# Autism as a Developmental Disruption in the Brain Mechanisms for Learning and Memory: Evidence from Analysis of Free-Swimming Behavior in Shank3 Mutant Zebrafish

*S. Iyer[1,3], N. Najib[1], S. Asdjodi[1], E. Glasgow[2] and J. S. Kanwal[1]

Departments of [1]Neurology, [2]Tumor Biology, Georgetown University Medical School, Washington, D.C. 20057, [3]Thomas Jefferson High Sch. for Science and Technology, VA

## Introduction

**Objective**: Test the role of Shank3 in motivating social interactions and on short-term memory, employing a free-swimming spatial task using visual proximity to conspecifics as a motivating cue. Develop behavioral profiling as a quantitative diagnostic tool for early detection of those on the autism spectrum.

**Background**: The Shank3 protein, present within post-synaptic densities, has been linked to autism in humans. Zebrafish are cost-efficient models for neurological studies (Norton and Bally, 2010, p.1471). Shank3 mutant zebrafish lack dominance in social hierarchies and exhibit more anxiety than wild type fish. The developmentally-mediated lack of interest for social interactions in humans with either condition is also expressed in Shank3 mutant zebrafish (Uchino & Waga, 2013, p.106-107). Working memory has been impaired for for object recognition and social transmission of food preference tests in Shank3 mice (Durand et al., 2007; Moessner et al., 2007).

**Hypotheses**: Compared to wild type fish, Shank3 fish…

1. Have distinct socially-motivated free-swimming behavioral profiles, including socially-motivated swim velocities.
2. Spend less time swimming closely around an enclosure with conspecifics relative to one containing inanimate zebrafish-sized objects.
3. Fail to associate conspecifics with visuo-spatial cues.

## Methodology

Zebrafish were bred and reared in 14:10 light:dark cycle at 28°C. They were fed daily with brine shrimp and dried flake food and housed in one-liter habitat tanks with filtered and aerated water, containing 5 male and 5 female 3- to 5-month-old adult zebrafish. Fish were transported to the laboratory in habitat water and scooped out with a small container to avoid stress from hypoxia.

**Experimental Design**:

1. Examine zebrafish social behavior to create a baseline for normal social interactions.
2. Create a free-swimming choice test using social interaction as a motivator.
3. Collect and analyze data for our Shank3 v. WT behavioral profiles (n=3).

**Social Behavior Study**:

- Dominant fish display aggressive behaviors towards subordinates like chasing and biting.
- Dominant fish remain primarily isolated while subordinates remain in groups.
- Proportions of wins and losses can be used to determine social hierarchies.

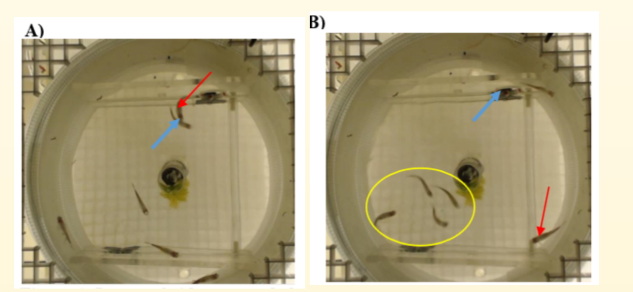



*Fig. 1.* **Development of socially-motivated dominance hierarchies.** A dominant individual (red arrow) tends to observe conspecifics (within yellow oval) from its "personal" space (B). This is preceded by "dashes" (high speed swimming) and chases towards conspecifics (A).

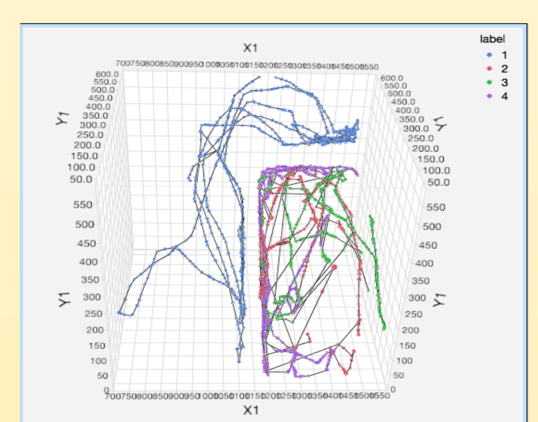


*Fig. 2.* **3D scatterplot generated form tracking data using iDTracker software** (Pérez-Escudero et. al., 2014)**.** Tracks show attraction of a test fish (blue trace) to 3 target fish (conspecifics) placed within a sub-enclosure (red, green & purple tracks). Social behavior studies suggested development of a social motivation paradigm for zebrafish.

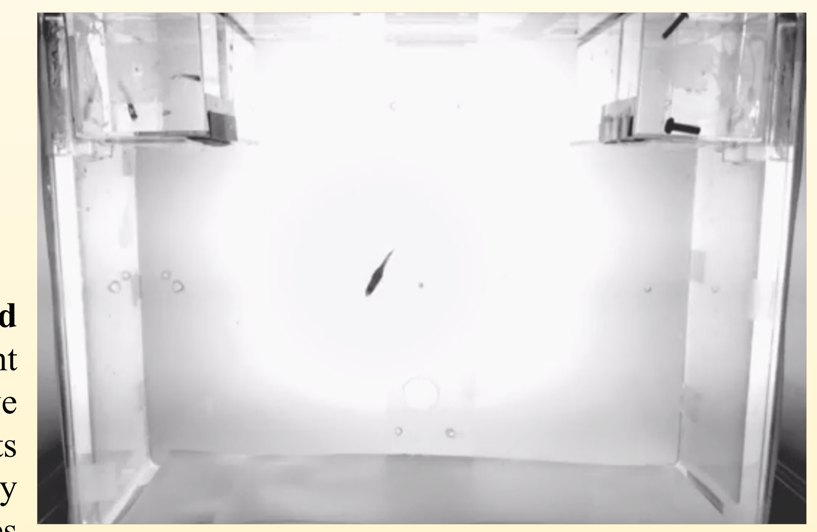


*Fig. 3.* **Photographic representation of the free-swimming choice test.** Each Plexiglass sub-enclosure was marked with different colored plastic strips to create visual cues for associative memory (Blaser & Vira, 2014). Conspecifics were placed in the sub-enclosure on the side opposite to the animal's preference and two artifacts (metal screws) on the non-rewarded side. In this case, conspecifics were in the right sub-enclosure and artifacts on the left. Ten minutes each were provided for subject acclimation, social interaction, and associative memory tests. For the first, the sub-enclosures were empty. For the second, sub-enclosures contained conspecifics and artifacts. For the third, view inside sub-enclosures was obstructed for the fish. Subsequent iDTracking produced density plots of fish location within test tank to identify sub-enclosure preferences.

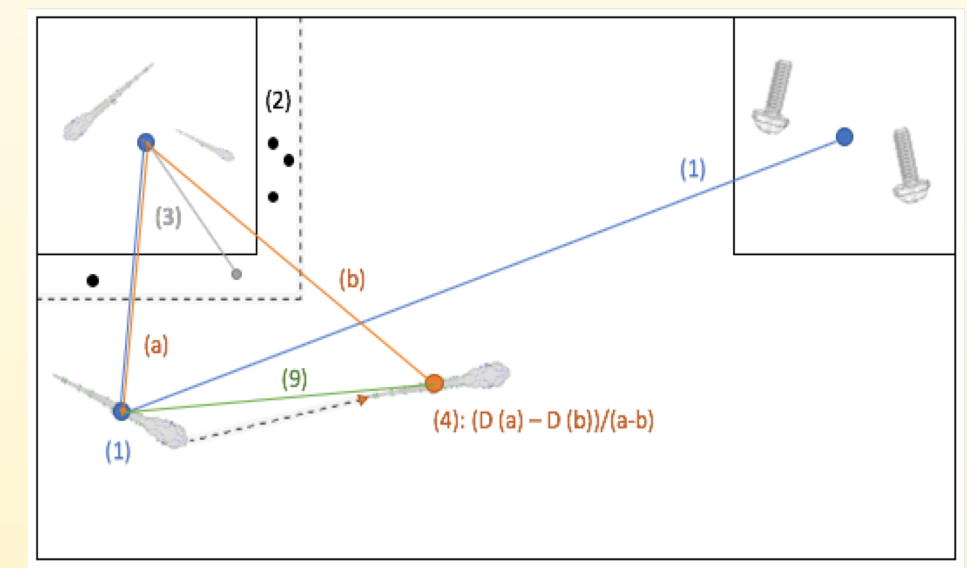



*Fig. 4.* **Analyzed measures labeled within the paradigm.** Coordinates were extracted per video frame using idTracker. Average distance of the test subject per frame from the center of each sub-enclosure was calculated (1). The number of data points within 6 cm (around the perimeter) from the rewarded sub-enclosure center was found (2). The average distance of these data points from the rewarded sub-enclosure center, at or less than 6 cm, was noted (3). Average swim velocity (cm/ms) towards the rewarded sub-enclosure center was calculated (4). Positive velocities were labeled with a '1' for approach and negative with '0' for retreat. Five or more consecutive '1's resulted in an "episode", for which we then found the number (5), average time (6), distance (7), and speed (8). Average swimming velocity between frames was calculated (9). All measures were calculated for seven time bins of 2700 frames or 1.5 minutes each. Values were averaged by genotype and weighted appropriately to create a social interaction index per time bin. 95% confidence intervals of each measure for wild type and Shank3 mutant fish were found for behavioral profiles. Scatterplots of proximity to conspecifics and swim velocity per approach episode between genotypes were also generated.

## Results

*Fig. 5 (left).* **Density plots of the location of wild type (A) and Shank3 (B) fish in the test tank during trials of social motivation.** Xy-coordinates of fish were obtained using iDTracker over a period of 16,200 frames, or 9.5 minutes. Down-sampled data to show fish locations (recorded at 30 fps). Wild type fish show preference for conspecifics, while Shank3 fish spend significant time away from sub-enclosures and even when near, without preference for either.

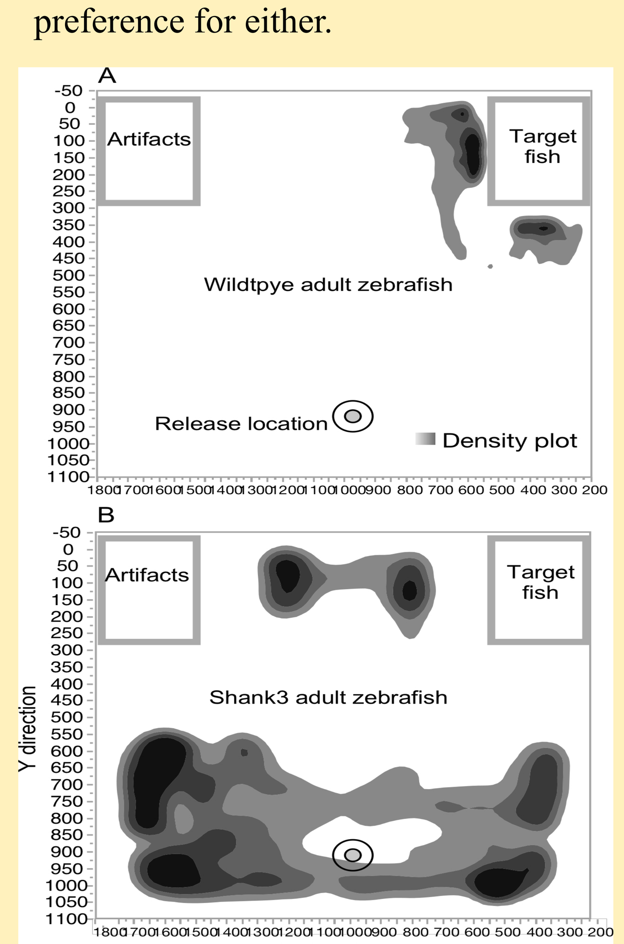
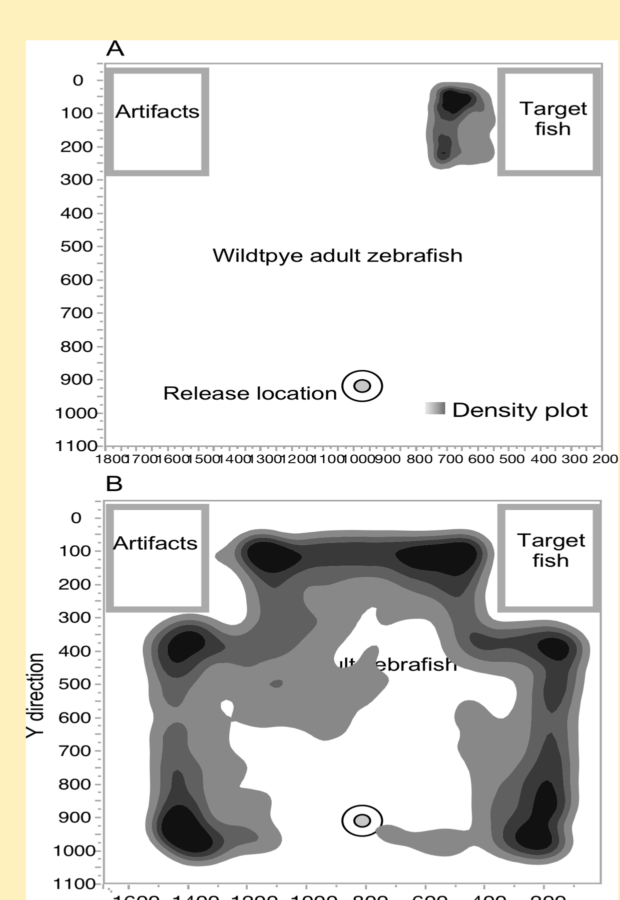



*Fig. 6 (left).* **Density plots of the location of wild type (A) and Shank3 (B) fish in the test tank during trials of associative memory.** Wild type fish show clear preference for sub-enclosure with conspecifics. Shank3 fish are noticed at all locations in the tank, even near artifacts for numerous frames.



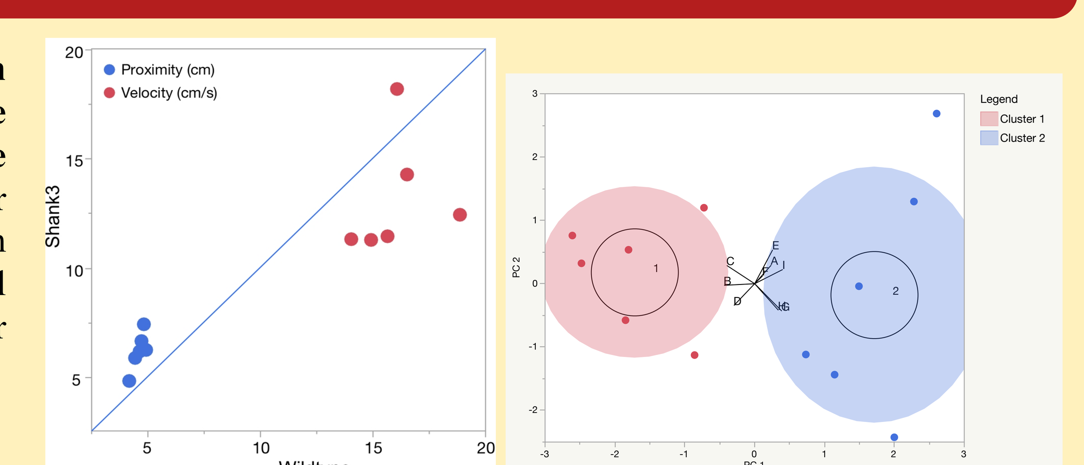




*Fig. 7 (left).* **Scatterplot of proximity to conspecifics (blue) and swim velocity per approach episode (pink) of Shank3 vs. wildtype fish.** *(right).* **Cluster analysis of structural and temporal parameters in the behavioral profile of Shank3 vs. wild type adult zebrafish.** Altogether nine parameters (labeled in the figure from A to I) were included in the model. The ray plot in the middle shows the orthogonal contributions of each parameter, indicating minimal correlation between most of the measured parameters. Solid circles indicate the location along the first two principal components of the overall behavioral profile captured within seven successive time bins included in the model. Data was obtained from three Shank3 and three wild type fish. Circles and shaded areas indicate 50% and 95% confidence intervals, respectively, of the multiparametric boundaries of each cluster. Non-overlapping boundaries indicate significant differences between the two clusters. Blue = wild type, pink = Shank3. Note the relatively smaller spread (a more stereotypic behavioral profile) between behavioral measures in Shank3 vs. wild type fish.

## Conclusions

All hypotheses were satisfactorily tested:

- Distinct behavior profiles exist for Shank3 and wild type adult zebrafish
- Less activity in "social cells" localized to ventromedial region of telencephalon reduces the value of social interaction for Shank3 fish = lack of socially-motivated mobility and associative memory (Lal et al., 2018).

Applications of our high-throughput screening paradigm:

- Automation to test with other aquatic and mammalian species
- Behavioral profiling for mutations in the variant synaptic proteins (e.g. Shank3) that cause Autism Spectrum Disorders (ASDs) and Schizophrenia (SCZ), drugs, or lesions.

## References


- Blaser, R. E., & Vira, D. G. (2014). Experiments on learning in zebrafish (Danio rerio): A promising model of neurocognitive function. *Neuroscience and Biobehavioral Reviews*, *42*, 224-231.
- Durand, C. M., Betancur, C., Boeckers, T. M., Bockmann, J., Chaste, P., et al (2007). Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nature Genetics*, *39*(1), 25–27. https://doi.org/10.1038/ng1933
- Lal, P., Tanabe, H., Suster, M. L., Ailani, D., Kotani, Y., Muto, A.,…Kawakami, K. (2018). Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. *BMC Biology*, *16*(45), 1-18. https://doi.org/10.1186/s12915-018-0502-y
- Moessner, R., Marshall, C. R., Sutcliffe, J. S., Skaug, J., Pinto, D., Vincent, J., Zwaigenbaum, L., Fernandez, B., Roberts, W., Szatmari, P., Scherer, S. W. (2007). Contribution of SHANK3 Mutations to Autism Spectrum Disorder. *American Journal of Human Genetics, 81*(6), 1289–1297. https://doi.org/10.1086/522590
- Norton, W., & Bally-Cuif, L. (2010). Adult zebrafish as a model organism for behavioural genetics. *BMC Neuroscience*, *11*(90), 1471-2202.
- Pérez-Escudero, A., Vicente-Page, J., Hinz, R. C., Arganda, S., & de Polavieja, G. G. (2014). idTracker: Tracking individuals in a group by automatic identification of unmarked animals [Computer software].
- Uchino, S., & Waga, C. (2013). SHANK3 as an autism spectrum disorder-associated gene. *Brain and Development*, *35*(2), 106-110.


## Acknowledgements

Supported by BGRO, Georgetown University, and a grant from Pioneer Academics, LLC. I would like to thank Riya Dabbi for her contribution with programs for data analysis and Dr. Glasgow for breeding, genotyping and maintaining zebrafish.