Optogenetic regulation of leg movement in midstage chick embryos through peripheral nerve stimulation

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Sharp AA, Fromherz S. Optogenetic regulation of leg movement in midstage chick embryos through peripheral nerve stimulation. J Neurophysiol 106: 2776–2782, 2011. First published August 31, 2011; doi:10.1152/jn.00712.2011.—Numerous disorders that affect proper development, including the structure and function of the nervous system, are associated with altered embryonic movement. Ongoing challenges are to understand in detail how embryonic movement is generated and to understand better the connection between proper movement and normal nervous system function. Controlled manipulation of embryonic limb movement and neuronal activity to assess short- and long-term outcomes can be difficult. Optogenetics is a powerful new approach to modulate neuronal activity in vivo. In this study, we have used an optogenetics approach to activate peripheral motor axons and thus alter leg motility in the embryonic chick. We used electroporation of a transposon-based expression system to produce ChIEF, a channelrhodopsin-2 variant, in the lumbosacral spinal cord of chick embryos. The transposon-based system allows for stable incorporation of transgenes into the genomic DNA of recipient cells. ChIEF protein is detectable within 24 h of electroporation, largely membrane-localized, and found throughout embryonic development in both central and peripheral processes. The optical clarity of thin embryonic tissue allows detailed innervation patterns of ChIEF-containing motor axons to be visualized in the living embryo in ovo, and pulses of blue light delivered to the thigh can elicit stereotyped flexures of the leg when the embryo is at rest. Continuous illumination can disrupt full extension of the leg during spontaneous movements. Therefore, our results establish an optogenetics approach to alter normal peripheral axon function and to probe the role of movement and neuronal activity in sensorimotor development throughout embryogenesis.

channelrhodopsin-2; PiggyBac; transgenic; gain-of-function; electroporation

EPISODEIC EMBRYONIC AND FETAL motility is a prominent feature of normal development. Such movements occur spontaneously without requiring input from the brain and constitute part of an emerging network of sensorimotor connections necessary for proper, coordinated movement. Many environmentally and/or genetically based developmental disorders involve disruption of normal movements during embryonic or fetal development. It is known that proper neuronal activity patterns are required for normal sensorimotor development (e.g., Kastanenka and Landmesser 2010; Menelaou and Svoboda 2009; Oppenheim et al. 2008). Indeed, a growing array of evidence suggests that altered neuronal activity patterns underlie many developmental movement disorders. The chick embryo has a long history of being an excellent model system for studies of sensorimotor development. The ability to modify movement episodes in chick embryos in a controlled manner both acutely and chronically would greatly facilitate our understanding of the connection between embryonic movement and neuronal activity during development.

Recently, optogenetic approaches have allowed for the activation or inhibition with light of specific neuronal populations in the central nervous system (Boyden 2011). In addition, a light-emitting diode (LED) nerve cuff was recently employed to activate motor axons in peripheral nerves of mice expressing channelrhodopsin-2 (ChR2), a light-driven cation channel, resulting in motor unit recruitment more similar to normal muscle activation than is achieved via electrical stimulation of nerves (Llewellyn et al. 2010). Optogenetic approaches have been applied to the study of early chick embryonic development, where it has been shown that electroporation of nonintegrating plasmid DNA encoding ChR2 into the embryonic chick spinal cord at stage 16 [embryonic days (E) 2-3] gives rise to transient ChR2 expression in spinal neurons (Li et al. 2005). Brief flashes of light delivered to the E5 embryo in ovo cause waves of excitation in the spinal circuitry and axial movements of the embryo similar to those produced spontaneously at this age (Kastanenka and Landmesser 2010; Li et al. 2005). Patterned stimulation of ChR2 with light on E4 and E5 has revealed the need for properly timed activity in spinal circuitry for normal pathfinding and cell adhesion molecule expression (Kastanenka and Landmesser 2010).

To understand better the role of movement and sensorimotor activity in proper development, we sought to extend the use of optogenetic approaches in chicks to later stages of development, E7–E13, during the time when sensorimotor integration occurs (Sharp and Bekoff 2001). Therefore, we combined a transposon-based expression system using PiggyBac (Lu et al. 2009) together with an improved ChR2 variant, ChIEF (Lin et al. 2009), in the chick to allow stable expression of ChIEF in spinal neurons throughout development. After electroporation of our construct, pPB-ChIEF-Tom, into the lumbosacral region of the spinal cord, it was possible to observe expression in the peripheral nerves as they innervate the leg. We were able to use blue light to activate ChIEF in these nerves and thereby evoke leg movements or disrupt normal spontaneous leg motility. Our data demonstrate the ability to regulate activity noninvasively in peripheral motor axons and to alter embryonic limb movement in ovo as a means to explore the connection between motor activity and nervous system development.
MATERIALS AND METHODS

DNA construct. PB-CAG-dGFP and CAGPBase were a kind gift from Drs. Yanyan Lu and Xiaohong Wang (Lu et al. 2009). Standard methods were used to generate pPB-ChIEF-Tom encoding a variant ChR2-Tomato fusion protein. Briefly, the coding region for ChIEF-Tomato was PCR-amplified using pCAGGS-I-oChIEFTom-Tomato-I-WPRE (a kind gift from the Tsien laboratory; Lin et al. 2009) as the template. Primer 1 (5′-gtatcagcggagacactc-3′) lies upstream of the ChIEF coding region, and primer 2 (5′-eggtatccgacagcttacgac-3′) introduces a BamHI I just downstream of the Tomato open reading frame (ORF). The PCR product was subcloned into the AgeI and BamHI I sites of PB-CAG-dGFP in lieu of the green fluorescent protein (GFP) ORF.

Electroporation. All embryonic procedures were consistent with policies of the Southern Illinois University Institutional Animal Care and Use Committee. Electroporation was conducted using standard procedures (see Lu et al. 2009). Fertile white leghorn chicken eggs were incubated until Hamburger-Hamilton (HH) stage 16–18, 5 ml of albumin was removed, and a 1.5-cm diameter hole was cut in the shell to expose the embryo. The DNA mixture, consisting of pPB-ChIEF-Tom and CAGPBase in a 2:5:1 ratio by mass along with 0.1% Fast Green in PBS, was pressure-injected into the neural tube of the embryo via a pulled micropipette until the length of the spinal cord was filled with solution. Sterile L-15 medium (5 ml) was gently placed over the embryo, and the electrodes (Pt-Ir, 0.25-mm diameter, 2-mm length) were placed on either side of the embryo covering the lumbosacral region. The DNA was delivered to the right side of the spinal cord by seven voltage pulses (30–40 V, 50-ms duration, 250-ms intervals). Embryos typically lie on their left side thus exposing their right leg for direct observation and experimentation. For electroporation of two different DNA constructs into the two opposite sides of the spinal cord, the first side was electroporated as described above. After a waiting period of ≈1.5 h, the procedure was repeated with the second DNA using voltage pulses of reversed polarity to direct the DNA to the opposite side of the cord.

ChIEF observation and imaging. Observations were made using a fluorescence stereomicroscope (Leica MZ10 F) equipped with filter sets optimized for GFP and Texas Red and a metal-halide illuminator. Tomato has a broad excitation range that allowed visualization with both filters, whereas GFP could only be excited with the proper filter set. After observation, eggs could be resealed and returned to the incubator for later observation or experimentation. Images of living embryos were obtained using a color digital camera (QImaging). Beginning on E7, extraembryonic membranes begin to obstruct the ability to observe embryos clearly. Therefore, these membranes were removed before imaging of leg innervation at E7 and later.

For observation and imaging of spinal cross-sections, embryos were eviscerated and immersion-fixed overnight in 4% paraformaldehyde. The lumbosacral spinal column was then removed, cryoprotected, and cryosectioned transversely at 16 µm. Sections were observed and imaged on a Zeiss Axioscope equipped with filters optimized for Alexa Fluor 488 and 546 and a monochrome digital camera (Zeiss AxioCam MRm). Optical sections were obtained using structured illumination (Zeiss ApoTome). Composite images were constructed with Adobe Photoshop software. Blank areas of the composite images (areas lying outside of the original images) were set to black to reduce distraction.

Activation of ChIEF. For activation of ChIEF where limb movements were not analyzed in detail, blue illumination was provided via the normal light path of the stereomicroscope by manually rotating the filter wheel between the green and blue excitation filter sets for the desired length of time. For quantification of limb movements, embryos were illuminated with a red LED (627 nm, 12,000 mcd/°; Super Bright LEDs; http://www.supeerbrightleds.com) to allow for continuous video recording of embryonic movement without activation of ChIEF. A blue LED (470 nm, 5,500 mcd/°; Super Bright LEDs) was positioned 5 mm above the thigh and switched on manually to activate ChIEF.

Kinematic recordings. Embryos were prepared on E9 as previously described (Sharp et al. 1999). Movements were video-recorded directly to a personal computer at 60 frames per second. An in-house routine for MATLAB was used to manually capture joint positions and to calculate joint angles. When joint markers were obscured by the blue LED, segmental lengths obtained from unobstructed video sequences were used to determine joint positions.

RESULTS

ChIEF expression. Previously, it was shown that it is possible to express transiently functional ChR2 in early embryonic chicks (E3–E5; Li et al. 2005). We employed a transposon-based system to obtain long-term expression of ChR2 as previously shown in chicks for other transgenes (Lu et al. 2009). Within 24 h after electroporation of pPB-ChIEF-Tom into the lumbosacral spinal cord on E2–E3, ChIEF could be visually observed in the living embryo in ovo. This allows rapid screening for embryos that express ChIEF in the lumbosacral spinal cord as seen in Fig. 1A and can allow for early stimulation of neurons in the spinal cord as previously reported (Li et al. 2005).

As the leg becomes innervated, it is possible to visualize ChIEF expression in the axons as they grow toward their peripheral targets. For example, Fig. 1B shows an image from a living E6 embryo encompassing the lower torso and upper leg. ChIEF expression can be clearly seen in the lumbosacral spinal cord as well as the nerves as they extend down the thigh and past the knee. By E9 (e.g., Fig. 1C), elaborate expression patterns can be seen in the axons as they innervate the leg musculature. Small clusters of punctate innervation can be observed in patches of the skin (arrowhead in Fig. 1C). Observation of detailed expression after E6 requires incision and retraction of the extraembryonic membranes as would be done in a typical behavioral experiment at these ages (Sharp et al. 1999). It is important to note that peripheral expression of ChIEF is restricted to axons and possibly Schwann cells and is not expressed in muscle fibers (Fig. 1D). This is due to the lack of progenitor cells for other peripheral tissues next to the neural tube at the time of electroporation. Starting at E11, it is not possible to observe detailed innervation patterns in the living embryo with standard illumination, although diffuse glows can be seen in the areas overlying large nerves.

Examination of spinal cord cross-sections from E6, E9, E11, E13, and E18 embryos revealed extensive expression of ChIEF in neurons and glia throughout one side of the spinal cord (Fig. 2). High-magnification optical sections revealed that ChIEF is localized primarily to the cell membrane (Fig. 2B). The contralateral side of the spinal cord shows expression of ChIEF in projections from the neurons, for which somata are located in the transgenic side of the spinal cord (Fig. 2C). This is not readily apparent in the image in Fig. 2A due to the disparity in the intensity of fluorescence.

The ability to direct transformation to the somata of cells on one side of the cord allows expression in cells on opposite sides of the spinal cord of different constructs (Fig. 2, D–F). We found that waiting 1.5 h between DNA injections was sufficient to prevent electroporation of the first DNA into the second target region. We did not optimize this timing, and it may be possible to use a shorter waiting period.
Innovative Methodology

OPTOGENETIC REGULATION OF EMBRYONIC CHICK MOTILITY

Fig. 1. Channelrhodopsin-2 variant ChIEF expression in ovo in the living embryo. A: overlaid images, white light and fluorescence, of an embryonic day 4 (E4) embryo showing the localization of ChIEF. The bracket indicates the region in the caudal spinal cord where the electroporation electrodes were placed and where ChIEF can be seen. B: photomicrograph showing ChIEF in the spinal cord and axons innervating the leg (bracket) of an E6 embryo. C: ChIEF can be seen in the axons innervating the lateral side of the right leg of an E9 embryo. This is typical resolution for an embryo imaged in ovo after a behavioral experiment where the extraembryonic membranes have been retracted. The arrowhead indicates small punctate axonal terminations in the skin. D: cross-sectional image of the femorotibialis muscle. Notice that ChIEF is only expressed in the axons. A, ankle; E, eye; K, knee; L, leg; S, spinal cord; W, wing. Scale bar = 1 mm (A–C) and 25 μm (D).

Embryonic movement evoked by ChIEF activation. Previous studies have shown that brief pulses of light delivered to E4 and E5 embryos expressing ChR2 in the cervical spinal cord cause axial movements that propagate along the entire length of the embryo and mimic spontaneous waves of activity at this age (Kastanenka and Landmesser 2010; Li et al. 2005) and that bursts of action potentials could be generated in lumbosacral nerve roots of isolated spinal cord and hindlimb preparations with either lumbosacral or cervical expression of ChR2 (Li et al. 2005). We found that back flexures in the lower back could be induced by brief pulses of blue light on E4 and E5 in embryos expressing ChIEF in the lumbosacral spinal cord when presented between bouts of spontaneous movement. Interestingly, 43% (3/7) of E5 embryos displayed propagating waves of contraction that were initiated within 1–2 s of the initial local contraction similar to spontaneous waves of axial movement. ChIEF activation on E6, when innervation of leg muscles has begun (e.g., Fig. 1B), caused contraction of muscles in the thigh that were visualized as bunching of the skin in the thigh in addition to local contraction of back muscles. Examination of evoked movements after E6 required incision and retraction of extraembryonic membranes. Pulses of light presented to embryos on E7 (3/5), E9 (6/10), and E11 (5/5) resulted in flexions of the right leg with no movement in other parts of the embryo. It was not possible to evoke leg movements in E13 or E18 embryos with our methodology. However, light-activated contraction of intercostal muscles was observed in two E18 embryos after evisceration while being dissected for sectioning.

Since embryonic motility has been well-described on E9 (Bradley and Bekoff 1990; Sharp et al. 1999; Sharp and Bekoff 2001), we chose to examine light-evoked movement more carefully at this age. Brief (~1-s) pulses of light centered over the thigh and presented between episodes of spontaneous movement produced flexions of the leg that were very reproducible when presented with a period >0.5 Hz. Extension of the leg was not observed as a result of light stimulation. Evoked movements were largest at the ankle, smaller at the hip and knee, and maintained until the light was turned off (Fig. 3A). However, the joints relaxed back toward resting position during stimulation lasting more than 2–3 s. Relaxation was likely the result of inactivation of ChIEF with prolonged exposure to light (Lin et al. 2009). This is supported by the fact that several minutes of continuous light inhibited responses to subsequent ~1-s pulses after continuous illumination had been terminated (Fig. 3B), but response amplitude recovered after several minutes of darkness (Fig. 3C). The inhibition of light-evoked movement varied between embryos and may represent differences in ChIEF expression levels within the axons or the number of axons expressing ChIEF.

To determine whether light stimulation caused activation of motor axons in the peripheral nerve or neurons in the spinal cord, the stimulating LED was centered over several different locations: the spinal cord, the thigh, and the foot. Only placement of the LED over the thigh resulted in evoked movements. Taken together with the lack of generalized body movements seen in embryos before E7, this supports the conclusion that light-evoked movements result from activation of ChIEF in the peripheral motor axons.

Given the inactivation of ChIEF with prolonged exposure to light, we did not anticipate seeing a change in spontaneously generated leg motility when we exposed embryos to constant illumination. Surprisingly, three of the animals showed a marked decrease in the maximum joint angle achieved during the first spontaneous bout of movement (Fig. 3, D, E, and H). This was most easily observed for the ankle and corresponded to the inability for these embryos to extend their leg beyond resting positions. However, the maximum joint flexion (minimum joint angle) was not altered by continuous illumination. The ability to extend the leg recovered over several minutes under constant illumination (Fig. 3, F and H), and the rate of this recovery corresponded to the sensitivity of evoked behavior to light-pulse length for each animal. A normal range of movement was displayed in the first spontaneous movement bout after cessation of light stimulation (Fig. 3, G and H) indicating that changes in limb extension were the result of ChIEF activation. These results suggest that prolonged ChIEF activation causes a low level of tonic activity in the flexor muscles that is sufficient to counteract extensor activity but is not sufficient to cause flexion of the leg directly. Additionally,
constant illumination did not decrease the excitability of motor axons or flexor muscles to centrally generated activity.

DISCUSSION

The middle stages of embryogenesis in the chick, E7–E13, constitute a critical time period for sensorimotor development, and actions that disrupt normal movement interfere with normal development. For example, it has long been known that disruption of movement with neuromuscular blockade interferes with the normal progression of programmed cell death in motor neurons and causes hyperinnervation of the muscle (see Oppenheim et al. 2008). The purpose of this study was to utilize optogenetic approaches as a noninvasive, temporally and spatially controlled means to probe the role of motor neuron activity during this time period.

Electroporation of plasmid DNA into the spinal cord on E2–E3 has become a fairly routine method for introducing transgenes to study early developmental processes in the chick (Scott et al. 2010). The development of the PiggyBac transposon system by the Wang laboratory (Lu et al. 2009) has overcome the typical limitation of transient gene expression for plasmid electroporation by allowing for the integration of transgenes into the genomic DNA of chick cells. We used the PiggyBac expression cassette to express successfully the ChR2 transgenes into the genomic DNA of chick cells. We used the PiggyBac system allows for stable transgene expression and could likely be used to study the effects of altered gene expression in posthatch animals. Additionally, we demonstrated that it is possible to utilize more than one DNA construct targeted to opposite sides of the spinal cord in the same embryo.

To utilize optogenetic methods in developing chicks, it is extremely desirable to be able to activate light-driven conductances using noninvasive approaches, i.e., illumination from outside of the embryo. Embryonic tissues are very fragile, and although it is possible to perform somewhat invasive procedures acutely, e.g., electromyography, it is unlikely that indwelling fiber-optic stimulation of the spinal cord over the course of days would be tolerated at these early stages. The Landmesser laboratory has shown that embryonic tissues are sufficiently transparent to allow for exogenous light activation and modulation of embryonic movements at E4–E5 (Kastanenka and Landmesser 2010; Li et al. 2005). The use of pPB-ChIEF-Tom has allowed us to demonstrate that embryonic tissues remain sufficiently transparent until E11 to allow for the activation and modulation of leg movements with a simple LED. It is likely that a brighter light source would extend the useful range of this type of procedure to later stages of development.

One of the advantages of using pPB-ChIEF-Tom for in ovo experimentation over the ChR2 construct used by the Landmesser laboratory (Kastanenka and Landmesser 2010; Li et al. 2005) is the Tomato reporter. Tomato is excited by light that is red-shifted relative to the GFP reporter previously used. Since red-shifted light penetrates tissue to a greater depth, it allows for greater ability to visualize expression of ChIEF in the living embryo. Additionally, green light can be used to excite Tomato without exciting ChIEF at the same time. Use of a reporter molecule that is excited by light of even longer wavelength would enhance the depth of visualization, and numerous reporter molecules with various excitation properties are available (Day and Davidson 2009).

In our experiments, we targeted expression of ChIEF to the lumbosacral and low thoracic segments of the chick embryo. We found that light activation of ChIEF in this region on E5
induced local contraction of back muscles that are innervated by transformed motor neurons and sometimes initiated axial movement along the entire length of the embryo. These findings complement and extend prior reports by the Landmesser laboratory that it is possible to activate motor neurons expressing ChR2 in the cervical spinal cord on E5 and thus light-activate axial movements in ovo (Kastanenka and Landmesser 2010; Li et al. 2005). Although they report that bursts of action potentials could be generated in lumbar motor nerves after light stimulation in isolated spinal cord preparations expressing ChR2 either in the cervical or lumbar regions (Li et al. 2005), they do not state if this also holds true for intact embryos expressing ChR2 in the lumbar region.

To activate motor neurons and avoid other ChIEF-expressing cells in the spinal cord selectively, we sought to stimulate motor axons peripherally in the leg. We were able to visually observe good innervation patterns from E6 through E10 in the living embryo and found that this correlated nicely with the...
ability to evoke leg flexures with light stimulation. It is likely that the progressive thickening of tissues begins to block sufficient amounts of light by E11 that it is no longer possible to evoke movement with a single LED. This interpretation is supported by our findings that it was still possible to evoke intercostal contractions on E18 when stimulating thoracic motor neurons in partially dissected preparations. The use of brighter illumination or longer-wavelength multiphoton stimulation may allow for light-evoked movements at later stages. We observed that exposure of the entire thigh on E9 to blue light resulted in highly reproducible flexure of the leg in a given embryo. We did not observe extensions of the leg as a result of light stimulation. This is likely due to the large representation of flexor-innervating motor axons in the lateral thigh. However, it is possible that some extensor activity was evoked but masked by stronger flexor activation. This will have to be resolved with electromyography. Since nerves providing innervation to different muscles traverse somewhat different pathways, this difference could be used to activate different muscle groups selectively. For example, it is unlikely that muscles on the medial side of the leg would be activated by light from the lateral side of the embryo, and this difference could be utilized for differential modulation of muscles in the same leg.

The variability in amplitude of evoked joint flexions that we observed between animals is likely due to differential expression levels in the motor neuron pools for each animal but could also reflect basic biomechanical differences between the animals themselves. However, it is important to stress that the qualitative features of the light-evoked leg movements were the same. For future acute experiments, it would be helpful to use a focused light source that could be limited to a particular nerve or muscle location to limit which motor neuron and muscle groups are being activated.

ChIEF was developed in part to overcome the fact that constant illumination causes an inactivation of ChR2. Under constant illumination, 60–70% of the ChIEF conductance remains available in contrast to only ~25% for ChR2 (Lin et al. 2009). Nonetheless, the decrease in amplitude of evoked movements that we observed after longer pulses of light or constant illumination is more likely due to ChIEF inactivation than some form of neuronal or muscle fatigue. This conclusion is supported by the fact that the amplitude of spontaneous leg flexures is not decreased under constant illumination as would be expected if motor axons or muscle fibers had been rendered unresponsive due to prolonged ChIEF excitation. Given the decrease in the amplitude of evoked movements after prolonged stimulation with light, it was somewhat surprising that constant illumination decreased the amplitude of spontaneous extension of the leg in some embryos. One interpretation of this result is that low levels of tonic firing of at least some motor neurons is caused during constant illumination, and this is able to generate sufficient tension in flexor muscles to counteract spontaneous extensor muscle contraction. Such low level tonic activity of the flexors would not be expected to alter leg flexion as central excitation of motor neurons in the spinal cord should be minimally effected by low rates of back-propagating action potentials.

Although we used constant illumination in this study for the sake of simplicity, it would be desirable to use more physiological patterns of stimulation. For example, bursts of light pulses at 1–2 Hz would mimic the normal spontaneous activity of motor neuron discharge. The ability for ChIEF to follow high-frequency light pulses was part of the motivation for its development (Lin et al. 2009). Physiologically patterned bursts of light can be accomplished by rapidly pulsing current to LEDs or by rapid shuttering of lasers. Such stimulation would likely reduce the inactivation of ChIEF observed in this study and allow for exploration of the patterning of neuronal/muscle activity during neural circuit stabilization.

We have demonstrated that ChIEF combined with a transposon-based approach can be used to evoke and modulate activity of motor neurons and limb movements in chick embryos through at least E11. In future studies, it should be equally possible to inhibit neuronal activity in peripheral axons using light-driven chloride or proton pumps (Arrenberg et al. 2009; Chow et al. 2010). Importantly, we have shown that tonic activation of motor neurons innervating flexor muscles can disrupt normal extension of the leg. This should in turn alter normal sensory feedback and motor neuron activity in spinal circuitry. Our approach provides a noninvasive probe of the role of movement and neuronal activity during establishment of neural circuitry and may provide insights into developmental movement disorders.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: A.A.S. and S.F., conception and design of research; A.A.S. and S.F., performed experiments; A.A.S. and S.F., analyzed data; A.A.S. and S.F., interpreted results of experiments; A.A.S., prepared figures; A.A.S. and S.F., drafted the manuscript; A.A.S. and S.F., edited and revised the manuscript; A.A.S. and S.F., approved the final version of the manuscript.

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