Title: Touch responsiveness in zebrafish requires voltage-gated calcium channel 2.1b

Abbreviated title: fakir is caused by a mutation in CaV2.1b

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The molecular and physiological basis of the touch-unresponsive zebrafish mutant *fakir* has remained elusive. Here we report that the *fakir* phenotype is caused by a missense mutation in the gene encoding voltage-gated calcium channel 2.1b (*CACNA1Ab*). Injection of RNA encoding wild type CaV2.1 restores touch responsiveness in *fakir* mutants, whereas knockdown of *CACNA1Ab* via morpholino oligonucleotides recapitulates the *fakir* mutant phenotype. *Fakir* mutants display normal current-evoked synaptic communication at the neuromuscular junction, but have attenuated touch-evoked activation of motor neurons. NMDA-evoked fictive swimming is not affected by the loss of CaV2.1b, suggesting that this channel is not required for motor pattern generation. These results, coupled with the expression of *CACNA1Ab* by sensory neurons, suggest that CaV2.1b channel activity is necessary for touch-evoked activation of the locomotor network in zebrafish.
Introduction

Sensory-evoked motor behaviors, common to most animals, are typified by running or flight in terrestrial organisms, and swimming in aquatic organisms. Although these motor behaviors are seemingly different, much of the underlying neural circuitry is similar. Stimuli are first perceived by sensory neurons tuned to particular modalities. These sensory neurons relay inputs to interneurons, which then activate motor neurons and induce muscle contractions resulting in locomotion. While much has been learned about the circuitry underlying sensory-evoked motor behaviors in vertebrates through comparative anatomy and in vivo electrophysiology, proportionally less is known about the contribution of specific genes to the formation and function of these circuits. To overcome this deficit within the context of touch-evoked motor behaviors we and others have turned to the model organism zebrafish.

Zebrafish are ideally suited to address these questions as embryos develop externally and possess a relatively simple nervous system amenable to both in vivo electrophysiology (Drapeau et al. 1999) and optical techniques (McLean and Fetcho 2011). In addition, recent advances have rendered the zebrafish genome modifiable via forward and reverse genetic techniques (Lawson and Wolfe 2011). Finally, zebrafish mature quickly, going from a fertilized egg to an embryo capable of responding to tactile stimuli with highly stereotyped motor behaviors within the first days of life (Saint-Amant and Drapeau 1998). These features have fostered the use of zebrafish in large scale mutagenesis screens aimed at identifying mutations which affect touch-evoked motor behaviors (Granato et al. 1996).

Subsequent work with zebrafish mutants isolated from forward genetic screens have identified genes necessary for touch-evoked motor behaviors at the level of skeletal muscle (Hirata et al. 2004;
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Hirata et al. 2007; Hirata et al. 2012; Ono et al. 2002; Schredelseker et al. 2005; Westerfield et al. 1990), premotor elements (Burgess et al. 2009; Cui et al. 2004; Hirata et al. 2005; Low et al. 2010b; McKeown et al. 2012) and sensory neurons (Low et al. 2011; Nakano et al. 2010). Here we report that the touch-unresponsive zebrafish mutant fakir results from a hypomorphic missense mutation in the gene encoding voltage-gated calcium channel 2.1b (CACNA1Ab). Our findings reveal that CACNA1Ab is expressed by sensory neurons, and demonstrate that the locomotor network that underlies swimming in zebrafish is normal in fakir mutants. Collectively these results suggest that normal CaV2.1b channel activity is required within mechanosensitive neurons for touch-evoked activation of the zebrafish locomotor network.
Materials and Methods

Animal care and use

Zebrafish were bred and raised according to approved guidelines set forth by the Animal Experimentation Ethics Committee, University of Montréal, and the Office of Animal Resources, Harvard University. Staging of embryos was performed as described previously (Kimmel et al. 1995).

The fakir allele tm154 (far<sup>tm154</sup>) was identified in a screen for mutations affecting locomotor behaviors (Granato et al. 1996).

Mapping

A mapping family for fakir was established by crossing a fakir carrier with a wild type zebrafish from the WIK genetic background (Zebrafish International Resource Center, Oregon). Offspring from this mapping family were subjected to high resolution mapping using the previously described mapping procedure (Talbot and Schier 1999), and the following SNP primer sets:

SNP-1
Forward: 5’ – GCGCAACTCACTCAGTCATC – 3’
Reverse: 5’ – AAGACGGACAAGCGGCTAC – 3’

SNP-2
Forward: 5’ – TCGCTGTGGAGACTGAGACTT – 3’
Reverse: 5’ – CGACTTGGTCCATGTTTCCT – 3’
Behavioral analysis

Embryos beginning ~17 hours post-fertilization (hpf) were dechorionated with pronase, placed individually into chambers of 24 – well plates, and raised in a water bath at 28.5°C until indicated time points. Spontaneous coiling in embryos was monitored using a custom-made acrylic 100 well dish (3 mm in diameter, and 5 mm deep) over two minutes. Tactile stimuli were applied by striking the tail of embryos with a pair of No.5 forceps up to three times. Zebrafish were scored according to their greatest response to touch at 48 hpf as either wild type (embryos exhibiting greater than five body lengths of touch-evoked swimming), intermediate (embryos that responded to touch, but swam less than five body lengths), or unresponsive (no visible response to touch). Behaviors were captured with PGR Flycap at 30 and 200 Hz using Flea®-2 (FL2-20S4M-C) and Grasshopper™ (GRAS-03K2M-C) cameras, respectively (Point Grey Research, Richmond, BC), mounted to an Olympus dissecting microscope (SZX7). Images were analyzed off-line using ImageJ (http://rsbweb.nih.gov/ij/).

Immunohistochemistry, alpha-bungarotoxin labeling, and in situ hybridization

Labeling was performed at room temperature (22°C) on larvae 48 – 52 hpf that were raised in 200 µM 1-phenyl-2-thiourea beginning at ~20 hpf to prevent pigmentation using the following procedures. For immunohistochemistry, larvae were fixed in 4% paraformaldehyde for 30 min and then washed five times (5X) for 10 min in washing buffer (phosphate buffered saline (PBS) containing 0.1% Triton X-100), and 2X for 10 min in blocking buffer (wash buffer containing 2 mg/ml bovine serum albumin and 5% heat-inactivated sheep serum). Primary antibodies purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) were bound overnight in blocking buffer at the following dilutions: anti-Islet (1:100) which labels sensory neuron cell bodies, 3A10 (1:100) which labels Mauthner cells, and anti-SV2 (1:100) which labels synaptic vesicles within neurites. Thereafter larvae
were washed 5X for 10 min in washing buffer, 2X for 10 min in blocking buffer. Antibody staining was visualized using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) and 0.15 mg/ml diaminobenzidine to produce a brown precipitate according to the manufacture’s guidelines. Peripheral neurites were labeled using anti-acetylated tubulin antibody (1:1000) from Sigma-Aldrich (St. Louis, MO). These embryos were washed 5X for 10 min in washing buffer, 2X for 10 min in blocking buffer, and then incubated for 2 h at room temperature with anti-mouse Alexa-488 secondary antibody. All processed larvae were washed 6X for 10 min with washing buffer and then mounted in 70% glycerol in PBS for imaging.

Nicotinic acetylcholine receptors were labeled using Alexa-594 conjugated alpha-bungarotoxin (Invitrogen, Carlsbad, CA). Fixed embryos were washed 3X for 10 min with PBS, treated with collagenase at 1 mg/ml in PBS for 15 min, and then washed 3X for 10 min with PBS. Embryos were then pre-incubated in binding buffer (2% BSA, 0.5% Triton X-100, 0.1% sodium azide, in PBS) for 30 min, followed by incubation with Alexa-594 conjugated alpha-bungarotoxin at 10 µg/ml in binding buffer for 30 min. Thereafter embryos were washed 3X for 10 min with binding buffer and mounted in 70% glycerol in PBS. Images were captured with a spinning disk confocal (Quorum, Guelph, Ontario) microscope (Olympus BX-51, Center Valley, PA).

*In situ* hybridization was performed using variations of a previously described approach (Westerfield 2000). In brief, a cDNA fragment of *CACNA1Ab* was amplified using the following set of primers, and subcloned into pGEM®-T Vector (Promega, Madison, WI).

**CACNA1Ab:**

Forward 5’ – CCCAGGAGCGAAGCGAAGAACA – 3’

Reverse 5’ – GCTGGAGTCTGTCAGAATGAGACTGC – 3’
1 µg of linearized plasmid was used as template to synthesize DIG-labeled sense and antisense riboprobes. Probe integrity was checked by gel electrophoresis. Embryos were dehydrated with an increasing percentage of methanol in PBS and stored at −20°C for 30 min in methanol. Thereafter embryos were rehydrated by decreasing the percentage of methanol in PBST (PBS containing 0.1% Tween), incubated at 37°C with proteinase K for 15 min to increase the penetration of riboprobes, and fixed again for 30 min at room temperature. Antisense and sense control riboprobes were hybridized for ~16 h at 65°C. Anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science, Indianapolis, IN) was bound at room temperature for 2 h followed by chromogenic detection using a NBT/BCIP solution (Roche Applied Science). The chromogenic reaction was quenched at various time points for both antisense and sense control conditions. Staining for CACNA1Ab was considered specific when corresponding staining was absent in sense control.

**Recording methods**

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Electrophysiological recording from zebrafish (48 – 60 hpf) were obtained from axial skeletal muscle and neurons at room temperature using methods similar to those previously described (Buss and Drapeau 2000; Drapeau et al. 1999). In brief, larvae were anesthetized and dissected in Evans recording solution (in mM): 134 NaCl, 2.9 KCl, 2.1 CaCl2, 1.2 MgCl2, 10 glucose, 10 HEPES, pH 7.5 with NaOH containing 0.02% (w/v) tricaine. The skin of a larva pinned laterally to a 35 mm Sylgard® coated dish was removed with a pair of No.5 forceps. The solution was exchanged throughout the recording session at ~1 ml/min with Evans containing curare at 3 and 15 µM for skeletal muscle and neuron recordings, respectively. To gain access to the spinal cord the bath solution was replaced with recording solution.
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containing 1 mg/ml collagenase Type XI and incubated until the muscle started to separate at the somatic boundaries (~10 min). Thereafter the muscle was peeled away using suction applied to a broken pipette (~50 µm). The internal recording solution contained (in mM): 116 K - gluconate, 16 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, at pH 7.2 with KOH and 0.1% Sulforhodamine B for cell type identification. Borosilicate glass electrodes had resistances of 3 – 4 MΩ and 5 – 8 MΩ for muscle and neuron recordings when filled with internal recording solution, respectively. Paired motor neuron – muscle recordings were performed in the absence of curare, and in the presence of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 40 µM d-2-amino-5-phosphonovaleric acid (APV) to block excitatory inputs into motor neurons. Recordings were made with Axopatch 200B amplifiers (Axon Instruments, Union City, CA) low passed filtered at 1 – 5 kHz, and sampled at 1 – 10 kHz. Tactile stimulation delivered as a jet of water to the tail (20 - 40 psi, 20 ms), and the application of acetylcholine to skeletal muscle (20 psi, 50 - 100 ms) were delivered through borosilicate glass pipettes using a Picospritzer III (Parker Hannifin, Cleveland, OH). Data acquisition was controlled by pClamp 10 software using a Digidata 1440A interface.

Electrophysiological recording from the previously described stable HEK293T cell line (Piedras-Renteria et al. 2001) expressing the β₁c and α₂δ subunits were obtained at room temperature using methods similar to those previously described (Hamill et al. 1981). Flag tagged human CaV2.1 (3 µg) was co-transfected with enhanced Green Fluorescent Protein (eGFP, 1 µg) into cells growing in 60 mm diameter dishes coated with Matrigel™ (BD Biosciences, Mississauga, ON Canada) with Lipofectamine™ 2000 (Invitrogen, Burlington, ON Canada). The following day cells were split, reseeded on broken glass coverslips coated with Matrigel™, and recorded from 4 – 12 h later. Whole-cell recordings were obtained using borosilicate glass electrodes with resistances of 5 – 8 MΩ when filled with internal recording solution (in mM): 145 CsAsp, 10 EGTA, 10 HEPES, 5 NaCl, and 1 MgCl₂,
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at pH 7.4 with CsOH. The external recording solution contained (in mM): 140 NaCl, 2 KCl, 10 CaCl₂, 1
MgCl₂, 10 HEPES, pH 7.5 with NaOH. Recordings were made with an Axopatch – 1D amplifier (Axon
Instruments, Union City, CA) low passed filtered at 5 kHz, and sampled at 10 kHz. Data acquisition was
controlled by pClamp 8 software using a Digidata 1200B interface. The initial data analysis was done
with Clampfit 10, and figures were prepared using Sigma Plot 11.0 and Adobe Illustrator CS2.

Site-directed mutagenesis

The substitution of leucine 356 with a valine was performed through splice by overlap extension
using the primer sets below.

Set 1:
Forward 5′ – AGCTGGCTAGCGTTTAAACTTAAGCTTGTTACCGAGCTCGGATCCATG – 3′
Reverse 5′ – CTCCCCTGACAGCACACCCAGTACTTCAGCATAAAA – 3′

Set 2:
Forward 5′ – TTTTATGCTGAACGTACTGGGTGCTGTCAGGGGAG – 3′
Reverse 5′ – AAAGGGCGAAGACGACAATGAACAGGAAAAGGAGAAACAACAGGCTGAT – 3′

The underlined nucleotides indicate the base pair substitutions used to generate the missense mutation.
PCR product was then digested and subcloned into wild type cDNA using BsmBI. Mutagenesis was
confirmed by DNA sequencing (Institute for Research in Immunology and Cancer Sequencing Core,
University of Montréal) and is referred to using the one-letter code, wherein L356V represents the
substitution of leucine 356 with a valine.
Morpholino, RNA and plasmid injections

A splice blocking morpholino (Gene Tools, Philomath, OR) was raised to cause the retention of intron 7 of CACNA1Ab: 5'- GATAGATCTTACCCTGAGAGAACAC - 3', resulting in a premature stop codon at position E358, which is equivalent to position E362 in the human sequence. Embryos for morpholino injections were of the TLAB genetic background (derived from adults from a TL x AB cross). The CACNA1Ab morpholino, diluted from a stock concentration to 250 µM in 1 nl, was injected into embryos between the one and two – cell stage using a PicoPump (World Precision Instruments). For each injection condition, cDNA was synthesized using Superscript II (Invitrogen, Carlsbad, CA) from total RNA extracted from ten embryos at 48 hpf with TRIzol® (Invitrogen, Carlsbad, CA) following manufactures guidelines. Quantitative PCR of CACNA1Ab transcripts was performed using SYBR green (Qiagen, Valencia, CA), and normalized against an amplicon for zebrafish beta-actin.

For RNA injections, capped RNA encoding wild type human CaV2.1 (CaV2.1 ancestry), human CaV2.1 harboring the L356V substitution (CaV2.1 ancestry), were synthesized using a mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). A 1 – 2 nl solution of RNA diluted to 200 pg/nl in DEPC-H20 containing 0.1% fast green was injected into embryos at the one to four-cell stage using a Picospritzer III (Parker Hannifin, Cleveland, OH). Embryos were then sorted 6 h later for uptake of dye.

Prior to the beginning of an experiment, embryos were dechorionated and staged as described above. Embryos were scored for their behavior at 48 hours, and a chi-square analysis was performed on the results.

Statistical analysis

Data were analyzed using Microsoft Excel. In text and in all figures, data are presented as the average ± SEM. Asterisks (*) in figures denotes a Student t-test p value < 0.05.
Results

Fakir mutants exhibit a progressive loss of touch-evoked motor behaviors

The zebrafish mutant fakir was originally isolated in a screen for mutations affecting touch-evoked motor behaviors (Granato et al. 1996), due to its diminished ability to respond to tactile stimuli on the third day of development (Figure 1). To expand upon the initial characterization of the fakir mutant phenotype, we examined the two additional motor behaviors displayed by zebrafish embryos: spontaneous and touch-evoked coiling.

Spontaneous coiling, the first motor behavior exhibited by zebrafish embryos, is characterized by slow alternating contractions of the trunk and tail towards the head (Figure 1A). Spontaneous coiling in zebrafish is neurogenic in nature (Westerfield et al. 1990), and intrinsic to the spinal cord as they persist following spinalization (Saint-Amant and Drapeau 1998). When compared to wild type siblings, fakir mutants were found to exhibit a similar frequency of spontaneous coiling (Table 1).

At ~21 hpf tactile stimuli delivered along the body axis often evokes 1–3 coils of the trunk and tail (Figure 1B). Touch-evoked coils are faster than spontaneous coils, and require rostral elements of the spinal cord as they are lost following lesions caudal to somite 10 (Downes and Granato 2006; Pietri et al. 2009). When compared to wild type siblings, fakir mutants were also found to display a similar distribution of touch-evoked coils (Table 1). Thus spontaneous and touch-evoked coiling behaviors are normal in fakir mutants.

At 27 hpf, tactile stimuli begin to evoke bouts of swimming capable of propelling embryos at least one body length. Touch-evoked swimming increases such that at 48 hpf embryos are capable of generating swimming bouts that cover multiple body lengths (Figure 1C). In agreement with the initial report of the fakir mutant phenotype (Granato et al. 1996), a proportion of embryos consistent with a
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recessive mutation never produced episodes of swimming ($23.0 \pm 1.1\%$; $p = 0.64$, $X^2$ test, $n = 224$ total embryos from 3 clutches). However, a closer examination of the remaining touch-responsive embryos revealed that the majority of embryos initially responded to tactile stimuli, but thereafter failed to display sustained bouts of swimming. Segregation of the touch-responsive embryos according to their extent of swimming ($0 – 5$ vs. $> 5$ body lengths) revealed a ratio consistent with a 2:1 Mendelian inheritance of the mutant allele within the sibling population ($49.7 \pm 1.0\%$ vs. $27.3 \pm 0.2\%$, $p = 0.73$, $X^2$ test), suggesting that the reduced bouts of swimming represent a heterozygous “intermediate” phenotype, a finding further addressed below. Embryos displaying the intermediate phenotype were excluded from subsequent analysis unless otherwise indicated.

**Elements of the touch-evoked circuit are present in fakir mutants**

In an attempt to understand the ontogeny of the *fakir* phenotype, we first examined whether the *fakir* mutation caused gross disruptions in the morphology and distribution of neurons and proteins known to participate in zebrafish touch-evoked motor behaviors. In zebrafish, sensitivity to touch along the trunk and tail is conferred by Rohon-Beard (RB) neurons (Low et al. 2010a), a population of sensory neurons located within the dorsal spinal cord (Bernhardt et al. 1990). RBs extend peripheral neurites into the overlying skin, and send a central process rostrally and caudally within the dorsal spinal cord. The rostral processes of RBs project ipsilaterally into the hindbrain (Bernhardt et al. 1990), where they lead to the activation of ~90 bilateral pairs of reticulospinal neurons during motor behaviors (Gahtan et al. 2002). Of the ~90 bilateral pairs of reticulospinal neurons activated following touch, the contribution of Mauthner cells in fish are best understood (Korn and Faber 2005). Mauthner cells receive input from mechanosensitive neurons (Zottoli and Faber 1979), and in turn make monosynaptic contacts with motor neurons (Jontes et al. 2000), which are most likely excitatory.
Fakir is caused by a mutation in CaV2.1b (Mongeon et al. 2008). Motor neurons drive activity within axial skeletal muscle via cholinergic neurotransmission (Westerfield et al. 1990), resulting in locomotion. Thus RBs, Mauthner cells and motor neurons represent a minimal three neuron circuit with the potential of translating touch into motion (Figure 2A). When examined, fakir mutants were found to have RBs that extended peripheral neurites into the overlying skin (Figure 2B). In addition, fakir mutants also possessed Mauthner cells whose axons crossed the midline and projected into the contralateral spinal cord (Figure 2B), and motor neurons that extended axons into the axial skeletal musculature (Figure 2B). Finally, labeling of nicotinic acetylcholine receptors (nAchRs) with alpha-bungarotoxin revealed a similar presence of nAchRs in fakir axial skeletal muscle (Figure 2B). These findings indicate that the fakir mutant phenotype cannot be explained by a loss of one or more of these basic elements.

**Fakir mutants lack touch-evoked synaptic drive to skeletal muscle**

As components of the escape circuit were present and appeared normal in fakir mutants, we next examined activity within these elements in vivo, beginning with touch-evoked activation of axial skeletal muscle. In zebrafish, axial skeletal muscle is comprised of a single lateral layer of slow twitch fibers, and multiple medial layers of fast twitch fibers (Devoto et al. 1996), both of which are recruited during swimming (Buss and Drapeau 2000). Whole-cell current clamp recordings from slow twitch fibers in the presence of 3 µM curare, a concentration sufficient to attenuate membrane depolarizations below the level necessary to trigger excitation-contraction coupling, revealed that tactile stimuli routinely evoked a bout of fictive swimming in wild type embryos (Figure 3A; n = 5/5). The frequency of fictive swimming in wild type embryos (36.3 ± 0.9 Hz) was consistent with previous reports of touch-evoked fictive swimming (Buss and Drapeau 2001). In contrast, similar tactile stimuli failed to evoke bouts of fictive swimming in fakir slow twitch fibers (Figure 3A; n = 0/5).
To determine whether the absence of touch-evoked responses in *fakir* axial skeletal muscle was the result of non-functional nAchRs we examined the responsiveness of slow twitch skeletal muscle to applied acetylcholine under whole-cell voltage-clamp. Slow twitch fibers from both wild type and *fakir* mutants responded to applied acetylcholine with pronounced inward currents (Figure 3B; $n = 5$ for each), while neighboring fibers visibly contracted. When compared, the current responses were similar between wild type and *fakir* mutants (wt $26.5 \pm 4.6$ vs. *far* $38.0 \pm 11.8$ pA/pF; $p = 0.4$), indicating that the nAchRs in both were functional and capable of causing muscle contractions when activated.

As *fakir* skeletal muscle was capable of responding to acetylcholine, we next investigated whether the mutant phenotype was caused by a defect in transmitter release at the neuromuscular junction, starting with spontaneous transmitter release. In the presence of tetrodotoxin (Figure 3C), which blocks spiking in zebrafish motor neurons (Cui et al. 2004), spontaneous miniature end-plate currents (mEPCs) were observed in slow twitch axial skeletal muscle from both wild type and *fakir* mutants. A comparison of mEPCs revealed that the overall frequency of mEPCs were indistinguishable between wild type and *fakir* mutants (wt $3.7 \pm 0.2$ vs. *far* $3.0 \pm 0.6$ Hz; $p = 0.3$), however events $> 70$ pA were slightly reduced in *fakir* mutants (Figure 3D). Thus *fakir* mutant motor neurons are capable of releasing acetylcholine spontaneously.

Finally, activity dependent transmitter release at the neuromuscular junction was examined in *fakir* mutants through paired recordings between primary motor neurons and fast twitch axial skeletal muscle. An initial analysis of membrane properties revealed that motor neurons from *fakir* mutants exhibited similar resting membrane potentials when compared to motor neurons from wild type embryos (Table 2). In addition, motor neurons from *fakir* mutants initiated action potentials in response to depolarizing current injections (Figure 3E), with no significant difference in action potential threshold, and amplitudes of over- and undershoot. Likewise activation of motor neurons by
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current injection routinely evoked end-plate currents in both wild type and fakir skeletal muscle (wt 99.2 ± 0.8 vs. far 95.8 ± 4.2 %; p = 0.5). An analysis of the amplitude distribution of evoked end-plate currents failed to uncover a difference between wild type and fakir mutants (Figure 3F). These findings indicate that evoked synaptic communication at the neuromuscular junction is normal in fakir mutants, suggesting that the fakir phenotype is likely caused by a defect upstream of the neuromuscular junction.

**Fakir motor neurons exhibit abbreviated responses**

As motor neurons in fakir mutants spiked and released neurotransmitter, we explored whether they were being activated in response to touch. In zebrafish embryos, a tactile stimulus evokes a bout of bursting in motor neurons (Drapeau et al. 1999), which is characterized by a prolonged train of action potentials atop a depolarized synaptic plateau. Touch-evoked bouts of bursting appear to be synaptically driven, rather than a consequence of intrinsic motor neuron membrane properties, as bouts of bursting in individual motor neurons are not terminated by hyperpolarizing current injections (Cui et al. 2004). We found that tactile stimuli, applied as a 20 psi puff of water to the tail of an embryo, reliably evoked sustained bouts of bursting in wild type motor neurons (Figure 4A; n = 9/10 trials, from 5 wild type fish). In contrast, the same stimulus delivered to fakir embryos failed to evoke sustained bouts of bursting (n = 0/12 trials, from 6 mutants), often generating instead a very brief subthreshold synaptic event (Figure 4B). When expressed as a number of spikes per stimulation fakir mutants were found to exhibit 0.25 ± 0.2 spikes per stimulation at 20 psi (n = 12), compared to 37.1 ± 8.5 spikes per stimulation in wild type embryos (n = 10).

We next examined whether increasing the stimulation amplitude might increase the number of spikes per stimulation in fakir mutants. However, increasing the stimulation amplitude to 30 and 40 psi
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resulted in only a modest increase in the probability of observing action potentials in fakir mutants; 0.33 ± 0.3 and 0.70 ± 0.3 spikes per stimulation, respectively (Figure 4B). These findings indicate that the fakir mutant phenotype arises from attenuated touch-evoked activation of motor neurons.

The fakir phenotype arises from a missense mutation in the gene encoding voltage-gated calcium channel 2.1b

The fakir locus was previously mapped to Chromosome 11 (Geisler et al. 2007). To further pinpoint the fakir locus we performed high resolution mapping from more than 5000 meioses, which uncovered two tightly linked single-nucleotide polymorphic markers (Figure 5A). Assessment of the genes near these markers revealed the presence of a zebrafish paralog for the pore-forming (alpha) subunit of voltage-gated calcium channel 2.1 (CACNA1Ab), classically referred to as the P/Q-type calcium channel (Catterall et al. 2005). Considering that mutations in human CACNA1A have been linked to movement disorders (Pietrobon 2010), CACNA1Ab was identified as a candidate for fakir. Sequence analysis uncovered a thymine to guanine nucleotide substitution in fakir homozygous mutants (Figure 5B), resulting in a leucine to valine missense mutation at amino acid residue 356 (L356V). Examination of all publicly available zebrafish sequences revealed a thymine to be the reported nucleotide at this position. In addition, sequencing of the corresponding region from several wild-type strains of zebrafish uncovered a thymine in each line, suggesting that the thymine to guanine nucleotide substitution was mutagenically induced.

To determine whether the loss of CACNA1Ab was the cause of the fakir mutant phenotype we performed RNA rescue and morpholino mediated knockdown experiments. Injection of RNA encoding wild type human CaV2.1 into three separate clutches of embryos obtained from incrosses of fakir carriers decreased the percentage of embryos exhibiting the homozygous fakir phenotype from 26.0 ±
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2.0% in sham injected controls \((p = 0.97, \chi^2\) test, \(n = 39\) embryos), to 7.3 ± 4.9% in RNA injected embryos \((p = 0.01, \chi^2\) test, \(n = 55\) embryos).

To determine whether knockdown of CaV2.1b would recapitulate the fakir mutant phenotype we injected a splice blocking morpholino predicted to truncate CaV2.1b at amino acid E362 into wild type embryos. RT-PCR analysis of \(CACNA1Ab\) morphants revealed a significant reduction in mature \(CACNA1Ab\) transcripts, and a corresponding increase in unspliced \(CACNA1Ab\) RNA (Figure 5C).

Behaviorally, the injection of the splice blocking morpholino was found to eliminate touch-evoked motor behaviors (Figure 5D). Collectively these results indicate that \(CACNA1Ab\) is the causative gene in fakir.

The L356V substitution alters CaV2.1 channel activity, and exerts a dominant negative effect on zebrafish touch-evoked behaviors

Leucine 356 is located within the sixth transmembrane segment of the first domain (S6 – DI) of CaV2.1 (Figure 6A). Sequence analysis of CaV2.1 from several different species, including invertebrates, found leucine to be conserved at this position in CaV2.1 (Figure 6B), and within the family of voltage-gated calcium channels from zebrafish as a whole (Flicek et al. 2012). The functional consequence of the valine substitution was examined by comparing whole-cell voltage-clamp responses of CaV2.1 harboring the L356V substitution (CaV2.1\textsubscript{L356V}) to responses of wild type CaV2.1 (CaV2.1\textsubscript{WT}) in a stable HEK293T cell line expressing the auxiliary \(\beta_{1c}\) and \(\alpha_2\delta\) subunits (Piedras-Renteria et al. 2001). In response to membrane depolarization, a rapidly activating inward current was observed in both CaV2.1\textsubscript{WT} \((n = 19)\) and CaV2.1\textsubscript{L356V} \((n = 12)\) transfected cells (Figure 6C). Closer examination of the voltage-gated currents from HEK293T cells expressing CaV2.1\textsubscript{L356V} revealed a
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severe reduction in current density, and a ~10 mV depolarization of $V_{\text{max}}$ when compared to CaV2.1$_{\text{WT}}$ (Figure 6D). Thus, the substitution of leucine 356 with a valine impairs CaV2.1 channel activity.

The observation that approximately half of the embryos from fakir carrier incrosses showed a partial reduction in touch responsiveness raised the possibility that embryos with reduced touch responsiveness were heterozygotes. Indeed, genotyping revealed that embryos displaying the intermediate touch-evoked motor phenotype were almost always heterozygotes (Figure 6E), with a lone false positive originating from a wild type embryo displaying reduced swimming. Considering this finding we next examined whether reduced touch responsiveness in fakir heterozygotes was the cause of a dominant negative effect of the L356V mutation. To this end we injected increasing doses of RNA encoding CaV2.1$_{L356V}$ into wild type embryos, wherein we observed a concomitant increase in the percentage of wild type embryos exhibiting the intermediate touch-evoked phenotype (Figure 6F). These findings are consistent with the L356V missense mutation inducing the intermediate phenotype of heterozygotes via a dominant negative effect.

CACNA1Ab is expressed by sensory neurons, and is dispensable for NMDA-evoked fictive swimming

In an effort to identify the zebrafish neurons in which CaV2.1b is required for normal touch-evoked behaviors we examined the expression pattern of CACNA1Ab at 48 hpf. Whole-mount in situ hybridization revealed expression of CACNA1Ab by dorsal spinal cord neurons (Figure 7B). The position and size of the dorsal spinal cord neurons is consistent with the reported location of RB sensory neurons (Bernhardt et al. 1990), and our previous staining of RBs (Figure 2B), and thereby suggests that the fakir phenotype arises from a defect in sensory neurons.

Previous genetic and pharmacological studies have implicated CaV2.1 in a range of cellular activities ranging from neuronal excitability (Pineda et al. 1998), to gene expression (Sutton et al. 1999).
However, the best characterized role for CaV2.1 within neurons is in the conduction of calcium into presynaptic terminals, an event necessary for activity-dependent neurotransmitter release. This contribution has been examined in detail in several naturally occurring CaV2.1 mutations in mice (Fletcher et al. 1996; Mori et al. 2000; Ophoff et al. 1996; Zwingman et al. 2001). Given the expression of CACNA1Ab by sensory neurons in zebrafish, we postulated that normal CaV2.1b channel activity is necessary for tactile stimuli to reach and activate neurons within the locomotor network. If true then NMDA, which has been shown to drive patterned activity of the locomotor network similar to touch (Cui et al. 2004), might induce patterned activity in zebrafish lacking CaV2.1b. To address this point, touch-unresponsive CACNA1Ab morphants were chosen to remove any confounding effect of the L356V allele. From whole-cell current clamp recordings of skeletal muscle, we found that bath application of NMDA induced repetitive bouts of fictive swimming in both wild type and CACNA1Ab morphants (Figure 7C). A closer examination of NMDA-evoked fictive swimming (Figure 7D) revealed that the distribution of episode periods, which reflect the propensity of the locomotor network to activate in response to NMDA, were similar between wild type and CACNA1Ab morphants (Figure 7E). Likewise the distribution of episode durations, or the length of activity within the locomotor network once triggered, were also comparable between wild type and CACNA1Ab morphants (Figure 7F). Finally, the average fictive swimming frequency between CACNA1Ab morphants and controls was indistinguishable (Figure 7G), indicating that the locomotor network that underlies swimming in zebrafish does not require CaV2.1b to generate normal patterns of fictive swimming. Collectively these findings are consistent with a role for CaV2.1b in allowing tactile stimuli to activate the zebrafish locomotor network.
In this study we demonstrate that the zebrafish mutant *fakir* harbors a missense mutation in the gene encoding for CaV2.1b (*CACNA1Ab*). When examined *in vitro* the missense mutation was found to result in a hypomorphic CaV2.1 channel, a finding that suggests that the homozygous *fakir* mutant phenotype is caused by a reduction in CaV2.1b channel activity. Accordingly, injections of RNA encoding wild type CaV2.1 into clutches of *fakir* incrosses reduced the number of fish displaying the homozygous touch-unresponsive phenotype, while the injection of a splice blocking morpholino that truncates CaV2.1b prior to the second domain (E362stop) was found to induce the homozygous touch-unresponsive *fakir* phenotype. Unexpectedly, we also noticed a previously unreported “intermediate” phenotype in genetically identified *fakir* heterozygotes (Granato et al. 1996). As the injection of RNA encoding the mutant CaV2.1 isoform into wild type embryos induced the intermediate phenotype we conclude that the intermediate *fakir* phenotype is most likely the result of a dominant negative effect by the mutant allele, rather than the consequence of haploinsufficiency. Collectively these results indicate that *CACNA1Ab* is the causative gene in *fakir*, and suggest that normal CaV2.1b channel activity is required for proper touch-evoked motor responses in zebrafish.

In attempt to understand the physiological basis of the touch-unresponsive *fakir* phenotype we examined the expression profile of *CACNA1Ab*, and assessed electrophysiological activity within several cells belonging to the touch-evoked escape circuit. Our results indicate that CaV2.1b is not necessary within the locomotor network that underlies motor behaviors in zebrafish, as demonstrated by the ability to generate normal patterns of fictive swimming when the network is exogenously activated by bath application of NMDA. However, touch-evoked activation of these cells was either absent, or severally diminished in homozygous *fakir* mutants. These results, coupled with the
expression of \textit{CACNA1Ab} by sensory neurons, suggest that CaV2.1b channel activity is required specifically in touch-sensitive neurons to trigger the zebrafish locomotor network.

Arguably the best understood cellular role for CaV2.1 is in conduction of calcium into presynaptic terminals during activity-dependent neurotransmitter release (Catterall et al. 2005). This raises the possibility that the \textit{fakir} mutant phenotype might result from reduced synaptic communication at the RB – interneuron synapse; a hypothesis that could account for both the delayed onset of touch-unresponsiveness in homozygous \textit{fakir} mutants, and the presence of an intermediate heterozygous phenotype. In homozygous \textit{fakir} mutants a reduced amount of activity-dependent transmitter released from sensory neurons might be sufficient to activate downstream interneurons early in development when neurons are small, and therefore have high input resistance. However, as the input resistance of zebrafish neurons decreases during development (Saint-Amant and Drapeau 2000), the residual amount of transmitter released by sensory neurons in \textit{fakir} homozygous mutants might become insufficient to activate the downstream interneurons necessary for transient and sustained activity within the zebrafish locomotor network. In a similar fashion, heterozygous RBs possessing a mixed population of wild type and mutant CaV2.1b channels would be expected to release more transmitter than homozygous mutants, but still less than wild type embryos. This amount of transmitter released could be sufficient to trigger transient activity, but insufficient for sustained activity in heterozygous embryos.

Interestingly, a recent pharmacological-mediated investigation into the identity of voltage-gated calcium channels expressed by zebrafish RBs was conducted (Won et al. 2011). This work found that RBs possessed voltage-gated calcium currents attributable to several members of the family, findings that raise questions regarding the apparent inability of other voltage-gated calcium channel family members to compensate for CaV2.1b in \textit{fakir} mutants. One possible explanation for this phenomenon
fakir is caused by a mutation in CaV2.1b

involves the calcium channel “slot” hypothesis (Cao et al. 2004), which postulates that although neurons may express several types of voltage-gated calcium channels, a particular subtype receives preferential inclusion at a fixed number of calcium channel slots. These calcium channel slots are presumably near active zones where calcium enters during activity-dependent transmitter release. Thus the presence of CaV2.1bL356V in fakir heterozygous and homozygous mutants would prevent other voltage-gated calcium channel family members expressed by RBs from compensating for mutated CaV2.1b. This hypothesis is supported by our finding that injection of RNA harboring the hypomorphic L356V mutation was capable of inducing the heterozygous intermediate fakir phenotype in wild type zebrafish. Another possible explanation for the apparent lack of compensation by other calcium channels consistent with the results obtained from the pharmacological investigation (Won et al. 2011) could be the presence of a retention motif which precludes their movement away from the cell body, as these recordings were made from the cell bodies of RBs.

The ability to examine whether zebrafish RBs exhibit a slot preference for CaV2.1b, as well as test whether the amplitude of activity-dependent transmitter release varies within fakir clutches in a predicted fashion (wild type > heterozygote > homozygote), will require the development of a paired recording preparation that facilitates whole-cell voltage clamp recordings from neurons directly postsynaptic to RBs. Development of such a recording preparation would also provide researchers with a genetically amenable in vivo model in which to examine the functional consequence of novel mutations in human CaV2.1, a channel linked to several neurological disorders in humans (Pietrobon 2010).
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Acknowledgements

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*fakir* is caused by a mutation in CaV2.1b

**Figure Legends**

**Figure 1:** *Fakir* mutants exhibit a loss of touch-evoked swimming. (A) Time-lapse images of spontaneous coiling at 20 hours post-fertilization (hpf). Scale bar 1 mm. (B) Time-lapse images of touch-evoked coiling from 26 hpf wild type (wt) and *fakir* (far) mutant embryos. Scale bar 500 µm. (C) Time-lapse images of touch-evoked responses from 48 hpf wild type, intermediate, and *fakir* mutant larvae. Scale bar 1 mm.

**Figure 2:** A minimal touch-evoked escape circuit is morphologically present in *fakir* mutants. (A) Lateral (left) and dorsal (right) schematics of the minimal touch-evoked escape circuit. Hindbrain (HB), Spinal Cord (SC), Peripheral Neurites (PN), Rohon-Beard (RB), Mauthner cell (M cell), Motor Neuron (MN). (B) Immunohistochemical labeling of RB peripheral neurites innervating the skin (acetylated tubulin), RB cell bodies (Islet-1), Mauthner cells (3A10), motor axons (SV2), and nicotinic acetylcholine receptors (Alexa-594 conjugated alpha-bungarotoxin) highlighted by arrows in wild type and *fakir* mutants. All images are lateral views with rostral to the left except for Mauthner cells, wherein (†) denotes a dorsal view with rostral towards the top. Scale bar 50 µm.

**Figure 3:** Synaptic transmission at the neuromuscular junction is present in *fakir* mutants. (A) Touch-evoked synaptic drive to wild type and *fakir* mutant axial skeletal muscle elicited by a water puff to the tail (100 ms; 20 psi), under current-clamp and in the presence of 3 µM curare. Gray bars here and in subsequent figures denote time and duration of stimulus. (B) Responsiveness of wild type and *fakir* mutant slow twitch skeletal muscle held at -60 mV to applied acetylcholine (10 s, 100 µM). (C) Several seconds of miniature end-plate currents (mEPSCs) from wild type and *fakir* mutant slow twitch skeletal
fakir is caused by a mutation in CaV2.1b

muscle held at -60 mV in the presence of tetrodotoxin (1 µM). (D) A cumulative frequency distribution of mEPSCs amplitudes from wild type and fakir mutant slow twitch skeletal muscle. Error bars represent SEM. (E) Paired Caudal Primary (CaP) motor neuron - fast twitch skeletal muscle recordings from wild type and fakir mutants wherein a depolarizing current injection (2 ms) to the cell bodies triggered action potentials in motor neurons, and evoked end-plate currents in wild type and fakir mutant skeletal muscle. (F) Cumulative frequency distribution of evoked end-plate current amplitudes from skeletal muscle.

Figure 4: Tactile stimuli triggers attenuated synaptic bouts in fakir mutant motor neurons. Touch-evoked bursting in motor neurons under current-clamp from a wild type (A) and a fakir mutant (B) caudal primary motor neurons in response to tactile stimuli (50 ms; 20 – 40 psi as indicated).

Figure 5: Fakir mutants harbor a missense mutation in cacna1ab. (A) Location of the tightly linked single nucleotide polymorphic markers (SNP) on chromosome 11 identified by meiotic mapping, and the gene encoding voltage-gated calcium channel 2.1b (cacna1ab). (B) Nucleotide sequence alignment of fakir homozygous mutant DNA highlighting the T > G nucleotide substitution compared to DNA sequences from an Expressed Sequenced Tag (EST), a Bacterial Artificial Chromosome (BAC), from the Tuebingen genetic background (TU), the Tupfel long fin genetic background (TL), the WIK and AB genetic backgrounds, and from twenty-one Whole Genome Sequences (WGS). (C) Target location of the splice blocking morpholinos (MO), and effect on the production of mature RNA and presence of immature RNA. Arrows are representative of the PCR primers used to assess extent of splice blocking. (D) Time-lapse images of touch-evoked motor behaviors from uninjected, control injected (CTL), or splice blocking (SB) morpholino injected embryos.
**fakir** is caused by a mutation in CaV2.1b

Figure 6: The **fakir** missense mutation reduces CaV2.1 channel activity, and exerts a dominant negative effect on touch-evoked motor behaviors. (A) A graphic representation of Cav2.1b indicating the location of the L356V missense mutation within Segment 6 (S6), Domain I (DI) found in **fakir** mutants. (B) Sequence comparison of zebrafish CaV2.1b to CaV2.1 from other organisms highlighting the conservation of amino acids at, and around position 356. (C) Typical currents evoked from HEK293T cell expressing either wild type human CaV2.1 (CaV2.1<sub>WT</sub>), or human CaV2.1 harboring the leucine 356 to valine mutation (CaV2.1<sub>L356V</sub>), following membrane depolarization. (D) Average voltage-current density responses of CaV2.1<sub>L356V</sub> compared to CaV2.1<sub>WT</sub>. (E) Probability of predicting genetic identity from touch-evoked response in three phenotypically distinct groups of at least 30 embryos isolated from three different clutches. (F) Effect of RNA harboring the L356V substitution injected into wild type embryos (n > 30 embryos each, from 3 clutches).

Figure 7: **cacna1ab** is expressed by sensory neurons in zebrafish and is dispensable for NMDA-evoked fictive swimming. (A) Lateral representation of a 48 hpf zebrafish embryo. (B) Enlarged view of spinal cord, displaying anti-sense labeling of **cacna1ab** by RB sensory neurons (arrowheads). Dashed line indicates ventral edge of the spinal cord. Scale bar 100 µm in each. (C) A slow sweep of NMDA-evoked fictive swimming in the presence of 3 µM curare recorded under current clamp. Boxed area highlights enlarged area shown below. (D) A faster sweep of NMDA-evoked fictive swimming from boxed area above. Episode period and episode duration is demarcated by bracketed areas. (E) Cumulative distribution plots of episode period, and (F) episode duration from 2 min of NMDA-evoked fictive swimming (n = 5 fish for each. Average episode period: wt 1473 ± 110 vs. **far** 1433 ± 100 ms; p = 0.80. Average episode duration: wt 707 ± 49 vs. **far** 606 ± 66 ms; p = 0.25). (G) Fictive swimming frequency
*fakir* is caused by a mutation in CaV2.1b

587 (frequency of endplate potentials within an episode) from control and *cacna1ab* morphants.
Table 1: Analysis of spontaneous and touch-evoked coiling in wild type and fakir mutants ($n = 18$ for each, from three clutches)

Table 2: Analysis of motor neuron membrane properties from wild type ($n = 18$) and fakir mutants ($n = 13$).
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References


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Figure 1
Low et al., 2011
Figure 2
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Figure 5
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