The shocked Gene is Required for the Function of a Premotor Network in the Zebrafish CNS

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running head: Zebrafish CNS function mutation

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ABSTRACT

The analysis of behavioral mutations in zebrafish can be a powerful strategy for identifying genes that regulate the function and development of neural circuits in the vertebrate CNS. A neurophysiological analysis of the shocked (sho) mutation that affects the initiation of swimming following mechanosensory stimulation was undertaken to identify the function of the sho gene product in the developing motor circuitry. The cutaneous Rohon-Beard (RB) mechanosensory neurons responded normally to stimulation and muscle fibers were unaffected in sho embryos suggesting that the output of the CNS is abnormal. Indeed whole cell patch recordings from mutant muscle cells showed normal spontaneous miniature endplate potentials, but abnormal touch-evoked endplate potentials. Furthermore, motor neuron recordings showed that bursts of rhythmic action potentials from synaptically-dependent depolarizations are initiated in wildtype motor neurons following sensory stimulation or bath application of NMDA. These bursts presumably correspond to bouts of swimming. In sho motor neurons the touch-evoked depolarizations were not sustained resulting in an abbreviated burst of action potentials. The defective responses were not due to any obvious defect in sho motor neurons since their basic properties were normal. These results suggest that in sho embryos there is aberrant motor processing within the CNS and that normal motor processing requires the sho gene product.

Key words: zebrafish, mutation, neural circuit, NMDA, behavior
INTRODUCTION

The genetic analysis of zebrafish has been highly useful for the examinations of a variety of biological issues, most notably in early development and organogenesis (Driever et al. 1996; Haffter et al. 1996). Genetic analysis has also been applied to study various aspects of behavior and behavioral development in zebrafish (Baier 2000; Granato et al. 1996)). In fact, locomotory behaviors such as the escape response and swimming have been well characterized in fish.

The escape response in zebrafish consists of one or more very fast coils immediately following a touch (Eaton and Farley 1975; Saint-Amant and Drapeau 1998). The escape response initially appears at 21 hour postfertilization (hpf) and requires the hindbrain (Saint-Amant and Drapeau, 1998). After 28 hpf embryos respond to touch with a bout of swimming with approximately 50% and 100% of embryos swimming following a tail touch at, respectively, 29 and 36 hpf. As with the early response, swimming following touch requires the hindbrain but not necessarily the more anterior brain regions.

The fact that the early behaviors require only the spinal cord and hindbrain suggests that the neural circuits responsible for these behaviors are localized to these regions of the CNS. Since the early hindbrain and spinal cord contain a relatively small number of neurons many of which are well-characterized (Bernhardt et al. 1990; Eisen et al. 1986; Fetcho and Faber 1988; Kimmel et al. 1982; Kuwada et al. 1990; Mendelson 1985), these neural circuits are likely to be relatively simple ones amenable to neurophysiological analysis. In fact, zebrafish embryos can be analyzed with electrophysiological methods from the moment that neurons become functional (Buss and Drapeau 2000; Ribera and Nusslein-Volhard 1998; Saint-Amant and Drapeau 2000), and using them on fish, including zebrafish, has elucidated many elements of the neural circuits.

The fact that early zebrafish behaviors and their neural circuits are reasonably well characterized and that mutations in these behaviors have been generated suggest that the neurophysiological analysis of these mutants will be useful for understanding the genetic basis for behavior. One such mutation, shocked, was identified by the lack of swimming following touch at 48 hpf (Granato et al. 1996). Normally 48 hpf zebrafish respond to a mechanosensory stimulus with an escape response consisting of a massive contraction of the trunk muscles on one side followed by swimming. sho larvae have normal looking muscles and respond with a tail flip but fail to initiate swimming in response to touch. Here we report that sho embryos are defective in their escape response as well as swimming. Furthermore, using electrophysiological and pharmacological methods we show that motor processing within in CNS is perturbed in sho embryos suggesting that the sho gene product is required within the CNS for normal network function.

MATERIALS AND METHODS

Animals. Zebrafish were bred and raised according to established procedures (Westerfield 1993), which meet the guidelines set forth by the University of Michigan animal care and use protocols. The developmental staging of the embryos was determined by counting somites as described in (Kimmel et al. 1995). The mutant line sho was provided by Dr. Michael Granato at the University of Pennsylvania.
**Behavioral observation and video capturing.** Embryonic and larval behaviors were observed using a Leica stereo dissection microscope. Mechanosensory stimuli were delivered with a thin tungsten wire probe. Videos were captured using a Panasonic wvbp-330 CCD camera and a Scion LG-3 frame grabber (Scion Corp, Frederick, MD) and analyzed with NIH Image on a Mac G4.

**Immunohistochemistry.** Whole-mount antibody labeling was performed as described in Westerfield (1993). The following antibodies and dilution concentrations were used: F59 (1:10) that labels slow-twitch skeletal muscles (Devoto et al. 1996), anti-acetylated α-tubulin (1:10,000) that labels axons by recognizing microtubules (Bernhardt et al. 1990), znp-1 (1:100) that labels primary motor axons (Westerfield 1993), zn-5 (1:500) that labels a subset of neurons including cerebellar neurons, hindbrain commissural neurons, branchiomotor neurons and secondary spinal motor neurons (Chandrasekhar et al. 1997; Chandrasekhar et al. 1999), zn-12 (1:2000) that labels the RB peripheral axons (Trevarrow et al. 1990), 3A10 (1:100) that labels the Mauthner cells and CoPA neurons (Hatta 1992), anti-SV2 (1:100) that labels synaptic vesicles (Feany et al. 1992), and Mab35 (1:100) that labels nAChRs (Tzartos et al. 1981). The antibody staining was visualized using the Vectastain ABC Kit from Vector Laboratories (Burlingame, CA) and 0.15 mg/mL diaminobenzidine to produce a brown peroxidase reaction product. Molecular Probes (Eugene, OR) Alexa fluorescent anti-mouse and anti-rat antibodies were used as secondary antibodies for the double-labeling experiments. Fluorescent images were collected and processed with a Zeiss confocal microscope (Carl Zeiss, Thornwood, NY) and LSM 500 software. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. All of the antibodies were purchased from the Developmental Studies
Hybridoma Bank (Iowa City, IA), except F59, which was provided by Dr. Frank Stockdale at Stanford University, and anti-acetylated α-tubulin from Sigma-Aldrich.

**Calcium Green-1 Imaging.** Muscle cells from 1.5-2 day old embryos were dissociated using a modified trypsin digestion protocol (Westerfield 1993) and cultured for 1 day. Intracellular calcium levels were visualized by adding Calcium Green-1 AM (Molecular Probes) to the bath solution 1-2 hours before the experiment in order to allow sufficient uptake of the dye. The bath solution contained (in mM): 10 HEPES, 145 NaCl, 5 KCl, 1 NaH$_2$PO$_4$, 2 MgSO$_4$, 10 Glucose, and 2 CaCl$_2$. Calcium transients were triggered by ejecting carbachol (1 mM, in bath solution) from a micropipette with its tip positioned approximately 100 µm from the muscle cell. The pressure (10 psi) and duration (100 ms) of the ejection were controlled by a Picospritzer II (Parker Hannifin, Fairfield, NJ). The calcium response was captured with a Scion frame grabber and analyzed with NIH Image on a Mac G4.

**Dissection.** The dissection protocols for *in vivo* patch clamping have been described previously (Buss and Drapeau 2000; Drapeau et al. 1999; Saint-Amant and Drapeau 2003). Briefly, a zebrafish embryo was anaesthetized and paralyzed in recording solution (in mM: 134 NaCl, 2.9 KCl, 2.1 CaCl$_2$, 1.2 MgCl$_2$, 10 glucose, 10 HEPES, 0.0075 d-tubocurarine, 290 mOsm, pH 7.8) that contained tricaine (0.02%). The embryo was immobilized by inserting tungsten wires (25 µm diameter) through the notochord. For muscle recordings, target cells were exposed by peeling off the skin overlying several segments. When recording from neurons, the muscle mass was removed by treating embryos briefly with a dilute solution of collagenase (0.02%) followed by gentle suction through a broken pipette in order to expose the spinal cord.
Electrophysiology and motor neuron recordings. The dissected embryo was under constant perfusion of recording solution (2-5 mL/min). The intracellular solution consisted of (in mM): 116 potassium gluconate, 16 KCl, 2 MgCl$_2$, 10 HEPES, 10 EGTA, 4 Na$_3$ATP, 273mOsm, pH 7.2. In experiments that require the removal K$^+$ conductance, KCl and K-gluconate were replaced with cesium chloride and Cs-gluconate, respectively, in the intracellular solution. A small amount of sulforhodamine (0.2%) was also included in the intracellular solution to facilitate the identification of patched cells. Patch pipettes were pulled from borosilicate silicate glass to yield electrodes with resistances of 6-10 MΩ for muscle and 10-30 MΩ for neuron. The pipette junction potential has been measured to be 6.4 mV, and all potentials were corrected for this junction potential. Recordings were made with an Axopatch 200 amplifier (Axon Instruments, Union City, CA), low-pass filtered at 5 kHz and sampled at 10 kHz. Data was collected with Clampex 8.2 software (Axon Instruments) and analyzed with Clampfit 9.0 software (Axon Instruments). Fictive swimming was elicited by a sudden change in light intensity, or by ejecting bath solution from a pipette with a 15-30 µm tip opening. The duration (10-30 ms) and pressure (20-50 psi) of the stimulation were regulated by a Picospritzer.

The primary motor neurons were identified by their position, size, and axonal projections to muscle. The input resistance, R$_i$, was determined by recording the changes in membrane potential in response to incremental current injections. Each stimulation lasted 300-500 ms and was repeated every 5 seconds. For the study of action potential properties, step-wise current injections were made near the threshold, and the spike generated from the smallest current stimulation was used for the analysis. For the analysis of current/voltage relationships, the voltage-dependent currents induced by incremental voltage steps were recorded. In these voltage
clamp recordings of the motor neurons, QX-314 (2 mM) was included in the intracellular solution to block the train of unclamped action potentials that were invariably induced by the supra-threshold depolarizing voltage steps in the absence of QX-314. This also reveals other conductances normally masked by the voltage-dependent sodium currents. Clampfit 9.0 software was used for the analysis of the membrane properties and action potential properties of the motor neurons.

**RB recordings.** Similar to the motor neurons, we were able to obtain whole-cell recordings of the RB neurons. These neurons were identified by their position, size, and axonal projections in the dorso-lateral fasciculus (DLF) of the spinal cord. Action potential properties and membrane properties were studied as those in the motor neurons, with the exception that QX-314 was not included in the intracellular solution for any recording. At supra-threshold voltage steps, only a single action potential was observed at the onset of the steps, instead of the continuous trains of spikes observed in motor neurons.

**Miniature endplate potential analysis.** Tetrodotoxin (1 mM) was added to recording solution lacking d-tubocurarine. Spontaneous release events were detected automatically using the template search function in Clampfit 9.0. Events with amplitudes lower than 0.5 mV and other erroneous events were excluded after visual inspection.

**Exogenous muscle stimulation in paralyzed animals.** Embryos were prepared as described above for physiology. A higher concentration of d-tubocurarine (15 µM) was used to block any CNS triggered activity. A constant positive pressure was applied to a micropipette containing either
caffeine (6 mM) or KCl (20 mM). The resulting stream was targeted to the exposed musculature to induce contractions, which were videotaped. Contractions were also triggered by exogenous depolarization of single muscle cells under whole cell configuration. Steps of depolarizing current (3-6 nA) at varying frequencies (10-40 Hz) were applied to muscle in the current clamp mode. The resulting contractions were videotaped.

*NMAD induced activity.* Current clamp recordings from muscle cells or neurons were maintained for 25 min. During each recording, NMDA (10-100 µM) was introduced to the recording solution by perfusion at 5 min and continued for 10 min. At 15 min, TTX (1 µM) was introduced to the bath in addition to NMDA until the end of the recording. For each recording, three intervals were analyzed. Interval 1, from 0-5 min, was the pre drug control. Interval 2, 10-15 min, was used to study the effect of NMDA on each cell. Interval 3, 20-25 min, was used to study the TTX-resistant response of the cell to NMDA.

**RESULTS**

*sho embryos are defective in the escape response and swimming*

*sho* mutants, which were originally identified because they failed to initiate swimming following touch at 48 hpf (Granato et al. 1996), are also deficient in the escape response. At 24-26 hpf *sho* embryos failed to respond to touch while wildtype embryos responded with an escape response (Fig. 1). Wildtype sibling embryos respond to head touch with 1, 2 or 3 vigorous contractions that reorient the embryos in, respectively, 14.6%, 66.7%, and 18.8% of cases (n=48) with 98% of multiple contractions alternating from side to side. Tail touch lead to 1, 2, 3, or 4
contractions that projected the embryos forward in 14.6%, 61%, 19.5% and 4.9% of cases, respectively (n=41) with 100% of multiple contractions alternating from side to side. On the other hand, *sho* embryos fail to respond normally to both head touch and tail touch in all cases (n=13) at 24-26 hpf. At 48 hpf wildtype siblings swim away following touch (n=20) while *sho* embryos respond with a few uncoordinated trunk contractions or not at all (n=13) (Fig. 1).

**Mechanosensory neurons are not obviously aberrant in *sho* embryos**

Defective distribution or morphology of sensory cells could potentially account for the behavioral defects observed in *sho* mutants. However, the distribution and morphology of the trigeminal, RB, and posterior lateral line neurons were normal in *sho* embryos. The distribution of large, Islet-positive RB in the dorsal spinal cord and trigeminal neurons were comparable between mutants (3.2 ± 0.1 RB neurons per hemisegment, n=6 embryos; mean ± standard error) and wildtype sibling (3.2 ± 0.1 RB neurons per hemisegment, n=5 embryos) embryos at 23 hpf as were the peripheral axons of trigeminal, RB, and posterior lateral line neurons labeled with anti-acetylated-α-tubulin and MAb zn-12 at 27 hpf (n=8 for mutant and n=8 for wildtype sibling embryos; Fig. 2). Thus there appears to be no obvious anatomical defects of sensory neurons observable at the light level that can explain the aberrant behavioral responses of the mutant embryos.

Defective sensory processing could potentially account for the behavioral defects. However, several findings argue against this possibility. First, the thresholds for mechanosensory activation of a response at the neuromuscular junction at 48 hpf were comparable for *sho* and wildtype sibling embryos. Stimulation of the tail with a spurt of bath solution required 30 ± 4 psi to induce a consistent electrophysiological response at the neuromuscular junction in *sho*
embryos (n=5) compared with 34± 3 psi in wildtype sibling embryos (n=5). Second, the spike threshold to intracellularly injected current, overshoot of the action potential, maximum slope of the rising phase of the action potentials, and the input resistance of RB neurons were not obviously perturbed by the mutation (Table 1). Thus it appears that that the morphology and several measures of excitability of the mechanosensory neurons are not aberrant in the mutants. We were not able to directly examine sensory transduction by the RB neurons because the mechanosensory stimulation caused too much disturbance for effective recordings from RB neurons, which typically have peripheral axons that innervate the skin of nearby segments (Metcalf et al. 1990). However, our finding that the threshold for mechanosensory stimulation for responses in muscles at 48 hpf suggests that sensory transduction in RB neurons is not obviously disturbed in sho embryos.

It is possible that sensory input from the posterior lateral line could have contributed to the muscle response. If the lateral line was dysfunctional in sho embryos, then it could account for the aberrant muscle response to sensory stimulation in our experiments. This possibility was not likely since our procedure of pinning the embryos and removing the skin for muscle recordings likely destroyed the posterior lateral line nerve. We confirmed that this was the case by processing sho embryos that had been recorded from with anti-acetylated-α-tubulin to label the posterior lateral line nerve. In all cases (n=19) the posterior lateral line nerve had been disrupted by the pinning and eliminated from segments in which the skin had been removed (not shown). Although we could not prove the posterior lateral line is entirely normal, the abnormal muscle response to sensory stimulation cannot be explained by a possible dysfunction in the lateral line due to its removal in our electrophysiological protocols.

**Muscles in sho embryos are normal**
The axial muscles in *sho* embryos were examined to see if they contributed to the observed behavioral defects. First, we confirmed that the organization and structure of the axial muscles were normal when examined with differential interference contrast optics (not shown; (Granato et al. 1996) and that the expression and distribution of muscle myosin was normal in *sho* embryos (not shown). Second, the ability of *sho* muscles to contract was tested pharmacologically and physiologically. Muscles from 48 hpf *sho* embryos (n=5) as in wildtype siblings (n=5) contracted in response to focal application of caffeine (6 mM) from a micropipette (not shown). Since caffeine causes the release of Ca\(^{2+}\) from intracellular stores, the resulting *sho* muscle contractions suggest that the site of defect is not in the Ca\(^{2+}\)-dependent contractile machinery of muscle. The ability of *sho* muscles to contract upon depolarization of their membrane was examined by focal application of 20 mM KCl. *sho* muscles (n=10) as wildtype sibling muscles (n=5) contracted following application of KCl (not shown). Furthermore, *sho* muscles (n=4) as wildtype sibling muscles (n=2) followed repetitive stimulation by direct injection of a pulse of depolarizing current at frequencies up to 35-40 Hz when the muscles reached tetanus. Thus, it appears that the excitation-contraction coupling mechanism involving the sensing of membrane depolarization to Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) is functional in the mutants. To directly examine if Ca\(^{2+}\) is released properly from the SR of mutants, dissociated muscles were loaded with Calcium-Green-1 AM, a Ca\(^{2+}\) indicator dye, and analyzed following application of an acetylcholine agonist, carbachol, *in vitro*. As expected from the normal response of muscles to depolarization, *sho* muscles (n=6) as wildtype sibling muscles (n=5) responded to carbachol with an increase in intracellular Ca\(^{2+}\) (Fig. 3). These results suggest that *sho* muscles respond normally to the neurotransmitter and that the
excitation/contraction mechanism is normal. Thus it appears that muscles in sho mutants are normal.

**Neuromuscular junctions in sho embryos are functional and motor output from the CNS is aberrant**

Since sensory processing and muscle function were not obviously abnormal in sho embryos, outgrowth by motor axons and development of the neuromuscular junction were examined in the mutants. Outgrowth by the presynaptic primary motor axons labeled with diI or MAb zn-1 in sho embryos (n=10) was normal as was the pattern of neuromuscular junctions (Fig. 4). Simultaneous labeling for both the presynaptic synaptic vesicle antigen, SV-2, and the postsynaptic nicotinic acetylcholine receptors showed that presynaptic and postsynaptic elements were juxtaposed in the axial muscles at the light level and that the pattern of neuromuscular junctions was indistinguishable between mutant (n=10) and wildtype siblings (n=10).

Since our anatomical analysis suggested that the neuromuscular junctions were in place in the mutants, the electrophysiological response of sho muscles to touch was examined in order to analyze the functional state of the neuromuscular junction and the output by the motor neurons innervating the muscles. The axial muscles are composed of a superficial layer of slow twitch muscles with deeper fast twitch muscles (Devoto et al. 1996). Both slow and fast twitch muscles contract during swimming (Buss and Drapeau 2002). Slow twitch muscle recordings from partially curarized 48 hpf wildtype sibling embryos (n=11) showed a long burst (1994 ± 288 ms) of rhythmic depolarizations in response to mechanosensory stimulation of the tail or visual stimulation (light dimming) (Fig. 5). The frequency of the rhythmic depolarizations (28.6 ± 1.0 Hz) corresponded to the frequency of swimming of approximately 33 Hz observed at 48 hpf.
(Buss and Drapeau 2001). In 48 hpf sho embryos (n=11) a much shorter (124 ± 53 ms), irregular depolarization was recorded following mechanosensory or visual stimulation. Thus the slow twitch muscle recordings correlate with the behavioral response at 48 hpf and suggest that there is a defect in the CNS output to the muscles and/or the function of the neuromuscular junction in sho embryos.

To examine the state of the neuromuscular junction, we examined the amplitude and frequency of spontaneous miniature endplate potentials (mepps). Both the amplitude and frequency of mepps were unperturbed in sho embryos (n=12 for sho, n=10 for wildtype sibling) suggesting that, at least, spontaneous release of transmitter by the motor terminals and the electrophysiological response of the postsynaptic muscle membrane at the neuromuscular junction was normal (Fig. 6). Although the state of evoked release to direct motor neuron stimulation is unknown, these results suggest that the function of the postsynaptic portion of the neuromuscular junction is normal and that the output from the CNS is aberrant in sho mutants.

There is a functional defect in the CNS of sho embryos

The nature of the CNS defect in sho embryos was investigated by a combination of anatomical and physiological methods. The overall patterning of neurons and axonal tracts analyzed with anti-acetylated-α-tubulin, MAb zn-5 and MAb 3A10 antibodies that label all or subsets of neurons were normal in sho embryos (not shown). The distribution and development of many spinal and hindbrain neurons such as the Mauthner cell, the command neuron for the escape response, and the CoPA and VeLD spinal interneurons showed no obvious defects.

Electrophysiological analysis of primary motor neurons demonstrated that the response of primary motor neurons to sensory stimulation was aberrant in sho embryos (Fig. 7). Normally a
long burst of rhythmic action potentials initiated from a sustained depolarization is recorded in response to touch in wildtype sibling motor neurons (2360 ± 41 ms, n=6) at 48 hpf. The frequency of action potentials in the bursts (28.5 ± 1.1 Hz) corresponded with the frequency of the swimming of approximately 33 Hz at 48 hpf (Buss and Drapeau 2001). In sho embryos, however, the initial depolarization is not sustained and only a short burst of action potentials is evident following stimulation (217 ± 53 ms, n=5). The aberrant response of motor neurons in the mutants correlates with the abnormal response at the neuromuscular junction, and suggests that a major component of the defect in sho embryos is due to a defect in the CNS.

To see if the aberrant motor responses were due to changes of passive membrane properties and/or voltage-sensitive currents, we examined the intrinsic properties of motor neurons in sho embryos. First, I-V curves were determined for primary motor neurons using voltage clamp. The voltage-sensitive Na\(^+\) currents were blocked with 2 µM QX-314 within the pipette during voltage clamp since the adjacent axonal membrane was not adequately spaced clamped to avoid artifacts due to initiation of action potentials by the axon. Under these conditions the I-V curves were comparable between the primary motor neurons of sho embryos (n=6) and wildtype siblings (n=6) (Fig. 8) suggesting that membrane properties other than the voltage-dependent Na\(^+\) conductance were unaffected by the mutation. Second, the spike threshold to injected current, overshoot of the action potential, and maximum slope of the rising phase of the action potential in motor neurons were unperturbed (Table 1) suggesting that voltage-sensitive Na\(^+\) currents were also normal in sho motor neurons. Third, there was no significant difference in the input resistance of the motor neurons between sho and wildtype sibling embryos. Fourth, a brief depolarization can induce a plateau-like, prolonged afterdischarge that is dependent on Ca\(^{2+}\) when K\(^+\) currents are blocked pharmacologically via
intracellular cesium (Buss et al. 2003). Comparable afterdischarges can be initiated in both sho and wildtype sibling motor neurons following brief depolarizations from a patch electrode filled with a solution in which K$^+$ was replaced by cesium (Fig. 9). These afterdischarges are blocked by extracellular cobalt (1 mM) in both sho (n=4) and wildtype sibling (n=6) motor neurons. This suggests that the Ca$^{2+}$-dependent currents that underlie the afterdischarge are not compromised by the mutation. Fifth, direct hyperpolarization of the motor neuron during fictive swimming should terminate a plateau potential if it is dependent on the intrinsic properties of motor neurons. On the other hand, if the sustained depolarization requires synaptic input, then hyperpolarizing the motor neurons during a fictive swimming episode should not terminate the depolarization. We found that hyperpolarizations did not terminate mechanosensory-induced depolarization in wildtype motor neurons (n=4; Fig. 10) suggesting that the sustained depolarizations during fictive swimming are not due to intrinsic properties of motor neurons. Thus, it appears that the membrane properties of motor neurons are normal and that synaptic input to motor neurons is defective in the mutants.

**NMDA-dependent synaptic drive to motor neurons is abnormal in sho embryos**

The functional defect was further characterized by analyzing the synaptic input to the motor neurons in sho embryos. Since one major deficit is the lack of a sustained depolarization in the mutant motor neurons (see above), the source of the sustained depolarization was examined more closely. Glutamatergic synaptic transmission via NMDA receptors is a good candidate for the generation of sustained depolarizations because they are activated by glutamate only at depolarized membrane potentials and, therefore, could be responsible for sustaining the depolarization. In fact, in the lamprey CNS plateau potentials are NMDA-dependent (Di Prisco
et al. 1997). Indeed APV (50µM), a NMDA receptor inhibitor, blocked the sustained depolarization in wildtype motor neurons normally induced by mechanical stimulation so that only a short burst similar to that seen in sho motor neurons was evoked (Fig. 11; before APV, 1993 ± 494 ms, n=4; following APV, 248 ± 27 ms, n=4). Furthermore, application of NMDA (10-100 µM) to embryos induced wildtype sibling motor neurons to depolarize slowly (25 µM NMDA depolarized motor neurons by 5-13 mV, n=4) and exhibit episodes of plateau-like depolarization, in addition to the slow depolarization, that gave rise to bursts of action potentials. These transient plateau-like depolarizations induced by NMDA could be studied without action potentials in the motor neurons by intracellular application of 2 µM QX-314 from the patch pipette (Fig. 12).

Since there are likely to be NMDA receptors on both the motor neurons and other neurons in the CNS, the effect of APV and NMDA could be a direct one on motor neurons and/or an indirect one via other CNS neurons. To examine this, the response of motor neurons to NMDA (25 µM) was examined in the presence of TTX (1 µM) in order to cause a generalized blockade of sodium-dependent action potentials. This should synaptically isolate the recorded motor neuron and allow one to assay the direct response of individual motor neurons to NMDA. NMDA alone caused wildtype sibling motor neurons (n=4) to slowly depolarize (5-13 mV) and initiate plateau-like potentials. However, NMDA plus TTX eliminated the plateau-like potentials but not the slow depolarization (Fig. 13). This suggests that the slow depolarizations are due to activation of NMDA receptors on the motor neurons, but plateau-like potentials are due to NMDA activation of neurons that synaptically activate the motor neurons. Interestingly, application of NMDA alone to sho embryos (n=4) resulted in the slow depolarization (5-18 mV) of motor neurons with no plateau-like potentials much like the synaptically isolated wildtype
sibling motor neurons in TTX and NMDA. These findings suggest that the response of NMDA receptors, at least on the motor neurons, to direct application of NMDA is normal, but the synaptic drive onto motor neurons from a premotor network that is activated by NMDA is defective in sho embryos.

DISCUSSION

Our analysis of the phenotype of sho mutants establishes that they are defective in early behaviors exhibited by zebrafish embryos: the escape response and touch-induced swimming. Although we have not directly examined sensory transduction by mechanosensory neurons, our analysis suggests that the properties of mechanosensory neurons are not obviously affected by the mutation. Likewise, muscle function appears to be normal. Furthermore, at least some of the presynaptic and postsynaptic components are properly expressed at the neuromuscular junction. Although the status of evoked muscle response to direct stimulation of motor neurons is unknown, analysis of spontaneous mepps suggests that function of the postsynaptic membrane at the neuromuscular junction and spontaneous release of transmitter by the motor terminals appear to be normal in the mutants. However, abnormal recordings from motor neurons following sensory stimulation coupled with the finding that RB neurons appear to be normal suggest that the CNS is functionally compromised in the mutants during swimming and that sho encodes a product required for proper CNS signaling. Since the escape response is also defective, the CNS defect in sho embryos is also responsible for defects in this behavior as well.

The CNS physiology defects exhibited by sho mutants could be due to morphological abnormalities in neurons or to neurophysiological defects within the CNS. Previous analysis of
zebrafish behavioral mutants has identified a number of mutations that exhibit aberrant physiology and morphology in the nervous system. *space cadet, unplugged,* and *diwanka* embryos exhibit defects in the morphology of specific neurons (Lorent et al. 2001; Zeller and Granato 1999; Zhang and Granato 2000) and abnormal behavior (Granato et al. 1996). On the other hand, touch insensitive mutants such as *macho, alligator,* and *steifftier* exhibit aberrant voltage-sensitive Na\(^+\) currents in mechanosensory neurons that result in abnormal behavioral responses (Ribera and Nusslein-Volhard 1998). Mutations have also been identified that affect the function of the neuromuscular junction. One such mutation, *twitch once,* is a loss-of-function mutation of the gene encoding for rapsyn (Ono et al. 2002).

Our behavioral, morphological, and physiological analyses suggest that the *sho* gene encodes a product that is required for proper morphological and/or physiological development within the CNS. Although our analysis was far from comprehensive, so far we have not detected any morphological defects. The overall pattern of neurons and axons within the CNS appears normal and morphological development of early, identifiable neurons such as the RB sensory neurons, CoPA and VeLD spinal interneurons, Mauthner cell, and primary motor neurons appears normal. The apparent normal morphology of several different neurons rules out the possibility that *sho* encodes for a factor necessary for process outgrowth by all neurons. It is still very possible, however, that a morphological abnormality at the ultrastructural level or in the many neurons not yet examined closely could be the basis for the CNS defect.

Several of our results are compatible with a defect in a NMDA-sensitive process within the CNS of *sho* embryos. First, application of APV onto wildtype motor neurons phenocopies the mutation. Second, NMDA initiates fictive swimming activity in wildtype but not *sho* motor neurons. These results also suggest that NMDA is critical for the generation of sustained
depolarization during swimming in zebrafish as it is in other organisms (Brownstone et al. 1994; Di Prisco et al. 1997; Schmidt et al. 1998). In fact several of the key synapses in the Xenopus tadpole swim circuit are known to be glutamatergic (Roberts 2000). In Xenopus the excitation of two classes of excitatory and interneurons in the dorsal spinal cord by RB neurons is primarily mediated by AMPA receptors. These dorsal interneurons in turn activate, via AMPA and NMDA receptors, the interneurons that constitute the spinal pattern generator ((Li et al. 2004; Li et al. 2003). Additionally, the descending interneurons that are thought to provide excitatory synaptic drive for the pattern generator are mediated by NMDA and kainate receptors (Dale and Roberts 1984; Roberts and Alford 1986). Although the swim circuit is not well characterized in zebrafish compared with Xenopus, the MCoD interneurons are active during swimming and likely to be glutamatergic (Higashijima et al. 2003; Ritter et al. 2001) and could potentially be a source of synaptic drive for the swimming pattern generator.

One possibility is that synaptic transmission mediated via NMDA receptors is defective in mutants. sho embryos could be defective in release of glutamate at glutamatergic synapses. However, the fact that exogenous NMDA initiates swimming in wildtype embryos but does not rescue sho embryos suggests that a defect over and above potential defects in glutamate release within the CNS must exist in mutants. Could the defect be one in the NMDA receptor itself? The comparable responses of sho and wildtype motor neurons to direct application of NMDA in the absence of synaptic transmission argue against this point. However, it remains possible that the voltage dependency of NMDA receptors may be defective in mutants.

Another possibility is that the membrane properties of neurons that are activated by NMDA action are aberrant in sho embryos. These could be neurons that directly or indirectly (polysynaptically) respond to NMDA. Analysis of the membrane properties of RB and motor
neurons in mutants suggest that at least some of the passive membrane and voltage-gated properties are normal. However, the membrane properties of interneurons that receive sensory information that are presumptive analogs to the dorsal interneurons in *Xenopus* (Li et al. 2004; Li et al. 2003); (Roberts 2000) and/or those neurons that make up the swimming pattern generator could be defective in *sho* embryos. In lamprey and *Xenopus*, spinal neurons exhibit intrinsic, voltage-dependent oscillations of the membrane potential that act in concert with NMDA mediated activation to mediate swimming (Aiken et al. 2003; Prime et al. 1999; Reith and Sillar 1998; Wallen and Grillner 1987). Motor neurons are thought to be part of the pattern generator in organisms such as *Xenopus*. At present, however, the status of potential intrinsic oscillatory mechanisms in zebrafish and whether they might be compromised in *sho* embryos is unclear. Thus, *sho* is not likely to encode for a ubiquitous membrane property protein or a protein that regulates the synthesis, stability or localization of such a protein found in many neurons including RB neurons, but could encode for one expressed in a subset of CNS neurons that are normally activate during swimming. Furthermore, since the escape response and swimming are aberrant in *sho* embryos, the molecular factor is likely to be common to the neural circuits for both behaviors ((Fetcho and Faber 1988; Grillner 2003; O'Malley et al. 1996)). Given the similarities of how locomotion is regulated from mammals to lamprey, it would not be surprising if the *sho* product played similar roles in the generation of synaptic drive to motor neurons in a wide variety of organisms(Grillner 2003; Kiehn and Butt 2003). Ultimately definitive elucidation of the function of the *sho* product will require the molecular identification of the *sho* gene.
ACKNOWLEDGEMENTS

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Table 1. Membrane properties of the Rohon-Beard and primary motor neurons in sho embryos are comparable to those in wildtype sibling embryos.

<table>
<thead>
<tr>
<th>Rohon-Beard</th>
<th>Wt (n=5, 43-50hpf)</th>
<th>Sho (n=5, 46-52hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_m ) (mV)</td>
<td>-74.4±3.3</td>
<td>-73.6±3.9</td>
</tr>
<tr>
<td>( R_I ) (-100 to -60 mV) (M( \Omega ))</td>
<td>2268±433</td>
<td>2015±512</td>
</tr>
<tr>
<td>( R_I ) (-60 to -20 mV) (M( \Omega ))</td>
<td>190±24</td>
<td>172±21</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-35.7±2.8</td>
<td>-36.0±2.2</td>
</tr>
<tr>
<td>Spike overshoot (mV)</td>
<td>44.8±6.1</td>
<td>49.4±14.0</td>
</tr>
<tr>
<td>Maximum rise slope (mV/ms)</td>
<td>136±35</td>
<td>166±21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Motor neuron</th>
<th>Wt (n=5, 43-48 hpf)</th>
<th>Sho (n=6, 42-49 hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_m ) (mV)</td>
<td>-68.6±2.3</td>
<td>-69.7±2.2</td>
</tr>
<tr>
<td>( R_I ) (-100 to -40 mV) (M( \Omega ))</td>
<td>476±45</td>
<td>433±22</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-41.0±1.9</td>
<td>-39.2±3.2</td>
</tr>
<tr>
<td>Spike overshoot (mV)</td>
<td>-5.7±3.0</td>
<td>-2.5±3.9</td>
</tr>
<tr>
<td>Maximum rise slope (mV/ms)</td>
<td>95±9</td>
<td>106±10</td>
</tr>
</tbody>
</table>

The values are represented as mean ± S.E.M.; none of the values were significantly different between sho and wt embryos (Student’s t-test), with all p-values > 0.35.
FIGURE LEGENDS

**Fig. 1.** The escape response and touch-induced swimming is aberrant in *sho* embryos. Shown are selected frames from a video (time of the frame in ms is shown in the upper left portion of the panels A and C). *A.* 26 hpf wildtype embryos respond to mechanosensory stimulation with two vigorous coils. Each coil is denoted by arrow. Asterisk marks return of the trunk to a straightened position prior to the 2nd coil. *B.* 26 hpf *sho* embryos fail to respond to touch. *C.* 48 hpf wildtype embryos respond to touch with a fast C-bend followed by a short bout of swimming. In each frame two fields are captured 16 ms apart giving rise to two superimposed images per frame. *D.* 48 hpf *sho* embryos respond to touch with several uncoordinated movements (33 and 67 ms, arrows) but no swimming. *E* and *F.* Higher magnification view of frames taken at 33 and 67 ms to illustrate the uncoordinated movements of the mutant. Scale, 1 mm (*A, B, E* and *F*); 3 mm (*C* and *D*).

**Fig. 2.** The distribution and morphology of *sho* RB neuron somata and their peripheral axons are normal. The distribution of dorsally located RB neurons labeled with anti-Islet are similar in *sho* embryos (*B*) and wildtype siblings (*A*) at 23 hpf. The pattern of the peripheral axons of RB neurons labeled by MAb zn-12 in the skin of the wildtype sibling (*C*) and *sho* embryos (*D*) are indistinguishable at 27hpf. Scale, 40 µm.

**Fig. 3.** Release of Ca\(^{2+}\) from the sarcoplasmic reticulum upon stimulation follows a normal time course in *sho* muscles. Cytosolic Ca\(^{2+}\) was measured with the fluorescent calcium indicator dye, Calcium Green-1 AM. The fluorescent images are pseudo-colored (red: high intensity; blue:
low). Both dissociated wildtype sibling \((A, B, E)\) and \(sho (C, D, F)\) muscles responded to a 10 ms pulse of carbachol (arrow) with a transient elevation of their cytosolic \(Ca^{2+}\) levels. \(A\) and \(C\) are before carbachol, and \(B\) and \(D\) after. \(E\) (wildtype) and \(F (sho)\) are graphs of the relative fluorescence intensities of two representative cells.

**Fig. 4.** The distribution of key pre- and post-synaptic elements of the neuromuscular junction is normal in \(sho\) embryos. Panels \(A-D\) are identical views of the same wildtype sibling embryo and \(E-H\) the same \(sho\) embryo. The pattern of nACh receptors on the axial muscle, labeled with MAb 35 (red), is comparable in wildtype \((A)\) and \(sho (E)\) embryos (48 hpf). The distribution of synaptic vesicles, labeled with anti-SV2 (green), in motor terminals is comparable in wildtype \((B)\) and \(sho (F)\) embryos. The arrow denotes labeling of the posterior lateral line axons. The synaptic vesicles and nAChR are juxtaposed (yellow) in both wildtype \((C)\) and \(sho (G)\) embryos. Panels \(C\) and \(G\) are merged images of, respectively, \(A\) and \(B\), and \(E\) and \(F\). Higher magnification view of areas outlined in \(C\) and \(G\) are seen in \(D\) and \(H\), respectively. Scale bar, 50\(\mu m\) for \(A-C\) and \(E-G\); 15 \(\mu m\) for \(D\) and \(H\).

**Fig. 5.** The voltage response of \(sho\) muscles is defective following tactile stimulation at 48 hpf. \(A\). Shortly following a mechanical stimulus (arrowhead), wild type sibling muscle responded with a burst of rhythmic depolarizations that likely represented swimming. \(B\). \(sho\) muscles responded with a short, non-rhythmic depolarization, which correlates with the uncoordinated behavior seen in the mutants.
**Fig. 6.** Spontaneous synaptic transmission is normal in sho embryos (48 hpf).  
*A.* A representative trace of spontaneous mepps recorded in a wildtype muscle.  
*B* and *C.* The frequency distribution of mepps is comparable between wildtype sibling (*B*) and *sho* (*C*) muscles.

**Fig. 7.** The response of *sho* primary motor neurons to tactile stimulation is aberrant at 48 hpf.  
*A.* Upon mechanical stimulation (arrowhead) wildtype sibling motor neurons responded with a sustained, plateau-like depolarization (bracket) from which a burst of action potentials was initiated.  
*B.* In *sho*, the initial depolarization was not sustained, and the burst of action potentials was prematurely terminated.

**Fig. 8.** Membrane properties of wildtype and *sho* primary motor neurons are comparable.  
*A.* Current records from whole cell voltage clamp analyses of wildtype sibling (top) and *sho* motor neurons. For these analyses 2 μM QX-314 was included in the intracellular solution to block voltage-dependent Na\(^+\) currents.  
*B.* I-V curves of the peak amplitudes of the voltage-dependent outward currents in wild type (n=6) and *sho* (n=6) motor neurons recorded with QX-314 in the pipette. Voltage steps in 10 mV increments from a holding potential of –60 mV were applied. There is no significant difference between the amplitude of the outward currents between wildtype and mutant motor neurons. Each data point is represented as mean ± S.E.M.

**Fig. 9.** Motor neuron properties underlying plateau-like, afterdischarges are not aberrant in *sho* embryos.  
*A.* A 2 ms depolarizing current pulse initiates a plateau-like, afterdischarge from which a burst of action potentials arise when K\(^+\) currents are blocked by intracellular cesium
from the patch electrode in a motor neuron of a wildtype sibling (48 hpf). +Co denotes that the afterdischarge is eliminated by the addition of Co$^{2+}$. B. A 2 ms depolarizing pulse initiates a plateau-like, Co$^{2+}$-sensitive afterdischarge in sho embryos (48 hpf) that is indistinguishable from that seen in wildtype siblings.

**Fig. 10.** The sustained depolarizations and action potential bursts appear to be independent of intrinsic motor neuron properties in wildtype embryos. Mechanosensory stimulation (arrowhead) initiates a sustained depolarization and a long burst of action potentials that are not terminated by injection of pulses hyperpolarizing current into a wildtype motor neuron (48 hpf).

**Fig. 11.** Blocking NMDA receptors shortens the touch-evoked depolarization and burst of action potentials in wildtype motor neurons. A. The response of a wildtype motor neuron at 48 hpf to mechanical stimulation (arrowhead), included a sustained depolarization (bracket) and a long burst of action potentials. B. In the same motor neuron, tactile stimulation elicited a shortened depolarization and shortened burst of action potentials when NMDA receptors were blocked with the addition of APV (50 µM).

**Fig. 12.** NMDA-dependent synaptic drive to sho motor neurons is abnormal at 48 hpf. A-C are three segments of a continuous recording from a wildtype sibling motor neuron, and D-E three segments of a continuous recording from a sho motor neuron. Between the panels are time breaks of 5 min. The voltage records show that activity of motor neurons before application of 25 µM NMDA (A, D), during NMDA exposure (B, E), and during exposure to NMDA and 1 µM TTX (C, F). Both wildtype and sho motor neurons responded to NMDA with a slow
depolarization (8 mV), but wildtype motor neurons also showed episodes of long lasting plateau-like potentials (bracket), which was not present in sho. The NMDA initiated plateau-like potentials were eliminated upon addition of TTX that presumably blocked synaptic transmission within the CNS. In these records, action potentials were blocked in the motor neurons at all times by intracellular application of 2 µM QX-314 from the patch pipette.
Figure 1.
Figure 2.
Figure 3.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 11.
Figure 12.