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Neuronal cell-type-specific alternative splicing: A mechanism for specifying connections in the brain?

Joshua Shing Shun Li, Grace Ji-eun Shin, and S Sean Millard*
School of Biomedical Sciences; The University of Queensland; Brisbane, QLD, Australia

Alternative splicing (AS) allows a single gene to generate multiple protein isoforms. It has been hypothesized that AS plays a role in brain wiring by increasing the number of cell recognition molecules necessary for forming connections between neurons. Many studies have characterized isoform expression patterns of various genes in the brain, but very few have addressed whether specific isoforms play a functional role in neuronal wiring. In our recent work, we reported the cell-type-specific AS of the cell recognition molecule Dscam2. Exclusive expression of Dscam2 isoforms allows tightly associated neurons to signal repulsion selectively within the same cell-types, without interfering with one another. We show that preventing cell-specific isoform expression in 2 closely associated neurons disrupts their axon terminal morphology. We propose that the requirement for isoform specificity extends to synapses and discuss experiments that can test this directly. Factors that regulate Dscam2 cell-type-specific AS likely regulate the splicing of many genes involved in neurodevelopment. These regulators of alternative splicing may act broadly to control many genes involved in the development of specific neuron types. Identifying these factors is a key step in understanding how AS contributes to the brain connectome.

Keywords: alternative splicing, cell-type-specific, Dscam2, Dscam1, Dscam, neuronal wiring

Abbreviations: AS, alternative splicing; Dscam2, Down syndrome cell adhesion molecule 2; L1, lamina neuron 1; L2, lamina neuron 2; R cells, photoreceptor cells

*Correspondence to: S Sean Millard, Email: s.millard@uq.edu.au

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In 1978, Walter Gilbert postulated that, through RNA splicing, multiple proteins of related or diverging functions could potentially arise from only a single transcription unit.[1] Accordingly, this idea was validated by the discovery that both a secreted and a membrane-bound form of an antibody were being produced from a single gene.[2-5] Since then, more examples of the same phenomena have emerged and this mechanism is what we now refer to as alternative splicing (AS). More recent studies indicate that approximately 95% of human multi-exon genes are alternatively spliced.[6,7] AS remains a crucial mechanism for increasing proteome diversity, but little is known about its functional implication.

Proteome diversity is much needed in the nervous system where multifaceted cellular processes, such as neurotransmission and synaptic plasticity, are coordinated with only a modest number of genes. It is also unclear how this limited set of genes specifies $\sim 10^{15}$ neuronal connections that are found in the human brain. Neurodevelopmental studies conducted over the past few decades have elegantly uncovered protein-protein interactions that govern neuronal connectivity.[8,9] Among these are families of genes that generate extreme biochemical diversification of receptor isoforms through alternative promoter usage, AS or a combination of the 2.[10-13] Two dramatic examples of this are the mammalian clustered *protocadherins* and *Drosophila Dscam1*. Both genes produce multiple isoforms with unique binding specificities, whereby identical isoforms mediate homophilic recognition.[14,15] If this diversity were required for specifying neuronal connections, one would expect isoform expression to be invariable between different animals. Contrary to this, the expression profile of single neurons revealed that numerous isoforms of both protocadherins and Dscam1 are expressed stochastically in each cell.[16-19] This type of probabilistic isoform expression is not suited for specifying connections between different neurons, but rather establishes a

unique neuronal identity critical for self-avoidance.[20,21] Self-avoidance plays a crucial role in organizing the nervous system by promoting the segregation of branches from the same cell and thus maximising the surface area that an individual neuron can cover.[22-24]

If AS plays a role in specifying connections in the nervous system, it needs to be regulated in a cell-type-specific manner. Recent work from our lab has shown that this is the case for another Dscam family member, called Dscam2. We demonstrated that alternative isoforms of *Dscam2* are expressed in distinct neuronal cell types and that this expression pattern is crucial for proper neuronal wiring in the *Drosophila* brain.[25]

Dscam2 encodes a cell recognition molecule of the immunoglobulin (Ig) superfamily; through mutually exclusive AS of exon 10, this gene produces 2 biochemically distinct isoforms (Dscam2A and Dscam2B). These two proteins that only differ by a single Ig domain, exhibit isoform-specific homophilic binding: A only binds to A, not B, and vice versa (**Fig. 1A**). Similar to Dscam1, homophilic interactions between identical isoforms result in repulsion, at least in a subset of neurons. Dscam2 repulsion plays an important role in generating boundaries in the visual system and also in specifying the postsynaptic composition of photoreceptor synapses.[26,27] Given that there are only 2 Dscam2 variants, stochastic expression of multiple isoforms cannot confer a neuronal identity like it does for Dscam1. Instead, these biochemically distinct proteins may be used to perform similar functions in different neurons, analogous to how 2 genes could produce 2 independent repulsive proteins. If Dscam2 were used in this way, isoform expression would be expected to be tightly regulated. We investigated this problem in the visual system where Dscam2 mutant phenotypes have been well characterized.

The fly eye is comprised of ~750 ommatidia, each of which houses 8 photoreceptor neurons (R cells). R1-R6 axons target to the lamina where they form synapses with 3 of the 5 lamina neurons (L1-L3), whereas R7-R8 project to the medulla neuropil and target within 2 distinct synaptic layers. The medulla is topographically arranged where a repeated array of neuronal columns corresponds to the number of ommatidia in the retina. Each column comprises ~60 cell types, among which, L1 and L2 are 2 tightly associated neurons important for motion detection.[28,29] The cell bodies of these neurons reside in the proximal region of the retina and form stereotypical "bottlebrush"-like dendritic arrays that span the lamina cartridge. L1 and L2 axons form output synapses at specific layers in the medulla (**Fig. 1B**). Each column contains one L1 and one L2 and each neuron makes connections only in its column of origin. During early development, L1 axons send out processes as they search for their synaptic targets. L1 processes in adjacent columns overlap with one another at this stage and Dscam2-mediated repulsion ensures that connections are made only in a single column. This process is referred to as tiling, which describes the complete but non-overlapping coverage of a synaptic area by the same type.[26,30-32] Accordingly, L1 clones that lack *Dscam2* show a tiling defect because they fail to recognize adjacent L1 axons and thus make additional connections to neighboring columns. Interestingly, L2 neurons that lack Dscam2 exhibit a similar defect in their dendrites, where the arrays breach their boundaries and project into neighboring cartridges. The functional requirements for Dscam2 repulsion in L1 and L2 neurons raised the question of how 2 neurons expressing the same repulsive protein can stay physically associated without repelling one another. In our study, we envisioned that regulated AS could resolve this paradox. Distinct Dscam2 isoform expression in L1 and L2 neurons would permit these neurons to repel other cells of the same type, but not each other.

To test this, we generated Dscam2 isoform-specific reporters to visualize splicing choice *in vivo*. These reporter strains revealed that L1 and L2 exhibit exclusive Dscam2 isoform expression with L1 expressing Dscam2B and L2 expressing Dscam2A. Cell-specific and exclusive Dscam2 isoform expression was also observed in lamina neurons L3 and L5, whereas dynamic Dscam2 isoform expression was observed in L4, with Dscam2B expressed at early stages and both isoforms expressed later in development.[25] To address whether regulated isoform expression was functionally required for neuronal wiring, we engineered *Dscam2* alleles with reduced isoform diversity. Using a recombinase-mediated cassette exchange approach, we generated flies that expressed a single Dscam2 isoform from the endogenous locus (isoform A and isoform B strains). Despite a significant reduction in animal viability in these single isoform lines, the organization of the visual system was overtly normal. This is surprising given that a large number of cells in the optic lobe express Dscam2, and suggests that multiple mechanisms must exist to maintain its stereotypic arrangement. The lack of tiling phenotypes in L1 axons also suggests that either Dscam2 isoform is sufficient to mediate repulsion. However, a detailed analysis of L1 and L2 axon morphology demonstrated that cell-specific isoform expression is necessary in these cells. Specifically, we observed a reduction in L1 and L2 axon arbor size in single isoform mutants. To confirm that this was due to the specific interaction between L1 and L2, as opposed to interactions with the other cells in the same fascicle in our whole animal single isoform lines, we conducted mosaic analysis with a repressible cell marker (MARCM).[33] We generated L1 or L2 clones, homozygous for a single Dscam2 isoform, in an otherwise heterozygous background. Using this approach, different isoform-expression combinations of L1 and L2 neurons within a single column were generated allowing us to assess the functional consequences of disrupting isoform specificity. Since we knew which isoform each lamina neuron in the fascicle expressed, we could predict the isoform composition of unlabeled cells surrounding the labeled cell. For example, labeled L1 clones expressing Dscam2A would encounter unlabeled L2 neurons also expressing Dscam2A. In this case, a severe reduction in arbor size was observed as expected. On the other

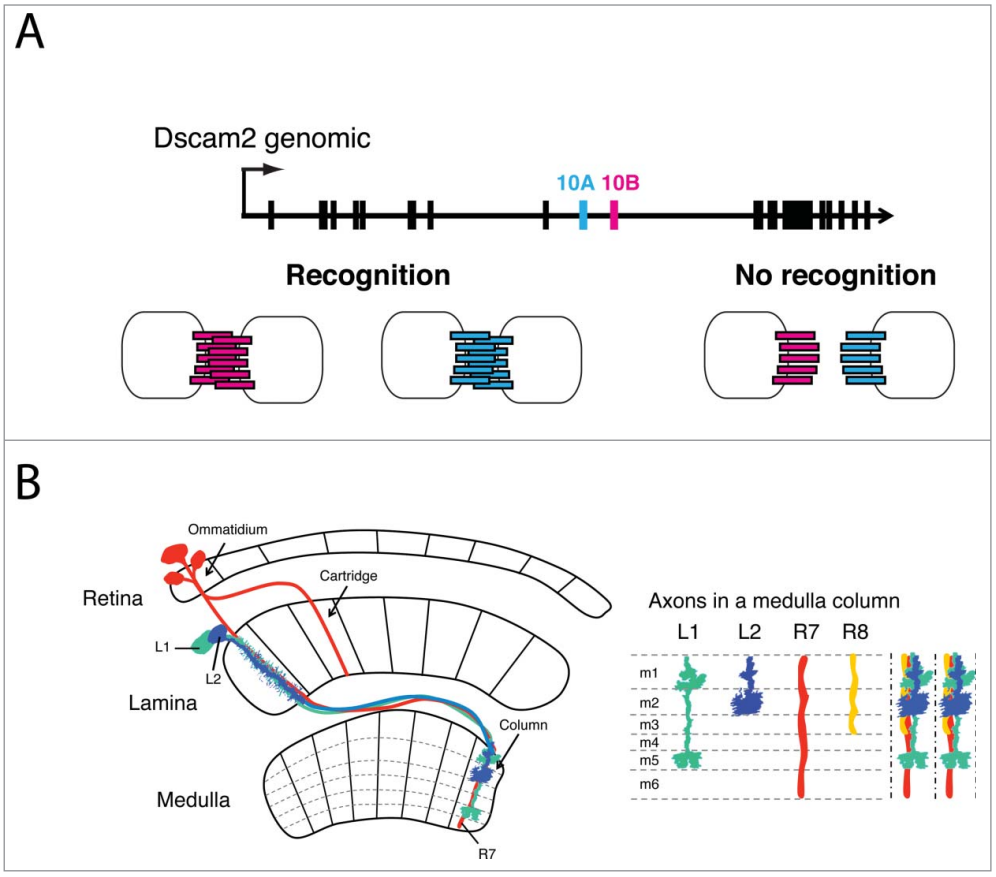

Figure 1. (**A**) Schematic of the *Dscam2* genomic region highlighting the alternatively spliced region: exon 10A (blue) and 10B (pink). Both isoforms bind in an isoform-specific manner where recognition is elicited when identical isoforms on opposing membranes meet. (**B**) Schematic depicting the *Drosophila* visual system. The retina consists of ~750 ommatidia housing 8 photoreceptors (R cells, R1-R8). R1-R6 project to the lamina neuropil to form synapses with L1 and L2 neurons. These further connect to the medulla neuropil where axon arbors are formed at specific layers.

hand, using the same Dscam2A MARCM strategy, L2 clones expressing Dscam2A were engineered to meet unlabeled heterozygous L1 neurons expressing both Dscam2A and Dscam2B. Interestingly, an intermediate reduction in arbor size was observed with this scenario. This data suggests that Dscam2 repulsion can be modulated by the degree of isoform similarity. Cells expressing identical isoforms exhibit more repulsion than cells expressing different combinations of isoforms. This could serve as a way to modulate Dscam2 recognition and repulsion, thus providing additional mechanisms for neurons to interact in a Dscam2-dependent manner. For instance, Dscam2 isoforms could form 3 distinct cis-dimer complexes, A-A, A-B and B-B. Under this premise, 2 opposing neurons expressing Dscam2 can form up to 6 different combinations of interactions and this could result in different degrees or types of Dscam2 signaling. The potential alternative binding partners and distinct downstream signaling effectors in different cells may also add to this complexity.

Concluding Remarks

Our study exemplified the importance of cell-type-specific AS in the proper wiring of neurons. We propose that L1 and L2 neurons express distinct Dscam2 isoforms so that they can repel neighboring cells of the same type, but not each other. Differential isoform expression allows for normal axon-terminal development in L1 and L2 neurons by avoiding inappropriate repulsion. This mechanism is likely also important for photoreceptor synapses where L1 and L2 are invariantly paired in each postsynaptic compartment. Previous work demonstrated that Dscam1 and Dscam2 redundantly mediate self-repulsion in these cells to prevent sister neurites from incorporating into the same synapse. It is likely that expression of different isoforms in L1 and L2 cells is also necessary for this postsynaptic pairing. It will be interesting to analyze the Dscam2 single isoform strains of flies for changes in photoreceptor synapses and visual-related behaviors to test this possibility.

While we have only begun to demonstrate the functional requirements of cell-type-specific AS in the fly visual system, we predict that this is a common mechanism in establishing proteome diversity required for neuronal wiring. Consistent with this, a recent study showed that the mammalian Neurexins exhibit brain

region-specific AS and linked this to cellular diversity in the nervous system.[34]

Finally, the mechanism underlying the cell-type-specific and exclusive AS of Dscam2 is a question that remains unresolved. Most splicing regulators are expressed ubiquitously[35–38] and although their abundance can vary depending on the biological context, current understanding favors preferential rather than exclusive expression of isoforms.[39] Given our exclusive isoform expression data, we predict that unidentified RNA binding proteins regulate cell-type-specific AS of Dscam2 either through the activation or suppression of an alternative exon 10. These splicing factors may be expressed in a cell-type-specific manner or alternatively, other cell-specific proteins may regulate this process through protein-protein interactions with the spliceosome. Since cells typically use their resources efficiently, it is likely that these cell-specific factors will regulate other alternatively spliced genes. One possibility is that many alternatively spliced products contribute to the development of the same neuron, so the factors that control this process will represent "hubs" of neurodevelopment. Using our isoform reporter strains in combination with molecular tools for knocking down gene expression, we hope to identify these regulators in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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