Function of Neuromuscular Synapses in the Zebrafish Choline-Acetyltransferase Mutant *bajan*

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Wang M, Wen H, Brehm P. Function of neuromuscular synapses in the zebrafish choline-acetyltransferase mutant *bajan*. J Neurophysiol 100: 1995–2004, 2008. First published August 6, 2008; doi:10.1152/jn.90517.2008. We have identified a zebrafish mutant line, *bajan*, in which compromised motility and fatigue result from a point mutation in the gene coding choline acetyltransferase (ChAT), the enzyme responsible for acetylcholine (ACh) synthesis. Although the mutation predicts loss of ChAT function, *bajan* inexplicably retains low levels of neuromuscular transmission. We exploited this residual activity and determined the consequences for synaptic function. The attenuated synaptic responses were a direct consequence of a decrease in both resting mean quantal size and quantal content. To replicate behavioral fatigue in swimming, motorneurons were stimulated at high frequencies. A prominent reduction in quantal content, reflecting vesicle depletion, was coincident with a small additional reduction in quantal size. In humans, defective ChAT leads to a decrease in both resting mean quantal size and quantal content. To establish the early circuitry (Svoboda et al. 2001). Historically pharmacology has served as the principal approach to altering activity levels. However, the advent of genetic knockout models offered an entirely new means of altering the components underlying membrane excitability. For example, in mice, complete loss of synaptic activity was achieved through elimination of transmitter synthesis. The results revealed compromised synaptic development in the forms of excessive intramuscular nerve branching and widened endplate bands (Brandon et al. 2003; Misgeld et al. 2002), pointing to a direct involvement of acetylcholine (Ach)-mediated activity in neuromuscular development. The complete elimination of the transmitter ACh in these animals, however, prevented any direct assessment of the role of synaptic activity on either pre- or postsynaptic function. Likewise, the complete loss of postsynaptic ACh receptors results in early embryonic lethality in mammals, thus once again precluding functional measurements.

Zebrafish offer a unique opportunity to study the role of synaptic transmission in development of vertebrate synapses through the unexpected longevity of ACh receptor-less mutant lines (Etard et al. 2005; Ono et al. 2001; Westerfield et al. 1990). Studies of these postsynaptic receptor nulls have indicated a largely normal synaptic morphology. Also in the only study of its kind, receptor-less synapses were compared with wild-type synapses for differences in presynaptic function by use of sterol dyes (Li et al. 2003). The in vivo measurements of depolarization-evoked exocytosis from presynaptic nerve terminals indicated no difference between wild-type and receptor-less fish, arguing against the idea that activation of postsynaptic receptors is required for presynaptic differentiation. Further interpretations were limited because, once again, there was no means to record pre and postsynaptic function. In the present study, we report a zebrafish ChAT mutant *bajan*, which represents the first line in which presynaptic cholinergic function is disrupted. Recordings from larval *bajan* fish reveal the presence of weak acetylcholine-mediated synaptic transmission, providing a window into the consequences of greatly reduced transmitter for synapse development and function.

**METHODS**

Brian’s wild-type strain was obtained locally and baj tf247 (Granato et al. 1996) was provided by the Max Planck Institute (Tübingen, Germany). Analysis of touch-induced escape responses utilized a Photon Fastcam high-speed CCD camera (San Diego, CA). Images (512 × 512 pixels) were captured continuously at either 1,000 or 2,000 frame/s over a period of 8 s.

For electrophysiological recording, larval fish were used between the ages of 48 and 96 h postfertilization (hpf). Individual embryos used in recordings were selected based on motility phenotype and later confirmed by genotyping to be mutant homozygotes (genotyping methods in supplemental material). Prior to recording the fish were anesthetized by a 2-min treatment with 10% Hanks solution containing 0.08% tricaine methanesulfonate (MS222; Western Chemicals, Scottsdale, AZ). The fish were decapitated, transferred to a siliconeelastomer (Sylgard)-coated chamber (Dow-Corning, Midland, MI) that contained bath recording solution (in mM: 134 NaCl, 2.9 KCl, 2.1 CaCl2, 1.2 MgCl2, 10 Glucose, 10 Na-HEPES, pH 7.4; ~290mosM). The fish were immobilized by inserting two tungsten pins through the

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notochord at approximately tail segments 5 and 14 after which the skin between the two pins was removed. In some cases, larvae were treated with 2 M formamide for 5 min to prevent muscle contraction followed by an extensive wash with bath solution. The chamber was mounted on an Axioskop FS upright microscope and viewed with an achroplan ×40 water-immersion objective (Zeiss, Oberkochen, Germany). The superficial slow muscle layer on the ventral side was removed to gain access to the deeper fast muscle cells by gentle suction using a ~15-μm OD glass pipette. For recording of spontaneous miniature endplate currents (mEPCs), 1.5 μM tetrodotoxin (Alomone labs, Jerusalem, Israel) was present in the bath solution throughout the recording to block nerve action potentials. The dorsal muscle was removed to expose the spinal cord for simultaneous nerve-muscle recordings of evoked synaptic currents (Wen and Brehm 2005). An EPC 10/2 dual patch-clamp amplifier (List Electronics, Darmstadt-Erstadt, Germany) was used to sample data at 50 kHz. Synaptic currents were analyzed off-line using MiniAnalysis (Synaptosoft, Decatur, GA), Pulsefit (HEKA Elektronik, Lambrecht, Germany) or IGOR Pro (WaveMetrics, Lake Oswego, OR) software.

Confocal imaging of larva was performed using a Zeiss LSM 510 Meta System configured on an Axiovert 200 inverted microscope and C-Apochromat ×40 water-immersion objective. To label ACh receptors, decapitated and skinned larvae were placed in 1% Hanks, and the skin along one side of the tail was removed. This was followed by a 15-min incubation in 0.1 μM alpha-bungarotoxin conjugated to either Alexa Fluor 647 or tetramethylrhodamine (Molecular Probes). The DNA was extracted from 20 individual siblings (BSA) was used to determine the chromosomal linkage. Genomic DNA of agarose scorable microsatellite markers (Mappairs, Clontech) was then sequenced. Some cases, the ACh receptors were labeled with 0.1 μM M alpha-bungarotoxin conjugated to Alexa Fluor 647 or tetramethylrhodamine (Molecular Probes). The larvae were then washed in toxin-free 100% Hanks for 2 h with frequent changes of solution to remove nonspecific binding of toxin. For staining of acetylcholinesterase, decapitated and skinned larvae were incubated for 1 h in a 100% Hanks solution containing 0.1% BSA and ~0.12 μM Fasciculin II (Alomone Labs) that we conjugated to Alexa Fluor 633 (Molecular Probes). Fish were then washed for 1.5 h in toxin-free 100% Hanks. For imaging, the labeled embryos were mounted in 1% agarose with the intact side (nonskinned) flush against the bottom of a glass coverslip. For comparative purposes, imaging on wild-type specimens was optimized first and reapplied to subsequent specimens. Dissociated muscle was obtained from bungarotoxin labeled, skinned larvae by treatment with 10 mg/ml collagenase (Gibco) for 30–45 min. The tail was gently triturated to release muscle cells into glass-bottom dishes for microscopy.

For low-resolution positional gene mapping, bulk segregant analysis (BSA) was used to determine the chromosomal linkage. Genomic DNA was extracted from 20 individual bajan homozygote mutant and 20 wild-type/heterozygote sibling embryos between 72 and 96 hpf. The DNA was used as template in PCR amplification with 214 pairs of agarose scorable microsatellite markers (Mappairs, Clontech) to scan the genome for linkage. The results of BSA were analyzed with the use of linkage analysis (BSA) was then used to quickly assign the genetic map position of the candidate gene. 

Switching Mechanism at 5′-rapid amplification of cDNA ends (RACE) were performed using Switching Mechanism at 5′-end of RNA Transcript (SMART, Clontech) kit. The PCR products were then gel purified and TOPO cloned for sequencing. After acquiring the complete encoding sequence of zebrafish ChAT from the RACE results, the full-length ChAT cDNA (Gene Bank accession number EU660883) was amplified using primer pairs: 5′-TCTCTAATTTTGGATGTCGTTTTTTC-3′ and 5′-TCTCGGCTTCTTTATGACCTTCTCC-3′. Two morpholino oligonucleotides directed against ChAT were tested for their ability to phenocopy bajan motility dysfunction. One morpholino was directed against the translational start site (5′-GAAACTGGCATCTCCTCAAGTTGGAA-3′) and a second against the splice site where the bajan mutation occurred (5′-ATCATACACTAACACAAATGATCAG-3′). Prior to injection, the morpholino stock solution was heated at 65°C for 5 min and mixed with Fast Green dye (Sigma) before diluting to a final concentration of 2 mM. For the splice site morpholino, 0.5 nl DNA was pressure injected into the yolk of one- to four-cell stage wild-type embryos. At 48–72 hpf, injected embryos were sorted based on their motility phenotype and further analyzed with RT-PCR and sequencing. Rescue of the bajan phenotype was performed by injecting synthetic RNA. All the eggs born to a pair of bajan heterozygous parents were injected with ~100 pg of wild-type ChAT mRNA. At 48–72 hpf, embryos were assessed and counted based on their motility phenotype. All embryos that displayed normal escape response were genotyped (see Supplemental material).

For immunohistochemistry, larvae were fixed overnight at 4°C in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Larvae were then decapitated to increase accessibility to the tissue and washed in distilled water to dissolve salts. Following acetone treatment for 7 min at −20°C, larvae were rinsed in distilled water and transferred to a PBS solution containing 0.5% Triton-X, 2% goat serum and the antibody of choice for overnight incubation at 4°C. The larvae were then washed extensively in PBS containing 0.5% Triton-X for 2 h after which 1:1000 Alexa 488-conjugated secondary antibody (Jackson Labs) was added. Following overnight incubation at 4°C the larvae were washed extensively in PBS containing 0.5% Triton-X. Antibodies utilized were anti-ChAT (1:50, Millipore, Billerica, MA.), anti-SV2 (1:100) and mixed anti-znp-I/zn-I (1:10, Developmental Studies Hybridoma Bank; University of Iowa). In some cases, the ACh receptors were labeled with 0.1 μM alpha-bungarotoxin which was added to the secondary antibody incubation 15 min prior to the final wash series.

All data presented was expressed as means ± SD and all statistics utilized unpaired t-test.

RESULTS

Identification of the bajan mutation

Identification of the bajan mutation utilized a combination of genetic and electrophysiological approaches. Electrophysiological recordings indicated that spontaneous synaptic currents were unusually small, pointing to a potential defect in synaptic transmission. Because initial analysis of postsynaptic receptors by fluorescent conjugated alpha-bungarotoxin showed no obvious defect, the likely mutation was presynaptic. Candidate proteins were vesicular acetylcholine transporter (VACH) and choline acetyltransferase (ChAT), both of which would be expected to reduce quantal size. Bulk segregant analysis (BSA) was then used to quickly assign the bajan allele to a specific chromosome. For this purpose, 214 simple se-
quence length polymorphism (SSLP) markers were screened by PCR from genomic DNA pools composed of either 20 identified motility mutants or 20 normal siblings born to the same bajan heterozygous parents. Marker Z17223 showed nonrandom segregation of the mutant allele (TL) in the mutant group and sibling groups, indicating linkage between the bajan mutation and chromosome 13. Examination of the zfin database confirmed that the VAChT and ChAT genes were both located on chromosome 13.

Sequence of the VACHT DNA in bajan mutants revealed no differences in the predicted coding region when compared with wild-type. However, in the process of sequencing zebrafish ChAT, the reverse transcription polymerase chain reaction (RT-PCR) amplicons showed completely different patterns between mutants and wild-type fish at 72–96 hpf. A single product was amplified from the wild-type cDNA, whereas three different amplicons appeared in the mutant, none of which corresponded precisely to the wild-type product in size (Fig. 1). Sequence analysis of the PCR products revealed a single point mutation (A to C) at the splice acceptor site of (Fig. 1). Sequence analysis of the PCR products revealed a single point mutation (A to C) at the splice acceptor site of intron 2 in bajan. The mutation created three different mutant transcripts, all of which introduced a premature stop codon. The 3.2Kb transcript predicts a truncated 80 amino acid product. The second (1.8 kb) and the third transcripts (1.7 kb) utilized a splice acceptor site downstream of the mutation, and predicted truncated protein products of 102 and 83 amino acids, respectively (Fig. 1).

Two different stereotypic motility responses were quantitated to characterize the bajan motility defect. First, the spontaneous coiling responses from dechorionated wild-type and mutant embryos were compared between 23 and 30 hpf. All of the wild-type embryos (n = 89) displayed at least one spontaneous tail contraction during the 5-minute observation period. When the offspring of bajan heterozygotic parents were examined approximately one of four larvae lacked coiling movements. All of these larvae were subsequently confirmed to be mutant homozygotes (data not shown). Second, high-speed imaging of escape responses in 48- to 72-hpf wild-type and bajan fish were compared for differences in touch avoidance and subsequent swimming behavior (Fig. 2). On a single touch on the tail, wild-type larvae responded with an initial C-bend, which peaks at ~20 ms after stimuli, followed by a more variable second bend to the opposite side and alternating tail beats. The delay between the stimulus and onset of the response appeared to be normal in bajan fish. However, in mutant fish the fast stereotypic initial C-bend was greatly attenuated and the alternating tail beats were barely visible, resulting in an abortive swimming. The escape responses of bajan heterozygotes were indistinguishable from those of wild-type fish.

To determine whether the ChAT mutation underlies the bajan phenotype, we injected wild-type fish with morpholino DNA at the single cell stage to knockdown ChAT expression. The ChAT morpholino was designed based on the intron-exon border sequence that flanks the bajan point mutation (www.gene-tools.com). The high-speed images of touch-elicited escape response from the splice morpholino injected 48- to 72-hpf wild-type fish showed incomplete initial bend and weakened subsequent tail contractions, both characteristic of bajan (Fig. 2). RT-PCR analysis from the splice morpholino injected fish showed successful disruption of pre-mRNA splicing and absence of wild-type product. Different from the mutant pattern, the splice morpholino created a single mis-splided product, corresponding to the smallest-sized band in the mutant (Fig. 1). Sequencing the product showed a complete match to the shortest mutant transcript. We also tested a morpholino directed against the translational start site of ChAT and obtained different results. A large number of experiments were undertaken to optimize the amount of morpholino injected into wild-type. There appeared to be threshold of ~2.0 ng for the injection, below which the injection produced no phenotype in any of the injected embryos while amounts above the threshold killed more than half of the embryos and induced extensive morphological defects in the survivors.

As independent evidence that the bajan phenotype results from the disruption of the ChAT gene, wild-type ChAT mRNA was injected into 163 embryos born to heterozygote parents. Injected larvae were evaluated for touch response at 48–72 hpf by high-speed imaging. More than 90% (147/163) of the larvae showed normal touch response with multiple strong alternating bends (Fig. 2) as compared with 75% predicted by Mendelian genetics, suggesting successful behavioral rescue. The remaining 10% (16/163) of the larvae displayed a motility phenotype much less severe than the bajan homozygotes. Genotyping all the individual larvae that showed normal escape response...
revealed the existence of mutant homozygotes among them, confirming successful behavioral rescue.

All three transcripts in *bajan* predict truncated protein products due to premature stop codons, resulting from frame shifts. All of these truncated forms would be predicted to be nonfunctional due to the absence of the catalytic site. Whole-mount immunohistochemical labeling of wild-type fish with a polyclonal ChAT antibody showed strong labeling of spinal motorneuron cell bodies and their projecting axons (Fig. 3). These labeled nerve terminals overlapped with alpha-bungarotoxin labeled ACh receptor clusters. By contrast, no ChAT signal was detected in *bajan* spinal cord or tail (Fig. 3).

**Postsynaptic development in bajan**

To determine potential consequences of the ChAT mutation on synapse development, 72- to 96-hpf *bajan* and wild-type larvae were labeled with both presynaptic and postsynaptic markers (Fig. 4A). Presynaptic terminals were labeled effectively with an antibody directed against the vesicular protein SV2 and postsynaptic ACh receptor clusters were labeled using fluorescent-conjugated alpha-bungarotoxin. Both *bajan* and wild-type larva showed well-developed postsynaptic receptor clusters that were coincident with the presynaptic SV2 label. To the eye, there were no discernable differences between wild-type and *bajan* in terms of numbers or sizes of clusters (Fig. 4A). Additionally, there was no obvious difference in the staining patterns of motor neuron terminals (Fig. 4B). However, it was not possible to determine which clusters were associated with an individual muscle cell in the intact tail.

Therefore to better determine whether differences exist, quantitation of both receptor cluster number and cluster size was performed on acutely dissociated muscle cells labeled with fluorescent alpha-bungarotoxin (Fig. 4C). For the purpose of direct comparisons between paired wild-type and *bajan* muscle, the confocal settings were held constant and z stacks were generated for each cell. No significant difference in cluster number (P > 0.05) was found between wild-type (7.19 ± 1.33; n = 6) and *bajan* (5.49 ± 2.02; n = 14) fish. However, a small but significant increase in mean area for individual clusters (P = 0.0018) was observed for *bajan* (2.45 ± 1.89 μm²; n = 144) compared with wild-type (1.79 ± 1.13 μm²; n = 102) larvae (Fig. 4D).

**Synaptic currents in bajan**

Whole cell voltage-clamp recording from fast skeletal muscle of the tail was used to test for functional defects in neuromuscular transmission associated with the ChAT mutation. Spontaneous mEPCs from *bajan* and wild-type fish at 72–96 hpf (Fig. 5A) both exhibited a fast rising phase and a slower single-exponential decay. To determine whether quantitative differences existed, the 10–90% rise time and decay time constant for each mEPC were determined. The frequency histograms for rise times and decay time constants for the representative wild-type and *bajan* fish indicated no differences (Fig. 5A). By contrast, the distribution for peak amplitudes indicated an ~2.5-fold difference between the mutant and wild-type (Fig. 5A).

**FIG. 2.** Comparisons of wild-type and *bajan* escape responses. Escape responses were elicited in 48- to 72-h post-fertilization (hpf) fish by a gentle tail prod and imaged at the rate of 1,000 frame/s. Every 10th frame is shown for wild-type, *bajan* homozygote, morpholino phenocopied and ChAT mRNA rescued fish. The phenocopy was performed by injecting morpholino oligonucleotides directed against the splice site of ChAT into wild-type eggs. The rescue represents injection of wild-type ChAT RNA into *bajan* homozygous eggs.

**FIG. 3.** ChAT label is not detected in 72- to 96-hpf *bajan* larvae by immunohistochemistry. The cell bodies of spinal neurons (top) and neuromuscular junctions (bottom) of 72- to 96-hpf wild-type fish are strongly labeled by anti-ChAT antibody (in green). This label is coincident with many of the receptor clusters labeled by tetramethylrhodamine-conjugated α-bungarotoxin (in red). By contrast no ChAT signal was detected in the spinal cord or neuromuscular synapses of *bajan* fish (n = 10). A labeled motorneuron in wild-type fish is indicated (*).
The mean values for amplitude, rise time, and decay time constants for each individual recording were then compared for wild-type and bajan (Fig. 5B). Overall, rise times averaged 0.211 ± 0.034 ms in wild-type (n = 9 recordings) and 0.201 ± 0.029 ms in bajan (n = 14 recordings), which were not significantly different (P = 0.46). The time constants of decay, determined by exponential fit to each mEPC were not significantly different between bajan (0.566 ± 0.083 ms) and wild-type fish (0.529 ± 0.089 ms; P = 0.32). By contrast, mean amplitude values for bajan and wild-type fish corresponded to 205 ± 73 and 536 ± 90 pA, respectively, and the differences were highly significant (P < 0.001). The frequency of mEPCs was compared between wild-type and bajan. While the values varied considerably between individual cells, the average mEPC frequency in bajan (11.0 ± 11.3 events min⁻¹) was not different from the wild-type (10.8 ± 7.4 events min⁻¹, P = 0.96).

Simultaneous recordings from primary motor neuron and target skeletal muscle were performed to compare EPCs in wild-type and bajan fish (Wen and Brehm 2005). Comparisons of the action potential waveforms of primary motorneurons revealed no obvious differences between bajan and wild-type fish (Fig. 6A). Also there was no difference in the mean rise time (bajan = 0.273 ± 0.035 ms, n = 11 recordings; wild-
type = 0.257 ± 0.045 ms, n = 23 recordings; P = 0.28) or
decay time constant (bajan = 0.623 ± 0.095 ms, wild-type =
0.693 ± 0.091 ms; P = 0.06). The average amplitude of EPCs
was significantly smaller for bajan (1.20 ± 0.54 nA) than
wild-type (4.90 ± 1.87 nA, Fig. 6B, P < 0.001). To determine
whether quantal content was altered in response to the reduced
quantal size, the mean quantal content was determined by the
direct method of dividing EPC amplitude by the mEPC am-
plitude. The mEPCs in vivo are distributed in a simple bell-
shaped distribution and most if not all can be resolved due to
near isopotentiality and large amplitude (Fig. 7A). Therefore
this method provides a reliable indicator of the number of
unitary events that compose a single evoked response. The
mean quantal content was significantly reduced in bajan (5.8 ±
2.6, n = 11 recordings) compared with wild-type (9.1 ± 3.5,
n = 23; P = 0.005).

Bajan fish undergo use-dependent fatigue in swimming. To
examine the underlying basis for the fatigue, we tested the
responses to high-frequency stimulation. In both bajan and
wild-type fish, motorneurons were able to follow 100-Hz
stimulation by generating action potentials (not shown), but
within 10 s of stimulation, the endplate currents exhibited
failures associated with vesicular depletion (Fig. 8A). To
determine whether quantal size changed during depletion, we
compared the distribution of spontaneous mEPCs at resting
state to that obtained during the final phase of stimulation, a
time corresponding to frequent failures (>95% action poten-
tials do not elicit any EPC responses). Measurements of mean
quantal size from the overall wild-type distribution corre-
sponded to 536 ± 90 pA (n = 9 cells, Fig. 5B) with no
stimulation and 542 ± 135 pA (n = 13 cells) during the final
phase of the depletion protocol (Fig. 8B). These values were
not significantly different. For bajan, the values corresponded
to 205 ± 73 pA (n = 14 cells) prior to stimulation and 149 ±
48 pA (n = 12 cells) after stimulation. These values reflected
a small but significant difference (P = 0.03). As an alternative
approach, we measured the amplitudes of the first EPC to
recover following depletion for comparison. For bajan a mean
value of 168 ± 85 pA (n = 9) also appeared slightly smaller,
however, was not significantly different from the resting mean
size (P = 0.29).

Source of neurotransmitter in bajan

All three ChAT transcripts in bajan are truncated and pre-
dicted to result in nonfunctional peptides. Thus the expectation
on the basis of the mutation would be elimination of synaptic
transmission and paralysis in bajan homozygotes. However,
neurotransmission and limited motility were observed for all
bajan mutants tested. Treatment with 1 μM alpha-bungaro-
toxin completely eliminated synaptic currents in bajan, indi-
cating that all transmission was cholinergic. One potential
alternative mediator of neuromuscular transmission was the
weak agonist choline. Unlike the native transmitter, choline
would be resistant to hydrolysis by cholinesterase. Therefore
to directly test whether ACh was responsible for mEPCs and
EPCs in bajan, synaptic responses were recorded prior to and
after inhibition of acetylcholinesterase (AchE; Fig. 9). To
inhibit AchE function, muscles were treated with fasculin II
toxin (Fas II), a potent inhibitor of AchE. Fluorescent-conju-
gated Fas II colocalized with alpha-bungarotoxin-labeled syn-
apses in both bajan and wild-type fish (Fig. 9A). Therefore we
tested the ability of Fas II treatment to alter the synaptic current
kinetics. Examination of the scatter plots for decay time con-

FIG. 6. Paired neuron-muscle recordings show a significant
reduction in evoked postsynaptic current amplitude in bajan.
A: sample traces of motor neuron action potentials (top) and
associated EPC (bottom) from fast skeletal muscle of wild-type
(left) and bajan (right) 72- to 96-hpf larvae. B: histograms of
evoked current amplitudes (left), rise times (middle), and decay
time constants (right) for wild-type (■) and bajan (□). The
EPCs were recorded at a muscle holding potential of –50 mV.
Data in B represents mean values from 23 wild-type and 11
bajan recordings.
stant from fast muscle cells revealed a pronounced prolongation of the distributions for both wild-type (1.8 fold; n/H1100514) and bajan (1.7 fold; n/H1100512) larvae following FAS II treatment (Fig. 9B). Similar results were obtained using the irreversible ACHE inhibitor methanesulfonyl fluoride (data not shown).

The source of the ACh is unclear in light of the fact that none of the ChAT transcripts predict a functional enzyme. One potential source of transmitter is maternally derived ChAT protein and/or transcript, and this idea is consistent with electrophysiological measurements over the first 3 days of development. The average mEPC amplitude recorded at 48, 72, and 96 hpf was relatively constant in wild-type fish (Fig. 9C), whereas bajan larvae exhibited a significant decrease in peak amplitude between 72 and 96 hpf (Fig. 9C). A weakening in neurotransmission was further reflected in the decreasing mEPC frequency in bajan over the same time period (Fig. 9C). However, immunohistochemical staining of ChAT protein failed at 48 hpf as did Western blot measurements at all ages precluding quantitative determination of protein levels. As an alternative approach, semi-quantitative RT-PCR was used to detect full-length ChAT mRNA. A weak band was detected in larval bajan, corresponding to an estimated 0.4% of the levels seen in wild-type fish (Fig. 9D; see Supplemental material for methods). Transcripts were not detected at either the 1- or 16-cell stage and appeared to increase ≤48 hpf, inconsistent with maternally derived transcripts. These measurements were only semi-quantitative because real-time qPCR amplification requires a single product, which was not possible in our case. Thus it appears likely that low levels of functional ChAT were being synthesized in bajan due to a small amount of wild-type ChAT transcript that was correctly spliced from the mutated gene.

**DISCUSSION**

Our finding that a mutation in ChAT is causal to the bajan phenotype adds another locomotory mutant to the rapidly growing list of fish with neuromuscular defects. To date,
mutants isolated on the basis of motility dysfunction have been shown to affect diverse aspects of neuromuscular transmission including genes required for receptor expression (Ono et al. 2001, 2004; Westerfield et al. 1990), receptor clustering (Ono et al. 2002), transmitter release (Woods et al. 2006), and now transmitter synthesis. Unlike most of the motility mutants identified thus far, however, **bajan** appears to be a hypomorph and retains low levels of ChAT function. The function is preserved despite the fact that **bajan** contains a splice site mutation and all of the ChAT transcripts that were sequenced are predicted to be functional nulls. The possibility that choline mediates the transmission in **bajan** was ruled out by our demonstration that cholinesterase plays an active role in shaping the synaptic kinetics. The possibility that additional ChAT genes might be present in the zebrafish genome is also unlikely. Blasting a highly conserved portion of the protein sequence surrounding the enzymatic center in ChAT against the entire zebrafish genome yielded a single predicted gene. One of the family members, carnitine acetyltransferase, has been shown in vitro to catalyze the acetylcholinesynthesis reaction with ~100 times less affinity for choline (Goodman and Harbison 1981). However, the lack of choline acetylating activity by the brain lysates and the complete absence of synaptic responses in ChAT knockout mice argue against the involvement of an enzyme other than ChAT (Misgeld et al. 2002).

Maternal contribution of ChAT was also considered as a possible source of residual transmission. However, no direct support for this idea was provided by RT-PCR analyses using multiple primers that were designed to detect full-length ChAT mRNA. Instead the primers identified a weak band in **bajan**, corresponding to an estimated 0.4% of the levels seen in wild-type fish. Furthermore, the levels of ChAT mRNA rose during development and this could only happen if the homozygous **bajan** fish were able to transcribe their own ChAT mRNA. So it appears likely that low levels of functional ChAT were being synthesized in **bajan** due to a small amount of wild-type ChAT transcript that was correctly spliced from the mutated gene. **Bajan** motility was phenocopied by a morpholino directed against the splice site of ChAT, but morpholinos directed against the ChAT start site were lethal, indicating that total loss of function is likely not tolerated. This may explain why only a single allele of **bajan** was isolated in the Tuebingen motility screen (Granato et al. 1996) and also why there have been no ChAT zebrafish mutants identified in any other screens thus far. Our data further indicate that only small levels of ChAT activity are required to support synaptic transmission.

We realized that the effects of reduced activity could provide new information on synapse development. There has been a long history of studies that sought to link neurotransmission to synapse development (Sanes and Lichtman 1999). In general, studies in mammals have been hampered by an inability to completely block neuromuscular activity for sustained periods of time. For example, postsynaptic receptor nulls have never...
been generated in mammals, and chronic pharmacological block of receptors is not well tolerated in vivo. However, neuromuscular synapses in mice were recently silenced by genetic elimination of ChAT and therefore acetylcholine (Brandon et al. 2003; Misgeld et al. 2002). Unexpectedly, excessive branching occurred resulting in increased size and numbers of synapses in diaphragm muscles, pointing to a direct role for ACh. Likewise, bajan showed evidence of increased postsynaptic area, but the numbers of synapses were not increased. One possibility to explain this difference is that zebrafish tail muscle is normally multiply innervated. Alternatively, it is possible that 40% of residual synaptic activity in bajan is sufficient to promote proper synapse number. In support of the latter idea are many studies showing that low levels of synaptic transmission are adequate to prevent ACh supersensitivity, a process known to be activity regulated (Cangiano 1985).

Of particular interest is the role of ACh-mediated activity in establishing presynaptic function. Activity-dependent modulation of presynaptic release machinery at the neuromuscular junction has been well described in *Xenopus* nerve-muscle co-cultures (Fitzsimonds and Poo 1998). ACh release from the developing spinal neurons appeared greatly potentiated once the growth cones came into contact with the myocytes and the potentiation was inhibited by transient blockade of postsynaptic AChR activity (Liou et al. 1997, 1999; Wan and Poo 1999). Neuromuscular formation plays a further role in altering the ion channel composition of spinal neurons (Nick and Ribera 2000). Activity-dependent adaptive mechanisms have also been described in vivo wherein chronic injection of alpha-bungarotoxin leads to an inverse relationship between quantal content and the amplitude of the synaptic potential (Plomp et al. 1992). Similarly, mice that have been genetically altered to have reduced postsynaptic AChR density exhibit a compensatory increase in quantal content (Sandrock et al. 1997). Thus much evidence exists in support of postsynaptic ACh receptors serving in a feedback role to alter presynaptic release. Bajan offers the unique opportunity to explore this issue due to the residual Ach-mediated synaptic activity. Measurements of rise and decay times revealed no differences in either spontaneous or evoked synaptic currents. Additionally, in contrast to expectations, we found a slight reduction rather than increase in the quantal content. Overall, it appears that a 60% reduction in synaptic response is well tolerated in terms of synaptic function despite the fact that motility is severely compromised.

Humans with defective ChAT suffer from episodic apnea, a form of congenital myasthenic syndrome (Miselli et al. 2003; Ohno et al. 2001; Schmidt et al. 2003). Like bajan, a single mutant copy is asymptomatic but double mutants exhibit fatigue in response to repetitive stimulation or exercise. In both, progressive weakness associated with exercise results from a decrease in EPC amplitude during repetitive firing of motor-neurons. However, the findings from symptomatic humans and bajan disagree with respect to the underlying mechanisms. In CMS patients, recordings from biopsied muscle pointed to a near normal resting quantal size, which dropped sharply in response to repetitive stimulation (Miselli et al. 2003; Ohno et al. 2001). These findings from human subjects have led to the proposal that the major adverse effect of reduced ChAT activity is vesicle refilling, which is too slow to keep up, even under modest stimulus conditions. In bajan, however, the initial resting quantal size was greatly reduced compared with wild-type fish. Furthermore, unlike humans, measurements of quantal size during high-frequency stimulation, adequate to deplete vesicles, indicated little or no further decrease. Instead we observed a drop in quantal content, reflecting reduced probability of exocytosis. So the combination of a smaller but fixed quantal size along with frequency-dependent reduction in quantal content is causal to the reduction in EPC amplitude and associated fatigue in bajan.

Because there is no a priori reason why vesicle filling should be different in fish and humans, we considered possible underlying explanations. Normally, ChAT is present in amounts that are in excess to those required for effective conversion of choline to acetylcholine. In fact, synthesis of ACh is thought to be rate limited by the availability of choline, not the rate of conversion (Kuhar and Murrin 1978). So normally ChAT is able to keep up with the supply provided by the plasma membrane choline transporter. Thus a 50% reduction in ChAT is without effect in fish and humans (Miselli et al. 2003; Ohno et al. 2001; Schmidt et al. 2003). However, homozygotic bajan fish likely experience a much greater reduction in ChAT than thought to occur in perhaps all but the most severely affected human patients. In fact, ChAT levels in bajan are likely to represent <1% of normal, and such low levels might be required before effects on resting quantal size are seen. The question then arises as to why the vesicles are unable to increase their contents beyond 40% of normal over time in bajan. The distributions of mEPC amplitudes for wild-type and bajan fish are nonoverlapping, and large-amplitude events are missing entirely in all recordings from bajan. A potential explanation may lie in relative amounts of choline and ACh in the terminals. The reduced quantal size may reflect vesicles that are actually filled to capacity but contain an acetylcholine and choline mixture. This may be exacerbated by increases in the levels of the plasma membrane choline transporter as seen in ChAT knockout mice (Brandon et al. 2004). Thus in contrast to studies on human ChAT subjects, vesicular filling by ACh is incomplete in bajan and is rapid relative to vesicle recycling. Similar manifestations of lowered quantal size may be present in CMS individuals that harbor the most severely compromised mutations in ChAT.

GRANTS

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REFERENCES


Etard C, Behra M, Ertzer R, Fischer N, Jesuthasan S, Blader P, Geisler R, Strähle U. Mutation in the delta-subunit of the nAChR suppresses the...


