). We then connected the EMG electrodes and laser motion sensor and began collecting motor maps. These EMG experiments were typically conducted using increased laser power (40-600 mW mm\(^{-2}\)) and duration (up to 35 ms), with stimulus parameters adjusted to supra-threshold levels.

Animals and surgery

Adult mice aged 2-3 months and weighing 25-30 g were used for these experiments, and were maintained on a 12:12 hour light:dark schedule. Anesthesia was induced with isoflurane (1.5 % in air) and body temperature was maintained at 37° C ± 0.5° C using a feedback-regulated heating pad. A craniectomy was made over the right
sensory-motor cortex while the anesthetized mouse was supported by ear and tooth bars. The skull was then fastened to a stainless steel plate (Kleinfeld & Denk, 2000) with cyanoacrylate glue and dental cement, and the plate attached to 25.4 mm posts mounted on an aluminum plate that could be bolted to a stage. The exposed brain was covered with 1-1.5 % agarose (Type 3-A Sigma; A9793) dissolved in a HEPES buffered (pH 7.3) physiological salt solution (in mM): 135 NaCl, 5.4 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, and sealed with a custom cut glass coverslip. Isoflurane anesthesia was maintained during IOS imaging of somatosensory representations, but was replaced by ketamine/xylazine prior to LBM. Consistent with previous work (Ferezou et al., 2007; Hosp et al., 2008), we found it easier to elicit an evoked response in ketamine/xylazine anesthetized animals. Ketamine/xylazine was administered in doses of 0.02 mL (20 mg mL⁻¹ ketamine, 2 mgmL⁻¹ xylazine) approximately every 30 minutes or as necessary to maintain a constant level of anesthesia.

**Optical imaging and photoactivation**

To perform IOS imaging and to create maps of the surface vasculature a Dalsa 1M60 camera was used (Waterloo Ont. Canada). The frame grabber for the camera was an E1DB from EPIX (Buffalo Grove IL USA) and was running EPIX XCAP version 2.2 software. The camera was mounted on a vertical milling machine (Sherline Tool #5430, Miami FL USA), and images were taken through a macroscope composed of front-to- front video lenses coupled with a 52 mm threaded adaptor ring (BH Photo, New York NY USA). The top lens (closer to the camera) was a 135 mm F2.8 Nikor and the lower lens was a 50 mm F1.4 Nikor lens. To direct the 473nm photoactivation laser
(CrystaLaser BCL-473-050, Reno NV USA) a hole was cut in the side of the Dalsa 1M60 camera F-mount adaptor and a dichroic mirror was installed between the CCD camera and the first video lens. The dichroic mirror was an Olympus DM500 (500nm cut-off). To direct the blue laser light an optical cage was constructed using Thorlabs 5 mm rods and microbench parts similar to that previously reported by us for photoactivation of rose bengal (Sigler, Goroshkov, & Murphy, 2008).

There are several possible methods for photoactivation of ChR2 transgenic mice (Arenkiel et al., 2007). Fiber optic systems are the best option for targeting subcortical structures, but are not ideal for stimulating the cortical surface because of light divergence (Aravanis et al., 2007). Divergence becomes a considerable problem in mapping, where curvature of the brain results in a variable distance between the light source and cortical surface. By using lens based beam-conditioning optics we were able to generate a relatively collimated beam that could be varied in size from 100 to 220 μm in diameter by changing lens focal lengths and/or lens positions. Within the optical cage, a 25.4 mm plano convex 50 mm focal length lens (LA1131, Thor Labs, Newton NJ USA) could be moved relative to the first video lens (typically placed 145 mm from the first video lens) to alter the laser spot size on the brain surface. The beam XY position within the video image field could be adjusted by moving both the plano convex lens within its mount using a Thorlabs XY translator lens mount (HPT1) as well as a right angle silver mirror mounted on a XY adjustable holder (Linos 065087, Goettingen, Germany) within a 30 mm Linos cube (061081). A final level of adjustment was achieved using a Linos XY adjustable holder (065087).
The XY stage used to move the animal relative to the laser was driven by XY LS50 high-velocity motors and controlled by an MS2000 2-axis stage controller (Applied Scientific Instrumentation, Eugene OR USA). All maps were created based on a random sequence of movements to a series of positions outlined in a grid of stimulus locations superimposed over a map of the brain (see Software section below for details). We chose a stage scanning system since it was capable of repeated optical stimulation at intervals of < 1 s and ensured accurate XY positioning. An advantage of a mechanical scanning system is that all movements are based on absolute distance with respect to the excitation laser, and therefore it is inconceivable that photoactivation power or position would be subject to errors due to lens aberration that may occur near the edges of an image field. Although it would be possible to reduce the time between stimulation points by using a galvanometer and mirror based beam steering system, shorter (< 1 s) interstimulus intervals may lead to unexpected interactions between stimulus pulses. For both EEG and EMG maps, stimulus parameters (especially duration) were increased gradually until deflections in the recorded traces became apparent upon visual inspection. Once this threshold was reached, we would increase the stimulus duration by an additional 50 % to ensure adequate stimulation.

Testing for the effect of photodamage on motor maps

To test for photodamage, we compared processed forelimb EMG responses from two animals evoked by stimulation within a region of interest (a square of 36 pixels, each pixel 300 μm²). We compared EMG responses from stimulus parameters for trials at the beginning and the end of an experiment. In one animal, there was no significant
difference in evoked EMG amplitude after 143 intervening stimulus trials (P = 0.1220, paired t-test). The other animal showed an increase in EMG amplitude after 103 trials of stimulation (P = 0.0004, paired t-test), which is explainable by a decrease in anesthetic depth or stimulation-induced plasticity (Figure 10).

The laser powers used (40-600 mW mm\(^{-2}\), 1-35 ms) were within the limits of the maximum permissible exposure to the human cornea (Lund, Stuck, & Edsall, 2006) (for 1 W mm\(^{-2}\), the maximum permissible exposure time specified by IEC 60825 standards is 1ms for lasers with wavelengths 400-700 nm).

**IOS**

Prior to each motor mapping session, we conducted IOS imaging to define the locations of the somatosensory forelimb and hindlimb representations. Following a protocol described previously (Winship & Murphy, 2008), we used piezoceramic bending actuators (Piezo Systems Q220-AY-203YB, Cambridge MA USA) to deliver 1 s trains of 100 Hz vibrations to the forelimb and hindlimb alternately. 15 baseline images were compared to 15 images captured over a 1.5 s period following stimulation, and a custom-written ImageJ (NIH, Bethesda MD USA) plugin was used to calculate the percentage change in reflectance of 635 nm light. A 50 % threshold was then applied and the resulting maps color-coded.
**ICMS**

ICMS was performed using a glass pipette (2-3 MΩ, made on a Narashige P_83 vertical electrode puller) containing a 0.25 mm bare silver wire and filled with 3 M sodium chloride, with fast green (Sigma) added in order to facilitate visualization under the microscope. Five to ten 125 ms trains of stimulation, each with five 240 µs stimuli at maximum intensities of 200 µA, were delivered at a frequency of 40 Hz to a depth of 400-500 µm to target layer 5 motor neurons. Impalement sites were guided by somatosensory IOS maps, and spacing between sites was approximately 500 µm. EMG latencies for ICMS and LBM were calculated by measuring from stimulus onset to the point where a pre-defined threshold was exceeded (three times the standard deviation of the baseline noise).

**Motor output recordings**

Hindlimb EMGs were recorded from the biceps femoris and the vastus lateralis muscles using electrodes similar to those described by others (Pearson, Acharya, & Fouad, 2005). These were constructed by twisting together a pair of 0.125 mm teflon coated silver wires and stripping the insulation from two non-overlapping contacts. The twisted bipolar electrodes were then inserted into the muscle using a 22.5-gauge needle. The forelimb EMG recordings were made from the triceps brachii and the extensor carpi radialis brevis muscles using single 0.125 mm teflon wires bared 2 mm from the end and inserted with a 26-gauge needle. The insulated tips of the wires were then bent over to secure them in place. A common ground for the two forelimb electrodes was inserted into a small incision in the footpad. The larger twisted bipolar
electrodes were used exclusively in the hindlimb because of the small size of the forelimb muscles. Forelimb movements were also quantified using a laser motion sensor (LK-2000, Keyence, Osaka Japan).

**EEG**

To examine stimulation-evoked EEG responses at the cortical surface, we used a razor blade to bare ~1 mm from the tip of 125 μm diameter silver wires. The electrodes were inserted into the agarose near each of the four corners of the craniectomy. We then mapped the entire surface of the exposed cortex, performing 3-5 repetitions per map. The averaged maps recorded by each electrode were then normalised and a mean map incorporating the information from all electrodes was created. The duration of individual EEG depolarisations was measured from stimulus onset to the time point where the trace returned to 85 % of the pre-stimulation baseline. EEGs and EMGs were sampled at 5 kHz. An unpaired t-test was used to compare stimulus duration vs. EEG depolarization duration.

**Effects of glutamate receptor antagonists on EMG maps**

Although light activation may be targeted to a selected region, adjacent areas of cortex could be activated through intra-cortical synaptic interactions. We have addressed this possibility by applying AMPA/NMDA glutamate receptor antagonists to the surface of the cortex at high concentrations that have previously been shown to completely block sensory stimulation induced intrinsic optical signal maps (Figure 8).
These maneuvers would be expected to block intracortical synaptic transmission and potentially the spread of excitation. Despite using these antagonists, we found only modest change in the size area or amplitude of the light activated maps within the first 30-60 min. These experiments were performed as follows. After obtaining a set of baseline maps as described above, we applied CNQX (4.5 mM) and MK801 (300 μM, both in physiological saline solution) to the open craniectomy (with dura intact) and allowed the drugs to incubate for 30 minutes. Motor mapping resumed following this period and lasted for up to two hours after the incubation period. The drugs were reapplied (at the same concentrations) to the cortical surface at intervals of approximately 30 min. To compare EMG amplitudes before and after application of the drugs, we calculated mean amplitudes for a region of 12 pixels (3 × 4) at the center of the motor map (as defined by a two-dimensional Gaussian fit). Paired t-tests were used to compare EMG and EEG amplitudes at time points after drug application (30-60, 61-90 min) to pre-drug EMG and EEG amplitudes. Glutamate antagonists failed to have a significant effect on motor map amplitude (P = 0.1393, n = 14 maps from four mice, paired t-test), or the cortical EEG (P = 0.0595, n = 10, paired t-test) elicited by light stimulation within 30-60 min of application in 4 of 5 animals examined (Figure 8). At later time points (90 min) map amplitude was depressed (P < 0.0001, n = 12 maps from four mice, paired t-test) (possible due to more distant drug action), but map boundaries were in part retained. A total of 5 animals were studied for comparison of EEG and EMG sensitivity to antagonists; one animal was not included since the EMG amplitude decreased by over 80 % within the first 30 min of antagonist application and the EEG was of poor quality compared to the results observed in the other 4 animals. These
observations suggest that light-based motor maps are not necessarily dependent on intracortical synaptic activity. At longer time points (90 min after MK801 and CNQX addition) we did observe an 80% depression of map amplitude. We are currently exploring why the drugs had a delayed effect on amplitude, but presumably this reflects a more distant site of action, perhaps near the layer 5 somata or within the striatum or spinal cord. Nonetheless, despite pharmacological diminution of the maps by a factor of 3 in the amplitude, their general boundaries are still apparent (Figure 8). This result is consistent with light-based mapping directly activating layer 5 output neurons leading to muscle potentials rather than a model where light-based pulses activate intracortical synaptic transmission, which does not necessarily reflect direct connections from motor cortex.

**Characterization of photoactivation area**

We used IOS imaging using 630 nm illumination as described above to assess the spread of laser excitation following photostimulation. We compared 15 baseline images to 2 images collected 200-400 ms after photostimulation (100ms burst of 5 ms pulses delivered at 100 Hz with laser power between 156 and 469 mW mm\(^{-2}\)). We also used an intracortical microelectrode (see ICMS section above) to deliver a 100 ms 100 Hz train of 200 µA of stimulation pulses using the parameters employed for ICMS. To obtain reliable IOS activation using the ICMS electrode we needed to increase the pulse duration to 5 ms. In analysis of 2 animals we found that varying the ICMS pulse duration from 0.5-5 ms did not strongly affect the width of the IOS activation ($r^2 = 0.325$). The
photoactivation profile was estimated from average images of changes in light reflectance and measuring the full width at half maximal amplitude of the response as well as a contour plot analysis of averaged group data (Figure 9).

**Software**

Custom software in Igor Pro (Wavemetrics) running on a standard PC controlled the scanning stage using serial commands, while a National Instruments board (PCI-6036E) triggered the 473 nm laser with a TTL pulse and acquired analog outputs (5 kHz) from EEG, EMG, and the laser motion sensor. The software package includes a graphical user interface that allows the experimenter to modify all parameters of interest (e.g. stimulus duration, number of repetitions, inter-stimulus delay, channels recorded, sampling rate etc.). Within each repetition, stimuli were always delivered in a randomised fashion. Randomisation was achieved by sorting the list of desired stimulation points by a list of random numbers generated by the Igor Pro random number function. At the beginning of each experiment, the number and location of stimulation points were defined with reference to an image of the exposed brain.

**EMG and pixel based motor map analysis**

EMG records were sampled at 5kHz, and band-pass filtered (0.5-500 Hz), full-wave rectified, the mean of the pre-stimulation baseline subtracted, and integrated to give the array of values displayed in pixel-based maps. In order to quantify the size of motor maps and locate their centers, we fitted a two-dimensional Gaussian curve to the pixel-
based maps. Motor and sensory map areas were estimated from contour lines of Gaussian fits at 50% of peak value. The mean areas of the Gaussian-fit cortical representations of the four muscles studied were then determined (n=9 animals), and a one-way ANOVA was performed (P = 0.0006) followed by the Tukey-Kramer multiple comparisons test. The baseline offset of each Gaussian fit (z0) was defined as the mean of the background noise. This value was obtained from trials in which stimulation was targeted over thick bone, where no response should be evoked. In approximately 5% of cases, the maps were of relatively poor quality and could not be fit by the Gaussian function. Maps were excluded from further analysis if any of the following three empirically established criteria were not met: peak amplitude of map is more than five times greater than the standard deviation of the baseline noise; map width is at least 300 μm (typical size of one pixel) in the X and Y dimensions; and calculated map center must be within the area selected for photoactivation and imaging. Poor map quality could generally be attributed to imperfect craniectomies or anesthesia. The X and Y values of the map centers and widths were then averaged across several maps (3-6 per animal), and the means of these values were compared statistically. Before performing one-way ANOVA on the map area values of the different muscles, we tested the variation of the standard deviations and found that it was not significant (P = 0.131, Bartlett test). Because we did not record from all four muscles in some animals and because some maps were excluded, not all comparisons were made using the same number of animals. To generate maps based on laser motion sensor data, a two-sided Gaussian fit was applied to each trace and the peak displacement was plotted for each point of stimulation.
Histology

Brains were fixed for histology by transcardial perfusion with 4% paraformaldehyde and, coronal slices 100µm thick were sectioned by vibratome and examined under epifluorescence as described by Brown et al., (2007). Confocal microscopy image analysis was performed with 16 bit, 20 and 40x magnification at 1.6 and 3.2 µm pixel$^{-1}$. 
Results

Automated mapping of motor cortex using laser light

For automated ChR2-based motor mapping a relatively collimated 473 nm laser targeted through a simple microscope was used (see Methods and Figure 1a). As a check of the beam profile at the level of the brain tissue the beam was directed into the cortical surface of a fixed brain section (Figure 1b). The beam width (measured using a monochrome camera, see Methods) was 170 ± 3.7 μm at the cortical surface, and 640 ± 220 μm at 250 μm of depth (n = 7 measurements, Fig. 1c). All values are reported as mean ± standard deviation. Examination of light intensity at depth indicated that it decreased exponentially with a decay constant of ~450 μm.

For light-based mapping (LBM) the animal moved relative to the laser using a fast scanning stage (13 mm s⁻¹) (Callaway & Katz, 1993). The stage was moved in random order to each of the predefined stimulation locations superimposed on the cortical map (Figure 1a), and a flash of laser light was delivered to each point while electromyogram (EMG) and cortical electroencephalogram (EEG) were collected. The intensity and duration of photostimulation were selected based on their ability to elicit a suprathreshold EMG response (see Methods).
Automated LBM of the mouse cortex. (a) Experimental setup. Anesthetised mice were placed on a scanning stage and an array of cortical points (inset) was stimulated by a 473 nm collimated laser beam directed through a video microscope objective. Motor output was detected by EMG electrodes in forelimb and hindlimb muscles, and by a laser motion sensor fixed to the stage. (b) Photograph of a stimulation laser targeted at a coronal slice of fixed brain tissue embedded in carboxyfluorescein-containing agarose. (c) Intensity profile of the illuminated area as the beam passes through fluorescent agarose above the surface of the brain and 250 µm under the cortical surface (peaks were normalised for comparison). Images used for analysis were acquired using a high-resolution monochromatic camera. Scale bars, 1mm (a), 2mm (b), and 400 µm (c).
Photostimulation elicits homogeneous cortical excitation

After verifying that the stage scanning laser system was accurate in positioning, the ability to evoke local excitation of cortex was tested by placing surface EEG electrodes made of silver wire in the four corners of the craniectomy. Mapping EEG responses over areas of up to $20 \text{ mm}^2$ divided into activation sites of $\sim 0.09 \text{ mm}^2$ (300 $\mu$m spacing) demonstrated that photostimulation excited all regions of the exposed cortex (Figure 2a-c). Homogeneity of cortical excitation ensures that differences in motor maps reflect local motor output circuitry, and not the distribution of ChR2 responsiveness.

Figure 2 ChR2-mediated EEG responses

ChR2-mediated EEG responses can be elicited from all regions of the exposed cortex. (a) Mean EEG responses evoked when the laser stimulated that cortical location from all four electrodes at the cortical surface. EEG amplitudes were normalised to the maximum value (within an electrode), and then the mean values from all four electrodes were averaged. Lighter colours signify a larger response. The linear scale was set to emphasise variations in cortical response. At points of stimulation where the cortical surface was obstructed by blood vessels or bone (coloured red and green respectively),
responses were diminished or absent. Scale bar, 1mm. (b) Raw EEG traces from a single electrode. (c) traces (boxed in b) showing a representative EEG response evoked by stimulation over bone (top) and of exposed cortex (bottom). Optical stimulation began at the point marked by the asterisk. (d) The relative time courses of ChR2-evoked EEG and EMG responses are shown after a single 5 ms pulse of 160 mW mm$^{-2}$ laser light (blue bar). Note the prolonged EEG depolarisation relative to stimulus duration.

In evaluating EEG recordings it was found that photostimulation durations as short as 1-5 ms were able to evoke a response. These brief light flashes produced cortical depolarisations that were significantly longer than the stimulus duration (31.4 ± 5.4 ms, P < 0.0001, n = 15 trials in four animals, t-test, see Figure 2d). It was also found that targeting the laser at the exposed EEG electrode caused a large photoelectric artifact that was different in kinetics from the results of cortical tissue excitation and was restricted to periods when the laser was activated. As expected, ChR2$^{-/-}$ mice showed no response to photostimulation (n = 6), but did show the photoelectric artifact (Figure 3).

Figure 3 ChR2-negative animals
ChR2-negative animals show no response to photostimulation. (a) Stimulation was delivered to an array of points (red crosses), and cortical activity was
recorded by an EEG electrode (at right). (b) Each pixel represents the response evoked when the laser stimulated that cortical location, with lighter colors signifying a larger response. Scale bars in a and b 1 mm. (c) Raw EEG traces. Scale bars 3 mV, 200 ms. (d) selected traces enlarged from c. Note the large stimulation artifact produced when the laser strikes the recording electrode (bottom trace), which has amplitude and time kinetics dissimilar to genuine EEG responses. Scale bars 1 mV, 200 ms.

To confirm the expression of ChR2-YFP protein reported by the developers of the mouse (Arenkiel et al., 2007) and the distributor (Jackson Labs), a histological examination of ChR2-YFP fluorescence in a subset of animals was performed (n = 3, Figure 4). We corroborate the homogeneous distribution of protein throughout the sensory-motor cortex and the restriction to tufted layer 5 neurons as originally reported, and consistent with other Thy-1 promoter driven mouse lines (Arenkiel et al., 2007; Feng et al., 2000). In two animals examined by confocal microscopy we saw no labeling of neuronal cell bodies in more superficial layers (Figure 4).
Figure 4 ChR2 Expression

ChR2 is expressed throughout the sensory-motor cortex in layer 5 cells. Expression of YFP (a,b) and ChR2-YFP fusion protein (c-f) in fixed coronal sections of mouse cortex. Expression was under control of the Thy1 promoter in both cases. (a) Low magnification wide-field fluorescence micrograph of a YFP-H mouse cortex. Medial is to the right and dorsal is to the top. Borders between primary motor cortex (m1), secondary motor cortex (m2), and primary somatosensory cortex (s1) are marked with arrows. Scale bar equals 500 µm, and also applies to c. (b) Higher magnification view of the border between m1 and m2. Apical dendrites of YFP-expressing neurons in layer 5 can be seen ascending to layer 1. Scale bar 250 µm. (c) Low power wide-field fluorescence micrograph from a mouse expressing the ChR2-YFP fusion protein. The areal and laminar expression pattern is similar to that shown in a for YFP expression in YFP-H line mice. (d,e) Maximum intensity projections over 20 µm from coronal slices of 2 mice expressing ChR2- YFP. Apical dendrites of layer 5 pyramidal neurons expressing ChR2-YFP extend into layer 1. No pyramidal neurons expressing YFP-ChR2 are seen in layer 2/3. Arrow in (d) shows an axon entering the white matter (wm). Scale bar in e equals 200 µm and applies to f. (f) Higher magnification maximum intensity projection of 3 µm through layer 5 from the same
coronal slice illustrated in d. Examples of individual neurons expressing ChR2-YFP are indicated with arrows. Scale bar 100 µm. (g-j) Coronal sections (100 µm thick), anterior to posterior, of mice expressing ChR2-YFP, scale bar 1mm.

**Mapping light evoked muscle potentials in ChR2 mice**

By implanting silver EMG electrodes in the triceps brachii (extensor) and extensor carpi radialis brevis muscles of the forelimb and the biceps femoris (flexor) and vastus lateralis (extensor) of the hindlimb, the parameters of LBM necessary to evoke contralateral EMG responses were established. The effect of different power levels (40-600 mW mm$^{-2}$) and stimulus durations (1-35 ms) was assessed, and it was found that these parameters were sufficient to produce a motor response (Figure 2d). Photoactivation areas of 170 µm in diameter were reliably able to evoke a motor cortex EEG response and a delayed EMG response in contralateral forelimb (cFL) and contralateral hindlimb (cHL) muscles. Smaller photoactivation areas were not studied because the arbors of layer 5 neurons are at least 300 µm across, and therefore would not expect any increase in detail with reduced photoactivation areas.

Processed EMG responses were assigned a grayscale value on a linear scale from black (zero) to white (maximum response) to form a pixel-based map, typically created with grids of stimulation points using 300 µm spacing (Figure 5a,b and see Methods). Given some scattering of blue light by tissue (Aravanis et al., 2007), this spatial frequency should efficiently excite the cortex between each of the points and is consistent with photoactivation areas used in previous brain slice and in vivo work (Arenkiel et al., 2007; Aravanis et al., 2007).
Figure 5 High-resolution Motor Maps

High-resolution optically stimulated motor maps. (a, b) Forelimb triceps brachii (a) and hindlimb biceps femoris (b) motor maps created with 320 μm spacing between laser stimulation points (single 15 ms pulses at 160 mW mm$^{-2}$). Each map is the average of three repetitions. Absolute grayscale values are not equivalent for a and b. M, medial; L, lateral; R, rostral; and C, caudal. (c,d) One repetition of raw EMG traces for forelimb (c) and hindlimb (d), with individual traces arranged according to the cortical locations from which they were evoked by photostimulation. (e,f) Boxes in c and d identify expanded forelimb (e) and hindlimb (f) EMG traces with an asterisk indicated onset of the laser stimulation. Responses to optical stimulation of points outside the motor maps (top traces) and inside the motor maps (bottom traces) are shown. Scale bars, 1mm (a,b), 200 ms (c,d) and 20 ms (e,f).

Photostimulation within the center of motor representations yielded muscle excitation after a delay from the photostimulation onset of 10.8 ± 1.0 ms for cFL and 19.4 ± 1.0 ms for cHL EMG (n = 4 mice). Analysis of the relationship between cortical EEG depolarization and evoked EMG signals (Figure 2d) revealed the latency between
cortical excitation and muscle excitation. As expected, optically evoked EMG responses exhibited latencies comparable to those of EMG responses produced by direct electrode based stimulation of motor cortex in mice and other animals (Figure 5e,f) (Rho, Lavoie, & Drew, 1999). In intra-cortical micro-stimulation (ICMS) experiments, we found the latency of ICMS-evoked EMG responses to be 11.1 ± 1.1 ms for cFL and 19.5 ± 0.9 ms for cHL (n = 4 mice), consistent with values from photostimulation. Cortical regions from which LBM evoked larger EMG responses tended to also produce responses with shorter latencies (Figure 6).

Figure 6 Motor Map Response Latency 1
Response latency is inversely related to EMG amplitude. (a) High resolution forelimb motor map. White pixels are maximum EMG response, black is no response. Scale bar 1 mm. (b) Corresponding EMG latency map. Pixel values represent latency of EMG response from stimulus onset. Black pixels represent latencies greater than 40 ms or the absence of any response, white pixels represent latencies of less than 10 ms. Scale bar 1 mm.
In an animal where we performed both ICMS and LBM (Figure 7a), the positions and sizes of motor maps were generally in agreement. In this combined ICMS and LBM experiment we performed 26 penetrations to map the motor cortex, completing the ICMS map in approximately one hour. In the same time, we could map more than 2000 points using LBM.

Figure 7 ICMS and LBM motor maps
ICMS and LBM motor maps obtained from the same ChR2-positive mouse. (a) Points of electrode-based ICMS trains are displayed in blue (forelimb movement) and white (no forelimb movement). Purple contour lines represent the ChR2-derived LBM forelimb motor map created with single 20 ms, 160 mW mm$^{-2}$ laser pulses (90% and 50% of peak response). IOS sensory maps are displayed in yellow for sensory forelimb
sFL and red for sensory hindlimb sHL. Scale bar 1mm. (b,c) Raw EMG (top), full wave rectified response (bottom, solid line) and integrated response (bottom, dashed line) for the ICMS point of stimulation in a by the square (b) and the oval (c). Electrode symbol indicated stimulus onset.

Given that layer 5 neurons make cortico-spinal projections it is likely that LBM does not require intra-cortical excitatory synaptic activity to stimulate muscles. Application of AMPA and NMDA-type glutamate receptor antagonists (Figure 8) directly to the sensory-motor cortex at concentrations and durations previously shown to block sensory signals (Murphy et al., 2008) suggested that LBM activates corticofugal projections directly, and not antagonist-sensitive circuitous intracortical routes of motor activation.
Figure 8 Effect of NDMA and AMPA block 1

Cortical application of glutamate receptor antagonists have little initial effect on light-evoked EMG and EEG activity. (a) Experimental timeline for antagonist experiments (MK-801 0.3mM and CNQX 4.5mM, applied directly to the intact cortical surface, dura intact). (b) Forelimb motor map before antagonist application. Scale bar, 1 mm. (c) Forelimb motor map 50 min after initial antagonist application. (d) Forelimb motor map 75 min after initial antagonist application. Motor map amplitude, indicated by the gray scale with scale bar expressed in mV.s on a linear scale. (e) EMG (black bars) and EEG (red bars) amplitudes normalised to pre-antagonist values (error bars SEM, n= 4 animals). Group data indicates that cortical EEG responses and light-evoked muscle potentials are relatively resistant to blockade of excitatory transmission in the cortex consistent with EMG maps reflecting direct activation of cortical spinal neurons and not indirect intracortical circuits. (f) Motor map in d with scale increased 2.5 times to highlight area of map rather than EMG amplitude.
To estimate the area of cortex activated by light pulses we examined intrinsic optical signals (IOS) in response to 100 ms trains of light pulses and found them to spread over 1012 ± 316 µm (n = 4 mice, measured at full width at half maximal amplitude, see Figure 9 and Methods) consistent with the extent of light scattering observed at -250 µm (Figure 1c). In comparison, ICMS electrode activation widths of 690 ± 102 µm were observed (n = 3 mice), indicating that LBM activates an area only moderately larger than ICMS. Although one needs to consider that the map areas cannot be compared in a linear manner. The IOS response area within a contour plot drawn at 75% of the peak laser activation was considerably smaller (0.22 mm² or about 0.5 mm in diameter, Figure 9). These measurements suggest relative differences between ICMS and LBM activation areas. It is possible that the measured spread of the IOS signal is blurred due to out of focus signal emitted from deeper layers of the cortex, or due to brain curvature, making the exact area of activation difficult to determine. In addition, the use of this data to determine exactly what fraction of output neurons are activated with a single light pulse may be complicated by potential non-linearity associated with IOS measurements and uncertainty of where relevant firing thresholds lie.
Figure 9 Estimates of CHR2 and ICMS Area 1

Estimates of CHR2 and ICMS electrode based cortical activation spread using IOS imaging. (a) Image of brain surface with the location of blue laser light stimulation marked by a blue dot. A 100 ms train of 10, 5 ms laser pulses given at 100 Hz was used for optical stimulation. Intracortical microstimulation was performed in approximately the same area using a glass-stimulating electrode (see Supplementary Methods). (b) Image showing change in reflected light signal 200 ms after the onset of a train of blue light pulses. A small reduction in reflected light is observed consistent with
local brain activation. The scale for panel B is between -0.03 to +0.02 %; data is the average of 140 trials. (c) Change in reflected light signal in response to ICMS train stimulation, the average of 60 trials is shown. (d) Plot of change in IOS reflectance measured using a horizontal rectangle 180 μm in height placed across the centre of activation for both channelrhodopsin activation and ICMS. The data plotted is from panels b and c. No light activated changes in brain reflectance were observed in 2 wild type animals examined, or in animals killed by anesthetic overdose. (e) Average laser light-induced IOS response from normalised data (each animal scaled from -1.0 to 0) from n=4 animals using the parameters described above. Contour lines indicate 50, 75, and 90 % of the peak response in this panel and f. (f) Average (ICMS) stimulating electrode induced IOS response from normalised data (each animal scaled from -1.0 to 0) from n= 3 animals using the parameters described above.

Regarding phototoxicity, we observed no consistent decrease in the amplitudes of evoked EEGs or EMGs over the course of an experiment, and no gross histological evidence of damage. In two animals involved in particularly long experiments, EMG amplitude showed no significant reduction after > 100 stimulus repetitions made over the same areas (Figure 10). By making a sealed chronic cranial window and employing a non-invasive laser based measurement device (Ferezou et al., 2007) we found in two preliminary experiments that similar FL movement maps could be evoked in sessions made 7-10 days apart indicating that LBM does not lead to slowly developing toxicity (Figure 11). The laser motion sensor was more sensitive to paw movements than visual assessment and provided data on an absolute scale that agreed with EMG-based maps (Figure 12).
Focal and repeated photostimulation of motor cortex does not lead to degradation of motor map. (a) Image of cortex with region of focal and repeated stimulation displayed in red box. 103 repetitions of a 6 × 6 pattern of photostimulation (300 µm spacing between points) was delivered to the same region of cortex prior to motor mapping (up to 294 mW mm\(^{-1}\) and 50 ms). (b) Forelimb motor map created after focal and repeated photostimulation. There is a robust response in area corresponding to the position of focal stimulation after 100 repetitions (red box). Scale bars, 1 mm. (c) Plot of EMG amplitude over multiple repetitions of photostimulation. EMG amplitudes are taken as the average of multiple repetitions from the area corresponding to the red box in (a) and (b) (error bars are SEM). (d-f) Raw EMG traces corresponding to red box in (a) and (b) taken from the 1st, 50th, and 90th repetition of focal photostimulation. Scale bar 25 ms, 2 mV.
Figure 11 Long-term Motor Mapping 1

Motor maps can be evoked weeks apart within the same animals. Preliminary maps of forelimb movements evoked by ChR2 activation within sensory-motor cortex from two separate animals implanted with chronic cranial windows are shown (measurements made with a laser-based motion sensor). The animal in (a) has been mapped two times (b,c), with at least one week between mapping sessions. The animal shown in panel (d) has been mapped three times, the second (e) and third (f) maps are shown here. The map centers (defined by 2-DGaussian fit) are marked on each map and at the corresponding cortical location (in the top panel). The boundaries of the cranial windows are outlined in red. For all maps, black pixels represent cortical locations from which no movements were evoked, and white pixels represent the location of maximal response. Maps b and c are on the same scale, with black representing no evoked movement and white representing a limb displacement of 2.2 mm. Map e is similarly scaled from 0-4.6 mm, and f is scaled from 0-0.43 mm. Scale bar 1mm. Note that limb displacement is strongly dependent on anesthetic state and is highly variable.
Figure 12  Stimulation-evoked movements 1

Stimulation-evoked movements detected by EMG and laser motion sensor. (a) EMG-based motor map of the extensor carpi radialis muscle. Pixel values signify EMG amplitude, with white corresponding to peak response and black to the absence of any response. (b) Forelimb movement map from the same animal obtained using a laser motion sensor. Pixel values correspond to the displacement of the forelimb, with values ranging from 15 mm (white pixels) to no movement. Scale bars 1 mm. (c) EMG trace corresponding to pixel circled in a. Scale bars 100 mV, 100 ms. (d) Laser motion sensor trace corresponding to pixel circled in b. Scale bar 0.5 mm, 100 ms.
Fine motor map structure

Repeated LBM maps from the same animal indicated that spatial heterogeneity in EMG amplitude was not due to noise or poor sampling, but reflected the underlying properties of the motor representations (Figure 13).

Figure 13 Motor maps are stable 1
Motor maps are stable and repeatable. (a, b) Four consecutive replicates (numbered) of forelimb (a) and hindlimb (b) EMGs in response to laser stimulation using 10 ms, 160 mW mm$^{-2}$ blue-light pulses. The resulting four motor maps were generated
in ~100 s per repetition. In each array, individual EMG traces (200 ms long) are plotted according to the stimulation position from which they were evoked. These spatial relationships are preserved in the pixel-based maps of response amplitude. Stimulation was performed with 300 µm spacing between points, and each pixel represents a cortical area of 0.09 mm$^2$. Scale bars, 500 ms (top) and 1 mm (bottom).

To examine limb representations on a finer scale, the maps of two different muscles within a single limb were compared in size and center position by one-way ANOVA (P = 0.0007). Contralateral forelimb extensor muscle maps were similar in size when thresholded at 50% of maximal amplitude (as described in the Methods): carpi radialis brevis and triceps brachii muscles were 1.65 ± 0.61 mm$^2$ (n = 9 mice) and 1.60 ± 0.67 mm$^2$ (n = 7), respectively; P > 0.05. The same was true for the hindlimb biceps femoris flexor and vastus lateralis extensor (0.71 ± 0.30 mm$^2$, n = 5 and 0.61 ± 0.28 mm$^2$ n = 7, P > 0.05). Both cFL maps were significantly larger than either of the cHL maps (P < 0.05) which is consistent with epidural array-based mapping studies from rat (Hosp et al., 2008). Similar to map area, differences in motor map position were significant only when comparisons were made between cFL and cHL, and not between muscles within the same limb. Forelimb muscle representations had a mean center point that was separated from the center of the combined hindlimb map by an average distance of 0.46 ± 0.25 mm (P = 0.0005, n = 9 animals, t-test). The map analysis suggests that muscles working together to control a body part are represented in very similar regions of motor cortex, whereas muscles in different appendages overlap less. In eight animals map coordinates were defined with reference to bregma (Table 1).
<table>
<thead>
<tr>
<th>Muscle</th>
<th>Lateral from Bregma (mm)</th>
<th>Posterior from Bregma (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extensor carpi radialis brevis (FL extensor)</td>
<td>1.84 ± 0.37</td>
<td>0.42 ± 0.97</td>
</tr>
<tr>
<td>Triceps brachii (FL extensor)</td>
<td>1.96 ± 0.29</td>
<td>0.15 ± 0.66</td>
</tr>
<tr>
<td>Biceps femoris (HL flexor)</td>
<td>1.47 ± 0.32</td>
<td>0.55 ± 1.04</td>
</tr>
<tr>
<td>Vastus lateralis (HL extensor)</td>
<td>1.60 ± 0.25</td>
<td>-0.33 ± 0.97</td>
</tr>
</tbody>
</table>

Table 1 Motor Map Coordinates
Coordinates of the center point of cortical motor representations relative to bregma, as defined by two-dimensional Gaussian fitting. n= 8 animals, with 3-6 maps per animal. Note, both HL and FL muscles were not always assayed within the same animals and inter-animal variability can account for some variability in map centers.

The spatial relationships between sensory and motor representations of cFL and cHL were examined (n = 3 mice, Figure 14). Approximately 50% of cFL and cHL motor maps overlapped with sensory cortex (Table 2). Although there is some uncertainty about the motor map edge position (to within 500 µm), the motor and sensory map center positions are expected to be more precise. The distance between the centers of the forelimb motor and sensory maps were 1217 ± 669 µm, while centers of hindlimb motor and sensory maps were 540 ± 454 µm apart.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>% Motor map in sensory territory.</th>
<th>% Motor map in FL sensory territory</th>
<th>% Motor map in HL sensory territory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extensor carpi radialis brevis (FL extensor)</td>
<td>51 ±15</td>
<td>24 ±8</td>
<td>27 ±8</td>
</tr>
<tr>
<td>Triceps brachii (FL extensor)</td>
<td>51 ±12</td>
<td>22 ±10</td>
<td>29 ±4</td>
</tr>
<tr>
<td>Biceps femoris (HL flexor)</td>
<td>55 ±14</td>
<td>0 ±0</td>
<td>55 ±14</td>
</tr>
<tr>
<td>Vastus lateralis (HL extensor)</td>
<td>50 ±12</td>
<td>3 ±5</td>
<td>47 ±11</td>
</tr>
</tbody>
</table>

Table 2 Motor and Sensory Map Overlap
Overlapping sensory and motor representations of forelimb and hindlimb (n=3 mice).
Figure 14 Motor and Sensory Overlap 1

Motor and sensory cortical limb representations. Sensory forelimb (sFL) and sensory hindlimb (sHL) representations were visualised using IOS imaging (thresholded at 50% of maximal response). Contour lines at 50% of peak response are shown for the extensor carpi radialis forelimb muscle (mFL) and the hindlimb biceps femoris (mHL) and vastus lateralis (dark blue) motor maps derived from single 5 ms, 330 mW mm$^{-2}$ laser pulses. Scale bar, 1 mm.
Discussion

Using a software-controlled scanning stage combined with EMG recordings, we can optically map hundreds of sites within a few minutes. Given that electrode impalements require several minutes each, we estimate that laser-based mapping (LBM) is 2 orders of magnitude faster than electrode-based techniques. We anticipate that such an approach will be useful for determining changes in motor map structure before and after stroke or spinal cord injury (Raineteau & Schwab, 2001; Brown et al., 2009; Winship & Murphy, 2008).

Comparison to other motor mapping techniques

Although we have performed mostly acute experiments, LBM is ideally suited to longitudinal experiments and can be performed multiple times on the same animal through a chronic craniectomy (Trachtenberg et al., 2002) or a thinned skull preparation (Xu et al. 2007). Interestingly, we did observe some activation through thinned bone at the edge of the craniectomy (Figure 11). Repeated ICMS (on the same animal) has been conducted in the past (Kleim et al., 2003; Teskey et al. 2002), but the likelihood of damaging the brain makes LBM a better choice for longitudinal studies of reorganization following experimental manipulations.

Other advantages of LBM over penetrating electrodes are related to sampling. With LBM, stimulation points can be arrayed in a perfect grid, ensuring a more uniform sampling of the cortex than is possible with ICMS. We found that the presence of large blood vessels did not completely block the photoactivation of CHR2, and that motor
maps could be obtained even in areas occupied by large vessels, something that would not be possible with ICMS (Figure 2a).

**Characteristics of LBM maps**

LBM appears to detect motor representations selectively since the resulting forelimb and hindlimb motor maps were located medial to sensory maps (Figure 14) in the approximate location expected for the mouse motor cortex (Ferezou et al., 2007; Paxinos & Franklin, 2004) and in agreement with observations from rats (Hosp et al., 2008). Although the size and center of the forelimb and hindlimb motor representations were different, the two territories exhibited considerable spatial overlap. Possibly, motor map overlap between limbs could reflect activation at off-target sites due to light scattering or spatial overlap between axonal or dendritic arbors of forelimb and hindlimb motor cortex. Alternatively, map overlap may be physiologically relevant and would suggest that specificity in motor output is achieved through additional levels of regulation and not just the topographical layout of the motor cortex. Conceivably, LBM could be extended to single neurons to address whether excitation of individual neurons (Herfst & Brecht, 2008) within overlapping map areas can evoke both forelimb and hindlimb muscle excitation, or whether individual neurons are dedicated to specific limbs. Interestingly, we have shown previously that reorganization after a stroke can cause individual somatosensory neurons (normally preferentially activated by signals from a single limb) to process information from multiple limbs, suggesting that single neurons can assume multiple roles (Winship & Murphy, 2008). With regard to sensory maps, LBM shows that the centers of sensory and motor maps are generally ~0.5-1 mm
apart (Figure 14), supporting lower resolution studies using ICMS in rats that identify these areas as a mixed sensory-motor cortex (Hall & Lindholm, 1974; Sievert & Neafsey, 1986).

**Resolution limits of LBM**

The resolution of LBM depends on its ability to activate subsets of cortex despite the scattering of light and the presence of overlapping axons and dendrites from neurons with cells bodies outside of the activation area. Estimation of the cortical area LBM activates is a complex function of light scattering and depth dependent changes in excitability. However, we can define a lower limit based on the size of the hindlimb motor map we observe (~0.65 mm$^2$, or 0.9 mm in diameter). We have estimated the area of cortex activated by LBM pulses using IOS imaging. The area showing > 50 % of maximal activation was approximately 0.8 mm$^2$ (measured at full width at half maximal amplitude), about the size of the hindlimb motor map. Interestingly, IOS activation profiles of point-source ICMS electrodes were also relatively large, indicating that improvements in light delivery may not lead to large gains in resolution. Given that excitation of motor neurons would not be linearly related to IOS changes and that the method does not directly read out activity within output neurons, it is possible that the spatial resolution of LBM is substantially greater than estimated. Despite some uncertainty about map edge position (to within 500 µm), map center positions would be expected to be more precise and should accurately define the location of motor maps and potential changes after experimental manipulations. To improve the resolution of LBM, future work could employ red-shifted variants of ChR2 (Zhang et al., 2008) using
wavelengths of light that are less susceptible to scattering in tissue. Perhaps the largest gain in resolution would be from making a transgenic rat with ChR2 expression driven by the Thy-1 promoter, where the motor cortex would be at least 3 fold larger (Kleim et al., 2003).

**Future applications of LBM**

Most previous motor-mapping studies have been conducted in rats or other larger species, but the expanding range of available transgenic mice makes them an increasingly attractive experimental model. As other strains become available, it will be interesting to conduct motor or intracortical mapping studies using animals that express ChR2 in other cortical layers or groups of neurons. LBM could also be extended to more complex movements using patterned stimulation or multi-site activation (Graziano et al., 2004).

In summary, high-resolution motor maps are generated quickly, reliably, and accurately in mice expressing ChR2 using a stage scanning system and fixed laser. This new method offer investigators a new tool with greatly improved speed and precision to interrogate the motor cortex and address questions about sensory-motor processing both in the normal brain and after training, injury, or disease.
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