Casting a Genome-wide Net for Learning Mutants

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Wolman et al. (2015) report a forward genetic screen in zebrafish that implicated *pregnancy-associated plasma protein-aa* in habituation of the acoustic startle response. PAPP-AA is expressed in the underlying circuit, including Mauthner cells, and regulates habituation via IGF signaling.

Habituation is a non-associative form of learning characterized by a decremented response to repeated stimulation that cannot be explained by sensory adaptation or motor fatigue. It has been documented in diverse behavioral and cellular responses in organisms across phylogeny, from single-celled protists to humans. It is often considered a "cognitive building block" and the basis of selective attention. Consistent with this fundamental role, deficits in habituation are associated with a variety of neuropsychiatric disorders, including schizophrenia, autism, Tourette's, and obsessive-compulsive disorder. In a landmark paper in 1966, Thompson and Spencer (1966) outlined nine common characteristics of habituation, which have remained essentially unchanged ever since (Rankin et al., 2009). Despite a thorough behavioral characterization and conservation over hundreds of millions of years of evolution, the cellular and molecular processes underlying habituation remain poorly understood. Researchers have turned to a diverse set of model systems for a mechanistic explanation. This effort is exemplified by an article in this issue of *Neuron*, "A Genome-wide Screen Identifies PAPP-AA-Mediated IGFR Signaling as a Novel Regulator of Habituation Learning" (Wolman et al., 2015).

Since the pioneering work of Eric Kandel on habituation of the gill- and siphon-withdrawal reflex in *Aplysia*, the tractable nervous systems of invertebrates have been used as a powerful tool for unraveling the mechanisms of habituation. Despite being touted as the simplest form of learning, it has become apparent that habituation is mediated by multiple mechanisms operating at different levels of the circuitry and dependent on the nature and pattern of stimulation. In genetic model systems like *C. elegans* and *Drosophila melanogaster*, molecular components of habituation have been identified through both unbiased and candidate mutant screens (Table 1). Easily reared in the lab and capable of producing a large number of progeny, zebrafish offer many of the same advantages as invertebrates, but with a nervous system more similar to mammals. Larvae are especially appealing for large-scale screens, as they can be maintained in dense populations, and genes essential for adult viability can be studied. Furthermore, the larvae exhibit several stereotyped behaviors, like the acoustic startle response used by Wolman et al. (2015).

The acoustic startle response is an extremely fast evasive maneuver beginning with a C-shaped body bend (C-start) less than 10 ms after a brief auditory pulse, followed by a smaller counterbend and swimming. The response is highly stereotyped in terms of body angle, velocity, and duration (Burgess and Granato, 2007). Mediating the response are the Mauthner cells, a pair of bilaterally symmetrical command neurons in the hindbrain that receive multimodal sensory input and activate contralateral motor neurons. Although the kinematics of the response does not change, the likelihood of occurrence declines with repeated stimuli in a manner consistent with the key characteristics of habituation (Wolman et al., 2011). Retention of training can persist from minutes to hours, depending on the specifics of the stimulation protocol (Roberts et al., 2013).

Wolman et al. (2015) used an automated high-throughput system to perform a forward genetic screen for aberrant learning in a rapid habituation assay. They were able to isolate 14 mutants that had normal C-start kinematics, but slowed habituation kinetics. In naive animals, C-starts are more likely in moving than in stationary larvae (Burgess and Granato, 2007), and so it was essential to rule out elevated basal activity as the source of increased responsivity in the habituation assay. Importantly, none of the mutants traveled significantly further than controls over 160 s, although four mutants had reduced locomotion. It is worth noting that the majority of the mutants (10/14) were more responsive than control to subthreshold acoustic stimuli, suggesting increased sensitivity. This is relevant since one of the nine key characteristics of short-term habituation is that more intense stimulation results in a less pronounced response decrement (Rankin et al., 2009). Therefore, some of the affected genes may not be essential for mediating habituation per se, but rather setting the tone of the circuit. Interbreeding the strains for complementation testing revealed that the mutant alleles affected 13 genes (each designated most appropriately, e.g., *oops I did it again* and *ignorance is bliss*). The degree of the habituation deficit varied across strains. The most impaired, *unfiltered*, exhibited less than a 5% decrease in the probability of a C-start response at the end of the assay (compared to > 80% in controls), a deficit apparent over a range of interstimulus intervals.

With a three-generation mapping cross followed by whole-genome sequencing and homozygosity analysis, Wolman et al. (2015) identified a candidate single-nucleotide polymorphism for two of the mutants, *information overload* and *unfiltered*. In *information overload* the mutation resulted in a premature stop codon

Table 1. High-Throughput Testing for Habituation Mutants

Model System	Behavioral Response	Genomes Screened	Habituation Mutants	Genetic Loci	Molecular Identity	References
Zebrafish	acoustic startle	614	14	13	2	Wolman et al., 2015
Drosophila	olfactory startle	874	26	26	26	Eddison et al., 2012
Drosophila	olfactory startle	150	0	0	0	Sharma et al., 2009
C. elegans	tap withdrawal	33	2	2	1	Swierczek et al., 2011

in *pyruvate carboxylase a* (*pcxa*), which encodes a mitochondrial enzyme for synthesizing oxaloacetate, an intermediate in many metabolic pathways. Although not thought to be active in neurons, in astrocytes PCXA is essential for the biosynthesis of the neurotransmitter, glutamate. Further work on this gene may therefore identify a novel locus of plasticity for habituation, i.e., glia.

In *unfiltered* the mutation caused a premature stop codon in exon 3 of *pregnancy-associated plasma protein-aa* (*pappaa*), resulting in the loss of the catalytic and membrane tethering domains for the encoded extracellular metalloprotease. This was confirmed as the causative allele by the successful rescuing of the *unfiltered* habituation phenotype with injection of either zebrafish or human *pappaa* mRNA. Expression of a proteolytically inactive human PAPP-AA variant did not rescue the learning deficit, suggesting that metalloprotease activity of PAPP-AA is required for normal habituation. For a better understanding of PAPP-AA function, Wolman et al. (2015) did whole-mount in situ hybridization to define the expression pattern. *pappaa* mRNA was detected in several components of the circuitry underlying the acoustic startle response, most notably the Mauthner cells, but also sensory afferents and interneurons known to modulate Mauthner activity. Importantly, the *unfiltered* mutant had no apparent morphological defects in the circuit. It remains to be determined in which cell class PAPP-AA is functioning to promote habituation.

IGF binding protein 4 (IGFBP4) is a known target for cleavage by PAPP-AA, which results in increased bioavailability of IGF-1. Wolman et al. (2015) therefore tested if pharmacological activation of the canonical IGFR signaling pathway could compensate for loss of PAPP-AA. Indeed, the habituation deficit of the *unfiltered* mutant was improved by acute exposure to Akt activator, SC79, or PI 3-kinase activator, 740 Y-P. Furthermore, a pharmacological inhibitor of IGF1R, BMS-754807, recapitulated the habituation deficit of the *unfiltered* mutant. Interestingly, the pharmacological activators did not simply induce a habituated state in naive animals, but rather facilitated normal learning. This suggests that a PAPP-AA/IGF1R/PI3K/Akt pathway is essential, but not instructive, for habituation of the acoustic startle response.

With this entry point, it will be exciting to see just how IGFR signaling promotes habituation. Further novel insights will undoubtedly arise from cloning the other 11 mutants, some of which also have deficits in a visual habituation assay of another distinct turning maneuver, the O-bend. This paper highlights the value of a large-scale unbiased forward genetic screen for implicating genes without any assumptions about the underlying molecular mechanism. The specificity of the assay for finding learning mutants stems from the use of a high-speed camera and computer vision software for detailed behavioral analysis of every animal. It is more of an automated characterization than a traditional screen. The success of this approach to date should motivate continued screening to saturation. Habituation is a highly conserved phenomenon, but it is not a single process; rather, it comprises multiple mechanisms operating at different levels of the circuit. The gene highlighted here, *pappaa,* is vertebrate-specific; however, other molecular components implicated in habituation appear to be conserved across phylogeny. For example, the large conductance voltage- and calcium-activated potassium (BK) channel mediates short-term habituation in mice, flies, and worms (Engel and Wu, 1998; Typlt et al., 2013). The best chance for a complete characterization of mechanisms of habituation relies on the continued use of diverse genetic model organisms with tractable nervous systems.

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