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Brian H. Smith

Chelsea N. Cook

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Brian H. Smith
School of Life Sciences, Arizona State University, Tempe, AZ
Chelsea N. Cook
School of Life Sciences, Arizona State University, Tempe, AZ

Abstract

Behavior genetics, and specifically the study of learning and memory, has benefitted immensely from the development of powerful forward- and reverse-genetic methods for investigating the relationships between genes and behavior. Application of these methods in controlled laboratory settings has led to insights into gene–behavior relationships. In this perspective article, we argue that the field is now poised to make significant inroads into understanding the adaptive value of heritable variation in behavior in natural populations. Studies

of natural variation with several species, in particular, are now in a position to complement laboratory studies of mechanisms, and sometimes this work can lead to counterintuitive insights into the mechanism of gene action on behavior. We make this case using a recent example from work with the honey bee, Apis mellifera.

Keywords

Learning polymorphism; mechanism; adaptation; honey bee; latent inhibition

Introduction

Forward (behavior-to-gene) genetic screens are a powerful means for investigating bases for individual differences in learning and memory (McGuire, Deshazer, & Davis, [40]). Classically, these screens study individual differences either by identifying heritable variation among individuals (Chandra, Hosler, & Smith, [9]), or more commonly by generating differences among individuals using mutagenic techniques (Dudai, Jan, Byers, Quinn, & Benzer, [17]). Both means for establishing genetically-based variation in behavioral performance have been used to develop laboratory-maintained strains of animals that can be used for more detailed investigations into the neural and molecular bases of these behaviors. More recently, reverse genetic approaches have been used to explore causal relationships between identified genes and behavioral plasticity (Venken, Simpson, & Bellen, [63]). Thus the tremendous amount of work that has been done over the past decades in starting with behavior and moving to genes, or vice versa, has been of immense value in understanding mechanisms of behavior, and specifically of learning and memory, common to animals as diverse as the almost microscopic round worm *Caenorhabditis elegans* (Lau, Timbers, Mahmoud, & Rankin, [35]), the fruit fly *Drosophila melanogaster* (Davis, [15]), and mammals (Tonegawa, Nakazawa, & Wilson, [62]).

Troy Zars was a major contributor to this literature, and a strong point of his collective works was an abiding interest in detailed studies of both genetic and behavioral mechanisms. Several of his recent studies evaluated the effects of different identified genes on learning behaviors (LaFerriere, Ostrowski, Guarnieri, & Zars, [32]; LaFerriere, Ostrowski, et al., [33]; Mendoza et al., [41]). His behavioral studies evaluated different types of memory, and in particular place memory (Ostrowski, Kahsai, Kramer, Knutson, & Zars, [48]; Sitaraman & Zars, [55]), including how learned information can interact, and override, innate preferences (Baggett et al., [3]). This work extended into how natural variation in the foraging gene (Chen et al., [12]), and how multiple genetic loci across different inbred lines of fruit flies (Williams-Simon et al., [64]), affect behavior. This latter work, in particular, provides a segue to an important theme that is emerging in the literature on gene-to-behavior relationships.

The central point of this perspective article is that *we need an equally strong effort toward understanding the ecological conditions in which individual variation in learning and memory has evolved and been maintained*. Such an endeavor, we will argue, can provide equally important, and sometimes counterintuitive, insights into mechanisms that underlie learning and memory. Furthermore, those insights stand to in turn inform controlled, mechanistic studies in the laboratory.

An important means for studying natural variation has been to evaluate animals that differ in performance on a specific learning task. The standard approach would be to then investigate in a more reductionistic way the neural and genetic determinants of this variation. In addition, it is important to study the role of this genetic variation in natural environments. This would involve testing animals of different, defined genotypes under semi-natural or natural conditions to establish what the genetic variation might do to enhance the actual fitness of individuals that exhibit different traits. Work on the fruit fly clock neurons under natural conditions, after having been first isolated and studied in the laboratory (Konopka & Benzer, [30]), is a classic example of this kind of approach (Menegazzi et al., [42]; Noreen, Pegoraro, Nouroz, Tauber, & Kyriacou, [47]; Sawyer et al., [53]).

The approach advocated below with the specific example of honey bees, which is a focus on heritable, natural variation, can be as important as studies using mutagenic techniques, because *natural variation could reveal mechanisms of learning and memory that might not be readily revealed with mutagenic studies*. The latter requires random 'blind' mutation in the genome or targeting of a specific gene using molecular manipulation, which is then followed by establishing a phenotype for that gene. However, as powerful as these strategies are, they may identify mutations that cannot survive under natural conditions. Moreover, neither strategy would be guaranteed to land on a gene that has been subject to natural selection for learning polymorphisms.

There are guiding principles that could be used to develop expectations and exploration of why natural variation within a species might exist (see rev in (Mery, [44])). And there are examples of the integrated laboratory-to-nature approach advocated here. Work in learning under natural conditions (Mery, [44]) and on the foraging gene (Anreiter & Sokolowski, [2]) of the fruit fly, *Drosophila melanogaster*, provide some very good examples. Furthermore, there have been many recent studies using learning in the wasp *Nasonia vitripennis* (Koppik, Hoffmeister, Brunkhorst, Kieß, & Thiel, [31]; Liefting, Hoedjes, Le Lann, Smid, & Ellers, [36]; Liefting, Rohmann, Le Lann, & Ellers, [37]) and in different species of the bumble bee *Bombus* (Muth, Cooper, Bonilla, & Leonard, [46]; Riveros & Gronenberg, [52]; Wolf & Chittka, [65]) that address related areas. With the advent of new methods for generation of transgenic animals in these species (Adli, [1]), combined with different means for measuring neural activity under controlled conditions where learning mechanisms can be evaluated (Riveros & Gronenberg, [52]), work with several species now promises to advance the approach advocated here.

However, a comprehensive review of this literature is beyond the scope of this perspectives article (see Giurfa, [23]). The focus here, therefore, will be on recent work with the honey bee (*Apis mellifera*), which has been instructive for how a study of natural variation can give insight into a specific mechanism.

Studies of learning and memory in the honey bee, Apis mellifera

Karl von Frisch (Frisch, [21]) first demonstrated that honey bee foragers learn spatial locations, colors and odors of floral resources that their colonies need for survival. Since then many studies have trained honey bees to fly to feeders in order to investigate what honey bees learn and how they use that information in different contexts (Menzel, [43]). Foragers that depart the colony to visit a feeder comprise a subset of bees in any colony that are motivated to fly some distance to forage for whatever reward is offered at the feeders. Honey bees can also be captured in or around the colony and brought into the laboratory to evaluate learning performance. In contrast to collections at a feeder, honey bees collected from the entrance could differ in experience and/or motivational state. For example, they can be foragers that specialize in collecting nectar or pollen (Page, Erber, & Fondrk, [50]), they could be from different behavioral castes (e.g. guards vs foragers) (Seeley, [54]) or be of different ages (young bees making orientation flights) and levels of foraging experience.

Once in the laboratory, bees can be restrained in harnesses so that they can freely move antennae and mouthparts (proboscis). One widely used conditioning procedure for restrained bees is called Proboscis Extension Response conditioning (Smith & Burden, [57]). PER in the laboratory allows for much more control of variables that are important for studying several different forms of nonassociative, associative and operant conditioning (Bitterman, Menzel, Fietz, & Schafer, [6]). In the basic procedure, an odor is presented in a predictive way with sugar reinforcement presented to the mouthparts. Pairing of the odor with sugar reinforcement generates an expectation of food whenever the odor is encountered. This expectation causes a bee to extend its proboscis in anticipation of, and in preparation for, reinforcement when it is presented. PER has been used to evaluate neural mechanisms of plasticity under more controlled conditions that allow for bioimaging (Deisig, Giurfa, Lachnit, & Sandoz, [16]; Galizia, Joerges, Kuttner, Faber, & Menzel, [22]; Locatelli, Fernandez, & Smith, [38]), electrophysiological recordings (Strube-Bloss, Herrera-Valdez, & Smith, [60]; Strube-Bloss, Nawrot, & Menzel, [61]), pharmacological (Hammer & Menzel, [26]; Stopfer, Bhagavan, Smith, & Laurent,

[59]) and molecular (Farooqui, Robinson, Vaessin, & Smith, [18]; Fiala, Muller, & Menzel, [20]) manipulation of the nervous system.

Individual differences in learning performance in per conditioning

The learning capabilities that have been revealed in PER studies certainly have evolved in response to environmental contingencies – such as the stimulation a honey bee receives as it approaches, lands on and feeds from a flower – that honey bees have experienced under natural conditions in the field. In addition, PER has also revealed individual variation in learning performance that is not readily observed when highly motivated foragers are trained to feeders, because animals not motivated to forage for sugars typically presented at feeders may not show up at the feeder. On tasks ranging from acquisition of an odor-sucrose association (Benatar, Cobey, & Smith, [4]) to more complicated conditioning protocols such as Reversal Learning (Chandra et al., [9]), Blocking (Smith, [56]), Signaled Avoidance (Smith, Abramson, & Tobin, [58]) and Latent Inhibition (Chandra, Wright, & Smith, [11]), a fraction of bees perform well, whereas another set of bees fail to learn the task according to expected criteria.

This variation could arise among bees from the same colony first and foremost because honey bees collected from the colony entrance can be in widely different motivational states, as reviewed above. Many of these differences can be controlled via the experimental composition of 'single cohort' colonies comprised of workers of the same age. Under this condition, the workers still divide into behavioral castes that the colony needs. Thus, using single-cohort colonies, same aged workers can be evaluated in PER as they age and controlling for whether they are nurses, guards or foragers. However, an early study using single-cohort colonies failed to find any correlation of PER performance to either age or behavioral caste (Bhagavan, Benatar, Cobey, & Smith, [5]).

The next obvious variable that could underlie individual differences among bees from the same colony is the genotype. Honey bee queens are polyandrous (Page, [49]), meaning that they mate with up to 20 drones and use sperm from those drones throughout egg laying. Thus at any given time workers in a colony arise from the same queen but from different drones, which establishes up to 20 different patrilines in any colony.

Furthermore, honey bee queens and drones, as well as bumble bee drones (Wolf & Chittka, [65]), can be easily conditioned in PER (Benatar et al., [4]; Chandra et al., [9]; Chandra, Hunt, Cobey, & Smith, [10]; Gong, Tan, & Nieh, [24]) (Box 1), which presents a powerful means for studying the heritability of individual learning phenotypes. After conditioning in PER, sperm from drones can be used to instrumentally inseminate (Cobey, [13]) virgin queens that show the same 'good' or 'poor' learning phenotypes. These studies take advantage of drones being haploid, because they arise from an unfertilized egg laid by the queen. That is, a drone is essentially a queen's gamete, and that gamete also happens to have impressively sophisticated learning abilities.

Box 1.

Why would queen and drone honey bees learn? Maybe the developmental machinery for making a brain that can learn is just too difficult to disassemble in queens and drones (see Liefting *et al*., [36]). Alternatively, it seems also likely that there is much in the biology of queens and drones that we do not to this day understand, such as the need to recognize nestmates, their home colony location or potentially the need to pass learned information to offspring, for example, via DNA methylation (Gong *et al*., [24]).

Breeding genetic lines via crossing drone and queen honey bees with like learning performance has now demonstrated significant heritability of learning phenotypes within domestic (natural) honey bee populations for Discrimination Conditioning (Benatar et al., [4]), Reversal Learning (Ferguson, Cobey, & Smith, [19]) and Latent Inhibition (Chandra et al., [9]) (Box 2) (Brandes, [7]; Brandes, Frisch, & Menzel, [8]). Therefore, much of

the variation revealed in studies of PER is genetic, and natural colonies probably contain workers that represent several of the possible genotypes at any given locus. Furthermore, one would assume that this variation somehow serves the colony. But how it might serve the colony requires more understanding of the genes that underlie the trait.

Box 2.

Brandes *et al*. ([8], [7]) studied genetic variation for learning performance by using a genetic line of honey bees in which workers reproduce parthenogenically. They were able to establish that individual differences in learning performance in workers tracked to their parthenogenically produced offspring, thus demonstrating heritability. They also demonstrated how learning traits affected colony performance.

Mapping learning traits in the genome

Quantitative Trait Locus mapping has revealed a few loci in the honey bee genome that correlate to Latent Inhibition and Reversal Learning (Chandra et al., [10]) (Box 3). Once high and low lines have been established, queens and drones from the lines can be crossed to establish a queen that is a hybrid genotype. This queen will then produce drones that are various recombinants of the lines, and using high-throughput learning assays many (e.g. over a thousand) drones can be evaluated for learning performance (Chandra et al., [10]). Those showing extremes of high and low can be used for genome sequencing guided by established markers such as Single-Nucleotide Polymorphisms or RAPD primers (Hunt & Page, [27]; Hunt, Page, Fondrk, & Dullum, [29]). Using QTL mapping, one locus in particular – called LRN1 (Chandra et al., [10]) – has been correlated to heritability in Latent Inhibition in QTL mapping studies. Several genes lie around this locus, and studies to evaluate the nature of the effects of these genes are ongoing. For the present purpose, it is necessary to highlight an independent set of studies that involved QTL mapping of a different forager trait – pollen collection. Colonies can differ in their propensity to collect pollen, and early studies of established significant heritable variation for pollen collection (Page, Rueppell, & Amdam, [51]; R., 2013). Later QTL mapping identified a few regions in the genome, one of which was called 'PLN2' (Hunt et al., [29]), and it is the same region as LRN1.

Box 3.

In reversal learning, honey bees are first conditioned to discriminate an odor (A+) that predicts a sugar reward from a second odor (Xo) that is not associated with reward. All individuals that successfully learn to respond to A but not to X are then introduced to a second phase, during which the reinforcement is switched to Ao versus X+. During the second phase animals that learn to switch fast versus slowly can be identified. Latent Inhibition involves unreinforced exposure to an odor (Xo) without reinforcement. After 20–40 such exposures, animals typically learn X+ (now reinforced) slowly relative to a novel odor A+. As with Reversal Learning, animals can be separated according to whether they learned not to respond to X + versus animals that respond normally to X + in spite of the Xo treatment in the first phase.

Thus independent QTL studies evaluating heritable variation for learning performance and pollen collection, two presumably important traits for foragers, have been mapped in part to the same genetic locus. There are several genes at this locus, any of which, or any combination of which, could affect one or both behaviors (Hunt et al., [28]). Nevertheless, one gene for a tyramine receptor (*amtyr*) stands out, because it is a precursor to octopamine, which has been identified as an important component of reinforcement signaling in the honey bee brain (Hammer, [25]). However, at this point further study is needed to establish causal linkages between genes at PLN2/LRN1 and the behaviors.

There could, of course, be separate genes for learning performance and pollen collection in the LRN1/PLN2 region. Alternatively, it could be one gene with broad phenotypic effects. If that is the case, what exactly is that effect, and how does it serve the colony? It could be that this gene or gene (s) are not genes *for* Latent Inhibition or *for* pollen collection *per se*. There might be an overarching function that unifies and explains both behaviors.

Performance of selected lines under semi-natural conditions

More recently **Cook** et al (**Cook** et al., [14]) took learning lines selected in the laboratory, as described above, for performance on Latent Inhibition and used them as a treatment condition in creation of single-cohort field studies. They created colonies composed of a large batch of workers that were from the same 'high' or 'low' learning phenotype or mixed 50:50. Workers from those colonies were first evaluated for performance on the standard Latent Inhibition protocol in the laboratory. This experiment showed that the learning phenotype was not influenced by the colony composition. Animals from 'high' lines selected for strong Latent Inhibition (slower learning of X + than A + after Xo treatment) were still high in Latent Inhibition performance, and vice versa for low line bees, regardless of being housed with a number of background, unselected bees or with bees from a different genotype. All colonies were maintained singly in large enclosures where feeders could be presented to establish how the bees in the colony responded to different feeder configurations that involved a known feeder and one or more new feeders put into the tent just prior to testing. Specifically, using marked bees, it was possible to establish what bees found novel feeders placed into the enclosure after foragers had been feeding for some time at the known feeder.

Forgers from the high line tended to stay with the known feeder after new, novel feeders were introduced. Foragers from the Low line were the ones that most often found and exploited the novel feeders. Moreover, foragers from the High line recruited via the round dance more vigorously, and foragers from both lines preferentially follow dances from the High line foragers.

These results point to an almost counterintuitive effect of the gene (s), potentially at LRN1/PLN2, that could unify the learning and pollen phenotypes. One interpretation for Latent Inhibition is that it reflects a process of 'attention' (Lubow, [39]). Animals attend to new stimuli, and that attention is strengthened or attenuated once it is clear whether or not those stimuli are associated with anything of consequence. This allows animals to focus potentially limited neural resources that underlie attention to stimuli on stimuli that are meaningful. In this interpretation, High line foragers show strong Latent inhibition and concomitantly strong processes of attention. They thus intensively focus on the exploitation of good resources in the local environment. Low line foragers are 'less attentive' and possibly more likely to locate new resources. Although, as a caveat, this explanation of the results is still only a hypothesis. Furthermore, that there is a process like attention in honey bees, or in any insect, still needs to be investigated in much more detail to establish to what extent it is, or isn't, like that in mammals.

Conclusions

In summary, taking laboratory selected learning lines and evaluating them in the field has revealed a potentially important forager attribute that affects how foragers explore and exploit resources. Moreover, a recent combined modeling and empirical study has shown how colonies of different mixtures of these learning types might be more effective at exploiting environments with different resource distributions (Mosqueiro et al., [45]). And the attention interpretation for the effect of the gene (s) at LRN1/PLN2 provides a testable hypothesis for explaining the large effect on both learning and pollen collection at this locus. Foragers from the high line, who show stronger attention, might be more sensitive to needs of the colony, and thus when the colony is short of pollen they focus on pollen collection more so than foragers from the Low line. Accordingly, there is a correlation between high Latent Inhibition and high pollen collection (Latshaw & Smith, [34]).

In closing, we have reviewed recent work on genetic variation for learning performance in honey bees, and we have shown how field studies using laboratory selected lines can potentially reveal testable hypotheses about the mechanism. And, they can help to narrow down answers to why this genetic variation exists and the ways it affects neural networks in the brain. More studies from the laboratory to the field and back are sorely needed, especially with animals in which the ability to study and manipulate genomes is now well established.

Disclosure statement

No potential conflict of interest was reported by the authors.

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