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14. ABSTRACT
The broad long-term objective of this proposal is to develop a novel model of autism spectrum disorder (ASD) using the zebra finch songbird. Such a model would be useful in testing therapeutic interventions aimed at remediating autism endophenotypes related to language and social deficits. Songbirds such as the zebra finch, like humans but unlike typical lab animals, learn much of their vocalizations and do so through social interactions. The autism susceptibility gene, *Cntnap2*, is expressed in a similar pattern in zebra finch and human brain To develop a songbirds ASD model, we are determining Cntnap2 protein expression patterns over development. This will pinpoint the key stage and brain region in which to disrupt Cntnap2 expression using RNA interference techniques. Our major finding is that Cntnap2 protein is enhanced in a premotor song control region in young male and female birds. As development proceeds, enhancement persists relative to surrounding brain tissue in males but declines in females. This is significant because in this species, only males learn to sing. This pinpoints the developmental stage in which to interfere with Cntnap2 expression and determine the effects on male song learning and social interactions.

15. SUBJECT TERMS
Animal model, Autism susceptibility, Birdsong, Cntnap2, Communication, Social

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INTRODUCTION

The broad long-term objective of this proposal is to develop a novel model of autism spectrum disorder (ASD) using the zebra finch songbird. While no single model can capture all features of ASD, songbirds are one of the few in which the language subcomponent comprised by learned vocal communication can be studied. This is because songbirds, like humans and unlike traditional laboratory animals, learn a significant portion of their vocalizations (song) through social interactions with conspecifics. We previously showed that a key region of the brain song control system known as the robust nucleus of the arcopallium (RA) exhibits enhanced mRNA expression of the autism susceptibility gene contactin-associated protein-like 2 (*Cntnap2*) in male zebra finches (who learn to sing) at the onset of the sensorimotor phase of song learning (Panaitof et al., 2010). In contrast, in females of this species who do not learn to sing, *Cntnap2* levels decline in this same region at this time. This and other observations lead us to hypothesize that Cntnap2 contributes to the regional and functional specification of brain regions important for socially-learned vocal communication in humans and songbirds, a key phenotype affected in ASD. In experiments designed to test this hypothesis, we have now shown that FoxP2 protein in RA follows the mRNA pattern, with a striking change between young males and females at 50 days. This developmental switch now provides the timepoint at which to genetically intervene in male Cntnap2 expression and to determine the behavioral effects on song learning. We are fully on track to proceed with these experiments.

BODY

We proposed three aims to be completed across the three year funding period. During the first year, we completed all tasks enumerated under Aim 1 and made significant progress on Aim 2, task 5. Notably, we determined the developmental time frame at which Cntnap2 levels in RA diverge between male and female zebra finches (Figure 1). The major challenge we encountered was in identifying shRNA constructs that effectively knockdown zebra finch Cntnap2. During the second year, we have obtained evidence that 3 constructs do so in vitro, completing task 5 in Aim 2 (Figure 2). Additionally, we have discovered an AAV serotype and promoter which drive transgene expression effectively in vivo and plan to use this instead of the lentiviral constructs previously proposed. After careful testing, we found lentivirus to be much less effective in our hands. This insight largely completes task 6 in Aim 2. Once in vivo tests of the shRNA constructs are complete, we will be in an excellent position to complete tasks 7-9 in Aim 2 and embark on Aim 3. These advances are detailed below:

Aim 1 (estimated to occur during years 1 and 2 of funding)
A complete draft of a manuscript describing the developmental changes in Cntnap 2 levels in RA is ready and being finalized for submission to a neurobiology journal (Figure 1; Task 4). An abstract has been presented at the 2011 annual meeting of the Society for Neuroscience (Condro et al., 2011; see Appendix). Additionally, the PI wrote a review on songbirds as an animal model for human language disorders which was included in a book that is currently in press (White, 2013).

Aim 2 (estimated to begin in year 1 and be completed in year 3)
We designed 4 non-overlapping short hairpin RNAs (shRNAs) against zebra finch Cntnap2. Plasmid constructs of these were prepared along with control constructs for nucleofection into cell cultures. Using both immortalized and primary neuronal cell culture systems, we performed extensive testing these constructs. None were effective in knocking down zebra finch Cntnap2 relative to levels in cultures that received control constructs. Thus, in year 2 we redesigned six targeting constructs using new bioinformatic techniques (Task 5). We have tested 3 of these, 2 of which substantially knock down exogenously expressed zebra finch Cntnap2 when co-transfected into HEK cells (Figure 2). We are currently testing these in vivo. Following production of high titer lentivirus at the UCLA viral vector core, we tested transduction efficiency both in vivo and in vitro (Task 6). We found low levels of expression of a reporter gene in both cases, below that required to produce a detectable behavioral result. Given technological advances in producing high titer adeno-associated virus, we tested 8 serotypes and promoters to determine those that effectively transduced songbird neurons. We found that

AAV serotype 2/1 and the chicken beta actin promoter efficiently transduce songbird neurons both in culture and in vivo.

KEY RESEARCH ACCOMPLISHMENTS
Year One
Milestone 1: animal approval.
Milestone 2: identification of an antibody that specifically detects zebra finch Cntnap2 in brain tissue and cell culture.
Milestone 3: Detection of appropriate sized bands via Western analysis. Alternative validation was accomplished via exogenous expression of Cntnap2 in multiple cell lines.
Milestone 4: Collection of developmental series of male and female zebra finch brains.
Milestone 5: Identification of brain regions containing the key premotor song control nucleus, RA, as well as outlying cortical areas using Nissl staining.
Milestone 6: Determination of the age at which Cntnap2 protein becomes enriched in male RA and is diminished in female RA.
Milestone 7a: An abstract on this work has been published and will be presented at the 2011 meeting of the Society for Neuroscience (see Appendix). An additional review article is in press (see White SA, under references) and a full primary research article is in manuscript format in preparation for submission and publication.
Milestone 7b: in progress

Year Two
Milestone 7b: We have now identified 3 shRNA constructs that effectively knockdown zebra finch Cntnap2 in vitro, and a control construct that does not affect these levels.
Milestone 8: Lentivirus was produced and delivered. However it proved ineffective in driving robust transgene expression. This is not surprising given that since 2007, no publications have emerged that use lentivirus in songbirds to alter behavior. Thus, we have switched to adeno-associated viruses for this goal.
Milestone 9: Partially complete: we have identified tutors with complex songs and have paired them with females for producing male sibling pairs.

REPORTABLE OUTCOMES
We previously found that zebra finch Cntnap2 expression patterns highlight vocal control regions in zebra finch brain (Panaitof et al., 2010), similar to the enrichment of human *Cntnap2* mRNA in brain regions that support complex cognitive processes including language development (Abrahams et al., 2007). This led us to hypothesize that Cntnap2 contributes to the regional and functional specification of brain regions important for socially-learned vocal communication in both humans and songbirds, a key phenotype affected in ASD. In support of this hypothesis, we now find that Cntnap 2 protein, like the mRNA, is enriched in the premotor song control nucleus known as RA, relative to its levels in the adjacent cortical areas. This enrichment is observed in young birds of both sexes. However, as development proceeds, it intensifies in males whereas it declines in females (Figure 1). This switch in expression corresponds to the time at which young male, but not female, zebra finches embark on sensorimotor learning of their vocalizations. Experimentally, this observation provides the critical time point and region at which to intervene in male Cntnap2 expression and determine subsequent behavioral effects on vocal learning and social interactions.

CONCLUSION
We have met each milestones (1-7) and, in the process, overcome a major hurdle in identifying a viral construct that successfully drives transgene expression in vivo. A manuscript describing these findings in nearing submission for publication (milestone 7a). Due to the unexpected weakness of lentiviral transgene expression, we are slightly behind our timeline outlined in the Gantt Chart. However, now that this challenge has been overcome, we expect to make rapid progress in the upcoming third year.

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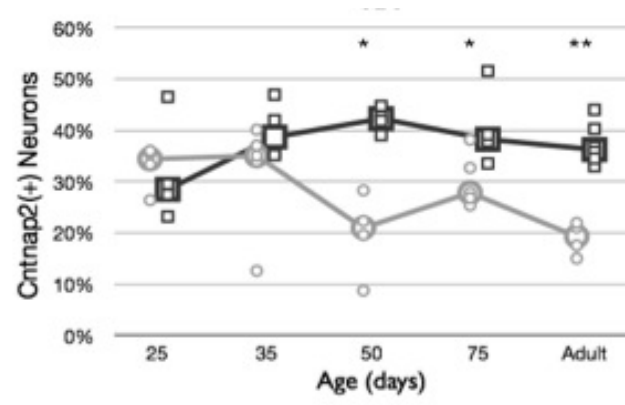
Figure 1. Quantification of Cntnap2 expressing cells in RA. Small squares (males) and circles (females) reflect the percent of NeuN(+) expressing Cntnap2 in individual animals. Large squares and circles reflect the means of each group. Statistical significance was determined by resampling T-test (* p<0.05; ** p<0.01).
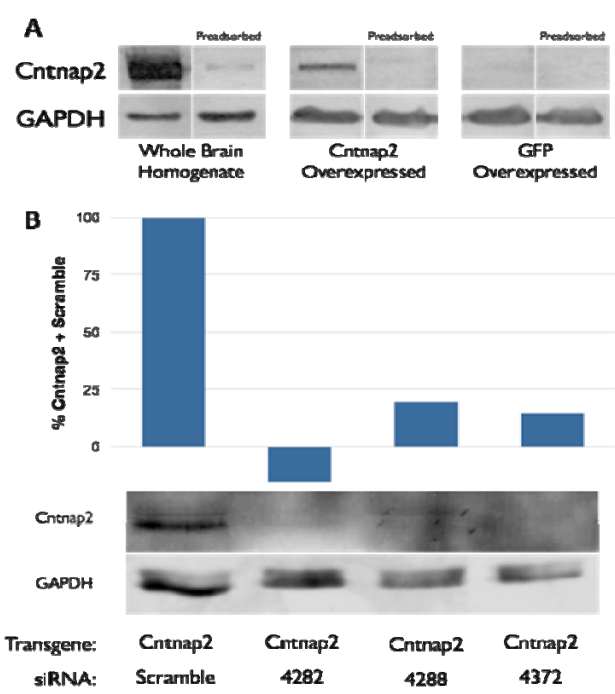

Figure 2. A) Anti-Cntnap2 antibody (Millipore ab5886) detects endogenous zebra finch Cntnap2 in whole brain homogenate (left). Overexpression of zebra finch Cntnap2 (GenBank: GU290551.1) in a zebra finch immortalized cell line (middle). Overexpression of GFP alone confirms no endogenous Cntnap2 expression in this line (right). In all controls, the antibody was preadsorbed with 30X antigen peptide. B) siRNA constructs designed to target zebra finch Cntnap2 were co-expressed in HEK 293 cells along with the construct containing coding sequences for zebra finch Cntnap2. All 3 constructs were able to prevent >75% of Cntnap2 from being expressed relative to a non-targeting scrambled control.

Abstract presented as a poster (Abstract 150.19) at the 2011 annual meeting of the Society for Neuroscience in Washington DC:

Autism Susceptibility Gene Contactin Associated Protein-like 2 Expression in a Songbird Model for Vocal Learning

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Contactin associated protein-like 2 (Cntnap2) is an exc iting molecule for the study of the genetic basis of language. In humans, Cntnap2 is a target of the FOXP2 transcription factor that is associated with verbal dyspraxia. Polymorphisms of Cntnap2 are linked to autis m spectrum disorder, specific language impairment, Gilles de la Tourette's sy ndrome, and additiona l neurological disord ers. Cntnap2 mutations correlate with neuroanat omical and functional abnormalities in language-related brain areas. Cntnap2 is among ~500 genes that have human-specific deletions in their regulatory regions relative to the chimpanzee suggesting that it plays a role in the evolutionary divergence of humans and apes. Since language is re stricted to humans, we must look to animal models that possess key aspects of language to study its cellular and molecular substrates. Songbirds such a s the zebra finch have been useful to study the genetic bas is of vocal le arning, a key aspect of language. This study focuses on zebra finch Cntnap2 expression in sexually dimorphic song nuclei responsible for vocal learning and production. We validated the use of a commercial antibody t o specifically detect Cntnap2 protein in the zebra finc h brain. Us ing this an tibody, we characterized Cntnap2 protein expression in both sexes at key time points during the critical periods for male song, and compared our findings to our prior mRNA studies. In the robust nucleus of the arcopallium (RA; a cortical nucleus critic al for vocal production) protein expression was enriched relative to the surrounding area and appeared restricted to a subset of cells within the nucleus. In males, this enrichment persisted throughout song development and into adulthood. In contrast, initially high levels at 25 days of age diminished in females to below levels in the surrounding region by 35 days of age. These observations parallel RA Cntnap2 mRNA expression. Like wise, protein levels parallel mRNA findings in the striatopallidal nucleus area X. There, Cntnap2 protein is lower than in the surrounding area throughout development in males. Females lack area X. In the lateral magnocellular nucleus of the nidopallium (LMAN), leve ls of protein were low, diverging from a high mRNA expression pattern. Ongoing experim ents will determine whether the enr ichment of Cntnap2 mRNA, but diminishment of protein is due transport of Cntnap2 pr otein away from the soma to LMAN nerve terminals in RA. Supported by 5 T 32 NS058280, NIH R21 HD 065271, and US Army AR093327.