INTRODUCTION

Mental retardation is a common condition affecting 3% of the population (Hagberg et al., 1981; Shea, 2006) and is caused by several etiologies (Aicardi, 1998; Raymond and Tarpey, 2006). Multiple single-gene syndromes have been identified in the last decade. Among them, Fragile X syndrome is the most frequent (Hinton et al., 1995; Skinner et al., 2005) and is caused by the absence of FMRP (Wohrle et al., 1993; De Boulle et al., 1993; Hornstra et al., 1993; Trottier et al., 1994), which is encoded by the FMR1 gene. As observed in human (Cianchetti et al., 1991; Cornish et al., 1999) and in several animal models, mutations in FMR1 homologs have yielded memory defects (Fmr1, 1994; Maes et al., 1994; Kooy et al., 1996; McBride et al., 2005; Bolduc et al., 2008) and long thin dendritic spines (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Comery et al., 1997; Irwin et al., 2002). Consistent with the latter observation, Drosophila FMRP homologue has been linked to known actin modifying molecules such as Rac in flies (Billuart and Chelly, 2003; Schenck et al., 2003), profilin in flies (Reeve et al., 2005), PAK in mice (Hayashi et al., 2007) and Ras in mice (Hu et al., 2008), but the molecular dysfunction underlying this defect in synaptic structure remains unknown.

Regulation of actin cytoskeleton is impaired in many human mental retardation syndromes (Inlow and Restifo, 2004) and appears crucially involved in synaptic plasticity in various cellular models of memory (Dillon and Goda, 2005). Similarly, induction of long-term potentiation in hippocampal neurons, a cellular model for synaptic plasticity, leads to modifications of dendritic spine shape (Engert and Bonhoeffer, 1999; Matus, 2000). The Filamin A gene is involved in actin cytoskeleton remodeling (Flanagan et al., 2001; Stossel et al., 2001). Filamin A is expressed in neurites of neurites (Herskowitz, 1991; Cornish et al., 1999), and these patients suffer various degrees of cognitive dysfunction and epilepsy (Battaglia et al., 1997; Fox et al., 1998). Finally, two patients with PNH were recently reported to have mutations in FMR1 rather than in Filamin A (Moroi et al., 2006).

Working on genetic mechanisms of memory in Drosophila (Bolduc and Tully, 2009), we too have noted a potential link between FMR1 and Filamin A. We recently have shown that disruptions of dFmr1, the fly homolog of FMR1, show (i) neuroanatomical, learning and memory deficits when disrupted early in development, and (ii) deficits specific to LTM formation when disrupted only in adults (Bolduc et al., 2008). Independently, a behavioral screen for LTM mutants identified the joy strain, which carries a genetic lesion in cheerio, the fly ortholog of Filamin A (Dubnau et al., 2003). Given these observations, we hypothesized that Fmr1 and Filamin A may interact in activity-dependent remodeling of actin cytoskeleton. We have tested this hypothesis by evaluating genetic interaction for either filamin A and cheerio, and they are associated with dendritic spine defects. In Drosophila, disruptions of the dFmr1 gene impair long-term memory (LTM), and the Filamin A homolog (cheerio) was identified in a behavioral screen for LTM mutants. Thus, we investigated the possible interaction between cheerio and dFmr1 during LTM formation in Drosophila. We show that LTM specifically is defective in dFmr1/cheerio double heterozygotes, while it is normal in single heterozygotes for either dFmr1 or cheerio. In dFmr1 mutants, Filamin (Cheerio) levels are lower than normal after spaced training. These observations support the notion that decreased actin cross-linking may underlie the persistence of long and thin dendritic spines in Fragile X patients and animal models. More generally, our results represent the first demonstration of a genetic interaction between mental retardation genes in an in vivo model system of memory formation.

Keywords: fragile X, filamin A, memory, mental retardation, dendritic spine

Fragile X mental retardation 1 and Filamin A interact genetically in Drosophila long-term memory

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The last decade has witnessed the identification of single-gene defects associated with an impressive number of mental retardation syndromes. Fragile X syndrome, the most common cause of mental retardation for instance, results from disruption of the FMR1 gene. Similarly, Periventricular Nodular Heterotopia, which includes cerebral malformation, epilepsy and cognitive disabilities, derives from disruption of the Filamin A gene. While it remains unclear whether defects in common molecular pathways may underlie the cognitive dysfunction of these various syndromes, defects in cytoskeletal structure nonetheless appear to be common to several mental retardation syndromes. FMR1 is known to interact with Rac, profilin, PAK and Ras, which are associated with dendritic spine defects. In Drosophila, disruptions of the dFmr1 gene impair long-term memory (LTM), and the Filamin A homolog (cheerio) was identified in a behavioral screen for LTM mutants. Thus, we investigated the possible interaction between cheerio and dFmr1 during LTM formation in Drosophila. We show that LTM specifically is defective in dFmr1/cheerio double heterozygotes, while it is normal in single heterozygotes for either dFmr1 or cheerio. In dFmr1 mutants, Filamin (Cheerio) levels are lower than normal after spaced training. These observations support the notion that decreased actin cross-linking may underlie the persistence of long and thin dendritic spines in Fragile X patients and animal models. More generally, our results represent the first demonstration of a genetic interaction between mental retardation genes in an in vivo model system of memory formation.

Keywords: fragile X, filamin A, memory, mental retardation, dendritic spine
Drosophila (Quinn et al., 1974; Tully and Quinn, 1985; Tully et al., 1994; Restifo, 2005). Here, we show that (i) *cherio* is expressed in the adult fly brain, (ii) *cherio* expression is upregulated in the joy mutant, (iii) LTM specifically is impaired in *cherio* mutants, (iv) LTM specifically is impaired in *dFmr1; cherio* double heterozygotes and (v) Filamin A is abnormally downregulated in the *dFmr1* mutant during LTM formation. These data show for the first time that regulators of protein translation (FMRP) and cytoskeletal structure (Filamin A) function together during LTM formation, thereby presenting a plausible molecular mechanism for a link between dendritic spine morphology and cognitive dysfunction in mental retardation syndromes.

MATERIALS AND METHODS

**DROSOPHILA STRAINS**

Flies were raised and disposed of as per Cold Spring Harbor Laboratory regulations under the supervision of Dr. Tim Tully. The *cher*mutant was previously generated in our laboratory as part of behavioral screen for LTM mutants (Cold Spring Harbor Laboratory) using P-element mutagenesis (Dubnau et al., 2003). The *cher*mutant carries a P-element insertion within the *cherio* gene. These mutants were “genotyped” using PCR primers (in bold).

12944000→ATTTTCATTTTGCTTCAACACTCACATTTTCATATTTCAGTAAAGTTGAGACGATTATTTTGGCGCACAACTTGCAAGCTTCATCCGAGCAATTTTCTCAGCTGATTTTTGTAGAATTTTTGCATCGTGAATAGAAGTTGAGATTTTTTTGTTAAAATATAACAGGTTTATACGAAAGTATGCACC

Thereby presenting a plausible molecular mechanism for a link between dendritic spine morphology and cognitive dysfunction in mental retardation syndromes.

**PAVLOVIAN LEARNING ASSAY**

In general, *Drosophila* were raised at 22°C and placed at 25°C overnight prior to behavioral experiments. Adult *Drosophila* less than 3-days old were subjected to Pavlovian olfactory conditioning for (i) one training session (learning), (ii) 10 training sessions without a rest interval (massed training) or (iii) 10 training sessions with 15 min rest between each (spaced training). After training, flies were stored at 18°C and then conditioned responses were tested at a 1 or 4-day retention intervals (Tully and Quinn, 1985). About 150 flies were trapped inside a training chamber, 95% of the inside of which was covered with an electrifiable copper grid. Flies were allowed 90 s to acclimate and then were exposed sequentially to two odors, 3-octanol (OCT) and 4-methylcyclohexanol (MCH), carried through the chamber in a current of air (750 mL/min). Flies were exposed to two converging currents of air from opposite arms of the T-maze, one carrying OCT and the other MCH. Flies were allowed 90 s to a second, control stimulus (CS-; either MCH or OCT), during which time they received the unconditioned stimulus index (PI). Double heterozygote+, *cher*/+*Fmr1Δ*, + flies were generated by mating *cher*/+, *cher*males and *Fmr1Δ*, + females.

**GENETIC CROSSES**

Double heterozygote+, *cher*/+*Fmr1Δ*, + flies were generated by mating *cher*/+*Fmr1Δ*, + flies were generated by mating *cher*/+, *cher*males and *Fmr1Δ*, + females.

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or 4 days after training as for immediate memory (above). PI and statistical test are performed using JMP software (SAS). All graphs depict mean ± SEM (PRISM).

SENSORIMOTOR CONTROLS

To rule out sensi-motor explanations for poor performance in the Pavlovian learning task, olfactory acuity and shock reactivity were assessed as in Boynton and Tully (Boynton and Tully, 1992) and Dura (Dura et al., 1993), respectively. For olfactory acuity, flies were placed in a T-maze and given the choice between an odor versus air. The odors are naturally aversive, and flies usually avoid the T-maze's arm containing odor. For shock reactivity, flies were placed in a T-maze and given a choice between an electrified grid in one T-maze arm and an unconnected grid in the other. After the flies distributed themselves for 2 min, they were anesthetized and counted, and the PI was calculated for post-training experiments.

WESTERN BLOT ANALYSIS

Approximately 2 mL of flies were collected in a 15 mL Falcon tube and flash-frozen in liquid nitrogen. Flies then were shaken, and fly heads were separated from bodies using 25 and 40 sieves. Fly heads were dispensed to a mortar and pylon on dry ice and pulverized, transferring the to a microcentrifuge tube to be homogenized using Invitrogen extraction buffer. Following homogenization, protein content was measured on Ependorf BioPhotometer. Protein solution was diluted 1:10 in water, and 10 mL of that solution was added to 1 mL of Bradford solution. Total protein was loaded per lane with extraction buffer. Electrophoresis was conducted as suggested in the Invitrogen manual for 55 min using a 3–8% gradient gel. Blotting was conducted for 1 h at room temperature. For FMRP staining, the 5A11 antibody 1:5000 (Developmental Studies Hybridoma Bank, University of Iowa) was used in combination with WesternBreeze kit. Hybridoma) was used in combination with WesternBreeze kit. For actin (Sigma) was as a loading control (1:5000). Quantification was done with six measurements per lane and obtained using ImageJ software. Flies were collected immediately after training.

IMMUNOHISTOCHEMISTRY

Two- to five-day-old flies were dissected and processed as described previously in Xia (Xia et al., 2005). For consistency, only females were selected. All genotypes were dissected similarly and processed in parallel. On day 1, flies were dissected in PBS and then brain were transferred to 4% PA for fixation at room temperature for 10 min and placed in a vacuum for 15 min in 0.2% Triton in 4% PA. Blocking and penetration were done in penetration/blocking buffer for 2 h at 4°C. Brains then were transferred to dilution buffer containing the primary antibody and placed overnight at 4°C. On day 2, brains were washed with wash buffer 4 times (10 min/time). Brains were transferred to secondary antibody and incubated overnight at 4°C in the dark. On day 3, brains were washed again 4 times (10 mins/time) and then mounted in a well made of 2 stacked reinforcer O-rings. The well was filled with approximately 7 uL of Focus-Clear solution and covered by a cover slip. Images were acquired using LSM software from Zeiss at 20×. The average thickness of a brain was around 100 um. For GFP imaging, brains were dissected, vacuumed and then left in penetration/blocking buffer overnight. On day 2, they were washed and mounted.

To assess mushroom body morphology, anti-FasII antibody 1D4 (Developmental Studies Hybridoma Bank, University of Iowa) was used at a concentration of 1:20 (Michel et al., 2004). Anti-FMRP antibody 5A11 (Developmental Studies Hybridoma Bank, University of Iowa) was used at a concentration of 1:100 (Inoue et al., 2002). Rat anti-Filamin A (c-terminal) (a generous gift from Dr. Lynn Cooley) was used at a concentration of 1:3000 (Sokol and Cooley, 2003). An equivalent cheerio imaging result was observed using the P-element enhancer-trap line, Kyoto 105280 crossed to UAS-GFP. The secondary anti-mouse Cy3 antibody (Jackson Lab) was used at a concentration of 1:200.

RESULTS

CHEERIO EXPRESSION IS ABERRANT IN MUTANTS

We took advantage of a cheerio mutant previously generated in a study of ring canal formation (Sokol and Cooley, 2003). The cherΔ mutant was derived from an imprecise excision of the EP(3) 3175 P-element insertion; homozygous females display defective germline cell packaging and border cell migration (Sokol and Cooley, 2003). We first asked if Cheerio was expressed in the adult fly brain (Figures 1A,B). With western blot analysis, we detected two isoforms of cheerio in adult fly brain, as was observed previously in egg chambers (Sokol and Cooley, 2003). Immunohistochemical analysis of wild-type adult brain revealed Cheerio expression mostly in the cytoplasm of cells lying at the base of the brain and midline (Figure 1B), highest in areas corresponding to the ventro-caudal region of the subesophageal ganglia and the median bundle. With both methods, Cheerio expression in brain was (i) decreased in the of the loss-of-function homozygous mutant, cherΔ and (ii) increased in the joy memory mutant (Figures 1A–C). As was reported originally by Sokol et al., who generated this antibody, we could detect some anti-cheerio signal with western blot but not with immunohistochemistry in cherΔ (Sokol and Cooley, 2003).

Considering the cerebral malformation in PNH patients, we examined, in wild-type and mutant flies, the structural integrity of the mushroom body (alpha and beta lobes), a neuroanatomical site important for olfactory memory (Pascual and Preat, 2001; Didelot et al., 2006; Yu et al., 2006; Krashes et al., 2007; Lu et al., 2007; Qian et al., 2007). We did not observe any gross morphological defects in cherΔ/cherΔ or cher+/cher+ homozygous mutants. (Figure 1D).

LTM IS DISRUPTED IN CHEERIO MUTANTS

Previous studies in Drosophila have established that memory formation after Pavlovian olfactory learning proceeds through several genetically distinct temporal phases (STM: short-term memory, MTM: middle-term memory, ARM: anesthesia-resistant memory and LTM; Tully et al., 1994). One-day memory after spaced training (ten training sessions with a 15-min rest interval between each) is composed of a cycloheximide-sensitive LTM component and a cycloheximide-insensitive ARM component. In contrast, 1-day memory after massed training (ten training sessions with no rest intervals) is composed of only ARM (Tully et al. 1994; Yin et al., 1994).
Even though LTM and ARM both can be detected 1 day after spaced training, ARM is decremental while LTM persists; 4 days after spaced training only LTM is present in wild-type flies (Tully et al. 1994; Figures 2C,D). 4-day memory after spaced training in both cher\textsuperscript{55}/cher\textsuperscript{55} and cher\textsuperscript{Joy}/cher\textsuperscript{Joy} homozygous was significantly lower than normal (P = 0.0034 and P = 0.0165) and near zero (Figures 2C,D), suggesting the defect in 1-day memory likely was produced by the absence of LTM.

In both the cher\textsuperscript{55}/cher\textsuperscript{55} and cher\textsuperscript{Joy}/cher\textsuperscript{Joy} homozygous mutants, 1-day memory after spaced training was defective (P = 0.0208 and P < 0.0001, respectively; cf. Dubnau et al., 2003), while that after massed training was not (P = 0.701 and P = 0.074, respectively; Figures 2A,B). To rule out sensorimotor defects as a possible explanation for mutants’ abnormal behavioral performance, we assessed “task-relevant” olfactory acuity and shock reactivity and found no significant differences between mutants and control (Table 1).
The \( \text{cher}^{\Delta 5} \) and \( \text{cher}^{\text{joy}} \) mutations had opposite effects on cheerio gene expression (Figures 1A–C) though both disrupted LTM. This suggested the interesting possibilities either that \( \text{cher}^{\text{joy}} \) might act as a dominant negative mutation or alternatively that the two mutations might act additively in heteroallelic mutants. We found that 1-day memory after spaced training was normal in \( \text{cher}^{\text{joy}}/+ \) and \( \text{cher}^{\Delta 5}/+ \) heterozygotes and in the \( \text{cher}^{\Delta 5}/\text{cher}^{\text{joy}} \) heteroallelic (\( P = 0.3447; N = 8 \) PIs per genotype). See text for discussion.

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**Fmr1 AND CHEERIO INTERACT GENETICALLY DURING LTM FORMATION**

Because both \( \text{dFmr1} \) and \( \text{cheerio} \) mutants are defective in LTM, we sought to investigate a possible genetic interaction using double heterozygotes. Consistent with this hypothesis, the expression patterns of FMRP and Cheerio overlapped largely in the ventro-caudal suboesophageal ganglia and in the midline bundle.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Shock reactivity (PI+/−SEM) (N = 4 PI per genotype)</th>
<th>Octanol avoidance (PI+/−SEM) (N = 2–4 PI per genotype)</th>
<th>Methylcyclohexanol avoidance (PI+/−SEM) (N = 2–4 PI per genotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>84.5 (2.158)</td>
<td>61.9 (2.997)</td>
<td>66.0 (2.035)</td>
</tr>
<tr>
<td>Cher joy/joy</td>
<td>77.3 (3.442)</td>
<td>65.4 (4.610)</td>
<td>69.0 (2.619)</td>
</tr>
<tr>
<td>Cher D5/D5</td>
<td>83.8 (4.211)</td>
<td>59.8 (6.787)</td>
<td>58.3 (7.499)</td>
</tr>
<tr>
<td>Joy/wt</td>
<td>76.0 (4.813)</td>
<td>54.0 (8.000)</td>
<td>62.5 (6.500)</td>
</tr>
<tr>
<td>FmrB55/wt, joy/WT</td>
<td>76.5 (2.901)</td>
<td>59.5 (4.500)</td>
<td>56.5 (10.500)</td>
</tr>
<tr>
<td>fmrB55/WT, joy/WT</td>
<td>77.0 (3.894)</td>
<td>67.0 (7.000)</td>
<td>58.5 (1.500)</td>
</tr>
<tr>
<td>cherD5/WT</td>
<td>76.0 (2.483)</td>
<td>66.5 (11.500)</td>
<td>64.0 (3.000)</td>
</tr>
<tr>
<td>fmrD5/WT, cherD5/WT</td>
<td>79.8 (2.926)</td>
<td>55.5 (7.500)</td>
<td>575 (4.500)</td>
</tr>
</tbody>
</table>

FIGURE 2 | LTM is defective in cheerio mutants. (A) One-day memory after spaced training is lower than normal in the loss-of-function homozygous mutant, \( \text{cher}^{\Delta 5} \) (CherD5; \( P = 0.0208; N = 8 \) PIs per genotype). In contrast, 1-day memory after massed training is normal in the \( \text{cher}^{\Delta 5} \) mutant (\( P = 0.701; N = 8 \) PIs per genotype). (B) One-day memory after spaced training is lower than normal in the gain-of-function mutant, \( \text{cher}^{\text{joy}} \) (Joy; \( P < 0.0001; N = 8 \) PIs per genotype). In contrast, 1-day memory after massed training is normal in the \( \text{cher}^{\text{joy}} \) mutant (\( P = 0.4149; N = 8 \) PIs per genotype). (C) 4-day memory after spaced training is lower than normal in the \( \text{cher}^{\Delta 5} \) (CherD5) mutant (\( P = 0.0034; N = 4 \) PIs per genotype). (D) 4-day memory after spaced training is lower than normal in the \( \text{cher}^{\Delta 5} \) (Joy) mutant (\( P = 0.0165; N = 4 \) PIs per genotype). (E) One-day memory after spaced training is normal in \( \text{cher}^{\Delta 5}/+ \) or \( \text{cher}^{\Delta 5}/\text{cher}^{\text{joy}} \) heterozygotes and in the \( \text{cher}^{\Delta 5}/\text{cher}^{\text{joy}} \) heteroallelic (\( P = 0.3447; N = 8 \) PIs per genotype). See text for discussion.
(Figure 1E). We assessed LTM formation in two different double heterozygotes, cher<sup>55</sup>/+;Fmr1<sup>B55</sup> and cher<sup>ps</sup>/+;+;Fmr1<sup>B55</sup>. In both cases, 1-day memory after spaced training was significantly reduced (P < 0.0001 and P < 0.0001, respectively) but that after massed training was not (P = 0.7478 and P = 0.5314, respectively). In contrast, 1-day memory after both spaced and massed training were normal for each of the single heterozygotes (cher<sup>55</sup>/+, cher<sup>ps</sup>/+ and Fmr1<sup>B55</sup>/+) (Figures 3A,B). In the double heterozygotes, shock reactivity and olfactory acuity were normal (Table 1), and 4-day memory after spaced training was significantly reduced and near zero (Figures 3C,D).

**Discussion**

Guided by the clinical suggestion that FMRP and Filamin A might have an intersecting function in regulating changes in neuronal cytoskeletal structure (Moro et al., 2006), we have shown that aberrant expressions levels (increased in cher<sup>ps</sup> or decreased in cher<sup>55</sup>) of cheerio, the fly homolog of Filamin A, are associated with specific defects in LTM memory formation. Such an observation has been reported before in the MeCP2 mouse model of Rett syndrome. Whereas Rett syndrome is associated with a reduction in MeCP2 levels (Amir et al., 1999) and mouse MeCP2 null mutation recapitulates several Rett-like symptoms (Guy et al., 2001), overexpression also leads to neurological defects (Collins et al., 2004). We also have shown that both decreased or increased levels of FMRP are associated with memory defects in Drosophila (Bolduc et al., 2008).

The cheerio and dFmr1 genes functionally interact during LTM formation. This interaction was observed specifically after spaced training and not after massed training. Given FMRP’s role in the
regulation of protein translation (Li et al., 2001; Khandjian et al., 2004; Stefani et al., 2004), we took this clue to the molecular level to quantify Cheerio expression levels in dFmr1 mutants. After spaced training, Cheerio expression did not change in wild-type flies, but the Cheerio short isoform specifically was reduced in the dFmr1 mutant. After massed training, this Cheerio short isoform is reduced in both wild-type and dFmr1 mutants. Thus, spaced training appears normally to disinhibit regulation of Cheerio short-form, and this disinhibition appears aberrant in the dFmr1 mutant. Further work will be required to understand this molecular mechanism. Nonetheless, the genetic interaction observed here for LTM formation may explain the similarities between patients with periventricular nodules and Fragile X syndrome.

A role for cheerio and dFmr1 during LTM formation suggests a shared molecular mechanism among the clinically and etiologically different mental retardation syndromes (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis and Sklena, 1993), which show defects in dendritic spines morphology. Other examples could include dysregulation of PAK, observed in Neurofibromatosis 1 NFI and Fragile X syndrome (Tang et al., 1998; Hayashi et al., 2007), and an abnormality in mTOR signaling observed in Tuberous sclerosis complex TSC1, TSC2 and NFI (Johannessen et al., 2005).

In Fragile X patients, the usual mushroom shaped dendritic spines are decreased in number and density and, instead, an excess of elongated thin spines is observed (Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2002). Although no study has yet linked...
Filamin A directly to the cytoskeletal structure of dendritic spines, cell membrane shape, in general, has been shown to depend on Filamin A. Serum starved melanoma cells lacking Filamin A fail to form a three-dimensional orthogonal network of cytoskeletal elements, for instance, after serum application. Instead, they form a dense mat of long actin filaments (Flanagan et al., 2001) – reminiscent of the long thin dendritic spines. Our results suggest that aberrant levels of Cheeiro expression during LTM formation could lead to decreased actin cross-linking, thereby generating abnormally shaped dendritic spines in Fragile X patients. Interestingly, a case of PNH and severe mental retardation has been reported to result from a duplication of Filamin A (Fink et al., 1997). At abnormally high concentrations, Filamin A causes actin arrangements into parallel, instead of orthogonal, arrays (Hartwig and Stossel, 1975). Further studies with high resolution imaging, such as the use of two photon-mediated release of caged glutamate, will be needed to look at dendritic spines in animal models of Fragile X and PNH. In addition, pharmacological rescue of Fragile X mutants using other cross-linkers could be tested. Indeed, alpha-actinin has been shown to interact with Filamin in a recent report by Esue et al. (2009).

ACKNOWLEDGMENTS

This project was supported by NIH and Dart Neuroscience LLC (to T.T.) and a CCHCSP Career Development Award (to F.B.).

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Received: 03 June 2009; paper pending published: 20 August 2009; accepted: 03 December 2009; published online: 08 January 2010.


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