JOURNAL OF NEUROGENETICS
https://doi.org/10.1080/01677063.2020.1715976

Taylor & Francis
Taylor & Francis Group

TRIBUTES

Memory, anticipation, action – working with Troy D. Zars

Reinhard Wolf[a], Martin Heisenberg[a], Björn Brembs[b], Scott Waddell[c], Aditi Mishra[d], Abigail Kehrer[d] and Angelynn Simenson[d]

[a]Rudolf-Virchow-Zentrum, University of Würzburg, Würzburg, Germany; [b]Institut für Zoologie-Neurogenetik, University of Regensburg, Regensburg, Germany; [c]Centre for Neural Circuits and Behaviour, University of Oxford, Oxford, UK; [d]Division of Biological Sciences, University of Missouri, Columbia, MO, USA

ABSTRACT

We present here our reflections on the scientific work of the late Troy D. Zars (1967 – 2018), on what it was like to work with him, and what it means to us. A common theme running through his work is that memory systems are not for replaying the past. Rather, they are forward-looking systems, providing whatever guidance past experience has to offer for anticipating the outcome of future actions. And in situations where no such guidance is available trying things out is the best option. Working with Troy was inspiring precisely because of the optimism inherent in this concept and that he himself embodied. Our reflections highlight what this means to us as his former mentors, colleagues, and mentees, respectively, and what it might mean for the future of neurogenetics.

ARTICLE HISTORY
Received 5 January 2020
Accepted 6 January 2020

KEYWORDS
Troy D. Zars; *Drosophila melanogaster*; engram; place learning; operant learning; mushroom body; temperature sensation

Memory mapping – Troy's impact

By Reinhard Wolf and Martin Heisenberg

Drosophila neurogenetics was announced in 1967 by Seymour Benzer (Benzer, 1967). In the same year Troy was born. Neurogenetics in its first 25 years unfolded as its own research field, distinct from for example neurology, neuroethology or developmental neurobiology. With genetics one looks at brain and behavior in the evolutionary perspective. Living creatures, while highly autonomous, need to interact with the world. Animals have their behavior for these interactions. Neurogenetics offers the dissection of brain and behavior. Behavior can be good or bad, beneficial or harmful for the animal, depending upon circumstance. Moreover, the study of behavior extends to the investigation of mental functions (Heisenberg, 2018). One may, for instance, categorize attention, motivation or learning as 'mental', because these are indirect modes of behavioral organization, the behavior in question may never be executed. The brain is the organ that throughout life organizes this ensemble of behavioral and meta-behavioral modules.

Studying biology Troy realized that understanding how brains generate behavior and mind is one of the outstanding challenges of the life sciences. *Drosophila* flies with their advanced genetic record offer an abundance of approaches in this field. Troy wanted to study brain and behavior. He applied for a Humboldt Fellowship to come to Germany and join our laboratory from 1997 to 2002.

Troy got interested specifically in learning and memory. This branch of *Drosophila* neurogenetics had started only in 1974 (Quinn, Harris, & Benzer, 1974) while in the 1960s it had not even been clear yet whether flies could learn at all. When Troy entered the field, several learning paradigms had been established and about half a dozen mutants with learning defects had been discovered and partially characterized.

Troy was not satisfied with the plain mutant approach, however. In a mutant with a defect in memory the corresponding protein would be altered or missing. If in the wild type the protein would be histologically local, this would limit the potential locations of the learning circuit or memory trace to some of the places where the protein is found. However, the protein could also serve other memory traces or entirely different unknown functions throughout the brain. Troy wanted to understand which was the case.

For his first project with us he designed a new localization strategy, the two-component rescue system. He chose the gene *rutabaga (rut)*, coding for an adenylate cyclase required in most learning/memory paradigms, including classical (Pavlovian) odor learning and operant place learning. The gene *rut* is widely expressed in the nervous system. In the loss-of-function mutant rut^{2080}, flies are severely impaired in learning/memory. Using tissue- and cell-specific promoter elements Troy targeted the wild type cDNA of rut^{+}

CONTACT Author contacts are listed below the article's main sections.

Supplemental data for this article can be accessed here.

to certain neurons or groups of neurons in the mutant and tested the memory performance of these flies. He found different rescue locations in different learning tasks. For classical odor avoidance learning he localized the *rut*-dependent associative short-term memory trace in Kenyon cells of the mushroom bodies (MBs), and indeed to a certain domain of the MB gamma-lobes (Zars, Fischer, Schulz, & Heisenberg, 2000). In operant place learning, the rut^{+} cDNA was required for rescue of the memory trace in the ventral ganglion, antennal lobes, and/or median bundle, but not in the mushroom bodies (Zars, Wolf, Davis, & Heisenberg, 2000). These two learning tasks are both associative but otherwise differ profoundly from each other. In operant place learning, the fly learns about the consequences of its walking actions at certain places in the heat box. In classical odor learning, it notices and stores relations in the world independent of its own behavior and of places.

After Troy's return to the USA, a close transatlantic collaboration developed between Germany and Columbia, Missouri, where Troy established his laboratory. Soon the two-component rescue approach with the *rut* gene was applied to visual learning, too (Liu *et al.*, 2006). Flies were studied in stationary flight in the flight simulator. In this apparatus, a single fly was attached to a torque meter in the center of a vertical drum (panorama). The fly's yaw torque drove the horizontal rotations of the drum. The panorama presented four visual landmarks in the centers of its four quadrants. The landmarks differed in one particular visual feature. In one experiment this was the height in the panorama, in the other the inclination of the contours. Orientation of the fly towards one pattern type led to dangerous heating, while orientation towards the other one was safe. Wild type flies quickly learned to avoid being heated. Troy's two-component rescue approach revealed the memory traces for both features to be located in the fan-shaped body (FB) of the central complex. The *rut*-dependent memory trace for 'contour inclination' resided in layer 1, and that for 'height' in layer 5 of the FB (Liu *et al.*, 2006). This type of visual learning is classical. The fly associates heat with certain orientations of the panorama marked by certain features of the landmarks. Also operant learning/memory can be tested in the flight simulator. In this experiment the fly is surrounded by a panorama without landmarks or texture. The fly's yaw torque is coupled to heat, in that torque to one side (e.g. clockwise) switches heat on, and torque to the other side (counterclockwise) switches it off. The fly quickly learns that its own actions are switching on the heat and suppresses yaw torque to the side that would raise the temperature. It keeps this behavioral restriction for a while, even after heat is switched off for good (test). The fly learns about the consequences of its yaw torque maneuvers. One would be eager to localize the memory traces for this operant behavior and to compare it to that of operant place memory in the heat box. This would be of particular interest, as the *rut* gene function seems not to be involved in operant learning in the flight simulator (Brembs, 2009).

To study classical conditioning in the flight simulator without visual landmarks, one can add colors to this type of conditioning, one at high temperature and a different one at normal temperature. If one now switches off the heat for the memory test, the fly keeps the color preference it had acquired during training. To probe whether this is indeed based on classical learning, one can switch off both, heat and colors during the test. Now the fly shows no yaw torque suppression for the turning direction, which during conditioning had also been correlated with heating. This means that the fly had not even acquired an operant memory, which it now could use. Classical color learning in this case had made operant yaw torque learning dispensable (Brembs, 2009). Again, it would be most interesting to localize the memory trace for color avoidance in this experiment.

A further collaboration addressed extinction of classical odor memory. As described above, the *rut* gene had been used to localize the memory trace of aversive odor learning to Kenyon cells in a certain region of the gamma-lobes. In the new study, the fly was first aversively conditioned for a certain odor and subsequently exposed to the same odor without reinforcement to gradually extinguish the memory. During the extinction phase, the chemical output synapses of the Kenyon cells were blocked. Whatever happened to the memory trace thus had to happen intracellularly in the Kenyon cells. Indeed, when the output was restored the performance of the fly showed that the odor stimulus without reinforcement had reduced the memory trace. Kenyon cell output had not been necessary during this phase (Schwaerzel, Heisenberg, & Zars, 2002) (for exciting new findings on extinction, see Felsenberg *et al.*, 2018).

Early in the era of fly behavioral research the field was dominated by the stimulus-response (S-R) relationship. Phototaxis, chemotaxis, anemotaxis, negative geotaxis, etc., were all considered 'hard-wired' responses to naturally occurring stimuli. Troy asked how firmly ingrained these responses really were. Would operant place learning over-ride an innate behavior? Indeed, phototactic and anemotactic responses in the heat box were entirely suppressed by operant place learning/memory. Regarding negative geotaxis, a moderate inclination of the chamber did not lead to a measurable preference for the elevated side. To the contrary, operant place learning/memory was positively enhanced (Baggett *et al.*, 2018).

Troy discovered that *Drosophila* has two types of thermosensors with different functions, one on the antennae for quick orientation in low-temperature gradients, and another for higher temperatures somewhere else in the body. The latter ones typically mediate the reinforcing stimulus in operant place learning in the heat box (Zars, 2001). This study revealed an interesting behavioral property of place learning. Exposure to high temperature (41 °C) for 1 min sensitizes the fly to take a subsequent mild heat pulse as reinforcing and to build a memory in place learning. Without sensitization, this mild heat pulse (30 °C) would be largely ineffective as reinforcement (Sitaraman, Zars, & Zars, 2007). To cause the sensitization, the 41 °C heat exposure has to be unexpected and out of control of the fly, as in learned helplessness (Sitaraman & Zars, 2010). The heat pre-exposure in operant place learning has a further behavioral effect that it shares with learned helplessness: it increases escape latencies.

Troy and his group also studied the role of biogenic amines. They found that serotonin, but neither dopamine nor octopamine, are necessary for operant place memory (for an interesting new aspect to this story see Mishra *et al*., this issue). Using the meanwhile advanced genetic tool kit, they were able to replace the reinforcement via heat by the direct stimulation of the roughly 80 serotonergic neurons in the brain to generate operant place memory in the heat box. They even elicited an increase in escape latencies by stimulating subsets of these neurons during the pre-exposure phase (Sitaraman *et al*., 2008; Sitaraman, Kramer, Kahsai, Ostrowski, & Zars, 2017).

This review of Troy's scientific achievements does not intend to be complete. Instead, it tries to show how productive Troy's approach to neurogenetics was, with the two-component rescue system for memory mapping and the two learning paradigms he chose (Zars, Fischer, *et al*., 2000; Zars, Wolf, *et al*., 2000).

If the main task of the brain is to organize behavior, the first step in neurogenetics should be to choose a behavior and to ask how it can be evolutionarily successful. In the second step one may want to apply genetics, physiology, anatomy, histology, and circuit analysis to understand how the brain does it. As stated in the opening paragraph, behavior should have beneficial consequences for the actor. This is a difficult demand, given that the brain has to deal with the future and the future is open. The world and the actor are undergoing continuous change, gradual and stochastic. With his choice of learning/memory and the 25 years he was given, Troy pioneered a branch of neurogenetics centrally significant for our understanding of brain, behavior and mind.

Reinhard Wolf and Martin Heisenberg. Rudolf-Virchow-Zentrum für Experimentelle Biomedizin, Universität Würzburg, Germany. reinhard.wolf@virchow.uni-wuerzburg.de and heisenberg@biozentrum.uni-wuerzburg.de

How flies contribute to understanding the evolution of language, or: how Troy Zars anticipated FoxP function

By Björn Brembs

Verbal behavior

"Sshh, don't tell anybody" Professor Troy Zars shushed me: "I have them!". He was of course referring to fly stocks mutant for the CG16899 gene, now known as *FoxP* (*forkhead box P*). While this after-hours discussion took place in 2007 at the International Congress of Neuroethology in Vancouver, Canada, the origin of the ensuing collaboration can be traced back to a 1957 book entitled "Verbal Behavior" (Skinner, 1957). In this tome, B.F. Skinner proposed that language might be acquired through an operant learning process: the first more or less random utterances (babbling) of infants are rewarded by their parents and correct utterances more so than incorrect ones. Moreover, just as imitating any movement, the ability to correctly imitate the words of others might be inherently rewarding. Eventually, the infants learn to correctly speak the words and sentences required to communicate their needs and affections.

Chomsky's critique

Two years after "Verbal Behavior", 29 year-old Noam Chomsky published a scathing critique of the book (Chomsky, 1959), shaking the standing of the towering behaviorist. The 23-page review is today widely considered as one of the starting points for the demise of behaviorism as the dominant school of thought in psychology and as one of the origins of the so-called "cognitive revolution" (Adelman, 2007; Bialystock, 1997; Harnish, 2002; Leahey, 1987; Palmer, 2006; Smith, 1999; Virués-Ortega, 2006; Watrin & Darwich, 2012). Among the numerous points of criticism in Chomsky's article is one that has received relatively little attention: Chomsky dismissed the idea of operant experiments conceptually paralleling language acquisition as "mere homonyms, with at most a vague similarity of meaning" (Chomsky, 1959). In other words, Chomsky very correctly pointed out that Skinner had never done any actual experiments on language acquisition, but had, instead, worked mainly on pigeons and sometimes rats which had to press levers and other manipulanda in one of his eponymous experimental chambers. With the astuteness, intellectual sharpness and polemic for which he has become famous, Chomsky chided Skinner for asserting that the same learning processes must be taking place in vocal learning as in lever pressing, without providing any evidence for this claim, other than conceptual similarity.

While Chomsky wrote that he would have expected psychological or anthropological evidence in support of Skinner's claims, psychological evidence was not available and obvious ethical considerations preclude experiments of the type Skinner commonly conducted on animals with infants (even though Skinner always promoted the use of his concepts in education). It was not until decades later that anthropological evidence started to become available, challenging Chomsky's later counter-proposal of inborn, universal grammar (Everett, 2005; Ibbotson & Tomasello, 2016; Reich, 2012). Biological evidence, while not specifically mentioned in the main part of Chomsky's book review is indirectly referenced by examples of imprinting and other forms of critical-period-based learning in animals. Chomsky realizes and carefully emphasizes that much of the required evidence is not available to anybody at the time: "there is little point in speculating about the process of acquisition without much better understanding of what is acquired." Besides the many other hard-hitting criticisms in Chomsky's review, this one must have been particularly stinging for Skinner, as he was keenly aware of the lack of understanding of what even the animals in his experiments were actually learning and how.

Multiple processes

There are many different learning processes taking place during operant learning in Skinner boxes and it was, at the

time, not possible to distinguish between these processes. What did the animal learn about the lever and what about the pressing behavior? Clearly, the animal's behavior changes over time and with various schedules of reinforcement, so on top of parallel processes there seemed to be sequential processes involved as well. It was completely open how separated or similar these processes were or how/if they interacted with each other.

These multiple processes were at the center of a debate between Skinner and a scholar of Pavlov's, Jerzy Konorski over several back-and-forth articles already in the late 1930s (Konorski & Miller, 1937a; Konorski & Miller, 1937b; Skinner, 1935, 1937). One core aspect around which much of the discussion revolved was the role of the lever as a stimulus. It was clear to both sides that the depressed lever may signal food to the animals (and the non-depressed lever no food) just as the ringing bell signaled food to Pavlov's dogs. Would this perspective be sufficient to subsume Skinner's experiments as merely a special form of Pavlovian learning? Skinner was not convinced and lamented that "the lever cannot be removed" in order to study his type of learning in the absence of such a stimulus.

In the subsequent decades, scholars designed countless experiments to solve the question of how similar or different these two types of learning really are (e.g. Balleine, 1994; Brembs & Heisenberg, 2000; Donahoe, Burgos, & Palmer, 1993; Donahoe, Palmer, & Burgos, 1997; Gormezano & Tait, 1976; Guthrie, 1952; Hebb, 1956; Hellige & Grant, 1974; Hoffmann, 1993; Rescorla, 1994; Sheffield, 1965; Trapold, Lawton, Dick, & Gross, 1968; Trapold & Overmier, 1972; Trapold & Winokur, 1967). However, no clear answer could be found. Towards the end of the 1960s/early 1970s the community seemed to have settled on the rather unsatisfying position that it is experimentally impossible to separate the different processes and that such multiple, parallel learning mechanisms apparently always occur together in an intertwined fashion, essentially leaving the issues debated in the 1930s addressed but unanswered - even long after Skinner's "Verbal Behavior" and Chomsky's demand for evidence.

New experiments

However, as so often in biology, a question that cannot be answered using one species can be answered by adapting the experiments to different species. In the 1990s, decades after the psychologists had abandoned the study of the confounding processes in operant learning, two preparations were developed independently of each other, one in *Drosophila* (Wolf & Heisenberg, 1991) and one in the marine snail *Aplysia* (Nargeot *et al.*, 1999a; Nargeot *et al.*, 1999b), that accomplished what Skinner had dreamed of: it was now possible to study the purely operant processes that take place in the absence of predicting stimuli such as the lever of a Skinner box. The common concept of both preparations was to provide operant feedback on behavior that occurred independently of any other stimuli or manipulanda. In flies, the behavior was turning behavior in tethered individuals (yaw torque, Wolf & Heisenberg, 1991) and in *Aplysia* the behavior was biting behavior (Brembs, Lorenzetti, Reyes, Baxter, & Byrne, 2002; Mozzachiodi, Baxter, & Byrne, 2013; Nargeot *et al.*, 1999a; Nargeot *et al.*, 1999b). Flies received feedback in the form of a punishing heatbeam and *Aplysia* received virtual food reward: stimulation of the esophageal nerve that would otherwise signal the presence of food in the buccal cavity. These types of feedback are not directional in any way or require consumption, respectively, eliminating any potential stimuli beyond the actual feedback itself.

Early experiments quickly showed that different biochemical processes were involved than those commonly found in Pavlovian and other learning tasks. In particular, protein kinase C (PKC) was found to be involved in this process, while manipulating the known biochemical pathways underlying synaptic plasticity in Pavlovian preparations had subtle, if any, effects (Brembs & Plendl, 2008; Mozzachiodi *et al.*, 2013).

Language gene

Almost at the same time, a pair of discoveries was about to accelerate putting important pieces of the decades-old puzzle together. First, in 2001, a gene was discovered to underlie a particular form of human disorder, verbal dyspraxia, characterized by difficulties to articulate speech properly. While at first some thought that the gene, FOXP2, was involved in communication (a "language gene"), a 'motor hypothesis' was also discussed at the same time, proposing that FOXP2 was, instead, an important gene for learning the fine motor control of orofacial movements required to produce speech and language (Balter, 2001; Dominguez & Rakic, 2009; Watkins, Dronkers, & Vargha-Khadem, 2002). Next, a few years after the discovery of human FOXP2, Constance Scharff presented work from her lab that knocking down FOXP2 in juvenile zebra finches interfered with their song learning (Haesler *et al.*, 2007). This particular form of learning had been described as a form of operant learning before, using the same arguments by analogy that Skinner had used to describe language acquisition in humans (Marler, 1991). First, juvenile male birds generate highly variable subsong, the equivalent of human babbling. Just like a human infant would attempt to match words they had heard before, the male songbirds compare their song to a song template they acquired when listening to adult male song. By using the auditory feedback to minimize the mismatch between template and actually produced vocalizations, both birds and humans are thought to slowly arrive at either crystallized song (birds) or speech (humans). Just as the experiments in invertebrates, also vocal learning took place in the absence of any other associated stimuli or manipulanda. Perhaps the two invertebrate learning processes, conceptually analogous to vocal learning, were also biologically related, homologous?

There was the opportunity to test Skinner's hypothesis: a gene both specific for speech in humans and important for song learning – and song learning had been shown previously to also involve PKC (Sakaguchi & Yamaguchi, 1997; Yoshida, Yamada, & Sakaguchi, 2003)! Maybe FoxP manipulations would lead to homologous defects in

vertebrates and invertebrates? Was this going to be the kind of evidence Chomsky had demanded Skinner to provide or wait for?

Testing Skinner

Troy and I had overlapped in Martin Heisenberg's laboratory during the mid-late 1990s, where we both had worked on different operant learning paradigms. At the 2007 ICN in Vancouver we met and I presented the idea to test Skinner's hypothesis from 1957 in flies, if only there was a way to find out if flies even had a *FoxP* gene? This was when he (jokingly, of course, Troy was never a peddler of secrecy) shushed me and disclosed that he was already studying several fly strains with insertion mutations in a gene that was homologous to the vertebrate FOXP2 gene. He had already anticipated the potential role *FoxP* may be playing for our understanding of operant learning processes and had begun to unravel the molecular function of *FoxP* in *Drosophila* even before the gene was properly annotated. I was thoroughly impressed.

He sent me his mutant and control flies and we quickly found out that this *FoxP* allele was specifically involved in the particular form of 'pure' operant learning that was conceptually analogous to language learning, but not in other forms of operant or Pavlovian learning. Immediately thereafter, we replicated these mutant-based findings with an RNAi construct targeting the last exon, where the most specific mutant had its insertion (Mendoza *et al.*, 2014). These results corroborated both the motor hypothesis of FOXP2 function and Skinner's 1957 conjecture that language acquisition indeed contained an operant process, at least for the speech component of language. The results suggested a kind of 'deep' homology binding the early stages of language acquisition in humans to much more ancient forms of more general motor learning in all bilaterian animals (Bolhuis, Okanoya, & Scharff, 2010; Scharff & Petri, 2011). Current evidence is thus consistent with the hypothesis that there is an operant process at the root of early language acquisition and that this process is an evolutionary conserved motor learning component, comprising PKC, FoxP and likely many as yet unknown genes.

Operant learning

While our findings are by no means the only ones challenging one of Chomsky's many intellectual contributions, they equally do not invalidate all of his many criticisms brought forth in his review of "Verbal Behavior". In fact, the mechanism by which we acquire language is, of course, inborn and in the case of humans, that particular form of motor learning may well be specialized for language in a way it is in no other animal. However, the historical tensions between those who see themselves aligned with one or the other side of the cognitive divide still run deep. Maybe it is therefore not too surprising that there was some initial resistance to the implications of our discovery. Perhaps the kind of attachment to one side of a historically entrenched intellectual debate is best exemplified by the prosaic comments of one of our reviewers: "the data are made to bear the weight of an elaborate hypothesis, and they are literally crushed by it, like a tiny matchstick house beneath a bowling ball". About 2.5 years later, in 2014, our discovery was finally formally published, seven years after Troy and I discussed *FoxP* in Vancouver (Mendoza *et al.*, 2014). Soon after, it was discovered that mice with humanized versions of their FOXP2 genes also showed subtle alterations in several forms of motor learning (Schreiweis *et al.*, 2014). PKC and FoxP are indeed components of an operant motor learning mechanism that appears to be conserved throughout bilaterian evolution. Now we just have to find the other components and sort them in the right order.

Björn Brembs, Institut für Zoologie - Neurogenetik, Universität Regensburg, Germany. bjoern@brembs.net

A short tribute to professor T

By Scott Waddell

Troy Zars was my contemporary in the field and a very dear friend. Funnily enough with working on memory, I cannot remember when I first met him. It was either the 2000 Biennial European Fly Neurobiology Conference, aka Neurofly, in Alicante, or when he hosted me with Martin Heisenberg in Würzburg that same year. In Alicante we both had such cool stories and spoke in the same session. I remember chatting beforehand and that we were both nervous as hell. Troy had used GAL4 UAS to rescue associative short-term olfactory memory by re-establishing *rutabaga* expression in the mushroom bodies of mutant flies and I had used it in my studies of *amnesiac* with Chip Quinn. That seemed really cool back then, we were both thrilled, but it sounds very blasé now.

However, Troy (Sean McGuire and Josh Dubnau) and I were also starting to use Toshi Kitamoto's UAS-*Shibire*^{ts1} transgene in our studies of fly memory. When Troy hosted me in Würzburg I remember chatting with him, Bertram Gerber and Martin Heisenberg about how region-specific rescue of memory mutants, and blocking output from these same neurons could be used to locate 'memory engrams'. This was a formative trip. I got to know Troy better and I'd never met Martin Heisenberg before. When sitting down to talk to Martin he said 'Don't tell me anything you wish me to keep a secret'. I have always been terrible at keeping secrets so I told him everything I knew! I had already told Troy anyway. I am delighted to realize, when reading other articles in this issue, that this Würzburg trip is the source of a Troy legend. Troy was a marvelous host and he drove us (myself and Doug Guarnieri, a college buddy of Troy's and in those days a postdoc studying alcoholism with Ulrike Heberlein), I think to the Miccelskeller of the Familie Scheckenbach in Sulzfeld am Main, to sample the regionally famous *Meterbratwurst* – as it sounds a

metre long sausage with *Sauerkraut* and *Knödel* - for lunch. Needless to say this was a task and Doug and myself did not have Troy's stamina. So he ate the remainder of ours too and then delighted all afternoon in ridiculing our meager efforts. I swear I would have made it if I had left the *Knödel* alone! We returned to Troy's place, cracked a few beers, grilled more food and hung out with his family, Melissa and the boys Ethan and Ben. I remember making tents and chasing those two around. Würzburg became an almost yearly pilgrimage for me. Although I missed Troy in Würzburg when he moved on to start his own group, I vividly remember being together for Neurofly in Würzburg in 2008.

We started our independent groups almost simultaneously. Troy in Missouri and myself at UMass Medical School. As soon as I could I invited Troy up to Worcester, Massachusetts to give a seminar and managed to time it perfectly so that he was the guest of honor at my first lab Christmas party. Again, legends were made: I instigated a lab Jenga competition played with toes and I learned years later that it had become a Zars' family tradition. We exchanged Secret Santa gifts, Troy's was Mr. T (aka B. A. Baracus), soap on a rope. He proudly wore it around his neck all evening (Figure 1).

I hung out with Troy whenever I could, at meetings in Houston, Roscoff, D.C., and fairly recently at *Learning and Memory: A Synthesis of Bees and Flies*, a meeting he organized with Dorothea Eisenhardt and Martin Giurfa at HHMI Janelia Research Campus. If I was not at a meeting, he hung out with people from my lab. In 2018 Troy had invited me to talk at Mizzou. However, a couple of months beforehand he emailed to ask if he could call me. He wanted to tell me that he had cancelled my trip because he had realized it overlapped with SfN and so there would not be enough neuroscientists to justify it! I protested that I was only coming to see him. However, he also told me he was ill, and he insisted that we reschedule my trip for Spring 2019. Knowing that my seminar was delayed and Troy was not well, I asked Melissa if I could come and make a surprise visit just before Christmas. Melissa and I secretly planned the trip and luckily the stars aligned. Troy returned home from hospital the afternoon I arrived. I can see him now, he was totally shocked as I walked into the front room, 'wha, wha, what the hell are you doing here!?' I spent a couple of days with Troy, Melissa, Ethan, Ben, and Troy's youngest son Jonathon who I met for the first time. Sometimes in life we make good decisions. This trip is one of mine. Troy passed less than a week later.

I honored Troy's invitation to Mizzou early in 2019. My talk was the hardest I have ever had to deliver. I had hoped he would be in the front row heckling me, but it had become the Troy Zars Memorial Lecture. The family, Troy's favorite colleagues, and friends of mine and Troy's from St. Louis were there. Ironically, I spoke about our recent work, led by Johannes Felsenberg, on memory extinction. I had spoken to Troy about extinction in 2000 in Würzburg and about his article with Martin Schwaerzel *et al.* (2002) on extinction at Christmas 2002 in Massachusetts. In the last paragraph of Felsenberg *et al.* (2018) I wrote about Troy's model for extinction that he had proposed in Schwaerzel *et al.* (2002). We had talked, argued and laughed about that in 2018 in his living room in Missouri.

Figure 1. Prof. T sporting Mr. T in Princeton, MA, 2002. Image copyright Scott Waddell.

Scott Waddell. Centre for Neural Circuits and Behaviour, University of Oxford, United Kingdom.
scott.waddell@cncb.ox.ac.uk

"Do it all": approaches to understand place learning and memory formation in the Zars lab

By Aditi Mishra, Abigail Kehrer and Angelynn Simenson

Neuroscientists are an amazing group of researchers who often rely on tried and tested approaches to build a consistent model, while simultaneously developing and using emerging technologies to design new assays to push the boundaries of our knowledge. Our late mentor Professor Troy D. Zars (fondly, Troy) believed that the greatest questions could be answered by taking some necessary risks. Under his tutelage, the three of us learnt the importance of interdisciplinary research. During our initial interactions with Troy, he made it clear that we worked "with" him and not "for" him. Troy fostered a welcoming

environment that made the Zars lab a very happy place to be in. Hence, in this article we have referred to the Zars lab as "our lab". It is a brief summary of the work that was ongoing in our lab at the time of his passing and not meant to be a thorough and comprehensive review of the rationale and body of work these ideas were based on. Rather, it is an insight into how Troy operated under a "Do it all" philosophy that encompasses the interdisciplinary spirit of his work.

Our lab had a primary focus on place learning and memory using the heat box paradigm. We utilized the existing strengths of *Drosophila* genetics and transgenic manipulation, molecular biology, and imaging, and brought to it emerging technologies in thermogenetics, single-cell molecular biology, and connectomics. Our long-term goal was to decipher the functional neural circuitry underlying learning and memory. In the heat box paradigm of place learning, an individual fly's own behavior changes the environment toward positive or negative experiences through operant conditioning. Specifically, heat punishment results as a consequence of the fly crossing an arbitrary midline to the designated "heat-associated side" of the chamber and only ceases when the fly crosses back across the midline to the "cool-associated side" (Supplementary Figure 1). During and after training each fly chooses which side of the chamber it prefers to occupy. The proportion of time spent in either side of the chamber is calculated and compiled into a Performance Index (PI). The PI during a training session provides insight into a fly's learning during the experiment. In our lab, we used the heat box extensively to investigate place learning and memory in wild-type and mutant flies, and flies representing human disease models. When faced with a crossroads of where to direct our scientific efforts, Troy would offer us a simple solution: "Do it all".

There were four major projects that dominated our lab meetings between 2015 and 2018: development of novel thermogenetic tools, deciphering the role of dopamine neurons and of dopamine signalling in place learning and memory, determining bases of learning deficits in a fly model of classic galactosemia, and uncovering the genetic components of place learning and memory with quantitative genetics. Since the latter is reviewed in an accompanying paper in this issue (Williams-Simon *et al.*), we focus our discussion on the rationale and approaches pertaining to the first three projects. The three of us either led or were deeply involved in the running these projects. We have tried to convey Troy's ideas and scientific rigour by providing examples of our experimental design, of some remaining questions and of possible future research directions.

Deciphering the role of biogenic amine systems in place learning and memory

A large body of work has established that biogenic amine systems play a crucial role in modulating functional connections between neurons that contribute to learning and memory in a number of animals, including humans (Meneses & Liy-Salmeron, 2012; Puig *et al.*, 2014). The neural effects of biogenic amine systems on learning, memory, and social behavior in insects have been extensively studied. For example, in bees, dopamine, serotonin, and octopamine systems modulate different aspects of the formation and retrieval of conditioned behavior in a proboscis extension reflex assay (Hammer & Menzel, 1998; Mercer & Menzel, 1982), and regulate social behavior (Schulz & Robinson, 1999; Wagener-Hulme, Kuehn, Schulz, & Robinson, 1999). When Troy started his lab in 2002 at the University of Missouri-Columbia, several studies had documented roles for biogenic amine systems in *Drosophila* olfactory learning and memory (a classical conditioning procedure) and in courtship learning (an operant conditioning procedure) (Neckameyer, 1998; Tempel, Livingstonet, & Quinn, 1984; Vaysse, Galissié, & Corbière, 1988). At that time, experiments had implicated dopamine and synaptic transmission from dopaminergic neurons as being a critical signal for aversive olfactory learning, while octopamine and the neurons releasing it were considered to be important for appetitive learning (Aso *et al.*, 2010; Aso *et al.*, 2012; Schroll *et al.*, 2006; Schwaerzel *et al.*, 2003). There was however a dearth of literature investigating the role of these biogenic amines and of the neurons producing them in spatial learning and memory in fruit flies (Liu, Wolf, Ernst, & Heisenberg, 1999; Wolf *et al.*, 1998) – and indeed the serotonin system was completely neglected. With this Troy built his niche using the heat box paradigm (Wustmann & Heisenberg, 1997; Zars, Wolf, *et al.*, 2000) to delve into understanding the neurobiological basis of place learning and memory and to address possible roles for dopamine, octopamine and serotonin systems.

Initially, our experiments combined pharmacological and genetic approaches with behavioral assays to identify functionally relevant clusters of biogenic amine producing neurons. We found the serotonin system to be necessary for place learning and memory, but that dopamine and octopamine systems were not essential (Sitaraman *et al.*, 2008; Sitaraman, Zars, & Zars, 2010). These studies targeted dopaminergic neurons using TH-Gal4. However, it later became apparent that TH-Gal4 does not express in all dopaminergic neurons and that it excludes the protocerebral anterior medial (PAM) cluster, which features neurons that are now known to reinforce appetitive olfactory memory (Burke *et al.*, 2012; Liu *et al.*, 2012). PAM dopaminergic neurons include neurons that confer gustatory reinforcement with sugar rewards (Burke *et al.*, 2012) in addition to others that encode nutrient value (Huetteroth *et al.*, 2015; Yamagata *et al.*, 2015). In contrast, TH-GAL4 expresses in other dopaminergic neurons that reinforce aversive memory using punishment such as electric shock or bitter tasting compounds (Aso *et al.*, 2010; Claridge-Chang *et al.*, 2009; Das *et al.*, 2014). Following the "discovery" of the role of PAM dopaminergic neurons for appetitive memory formation, we were determined to re-evaluate our notion regarding the contribution of signalling from dopaminergic neurons, and pin down the role of signalling from subsets of dopaminergic neurons

in place learning and memory formation, starting with the PAM cluster. Using our newly developed thermogenetic tools (Mishra *et al.*, 2018, see below), we have been activating subsets of dopaminergic neurons with transgenically expressed Gr28bD to determine their contributions to place learning and memory (see Mishra *et al.*, this issue). In addition, instead of using a combination of Ddc-Gal4 and TH-Gal80 to restrict transgene expression to serotonergic neurons, we conducted experiments to assess the importance of specific serotonergic neurons. We still do not know whether distinct or overlapping neural pathways are modulated by biogenic amine systems in place learning and memory. More recently we were pursuing the notion that signalling from dopaminergic and serotonergic neurons interacts to modulate place learning and memory.

Development of new thermogenetic tools

The dissection of neural circuitry that underlies specific animal behaviors has been revolutionized by the development of genetically-encoded tools to manipulate neuronal function. These tools include those that rely on light (optogenetics) and temperature (thermogenetics). The heat-box paradigm uses "heat" as the punishment. Hence, it seemed logical for us to use temperature sensitive tools to alter the properties of neurons during the place learning assays. Since their discovery in the late 1990s (Caterina *et al.*, 1997), thermogenetic stimulation with temperature sensitive Transient receptor channel (Trp) proteins has been used to activate neurons. In flies, thermogenetic tools allow for extrinsic activation of neurons without any invasive procedures, thus aiding in understanding the links between neural circuitry and behavior in freely moving animal. However, certain caveats of existing thermogenetic tools constrain their utility. First there are only a few temperature sensitive proteins that are used as thermogenetic tools (Bernstein, Garrity, & Boyden, 2012; Owald, Lin, & Waddell, 2015). Other than a small number of Trp proteins, specifically TrpA1 and TrpM8, most other Trps activate outside the physiological range of model organisms and preparations (Bernstein *et al.*, 2012; Hamada *et al.*, 2008; Hoffstaetter, Bagriantsev, Gracheva, *et al.*, 2018; Peabody *et al.*, 2009; Story *et al.*, 2003). Second, Trp channels respond to changes in voltage as well as temperature which warrants additional controls during experiments (Brauchi & Orio, 2011; Nilius *et al.*, 2005). Further, *Shibire*^{ts1} is the only known thermogenetic tool that reduces synaptic output by inhibiting reuptake and recycling of vesicles at the synapse (Kitamoto, 2001). A lack of thermosensitive proteins spanning a wide range of physiologically tolerable activation temperatures constrained our studies of fly behavior in the heat-box, that could otherwise be used to explore the nuances of neuronal circuits underlying place learning and memory.

To overcome these limitations of existing thermogenetic tools, a recent focus of our lab was to identify and utilize temperature sensitive proteins that could activate neurons for a long period with a short trigger stimulus. We started with the newly discovered Gr28bD (Budelli *et al.*, 2019; Ni *et al.*, 2013). Since the predicted structure of Gr28bD is very different to that of Trp channels, it was an excellent system to investigate temperature sensitivity of proteins. In an extensive collaboration with the laboratories of Drs. Lorin Milescu and Mirela Milescu at the University of Missouri-Columbia we established Gr28bD as a novel thermogenetic tool (Mishra *et al.*, 2018). We showed that Gr28bD is a non-selective cation channel that activates at temperatures $\geq$34 °C. Unlike Trp channels which when expressed in heterologous systems elicited changes in cellular activity in response to changes in both voltage and temperature, Gr28bD only induced cellular activity faithfully following changes in temperature alone (Caterina *et al.*, 1997; Mishra *et al.*, 2018). We characterized Gr28bD physiology using the heterologous system of expression in *Xenopus oocytes* and monitored physiological changes following Gr28bD activation *in vivo* in flies expressing calcium reporters. We have also identified several other thermosensitive proteins with unique temperature response properties (manuscript in preparation). Currently, we are working to understand the structural properties of these thermosensitive proteins as a first step towards making designer proteins with tailored temperature responses.

Inspecting place learning and memory in a fly model of classic galactosemia

Several experiments in our lab were centred around the question of: "are there alterations in gene expression or neuronal structure that are specific or exclusive to place learning and memory?". We aimed to use single-cell sequencing to identify possible changes in gene expression that accompany behavior. Recently, single-cell sequencing has been used to catalog gene expression in neural subtypes in the fly brain (Croset, Treiber, & Waddell, 2018; Davie *et al.*, 2018; Konstantinides *et al.*, 2018; Li *et al.*, 2017). These studies identified the different classes of monoaminergic neurons and many neurons that express specific biogenic amine receptors (Croset *et al.*, 2018). This seems like an exciting approach to apply to studies of place learning using the heat-box, especially if neuronal gene expression signatures can be matched to available connectomes (Franconville, Beron, & Jayaraman, 2018; Huang *et al.*, 2019; Shih *et al.*, 2015; Zheng *et al.*, 2018).

Between 2016 and 2018, a goal of our lab was to apply the most up-to-date 10x genomic single-cell approach to identify monoaminergic cells that modulate place learning and memory. Troy's approach towards this goal was fairly unconventional. Instead of just using mutant and transgenic fly strains that alter biogenic amine systems, he motivated us to understand how changes in biogenic amine production or use might fit into a bigger picture of developmental disorders. Hence, our lab made fly models of classic galactosemia ("GALT-mutants" from here on), a hereditary disorder characterized by loss of function of the GALT enzyme (Coelho, Rubio-Gozalbo, Vicente, & Rivera, 2017). We used the CRISPR/Cas9 approach to create small deletions in the *GALT* locus, and isolated two fly strains, each with a

different loss of function allele (manuscript in preparation). In human beings, loss of function of the GALT protein limits the conversion of galactose to its functional metabolites, glucose-1-phosphate and uridine diphosphate-galactose. Under normal conditions these metabolites are absorbed in other metabolic pathways (e.g. glycolysis) or used in glycosylation reactions. However, loss of GALT function results in accumulation of galactose and an intermediate metabolite resulting in cognitive disorders, gonadal dysfunction in females, impaired executive functions, and disrupted learning and memory (Bosch, 2011; Kushner *et al*., 2010; Rubio-Gozalbo *et al*., 2019). For those affected, treatment is often limited to dietary restriction of galactose and lactose. As the *GALT* gene is highly conserved and the GALT protein performs the same function in flies as in humans, the GALT-mutant flies serve as an excellent model to decipher whether specific brain regions are disrupted in galactosemia. In collaboration with the Fridovich-Keil lab (Emory University), a considerable portion of our effort was directed towards determining how place learning and memory, and courtship behaviors were affected in the GALT-mutant flies. We aimed to use the 10x genomics to sequence the single-cell transcriptomes from the GALT-mutant fly brains. We also planned to analyse their neuronal architecture using electron microscopy and compare the structure with that of the recent fly connectome. We were enthusiastic about identifying brain regions, specific neurons, synaptic partners and deciphering the minute details of neurons – axon length, synaptic architecture and number - that affected place learning and memory. A longer-term objective was to apply this knowledge to generate a genetic or pharmaceutical rescue model for classic galactosemia.

In the short term, we are investigating learning and memory formation in the GALT-mutant larval *Drosophila*. A seminal study from the Fridovich-Keil lab had found that the GALT deficient larval *Drosophila* created by imprecise excision of a P-element in the *GALT* locus do not survive to pupation upon exposure to galactose in early larval stages (Kushner *et al*., 2010). This prompted us to examine whether learning and memory deficits manifest in early developmental stages in GALT-mutant flies. Our preliminary results indicate a deficit in olfactory memory formation in GALT-mutant larval *Drosophila*. Currently, we are verifying our findings while simultaneously expressing the human *GALT* transgene in the GALT-mutant larvae to potentially rescue the observed memory deficit (manuscript in preparation).

Conclusion

Troy's approaches towards scientific questions were forward-thinking and fearless. His "do it all" philosophy encouraged us to constantly challenge the relevance of our questions and experiments and to construct and recite a story from good science. While the projects described here might seem disconnected, Troy's varied strategies from mutants to disease models and tool development were strategically designed to solve the puzzle of understanding place learning and memory (Supplementary Figure 2). It is difficult for a person to be an expert on everything. Troy collaborated extensively with other scientists including biophysicists, geneticists, and other neuroscientists to dig deeper into the genetic and neural basis of learning and memory. Through his words and his actions, he showed us that it is essential for a scientist to be open minded and have the wisdom to appreciate others' skills. Beyond his excitement for the science, he cared about his student's well-being, growth and success. He would always ask how we were doing, what we were doing outside the lab to further our career and suggest and encourage us to pursue opportunities that would contribute to our success. In these and many more ways, Troy was a phenomenal mentor to have, and helped shape us into stronger, and more confident scientists. He taught through example that the path to success required resilience and determination. He had one of the most brilliant scientific minds we have ever met and learning from him was a true privilege. We will forever miss his deep chuckles and countless stories about his post-doctoral time in Germany. This world lost a truly wonderful individual with Troy's passing, but his ideas, teachings, and legacies will live on for many years to come. Working with Troy was a memorable adventure.

Aditi Mishra, Abigail Kehrer and Angelynn Simenson.
Division of Biological Sciences, University of Missouri-Columbia, USA. *amn2c@mail.missouri.edu, alk8b4@mail.missouri.edu, aps455@mail.missouri.edu*

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Adelman, B.E. (2007). An underdiscussed aspect of Chomsky (1959). *The Analysis of Verbal Behavior*, *23*, 29–34. doi:10.1007/BF03393044

Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A.B., … Tanimoto, H. (2012). Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genetics*, *8*, e1002768. doi:10.1371/journal.pgen.1002768

Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., & Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. *Current Biology*, *20*, 1445–1451. doi:10.1016/j.cub.2010.06.048

Baggett, V., Mishra, A., Kehrer, A.L., Robinson, A.O., Shaw, P., & Zars, T. (2018). Place learning overrides innate behaviors in *Drosophila*. *Learning Memory*, *25*, 122–128. doi:10.1101/lm.046136.117

Balleine, B. (1994). Asymmetrical interactions between thirst and hunger in Pavlovian-instrumental transfer. *The Quarterly Journal of Experimental Psychology. B, Comparative and Physiological Psychology*, *47*, 211–231.

Balter, M. (2001). Genetics. First gene linked to speech identified. *Science*, *294*, 32. doi:10.1126/science.294.5540.32a

Bernstein, J.G., Garrity, P.A., & Boyden, E.S. (2012). Optogenetics and thermogenetics: Technologies for controlling the activity of targeted cells within intact neural circuits. *Current Opinion in Neurobiology*, *22*, 61–71. doi:10.1016/j.conb.2011.10.023

Benzer, S. (1967). Behavioral mutants of *Drosophila* isolated by countercurrent distribution. *Proceedings of the National Academy of Sciences of the United States of America*, *58*, 1112–1119. doi:10.1073/pnas.58.3.1112

Bialystock, E. (1997). Anatomy of a revolution. In D. M. Johnson & C. E. Erneling (Eds.), *The Future of the Cognitive Revolution* (pp. 109–113). New York, NY: Oxford University.

Bolhuis, J.J., Okanoya, K., & Scharff, C. (2010). Twitter evolution: Converging mechanisms in birdsong and human speech. *Nature Reviews Neuroscience*, *11*, 747–759. doi:10.1038/nrn2931

Bosch, A.M. (2011). Classic galactosemia: Dietary dilemmas. *Journal of Inherited Metabolic Disease*, *34*, 257–260. doi:10.1007/s10545-010-9157-8

Brauchi, S., & Orio, P. (2011). Voltage sensing in thermo-TRP channels. In M. S. Islam (Ed.) *Transient receptor potential channels* (pp. 517–530). Dordrecht: Springer Netherlands. doi:10.1007/978-94-007-0265-3_28

Brembs, B. (2009). Mushroom bodies regulate habit formation in *Drosophila*. *Current Biology*, *19*, 1351–1355. doi:10.1016/j.cub.2009.06.014

Brembs, B., & Heisenberg, M. (2000). The operant and the classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator. *Learning and Memory*, *7*, 104–115. doi:10.1101/lm.7.2.104

Brembs, B., Lorenzetti, F.D., Reyes, F.D., Baxter, D.A., & Byrne, J.H. (2002). Operant reward learning in Aplysia: Neuronal correlates and mechanisms. *Science*, *296*, 1706–1709. doi:10.1126/science.1069434

Brembs, B., & Plendl, W. (2008). Double dissociation of PKC and AC manipulations on operant and classical learning in *Drosophila*. *Current Biology: CB*, *18*, 1168–1171. doi:10.1016/j.cub.2008.07.041

Budelli, G., Ni, L., Berciu, C., van Giesen, L., Knecht, Z.A., Chang, E.C., … Garrity, P.A. (2019). Ionotropic receptors specify the morphogenesis of phasic sensors controlling rapid thermal preference in *Drosophila*. *Neuron*, *101*, 738–747.e3. doi:10.1016/j.neuron.2018.12.022

Burke, C.J., Huetteroth, W., Owald, D., Perisse, E., Krashes, M.J., Das, G., … Waddell, S. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature*, *492*, 433–437. doi:10.1038/nature11614

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., & Julius, D. (1997). The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature*, *389*, 816–824. doi:10.1038/39807

Chomsky, N. (1959). Verbal behavior. *Language*, *35*, 26. doi:10.2307/411334

Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., & Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell*, *139*, 405–415. doi:10.1016/j.cell.2009.08.034

Coelho, A.I., Rubio-Gozalbo, M.E., Vicente, J.B., & Rivera, I. (2017). Sweet and sour: An update on classic galactosemia. *Journal of Inherited Metabolic Disease*, *40*, 325–342. doi:10.1007/s10545-017-0029-3

Croset, V., Treiber, C.D., & Waddell, S. (2018). Cellular diversity in the *Drosophila* midbrain revealed by single-cell transcriptomics. *eLife*, *7*, e34550. doi:10.7554/eLife.34550

Das, G., Klappenbach, M., Vrontou, E., Perisse, E., Clark, C.M., Burke, C.J., & Waddell, S. (2014). *Drosophila* learn opposing components of a compound food stimulus. *Current Biology*, *24*, 1723–1730. doi:10.1016/j.cub.2014.05.078

Davie, K., Janssens, J., Koldere, D., De Waegeneer, M., Pech, U., Kreft, Ł., … Aerts, S. (2018). A single-cell transcriptome atlas of the aging *Drosophila* brain. *Cell*, *174*, 982.e20–998.e20. doi:10.1016/j.cell.2018.05.057

Dominguez, M.H., & Rakic, P. (2009). Review of *language evolution: The importance of being human*. *Nature*, *462*, 169–170. doi:10.1038/462169a

Donahoe, J.W., Burgos, J.E., & Palmer, D.C. (1993). A selectionist approach to reinforcement. *Journal of the Experimental Analysis of Behavior*, *60*, 17–40. doi:10.1901/jeab.1993.60-17

Donahoe, J.W., Palmer, D.C., & Burgos, J.E. (1997). The S-R issue: Its status in behavior analysis and in Donahoe and Palmer's learning and complex behavior. *Journal of the Experimental Analysis of Behavior*, *67*, 193–211. doi:10.1901/jeab.1997.67-193

Everett, D.L. (2005). Cultural constraints on grammar and cognition in Pirahã: Another look at the design features of human language. *Current Anthropology*, *46*, 621–646. doi:10.1086/431525

Felsenberg, J., Jacob, P.F., Walker, T., Barnstedt, O., Edmondson-Stait, A.J., Pleijzier, M.W., … Waddell, S. (2018). Integration of parallel opposing memories underlies memory extinction. *Cell*, *175*, 709–722.e15. doi:10.1016/j.cell.2018.08.021

Franconville, R., Beron, C., & Jayaraman, V. (2018). Building a functional connectome of the *Drosophila* central complex. *eLife*, *7*, e37017. doi:10.7554/eLife.37017

Gormezano, I., & Tait, R.W. (1976). The Pavlovian analysis of instrumental conditioning. *The Pavlovian Journal of Biological Science*, *11*, 37–55. 10.1007/BF03000536

Guthrie, E.R. (1952). The psychology of learning (2nd, revised ed.). New York, NY: Harper & Brothers.

Haesler, S., Rochefort, C., Georgi, B., Licznerski, P., Osten, P., & Scharff, C. (2007). Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus area X. *PLoS Biology*, *5*, e321. doi:10.1371/journal.pbio.0050321

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., & Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature*, *454*, 217–220. doi:10.1038/nature07001

Hammer, M., & Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learning and Memory*, *5*, 146–156. doi:10.1101/lm.5.1.146

Harnish, R.M. (2002). *Minds, brains, and computers: An historical introduction to the foundations of cognitive science*. Malden, MA: Blackwell.

Hebb, D.O. (1956). The distinction between classical and instrumental. *Canadian Journal of Psychology*, *10*, 165–166. doi:10.1037/h0083677

Heisenberg, M. (2018). Mind from matter – via brain and behavior. *Neuroforum*, *24*, A95–A100. doi:10.1515/nf-2018-A002

Hellige, J.B., & Grant, D.A. (1974). Eyelid conditioning performance when the mode of reinforcement is changed from classical to instrumental avoidance and vice versa. *Journal of Experimental Psychology*, *102*, 710–719. doi:10.1037/h0036341

Hoffmann, J. (1993). *Vorhersage und Erkenntnis: die Funktion von Antizipationen in der menschlichen Verhaltenssteuerung und Wahrnehmung*. Göttingen: Hogrefe.

Hoffstaetter, L.J., Bagriantsev, S.N., & Gracheva, E.O. (2018). TRPs *et al.*: A molecular toolkit for thermosensory adaptations. *Pflügers Archiv – European Journal of Physiology*, *470*, 745–759. doi:10.1007/s00424-018-2120-5

Huang, Y.-C., Wang, C.-T., Su, T.-S., Kao, K.-W., Lin, Y.-J., Chuang, C.-C., … Lo, C.-C. (2019). A single-cell level and connectome-derived computational model of the *Drosophila* brain. *Frontiers in Neuroinformatics*, *12*, 99. doi:10.3389/fninf.2018.00099

Huetteroth, W., Perisse, E., Lin, S., Klappenbach, M., Burke, C., & Waddell, S. (2015). Sweet taste and nutrient value subdivide rewarding dopaminergic neurons in *Drosophila*. *Current Biology*, *25*, 751–758. doi:10.1016/j.cub.2015.01.036

Ibbotson, P., & Tomasello, M. (2016). Language in a New Key. *Scientific American*, *315*, 70–75. doi:10.1038/scientificamerican1116-70

Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *Journal of Neurobiology*, *47*, 81–92. doi:10.1002/neu.1018

Konorski, J., & Miller, S. (1937a). Further remarks on two types of conditioned reflex. *Journal of General Psychology*, *17*, 405–407. doi:10.1080/00221309.1937.9918010

Konorski, J., & Miller, S. (1937b). On two types of conditioned reflex. *Journal of General Psychology*, *16*, 264–272. doi:10.1080/00221309.1937.9917950

Konstantinides, N., Kapuralin, K., Fadil, C., Barboza, L., Satija, R., & Desplan, C. (2018). Phenotypic convergence: Distinct transcription factors regulate common terminal features. *Cell*, *174*, 622.e13–635.e13. doi:10.1016/J.CELL.2018.05.021

Kushner, R.F., Ryan, E.L., Sefton, J.M.I., Sanders, R.D., Lucioni, P.J., Moberg, K.H., & Fridovich-Keil, J.L. (2010). A *Drosophila melanogaster* model of classic galactosemia. *Disease Models and Mechanisms*, *3*, 618–627. doi:10.1242/dmm.005041

Leahey, T.H. (1987). *A history of psychology: Main currents in psychological thought*. Englewood Cliffs, NJ: Prentice-Hall.

Li, H., Horns, F., Wu, B., Xie, Q., Li, J., Li, T., … Luo, L. (2017). Classifying *Drosophila* olfactory projection neuron subtypes by single-cell RNA sequencing. *Cell*, *171*, 1206–1e22-1220.e22. doi:10.1016/j.cell.2017.10.019

Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B.D., Aso, Y., Friedrich, A.B., … Tanimoto, H. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature*, *488*, 512–516. doi:10.1038/nature11304

Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., … Liu, L. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. *Nature*, *439*, 551–556. doi:10.1038/nature04381

Liu, L., Wolf, R., Ernst, R., & Heisenberg, M. (1999). Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature*, *400*, 753–756. doi:10.1038/23456

Marler, P. (1991). Song-learning behavior: The interface with neuroethology. *Trends in Neurosciences*, *14*, 199–206. doi:10.1016/0166-2236(91)90106-5

Mendoza, E., Colomb, J., Rybak, J., Pflüger, H.-J., Zars, T., Scharff, C., & Brembs, B. (2014). *Drosophila* FoxP mutants are deficient in operant self-learning. *PLoS One*, *9*, e100648 doi:10.1371/journal.pone.0100648

Meneses, A., & Liy-Salmeron, G. (2012). Serotonin and emotion, learning and memory. *Reviews in the Neurosciences*, *23*, 5–6. doi:10.1515/revneuro-2012-0060

Mercer, A.R., & Menzel, R. (1982). The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee Apis mellifera. *Journal of Comparative Physiology A*, *145*, 363–368. doi:10.1007/BF00619340

Mishra, A., Salari, A., Berigan, B.R., Miguel, K.C., Amirshenava, M., Robinson, A., … Zars, T. (2018). The *Drosophila* Gr28bD product is a non-specific cation channel that can be used as a novel thermogenetic tool. *Scientific Reports*, *8*, 901. doi:10.1038/s41598-017-19065-4

Mozzachiodi, R., Baxter, D.A., & Byrne, J.H. (2013). Comparison of operant and classical conditioning of feeding behavior in *Aplysia*. *Invertebrate Learning and Memory*, *22*, 183–193. doi:10.1016/B978-0-12-415823-8.00015-0

Nargeot, R., Baxter, D.A., & Byrne, J.H. (1999a). In vitro analog of operant conditioning in *Aplysia*. I. Contingent reinforcement modifies the functional dynamics of an identified neuron. *Journal of Neuroscience*, *19*, 2247–2260. doi:10.1523/JNEUROSCI.19-06-02247.1999

Nargeot, R., Baxter, D.A., & Byrne, J.H. (1999b). In vitro analog of operant conditioning in *Aplysia*. II. Modifications of the functional dynamics of an identified neuron contribute to motor pattern selection. *Journal of Neuroscience*, *19*, 2261–2272. doi:10.1523/JNEUROSCI.19-06-02261.1999

Neckameyer, W.S. (1998). Dopamine and mushroom bodies in *Drosophila*: Experience-dependent and -independent aspects of sexual behavior. *Learning and Memory*, *5*, 157–165. doi:10.1101/lm.5.1.157

Ni, L., Bronk, P., Chang, E.C., Lowell, A.M., Flam, J.O., Panzano, V.C., … Garrity, P.A. (2013). A gustatory receptor paralogue controls rapid warmth avoidance in *Drosophila*. *Nature*, *500*, 580–584. doi:10.1038/nature12390

Nilius, B., Talavera, K., Owsianik, G., Prenen, J., Droogmans, G., & Voets, T. (2005). Gating of TRP channels: A voltage connection? *Journal of Physiology*, *567*, 35–44. doi:10.1113/jphysiol.2005.088377

Owald, D., Lin, S., & Waddell, S. (2015). Light, heat, action: Neural control of fruit fly behaviour. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *370*, 20140211. doi:10.1098/rstb.2014.0211

Palmer, D.C. (2006). On Chomsky's appraisal of Skinner's verbal behavior: A half century of misunderstanding. *The Behavior Analyst*, *29*, 253–267. doi:10.1007/BF03392134

Peabody, N.C., Pohl, J.B., Diao, F., Vreede, A.P., Sandstrom, D.J., Wang, H., … White, B.H. (2009). Characterization of the decision network for wing expansion in *Drosophila* using targeted expression of the TRPM8 channel. *Journal of Neuroscience*, *29*, 3343–3353. doi:10.1523/JNEUROSCI.4241-08.2009

Puig, M.V., Rose, J., Schmidt, R., Freund, N., Gonzalez-Burgos, G., Gunturkun, O., & Wang, M. (2014). Dopamine modulation of learning and memory in the prefrontal cortex: Insights from studies in primates, rodents, and birds. *Frontiers in Neural Circuits*, *8*, 93. doi:10.3389/fncir.2014.00093

Quinn, W.G., Harris, W.A., & Benzer, S. (1974). Conditioned behavior in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, *71*, 708–712. doi:10.1073/pnas.71.3.708

Reich, E.S. (2012). War of words over tribal tongue. *Nature*, *485*, 155–156. doi:10.1038/485155a

Rescorla, R.A. (1994). Control of instrumental performance by Pavlovian and instrumental stimuli. *Journal of Experimental Psychology: Animal Behavior Processes*, *20*, 44–50. doi:10.1037/0097-7403.20.1.44

Rubio-Gozalbo, M.E., Haskovic, M., Bosch, A.M., Burnyte, B., Coelho, A.I., Cassiman, D., … Berry, G.T. (2019). The natural history of classic galactosemia: Lessons from the GalNet registry. *Orphanet Journal of Rare Diseases*, *14*, 1–11. doi:10.1186/s13023-019-1047-z

Sakaguchi, H., & Yamaguchi, A. (1997). Early song-deprivation affects the expression of protein kinase C in the song control nuclei of the zebra finch during a sensitive period of song learning. *Neuroreport*, *8*, 2645–2650. doi:10.1097/00001756-199708180-00002

Scharff, C., & Petri, J. (2011). Evo-devo, deep homology and FoxP2: Implications for the evolution of speech and language. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*, 2124–2140. doi:10.1098/rstb.2011.0001

Schreiweis, C., Bornschein, U., Burguière, E., Kerimoglu, C., Schreiter, S., Dannemann, M., … Graybiel, A.M. (2014). Humanized Foxp2 accelerates learning by enhancing transitions from declarative to procedural performance. *Proceedings of the National Academy of Sciences of the United States of America*, *111*, 14253–14258. doi:10.1073/pnas.1414542111

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., … Fiala, A. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Current Biology*, *16*, 1741–1747. doi:10.1016/j.cub.2006.07.023

Schulz, D. J., Robinson, G. E. (1999). Biogenic amines and division of labor in honey bee colonies: Behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *Journal of Comparative Physiology A*, *184*, 481–488. doi:10.1007/s003590050348

Schwaerzel, M., Heisenberg, M., & Zars, T. (2002). Extinction antagonizes olfactory memory at the subcellular level. *Neuron*, *35*, 951–960. doi:10.1016/S0896-6273(02)00832-2

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., & Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *Journal of Neuroscience*, *23*, 10495–10502. doi:10.1523/JNEUROSCI.23-33-10495.2003

Sheffield, F. D. Prokasy. (1965). *Relation between classical conditioning and instrumental learning*. In W. F. Prokasy (Ed.), Classical conditioning: A symposium. New York, NY: Appleton-Century-Crofts.

Shih, C.-T., Sporns, O., Yuan, S.-L., Su, T.-S., Lin, Y.-J., Chuang, C.-C., … Chiang, A.-S. (2015). Connectomics-based analysis of information flow in the *Drosophila* brain. *Current Biology*, *25*, 1249–1258. doi:10.1016/j.cub.2015.03.021

Sitaraman, D., Kramer, E.F., Kahsai, L., Ostrowski, D., & Zars, T. (2017). Discrete serotonin systems mediate memory enhancement and escape latencies after unpredicted aversive experience in *Drosophila* place memory. *Frontiers in Systems Neuroscience*, *11*, 92. doi:10.3389/fnsys.2017.00092

Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y.-C., Sable-Smith, A., Kitamoto, T., … Zars, T. (2008). Serotonin is necessary for place

memory in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 5579–5584. doi:10.1073/pnas.0710168105

Sitaraman, D., Zars, M., & Zars, T. (2010). Place memory formation in *Drosophila* is independent of proper octopamine signaling. *Journal of Comparative Physiology*, *196*, 299–305. doi:10.1007/s00359-010-0517-5

Sitaraman, D., Zars, M., & Zars, T. (2007). Reinforcement pre-exposure enhances spatial memory formation in *Drosophila*. *Journal of Comparative Physiology A*, *193*, 903–908. doi:10.1007/s00359-007-0243-9

Sitaraman, D., & Zars, T. (2010). Lack of prediction for high-temperature exposures enhances *Drosophila* place learning. *Journal of Experimental Biology*, *213*, 4018–4022. doi:10.1242/jeb.050344

Skinner, B.F. (1935). Two types of conditioned reflex and a pseudo type. *Journal of General Psychology*, *12*, 66–77. doi:10.1080/00221309.1935.9920088

Skinner, B.F. (1937). Two types of conditioned reflex: A Reply to Konorski and Miller. *The Journal of General Psychology*, *16*, 272–279. doi:10.1080/00221309.1937.9917951

Skinner, B. F. (1957). *Verbal behavior*. New York, NY: Appleton-Century-Crofts. doi:10.1037/11256-000

Smith, N. (1999). *Chomsky: Ideas and ideals*. Cambridge, UK: Cambridge University Press.

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., … Patapoutian, A. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, *112*, 819–829. doi:10.1016/S0092-8674(03)00158-2

Tempel, B.L., Livingstonet, M.S., & Quinn, W.G. (1984). Mutations in the dopa decarboxylase gene affect learning in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *81*, 3577–3581. doi:10.1073/pnas.81.11.3577

Trapold, M.A., Lawton, G.W., Dick, R.A., & Gross, D.M. (1968). Transfer of training from differential classical to differential instrumental conditioning. *Journal of Experimental Psychology*, *76*, 568–573. doi:10.1037/h0025709

Trapold, M.A., & Overmier, J.B. (1972). *The second learning process in instrumental conditioning*. In A. H. Black & W. F. Prokasy (Eds.), Classical conditioning II: Current research and theory (pp. 427–452). New York, NY: Appleton-Century-Crofts.

Trapold, M.A., & Winokur, S. (1967). Transfer from classical conditioning and extinction to acquisition, extinction, and stimulus generalization of a positively reinforced instrumental response. *Journal of Experimental Psychology*, *73*, 517–525. doi:10.1037/h0024374

Vaysse, G., Galissié, M., & Corbière, M. (1988). Induced variation of serotonin in *Drosophila melanogaster* and its relation to learning performance. *Journal of Comparative Psychology*, *102*, 225–229. doi:10.1037/0735-7036.102.3.225

Virués-Ortega, J. (2006). The case against B. F. Skinner 45 years later: An encounter with N. Chomsky. *The Behavior Analyst*, *29*, 243–251. doi:10.1007/BF03392133

Wagener-Hulme, C., Kuehn, J.C., Schulz, D.J., & Robinson, G.E. (1999). Biogenic amines and division of labor in honey bee colonies. *Journal of Comparative Physiology*, *184*, 471–479. doi:10.1007/s003590050347

Watkins, K.E., Dronkers, N.F., & Vargha-Khadem, F. (2002). Behavioural analysis of an inherited speech and language disorder: Comparison with acquired aphasia. *Brain*, *125*, 452–464. doi:10.1093/brain/awf058

Watrin, J.P., & Darwich, R. (2012). On behaviorism in the cognitive revolution: Myth and reactions. *Review of General Psychology*, *16*, 269–282. doi:10.1037/a0026766

Wolf, R., & Heisenberg, M. (1991). Basic organization of operant behavior as revealed in Drosophila flight orientation. *Journal of Comparative Physiology A*, *169*, 699–705. doi:10.1007/bf00194898

Wolf, R., Wittig, T., Liu, L., Wustmann, G., Eyding, D., & Heisenberg, M. (1998). *Drosophila* mushroom bodies are dispensable for visual, tactile, and motor learning. *Learning and Memory*, *5*, 166–178. doi:10.1101/lm.5.1.166

Wustmann, G., & Heisenberg, M. (1997). Behavioral manipulation of retrieval in a spatial memory task for *Drosophila melanogaster*. *Learning and Memory*, *4*, 328–336. doi:10.1101/lm.4.4.328

Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.-Y., Friedrich, A.B., Sima, R.J., … Tanimoto, H. (2015). Distinct dopamine neurons mediate reward signals for short- and long-term memories. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 578–583. doi:10.1073/pnas.1421930112

Yoshida, Y., Yamada, T., & Sakaguchi, H. (2003). Activation of protein kinase C by the error signal from a basal ganglia-forebrain circuit in the zebra finch song control nuclei. *Neuroreport*, *14*, 645–649. doi:10.1097/00001756-200303240-00025

Zars, T. (2001). Two thermosensors in *Drosophila* have different behavioral functions. *Journal of Comparative Physiology A*, *187*, 235–242. doi:10.1007/s003590100194

Zars, T., Fischer, M., Schulz, R., & Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. *Science*, *288*, 672–675. doi:10.1126/science.288.5466.672

Zars, T., Wolf, R., Davis, R., & Heisenberg, M. (2000). Tissue-specific expression of a type I adenylyl cyclase rescues the *rutabaga* mutant memory defect: In search of the engram. *Learning and Memory*, *7*, 18–31. doi:10.1101/lm.7.1.18

Zheng, Z., Lauritzen, J.S., Perlman, E., Robinson, C.G., Nichols, M., Milkie, D., … Bock, D.D. (2018). A complete electron microscopy volume of the brain of adult *Drosophila melanogaster*. *Cell*, *174*, 730–743. doi:10.1016/j.cell.2018.06.019