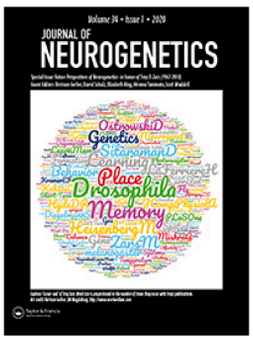

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REVIEW ARTICLE

Learning to collaborate: bringing together behavior and quantitative genomics

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ABSTRACT

The genetic basis of complex trait like learning and memory have been well studied over the decades. Through those groundbreaking findings, we now have a better understanding about some of the genes and pathways that are involved in learning and/or memory. However, few of these findings identified the naturally segregating variants that are influencing learning and/or memory within populations. In this special issue honoring the legacy of Troy Zars, we review some of the traditional approaches that have been used to elucidate the genetic basis of learning and/or memory, specifically in fruit flies. We highlight some of his contributions to the field, and specifically describe his vision to bring together behavior and quantitative genomics with the aim of expanding our knowledge of the genetic basis of both learning and memory. Finally, we present some of our recent work in this area using a multiparental population (MPP) as a case study and describe the potential of this approach to advance our understanding of neurogenetics.

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Learning; memory; MPP; genotype to phenotype mapping; complex traits

The genetics of learning and memory

Troy Zars was the kind of person who would get excited by all sorts of science, and he had the vision to recognize the potential links between his own research and areas that were sometimes fairly far afield from his own work. It was through this vision and his leadership that a collaboration began between the Zars lab, a neurogenetics lab, and the King lab, a quantitative genetics lab, with the goal of combining these research approaches to understand the genetic mechanisms determining individual-level variation in the phenotypes learning and memory. Two graduate students, Mathangi Ganesan and Patricka Williams-Simon, joined this collaboration and were co-advised between the two labs. Here, this research group discusses both how this research direction has influenced the field as a whole, and how working with Troy influenced our own scientific thinking and professional trajectories.

The ability of animals to respond to their environment via behaviors is critical to their survival and reproduction. One set of processes that are of particular importance are learning and memory. In this review we define learning as the change in an individual's behavioral capacity based on experience, and memory as the persistence of that changed capacity in the absence of the experience (Pearce, 2008). We also acknowledge that while an individual must learn in order to remember, the two phenotypes can to some extent be independent. These terms encompass a wide array of different processes, which have been categorized into different types of learning and memory. The most extensively studied type of learning is associative learning where a cue preceding an aversive or appetitive stimulus is associated with the onset of that aversive or appetitive stimulus (e.g. Busto, Cervantes-Sandoval, & Davis, 2010; Pascual & Préat, 2001; Pitman et al., 2009; Sitaraman et al., 2008). Additionally, cues that follow an aversive event signaling the offset of the aversive stimulus can be learned and approached later. Such a phenomenon is called relief or backward conditioning (Yarali & Gerber, 2010). Learning cues associated with environmental changes to alter one's own future actions toward these cues is called classical associative conditioning (Owald, Lin, & Waddell, 2015). In classical conditioning procedure, the animal is trained with a cue called the conditioned stimulus that is paired with an aversive (e.g. electric shock) or appetitive (e.g. sucrose) stimulus called the unconditioned stimulus (Kahsai & Zars, 2011; Pitman et al., 2009). The animal is then expected to either avoid or approach the conditioned stimulus and the animal's performance is considered as a measure of how well they have learned or do remember. For example, in aversive olfactory conditioning, especially in an odor-shock paradigm, groups of flies are trained to associate odor cues with the onset of an electric shock (Figure 1(B)). In the test phase, the flies avoid the odor associated with the electric shock indicating olfactory memory. Operant associative learning is where an animal learns how one's action helps attaining the best out of the environment (Kahsai & Zars, 2011). In an operant conditioning experiment, an animal's action determines stimulus outcome which then modifies the animal's subsequent behavior in an experience-dependent manner (e.g. heat box paradigm – Figure 1(C); (Wustmann, Rein, Wolf, &

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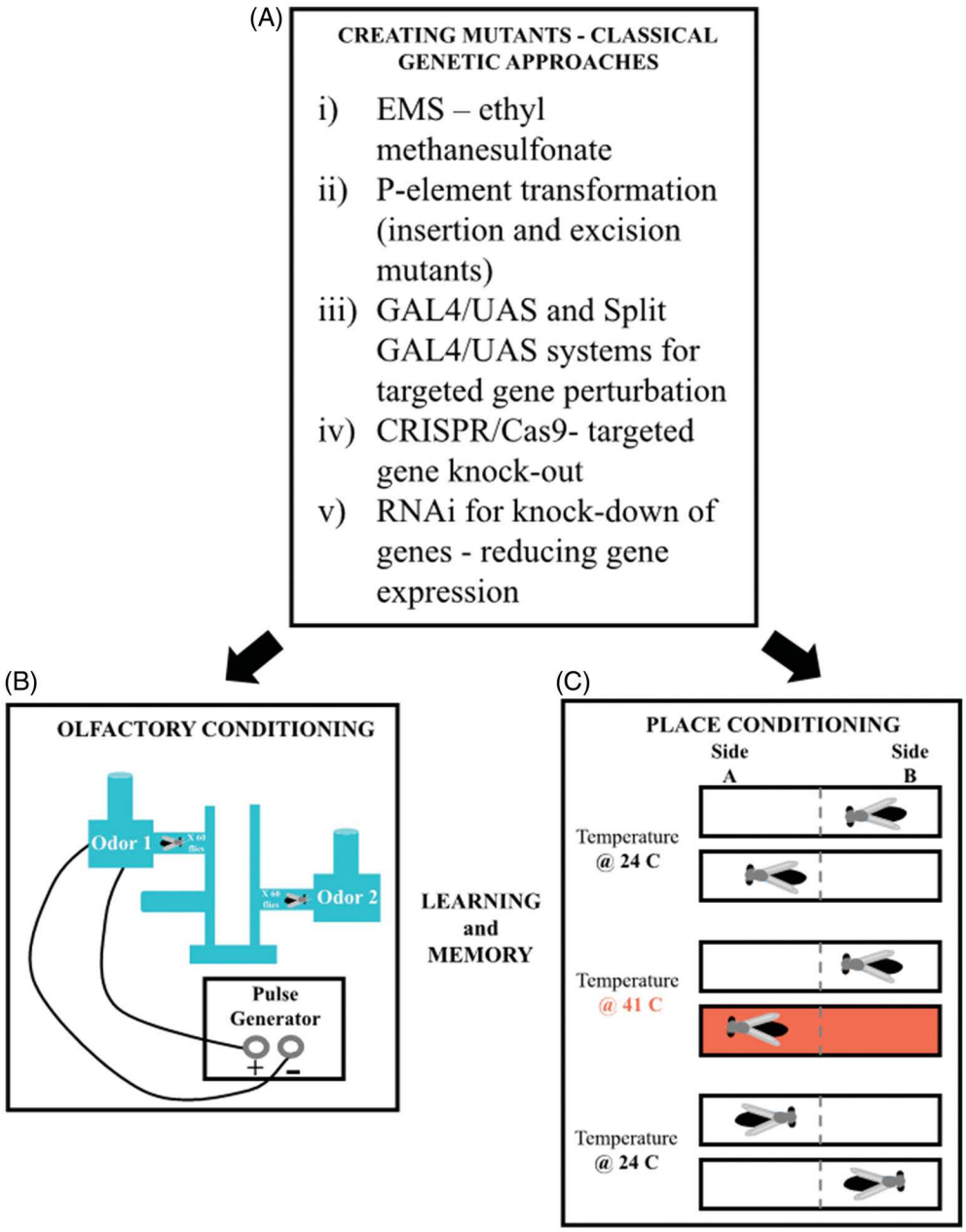

Figure 1. Mutagenesis – A classical genetic approach to study the genetic basis of behaviors. (A) Some of the mutagenesis methods used to study various behaviors including learning and/or memory are listed here. These techniques can also be used to target various parts of genes to identify critical sections of the genes contributing to a phenotype. The mutants generated using these methods can be tested in a wide variety of behavioral paradigms to test the necessity of the gene for the behavioral phenotype. Examples of commonly studied learning and memory paradigms are: (B) olfactory conditioning, specifically the discriminative olfactory conditioning, where flies are loaded into a T-maze set up where the flies learn to avoid an odor associated with electric shock (Tully & Quinn, 1985). (C) place conditioning where flies learn to avoid the side of the chamber associated with heat (Sitaraman et al., 2008; Wustmann et al., 1996).

Heisenberg, 1996; Zars, Wolf, Davis, & Heisenberg, 2000). When learning and/or memory varies among individuals, populations, and/or species, a major goal of neurogenetics is to identify the genetic mechanisms underlying that variation.

Several early experiments established that both learning and memory have a genetic basis and are heritable traits, including twin studies in humans (Galton, 1875), and selection experiments in both rodents and insects (Lofdahl, Holliday, & Hirsch, 1992; McGuire & Hirsch, 1977; Tolman, 1924). While these studies show that there are genes influencing learning and memory phenotypes, they do not tell us anything about which specific genes are involved. Early approaches to identifying the specific genetic determinants of learning and/or memory induced mutations to identify mutants that show a phenotypic effect. For example, exposure to a mutagen called Ethyl methane sulfonate (EMS) that induces mutations allowed Dudai, Jan, Byers, Quinn, and Benzer (1976) to discover *dunce* mutant flies which showed deficits in avoiding the shock associated odor suggesting that the *dunce* gene is necessary for learning. This same method was used to identify the *rutabaga adenylyl cyclase (rut)* mutant (Livingstone, Sziber, & Quinn, 1984) that has deficits in several learning and memory paradigms including the odor-shock and heat box paradigms (Han, Levin, Reed, & Davis, 1992; Wustmann et al., 1996). Several techniques allow for more targeted single gene mutagenesis, where

researchers perturb a particular gene and then study what effect/s that has on the phenotype (Godenschwege et al., 2004; Krashes & Waddell, 2008; LaFerriere et al., 2008; Ostrowski, Kahsai, Kramer, Knutson, & Zars, 2015; Zars, Wolf, et al., 2000). This approach has been commonly used across many organisms and have been pivotal to our understanding of many biological processes (De Souza, Hashmi, Horn, & Osmani, 2006; Hoppe, Chau, Flanagan, Reedy, & Schriefer, 2010; Howell et al., 2007; Lesuisse et al., 2005). In flies, the presence of transposons called P-elements have been advantageous for the development of other gene mutants by gene disruption through controlled excision or insertion of P-element (Han et al., 1992; Levin et al., 1992). This method has also been used to identify mutants with deficits in learning and/or memory (*rut*^{1} and *rut*^{2080}) Dudai & Zvi, 1985; Gervasi, Tchénio, & Preat, 2010; Han et al., 1992; Levin et al., 1992; Zars, Wolf, et al., 2000). Also in *D. melanogaster,* the development of binary genetic tools such as the GAL4/UAS, split GAL4/UAS and lexA/LexAop systems that target specific genes in specific tissues to modulate their function (Pfeiffer et al., 2010) have revolutionized the field of neurobiology and developmental biology (McGuire, Roman, & Davis, 2004), enabling studying neuromodulation down to a single neuron in fruit flies and other model organisms (Garrity, Goodman, Samuel, & Sengupta, 2010; Hige, Aso, Modi, Rubin, & Turner, 2015; Kitamoto, 2001; König, Khalili, Niewalda, Gao, & Gerber, 2019; Marella et al., 2006; Sitaraman, Aso, Rubin, & Nitabach, 2015; von Philipsborn et al., 2011). Finally, additional single gene approaches include the use of the RNAi system that specifically degrades the mRNA of the gene of interest, thus, reducing the gene expression significantly (Perrimon, Ni, & Perkins, 2010), and more recently the CRISPR/Cas9 system has been developed for targeted gene insertion or deletion (Jinek et al., 2012; Ran et al., 2013). Attributes such as high specificity, ease of design and cost efficiency have exalted CRISPR/Cas9 system to the forefront of genetic tools used to study genetic antecedents of various biological processes (Ekman et al., 2019; Gasparis, Przyborowski, Kała, & Nadolska-Orczyk, 2019; Lentsch et al., 2019; Prolo et al., 2019).

Troy incorporated several of the above approaches (listed in Figure 1(A)) to answer his research questions. His work is well-known for research that furthered the understanding of key genes and neuronal pathways in which their products function in *D. melanogaster* learning and memory. Zars, Wolf, et al. (2000) discovered that *rut* gene plays a critical role in only certain regions of the fly brain in the operant heat box paradigm. In addition, Zars, Fischer, Schulz, and Heisenberg (2000) were able to localize rut-dependent associative olfactory short-term memory in the fruit fly brains to specific sets of neurons in the mushroom body. The role of *rut* in memory has been further confirmed with a meta-analysis (Tumkaya, Ott, & Claridge-Chang, 2018). Additionally, Troy's work extended to studying other aspects of the odor-shock paradigm such as, memory extinction processes (Schwaerzel, Heisenberg, & Zars, 2002).

In more recent years, the Zars lab played a key role in developing novel modifications to the heat box paradigm for studying place learning and memory, which is a major contributor in expanding our understanding of behavior in fruit flies. The heat box is a specialized apparatus that uses temperature as a stimulus to determine the extent to which individuals learn or not. For example, a mutation in the *tribbles (trbl)* gene was found to reduce place memory and enhance olfactory memory, but had no effect on ethanol sensitivity, whereas a mutation in the *ethanol sensitivity with low memory* (*elm*) gene did not alter place memory, however, it reduced olfactory memory and strengthened ethanol sensitivity (LaFerriere et al., 2008). His research was the first to show that perturbation of the *radish* (*rad*) gene reduced place memory, but not place learning (LaFerriere, Speichinger, Stromhaug, & Zars, 2011). The lab also distinguished the role of *arouser* (*aru*) gene in place and olfactory memory formation, in the heat box and the odor-shock paradigms (LaFerriere, Ostrowski, Guarnieri, & Zars, 2011). *Arouser* mutations giving rise to increased and reduced expression of the gene was seen to elicit reduction in place and olfactory memory respectively. Several additional *D. melanogaster* genes are known to play crucial roles in multiple paradigms (for a summary see Kahsai and Zars 2011).

In addition, the Zars lab's work was the first step in identifying the roles of several biogenic amines in place learning and memory within the heat box (Figure 1(C)). His lab elucidated that place memory was not contingent upon a functional octopamine system (Sitaraman, Zars, & Zars, 2010), and that serotonin system function was essential for place memory formation, but that majority of the dopamine neurons relaying aversive signals were not (Sitaraman et al., 2008). At that time dopamine neurons signaling appetitive information (PAM neurons) were not identified and hence, were not covered in (Sitaraman et al., 2008). Recently, the lab investigated the role PAM neurons and found that they modulated fly behavior in the heat box (Mishra et al., – see current issue). The lab also investigated the role of different subsets of serotonin neurons and found that two subsets of serotonin neurons relayed the aversive reinforcement signal necessary for place memory formation (Sitaraman, Kramer, Kahsai, Ostrowski, & Zars, 2017). Flies when exposed to high temperatures before the conditioning protocol showed increased escape latency and memory (Sitaraman et al., 2017). The lab tested the necessity and sufficiency of the two subsets of serotonin neurons that relay aversive reinforcement by blocking and activating them respectively in a high temperature pre-exposure condition. These neurons were however, not sufficient to induce memory enhancement after pre-exposure to high temperature which was substituted by the activation of serotonergic neurons (Sitaraman et al., 2017). These studies as a whole provided insights into the underlying mechanisms determining how animals learn and how memories are formed.

While these types of approaches have been hugely informative in identifying genes that are critical to normal neuronal functioning, the connection between these studies and natural variation in learning and memory performance

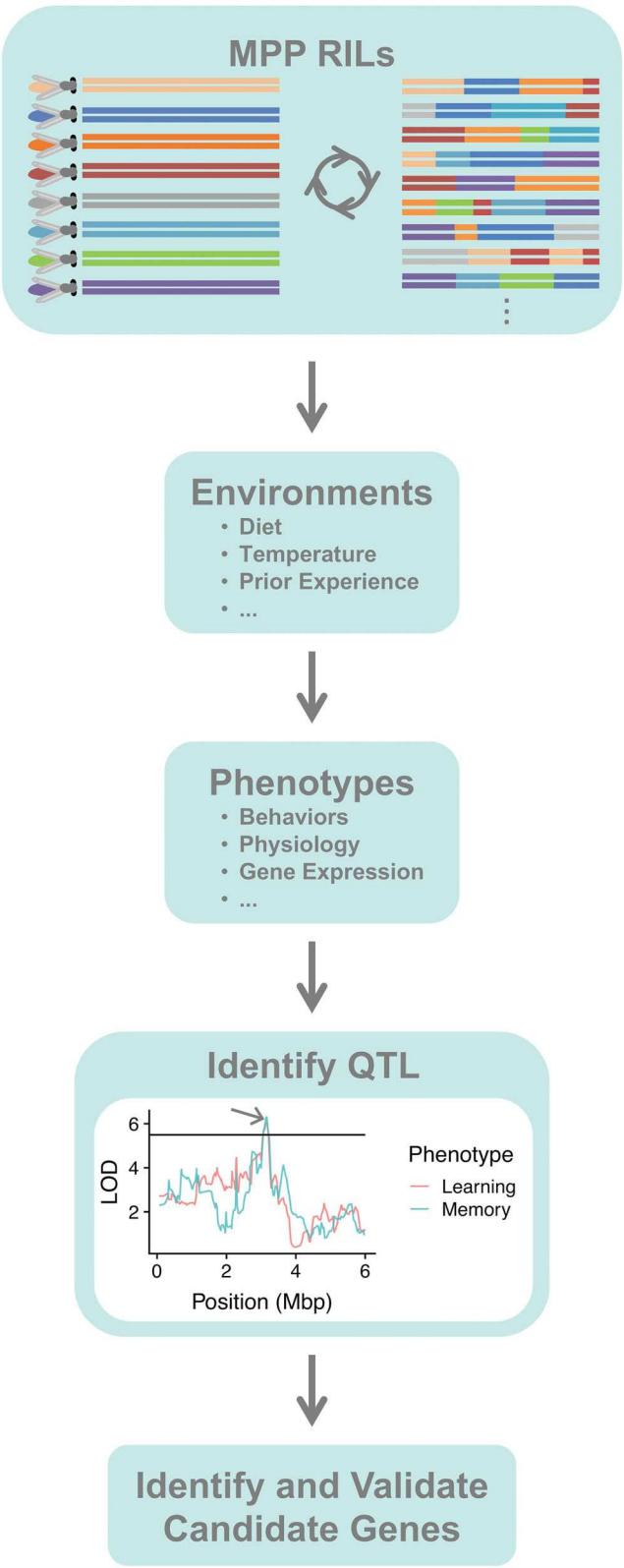

Figure 2. The multiparental population (MPP) approach to linking genes to complex phenotypes. The *Drosophila* Synthetic Population Resource (DSPR) MPP was created by crossing 8 inbred founder lines for 50 generations followed by 25 generations of inbreeding to generate a set of over 800 recombinant inbred lines (RILs; (King, Macdonald, et al., 2012; King, Merkes, et al., 2012)). These stable mosaic genotypes can be assayed in multiple environments for multiple phenotypes to map QTL across levels of biological organization, with the ultimate goal of identifying and validating the causal genetic variants.

has been elusive. There have been a few cases where a candidate gene approach has been successful in identifying a naturally occurring variant. The candidate gene approach aims to identify a relationship between segregating genetic variation and a phenotype focusing on genes where there is some information about the candidate gene's function from previous work (e.g. via the mutant-based approaches described above). This approach has been used successfully in *D. melanogaster* to identify naturally occurring genetic variants influencing behaviors such as learning (Anreiter & Sokolowski, 2019; Mery, Belay, So, Sokolowski, & Kawecki, 2007) circadian rhythm (Tauber et al., 2007); (Bauzer, Souza, Ward, Kyriacou, & Peixoto, 2002), and pupation (Zhang, Guy Reeves, & Tautz, 2019). However, overall, the majority of functional annotations ascribed to genes in the *D. melanogaster* genome have not led to discoveries of segregating variants that affect the natural phenotypic variation for that function within a population. This is not necessarily surprising as the major effect mutants identified by these screens are not necessarily the same variants segregating in populations and contributing to individual differences. In fact, for genes that have a critical role in the nervous system, we might expect that perturbations to those genes would be selected against, and that modifier variants elsewhere in the genome might be the major contributors to interindividual variability, as has been found for some other major effect genes (e.g. Liu et al., 2019). Identifying the source of this variation requires using a system with the ability to map naturally occurring variants.

Mapping the genetic variants contributing to complex traits in general has presented a major challenge due to the difficulty of characterizing the effect of a single variant when there are many other variants also affecting a phenotype and the effects at individual loci are subtle (Boyle, Li, & Pritchard, 2017; Rockman, 2012). If trait categories are viewed as a hierarchy, Garland and Kelly (2006) have argued that behavior is expected to be one of the most complex, because it will be influenced by physiology, morphology, etc. at the lower hierarchical levels, leading to the expectation that the genetic basis of most behaviors will be highly complex. In addition, the processes of learning and memory are themselves the products of many other processes, such as sensory and motor functions, which further argues for their expected complexity (Schultzhaus, Saleem, Iftikhar, & Carney, 2017; Dolan et al., 2019). Early quantitative genetic approaches to map genetic variants used two-way quantitative trait loci (QTL) mapping, in which two parental strains are crossed to create an F1, then the F1s are either crossed to themselves or backcrossed to one of the parents to create an F2 generation. This creates a population with recombination breakpoints at different positions throughout the genome, allowing one to identify the association between the genotype at a given position and the phenotype of interest. However, because the individuals are only crossed for just a few generations, resulting in large haplotype blocks, the resolution for identifying individual genes, rather than regions of the genome is low (Mackay, 2001; Slate, 2004), with mapping regions typically wider than 10 cM

(centiMorgans) and encompassing hundreds of genes. This has made it difficult to hone in on candidate genes that are influencing a particular phenotype.

With the advent of the genomics era, making sequencing of whole genomes feasible, additional approaches to mapping within population genetic variation have become possible, including genome-wide association studies (GWAS), which use a set of individuals to statistically associate single variants (e.g. a SNP) with the phenotype of interest (Long & Langley, 1999; Manolio et al., 2009; Zhang et al., 2019), and evolve and resequence approaches, which use artificial selection to produce populations that differ in the phenotype of interest and associate those differences with allele frequency differences (Baldwin-Brown, Long, & Thornton, 2014; Kofler & Schlotterer, 2014). Both GWAS (Papassotiropoulos et al., 2011), and evolve and resequence approaches (Mery, Belay, et al., 2007; Mery, Pont, Preat, & Kawecki, 2007) have successfully identified genetic variants not previously known to influence learning and memory phenotypes. A different approach that has the potential to elucidate linkages across the genotype to phenotype map is to use a multiparental population (MPP; Figure 2), which allows for multiple measurements of different phenotypes in different environments to be measured on the same set of genotypes. MPPs are similar in design to a traditional two-way QTL mapping approach, however they use multiple inbred founder lines, which is first crossed for multiple generations to create a fine-scale mosaic of the genome to allow for higher mapping resolution, and then mixed to create several recombinant inbred lines (RILs). One advantage of having a stable panel of RILs is the ability to measure numerous phenotypes across levels of organization to uncover the mechanisms determining complex traits. There are several MPP resources in different organisms and this approach has been used successfully to dissect an array of different phenotypes (de Koning & McIntyre, 2017) e.g. lifespan: (Highfill, Reeves, & Macdonald, 2016; Stanley, Ng'oma, O'Day, & King, 2017); gene expression: (Aylor et al., 2011); seed size and number: (Gnan, Priest, & Kover, 2014). As with all mapping approaches focused on complex traits, success depends on measuring a large number of lines (see Keele, Crouse, Kelada, & Valdar, 2019; King, Macdonald, & Long, 2012 for power analyses), with several hundred needed to identify QTLs that explain 5–10% of the variation in a phenotype. In addition, for phenotypes with a low heritability, it may be necessary to measure multiple individuals per genotype (Keele et al., 2019). Beyond this requirement of a fairly large scale study, there are few other limitations to what phenotypes might be investigated in this way. In the case study below, we discuss how this approach has been applied to understand the genetic basis of place learning and memory using an MPP in fruit flies.

Case study: place learning and memory in the DSPR

There is a movement toward recognizing the underlying complexity of the processes of learning and memory and developing approaches to study this complexity in realistic settings. These approaches provide the opportunity to characterize the genetic mechanisms underlying variation in learning and memory and other highly complex phenotypes. Here, we describe a collaborative project led by Troy that successfully took a quantitative genetics approach to understanding interindividual variability in a measure of place learning and memory to illustrate the potential of this approach.

In Williams-Simon et al. (2019), we used a large multiparent population, the *Drosophila* Synthetic Population Resource (DSPR), in combination with the high throughput heat box assay developed by the Zars lab to identify segregating variants that contribute to place learning and/or memory performance in *D. melanogaster*. The DSPR consists of two sets of over 800 RILs, each derived from 8 founders (parents), 50 generation cross (King, Macdonald, et al., 2012; King, Merkes, et al., 2012). We measured a huge number of flies, totaling almost 40,000 and identified 16 QTLs, with 5 QTLs affecting both learning and memory. One of the advantages of the DSPR and other stable multiparent mapping panels, is the ability to measure multiple phenotypes on the same genotypes. We also took advantage of this strength to characterize gene expression differences between pools of high performing and low performing RILs for learning and memory. After establishing which genes were significantly differentially expressed, we then looked specifically within the 16 QTLs' confidence intervals to identify which of the significantly differentially expressed genes located in that region. Additionally, we compared our dataset to a previous genome-wide eQTL dataset, which also used the DSPR, to identify the genes that have evidence for a cis eQTL in the DSPR (King, Sanderson, McNeil, Long, & Macdonald, 2014). Lastly, we manually examined the annotation for these genes in FlyBase and noting annotations such as: neurological process, behavior, or neurotransmitter, which might imply that the gene could be involved in the processes of learning or memory. Combining these datasets allowed us to identity 9 possible candidate genes. None of these genes have been previously implicated in either learning or memory, but all were identified in part due to some association with the nervous system. This study represents a critical first step towards expanding our understanding of the genetic mechanisms determining differences in both learning and memory performance in natural populations using the multiparent population approach. In addition, our study opens up the potential for any number of follow-up studies using these same lines, investigating intermediate phenotypes along the genotype to phenotype map in a highly controlled way. Only through these kinds of systems-level approaches can we hope to achieve a broader understanding of what genetic mechanisms make an individual perform well or poorly at a learning or memory task. We hope to ultimately uncover how genetic differences lead to changes in the brain that affect the processes of learning and the formation and retention of memories.

We have highlighted this project and its major findings both to advocate for this experimental approach, and to show what Troy's vision and scientific philosophy made

possible. It was Troy who recognized how the DSPR lines could be used in combination with his own lab's research to begin a new research direction for both his and the King lab. We plan to continue using this approach in our own research to further uncover the mechanisms determining place learning and memory performance by validating the candidate genes from our case study through a combination of genome editing approaches (Stern, 2014; Turner, 2014), and hope others will be inspired to take a similar, integrative approach to other problems in neurogenetics.

Learning the Zars model of science and life

Through our work with Troy, we not only learned scientific lessons, but we learned how to be better scientists and people through his example. Troy's attitude toward science was one of equal opportunity excitement and relentless optimism. When we would talk science with him, he was quick to focus on what new cool thing we might learn from a new study, rather than on potential shortcomings. This passion for all things science was contagious and infected us all. He taught us that if you love being curious and asking scientific questions, you will always get to an answer if you just keep searching.

This approach to science is what allowed him to see what kind of work was possible by combining several approaches and developing tools to answer a research question from a novel perspective. 'Do it all' was what he usually told us because he believed different experimental approaches from different perspectives were a part of one big puzzle. All of us had the experience of coming away from meetings with Troy having talked about a huge number of diverse project possibilities. He was a fearless researcher, eager to incorporate whatever new methods are best to answer a particular research question. The difficulty or complexity of a technique never deterred his quest to understand the ever fascinating field of neurogenetics. Troy has inspired us to be fearless, perpetually curious, and honest scientists and we will do our best to honor these lessons throughout our careers in science.

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