I Can't Watch: A Genetic and Circuit-Level Investigation of Observational Fear Learning

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https://doi.org/10.1016/j.neuron.2018.04.023

In this issue of *Neuron*, Keum et al. (2018) identify a *Nrxn3* variant that produces an enhancement of observational fear learning. Results suggest that *Nrxn3* loss of function, specifically within somatostatin-positive interneurons of the anterior cingulate cortex, is responsible.

Following learning, a previously neutral conditioned stimulus (CS) can elicit a fearful response. In order for this to occur, exposure to the CS must predict the experience of a naturally aversive unconditioned stimulus (US). This phenomenon has incredible adaptive value by permitting animals to leverage the CS within their environment to avoid threats and therefore facilitate their survival and reproduction. A learned fear response can be acquired directly or indirectly. For example, you may avoid driving through an intersection (CS) in which you have previously been in a car accident (US). However, you may also avoid an intersection in which you have observed others get into a car accident. This sort of vicarious learning is advantageous, as it permits learned avoidance of a threat without direct experience. How is it that the nervous system forms the association of an aversive US with a neutral CS simply through observation? In classical fear conditioning, representations of CS and US converge on the amygdala in order to form an association that enables learned behavior (LeDoux, 2000). Similarly to classical fear conditioning, observational fear learning (OFL), or learning through others' experience, is amygdala mediated (Jeon et al., 2010; Phillips and LeDoux, 1992: Olsson et al., 2007). A key difference is the unique requirement of anterior cingulate cortex (ACC) activity in OFL (Jeon et al., 2010). The ACC, which sends efferent fibers to the amygdala, is known to be engaged when observing others experiencing pain (Singer et al., 2004). Furthermore, enhanced synchronous activity between the ACC and the amygdala during OFL suggests that these two critical nodes may communicate to subserve this behavior (Jeon et al., 2010).

In this issue of *Neuron*, Keum et al. (2018) provide genetic and circuit-level insight into the involvement of the ACC in OFL. The authors begin by assaying 17 different inbred mouse strains on an observational fear learning task. In this task, an observer mouse witnesses a demonstrator mouse receive repetitive foot shocks. During the presentation of foot shocks to the demonstrator mouse, the observer mouse, despite not receiving foot shocks, shows freezing behavior. Twenty-four hours later, the observer mouse is placed in the same chamber to assay for learned fear behavior. Of these 17 mouse strains, the 129S1 strain showed an enhancement of freezing, both during the demonstration as well as during exposure to the same context the following day. The authors hypothesize that the unique genetic background of the 129S1 strain is the causal factor underlying its enhanced fear response. To determine which genes are responsible for this phenotype, they perform whole-genome sequencing and identify eight non-synonymous coding single nucleotide polymorphisms (SNPs) unique to the 129S1 strain. Based on gene expression in the brain, as well as predicted consequences of the mutation, authors subsequently decide to follow up on a SNP localized to the neurexin-3 (*Nrxn3*) gene that results in a putatively deleterious substitution of an arginine for a tryptophan at position 498. Neurexin-3 is a member of the neurexin family of presynaptic cell-adhesion molecules that are essential regulators of synaptic properties (Südhof, 2017).

In order to test whether the substitution found in *Nrxn3* contributes to the enhancement of OFL, the authors cleverly leverage CRISPR/Cas9 genome editing to induce the SNP within the B6J background. These mice indeed show an enhancement of freezing behavior during the demonstration, but not when assayed in the same context the following day. These data suggest that the *Nrxn3* SNP at least partially explains the phenotype of the 129S1 strain. The authors suggest that this partial recapitulation of the enhancement of OFL could be explained by differences in genetic modifiers involved in social fear memory between the 129S1 and B6J strains.

Next, they attempt to characterize the cell type and brain region, in which the *Nrxn3* SNP could be mediating its effects on OFL, by crossing a *Nrxn3* conditional knockout line ($Nrxn3^{f/f}$) with a series of cell-type-specific Cre-expressing driver lines in the B6J background. Given the reported significance of the ACC in OFL (Jeon et al., 2010), the authors first cross $Nrxn3^{f/f}$ with a forebrain glutamatergic cell driver line ($Emx1^{cre/cre}$). However, these mice fail to produce a detectable change in the expression of fear following OFL. Hypothesizing *Nrxn3*'s role within inhibitory neurons, the authors next restrict *Nrxn3* knockout to parvalbumin (PV^+), somatostatin (SST^+), or vasoactive intestinal peptide-expressing (VIP^+) interneurons. Strikingly, conditional deletion of *Nrxn3* selectively within SST^+ neurons produces an enhancement of OFL both during conditioning and when exposed to the same context on the following day, as seen in the 129S1 strain. Authors

also demonstrate that this enhancement, seen following brain-wide removal of *Nrxn3* from SST$^+$ neurons, can be partially recapitulated through selective knockout within SST$^+$ neurons of the ACC. These results collectively suggest that the enhanced OFL observed in the 129S1 strain may be explained in part by an effect of the *Nrxn3* SNP on SST$^+$ neurons in the ACC. However, it is important to note that this study does not address how the identified *Nrxn3* variant affects protein function. Therefore, comparisons made using *Nrxn3* knockout cannot be made without caveat.

Next, the authors carry out a set of *in vitro* electrophysiological experiments which show that removing *Nrxn3* from SST$^+$ neurons leads to a decrease in both spontaneous and evoked GABAergic transmission onto pyramidal neurons of the ACC. This reduction can be explained by diminished efficacy with which an action potential elicits GABA release from SST$^+$ neurons. These data suggest that a decrease in activity from SST$^+$ neurons drives enhancement of OFL. Indeed, optogenetic inhibition of SST$^+$, but not PV$^+$ neuron activity, within the ACC during OFL produced an increase in freezing in the observer mouse, both during demonstration and upon exposure to the arena on the following day. The opposite effect is observed upon an increase in SST$^+$ neuron activity within the ACC. Together, these data clearly implicate the activity levels of SST$^+$ neurons in the manifestation of OFL.

In summary, Keum et al. (2018) use a forward genetic approach to identify a *Nrxn3* variant that enhances OFL. They find the unique requirement of Nrxn3 expression in SST$^+$ neurons for the enhancement of OFL and further show that loss of expression within the ACC is at least partially responsible for the phenotype. Perhaps the most striking finding in this study is the robust bidirectional control of OFL through optogenetic manipulation of ACC SST$^+$ neurons. These data clearly implicate SST$^+$ neurons of the ACC in OFL. However, without experiments assaying the contribution of ACC SST$^+$ neurons in classical fear conditioning, the specificity of this neuronal population to OFL remains to be determined.

This study represents a significant advance in understanding of the neural underpinnings of OFL. It provides mechanistic insight into a relevant cell type for this behavior and confirms the importance of the ACC in OFL. The knowledge that SST$^+$ neurons are critically important for OFL offers a foothold into the understanding of the microcircuitry that mediates this complex behavior. Considering the ACC is engaged during both experienced (Hutchison et al., 1999) and observed pain in humans (Singer et al., 2004), a detailed circuit level analysis may reveal how the ACC is uniquely required for OFL (Jeon et al., 2010).

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