Mapping neurons and brain regions underlying sensorimotor decisions and sequences

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ABSTRACT

A fundamental property of the nervous systems across the animal kingdom is the ability to select appropriate actions and sequences of actions in response to sensory cues. Many behaviors are physically mutually exclusive so commitment to one requires complete suppression of others. Further, because many behaviors are serially organized into sequences of actions, mechanisms must also exist that control transitions from one action to the next. The circuit mechanisms by which nervous systems achieve behavioral choice, stability and transitions are still incompletely understood. A key step in understanding these functions is to identify neurons and brain areas involved in controlling behavioral choice, stability and transitions. To do this, we developed an approach where we combined a large-scale neuronal inactivation screen with an automated action detection of sensorimotor decisions and sequences in response to a sensory cue. We characterized the response of wild-type larvae to a mechanosensory stimulus and found they can respond to the stimulus with a probabilistic sequence of 4 possible actions (head-cast, head-retraction, back-up and stop). We analyzed behaviors from around three hundred thousand larvae ($N=2.9x10^5$) where we selectively silenced small numbers of neuron types and individual neuron types systematically across the nervous system using a library of *Drosophila* GAL4 lines and determined the effect of these manipulations on larval mechanosensory responses. We identified neurons and brain-regions that when inactivated affected *Drosophila* larval sensorimotor decisions and sequence transitions between the different actions. Specifically, we identified 51 candidate lines for sensory processing and 24 candidate lines for competitive interactions. We also detected phenotype categories for sequence transitions consistent with a model of sequence generation where transitions and reversals are independently controlled. These findings provide the basis for understanding how sensorimotor decisions and sequence transition are controlled by the nervous system.

1. Introduction

In order to enable reliable and coherent responses of organisms to sensory cues, the choice of one action must be accompanied by a full suppression of all competing physically mutually exclusive actions. Often, animals respond to stimuli, not with single actions but with sequences of actions in which case, the transitions between actions need to be precisely controlled. These actions can vary between individuals and also within individuals upon repetition of the stimuli and thus have a probabilistic component. The circuit implementation of competitive interactions between neurons that promote mutually exclusive actions and the circuit mechanisms that control action stability and transitions from one action to the next are still incompletely understood.

For example, whether the choice of a behavior, its stability and transition into different behaviors are implemented in specialized decision-making centers or distributed across the nervous system form the sensory to the motor side is still a heavily debated subject (Cisek, 2007; Cisek and Kalaska, 2010; Gaudry and Kristan, 2009; Gold and Shadlen, 2007; Kristan, 2008; Lebedev and Wise, 2002; Livant, 2007; Miller et al., 1960; Reyn et al., 2014)We have previously identified a circuit for behavioral choice (between 2 actions) and sequences in response to an air-puff at the earliest stage sensory processing site which supports a model in which competitive interactions are distributed across the nervous system (Cisek and Kalaska, 2010; Jovanic et al., 2016). To examine the generalization of this computing architecture, it is necessary to identify other sites of competitive interactions and understand how the outcomes of these putative circuits for behavioral choice are integrated to give rise to a unified decision about which action to perform.

In addition, when actions are executed in sequences, does the nervous system need to specify the order of the individual actions in the sequence? There have been different theoretical models of sequence generation proposed: parallel queuing (Lasley, 1951; Seeds et al., 2014), synaptic chains (Long et al., 2010) and more recently chains of disinhibitory loops (Jovanic et al., 2016). But how long flexible behavioral sequences are implemented in the nervous system remains an open question.

A first step towards understanding the circuit mechanisms of action selection and probabilistic sequence generation, is to identify neurons and nervous system areas involved in promoting, stabilizing and suppressing specific actions.

Here, we used the GAL4/UAS system available in the *Drosophila* to selectively and constitutively silence neurons in populations of behaving *Drosophila* larvae during behavioral responses to an air-puff stimulation using tetanus-toxin. The air-puff can evoke 4 different actions in the larva that can be organized in various sequences which makes it suitable for studying both sensorimotor decisions and sequences. We have previously used this assay to study the circuit mechanisms underlying the choice between the two most prominent actions that occur in response to air-puff and a sequence of these two behaviors (Jovanic et al., 2016). Here we expanded the study on the choice between

multiple behaviors and longer behavioral sequences (of up to 4 different actions). We designed a behavioral screen using a library of *Drosophila* lines (Jenett et al., 2012; Li et al., 2014). Based on anatomical pre-screen of a collection of images of the larval stages of the GAL4 *Drosophila* lines we selected those that target small subset of neurons. 567 driver lines were screened and the behaviors of tens of thousands of animals recorded.

To quantify larval responses, we used supervised machine learning to reliably detect the actions that can be evoked by air puff and their sequences. In addition to new sensory lines, we identified 75 candidate GAL4 lines that target neurons required for behavioral responses to mechanosensory stimuli. Amongst these, we identified candidate elements of functional circuit modules for sensory processing (51 GAL4 lines) and sensorimotor decisions (24 GAL4 lines). We also identified candidate hit lines for specific sequence transitions (127 GAL4 lines total). The hit lines span different regions of the ventral nerve cord and the brain which suggests multiple sites of competition that are distributed across the nervous system.

The candidate lines provide a valuable resource for those interested in studying neural mechanisms underlying sensorimotor behaviors as it can restrict the focus for those interested in specific aspect of behavior (i.e. sensory processing, decisions, sequence generation) from the exploration of all the neurons in the nervous system to specific neurons and brain regions. Therefore this database is a prerequisite for the comprehensive circuit studies underlying sensorimotor decisions and action sequences during behavioral responses to a mechanical sensory cues as the candidate neurons can be used as starting points for circuit mapping and analysis by combining the results of silencing behavioral genetics experiments with the different methods available in *Drosophila* larva to relate structure and function of neuronal circuits: EM reconstruction, optogenetics, electrophysiology, calcium imaging, immunohistochemistry and modeling (Heckscher et al., 2015; Jovanic et al., 2016; Ohyama et al., 2015; Zwart et al., 2016).

2. RESULTS

2.1 An assay for sensorimotor decisions and sequences, using machine-learning based characterization of the behavioral response to an air-puff

In order to functionally map neurons specifically to the different aspects of sensorimotor behaviors, we presented stimuli to populations of freely behaving wild type larvae and larvae with inactivated neurons and monitored their behavioral responses (Figure 1 A-B)

We used a supervised learning algorithm that relies on a limited set of features, able to handle low resolution images of larva, to specifically detect 5 action categories that we observed in response to air-puff: Head-retraction, Stop, Cast, Back-up and Forward Crawl. These different discrete actions emerge naturally as separate clusters in a simple embedding space derived from few features. The actions are mutually exclusive and non-overlapping (Supplementary figure 1 A).

We have previously studied and analyzed the immediate response of larvae to the air-puff using a length variation method (Jovanic et al., 2016; Ohyama et al., 2013), (see Materials and methods for the link between new action definitions and previous ones). Here we analyzed a dataset of wild type larvae (control: w;;attP2-TNT larvae, (progeny of male w;;attP2 crossed to female UAS-TNT-e flies (13 831 larvae), using the newly developed method and quantified previously undetected actions: Stops and Back-ups, in addition to Head Casts, Head Retractions and Forward Crawls (Fig 1 C-E,G). Each of these actions occurs at different probabilities and these probabilities vary over the time of the response (Figure 1 D-F)). For example, head-retractions have a higher likelihood of occurring early in the reaction while the Crawl occurs with a highest probability late in the reaction (Figure 1 E).
We computed behavioral probabilities for each of the five actions during first second, during five and during fifteen seconds after stimulation (Figure 1F) and because the head-retractions occurs primarily immediately after stimulus onset we focused the behavioral probability analysis of the screen data on the shortest time window.

We further investigated the effect on air-puff intensity on the behavior. While the probabilities of some actions vary significantly with stimulus intensity (i.e. higher head-retraction and lower stopping and crawling at high intensity of air-puff), the amplitude of actions doesn't seem to change with stimulus intensity (Supplementary figure 1 B)

We computed transition probabilities between the different actions during the first 3 seconds after stimulus onset and found that from the crawl the larvae will be transitioning strongly into a cast or a head-retraction (35.9 % and 31.4 %) respectively, less strongly into a stop and with a lowest probability into a back-up (Figure 3). Head-retraction can transition into each of the four other actions with the highest likelihood of transitioning into a cast (42 %) and lower probability of transitioning into crawl and back-up (27.1 % and 25.3 %) and a low transition probability to stop 5.6 %). The cast has the highest probability of transitioning into a back-up (45.8%), a moderate probability of transitioning into a crawl (29.7 %), a lower probability of transitioning into a stop (18.8%) and a lowest probability of transitioning into a head-retraction (5.6 %). The Stop has a high likelihood of transitioning into a cast and lower probability of transitioning into a crawl. From back-up there is the highest probability to transition into a cast (79.9% than into a crawl (13.6 %) and with lower probability into a Stop and Head-retractions (4.5 % and 2%). This suggest that the first action in a sequence is most frequently a head-retraction or a cast (Jovanic et al., 2016) and that if a head-retraction is the first action it will be followed by the cast with the highest probability. If cast is the first action in the sequence than there is higher likelihood that the second action will be a back-up (or stop or crawl) than a head-retraction. Because transition probabilities between cast and back up are high in both directions, it suggests that there are multiple transition events between cast and back-up in one sequence with higher probabilities of transitioning from the back-up onto the cast. The transitions between head-retractions and cast, head-retractions and back-ups and back-ups and casts are asymmetrical, meaning that the transitions are more likely in one direction that the other (head-retractions to cast and back-up and back-up to cast). In the transition probabilities computed across the entire duration of the stimulus (Figure 1 G right) the transition probability from back up to crawl increase while the

probabilities of transitioning form crawl to stop and head-retraction decrease. This is consistent with evolution of behavioral probabilities during stimulation (Figure 1 E)

In summary, in response to an air-puff, the larvae perform a probabilistic sequence that can be composed of up to four different actions where the head-retraction is more likely to occur immediately after stimulus onset that any other actions, followed by cast, back-up and stop later in the response. The behavioral response to an air-puff is thus well suited to use as an assay to study sensorimotor decisions between multiple actions and generations of longer behavioral sequences.

2.2. Inactivation Screen for mapping neurons and brain regions that underlie the behavioral response to air-puff

In order to identify neurons specifically involved in sensorimotor decisions and sequences we applied the automated algorithm for behavioral detection to a behavioral inactivation screen dataset of larval responses to an air-puff stimulus. We analyzed behavioral responses of larvae in which we silenced small subsets of neurons and individual neurons. We tested 567 GAL4 lines that we selected for sparseness and expression quality based on an anatomical pre-screen out of the collection of the JRC GAL4 lines (Li et al., 2014). Expression patterns of the GAL4 lines used in this study are characterized in details and neuroanatomical information are available on http://www.janelia.org/gal4-gen1.

Ethogram of the full behavioral response to the air-puff of all the lines in the screen (Supplementary figure 2A, left) shows that the dominant behavior immediately after the stimulus onset is head-retraction and the second dominant behavior is a cast. The head-retraction is most likely to be followed by a stop or a cast, while the second dominant behavior at the second position would be the back-up and crawl (stop if the first dominant in the second position is a cast (Supplementary figure 2 A, right). Head-retractions, Stops and Back-up occur only after stimulus. Crawl and cast also occur prior to the stimulus onset, as these actions are part of larval foraging behavior.

Some of the tested lines did not have the same hierarchy of behavior as the majority including the control prior to stimulus onset. This is most likely due to locomotor defects (Supplementary Spreadsheet_locomotor defect lines). We report these lines as these neurons are good candidates for examining circuitry underlying locomotion. For the purpose of studying stimulus evoked decisions and sequences, we excluded those lines from further analysis as their defects would interfere with animal's capacity to respond to stimulation.

We determined families of hits by comparing the probabilities of performing each of the actions that occur in response to air-puff between test lines and the control. We considered as candidate for strong hits the 10% of lines that had the lowest or highest behavioral probabilities in head-retractions, casting or backing-up and considered as hits only those that were significantly different from the control (see Material and methods for details)

We then examined the different types of phenotypes (Figure 2 B-D, Supplementary Spreadsheet add) and found group of hits where: 1) one or more actions decreased, 2) one or more actions increased compared to the control and 3) one action was increased while (at least) another was decreased compared to the control. We consider the latter category of hits "decision hits" (or "competitive interaction hits") as the phenotypes where the probability of one behavior is increased while that of others is decreased is consistent with the same neurons promoting one actions while inhibiting others. These lines are the candidate hits for studying the circuit mechanisms underlying competitive interactions between different (mutually exclusive) behaviors (see section 2.5).

In addition to the behavioral probabilities, other features of behavioral responses, for example the amplitude of individual actions could also be affected. We quantified the amplitudes of head-retractions and casts (Supplementary figure 2. E-F). The amplitude of casting was on average inversely correlated to the probability, meaning that the stronger the casts, the least frequent they are (as it takes the animal longer to recover from the action and come back to neutral position). There were some exceptions. For example, the R15F02 that has both higher amplitude and probability of casting. Most of lines that head-retract little also do weaker head-retractions and those that do more head-retractions do that more strongly. An exception to this is the R11A07 that has a high head-retraction probability, but relatively low amplitude. Further investigating these outliers will help understand how the neurons control both the strength and probability of the response.

Because larvae usually crawl forward just prior to the stimulation we excluded the crawl from behavioral probability hit detection and considered it in the context of transition probabilities as it shows transitions from the baseline behavior into actions that occur in response to a mechanical stimulus (Supplementary table 2, Supplementary table 3)Similarly, we didn't include the stopping probability in the hit detection as phenotypes in stopping probability cannot be interpreted unambiguously. At lower intensity of stimulation larvae stop more (Supplementary figure 1 B and therefore) an increase in stopping (and decrease in other actions) might reflect impaired sensing of air-puff. Indeed, this type of phenotypes can be observed when silencing chordotonals-key sensory neurons for sensing air-puff (Supplementary table 1, see section 2.3). For all the hits, both the stopping and crawling probabilities are shown in Supplementary table 1 and Supplementary table 2 along with head-retraction, cast and back-up probabilities used for hit-detection.

2.3. At least 2 different types of sensory neurons mediate the air-puff response

We have previously identified chordotonal sensory neurons as sensory neurons that mediate the air-puff induced responses as silencing of these neurons resulted in severely impaired responses (Jovanic et al., 2016; Ohyama et al., 2013). While the response is severely impaired it is not completely abolished suggesting that some other sensory neurons could be involved in sensing air-current in addition to the chordotonals. In the Drosophila larva, there are multiple types of somatosensory neurons. Somatosensory neurons of type I, which are the monodendritic and are the chordotonal and the external

sensory neurons and multidendritic somatosensory neurons which can be subdivided into 4 classes I, II, III, IV depending on the level of their arborizations and the position on the body wall (Bodmer and Jan, 1987; Grueber et al., 2007; Merritt and Whitington, 1995).

Out of the lines screened we have identified 27 candidate hit lines that drive either only in sensory neurons (one or multiple types) or in sensory and some other neuron types (Figure 3A, Supplementary table 1). We found that chordotonal sensory neurons are present in at least 6 GAL4 lines (Supplementary table 1) including the positive control lines, R61D08 and iav-GAL4 lines that were described previously (Jovanic et al., 2016; Ohyama et al., 2013). Silencing of chordotonal neurons resulted in lower probability of head-retractions and casting and in some lines also of backing-up. Differences between the lines could be due to the different strength of the Gal4 drivers and to the different subtypes of chordotonal neuron targeted by the driver (Merritt and Whitington, 1995). Multidendritic (md) and external sensory (es) neurons appear in some of the lines with perturbed response to the air-puff (i.e. R30H09, R54A01) and thus are likely involved in air-puff sensing as well. While for the es neurons, we were not able to obtain a clean line, we obtained a line labelling multidendritic class III (md III) neurons and tested in the air-puff assay. We found that silencing of multidendritic neurons resulted in significantly less head-retractions (although less pronounced phenotype than for chordotonals) and backing-up, while the casting response remained unaffected (Figure 3, Supplementary table 1) suggesting that head-retraction and back-up are mediated also by the md III circuitry, while casts may be not. Md III have been shown to respond to light touch that induces similar response types: head-retractions, back-up, cast, and turn (away from the stimulus) (Tsubouchi et al., 2012; Yan et al., 2013). When silencing chordotonal and multidendritic class III neurons expressing tetanus-toxin using the NompC (a TRP channel that confers light touch sensitivity (Yan et al., 2013)) GAL4 driver that drives in both subsets of chordotonals and md III neurons the larvae showed an impaired response with less head-retractions, and casts, a phenotype consistent with a strong impairment in sensing air-current.

In summary, silencing of air-current sensing neurons resulted in lower probabilities of head-retractions and for some lines also in casting and/or backing –up. When silencing the chordotonal neurons, the probability of stopping was increased in several GAL4 lines (Supplemenary table 1), which suggested that the stopping response could be mediated by different sensory neurons and in absence of chordotonal sensory neurons this type of response becomes the primary response type. Alternatively, based on the finding that lower intensities of air-puff trigger more stopping than higher, in absence of chordotonals, the larvae could stop more because they sense the stimulus less.

2.4. Identification of central neurons involved in the air-puff response

Out of the lines driving in interneurons in the ventral nerve cord (VNC) and brain, we observed three main categories of hits that have significantly different behavioral probabilities from the control. The first categories are lines in which silencing of neurons results in lower probabilities of at least one behavior compared to the control and the second are the lines in which silencing of neurons results in higher probabilities of at

least one behavior. In both of these categories the other behaviors remain unaffected. In a third category, silencing a neuron, results in higher probability of one behavior and lower probability of at least one behavior or vice versa, suggesting that these neurons implement competitive interactions between the different behaviors.
If a neuron is required for sensory processing, the silencing of this neuron will result in a perturbed response where the larvae will react either more or less compared to a reference and the probabilities of performing an action could be affected across the behavioral repertoire (in one or more actions). Alternatively, a phenotype with higher or lower probabilities of behaviors could also be consistent with the modulation of behavioral response depending on the context or with neurons that control aspects of motor responses that will affect the speed, duration etc. of execution and affect behavioral probabilities indirectly.

Out of the hit candidate lines from the primary analysis, we identified the strong candidates in these categories that are significantly different than the control. We identified 51 such hits where the probability of at least one action was a) decreased or b) increased (Figure 3 B, Supplementary table 2).

Some of these lines have sparse expression patterns. We identified lines with more head-retraction (i.e. R11A07), more casting (i.e. R15F02) or more backing-up (R32E04). Silencing of neurons in R65B04 and R21A03 lines resulted in less head-retractions and casting respectively (Figure 3B). We didn't identify lines that back-up less, unless the phenotype was accompanied with less of head-retraction and/or cast.

2.5. Identification of neurons and brain regions involved in sensorimotor decisions

We determined that the detected actions in response to air-puff are mutually exclusive (Supplementary figure 1) and therefore we sought to identify neurons that could implement competitive interactions between these actions. We reasoned that if a neuron is required for sensorimotor decisions between mutually exclusive behaviors, inactivating that neuron may increase the probability of one action at the expense of another or several other actions.

We identified 24 hits in which larva perform less of at least one action and more of at least another action (Figure 4A, Supplementary table 2). This phenotype could be observed with different combinations of actions: head-retractions, casts and back-ups. Interestingly we never observed a decrease in back-up probabilities and increase in cast probability.

Twelve out of the 24 lines have sparse neuronal expression patterns (Figure 4B). For example, silencing of the neurons in R29F08 and R33D04 sparse lines results in more head-retraction and less casting and less head-retraction and more casting respectively. more back up, more casts and more casts and back-ups respectively (Figure 4).

These neurons are located in different areas of the nervous system (Supplementary table 2, Figure 4B-M) , which in line with distributed models of implementation of competitive

interactions and decision-making (Cisek, 2007; Cisek and Kalaska, 2010; Jovanic et al., 2016)

2.6. Identification of neurons and brain regions underlying sequence transitions

We computed transition probabilities between each pair of individual actions in all the different genotypes (Supplementary table 3. transition probabilities). As expected in a majority of candidate "decision hit" lines with affected behavioral probabilities, (except for R75G10, R45G05, R52B08) the transition probabilities were also perturbed (Supplementary table 3, Supplementary spreadsheet_TransitionProbabilities) and these 2 statistics are intricately linked.

We have previously proposed that lateral disinhibition promotes transitions between behaviors, while feedback disinhibition prevents reversal from one element in the sequence back onto the previous one (Jovanic et al., 2016) in a probabilistic sequence of two actions. We speculated that chains of such disinhibitory loops could be a general mechanism for generating longer behavioral sequences. We therefore sought to investigate whether for other pairs of actions: 1) transitions and reversals are separately controlled and 2) whether the maintenance of an action (through a positive feedback) allows the proper progression through the sequence and prevents reversals.

Consistent with our hypothesis that transitions and reversals are controlled independently, we identified neuronal lines where silencing of neurons affected only transitions or only reversals from one element in the sequence onto another (Supplementary table 3, in red transition hits are shown, in green reversal hit., Supplementary spreadsheet_TransitionProbabilities). We first analyzed hits with affected transition probabilities from the head-retraction to the cast and to the back-up (Figure 5 D, Supplementray table 3). We identified 62 such candidate hits (24 that affect head-retraction to cast transitions and 38 that affect head-retraction to back-up transitions). Interestingly while the head-retraction to cast hits had lower transition probabilities compared to the control, the majority of head-retraction to back hits had higher transition probability compared to the control (except for 5 lines). This suggest that the transitions from head-retraction to back-up are strongly inhibited in the wild-type larvae during early response to an air-puff.

We then analyzed candidate hit lines where both head-retraction to cast and head-retraction to back-up were affected to see whether there were competitive interactions that underlie the choice of the action to transition to, from the head-retractions. We found that out of 18 lines where both head-retraction to cast and head-retraction to back-up transitions were affected, only one had a decrease in both head-retraction to cast and head-retraction to back-up transitions. The remaining 17 lines had one type of transition increased and the other decreased compared to the control consistent with the existence of competition between the transitions into a back-up or a cast. In addition, the phenotypes were consistent with most of the lines promoting transitions from head-retraction to cast and inhibiting transition to back-up (Supplementary table 3, Figure 5 E).

Interestingly, in behavioral probability hits we never observed a decrease in back-up at the expense of cast or head-retraction (except a decrease in both back-up and cast and increase in head-retraction) (Figure 4A), which suggests that, at least in the early response to air puff the head-retraction and cast are dominant behaviors and that back-up response gets activated by a head-retraction (25.3%) or a cast (42%) possibly through disinhibition like we have shown for the two action sequence (Jovanic et al., 2016).

We then analyzed hits with affected transition probabilities from back-up to head-retractions and cast to head-retraction (reversals, these transitions occur rarely in the wildtype: 2 % and 5.6% respectively) and found 6 lines that affected only transitions from cast to head-retraction out of which 2 had increased transition probabilities consistent with their role in preventing reversals (Figure 5C, Supplementary table 3 sequence transition hits). We didn't identify any candidate hits that affected only the back-up to head-retraction transitions.

We next analyzed transitions between cast and back-up. The cast is more likely to occur after the first action in the sequence (the head-retraction) than the back-up (Figure 1. G). Unlike the reversal to head-retraction the reversal to cast occurs very frequently (in 79.8% of cases while the transition probability from cast to back-up is 45.8%). Out of 14 candidate lines that had only the back-up to cast transition probabilities affected (reversals) 1 had increased transitions (Figure 5C, Supplementary table 3.). We identified 27 candidate lines in which the cast to back-up transitions were affected, 16 of which show a decrease (promote transitions) and 11 show increased transitions from cast to back.

The fact that we find phenotypes where only one transition or reversal is affected between a pair of actions suggests that these processes are controlled separately.

3. DISCUSSION

In order to identify neurons and brain regions underlying sensorimotor decisions and probabilistic action sequences we performed a high-throughput inactivation screen where we silenced individual neurons and groups of neurons (using tetanus-toxin) in 567 genetic GAL4 lines in *Drosophila* larva and looked at the effects of these manipulations on decisions and sequences occurring in response to a mechanosensory cue.

We characterized the behavioral response of wild-type larvae to the stimulus (air-puff) and found that larvae perform a probabilistic sequence of four different actions. We developed and used an automated approach that detects and distinguishes 5 different discrete behaviors that larvae perform in response to the air-puff. Evidences suggest that the discrete action description is relevant when compared to a continuum approach as parameters associated to larva dynamics tend to naturally cluster. We used this analysis to describe phenotypes that result from manipulation of different populations of neurons or single neurons. We find phenotypes that are consistent with a specific role of neurons in sensory processing, sensorimotor decisions (competitive interactions) and sequence transitions. Neuronal expression data for all of the GAL4 lines used in this screen have been previously published (http://www.janelia.org/gal4-gen1). The number of neurons that were targeted in our tested lines varies from 2 to 7 pairs on average and smaller number of the GAL4 lines the driver is restricted to a single neuron type.

Our screen therefore identified candidate regulatory and control centers in the nervous system for sensorimotor decisions and probabilistic sequences. These candidate neurons and nervous system regions can restrict the focus for those interested in studying specific aspects of behavior: sensory-processing, sensorimotor decisions and sequences from the entire nervous system to a relatively small number of elements. For example, connectivity and activity mapping efforts can be focused just to the relevant subset of neurons.

We developed a framework for identifying circuit elements underlying decisions and sequence transitions. Sensory-processing, sensorimotor decisions and sequence generation are intertwined processes as the latter two will depend on how the sensory information is processed, and the sequence production mechanistically might depend on competitive interaction between distinct actions (competitive queuing, chain of disinhibitory loops (Jovanic et al., 2016; Lasley, 1951; Rosenbaum et al., 2007; Seeds et al., 2014) (Bullock, 2004). Nevertheless, we used the reasoning below to identify neurons selectively involved in competitive interactions that underlie decision-making and sequence generation.

We reasoned that, if the stimulus cannot be processed and thus perceived accurately the animals might respond less, by performing less of all or some of the actions. If the sensory processing is affected in the opposite way (hypersensitivity), animals might respond more, and perform more of all or some of the actions normally observed. Thus,

the neurons that gave such inactivation phenotypes (of less of one or more actions; or more of one or more actions) were assigned a candidate role in sensory processing.

On the other hand, inactivation of neurons involved in mediating competition between actions is expected to result in increased probability of one action and decreased probability of one or more other actions (or the converse).

We identified 24 hits (GAL4 lines) that affected competitive interactions that underlie sensorimotor decisions. These GAL4 lines drive in neurons that are located in the ventral nerve cord (both abdomen and thorax region), brain, suboesophagial ganglion. This suggests that the networks for sensorimotor decisions are distributed across the nervous system. In a previous study, we mapped a circuit for behavioral choice and sequence generation located in the early processing centers (Jovanic et al., 2016) suggesting that the choice between two types of responses is not centralized in higher-order centers but distributed in the nervous system. It is still unknown how the circuitry early at the sensory processing site interacts with other nervous system areas to produce coherent decisions. A perceptual decision could be made about the stimulus, categorizing it into a high-risk, very aversive, which would result in an escape type of response or a low risk or low aversive which would result into a startle type of response (stop and head-retraction). Alternatively, the decision about which behavior to perform could be modulated at the early sensory processing site by top-down pathways and no other site of competitive interactions would exist that would contribute to this type of decision to a somatosensory stimulus. Finally, competitive interactions between different sensorimotor pathways could be distributed across the nervous system (Cisek and Kalaska, 2010) and other sites in the brain, thorax or towards the motor side would exist that would contribute to the decisions. In this case the competitive interactions at the early sensory processing site would contribute to the decision-making process that is the result of the integration of competitive interactions across the nervous system, including other areas i.e. brain, thorax and pre-motor areas. The identification of candidate neurons that could mediate competitive interactions in other regions of the nervous system is in line with a distributed decision-making process and suggest that other sites of competitive interactions (in other regions of the nervous system) contribute to the decision of what to do in response to mechanosensory stimulus. The identification of candidate elements provides the groundwork for understanding how the decision-making process is organized in the nervous system.

The idea that decisions are made "through a distributed consensus that emerges in competitive populations" and that interactive behaviors require sensorimotor and selection system to function in parallel (Cisek and Kalaska, 2010) have emerged in various fields (Briggman et al., 2005; Cisek, 2007; Gaudry and Kristan, 2009; Ledberg et al., 2007; Pezzulo and Castelfranchi, 2009; Reyn et al., 2014)
, but it has been challenging to elucidate the neuronal architecture that would implement such decisions. The *Drosophila* larva, because of its numerical simplicity, small size and the existence of multiple experimental approaches for structural and functional connectivity studies, behavioral genetics, optogenetics etc. is an ideal system for investigating how the outcomes of these competitive interactions at the different sites are

integrated across the nervous system to give rise to coherent behavioral choices. This study provides the towpath for analyzing the functional neural architecture underlying decision-making.

The neural architecture that controls the productions of probabilistic action sequences and establishes the order of the individual elements in the sequence is also poorly understood. In our previous work on a two element sequence in response to an air-puff, we have proposed that transitions to the next element in the sequence and reversal to the previous element are controlled independently through two different motifs: lateral disinhibition from the neuron driving one behavior onto the neuron driving the following behavior and feedback disinhibition that provides a positive feedback that stabilizes the second behavior and prevents reversals back onto the previous actions. Based on this, in the screen we specifically looked at candidate lines with phenotypes where either only transitions or reversals between two actions were affected to test whether this may be a general mechanism involved in transitions between multiple actions. We have identified categories of phenotypes that inform us about the implementation of sequence transitions in the nervous system. We looked at transitions from the head-retraction to Cast and Back-up, and transitions from Cast to Back-up and the corresponding reversal transitions. We found phenotypes consistent with neurons that prevent reversals (from Cast to head-retraction and Back-up to Cast). In addition, we found phenotypes that suggest that asymmetric competitive interactions exist between transitions to cast and back-up (from head-retractions) where the Cast inhibits the back-up but the back-up doesn't inhibit the Cast.

In summary, our screen provides a starting point for identifying the mechanisms underlying the competitive interactions between behaviors as well as the transition between individual actions in probabilistic sequences. While the number of neurons that were targeted in our tested lines varies from 2 to 7 pairs on average, in the case when the lines label multiple neuron types, intersectional strategies can be used to further refine the expression patterns. In the larva, a volume of electron microscope data has been acquired and more than 60% of the nervous system has been reconstructed through collaborative efforts (Berck et al., 2016; Eichler et al., 2017; Fushiki et al., 2016; Heckscher et al., 2015; Jovanic et al., 2016; Ohyama et al., 2015; Schlegel et al., 2016; Schneider-Mizell et al., 2016; Zwart et al., 2016). The identified candidate neurons can therefore be reconstructed in the EM and the synaptic patterns of interactions between neurons involved in competitive interactions can be mapped. Combined with EM reconstruction, electrophysiology, and modeling the candidate lists of neurons can be used to relate structure and function and unravel the principles of how the nervous system makes decisions and produces behavioral sequences across the nervous system.

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Figure Captions

Figure 1. Characterisation of the behavioral response of wild-type larvae to an air-puff
A. Behavioral set-up **B**. Still from a movie of contours of larvae acquired with the Multiworm tracker software **C**. In response to air-puff larvae perform a probabilistic sequence of four actions **D**. Ethogram of the behavioral response to an air-puff (10s after stimulus onset). Here a subset of animals tested is shown for clarity (N larvae shown is 145). Different colors represent different actions: Blue-Head-retraction, Red-Cast B, Cyan-Back-up, Black-Crawl and in Green- Stop **E**. Behavioral probability (mean) before, during and after stimulation **F**. Behavioral probabilities in the first second, first 5 seconds and first 15 seconds after stimulus onset **G**. Transitions probabilities between five actions: Crawl to the Head-retraction 35.9 %, Crawl to Cast 31.4 %, from Head-retraction to Cast 42 % and Head-retraction to Crawl 27.1 %, to back-up 25.3% and to stop 5.6 %. The cast has the highest probability of transitioning into a back-up (45.8%), a moderate probability of transitioning into a crawl (29.7 %), a lower probability of transitioning into a stop (18.8 %) and a lowest probability of transitioning into a head-retraction (5.6%). The Stop has a high likelihood of transitioning into a cast and lower probability of transitioning into a crawl. From back-up there is the highest probability to transition into a cast (79.9 % than into a crawl (13.6%) and with lower probability into a Stop and Head-retractions (4.5 % and 2%).
The color of the transition represents the action that the animals are transitioning from. The color of the contour that the band lands on represents the actions that the animal is transitioning into. The colors are as in D and E. The plot on the left represents all the transitions in the first 3 s after stimulus onset and the plot on the right all the transitions during the entire duration of the stimulus of 38s.

Figure 2.
A. Experimental strategy and screen design **B**. Scatterplot of Head-retraction probability against Cast probability. The 10% of lines with lowest and highest probabilities of head-retraction are shown in red and blue respectively. The lines with low head-retraction and high cast probabilities are shown in green. The lines with high head-retraction and low cast probabilities are shown in magenta **C**. Scatterplot of cast probabilities against back-up probability. The 10% of lines with lowest and highest probabilities of casts are shown in red and blue respectively. The lines with low cast and high back-up probabilities are shown in green. The lines with high cast and low back-up probability are shown in magenta **D**. Scatterplot of Back-up probabilities against head-retraction probability. The 10% of lines with lowest and highest probabilities of back-up are shown in red and blue respectively. The lines with low back-up and high head-retraction probabilities are shown in green. The lines with high back-up and low head-retraction probabilities are shown in magenta **E**. Behavioral probability phenotype categories: 1) less response phenotype: lower probabilities in one or more actions 2) more response: higher probabilities in one or more actions 3) competitive interaction phenotype: lower probability of one action and higher probability of at least one action or vice versa

Figure 3. Phenotypic summaries using selected behaviors (head-retraction, cast and back-up) for the hits from the behavioral screen. The colors in the heatmap represent the

p-values for each behavior for the comparison between each line shown to the right of each plot to the w;;attP2-TNT control
A. 27 sensory lines **B**. CNS lines (51 lines). Left: Hits with lower probabilities in at least one behavior compared to the control (27 lines). Right: Hits with higher probabilities in at least one behavior compared to the control (24 lines)

Figure 4.
A. Phenotypic summaries using selected behaviors (head-retraction, cast and back-up) for the "decision" hits from the behavioral screen. The colors in the heatmap represent the p-values for each behavior for the comparison between each line shown to the right of the plot to the w;; attP2-TNT control. All the hits that have lower probabilities in at least one action and higher probabilities in at least one other action (24 lines) are shown **B-M**. Example of candidate hits with sparse expression patterns. **B-E** Hits where the competitive interactions between head-retractions and casts are perturbed **F-I**. Hits where the competitive interactions between head-retractions and back-ups are perturbed **K-M.** Hits where the competitive interactions between all three actions are perturbed
B-M. Green-GFP; Blue-N-cadherin

Figure 5.
A. Transition probability phenotype categories shown: 1) Only reversal affected, 2) Only transitions affected **B**. Transition probabilities for the Control: w;; attP2-TNT control **C.** Reversal hits: examples for Cast to Head-retraction and Back-up to Cast, **D**. Transition hits: Examples for Head-retraction to Back-up, Head-retraction to Cast and Cast to Back-up **E**. Example of a hit with an increase in transitions from head retractions to back-up and decrease in Head-retraction to Cast transitions

Supplementary figure 1.
A. Natural clustering. Discrete actions emerge naturally as separate clusters in a simple embedding space derived from few features. X and Y axis are arbitrary variables generated during the t-sne (van den Maaten and Hinton, 2008) computation.
B. left Behavioral probabilities (1st second) at high (6 m/s) and low (3 m/s) intensity of air-puff. P-values are all $p<0.001$ (***) Right Amplitude of casting and head-retractions and high and low intensity of air-puff

Supplementary figure 2.
A. Ethogram of the behavioral of all the lines from the screen before, during (38s) and after stimulation- dominant behavior is shown in the ethogram on the left and second dominant behavior in the ethogram on the right. Each line represents a neuronal line.
B Scatterplot of head-retraction against back-up probability. The 10% of lines with lowest and highest probabilities of head-retraction probabilities are shown in red and blue respectively. The lines with low head-retraction and high back-up probabilities are shown in green. The lines with high head-retraction and low back-up probability are shown in magenta **C.** Scatterplot of Cast probabilities against head-retraction probability. The 10% of lines with lowest and highest probabilities of cast are shown in red and blue respectively. The lines with low cast and high head-retraction probabilities are shown in green. The lines with high cast and low head-retraction probabilities are shown in

magenta **D**. Scatterplot of Back-up probability against Cast probability. The 10% of lines with lowest and highest probabilities of back-up are shown in red and blue respectively. The lines with low back-up and high cast probabilities are shown in green. The lines with high back-up and low cast probabilities are shown in magenta
E. Scatterplots of Amplitude of Casting (effective angle) and Cast probability **F**. Scatterplots of Amplitude of head-retractions (Relative length change) and head-retraction probability. **B-F**. Each dot represents the average value for a neuronal line.

Supplementary tables and spreadsheets

Supplementary spreadsheet_locomotor defects (abnormal behavior prior to stimulus onset)

Supplementary spreadsheet_behavioral probabilities

Supplementary spreadsheet_transition probabilities-Transition probabilities screen scale. For each transition probability a p-value for comparison with the corresponding transition probability in the control (w;; attp2) are shown. P-values <0.05 are shown in red

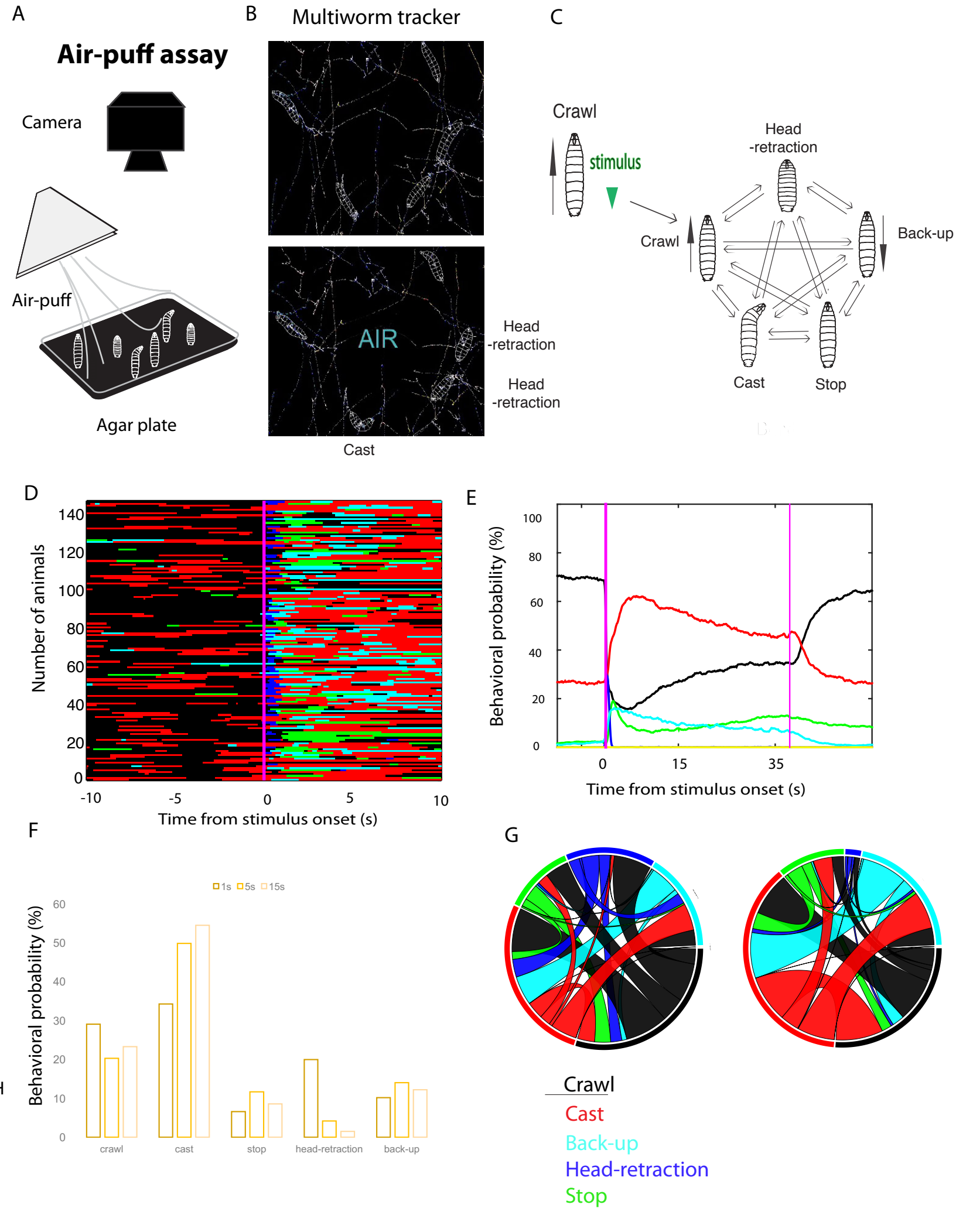
Supplementary table 1. Sensory line hits. Behavioral probabilities and p-values for comparison of each of the lines with the control

Supplementary table 2. CNS line hits. Behavioral probabilities and p-values for comparison of each of the lines with the control

Supplementary table 3. Transition probabilities hits. For each transition probability a p-value for comparison with the corresponding transition probability in the control (w;; attp2) are shown. P-values <0.05 for transition are shown in red, reversals are shown in green. The dark yellow labels the transition probabilities that are higher than the control

Reference:

van den Maaten, L.J.P., and Hinton, G.E. (2008). Visualizing Data using t-SNE. Journal of Machine Learning Research 2579–2605.


A
Air-puff assay
Camera
Air-puff
Agar plate
B
Multiworm tracker
AIR
Head
-retraction
Head
-retraction
Cast
C
Crawl
stimulus
Crawl
Head
-retraction
Back-up
Cast
Stop
D
Number of animals
Time from stimulus onset (s)
E
Behavioral probability (%)
Time from stimulus onset (s)
F
1s 5s 15s
Behavioral probability (%)
crawl
cast
stop
head-retraction
back-up
G
Crawl
Cast
Back-up
Head-retraction
Stop

A

Screen design

7000 lines JRC colection

↓

Anatomical pre-screen
Select lines with sparse expression patterns
in the larval brain and ventral nerve cord

↓

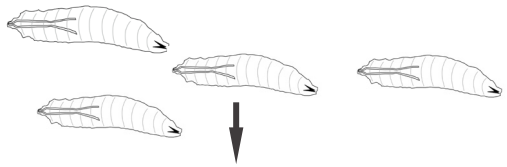
Screen 567 lines

X

♂ **567 GAL4 drivers**

♀ **UAS-TNT-e**

↓

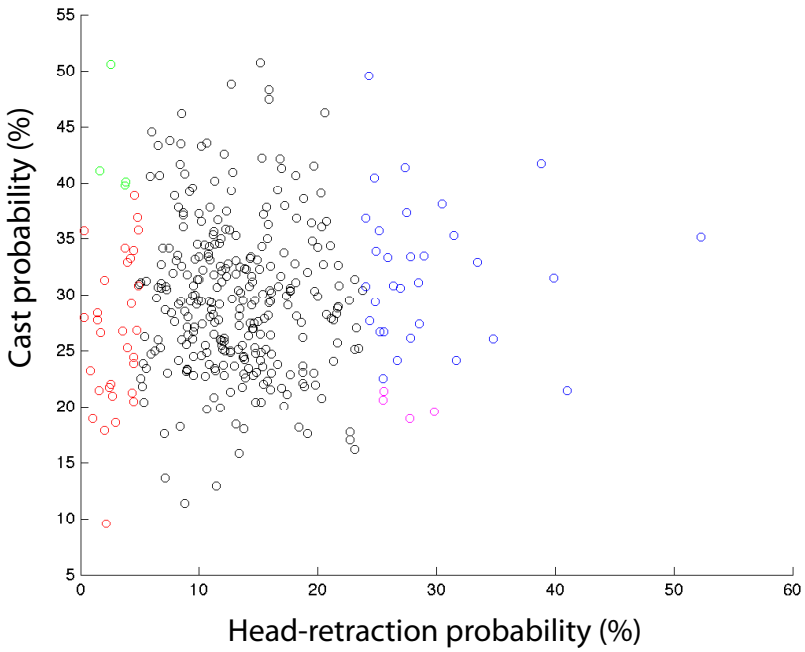


↓

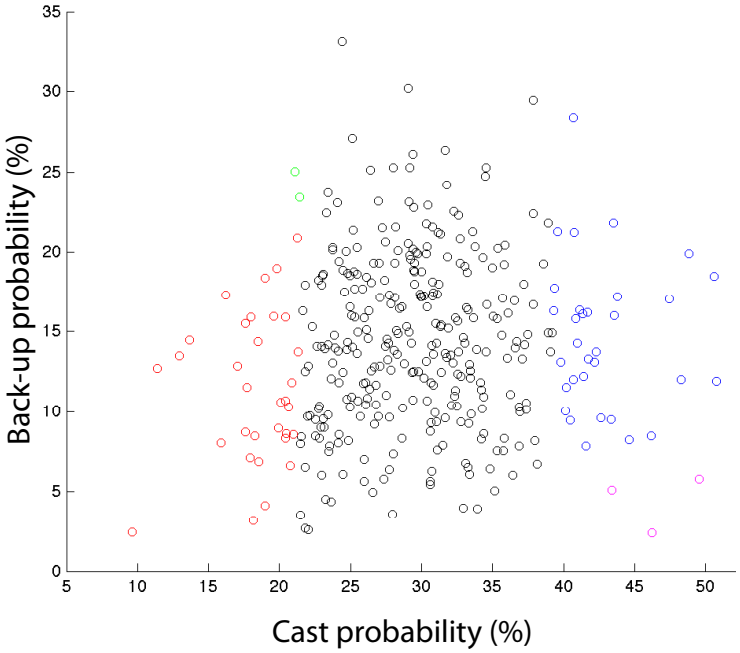
Detect changes in behavioral probabilities
Detect changes in transition probabilities

B

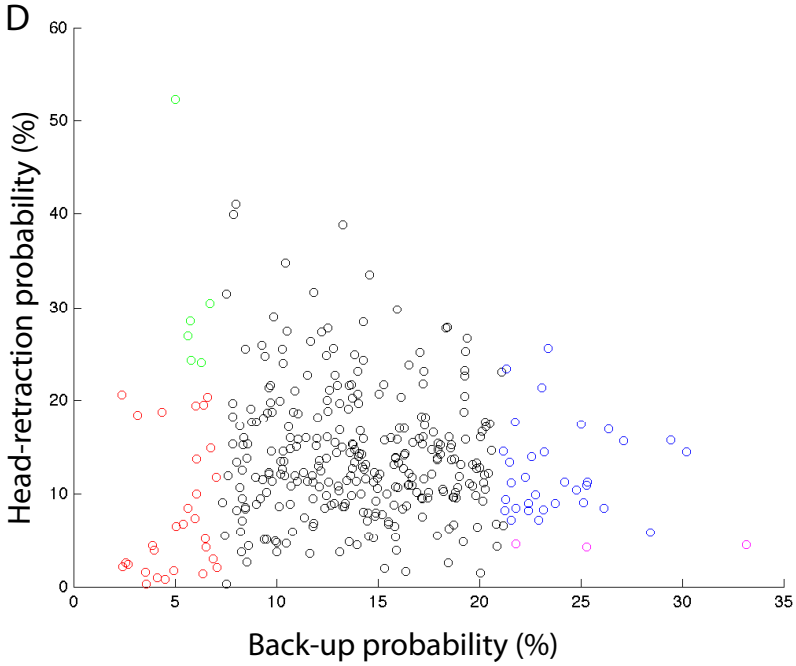
10 % lowest probability in probability on X
10 % highest probability on X
Low probabily on X and high probability on Y
High probabily on X and low probability on Y



C



D



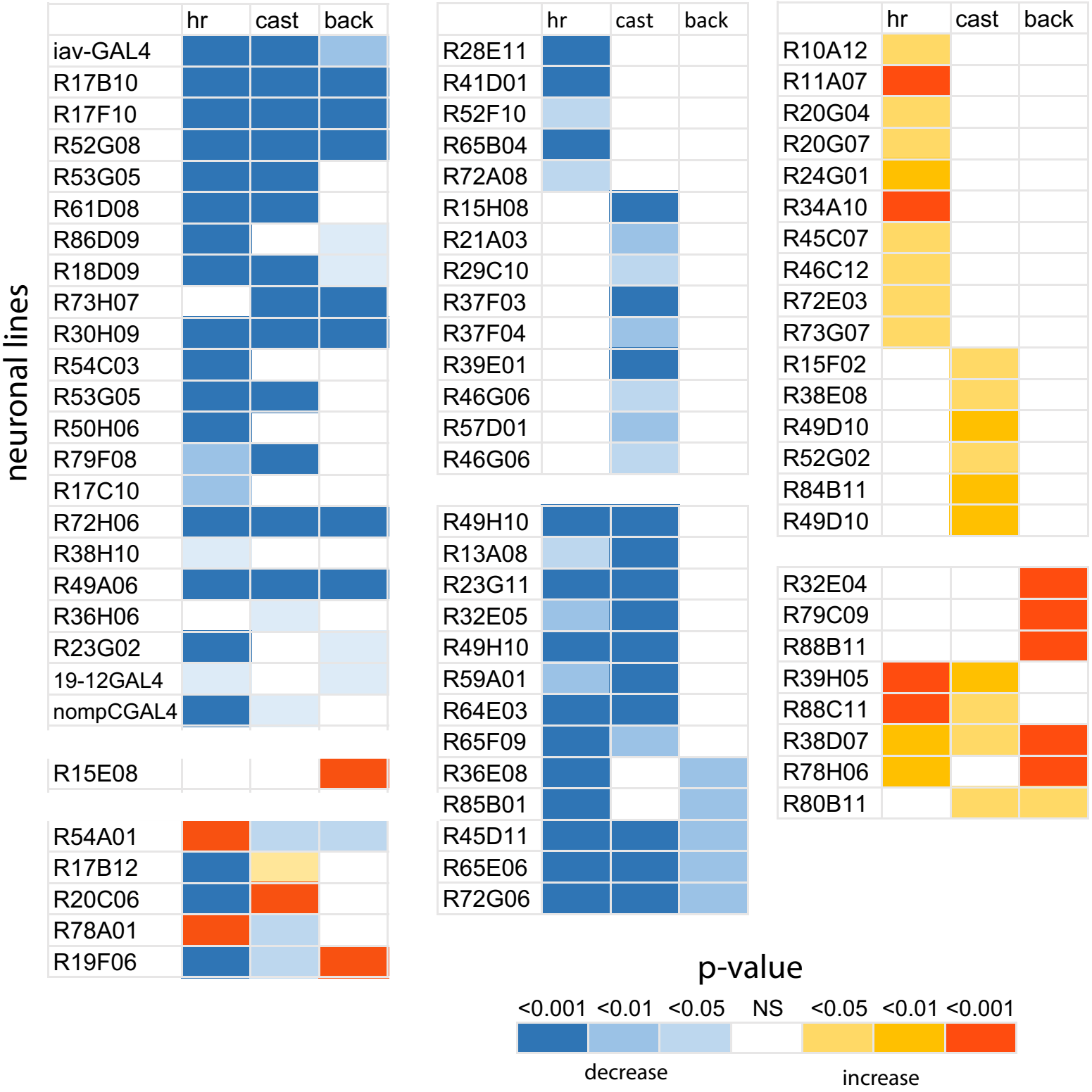
E Behavior probability phenotype categories

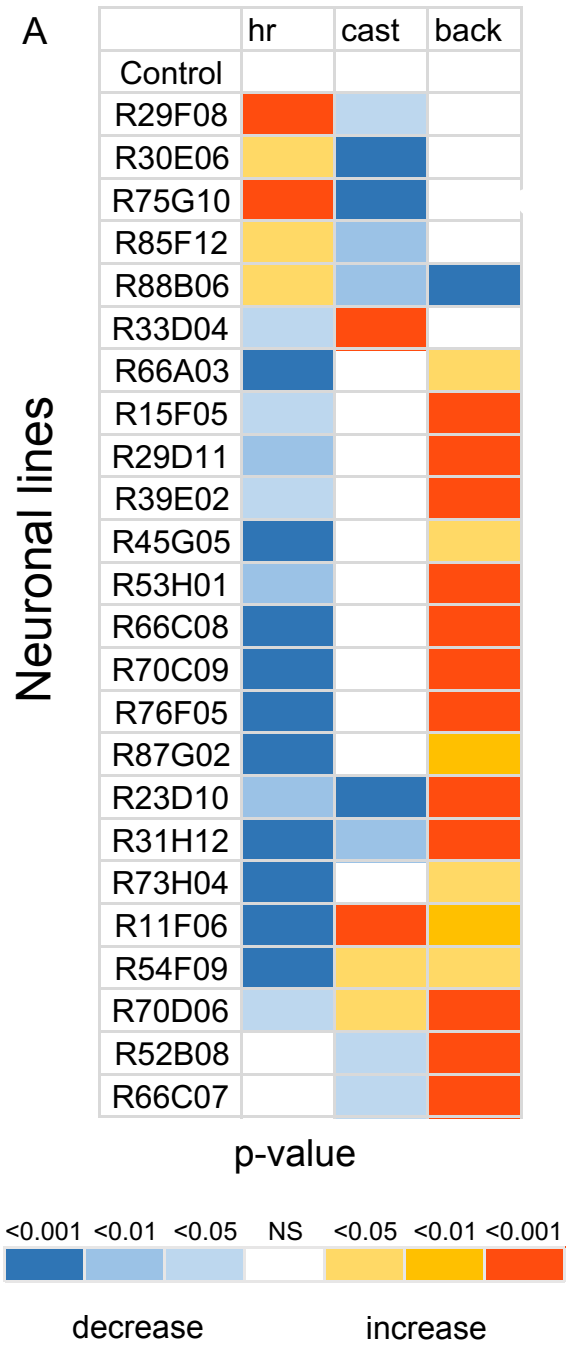
1) ACTION 1 ↓
....
ACTION n ↓

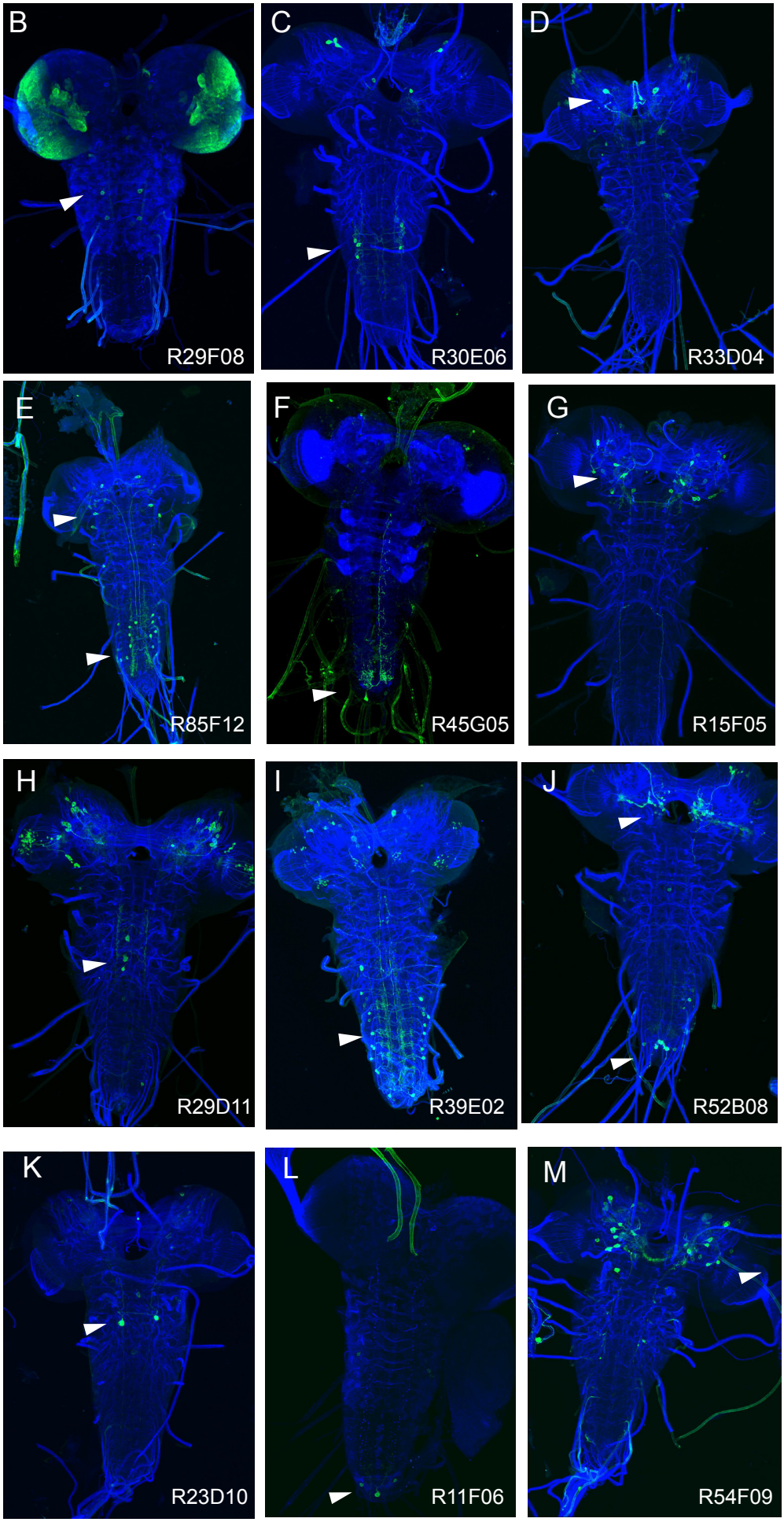
2) ACTION 1 ↑
....
ACTION n ↑

3) ACTION 1 ↓
and
ACTION 2 ↑
....
ACTION n ↑

ACTION 1 ↑
and
ACTION 2 ↓
....
ACTION n ↓


A
B
neuronal lines
hr
cast
back
iav-GAL4
R17B10
R17F10
R52G08
R53G05
R61D08
R86D09
R18D09
R73H07
R30H09
R54C03
R53G05
R50H06
R79F08
R17C10
R72H06
R38H10
R49A06
R36H06
R23G02
19-12GAL4
nompCGAL4
R15E08
R54A01
R17B12
R20C06
R78A01
R19F06
R28E11
R41D01
R52F10
R65B04
R72A08
R15H08
R21A03
R29C10
R37F03
R37F04
R39E01
R46G06
R57D01
R46G06
R49H10
R13A08
R23G11
R32E05
R49H10
R59A01
R64E03
R65F09
R36E08
R85B01
R45D11
R65E06
R72G06
R10A12
R11A07
R20G04
R20G07
R24G01
R34A10
R45C07
R46C12
R72E03
R73G07
R15F02
R38E08
R49D10
R52G02
R84B11
R49D10
R32E04
R79C09
R88B11
R39H05
R88C11
R38D07
R78H06
R80B11
p-value
<0.001
<0.01
<0.05
NS
<0.05
<0.01
<0.001
decrease
increase


A
hr
cast
back
Control
R29F08
R30E06
R75G10
R85F12
R88B06
R33D04
R66A03
R15F05
R29D11
R39E02
R45G05
R53H01
R66C08
R70C09
R76F05
R87G02
R23D10
R31H12
R73H04
R11F06
R54F09
R70D06
R52B08
R66C07
Neuronal lines
p-value
<0.001 <0.01 <0.05 NS <0.05 <0.01 <0.001
decrease
increase


B
R29F08
C
R30E06
D
R33D04
E
R85F12
F
R45G05
G
R15F05
H
R29D11
I
R39E02
J
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K
R23D10
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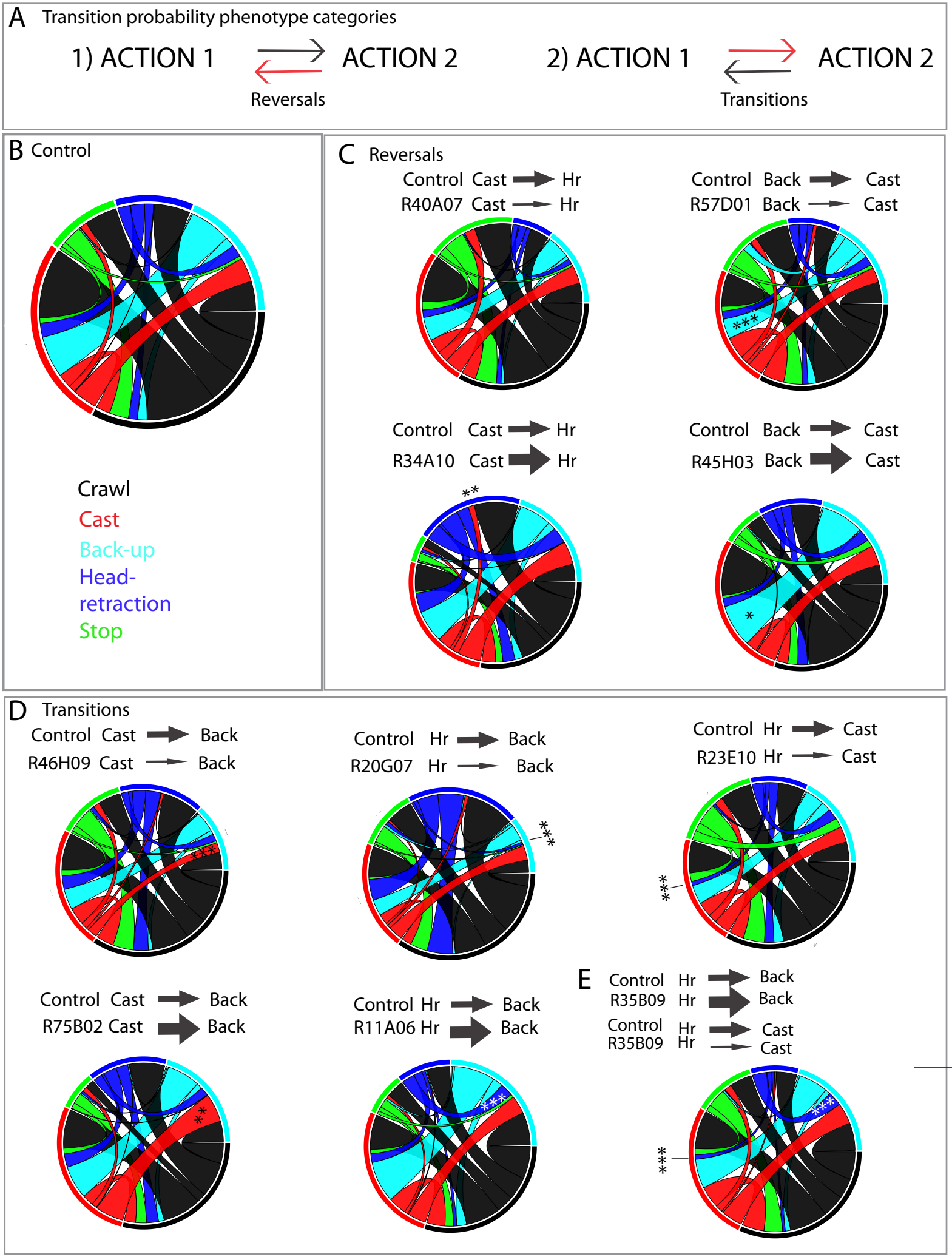
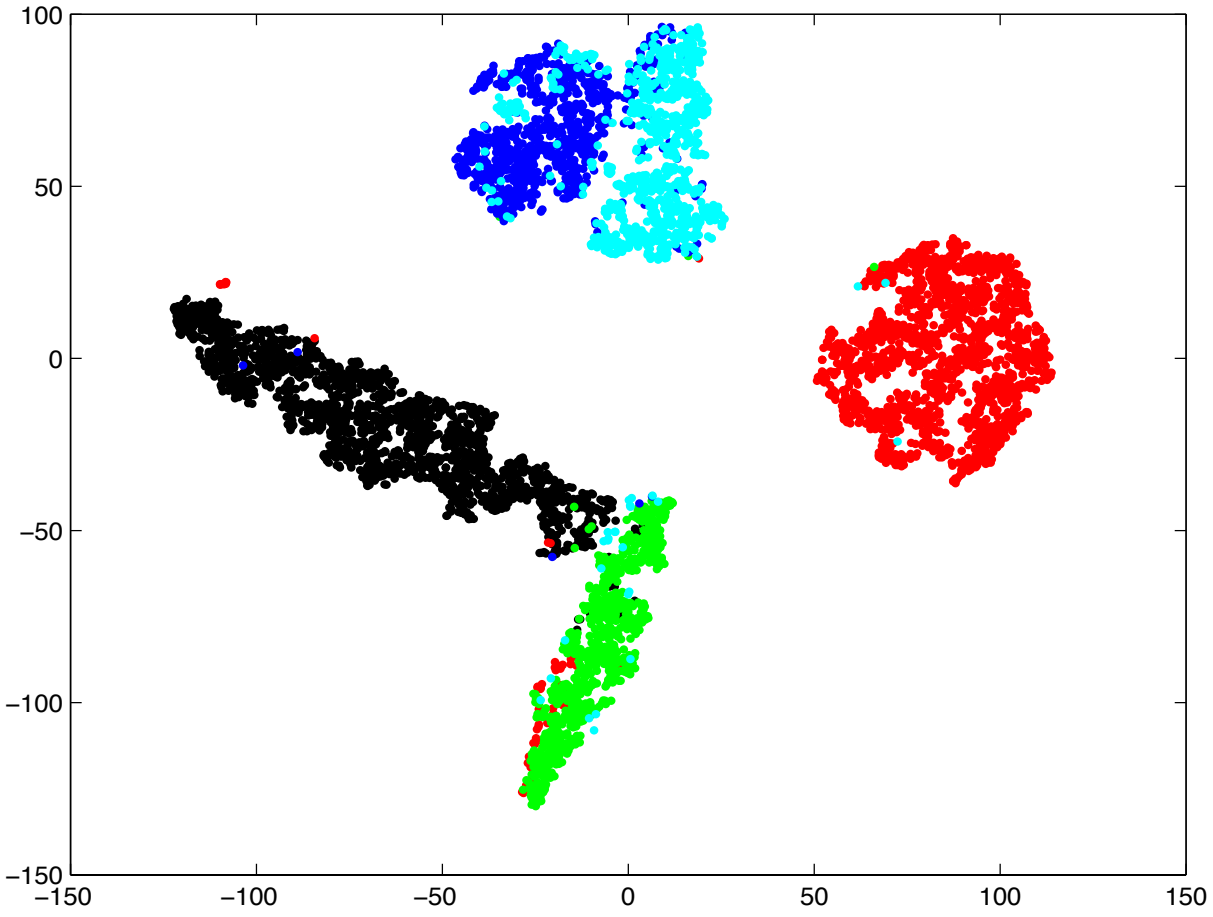
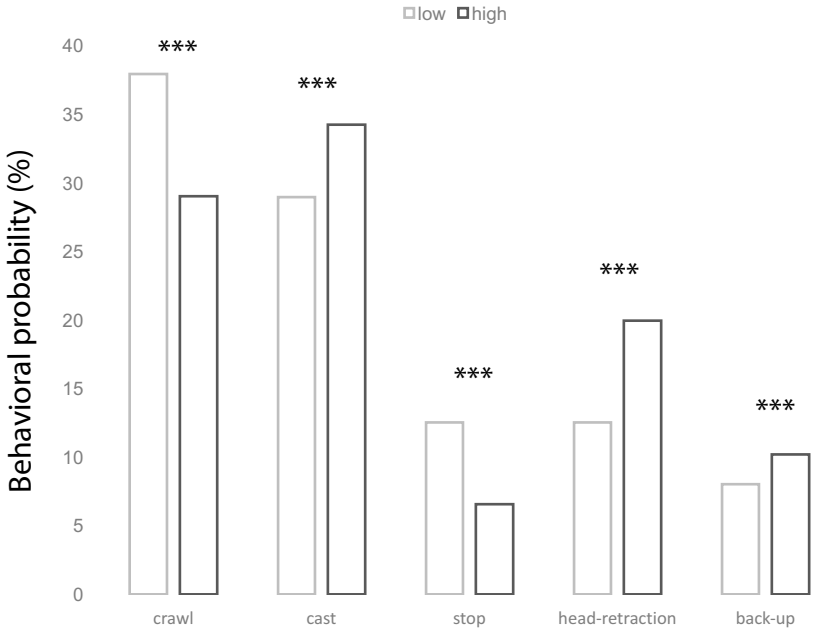
R11F06
M
R54F09


A Transition probability phenotype categories
1) ACTION 1
ACTION 2
Reversals
2) ACTION 1
ACTION 2
Transitions
B Control
Crawl
Cast
Back-up
Head-retraction
Stop
C Reversals
Control Cast Hr
R40A07 Cast Hr
Control Back Cast
R57D01 Back Cast
***
Control Cast Hr
R34A10 Cast Hr
**
Control Back Cast
R45H03 Back Cast
*
D Transitions
Control Cast Back
R46H09 Cast Back
***
Control Hr Back
R20G07 Hr Back
***
Control Hr Cast
R23E10 Hr Cast
***
Control Cast Back
R75B02 Cast Back
**
Control Hr Back
R11A06 Hr Back
***
E
Control Hr Back
R35B09 Hr Back
Control Hr Cast
R35B09 Hr Cast
***
***

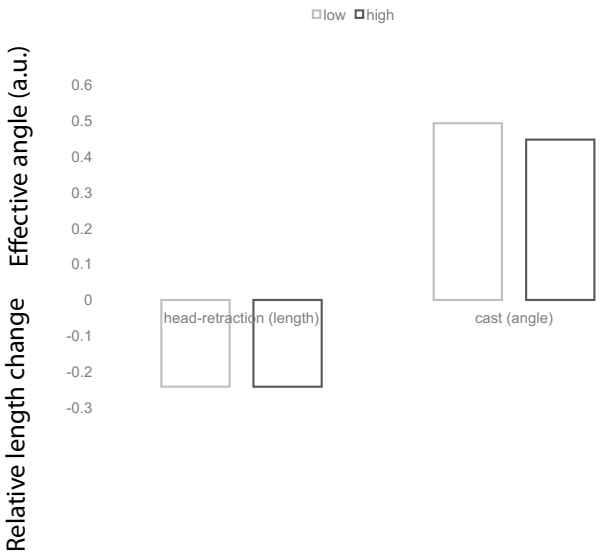
A

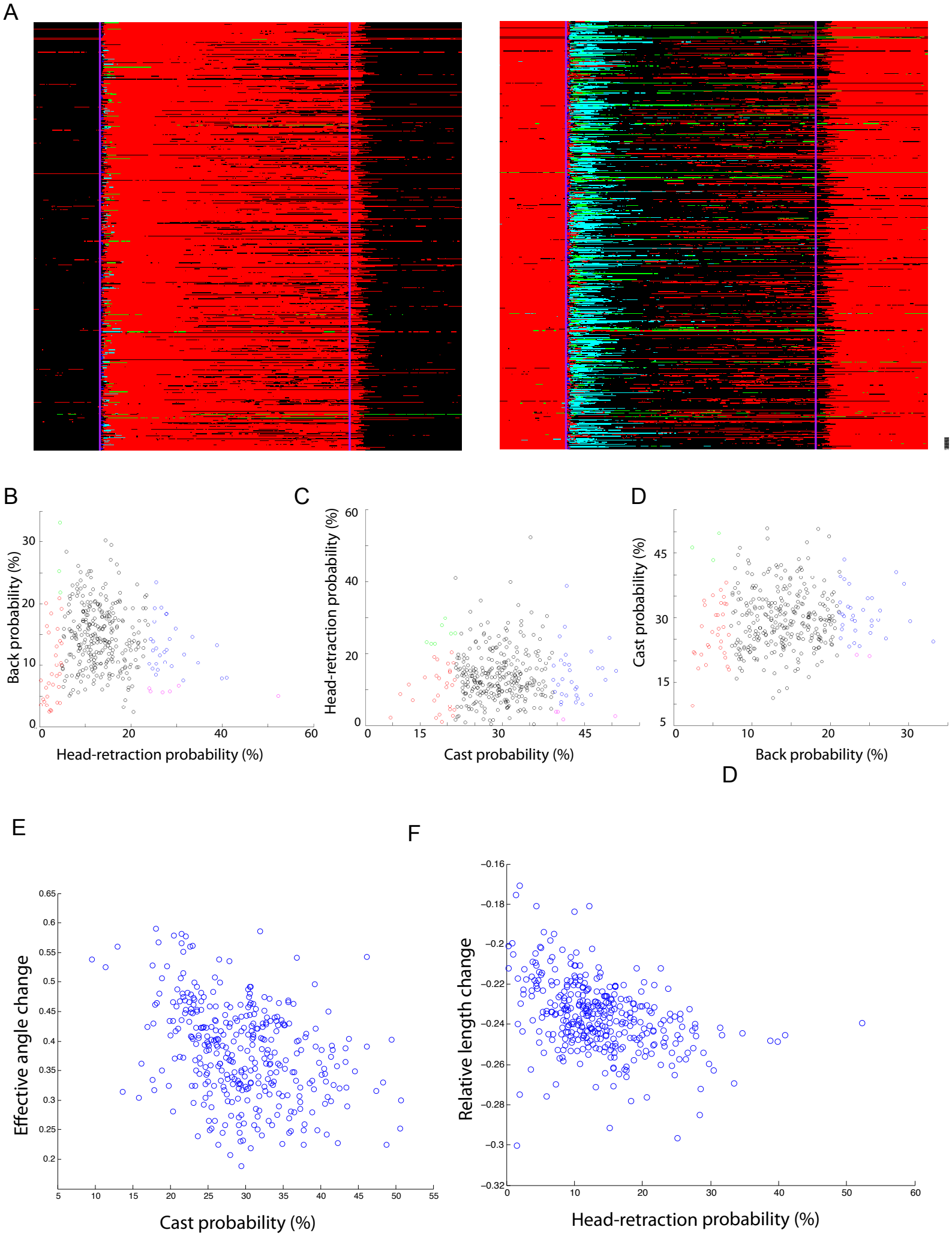

100
50
0
−50
−100
−150
−150
−100
−50
0
50
100
150

B


low
high
Behavioral probability (%)
40
35
30
25
20
15
10
5
0
***
***
***
***
***
crawl
cast
stop
head-retraction
back-up

C


low
high
Effective angle (a.u.)
Relative length change
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
head-retraction (length)
cast (angle)


A
B
Back probability (%)
Head-retraction probability (%)
C
Head-retraction probability (%)
Cast probability (%)
D
Cast probability (%)
Back probability (%)
D
E
Effective angle change
Cast probability (%)
F
Relative length change
Head-retraction probability (%)