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Serotonin and the search for the anatomical substrate of aggression

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All species of animals display aggression in order to obtain resources such as territories, mates, or food. Appropriate displays of aggression rely on the correct identification of a potential competitor, an evaluation of the environmental signals, and the physiological state of the animal. With a hard-wired circuitry involving fixed numbers of neurons, neuromodulators like serotonin offer adaptive flexibility in behavioral responses without changing the "hard-wiring". In a recent report, we combined intersectional genetics, quantitative behavioral assays and morphological analyses to identify single serotonergic neurons that modulate the escalation of aggression. We found anatomical target areas within the brain where these neurons appear to form synaptic contacts with 5HT1A receptor-expressing neurons, and then confirmed the likelihood of those connections on a functional level. In this Extra View article, we offer an extended discussion of these recent findings and elaborate on how they can link a cellular and functional mapping of an aggression-regulating circuit at a single-cell resolution level.

Keywords: 5HT1A receptor, aggression, behavior, PLP neurons, serotonin, ventrolateral protocerebrum

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Extra View to: Single Serotonergic Neurons that Modulate Aggression in Drosophila. Alekseyenko OV, Chan YB, Fernandez MP, Bülow T, Pankratz MJ, Kravitz EA. Current Biology; 2014; 24:2700–07.

Serotonin (5HT) has long been implicated in the regulation of aggression in a wide variety of animal species up to and including humans [1-4]. From relatively small populations of central nervous system neurons, 5HT ends up widely distributed throughout the nervous systems of most animal species, where it plays key roles in many physiological processes and essential behaviors like learning and memory, sleep, behavioral arousal and locomotion. Much progress has been made towards identifying the neuronal hard-wired circuitries concerned with such behaviors, but how and where serotonergic neurons fit into that circuitry remains relatively unknown. Serotonin actions are mediated by distinct types of receptors expressed on the surface of target neurons, where they commonly modulate the firing properties of neurons and/or change the effects of excitatory (glutamate) and inhibitory (γ-amino-butyric acid, GABA) signals to and from the cells.[5] In mammalian systems, at least 14 subtypes of serotonin receptors have been found. [6] To add to the complexity, in some brain regions 5HT receptors have been localized on neurons that have no direct serotonergic innervation, suggesting that 5HT can be diffusely released as well. In the simpler *Drosophila* brain only about 100 serotonergic neurons and 5 subtypes of 5HT receptors are found. As in other species, serotonergic neurons in the fly nervous system display arbors of processes that ramify widely in multiple neuropil areas. Earlier studies from several laboratories, including ours, showed that manipulating total populations of 5HT neurons in *Drosophila* influence a wide variety of different behaviors [7-12]. How do serotonergic neurons function to exert such a wide spectrum of behavioral actions? Do single serotonergic neurons exert generalized actions on multiple behaviors, selective actions on specific behaviors, or both? In either case, can we find the subsets of 5HT neurons involved in aggression?

In the *Drosophila* model of aggression, [13] the acute shut down of the entire population of serotonergic neurons with $Shi^{(ts1)}$ produced male flies that still engaged in fights but with a reduced ability to escalate aggression. [11] Similar results were reported using a less direct technique in an earlier study. [12] Induced dTrpA1-mediated activation of serotonergic neurons, in contrast, resulted in males that escalated fights faster and fought at higher intensities. [11]

In our most recent study[14] we used an intersectional genetics approach to restrict the population of serotonergic neurons that can be reproducibly manipulated in an attempt to identify those that selectively modulate aggression. We found that two serotonergic neurons per hemisphere from the posterior lateral protocerebrum (PLP) cluster are sufficient to enhance male aggression. Silencing these 5HT-PLP neurons with tetanus toxin light chain reduced, and activating them with dTrpA1 increased the display of high-intensity aggression in male flies. By manipulating either the entire serotonergic system[11] or this particular pair of individual 5HT neurons, [14] we observed an approximate two-fold change in the aggression display. This suggests that a substantial part of the serotonergic modulation of aggression comes from the proper functioning of just these specific pairs of 5HT-PLP neurons.

Examination of the anatomical profiles of these aggression-modulating PLP neurons revealed that they course through the entire brain, forming several dense innervation fields along the way. The cell bodies of the identified 5HT-PLP neurons and their axons are located close to the posterior surface of the brain, while their projections are directed anteriorly to form three distinct arborization zones: one in the ventrolateral protocerebrum; a second in close proximity to the fan-shaped body of the central complex; and a third around the peduncles of the mushroom body near the ellipsoid body of the central complex (**Figure 1A**). The densest arborization is in the ventrolateral protocerebrum, a region previously characterized as an integrative center for auditory, [15] visual, [16] and olfactory information processing. [17] More recently, this region also has been found to be important for courtship. [18] Aggression is a complex adaptable innate behavior that is likely to be regulated by multiple neuronal modulatory networks and influenced by multiple sensory cues. Thus, it may not be surprising that the branches of the 5HT-PLP neurons intermingle with other neuronal processes in well known integrative brain centers, where they likely form numerous input and output connections. To date, the neuronal pathways in fruit flies that trigger aggression remain largely unknown although manipulations of certain gustatory receptors do influence its display[19,20]. Both males and females show aggressive behavior towards individuals of their own sex, with behavioral patterns that are sexually dimorphic. [21] Only males display the high-intensity aggression pattern called "lunge" that is required to win the fight and only males establish dominance relationships. [13] These male-specific behavioral patterns are controlled by a subgroups of neurons expressing male forms of *fruitless* proteins (Fru^M). [22] A recent study reported that a group of male-specific Fru^M-positive tachykinin (Tk) neurons has been found that promote aggression. [23] The aggression-enhancing serotonergic PLP neurons that we have found do not co-express Fru^M (**Figure 2A**) or Doublesex (**Figure 2B**) and have similar morphological profiles in males and females (**Figure 2C**). Moreover, the arbors of processes of the 5HT-PLP neurons do not appear to overlap with the processes of Fru^M-Tk neurons, suggesting that alternative modulatory pathways exist that influence the display of male-specific high-intensity aggression. Additionally, in an earlier study, neuropeptide F (NPF), the invertebrate homolog of neuropeptide Y, has also been reported to influence aggression in male flies, [12] in a direction opposite to the Fru^M-Tk and 5HT-PLP neurons. The aggression-related part of NPF circuitry too is reported to be male-specific. The 5HT-PLP neurons do not co-express NPF (**Figure 2D**, posterior). However, NPF neurons have been reported to innervate the fan-shaped body of the central complex[24,25] and to partly surround the mushroom body neuropil regions[26] as well (also see **Figure 2D**, anterior). Both of these brain regions are nearby arborization fields of the 5HT-PLP neurons. In earlier studies, we identified aggression-modulating individual dopaminergic neurons from the PPM3 cluster and showed that they too send axonal terminal fields to the fan-shaped body of the central complex.[27] Thus, although several potential entry points into aggression-related modulatory networks have been reported recently, downstream anatomical targets of the neurons that make up such aggression-influencing elements remain poorly understood.

In an effort to identify downstream synaptic partners and possible targets of the 5HT-PLP neurons, we first examined the distribution and functioning of the five known subtypes of 5HT receptors within *Drosophila* brain neuropil regions. The 5HT1B receptor type is known to regulate circadian activity [8] and is highly expressed in the β and γ lobes of the mushroom bodies, but it is absent from the ventrolateral protocerebrum or the central complex. Not surprisingly, therefore, silencing of 5HT1B receptor-expressing neurons with Kir 2.1[28] or activating them with the dTrpA1 channel (data not shown) had no effects on male aggression. Members of the 5HT2 category (5HT2A and the recently discovered close homologue 5HT2B[29]) reportedly regulate larval heart rate[30], and larval feeding behavior also is regulated by 5HT2A receptors[29]. The brain distribution of 5HT2B receptors has not been determined, but 5HT2A[31], 5HT1A[32] and 5HT7[33] receptor subtypes are expressed in many neuropil regions where they mediate various behaviors in *Drosophila*. These three subtypes of receptors therefore are potential downstream anatomical targets of the processes of the 5HT-PLP neurons.

A variety of Gal4 driver lines presumably target 5HT receptor-bearing neurons, but none have been fully characterized since antibodies to specific subtypes of *Drosophila* 5HT receptors are not yet available. We attempted to utilize the GFP reconstitution across synaptic partners (GRASP) method [34] to search for putative synaptic contacts between 5HT neurons and 5HT receptor-expressing neurons. Four of eight candidate receptor Gal4 lines (one 5HT1A- and three 5HT2-Gal4 lines) showed no GRASP signal when examined for putative contacts with 5HT neurons, even though strong and broad Gal4 expression was found. This suggests that those GAL4 lines are either not reliable tools for studying expression patterns of 5HT receptors, or that some receptor neurons have no direct serotonergic innervation since 5HT can be diffusely released as well. Notably, the GRASP analyses did

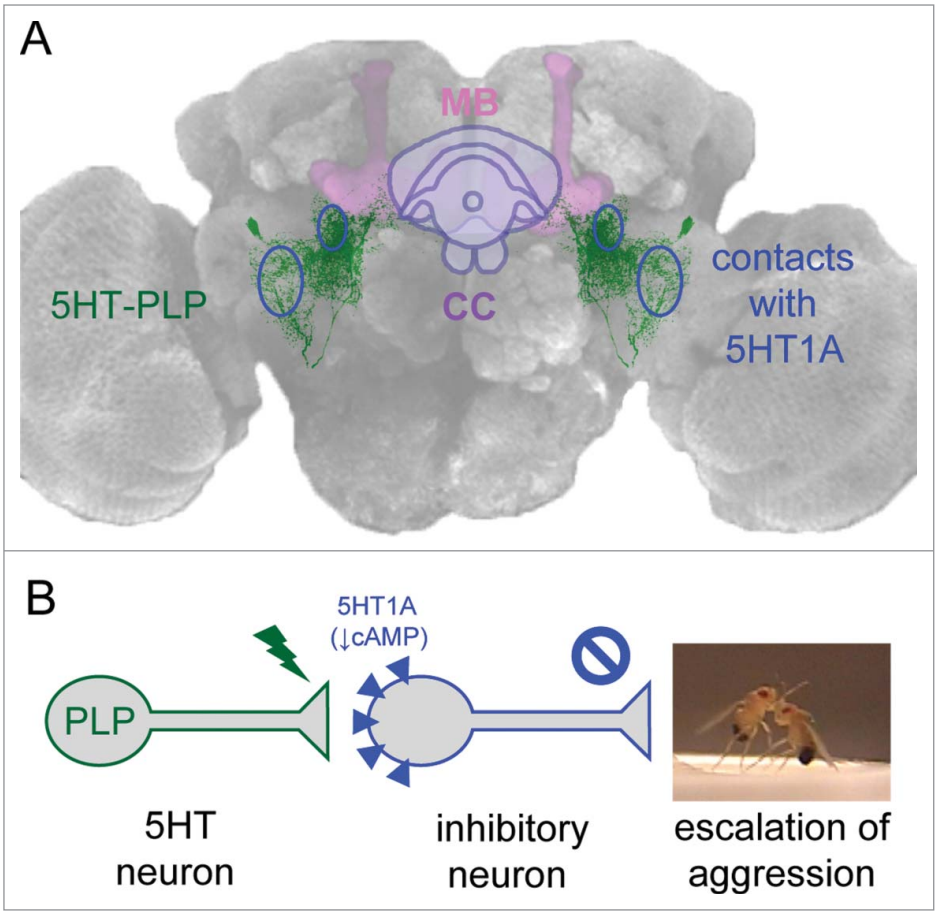

Figure 1. A symmetrical pair of serotonergic PLP neurons promotes the escalation of aggression in *Drosophila* males. **(A)** 5HT-PLP neurons and their anatomical target areas, where synaptic contacts with 5HT1A receptor-bearing neurons are formed (blue ovals). CC-central complex, MB-mushroom bodies. **(B)** Model of the inhibitory control of aggression. An activation of the 5HT-PLP neurons releases the inhibitory input from 5HT1A receptor-bearing neurons, which leads to the escalation of aggression.

show putative synaptic connections with serotonergic neurons within the terminal arborization areas of the aggression-modulating 5HT-PLP neurons for three of the tested Gal4 lines. Two of these were 5HT1A- and one was a 5HT7-receptor Gal4 driver line. It was of interest that the two anatomical regions where serotonergic neurons formed putative contacts with 5HT1A-expressing neurons – the ventrolateral protocerebrum and the area surrounding the peduncles of the mushroom bodies - were targeted by two different 5HT1A-Gal4 driver lines with non-overlapping expression patterns. A possible explanation for this result might be that the promoter region of the *Drosophila* 5HT1A gene has a complex structure in parallel with the repressor- and polymorphism-enriched transcriptional regulation of the mammalian Htr1a gene. [35] In that case different promoter regions chosen to generate the Gal4 lines might result in targeting different non-overlapping groups of neurons.

Further functional analysis revealed that dTrpA1-induced activation of neurons targeted by both 5HT1A-Gal4 drivers, but not by the 5HT7-Gal4 driver, resulted in reductions of aggression. This suggests that the 5HT1A-bearing neurons might themselves serve an inhibitory role in aggression pathways or might serve as direct links to a descending inhibitory control pathway. The former suggestion fits well with a model proposing that in vertebrate systems, activation of 5HT1A receptors, located postsynaptically on GABAergic interneurons, mediates hyperpolarizing responses to released 5HT, thereby reducing postsynaptic neuronal excitability and firing rates. [36] The authors of this study suggest that mechanisms of that type might be important links in releasing emotion-related behaviors. Mammalian 5HT1A receptors are implicated in the regulation of mood, emotions and stress responses and are candidate targets in the management of various neuropsychiatric disorders. [37] In *Drosophila,* [38] as in mammalian systems, [39] activation of 5HT1A receptors inhibits cAMP production, hyperpolarizes neurons, and reduces neuronal excitability. If the 5HT-PLP neurons do target inhibitory, possibly GABAergic, interneurons expressing 5HT1A hetero receptors, a similar "inhibition of inhibition" control mechanism might be operating to release the escalation of aggression in *Drosophila* upon activation of the 5HT-PLP neurons (**Figure 1B**).

It is probably an oversimplification to assume that the 5HT-PLP neurons, just as any other single aminergic or peptidergic neuron type, act as sole *control* element in the regulation of aggression in *Drosophila*. In our studies[14] we note small effects on locomotion and sleep when we reduce activity of the 5HT-PLP neurons, suggesting that these neurons likely serve roles in multiple behaviors including aggression. Also, little is known about the downstream 5HT1A receptor-bearing target neurons reported here. 5HT1A receptor mutant males have been shown to display significant sleep deficits, which can be rescued by reintroducing this receptor subtype into mushroom bodies[7]. We demonstrated that the 5HT-PLP neurons display three prominent sets of processes, one of them near the peduncles of the mushroom bodies where they form putative contacts with 5HT1A receptor-expressing neurons. It may be possible that via separated sets of processes and nerve endings, the 5HT-PLP together with the 5HT1A receptor neurons may influence two distinct behaviors - aggression and sleep. Another neuropil region associated with the 5HT-PLP neurons near the central complex could be the site where locomotion is influenced, as the central complex is a well-know locomotion integration center. [40,41] Future studies in which different arborization

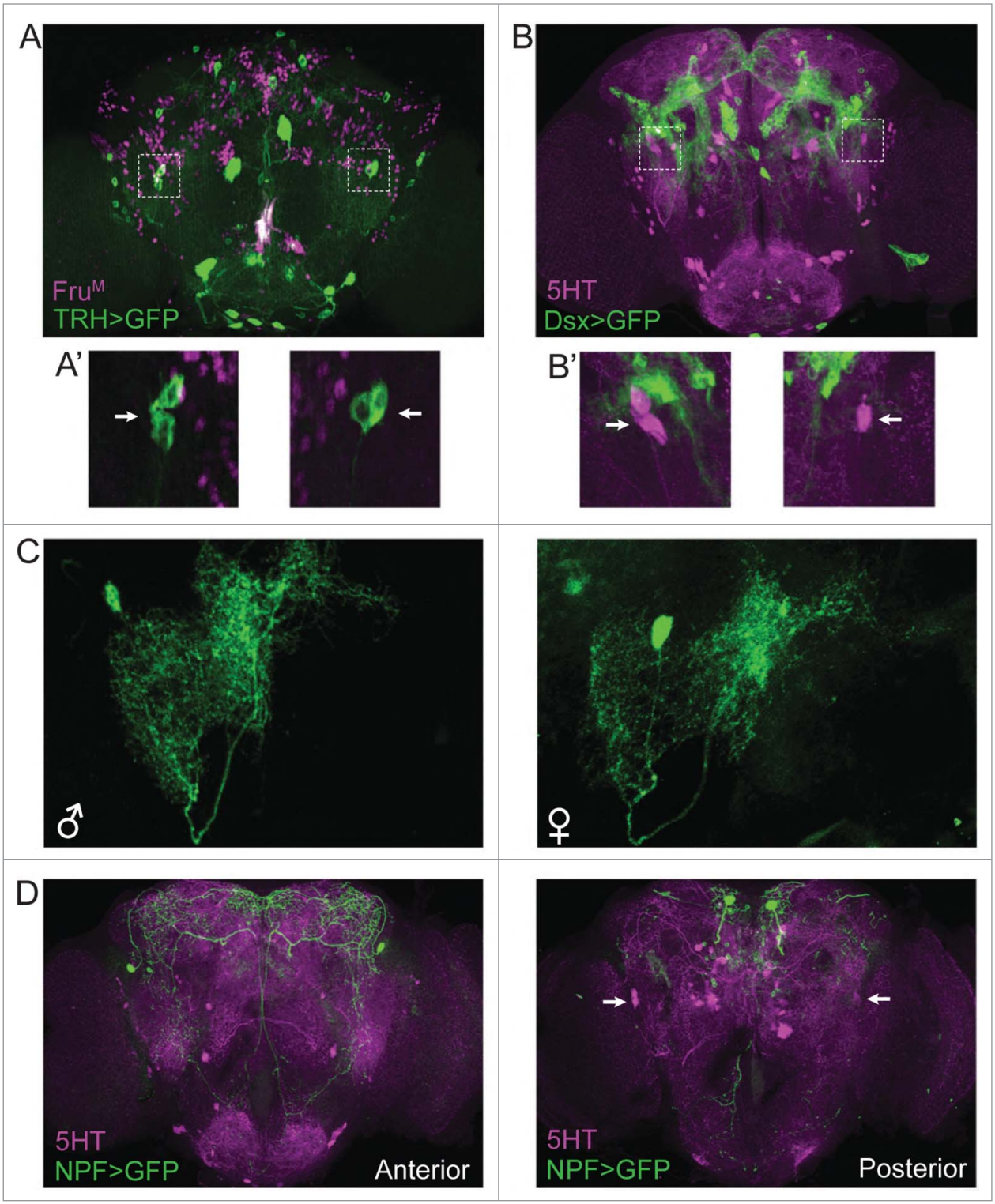

Figure 2. Characterization of the aggression-modulating serotonergic PLP neurons. **(A)** 5HT-PLP neurons do not co-express Fru^M. Anti-Fru^M staining is shown in magenta, serotonergic neurons are visualized by TRH-Gal4/UAS-CD8:GFP (green). The full z stack frontal projection is shown. A′ shows the optical section through the 5HT-PLP cell bodies (white arrows). **(B)** 5HT-PLP neurons do not co-express Dsx. Anti-5HT staining is shown in magenta, Doublesex-positive neurons are visualized by Dsx-Gal4/UAS-CD8:GFP. The full z stack frontal projection is shown. B′ shows the optical section through the 5HT-PLP cell bodies (white arrows). **(C)** 5HT-PLP neurons have similar morphology in males and females. Individual 5HT-PLP neurons are visualized by TRH-Gal4/UAS>stop>CD8:GFP in combination with 417-FLP. **(D)** 5HT-PLP neurons do not co-express NPF. Anti-5HT staining is shown in magenta, Neuropeptide F-positive neurons are visualized by NPF-Gal4/UAS-CD8:GFP. The anterior and posterior z stack frontal projections are shown separately. White arrows point to the 5HT-PLP cell bodies (posterior view).

regions of the same serotonergic neurons might be separately activated, perhaps by optogenetic means, would help in unraveling the functioning of these fascinating modulatory neurons.

In summary, the identification of bilateral pairs of aggression-promoting serotonergic neurons revealed the brain regions where likely anatomical connections are formed between these 5HT neurons and a subpopulation of 5HT1A receptor-expressing neurons, establishing key elements of an aggression-regulating circuit. Further experiments are needed to precisely identify the neurons specifically concerned with high-intensity aggression within the group of 5HT1A receptor expressing neurons and to reveal their neurochemical identity. These studies will be crucial next steps towards understanding at least one mechanism through which the high-intensity aggression necessary for the formation of dominance relationships is triggered in fruit flies.

Experimental procedures

The following fly lines were used: NPF-Gal4, UAS-CD8:GFP and UAS>stop>CD8::GFP from the Bloomington Stock Center (Bloomington, IN), Dsx-Gal4 was a gift from Stephen Goodwin (University of Oxford, Oxford, UK), TRH-Gal4 and 417-FLP were described previously.[11,14] Adult male and female brains were dissected, fixed, treated with primary and secondary antibodies, and prepared for confocal imaging as described previously.[14] The following primary antibodies were used: mouse anti-GFP (1:500) (Invitrogen, Carlsbad, CA), rabbit anti-5HT (1:1000) (Sigma-Aldrich, St. Louis, Missouri), mouse nc82 (1:20) (Developmental Studies Hybridoma Bank, Iowa City, IA) and rabbit anti-Fru (1:5000) (a gift from Barry Dickson, Janelia Research Campus, Ashburn, VA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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