Hox Activity Levels Governs the Evolution of Homologous Behaviors.

Authors: Jesús Rodriguez Curt[1,2], Meg Sambrook[2,3], Sara Deppieri[1,4], Katrin Nielsen[2], Nadezhda Velichkova[2], Richard Norris[5], Emiliano Merlo[6], Haluk Lacin[7], Simon A. Mitchell[5], James W. Truman[8], & Jimena Berni[1,2]*.

Corresponding author: j.berni@sussex.ac.uk

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Materials and Methods

Fly stocks: Fly strains were maintained on standard yeast–agar–cornmeal medium at 25 °C.

The following fly lines were used:

Short	Genotype	Source
NB 1-2	R16A01-DBD; R28H10-AD	H. Lacin
NB 2-4	*poxn*-Gal4	BDSC 66685
NB 3-5	GMR49C03-Gal4	H. Lacin
Nb 4-2	GMR81F02-Gal4	H. Lacin
NB 5-3	GMR86D02-Gal4, 86D02-Gal4	H. Lacin
NB 5-6	*Lbe(K)*-Gal4	S. Thor
NB 7-3	*eagle*-Gal4	M. Landgraf
UAS-CD4-GFP	w[1118]; P{w[+mC]=ppk-CD4-tdGFP}1b	BDSC 35842
UAS-CD8-GFP	w [*]; P{y[+t7.7] w[+mC] =UAS mCD8.GFP.UAS-rCD2i} attP2	BDSC 56170
UAS-myr: GFP	w[*]; P{y[+t7.7] w[+mC]=10XUAS-IVS-myr::GFP}attP2	BDSC 32197
UAS-GCamp6m	w[1118]; P{y[+t7.7] w[+mC]=20XUAS-IVS-GCaMP6m}attP40	BDSC 42748
UAS-*Abd-Bm*	w^{1}; P{ w^{+mC} =UAS-Abd-B.m.C}1.1	E. Sánchez -Herrero, BDSC 913
UAS-*Abd-A*	w^{1}; P{w^{+mC}=UAS-abd-A.G}21.8	S. Thor, BDSC 912
UAS-Ubx^{Ia1}	P{UAS-Ubx.Ia.R}	C. Alonso
UAS-*Antp*	w^{1118}; P{ w^{+mC} =UAS-Antp.Mb}W1	S. Thor, BDSC 7301
Multi-colour Flip-Out 2 (MCFO2)	hsFLPG5.PEST}attP3; {10xUAS(FRT.stop)myr::smGdP-OLLAS}attP2 {10xUAS(FRT.stop)myr::smGdP-HA}VK00005 P{10xUAS(FRT.stop)myr::smGdP-V5-THS-10xUAS(FRT.stop)myr::smGdP-FLAG}su(Hw)attP1	BDSC 64086
A18a/CLI2	w^{1118}; GMR15B07-GAL4} attP2	BDSC 48678

A18b3/CLI1	w^{1118}; +; P{ $y^{+t7.7}$ w^{+mC}=GMR47E12-GAL4}attP2, *Cha3.3*-Gal80	A. Nose
A18g5/Canon	w^{1118}; P{ $y^{+t7.7}$ w^{+mC} =GMR74F02-GAL4}attP2	BDSC 39861
A08e2	w^{1118}; GMR29G02-GAL4}attP2	BDSC 49496
A18c	w^{1118}; P{$y^{+t7.7}$ w^{+mC} =GMR17F06-GAL4}attP2	BDSC 47329
A23e	$w^{1118;}$ P{$y^{+t7.7}$ w^{+mC} =GMR15E05-GAL4}attP2	BDSC 48693
tsh-Gal4	w*; *tsh*-gal4/ CyO	M. Landgraf

Neuron naming

The name of neurons is based on the adult lineage that is generated from the NB. The name starts with an (A) because the neurons are abdominal, then the lineage number and a letter to determine different neuronal types form the lineage. If several neurons look very similar there is a number after the letter to indicate it is a different neuronal type i.e A18g3: A(abdominal)18(lineage) g(type) 3(sub-type). Certain neurons that were not initially associated to specific lineages were grouped by similarity of morphologies and numbered 25 and above; i.e. A30e. a few neurons were also assigned another name A18a = CLI2, A18b3 = CLI1 *(20)* and A18g5 = canon *(21)*. NB 1-2 generates the lineage 1; NB 2-4 lineage 18; NB3-5 lineage 9 and group 30; NB5-3 lineage 5 and a few neurons named 23; NB 5-6 group 26 and 27; NB7-3 group 26.

Multi Colour Flip Out protocol:

NB 2-4- identification of neurons and birth order.

For early neurons: Embryos were collected from 2-4pm at 25ºC, then kept at 18ºC until 2pm the next day. Embryos were transferred onto a mesh and rinsed to wash off any excess food. Embryos were heat shocked on the mesh in a water bath at 37ºC for 2-3 minutes. Embryos were transferred onto fresh agar plates (with yeast) and kept at 25ºC, until first instar larvae dissections.

For later neurons: Embryos were collected from 10-12am at 25ºC, then kept at 18ºC until 1pm the next day. Embryos were heat shocked in a water bath at 37ºC for 2-3 minutes. The plate was kept at 25ºC, until first instar larvae dissections.

For morphology analysis of wild type and Hox genes over-expressing neurons:

Embryos were collected from 12am-4pm at 25ºC, then kept at 29ºC until 9am the next day. Embryos were heat shocked in a water bath at 37ºC. The duration of heat shock given varied, depending on the line, and lasted between 3-15 mins. Plates were kept at 29ºC, until first instar larvae dissections on the afternoon. The over-expressing neurons were imaged in late first instar larvae to avoid further compensation mechanisms as consequence of the Hox-induced specification changes. Due to the low levels of expression of the line at embryonic stage 16, approximately 200 brains of each genotype had to be dissected.

Analysis of morphology

Neurons labelled using the multi colour flip out method were co-stained with anti-Neuroglian that reveals the main axons tracks in the VNC. We use this information to define segments boundaries and more generally to determine the morphology of interneurons. The presence/absence and change of direction of a primary dendritic or axonal branch was considered a change and was classified as posterior, anterior or commissural change. The length of a process was considered different only if they crossed to a new segment, and neurons whose dendrites shape changed in the same region of the neuromere were not considered to be variant.

Immunohistochemistry

Stage 16 embryos or nervous systems of newly hatched first instar larvae were dissected in External Saline (135 mM NaCl, 5 mM KCl, 4 mM MgCl2.6H2O, 2 mM CaCl2.2H2O, 5 mM N Tris (hydroxymethyl)methyl-2- aminoethanesulfonic acid and 36 mM sucrose, pH 7.15 [21]) and glued to a 0.1% poly-L-lysine-coated (Sigma P8920) cover slip (22x22mm). They were fixed with 4% formaldehyde in phosphate buffer (PBS) for 20 min at room temperature (RT) and rinsed in PBT (PBS + 0.3 % Triton X-100) 3 X 15 min at RT. Specimens were then incubated with the primary antibodies in PBT overnight at 4ºC in a wet chamber, washed in PBT 4 X 15 min at RT, and incubated with fluorescence-conjugated secondary antibodies at 1/500 in PBT for 3 hours at RT. Secondary antibodies were washed 4 X 30 min in

PBT at RT and specimens were mounted in Vectashield Antifade Mounting Medium (Vector Laboratories) between two aluminium-foil spacers, to avoid distortion of nerve cords, under a square cover glass (Brand, 22x22mm). Image stacks were captured on a Zeiss LSM880 confocal microscope.

Primary			
Antibody	Species	Source	Concentration
Anti-UBX	Mouse	Developmental Hybridoma Bank (DSHB), supernatant FP3.38	1:20
Anti-ABD-A	Goat	Santa Cruz Biotechnology, (dH-17): sc-27063	1:100
Anti-ABD-B	Mouse	DSHB, supernatant 1A2E9	1:50
Anti-ANTP	Mouse	DSHB, 8C11	1:50
Anti-ANTP	Rat	Stefan Thor lab	1:200
Anti-GFP	Chicken	Abcam, Ab13970	1:5000 1:400 embryo
Anti-ENGRAILED	Rabbit	Santa Cruz Biotechnology, (d-300): sc-28640	1:200 1:50 embryo
Anti-V5	Chicken	Bethyl Laboratories (A190-118A)	1:500
Anti-V5	Rat	Abcam, 46206571	1:2000
Anti-FLAG	Mouse	Sigma, F3165	1:8000
Anti-HA	Rat	Roche, 3F10 (11867423001)	1:400

Hox protein levels analysis

CNS of stage 16 embryos were dissected following the previously described protocol and stained with a-ANTP, a-UBX, a-ABD-A, a-ABD-B and a-GFP primary antibodies (See antibody list for reference). For quantification of Hox genes levels from confocal z image series, the images were loaded as composite images onto FIJI. Nuclei of neurons along the abdomen a1-a8 were identified and selected as regions of interest (ROIs) in respective focal planes. GFP-labelled neurons not located in the abdomen (e.g. thorax, SEG or brain lobes) were selected as ROIs for background fluorescence readings. Neurons with maximum intensity of Hox protein were quantified to obtain the 100% of intensity in the normalised data.

Fluorescence readings were obtained from both hemi-segments of 3 CNS and expressed as percentage of maximum fluorescence.

Kernel Density Estimation (KDE)

KDE was performed in Julia programming language using the MarginalHist function from StatsPlots.jl (https://github.com/JuliaPlots/StatsPlots.jl), which utilises KernelDensity.jl (https://github.com/JuliaStats/KernelDensity.jl). KDE analysis divided the probability mass into evenly spaced regions containing equal proportions of the probability density.

Behaviour

Differentiation experiments developmental protocol: Eggs were collected on standard apple juice agar plates supplemented with yeast paste for 3 hours at 25ºC. The oldest embryos will have just finalized cellularization. The plates were then transferred to an incubator at 18ºC for 12 hours. At 18ºC the amnioserosa becomes exposed after approximately 12 hs indicating that the embryos are in stage 12 and the third wave of NB proliferation has ended. At that stage the muscle precursors have already been formed[1]. Larvae were transferred at 29 ºC to release the repressive effect of gal80 onto gal4. Control larvae that do not over-express Hox genes eclosed after 30 hours of development from egg laying.

Locomotor analysis: Newly hatched first instar larvae (plates checked every 15 mins) were transferred to a 5 cm petri dish coated with 6 ul of 0.9% agarose. The plate was inverted to capture images from the ventral denticle bands. Two minutes movies were captured at 30 fps with a MQ013CG-On (Ximea) camera mounted on an M420 microscope (Leica) at 25X magnification. Movies were analyzed with the open-source software VCode (v.1.2.1; GitHub - microsoft/vscode: Visual Studio Code). The duration and number of forward and backward waves and turns performed by each animal was averaged and represent one data point.

Calcium imaging

Experiments were performed on first instar larvae, 0-4 h after hatching, at 23-25°C. Larvae were washed in H_2O and their nervous system dissected in external saline as above. The isolated nervous systems were then transferred to a 0.1% poly-L-lysine-coated cover slip to which they adhere and placed in a chamber filled with external saline. A DM-5000 (Leica) microscope with an APO 40x dipping objective was used for imaging. A 488 ± 15 nm wavelength light produced by a OptoScan Monochromator (CAIRN) passing through the epifluorescence port of the microscope was used to excite GCamp6m. Two thousand frames movies were captured using a cooled Orco-Flash-4 (Hamamatsu) camera at 5Hz at 512x512 pixel resolution. Intensity values were extracted on ImageJ from regions of interest (ROIs) placed over the neuronal soma. The data are presented as $\Delta f/f = (f_n-f_0)/f_0*100$ where f_0 is the average intensity. The number of events was empirically determined evaluating the $\Delta f/f$ curves across the abdominal segments.

Statistical Analysis

Statistical analysis was performed with SPSS (v29; IBM) or Prism (v.5.0b; GraphPad). Normality was tested with a Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for *p* values.

Reference

1. Bate, M. The embryonic development of larval muscles in *Drosophila*. *Development* **110**, 791-804, doi:10.1242/dev.110.3.791 (1990).

Movie 1. Control larva locomotion.
First instar control larva (*tsh*-Gal4/+ ; + /+) locomotion. The larvae alternates craws with turns.

Movie 2. *Abd-B* over-expression during differentiation affects larval balance.
Locomotor behaviour of a larvae over-expressing *Abd-B* (*tsh*-Gal4/+ ; UAS-*Abd-B*/ *tub*-Gal80^{ts}) in VNC uniquely during differentiation. The larva tumbles from left to right while crawling.

Movie 3. *Ubx* over-expression during differentiation drives the acquisition of a novel behaviour.
Locomotor behaviour of a larvae over-expressing *Ubx* (*tsh*-Gal4/+ ; UAS-*Ubx*/ *tub*-Gal80^{ts}) in VNC during differentiation. The larva lift and lower the thoracic segments during crawls and turns. This motor pattern is characteristic of abdominal segments during crawling and becomes incorporated into thoracic segments.

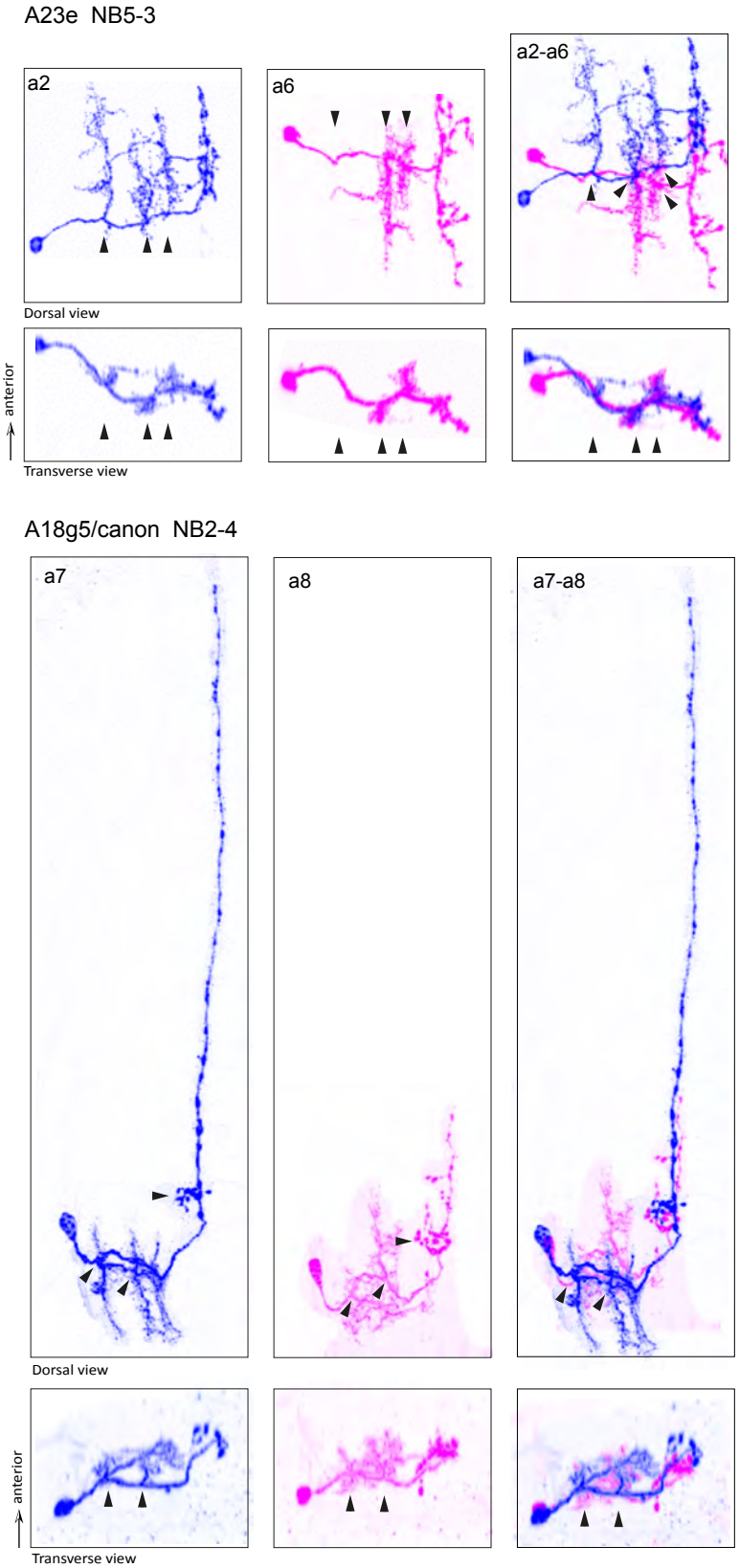
Movie 4. *Abd-A* over-expression during differentiation "abdominalises" the thoracic segments.
Locomotor behaviour of a larvae over-expressed *abd-A* (*tsh*-Gal4/+ ; UAS-*abd-A*/ *tub*-Gal80^{ts}) in the VNC during differentiation. The larva perform a high number of backward crawl bouts.

		a1	a2	a3	a4	a5	a6	a7	a8
NB 1-2	A01a	1	1	1	1	1	1	1	
	A01a2	1	1	1	1	1	1	1	
	A01b	1	1	1	1	1	1	1	
	A01b2	1	1	1	1		1	1	
	A01c	1	1	1		1	1	1	
	A01c2	1	1	1	1	1	1	1	
	A01d	1	1	1	1	1	1	1	
	A01h	1		1		1	1	1	
	A01j	1				1	1	1	
NB 2-4	A18a	1	1	1	1	1	1	1	
	A18b2	1		1	1	1	1	1	
	A18b3	1	1	1	1	1	1	1	1
	A18c	1	1	1	1	1	1	1	1
	A18g3a	1	1	1	1	1	1	1	
	A18g5	1	1	1	1		1	1	1
NB 3-5	A09a/ basin 1	1	1	1	1	1	1	1	
	A09b/ Basin 2	1	1	1	1	1	1	1	1
	A09c		1	1	1	1	1		
	A09d1	1		1	1	1	1	1	1
	A09d2	1	1	1	1	1	1	1	
	A09g/ Basin 3	1	1	1	1	1	1	1	
	A09l / A29c2	1	1	1	1	1	1	1	
	A09l2		1		1	1	1		
	A09z				1	1	1		
	A30a		1	1	1	1	1	1	1
	A30c		1		1	1		1	
	A30d	1	1		1	1		1	
	A30f	1	1	1	1		1	1	1

		a1	a2	a3	a4	a5	a6	a7	a8
NB 5-3	A23e / A23n		1	1	1	1	1	1	
	A23f1a /A23g		1	1	1	1	1	1	
	A23f1b	1	1	1	1	1	1	1	1
	A23f2			?	1	1	1	?	
	A05e	1	1	1		1	1	1	1
	A05k	1	1	1	1	1	1		
	A05n	1	1	1	1	1	1	1	
	A23k / A05p		1			1	1	1	
Nb 5-6	A26f	1	1	1	1	1	1	1	
	A26g / A26g2	1	1	1	1	1	1	1	
	A27k	1	1	1	1	1	1	1	1
NB 7-3	A26c		1	1	1	1	1		
	A26d	1	1	1	1	1	1	1	
	A26e	1	1	1	1	1	1	1	1
Nb 3-3	A08e2	1	1	1	1	1	1	1	

Supplementary Figure 1. Presence of neurons along the ventral nerve cord.

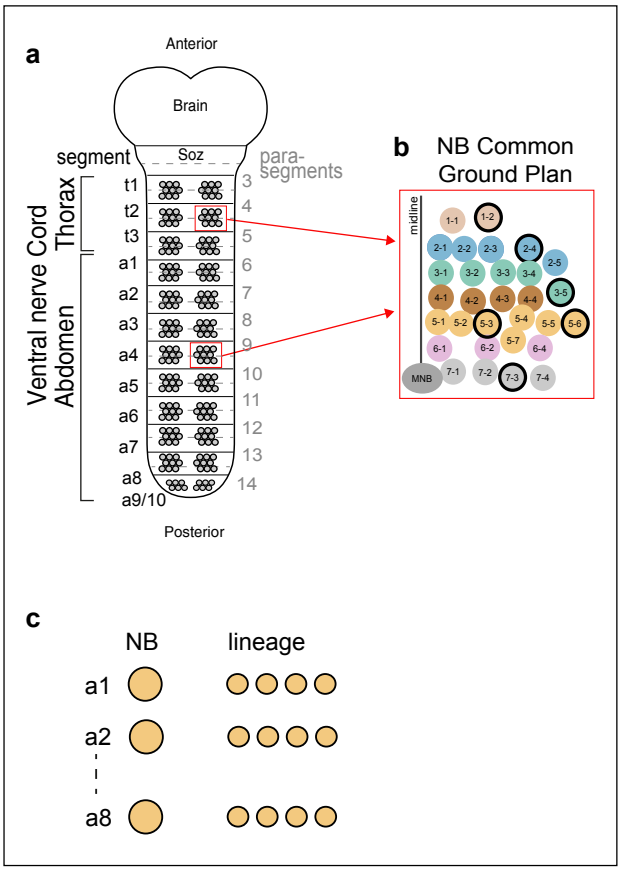
We have collated the information regarding the presence of homologous interneurons along the VNC. The “1” indicates that we have an image of the neuron either obtained by stochastic labelling, EM reconstruction, specific driver expression or from a publication. The orange-only box indicates that the presence of homologous neurons in all surrounding segments give us confidence that the neuron exist even if we are missing an image. Most neurons are present from a1 to a7 while the presence in a8 is more variable.



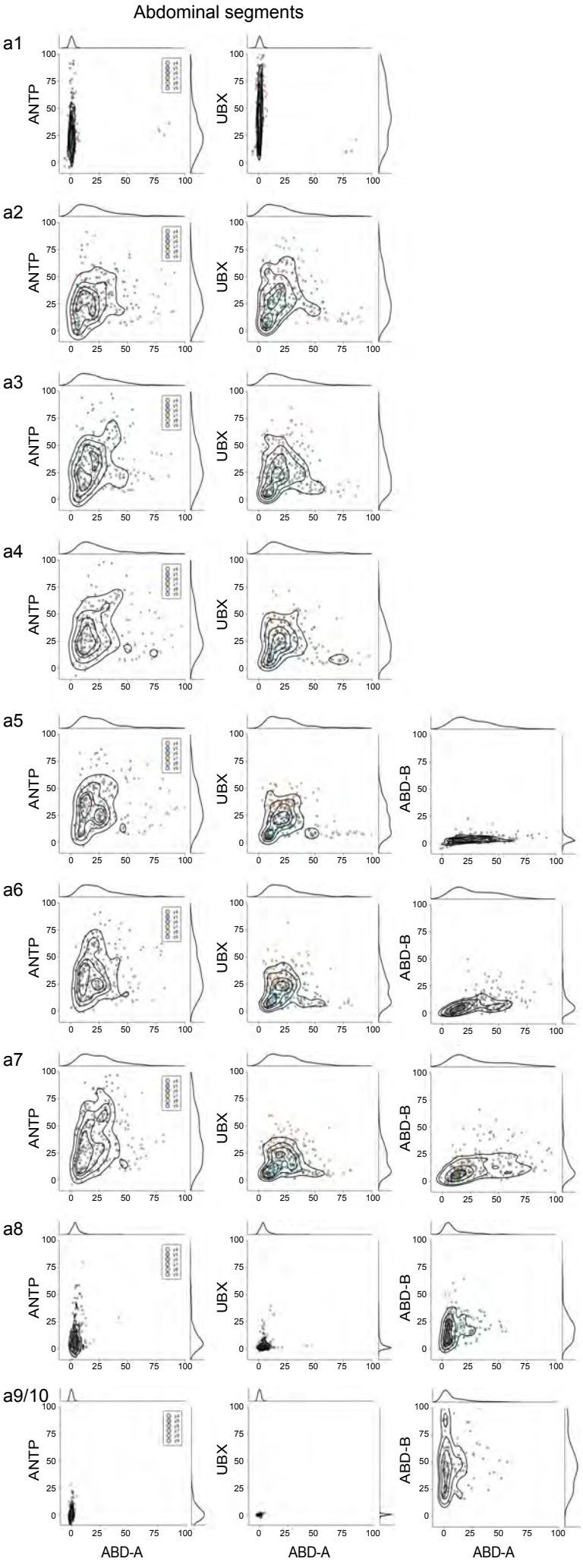
Supplementary Figure 2. Neuronal diversity is built onto a conserved main skeleton.

The dorsal and transversal view of two neurons with segmental diversity are shown. The main points of ramification of the primary neurites are highlighted with an arrowhead in neurons from two segments with clear diversity. The last panel show an overlayed image. The main skeleton of the neurons including the points of ramifications are unchanged even if the morphology is variant.

A



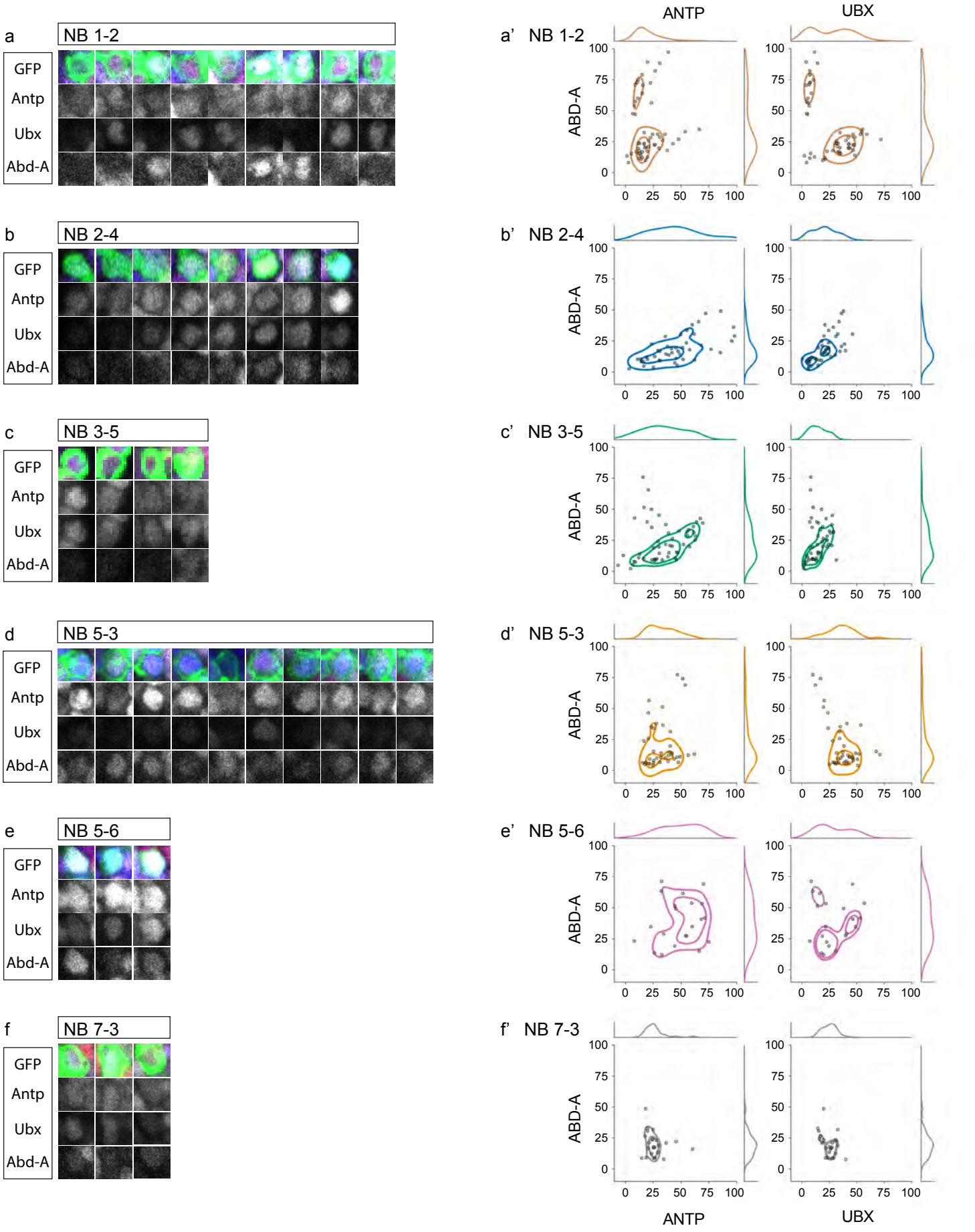
B



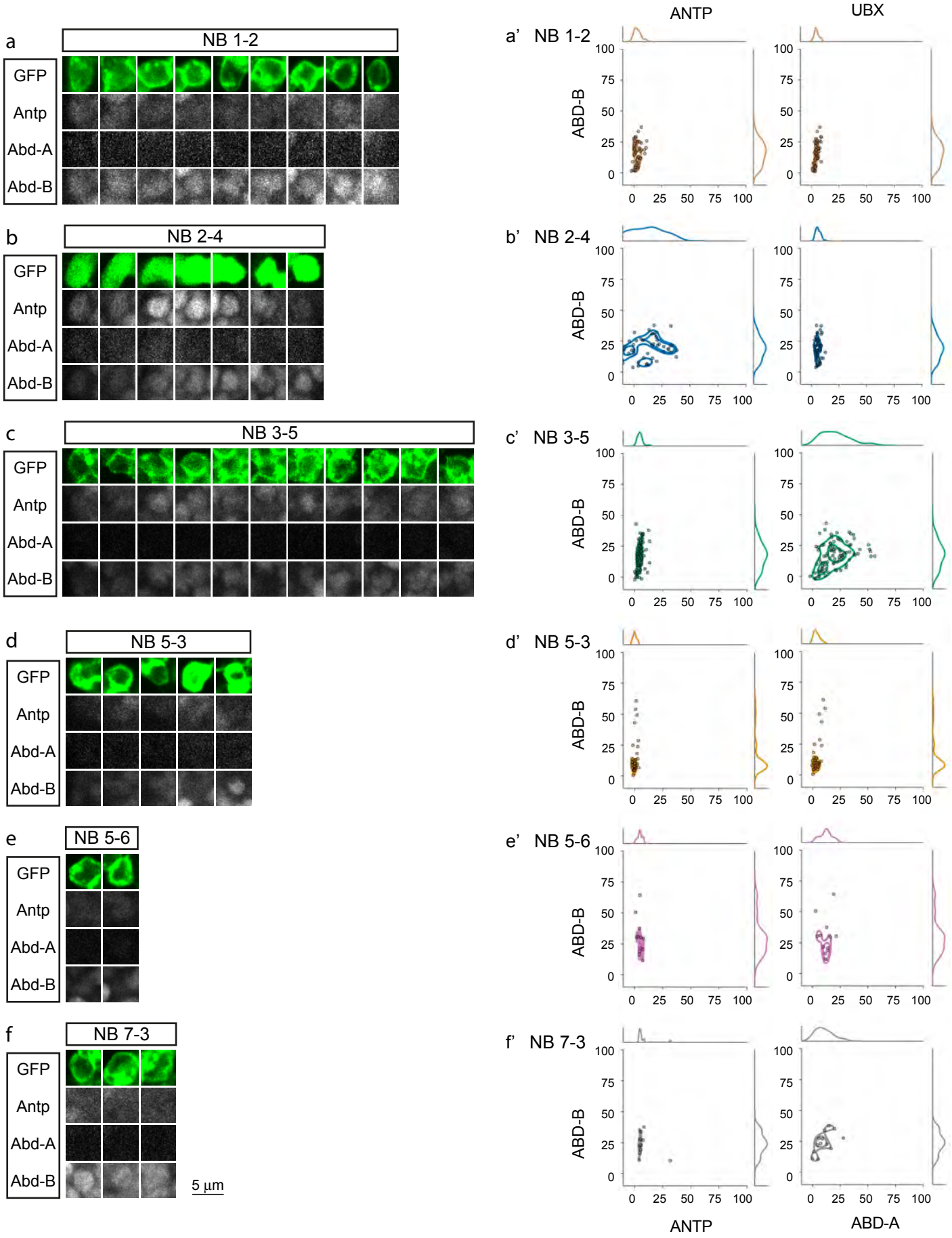
Supplementary Figure 3. Kernel Density Estimation (KDE) per segment.

A) Schematic of the NB lineages evaluated in this study. a) The neuronal progenitors called NeuroBlasts (NB) are distributed along the antero-posterior axis of the ventral nerve cord. b) They form a common ground plan of NBs that is highly invariant regardless of the segment where thye are located (apart in a9/10). Studied NBs are highlighted with a darker black line. c) Each NB gives rise to a neuronal lineage (they can also generate glial cells) where neruons are homolohogous across segments.

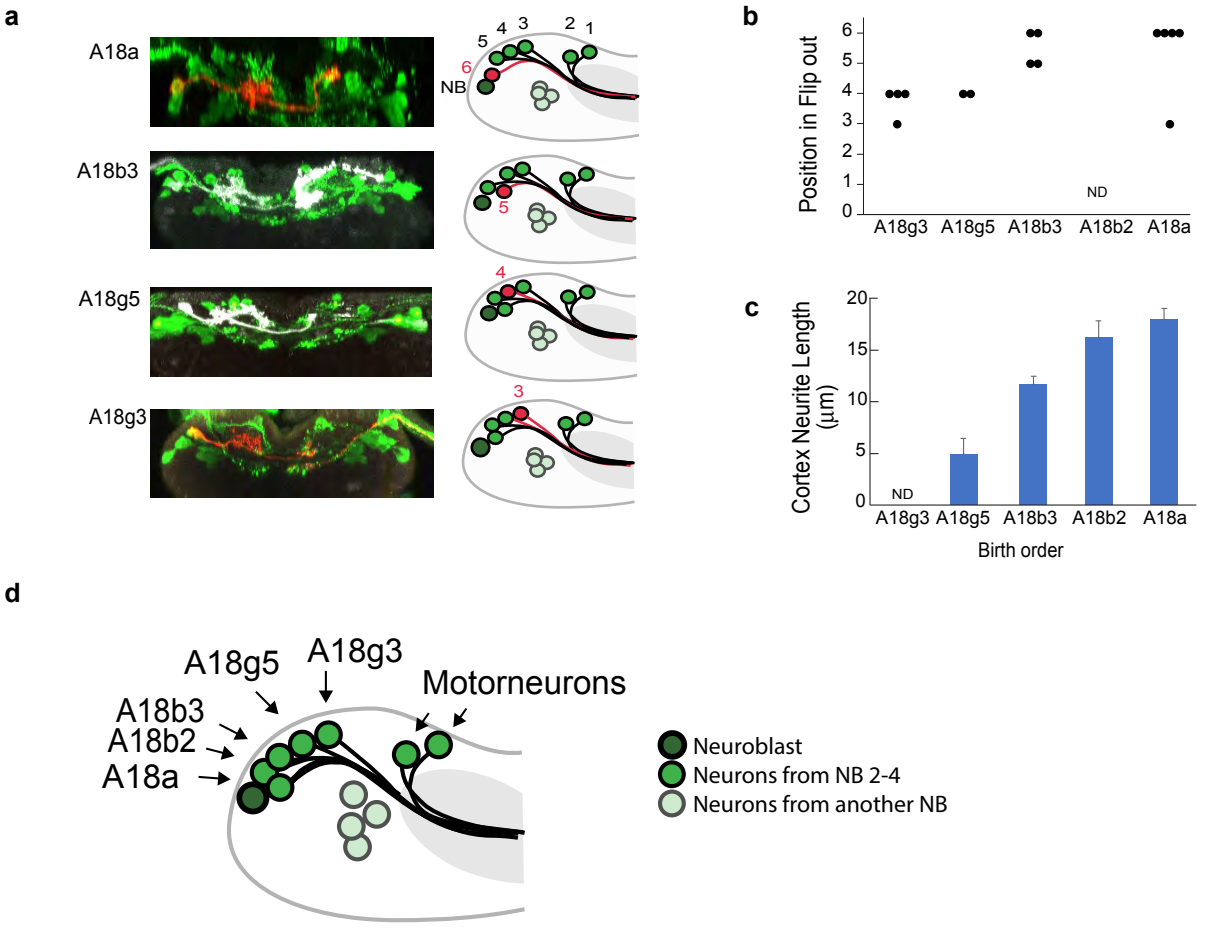
B) The normalised intensity of each Hox protein calculated as percentage from maximum levels of expression in the embryo nervous system was plotted and a KDE was performed dividing the probability mass in five evenly-spaced regions (20% density). In abdominal 1, UBX and ANTP were high and ABD-A almost 0% in all neurons. The distribution was therefore very steep in the ABD-A axis and the density peak was at around 20% in the UBX and ANTP axis. The KDE evaluating ANTP vs ABD-A and UBX vs ABD-A were very similar from a2 to a7. 80 % of the data were between 0% approximately 50%. The distribution was widening quite homogenously in all Hox protein levels directions. In the data comparing from UBX vs ABD-A the main density is often divided in 2 picks one of them with very low levels of Hox proteins. From abdominal 6 onward the levels of ABD-B start increasing but the majority of neurons had levels below 20% of ABD-B until a8. In a9/10 ABD-B levels are higher with an average of 50%.



Supplementary Figure 4. The variegation in Hox protein is present in all NB lineages studied (abdominal 4).
The hox expression and their analysis is shown for abdominal segment 4 but equivalent results were found in all segments. a-f. Single frame images of Hox expression in the neurons generated by the indicated NB. The images are presented in grey for better comparison and all images were acquired with the same confocal settings. a'-f'. Normalised quantification of Hox levels as percentage of highest expressing neurons in the same embryo. The left and right side of three individual nervous systems were analysed for each NB studied. A Kernel Density Analysis with lines representing 33% density are drawn.

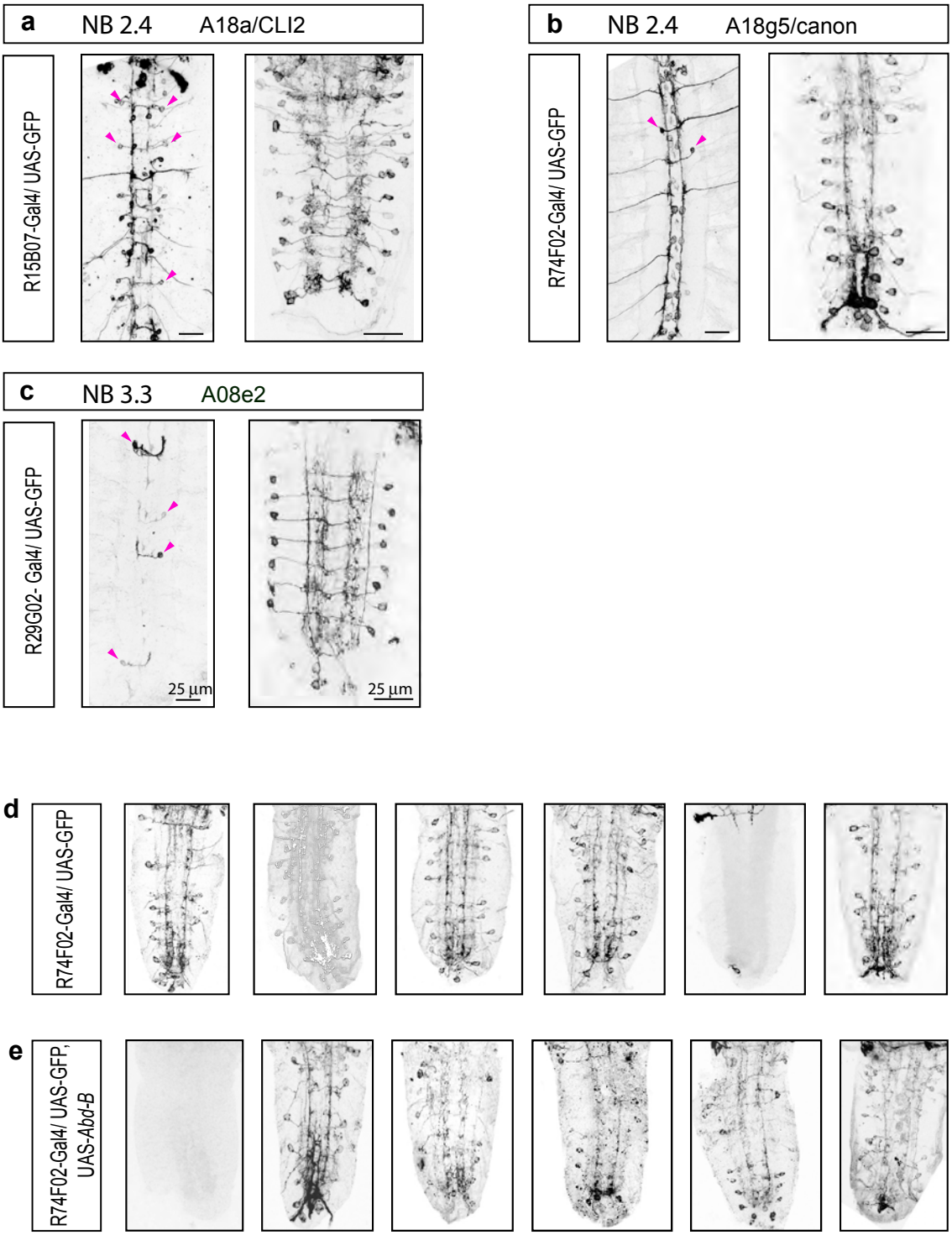


Supplementary Figure 5. The variegation in Hox protein is present in all NB lineages studied (abdominal 8). The Hox expression and their analysis is shown for abdominal segment 8. **a-f.** Single frame images of Hox expression in the neurons generated by the indicated NB. The images are presented in grey for better comparison and all images were acquired with the same confocal settings. **a'-f'.** Normalised quantification of Hox levels as percentage of highest expressing neurons in the same embryo. The left and right side of three individual nervous systems were analysed for each NB studied. A Kernel Density Analysis with lines representing 33% density is drawn.



Supplementary Figure 6. Characterization of the neuronal position of interneurons withing the NB lineage.

a. Flip out example images in first instar larvae showing the position of the different interneuron types, labelled in red or white, withing the NB lineage labelled in green.
b. Quantification of the number of Flipouts of neurons of each identity found at diffrent position in the lineage.
c. Birth order of neurons in NB 2-4 as predicted quantifying the length of the main neurite from the soma to its entry in the neuropile (virtualflybrain).
d. Final diagram showing the most commun position of the different neuronal types in NB 2-4 lineage.
ND indicates that no data are available.

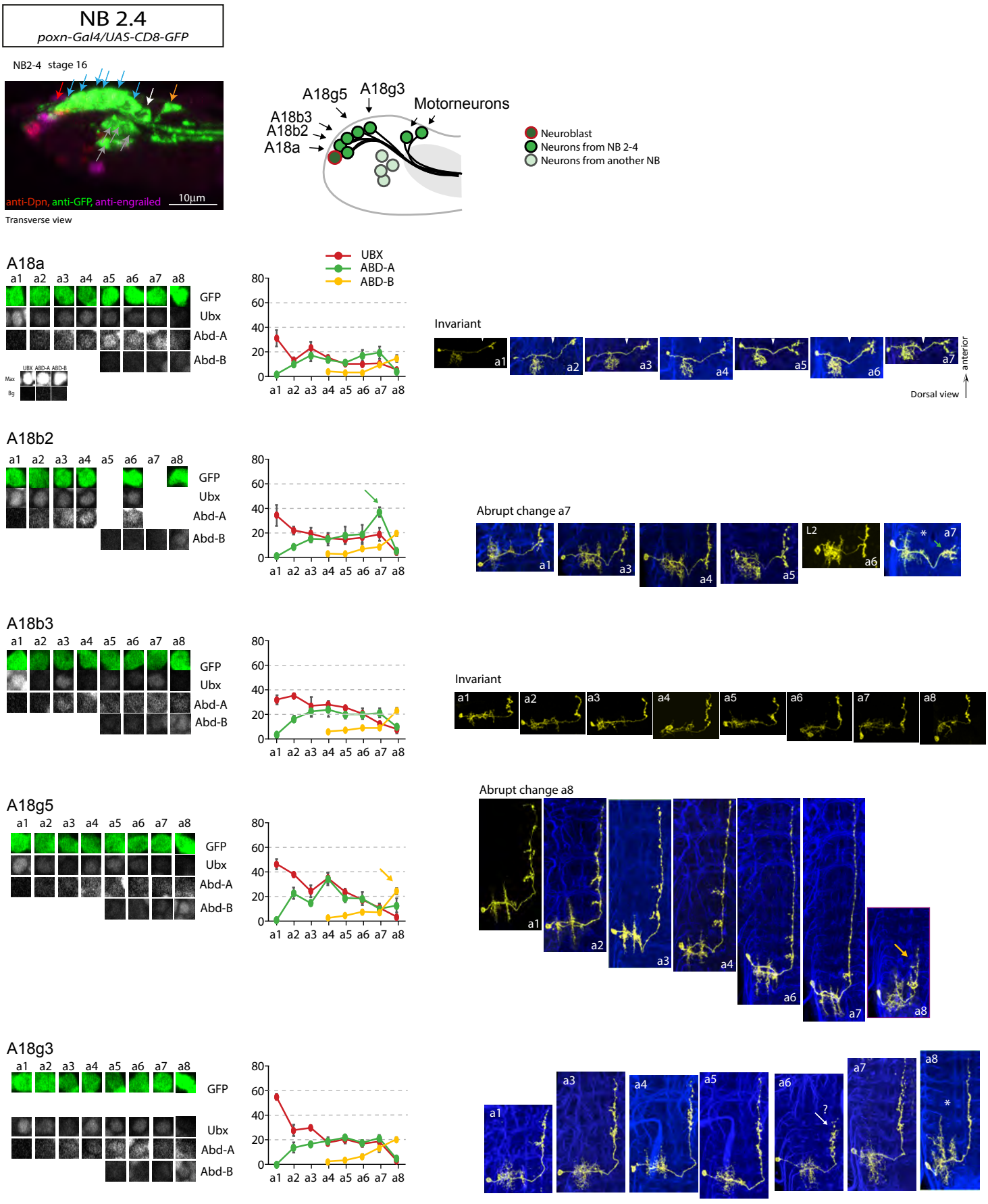


Supplementary Figure 7. Pattern of expression in embryo st16 and first instar larvae of the Gal4 lines for A18a, Canon and A08e2.

a-c. Each Gal4 line was crossed to UAS-myr-GFP and imaged at stage 16 and in newly hatched first instar larvae. At stage 16, GAL4 is not yet expressed in all segments and only a few specific neurons (labelled with magenta arrowheads) are targeted. At hatching, the patter of expression is more specific targeting the selected interneuron in all or almost all abdominal segments. A few unspecific neurons are labelled in R15B07 (A18a/C-LI2) and R74F02 (A18g5/canon). R29G02 is very specific and targets all segments.

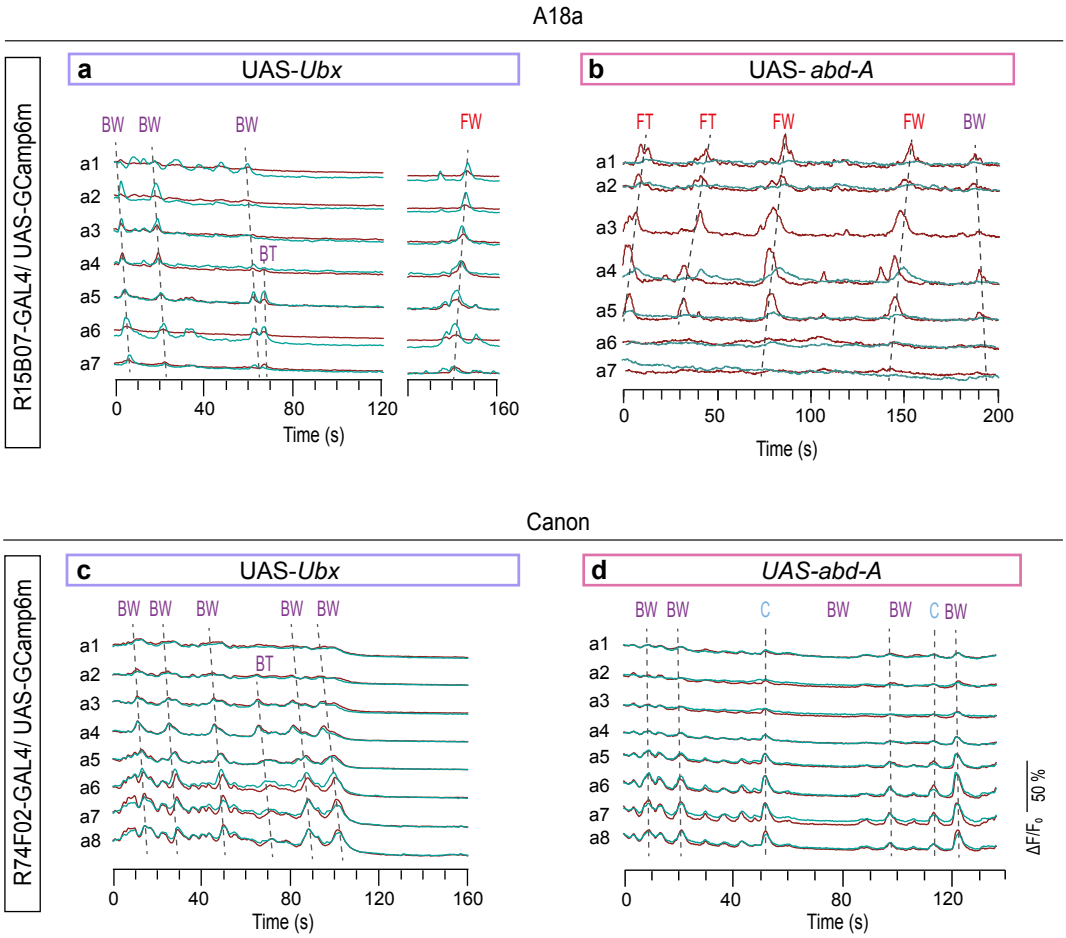
d-e. Examples of variability in the pattern of expression for the Canon driver line (R74F02) in first instar wildtype **(d)** and over-expresssing *Abd-B* **(e)** larvae**.** The proportion of brains without expression is under-represented as we rarely imaged CNS with no expression. When *Abd-B* is over-rexpressed the number of neurons is dicreased.

Note: We screened 101 Gal4 lines with positive expression in third instard larvae to only obtain these four Gal4 lines.



Supplementary Figure 8. Hox levels correlation with morphology changes in NB 2-4 lineage.

Left panel. Example of segmental Hox levels for the interneurons from NB 2-4 lineage.
Middle panel. Average Hox levels for each abdominal segment. N=6 for each data point. The arrows indicate the threshold above which changes of morphology appear.
Right panel. Segmental variants of the neurons quantified.



Supplementary Figure 9. Patterns of spontaneous activity in A18a and Canon neurons over-expressing Hox genes.

Calcium activity in spontaneous fictive locomotion across ventral nerve cord segments.
a. A18a/ UAS-*Ubx*; n=45 waves (14 CNS). **b.** A18a/ UAS-*abd-A*; n=99 waves (5 CNS). A18a activity is not affected by the over-expression of either *Ubx* or *abd-A.*
c. Canon/ UAS-*Ubx*; n=42 waves (11 CNS) . **d.** Canon/ UAS-*abd-A*; n=38 (9 CNS). Canon activity is not affected by the over-expression of either *Ubx* or *abd-A.*
All genotypes are heterocigotes.
FW = Forward Wave; FT = Forward Truncated; BW = Backward Wave; BT = Backward Truncated; C= Coincident.

presynaptic partners	number of synapses
canon_a5r	6
CM4 contralatral descending 1 right	4
pre-canon_17022828	4
MDN	3
canon_a4r	3
a08l	3
A27l?_a7r	3
pre-canon_15772978	2
CM4 ven ant 2 DNs_right	2
MDNa_right pair2	2
pre-canon_18569542	2
canon_a3r	1
DPMm2 descending right	1
UNK contralateral descending right	1
A27h_a6r	1
CM4 ven ant 1 right	1
others	21

MDN Backward command pathway
brain descending inputs
canon-canon synapses
forward crawl propagation necessary nueron
unknown function

Supplementary Figure 10. Synaptic contacts of Canon a6 right.

Neurons have been colour coded as a function of their known function.