Animals harbor a suite of innate fears (known as phobias in extreme cases), having seemingly learned these responses both before birth and in the absence of firsthand experience. These fears are rooted in evolution and are often species-specific. For example, mice respond defensively to odors related to foxes and cats, two of their most common predators in natural habitats.[1] Alternatively, humans respond defensively to snakes and spiders, both of which can be highly-lethal and are endemic to East Africa, the original range of genus *Homo*.[2] These factors strongly imply a genetic origin for these fears, though none has yet been investigated. Tightly-regulated genetic control over such an invariant set of characteristics strongly implies an underlying molecular basis for innate aversion. Innate fears comprise the majority of psychological stressors warfighters experience in the field: fears of loud noises, darkness, blood, injury, and death are all overwhelmingly innate in nature.[3] **Determination of the genetic phenomena responsible for these fears would permit a far deeper understanding of these psychological stressors and the molecular processes of soldiers' cognition under these conditions, a key part of the Army Research Office's Organismal Genetics thrust.**

Aversive responses have both behavioral and hormonal components. The two brain regions necessary and sufficient for innate aversive odor responses in mice are the cortical amygdala (CoA) and the amygdalo-piriform transition area (APir). The CoA mediates innate olfactory behavior, and is organized spatially based on the emotion a given odor evokes — neurons responsive to aversive odors are located in the anterior CoA, and neurons responding to all other odors are posterior.[4] APir controls the hormonal stress response to innately aversive odors, stimulating secretion of corticotropin-releasing hormone (CRH) by the hypothalamus, raising peripheral corticosterone levels.[5] Neither region has any other known functions. A distinct, spatial organization to neuronal populations that mediate specific behavioral functions, independent of individual experience, strongly implies genetic control over the developmental programs creating these pathways. For instance, a past study examining similar populations in other regions showed each one expresses a set of marker genes specific to their own population.[6] Thus, neuronal populations in CoA and APir may similarly express their own sets of marker genes. **I propose to identify the first set of marker genes for neurons controlling innate aversive responses to a set of specific odors.**

**<u>Aim 1:</u> Identify neurons specifically mediating innate olfactory aversion.**

<u>Hypothesis:</u> If these regions respond to specific innately aversive odors, then the specific neurons responding to each individual odor within these regions should be identifiable based on the population-level activity during odor exposure.

<u>Method:</u> I can mark these neurons using a transgenic mouse strain that will express sfGFP, a fluorescent reporter, in neurons active within a short window of time, such as a minutes-long odor exposure.[7,8] I will identify neurons responding to specific innately aversive odors by exposing mice within this window of time to either water, a negative control, or one of two well-validated innately aversive odors acting upon different olfactory subsystems: trimethylthiazoline (TMT), derived from fox glands and binding odorant receptors, or phenethylamine (PEA), derived from cat urine and binding trace amine-associated receptors.[9] The water-responsive group comprises neurons active at baseline, while the TMT- and PEA-responsive groups are independent neuronal populations activated by innately aversive odors, which also differ based on the specific content encoded. Differences in response between the aversive and neutral conditions (TMT/PEA vs. water) should reflect regional activity differences.

<u>Anticipated Results:</u> The sfGFP-expressing population should be enriched in CoA and APir in the TMT- and PEA-exposed mice compared to the water-exposed mice. The regions they innervate, the BLA, CeM, and the hypothalamus, which control general aversive responses,

should be enriched as well. Preliminary data from a pilot study of TMT- and water-exposed mice corroborates these predictions (*Figure 1A, 1B*).

**<u>Aim 2:</u> Identify genes specific to innate olfactory aversion neurons.**

<u>Hypothesis:</u> Neurons active during TMT or PEA exposure should express a common suite of marker genes not expressed in neurons active during water exposure, as well as unique suites of marker genes exclusive to neurons responding to an individual aversive odor. The sfGFP-expressing activated neurons can be dissociated on ice via an optimized combination of RNA polymerase inhibitors, physiological solutions, and extracellular matrix-specific cold-active proteases to maintain cell viability and eliminate gene expression artifacts.[10,11] I will combine this technique with dissection and fluorescence-activated cell sorting to isolate single live sfGFP-expressing cells from CoA and APir with high cellular yield and overall fidelity in gene expression.[12]

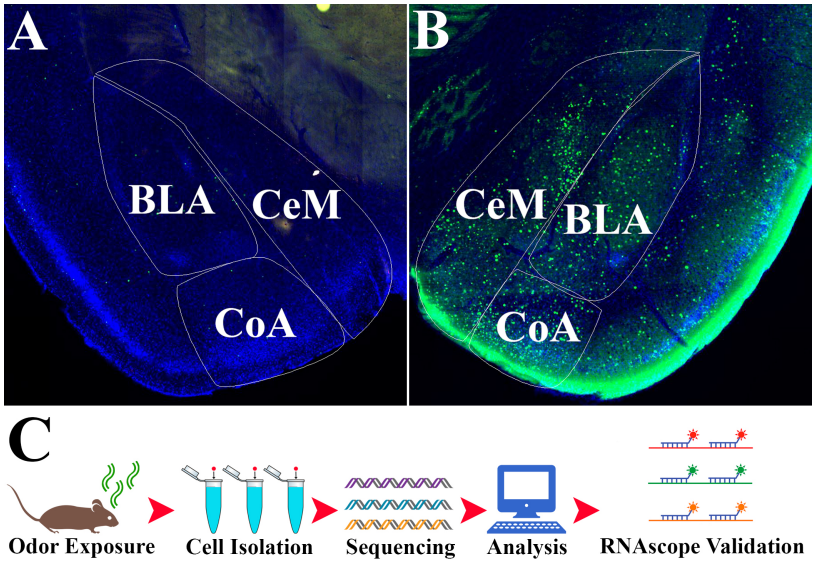


*Figure 1. Preliminary Results and Research Plan.* After odor exposure, cells (blue) in the amygdala express Arc (green). A. Amygdala neuron activity after water exposure. B. Activity after TMT exposure. C. Proposed research plan. BLA: basolateral amygdala nuclei, CeM: centromedial amygdala nuclei.

<u>Method:</u> I will use an efficient, well-validated, high-resolution form of single cell RNA-sequencing to precisely assay the expression of all genes in all isolated cells (*Figure 1C*).[13] This approach closely resembles the method used in a recent series of experiments in the neuroscience literature.[14] A custom computational pipeline purpose-built for this experiment will analyze the data. Machine learning algorithms will classify cells into groups based on similarities in underlying gene expression. Differential expression analysis will identify the most highly upregulated genes in each group of cells compared to all others. Gene ontology (GO) analysis will then identify these genes' functions. Using this functional analysis, the identity of each group of cells can be matched with known cell types, and novel cell types will be identified by unique functional patterns in gene expression. RNAscope, a multiplexed single-molecule RNA fluorescent *in situ* hybridization platform, will externally validate these results.[15] I collaborate with three groups across multiple disciplines and institutions to perform these techniques: in biology at the University of Cincinnati, biochemistry at UCLA, and bioengineering at UCSD.

<u>Anticipated Results:</u> Using this framework, I expect to identify at least one group of generally aversive neurons present in the TMT- and PEA-responding populations but not the water-responding population, with at least one corresponding suite of highly-expressed genes each. I also expect to identify at least one subpopulation specific to either innately aversive odor within this group, with their own marker genes as well. These results will all be confirmed via RNAscope. We expect these genes to display enriched neural development-related GO terms, resulting from a combination of neuronal identity and divergent histology.

**Aim 3: Manipulate specific innate odor aversion via identified marker genes.**

Hypothesis: Activation of light-activated ion channels (ChR2 and eNpHR) selectively expressed in TMT- and PEA-responsive cells in CoA or APir should be able to induce an artificial experience. Stimulation of excitatory ChR2 channels should induce innate aversion without odor, while stimulation of inhibitory eNpHR channels should block innate odor aversion.

Method: I will use the promoters of genes most strongly associated with the three populations responding to TMT, PEA, or water to conditionally express lentiviral Cre recombinase in the innate olfactory aversion neurons of interest. I will co-inject these lentiviruses into CoA or APir along with an adeno-associated virus encoding *ChR2* or *eNpHR* under control of a FLEx recombination switch to respectively stimulate or inhibit neurons expressing these genes.[16] I will inject these viruses into the double transgenic mouse from Aim 1 to determine specificity of expression. I will expose the mouse to one of the study's three compounds during optical stimulation in one of the given regions. I will measure the mouse's behavioral response during exposure using a four-quadrant behavioral attraction-aversion assay, and after exposure by extracting blood to determine the mouse's circulating corticosterone levels.[4,5]

Anticipated Results: Odor responsive population-associated genes will drive Cre-dependent light-activated ion channel expression only in neurons that express sfGFP in response to that specific odor, but not others. Quadrant-restricted optical stimulation of these ChR2-expressing neurons in the absence of odor will be sufficient to produce responses if the gene driving Cre expression is derived from a TMT- or PEA-responsive population, but not if the gene is from a water-responding population. Conversely, optical stimulation of eNpHR-expressing neurons under control of either innate odor responsive population-associated genes will be sufficient to prevent an aversive response to the innately aversive odor their population responds to, but not the other (e.g. TMT genes ablate a response to TMT but not PEA, and vice-versa). Water-responding population-associated genes will not block responses to either odor. While both CoA and APir will be examined, I expect effects to be restricted to the response controlled by the stimulated region (quadrant aversion for CoA, corticosterone release for APir).

This would be the first study to identify and validate the genetics of innate behaviors, the specific neurons mediating these behaviors, and their underlying genes. Finding such genes would allow the targeted stimulation and genetic access of these neurons for the first time, making modification or simulation of specific odor responses (even in the absence of prior experience) possible, a valuable future research tool. Using such tools, researchers could create far more precise experimental designs, allowing researchers to answer more specific hypotheses than ever before. The availability of such novel technologies at the intersection of psychology, molecular biology, and sensory neuroscience will have many implications for studies in all three fields and will further stimulate interdisciplinary research incorporating aspects of all three.

More importantly, such discoveries could open the door to numerous future advances in warfighting if translated to humans. Soldiers face many specific innate fears on that battlefield that have significant negative effects on overall cognitive performance, individually and as a whole. Fear extinction is time- and resource-intensive, and many individuals still reacquire these fears even after extensive training. The ability to genetically identify and microtarget the neural pathways underlying these fears could eventually lead to more efficient, more specific treatments to manipulate these involuntary responses, which could potentially lead to dramatic increases in combat effectiveness even under the worst of conditions. **This project would fulfill many of the objectives of the Army Research Office's Organismal Genetics thrust, characterizing the genetics of a neural pathway controlling key aspects of warfighter cognitive performance.**

**References**


1. Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A. & McGregor, I. S. The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci Biobehav Rev* **29**, 1123–1144 (2005).
2. Fredrikson, M., Annas, P., Fischer, H. akan & Wik, G. Gender and age differences in the prevalence of specific fears and phobias. *Behaviour Research and Therapy* **34**, 33–39 (1996).
3. Garcia, R. Neurobiology of fear and specific phobias. *Learn. Mem.* **24**, 462–471 (2017).
4. Root, C. M., Denny, C. A., Hen, R. & Axel, R. The participation of cortical amygdala in innate, odour-driven behaviour. *Nature* **515**, 269–273 (2014).
5. Kondoh, K. *et al.* A specific area of olfactory cortex involved in stress hormone responses to predator odours. *Nature* **532**, 103–106 (2016).
6. Kodama, T. *et al.* Neuronal Classification and Marker Gene Identification via Single-Cell Expression Profiling of Brainstem Vestibular Neurons Subserving Cerebellar Learning. *J. Neurosci.* **32**, 7819–7831 (2012).
7. Mo, A. *et al.* Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. *Neuron* **86**, 1369–1384 (2015).
8. Allen, W. E. *et al.* Thirst-associated preoptic neurons encode an aversive motivational drive. *Science* **357**, 1149–1155 (2017).
9. Pérez-Gómez, A. *et al.* Innate Predator Odor Aversion Driven by Parallel Olfactory Subsystems that Converge in the Ventromedial Hypothalamus. *Curr Biol* **25**, 1340–1346 (2015).
10. Wu, Y. E., Pan, L., Zuo, Y., Li, X. & Hong, W. Detecting Activated Cell Populations Using Single-Cell RNA-Seq. *Neuron* **96**, 313–329.e6 (2017).
11. Adam, M., Potter, A. S. & Potter, S. S. Psychrophilic proteases dramatically reduce single-cell RNA-seq artifacts: a molecular atlas of kidney development. *Development (Cambridge, England)* **144**, 3625–3632 (2017).
12. Hempel, C. M., Sugino, K. & Nelson, S. B. A manual method for the purification of fluorescently labeled neurons from the mammalian brain. *Nature Protocols* **2**, 2924–2929 (2007).
13. Torre, E. *et al.* Rare Cell Detection by Single-Cell RNA Sequencing as Guided by Single-Molecule RNA FISH. *Cell Systems* **6**, 171–179.e5 (2018).
14. Tasic, B. *et al.* Shared and distinct transcriptomic cell types across neocortical areas. *Nature* **563**, 72 (2018).
15. Wang, F. *et al.* RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *Journal of Molecular Diagnostics* **14**, 22–29 (2012).
16. Gompf, H. S., Budygin, E. A., Fuller, P. M. & Bass, C. E. Targeted genetic manipulations of neuronal subtypes using promoter-specific combinatorial AAVs in wild-type animals. *Front Behav Neurosci* **9**, (2015).