# Syndecan Affects Odor Response as well as Learning and Memory in Drosophila melanogaster

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## ABSTRACT

Syndecan (Sdc) is a transmembrane heparan sulfate proteoglycan that plays a crucial role in axon guidance and synapse formation during CNS development in *Drosophila melanogaster*. To further examine the effect of syndecan on CNS function, Sdc<sup>23</sup> mutant *D. melanogaster* larvae were used to examine odor preference and the capacity for learning and memory. A series of olfaction assays in both wild type and mutant larvae were performed to characterize naive odor responses before adding a training period to identify the capacity for associative learning. These results showed that Sdc<sup>23</sup> larvae prefer odors that wild type larvae do not respond to, suggesting a difference in odor receptor pathways and wiring. In addition, associative learning has been documented in wild type larvae, yet no evidence of associative learning in Sdc<sup>23</sup> larvae was found, suggesting that the syndecan also plays a role in learning and memory in *D. melanogaster* larvae.

## KEYWORDS

Syndecan; Proteoglycans; Neurodevelopment; Axon Guidance; Olfaction; Attraction Index; Associative Learning; Drosophila

## INTRODUCTION

Olfactory systems are critical in most organisms because chemical cues in the environment provide essential information about a wide range of stimuli such as food, predators, mates, and pathogens.<sup>1</sup> Many of these interactions are made possible because no physiological interaction is needed between organisms and these cues. This allows organisms to make behavioral decisions at longer distances from their cues.<sup>2</sup> Because olfaction is so important for organism survival, the development and function of the olfactory system is very highly regulated. In *Drosophila melanogaster*, the olfactory system gets organized twice; first, during development from the egg to larval stage, and then again when the larvae enters the pupal stage before becoming an adult.<sup>3</sup> Because this system is so well organized, it is a valuable area to study in order to better understand the distinct roles of genes, proteins, and compounds, such as proteoglycans, as they relate to basic neural processes such as axon guidance.

Insects provide a great platform through which to study olfaction because of the shared qualities their olfactory systems have with mammals. Most notably, both mammalian and insect olfactory systems operate under the principle that every glomerulus, or cluster of nerve endings in the olfactory bulb, receives input from one class of olfactory receptor neurons and each individual olfactory receptor neuron expresses a single odorant receptor.<sup>4</sup> At the level of the receptor, odorants primarily interact with transmembrane proteins through a signaling process in the cilia, which extend from olfactory receptor neurons.<sup>2</sup> Although the sizes of insect and vertebrate olfactory systems are highly variable, these core similarities are very well conserved. Maintaining so much similarity in their organization of the olfactory system with vertebrates, while also having a relatively simple nervous system, is what makes *D. melanogaster* an excellent model organism.<sup>5</sup> Furthermore, the different olfactory receptors and olfactory receptor genes expressed in *D. melanogaster* are very well characterized in both the larval and adult stages, making it an ideal model organism for studying olfaction as it relates to neurodevelopment.<sup>3</sup>

Proteoglycans are a particular topic of interest in neurodevelopment because of their crucial roles in neurogenesis, axon guidance, and synaptogenesis in the central nervous system.<sup>6-7</sup> They are expressed in the extracellular matrix (ECM) of many tissues, including the vertebrate spinal cord and the brain, where they have been implicated in controlling neural development by activating various growth factors.<sup>8</sup> Heparan sulfate proteoglycans (HSPGs) are abundant cell surface molecules in the ECM that have extremely important functions for ECM-mediated neurodevelopment, specifically axon guidance.<sup>9</sup> The structure of HSPGs consists of a core protein that is covalently bound to the highly charged glycosaminoglycan (GAG) side chain heparan sulfate (HS), a close structural relative of heparin.<sup>10</sup> These HS side chains are disaccharides that can exist on their own, but usually covalently attach to the HSPG core at a serine residue when they're found *in* vivo.<sup>11</sup>

While the functions of HSPGs are very diverse, one of their most important roles is in guiding developing axons along their proper neuronal pathways during development.<sup>6</sup> Studies in both vertebrates and invertebrates have shown that mutations in

HSPG core proteins lead to a variety of phenotypes with different neuronal connectivity, implying that they have a significant contribution in axon path guidance and synapse formation during development.<sup>11</sup> This study focuses on a specific type of HSPG called syndecan (Sdc) that localizes to developing axons and is necessary for axon growth cone navigation in the CNS.<sup>12</sup> Syndecan core proteins are highly evolutionarily conserved type 1 transmembrane proteins with multiple HS side chains. Syndecan is expressed at the cell surface in the nervous system of mammals and has homologs in a diverse array of species, including *Drosophila melanogaster, Caenorhabditis elegans*, and *Xenopus* laevis.<sup>13-14</sup>

In *Drosophila*, syndecan is a necessary component of Slit/Robo signaling pathway at the *Drosophila* central nervous system midline, and the axons of syndecan mutant embryos have frequent midline crossing defects.<sup>12,15</sup> Syndecan has been shown to facilitate the interactions between Slit and Robo,<sup>16</sup> allowing Slit and Robo signaling to regulate axon guidance in the CNS, including odorant receptor neuronal projections to glomerular targets.<sup>17</sup> Because Slit and Robo play crucial roles in regulating the development of the olfactory system in *Drosophila*, we hypothesized that Syndecan might also influence olfactory system development using a similar mechanism to how Sdc works in the developing embryonic central nervous system. To explore this on a behavioral level, we hypothesized that Sdc mutant *Drosophila* larvae will be attracted to different odorants than wild-type larvae due to a lack of proper olfactory wiring.

In addition, it is possible that Syndecan plays a role in other functions of the CNS, such as learning and memory, that have not yet been fully explored. Currently, there is some evidence that as a member of the ECM, HS signaling is important for learning and memory; HSPG knockout mice have been found to display an array of behavioral problems. Furthermore, mice that have been treated with heparinase (a substance that removes HSPGs locally) have demonstrated decreased LTP and hippocampal formation of contextual memories.<sup>18</sup> Therefore, it would be useful to study the effects of an HSPG mutation on learning and memory in a much simpler model organism. *Drosophila* possess the cellular machinery for learning and memory as early as the first instar larval stage, and can be trained to avoid an odor that is presented in tandem with a shock.<sup>19</sup> *Drosophila* larvae are also capable of associative learning and memory using either attractive (fructose) or aversive (quinine) gustatory cues to induce changes in naive odor preference.<sup>5</sup> However, the role of syndecan in *Drosophila* learning and memory by assessing changes in third instar Sdc<sup>23</sup> *Drosophila melanogaster* larvae odor preference following an associative conditioning training protocol. Given the previous literature showing that HSPGs regulate learning and memory in vertebrate systems, we hypothesize that Sdc mutants larvae will also show impaired associative learning and memory compared to control larvae for odors that are paired with gustatory cues.

## METHODS AND PROCEDURES

## Drosophila Stocks

*Drosophila* were reared in bottles of standard fly food at 25°C, and adult flies were transferred to new bottles every two days. Sdc<sup>23</sup> loss of function mutants have been previously described as a deletion that removes the start codon and the first two exons of Sdc.<sup>20</sup> Sdc<sup>23</sup> mutants were balanced over CyoGFP and reared in bottles of instant fly food (Formula 4-24; Carolina Biological Supply Company) on a 12:12 light dark cycle at 25°C. Homozygous Sdc<sup>23</sup> mutants were selected on a fluorescent dissecting microscope by selecting against GFP, and were transferred to odorant testing chambers.

## Larval Chemotaxis Assays

Olfaction assays were performed for ten different odorants: ethyl acetate, isoamyl acetate, pentanol, hexanol, hexan-3-ol, heptanol, hexanal, hex-2-enal, toluene, and ethyl proprionate. Pure odorants (2  $\mu$ L; Sigma Aldrich) were pipetted onto a small piece of Kimwipe in an Eppendorf tube cap on one side of a petri dish on a layer of 1.5% agar. Water (2  $\mu$ L) was pipetted onto a small piece of Kimwipe in an Eppendorf cap on the opposite side of the petri dish to serve as a control. Fifteen larvae per trial were placed in a straight line down the center of the Petri dish, and allowed to chemotax freely for two minutes. Larval position was marked at the end of the assay, and an "Attraction Index" was calculated by dividing distance from the odorant by the sum of their distance from the odorant and water (a/(a+b). Attraction Index values close to 0 indicated that larvae crawled toward the odor, while Attractive Index values close to 1 indicated that larvae per odor assay.



**Figure 1. Olfaction assay testing naïve odor versus water preference.** A) Olfaction assay setup at the beginning of a trial where fifteen larvae are placed in the center of a Petri dish, equidistant from an odorant on one side and water on the other. B) Olfaction assay following the 2 minute chemotaxis period, where *a* is the final distance of each larvae from the odorant and *b* is the final distance from the water. C) Schematic representation of a/(a+b) values for larval positions following chemotaxis.

Some of the odorants identified as significantly attractive were then tested against each other to assess larval preference for one odorant over another using the same protocol as before. To measure wild-type and Sdc<sup>23</sup> mutant ability for learning and memory, larvae underwent an associative learning training protocol.<sup>21</sup> Fifteen larvae were placed in the center of a "Training Plate" prepared with 2 M fructose dissolved in the 1.5% agar with odorant (2  $\mu$ L) in Eppendorf tube caps on either side of the dish. After one minute of chemotaxis, larvae were transferred to a second petri dish that was prepared without fructose and contained a different odorant (2  $\mu$ L) for one minute. Larvae were then transferred to a petri dish without odorant or fructose for one minute of rest. This protocol was repeated four times before larvae were transferred to a chemotaxis assay as described above, with the odorant they were pre-exposed to with fructose against the other. The training was done reciprocally by switching which odorant was paired with fructose to control for differences due to odorant preference. Odorants tested against each other were hexanol versus ethyl acetate, hexanol versus pentanol, and heptanol versus ethyl proprionate, and a total of 90 wild-type and 90 Sdc<sup>23</sup> mutant larvae were tested per each condition.

#### Quantification of Results

Larval position was marked on the Petri dish cap using a Sharpie marker immediately following the two-minute chemotaxis period. Larval position was used to calculate the Attraction Index for each odorant as described above. A two-tailed independent samples t-test of the average attraction index for each odorant vs. water condition was conducted against a water vs. water control in Excel. A two-tailed independent samples t-test of the average attraction index was conducted in Excel. All statistical analyses were subject to Bonferroni corrections for multiple samples.

#### RESULTS

#### Naïve Olfaction Assays

In order to determine if Sdc<sup>23</sup> mutant larvae have different odor preferences from control larvae, olfaction assays were performed to examine 90 wild-type and 90 Sdc<sup>23</sup> mutant larvae odorant preferences. A two-tailed independent samples t-test comparing responses of larvae to odorant versus water against control water versus water assays indicated that wild-type and Sdc<sup>23</sup> larvae are attracted to different odorants. Naïve odorant versus water assays indicated that wild-type larvae are significantly attracted to ethyl acetate, pentanol, hexanol, hexan-3-ol, and hex-2-enal (**Figure 2**). Sdc<sup>23</sup> mutant larvae demonstrate significant attraction to hexanol, hexan-3-ol, and ethyl proprionate (**Figure 2**). Two-tailed independent samples t-tests indicated that wild-type and Sdc<sup>23</sup> larvae are larvae exhibit significantly different responses to ethyl acetate, pentanol, hexanol and hex-2-enal (**Table 1**).



Figure 2. Wild-type and Sdc<sup>23</sup> larvae show attraction to different odorants. Attraction index for wild-type and Sdc<sup>23</sup> larvae in response to different odorants. Error bars denote the standard error of the mean and stars indicate attraction at the .005 significance level (significance was established by comparing responses to the water vs. water control with Bonferroni correction for multiple comparisons). The dashed line represents a water vs. water control condition with an average attraction index of .48 (SEM = .03, n = 265). Note: Attraction index values less than 0.5 indicate increased attraction to an odorant over the water control.

	Ethyl Acetate	Pentanol	Hexanol	Hexan-3-ol	Heptanol	Hex-2-enal	Ethyl Proprionate
Wildtype	~	~	<b>v</b>	~		<b>v</b>	
Sdc <sup>23</sup>			<b>v</b>	~			<b>v</b>
Significant Difference	Yes	Yes	Yes	No	No	Yes	No

Table 1. Attractive Odors for Wild-type and Sdc<sup>23</sup> Mutant Larvae. Check marks indicate the odorant was significantly attractive. The third row indicates whether or not the attraction index for each odor was significantly different between wildtype and Sdc<sup>23</sup> mutant larvae at the .007 significance level (significance was established by comparing behavioral responses between wildtype and Sdc<sup>23</sup> larvae with Bonferroni correction for multiple comparisons).

To determine naïve odor preference measures, wildtype and mutant larvae were put in an odor choice assay with two different odors to choose from. When given a choice between hexanol and ethyl acetate, wildtype larvae exhibit a slight preference for hexanol over ethyl acetate. Following an associative learning protocol where hexanol was paired with the gustatory reward fructose, wildtype larvae exhibited a significant increase in their preference for hexanol (p < .01). Sdc<sup>23</sup> larvae also display a slight naïve preference for hexanol over ethyl acetate, however, they displayed no increase in preference for hexanol following the same training protocol (**Figure 3**).



Figure 3. Wild-type but not  $sdc^{23}$  larvae learn to associate hexanol with fructose reward. Comparison between naïve odor preference behavior and odor preferences following fructose training. Error bars indicate the SEM, and stars indicate evidence of successful learning at the .05 significance level (p=.0022 for wild-type larvae, p=0.19 for  $sdc^{23}$  larvae).

Conversely, when ethyl acetate was paired with the gustatory reward fructose during the training protocol, wildtype larvae exhibited a significant shift in preference for ethyl acetate over hexanol. Again, Sdc<sup>23</sup> larvae exhibited no behavioral changes in response to this training **(Figure 4)**.



Figure 4. Wild-type but not  $sdc^{23}$  larvae learn to associate ethyl acetate with fructose reward. Comparison between naïve odor preference behavior and odor preferences following fructose training. Error bars indicate the SEM, and stars indicate evidence of successful learning at the .05 significance level (p=.0064 for wild-type larvae, p=.95 for  $sdc^{23}$  larvae).

The inability for Sdc<sup>23</sup> larvae to change their behavior in response to training could be due either to their inability to learn, or to their inability to smell the odorants. However, we knew that both wildtype and Sdc<sup>23</sup> mutant larvae could smell hexanol, since it was significantly attractive to both sets of larvae. To further examine the ability to train wildtype and Sdc<sup>23</sup> larvae, we conducted the same training protocol using another odorant previously demonstrated to be attractive to Sdc<sup>23</sup> larvae; ethyl proprionate. Both wildtype and Sdc<sup>23</sup> naïve larvae showed no significant preference for either odor. Following training where heptanol was paired

with fructose, there was no significant change in preference for either wildtype or Sdc<sup>23</sup> larvae (Figure 5). Similarly, no significant change in preference was observed for either genotype when ethyl proprionate was paired with fructose during training (Figure 6).



Figure 5. Wild-type and sdc<sup>23</sup> larvae do not learn to associate heptanol with fructose reward. Comparison between naïve and learned odor preferences. Error bars indicate the SEM. There is no evidence of successful learning at the .05 significance level (p=.13 for wild-type larvae, p=.24 for sdc<sup>23</sup> larvae).



Figure 6. Wild-type and sdc<sup>23</sup> larvae do not learn to associate ethyl proprionate with fructose reward. Comparison between naïve and learned odor preferences. Error bars indicate the SEM. There is no evidence of successful learning at the .05 significance level (p=.66 for wild-type, p=.92).

To verify the efficacy of the training protocol, wildtype larval preferences for hexanol and pentanol were tested. Naïve wildtype larvae show a significant preference for hexanol and there was a significant increase in this preference following training where hexanol was paired with fructose. After training where pentanol was paired with fructose, wildtype larvae exhibited a significant shift in preference for pentanol over hexanol (**Figure 7**).



Figure 7. Wild-type larvae learn to associate hexanol and pentanol with fructose reward. Comparison between naïve and learned odor preferences. Error bars indicate the SEM, and stars indicate evidence of successful learning at the .05 significance level (p=.0027 when hexanal is paired with fructose, p=.00065 when pentanol is paired with fructose).

## DISCUSSION

Wild-type larvae and Sdc<sup>23</sup> larvae exhibit different preferences for different odors, as wild-type larvae are significantly attracted to ethyl acetate, pentanol, hexanol, hexanol, and hex-2-enal while Sdc<sup>23</sup> larvae are significantly attracted to hexanol, hexanol, and ethyl proprionate. In addition, Sdc<sup>23</sup> larvae have a weaker behavioral response to all odors they find attractive, suggesting a mutation in the syndecan gene may result in altered wiring of the larval olfactory systems. One possible mechanism for such a wiring defect is that syndecan is known to be a necessary axon guidance receptor and its absence leads to improper Slit/Robo signalling.<sup>12,15</sup> Perhaps the absence of Sdc disrupts odorant receptor neuron projections to glomerular targets during neural development, or perhaps Sdc functions further downstream in the olfactory pathways. However, despite having less extreme attraction indexes than wild-type, Sdc<sup>23</sup> larvae still exhibit a significant preference for several odorants over water, indicating that Sdc<sup>23</sup> larvae are not simply anosmic.

Despite the fact that our associative learning training protocol was effective in training wild-type larvae, learning and memory was not exhibited by Sdc<sup>23</sup> mutants. Olfaction assays performed following the associative learning training protocol showed Sdc<sup>23</sup> larvae exhibit no change in odor preference after pre-exposure to odorants paired with the gustatory reward fructose. Conversely, wild-type larvae's attraction to either hexanol or ethyl acetate significantly increased after respective pairing with fructose, indicating that the absence of syndecan prevented mutant larvae from being able to either learn or recall a reward associated with an odor. The training protocol was repeated with heptanol and ethyl proprionate, once again, no evidence of learning and memory was observed for Sdc<sup>23</sup> larvae, however this time wild-type larvae also failed to respond to training. This is likely due to the fact that wild-type larvae are not sensitive to either odorant, as neither heptanol nor ethyl proprionate on its own produced a significant attraction index in wildtype larvae. To confirm the validity of this protocol for assessing learning and memory, wild-type larvae underwent a third training procedure with hexanol and pentanol, two attractive odorants. Significant increases in preference after training was observed in wildtype larvae, supporting the hypothesis that the training protocol was robust, and that syndecan mutants have deficits in learning and memory in *Drosophila melanogaster*.

Since the absence of syndecan changes larval olfaction-mediated behaviors significantly, it is possible that other complex cognitive processes are affected as well. Not surprisingly, previous research has shown impairments to vision in Sdc mutant adult *Drosophila*<sup>22</sup>, raising the possibility that Sdc<sup>23</sup> mutants also have defects in gustatory system function as well. In this case, Sdc<sup>23</sup> larvae would have been unable to detect the fructose in the associative training protocol, meaning their failure to respond to reward is due to gustatory rather than learning and memory deficits. Future studies could examine Sdc<sup>23</sup> mutants for associative learning capabilities using tactile aversive cues as opposed to attractive or repulsive gustatory cues. Previous work demonstrates that wild-type *Drosophila* are capable of learning to avoid odors that are paired with an electric shock.<sup>23</sup> In such an experiment, if Sdc<sup>23</sup> mutants do demonstrate learned aversion, then it would follow that syndecan plays an important role in gustatory system function, and that the failure to respond positively to fructose-paired odorants is due to an inability to detect fructose rather than a failure of learning and memory.

In order to further explore the mechanism of Sdc function in the olfactory system and in learning and memory, it would be useful to attempt to rescue the Sdc mutant phenotype by expressing a Sdc transgene in different spatial and temporal patterns in the developing larval brain. For example, if syndecan is only necessary during development to establish proper axonal connections, the syndecan mutant phenotype should be rescued with embryonic expression of a Sdc transgene, perhaps using a heat-shock promoter. Likewise, if syndecan is only involved in learning and memory, mushroom body expression of a Sdc transgene may rescue this mutant phenotype. Elucidating the spatial and temporal requirements of Sdc expression would provide insight into the molecular mechanisms of syndecan function in the developing olfactory system.

## CONCLUSIONS

Sdc<sup>23</sup> mutant *Drosophila* larvae find different food odors to be attractive compared to wild-type larvae, and have a less pronounced behavioral response to those odors. In addition, Sdc<sup>23</sup> mutant larvae cannot complete an associative learning task using gustatory cues. Together, this evidence indicates that the Syndecan gene mutation affects the wiring of the larval olfaction system and may be involved in the development of other cognitive processes, such as learning and memory. This finding is significant as previous studies have not explored the effects of Syndecan on memory, and further research should replicate these findings using different associative cues to determine if the Sdc<sup>23</sup> mutation impairs gustation in addition to olfaction or if it affects learning and memory directly.

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# ABOUT STUDENT AUTHORS

Dena Arizanovska and Jonathan King are neuroscience majors at Pomona College who graduated in May 2018. Dena plans on attending graduate school and having a career in research. Jonathan plans on pursuing an M.D.

## PRESS SUMMARY

This study examines the effects of the Syndecan (Sdc<sup>23</sup>) gene on the development of *Drosophila* larvae's sense of smell. It was found the larvae with a mutated Syndecan gene prefer different odors than larvae with an intact Syndecan gene, suggesting that the Syndecan gene is necessary for the proper development of the olfactory system. Furthermore, this study examined whether Syndecan is involved in learning and memory by exposing larvae to certain odors with fructose to see if this would enhance future attraction to the odorant. While wildtype larvae showed increased attraction to odorants following pairing with fructose, Sdc<sup>23</sup> larvae did not, suggesting an impairment learning and memory. This implies that the Syndecan gene is important for developing learning and memory abilities, and that it should be further studied for its role in higher cognitive functions.