Computational Tools for Behavioral Analysis of Zebra Finch Attractin Mutants

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Abstract

Zebra finch mutants for the attractin gene (*Atrn1*) had been shown to display defects in song learning, failing to reproduce a faithful copy of their tutor's song. However, it is not known whether these mutants have any other behavioral abnormalities such as hyperactivity or failure to form proper auditory song memories, a requirement for normal vocal learning. In this project, we developed a variety of computational methods to quantitatively characterize the behavior of *Atrn1*$^{-/-}$ mutants by fast and automated procedures. Zebra finch activity patterns were obtained through a MATLAB program designed to perform automated position tracking by extracting the animal's position from acquired video images. From records of the animal's position through time, we were able to derive heat maps, plots of cumulative distance travelled, and daily patterns, which can serve as objective standards of comparison of activities of mutant and wild-type finches. In addition, using automated song playback from two speakers, a phonotaxis experiment was conducted to examine the formation of auditory memories in these animals. For this purpose, we took advantage of the fact that wild type zebra finches naturally develop a preference for the tutor's song compared to an unknown song. Song preference of an animal was quantified by the time it spent in a pre-defined zone closer to the tutor song. Motion tracking and quantification of phonotaxis behavior are respectively automated and semi-automated, objective and reliable procedures, making them highly efficient tools for characterization of behavior of zebra finches in general.

I. Introduction

Verbal communication is a key function often affected in a number of neurodevelopmental disorders, including a subset of autism spectrum disorders (ASD). It has been hypothesized that the language impairments associated with these disorders may be due to perturbed assembly of brain areas involved in vocal communication[1]. Nevertheless, the precise contribution of developmental processes to vocal learning remains poorly understood. Studying the mechanism underlying vocal learning in animal models is therefore crucial to our better understanding of these diseases.

Insights into the function of neural circuits that control human speech development can be gained by using songbirds as a model organism. Songbirds serve as a valuable model because their song-development share several features with speech acquisition, including a critical period of vocal learning, the requirement of intact hearing, the existence of social contingencies for normal learning, and a set of circuits dedicated for learning and production of vocalizations[2]. Additionally, the songbirds' small size and the fact that they breed well in captivity makes them a tractable model for laboratory research. In the past few years, the Lois lab has developed a series of tools and techniques that enable the manipulation of specific cell types within the song system (Tarciso Velho personal communication), in addition to techniques such as lentivirus-mediated transgenesis, to study song learning in a transgenic setting[3,4].

In this study, we are interested in further studying a line of zebra finches in which the gene encoding the protein Attractin (*Atrn*) was mutated via insertional mutagenesis. *Atrn* mutant mice, called zitter (*zi*), show hypomyelination in the central nervous system, with a decrease in density

of myelin and abnormal formation of myelin sheath[5], hyperactivity[6], and dark coloration (in a brown background)[5]. Interestingly, our zebra finch mutants show abnormal song learning when compared to wild-type siblings, and, like the mouse mutant, finches homozygous for this mutation are dramatically darker than wild-type (Figure 1). Altogether, these observations suggest that *Atrn* plays similar roles in both species, and the zebra finch mutant thus presents a unique opportunity to study the role of myelination in vocal learning. We hypothesize that the deficiency in myelin production causes alterations in the bird's social behavior, which leads to the mutant's failure to reproduce a faithful copy the tutor's song.

Figure 1. Wild-type and Attractin mutant zebra finches.

Before examining the neuronal activity in mutants, we would first like to characterize their behavior to precisely isolate the behavioral components that are affected by *Atrn* disruption. Two important aspects of social interaction will be studied – the overall activity level and the preference for the tutor song, the latter is indicative of long-term auditory memories, complex acoustic signal processing, and social bond formation. More specifically, zebra finches exhibit phonotaxic behavior towards the tutor's song when presented with both the tutor's song and an unknown song[7]. This behavior requires the initial formation of long-term memories, followed by the processing of auditory information, recognition of the familiar song, and finally the expression of the preference. The disruption in any of these steps would lead to an unusual song preference behavior in *Atrn* mutants.

To measure these behavioral parameters, we have developed two main computational approaches in this study. Firstly, to quantify activity levels, we created an automated program to help us perform live tracking of the animal's motion. In addition, to quantify the degree of song preference, we adapted a protocol used in a previous study[10], and wrote a MATLAB script to control the sound presentation in a semi-automated manner. These two methods are fast, efficient and require minimal human supervision, which facilitates data collection and provides unbiased measures of behavioral performance.

II. Materials and Methods:

1. Description of mutants

Zebra finches carrying an insertion into the *Atrn* locus were generated through lentivirus-mediated transgenesis. Similar to the mouse *zi* mutants, these finches show a darker coat color compared to wild-type, demonstrating that the *Atrn* locus has in fact been disrupted. More importantly, these transgenic animals show impaired song learning compared to wild-type siblings (Figure 2). Curiously, mutant animals failed to faithfully copy specific syllables of the tutor's song, while the learning of other syllables is preserved, suggesting a specific learning deficit rather than a general sensory or motor problem.

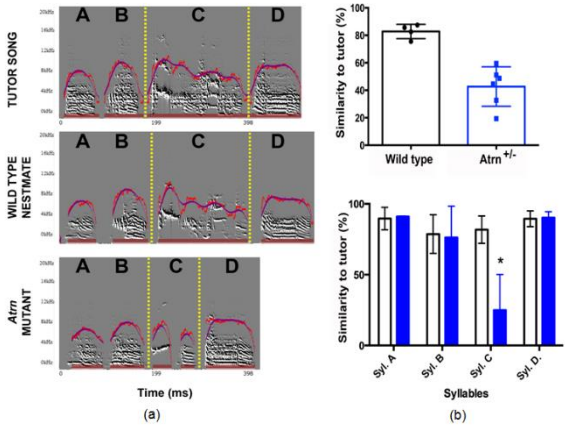

Figure 2. (a) Spectrogram of the tutor's song, (top) together with the song of wild-type (middle) and *Atrn* mutant (bottom) pupils at the end of the learning period.

(b) Top: percentage similarity between the pupil's song and the tutor's song.

Bottom: percentage similarity between the pupil's individual syllables and the corresponding syllables of the tutor's song. (white: wild-type, blue: *Atrn* mutant)

2. Real-time tracking of motion and behavior.

To facilitate the identification of the animal, we placed the bird in a light-colored environment so that its dark color stands out against the white background. A webcam was installed above each cage, acquiring images at approximately 25 frames per second.

A MATLAB script was written to track the animal's position by applying the following two-step procedure to each image frame (Figure 3A). The program first segments the image by identifying pixels whose intensities are lower than a specified threshold. Since the bird's color is dark and the environment is white by design, the segmentation yields a complete outline of the animal. Next, the centroid of this outline is computed, serving as a representation of the bird's location. Only the sequence of centroid positions is saved to retain the minimal information necessary for subsequent analysis. Each animal was tracked continuously for at least 3 days, each day from 7am to 9pm (we were unable to perform recording in the dark).

To investigate the movement profile in the presence of a companion bird, we introduced a white female in the same cage as the animal itself. Since this companion animal is white, its outline is not detected by the tracking software, thus allowing us to track the motion of only the dark-colored male.

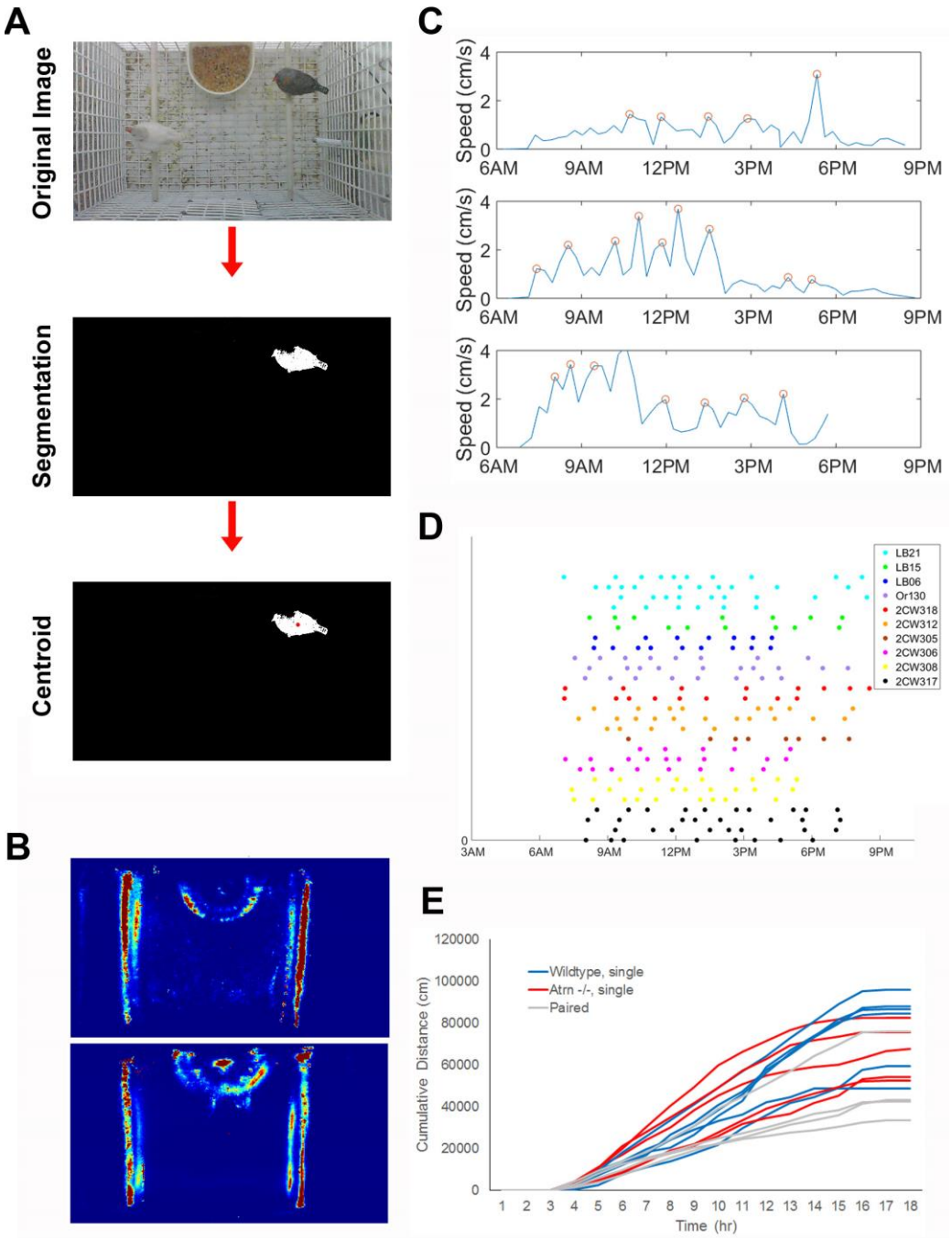

Figure 3. Activity patterns in zebra finches. A) Illustration of the position tracking algorithm. The algorithm is applied to every frame of the video stream to extract the position of the animal. Only the sequence of centroid positions is saved for further analysis (lower panel). B) Representative heat maps for a wild-type (top) and a mutant animal (bottom). C) Activity profile of animal in 3 consecutive days, revealing bouts of activity throughout the day (red circles). D) Activity peaks of all animals throughout the day. Each line represents one day of recording and each animal is represented by one color. LB21, LB15, LB06, Or130 are wild-type animals, and the rest are mutant animals. E) Average cumulative distance covered by individually housed wild-type (blue lines) and Attractin mutants (red lines) each day. Grey lines show activity profiles of a subset of birds (both wild-type and mutants) in the presence of a companion. Each line shows the cumulative distance profile of one animal, averaged across multiple recording days.

3. Song preference test.

Two speakers were installed on either side of a 120 cm x 45 cm x 45 cm cage (Figure 4A) to broadcast two different songs – one speaker plays the song of the tutor, while the other plays a song from an unknown conspecific. The cage was placed in an isolation chamber, and divided into three zones – a neutral zone in the middle flanked by two approach zones. Two wide-field cameras were placed directly above the boundary lines (dotted, figure 4A) to capture the bird's motion.

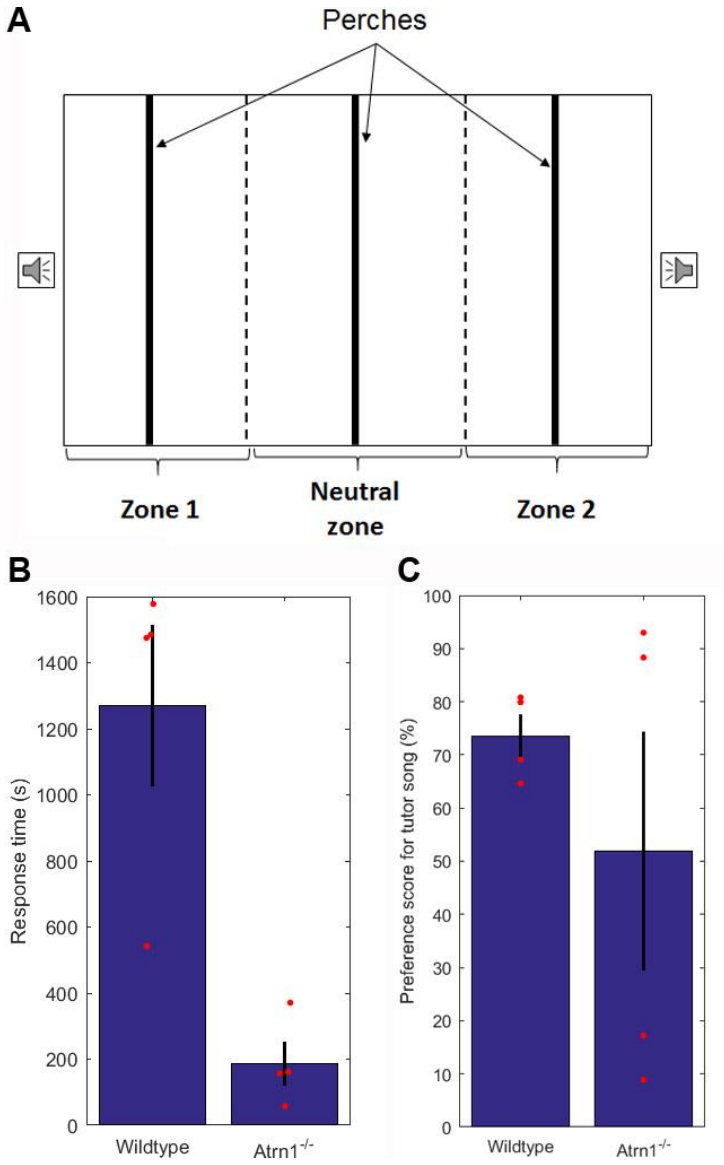

Figure 4. Song preference measurements. A) Schematic of song preference test setup. The diagram shows the top view of the cage, in which two speakers have been installed. The time spent in zones 1 and 2 was manually scored to determine whether a bird exhibits a preference to the tutor's song. B) Response time in wild-type and mutant zebra finches. C) Song preference score. The preference score was calculated by dividing the time spent in the tutor's zone by the total response time. In B and C, each bar indicates mean ± s.e.m, while each dot represents the preference score or response time of one animal.

Prior to testing, each bird stayed in the chamber for one day to acclimate to the setup while deprived of social interaction. Two test sessions were subsequently carried out using the protocol adapted from a previous study[10]. During 14 min, song A (tutor or novel; chosen randomly for each bird) was broadcast for periods of 1 min on the left side alternating with periods of 1 min during which song B (tutor song if song A was novel song and vice versa) was broadcast from the right side. On the next day, another session of 14 min was done in which the sides from which the two songs were played were reversed. Sound files were normalized using WavePad Editor. The songs were broadcast at 70 dB, measured at 20 cm from the speaker.

III. Results

1. Activity levels of Atrn1$^{-/-}$ mutants

Using the automated tracking software, we gathered activity profiles of 10 male finches (4 wild-types and 6 mutants). An example activity profile is shown in Figure 3C. These profiles revealed cyclical bouts of higher activity (red circles in Figure 3C, where each peak is defined as a maximum point in the speed profile which is at least 0.5 cm/s higher than the baseline). The timing and frequency of these movement peaks does not seem to vary between wild-type and mutant animals (Figure 3D).

Based on tracking data, we calculated the cumulative distance covered by the animals on each day, and averaged across recording days to obtain a representative profile for each animal (Figure 3E). These profiles showed that activity levels are highly variable across animals, and no discernable difference could be observed between Attractin mutants and wild-types. Indeed, the total distance travelled each day is not statistically different between mutant and wild-type animals ($p = 0.19$, Welch two sample t-test).

Finally, we considered the possibility that Atrn1$^{-/-}$ animals could have different spatial pattern of activity compared to wild-type animals. To test this possibility, we made use of activity heat maps, which show the areas of the cage the animals visit most frequently. Visual inspection of these heat maps did not reveal any discernable difference between animals. All animals spent most of the time near the food source or on the two perches (Figure 3B).

Altogether, our data suggest that when isolated, mutant animals do not show hyperactive behavior as we hypothesized, and they have a similar activity pattern to that of wild-type animals.

2. Activity levels in the presence of companion bird

To examine the influence of the social context in the animal's activity, we next measured the activity levels of individual males in the presence of a female companion. In 3 out of 4 animals, we observed a dramatic reduction in activity, such that their cumulative distance profiles are lower than all profiles of isolated animals (Figure 3E, grey lines). The remaining animal showed an activity level which is only 10% lower when the companion was introduced. Overall, the daily distance travelled is significantly decreased in paired animals compared to single animals ($p = 0.027$, Welch two sample t-test, one-tailed). Thus, even though the sample size is small ($n = 4$), the present data support the finding that activity levels decrease when the animal is paired with a female.

3. Song preference behavior of Atrn1$^{-/-}$ mutants

Eight female birds (four wild-type and four mutant) were tested using the song preference protocol. For each animal, the time spent in the tutor song's zone and the novel song's zone were manually scored by noting the time the animal crosses any of the two boundaries. Preference for the tutor's song was calculated by the dividing the time spent in the tutor song's zone by the total response time (the time spent in the approach zones).

Two significant differences could be observed in the phonotaxis behavior of Atrn1$^{-/-}$ mutants. Firstly, these animals spent significantly less time in the approach zones than wild-types ($p < 0.01$, Welch two sample t-test; Figure 4B), with an average response time that is 15% the average response time of wild-type animals. This reduction in response time could arise due to hearing or auditory processing deficits in Attractin mutants. Alternatively, this could also indicate a potential social deficit, since mutant animals could have a decreased motivation to approach and socialize while other birds are singing.

Secondly, we observed a change in the pattern of preference for the tutor's song in transgenic animals. While all wild-type animals showed preference for the tutor song (average tutor preference score of 72%), the mutant animals either strongly preferred the tutor song (tutor preference score >80%), or strongly preferred the novel song (tutor preference score <20%). This suggests that Attractin mutants can still discriminate between two songs and express a consistent preference for one of them, however, this preference could potentially be random and appears uninfluenced by the degree of familiarity with the song.

IV. Discussion

The automated tracking program revealed a highly variable profile of activity patterns across animals. This is not surprising since activity depends on the animal's state as well as environmental conditions, all of which can be highly variable between animals and recording days. This is particularly important in captive zebra finches as they are not an inbred species. Nevertheless, we did observe relatively consistent heat maps, indicating that all animals spent most of the time perching or feeding.

Another consistent pattern we discovered was the reduction in activity levels in the presence of a companion bird. We considered the possibility that this reduction might be due to a decrease in repetitive behavior (hopping back and forth between perches). Indeed, we quantified the amount of hopping by counting the number of times each animal crosses the midline, and found that the number of hops per day significantly decreases in paired animals ($p < 0.01$, Welch two sample t-test, one-tailed; the average number of hops for paired animals is only 38% that of singly housed animals). Thus, the lower activity levels can be attributed to a less repetitive behavior and could be an indication of lower stress levels and increased comfort when the animal is housed together with a partner. This consistent decrease in movement will be highly valuable in future studies to assess the level of anxiety of Atrn1$^{-/-}$ animals in social interactions. More specifically, if Attractin mutants are overly anxious, we would expect a lower extent of decrease, or even an increase in distance travelled, after the introduction of a female partner.

For the phonotaxis experiment, we observed a significant change in song preference in Attractin mutants as compared to wild-types. However, it is unclear whether this aberrant behavior is due to a defective long-term memory, which causes a failure in recognizing a familiar song, or due to an abnormal expression of preference despite intact auditory memory. Future experiments can dissociate these two causes by making use of a classical conditioning experiment as described previously[11], in which the bird has to differentiate between two different songs, one associated with a punishment and the other is not. The ability to learn to avoid the aversive stimulus will directly reflect the animal's capacity to form auditory memories and help us evaluate the cause of abnormal song preference in Attractin mutants.

Overall, the computational methods that we developed in this study, namely motion tracking and automated song presentation, have revealed novel findings about the behavior of both wild-type and Attractin transgenic animals. These results not only are a promising first step towards a better understanding of the song learning defect shown by Attractin mutants, but they also give us more confidence in the use of these protocols and methods in future characterizations of general behavior in zebra finches.

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