Variability in the number of abdominal leucokinergetic neurons in adult *Drosophila melanogaster*.

Javier Alvarez-Rivero¹, Marta Moris-Sanz¹, Alicia Estacio-Gómez¹, Merce Montoliu-Nerin¹, Fernando J. Díaz-Benjumea¹ and Pilar Herrero^{1,2}.

¹Centro de Biología Molecular Severo Ochoa (CBMSO), Madrid, ²Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain

Grant Sponsor: Spanish Ministerio de Ciencia e Innovación. Grant number: BFU2014-53761 (to F.J.D-B.)

Corresponding Author: Pilar Herrero, Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, E-28049 Madrid, Spain.

E-mail: pilar.herrero@uam.es

ABSTRACT

Developmental plasticity allows individuals with the same genotype to show different phenotypes in response to environmental changes. An example of this is how neuronal diversity is protected at the expense of neuronal number under sustained undernourishment during the development of the *Drosophila* optic lobe. In the development of the *Drosophila* central nervous system, neuroblasts go through two phases of neurogenesis separated by a period of mitotic quiescence. Although during embryonic development much evidence indicates that both cell number and the cell fates generated by each neuroblast are very precisely controlled in a cell autonomous manner, after quiescence extrinsic factors control the reactivation of neuroblast proliferation in a not as known way. Moreover, there is very little information about whether environmental changes affect lineage progression during postembryonic neurogenesis. Using as a model system the pattern of abdominal leucokinergetic neurons (ABLKs), we have analysed how changes in a set of environmental factors affect the number of ABLKs generated during postembryonic neurogenesis. We describe the variability in ABLK number between individuals and between hemiganglia of the same individual and, by genetic analysis, we identify the Bithorax-Complex genes and the Ecdysone hormone as critical factors in these differences. We have also explored the possible adaptive roles involved in this process.

INDEXING TERMS: Developmental plasticity; *Drosophila*; neuropeptide; Leucokinin; RRID:AB_2567205; RRID:AB_10805300; RRID:AB_528209; RRID:AB_528215; RRID:AB_528214; RRID:AB_430877; RRID:AB_2313707; RRID:AB_2567574; RRID:AB_2567931.

INTRODUCTION

The way that neurons display their axonal processes and network output through development, via cellular remodelling, and how spatial and temporal cues are integrated to

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/cne.24093

© 2016 Wiley Periodicals, Inc.

Received: Mar 25, 2016; Revised: Jul 25, 2016; Accepted: Jul 25, 2016

produce different cell types during neurogenesis have been investigated in several organisms (Veverlytsa and Allan, 2013; Yau and So, 2014; Kohwi and Doe, 2013). Much of the developmental plasticity is set up through morphological remodelling during postembryonic development and adult neurogenesis, which is a mechanism associated with functional or learning processes (Abrous et al., 2005).

The role of neuronal plasticity in *Drosophila* has been studied on the functional identity of photoreceptors (Sprecher and Desplan, 2008), in the integration of new memories during adult neurogenesis in mushroom bodies (Zhao et al., 2008) and more recently, in the optic lobes, where the hitherto undescribed adult neurogenesis contributes to regeneration after injury (Fernández-Hernández et al., 2013). All these studies have shown that the central nervous system (CNS) is endowed with potent and adaptive capacities which enable it to respond to environmental perturbations. In the larval neuromuscular junction (NMJ) and the visual system of *Drosophila*, synaptic plasticity constitutes a mechanism of control that adapts the presynaptic and postsynaptic strength to functional needs (Frank et al., 2013). Recently, the variation in proportion between the different neuronal classes as a result of alterations in nutritional conditions, have been described in the mushroom bodies (Lin et al., 2013). The increase of γ neurons under low protein/sugar diets suggests an adaptive capacity that expands the developing olfactory system as nutrient resources become scarce (Lin et al., 2013). Along these lines, an adaptive mechanism has been found in the Optic Lobe of *Drosophila*, by which neuronal diversity is preserved at expense of neuronal number and growth under dietary restriction (Lanet et al., 2013).

More recently, Truman's group has described variability in the interindividual morphology of postembryonic neurons in some lineages of *Drosophila* VNC (Mellert et al., 2016). This variability affects the pathfinding of neurite bundles and their connectivity. The variants showed correlation with the time of flight initiation and depended on genetic and environmental conditions.

In this report we have analysed the variability in the number of a specific type of neurons and a set of factors that can control this variability. To facilitate this we used the abdominal leucokineric neurons (ABLKs) of the *Drosophila* CNS as a model.

ABLKs characterize by the expression of the neuropeptide leucokinin (LK). Neuropeptides are signalling molecules involved in many different processes in development and metabolism (reviewed in Nassel 2002). LK was initially identified as a neurohormone that increases malpighian tubule fluid secretion in most invertebrate species (Nassel 2002) but it has been studied more extensively in *Drosophila* (Terhzaz et al., 1999). LK is expressed in three groups of neurons in *Drosophila* CNS: the lateral horn leucokineric neurons (LHLKs), the subesophageal leucokineric neurons (SELKs) and the abdominal leucokineric neurons (ABLKs) (de Haro et al., 2010). Recently, we have reported that there are two sets of ABLKs defined by the developmental stage in which they are specified. The first set corresponds to neurons generated during embryonic neurogenesis, these ABLKs start expressing LK in late embryo and appear in a fixed number in the abdominal segments A1-7, one per hemisegment (the left or right half of a segment). The second set is generated during postembryonic neurogenesis by the same progenitor neuroblast (NB) (Estacio-Gómez et al., 2013). However, it

has not been till now that they have been found to appear in a variable number and start expressing LK at different times during pupal development. ABLKs not only vary their number from larva to the adult stages but the number of ABLKs in adults varies between different individuals and between the left and right hemiganglion of the same individual. Thus, the pattern of ABLKs seems to be a good model system to study the mechanisms causing neuronal plasticity during the development of the CNS. We present data that support the hypothesis that genes of the Bithorax-Complex (Bx-C) and the 20-hydroxyecdysone steroidal hormone (hereafter referred to as ecdysone) are responsible for this postembryonic numerical variation and find evidence of an adaptative triggered by environmental forces such as humidity and nutrient availability.

MATERIALS AND METHODS

Fly strains

The flies were maintained on standard *Drosophila* medium at 25°C, 60% RH under 12-12h light-dark cycle except for the cell lineage experiments with tub-Gal80^{ts}, in which the flies were grown at 18°C and the temperature increased from 18°C to 29°C in the second instar larva.

The fly stocks used were as follows:

Canton S was used as the wild type.

Act5C>stop>βgalactosidase, (After recombination between FRTs >> the Act5C promoter drives expression of βgalactosidase constitutively) (Struhl and Basler, 1993)

Ubx^{MX6}, abd-A^{M1}, Abd-B^{M1}, (Sánchez-Herrero et al., 1985), *abdAlacz* from Bloomington Drosophila Stock Center (BDSC).

Ubx^{MX6} abd-A^{M1} Abd-B^{M8} / TM3 P{w Act GFP}JMR2 Ser and *Dp(3;1)P115/FM0;Dp(3;3)P5/TM6B* (female with two extra dose of Bx-C) (both gift from Sánchez-Herrero), (Sánchez-Herrero et al., 1985; González-Reyes and Morata, 1990).

Ecr³¹, Ecr^{K06210} (lacZ expression), *Ecr¹¹², Ecr^{M554fs} Ecr^{Q50S}* from BDSC.

The following Gal4 lines were used:

Lk-Gal4 (de Haro et al., 2010); *wor-Gal4* (NB driver), *elav^{C155}-Gal4* (panneuronal driver); *nab-Gal^{NP41316}* (Terriente-Felix et al., 2007).

Tub-Gal80^{ts} (Zeidler et al., 2004), this driver is constitutively expressed and inactivates Gal4 drivers at 17°C. At 29°C Gal80^{ts} is inactivated and Gal4 drivers are active.

UAS lines used:

UAS-GFP (Green Fluorescent Protein enhanced reporter gene), *UAS-flp* (Flippase recombinase permit s recombination between two FRTs), *UAS-Ubx^{JA1}*, *UAS-abd-A²⁰⁻¹⁰⁻¹*, *UAS-Abd-B^{m25G19}*, *UAS-Ubx-RNAi* (Monier et al., 2005), *UAS-abd-A-RNAi*, *UAS-Abd-B-RNAi* (from Vienna Drosophila RNAi Center VDRC).

UAS-EcRA, *UAS-EcRB1*, *UAS-EcRB2*, *UAS-EcRB1^{DN}*, from BDSC,

UAS-NaChBac, *UAS-Kir2.1* (channels that depolarize or hyperpolarize neurons) from BDSC.

Antisera and immunocytochemistry

The CNS of larvae and adults were dissected out in PBS and were fixed for 30-60 min in ice-cold 4% paraformaldehyde in PBT (PBS containing 0.3% Triton X-100, pH7.4). After several 10 minute washes and a methanol immersion at 4°C for 12 minutes, the CNS's were blocked in 3% normal goat serum (NGS) for 1 h and incubated overnight at 4°C in primary antibody diluted in PBT (pH 7.4) (Herrero et al., 2003).

The primary antibodies used were as follows (Table 1): rat anti-Lk1:1000 (Estacio-Gómez et al., 2013), rabbit anti-LK 1:2000 (gift from Dr. Nässel) (Cantera and Nässel, 1992), mouse anti-Ubx 1:20 (from Developmental Studies Hybridoma Bank, University of Iowa, DSHB FP3.38), mouse anti-Abd-B 1:20 (from DSHB 1A2E9), mouse anti-βgalactosidase 1:2000 (Promega), rabbit anti-βgalactosidase 1:250 (ICN Biomedicals GmbH), mouse anti-EcR1:10 (DSHB DDA2.7), mouse anti-EcRA1:10 (DSHB 15G1a), mouse anti-EcRB1 1:10 (DSHB AD4.4), rabbit anti D-caspase-1 1:200 (Cell Signaling Technology Inc., Dcp-1)

Following incubation, several washes of 10 min each in PBT were performed at room temperature. Secondary antibody incubations were carried out for 1.5 hours at room temperature.

After several washes in PBT for 1 hour the CNS's were mounted in Vectashield Mounting Medium (Vector Laboratories H-1000).

The secondary antibodies, including donkey anti-mouse, -rabbit or -rat conjugated with Alexa Fluor 488, 555 or 647 (Thermo Fisher Inc) were used at a 1:500 dilution.

Production and specificity of primary antisera

To generate rat anti-LK, two rats were immunized with the peptide NSVVLGKKQRFHSWGC, corresponding to the sequence of the mature peptide. The terminal Cys residue was added to couple the peptide to keyhole limpet hemocyanin carrier protein. After five immunizations, the rats were bled and the sera were preabsorbed against 24h *Drosophila* embryos. The resulting sera were tested for Lk specificity staining of the larval CNS (Estacio-Gómez et al., 2013).

Anti-LK I, was generated to LK-1 of the cockroach *Leucophaea maderae* and coupled to BSA in rabbit (Nassel et al., 1992). The antisera were tested for specificity by ELISA, nitrocellulose models and insect tissues, such as *Drosophila*, after preabsorption with synthetic peptides (Liu et al., 2015).

Microscopy and Image analysis

CNS preparations were scanned with a confocal microscope Zeiss LSM 510 META. The images scanned and the single optical sections were analyzed using the software Zeiss LSM and edited for contrast, brightness and coloring with Adobe Photoshop CS2 version 9.0.

Quantification of fluorescence

The level of Ubx expression was evaluated from different individuals with identical laser intensities and scan settings. The fluorescence was measured by calculating mean gray values (average gray values within selected areas) using Image J software (NIH). Integrated density (area x mean gray value) was finally determined by subtracting the intensity of the tissue background and used to compare the fluorescence intensities.

Sorbitol feeding and humidity assays

Canton-S egg lays were collected (60-70 eggs per vial of 37x100mm size) and reared at 25°C, on the usual medium to which sorbitol was added at final concentrations of 0.6 M, 1.2M, 1.8M or 2.4M.

Other Canton-S egg-lays were reared at 25°C under three different humidity conditions (30%, 60% and 99% RH).

All CNS dissections were performed on eight-day old males after eclosion. The number of individuals per assay was 30 males.

Diet restriction assays

For nutrient restriction experiments, hatching larvae were transferred to 20% sucrose and 5% dry yeast in 2% aqueous agar for 48 h until second instar larvae. Then larvae were transferred into medium containing 20% sucrose only (Luo et al., 2013). After a week the larvae (they did not undergo metamorphosis during this time) were again transferred to 20% sucrose and 5% dry yeast medium till 4 days old adults and then they were dissected. Control, flies were reared on food containing 20% sucrose and 5% dry yeast.

Desiccation resistance assay

Three days old males were transferred into groups of 25 in dry vials (without food or water) at the usual conditions (25°C and 60%RH, 12:12 hours light:dark). The number of dead flies was recorded every 4-8h. The experiments were carried out in three replicates for each genotype.

Capillary feeding (CAFE) assay

The amount of food eaten was measured by a capillary feeding assay (CAFE) according to Ja et al., 2007, and modified according to Luo et al., 2013. The males were placed in 1.5ml Eppendorf tubes into which a 5 ml capillary tube containing 5% sucrose, 2% extract yeast and 0.1% propionic acid was inserted. Several control tubes were set up with food-filled capillaries inserted but without flies. The food consumed was determined as the diminished food level minus the average decrease in control capillaries (caused by evaporation). The daily consumption was measured and calculated cumulatively over 4 consecutive days. These

experiments were carried out in three replicates with 5-6 males of each genotype for each replicate.

RESULTS

In the ventral nerve cord (VNC) of the *Drosophila* larva each of the 7 first abdominal hemisegments (A1-A7) contain one ABLK neuron which was detected by LK immunostaining (Fig.1A) (Cantera and Nässel, 1992). These neurons are generated during the embryonic neurogenesis in an invariant number and position of neurons. This pattern remains unchanged along the larval stages (de Haro et al., 2010). But this condition changes in the adult, when the number of ABLKs per hemiganglion increases and become very variable among individuals. We have recorded this variability and analyzed several genetic and extrinsic factors linked to it.

Variation in ABLK number in adults.

To accurately measure the variation in the number of ABLKs, we carried out an extensive study in different ages from young to adult. We used the transgenic line *lk-Gal4*, which reproduces the pattern of expression of LK (*lk-Gal4,UAS-GFP*) (de Haro et al., 2010), combined with LK polyclonal antibodies. This enabled us to identify ABLKs regardless of neuropeptide release (Fig. 1A-B). Match between GFP and LK expression was complete in both larval and adult stages (Fig 1D-E). The number of ABLKs found in adults ranged from 8 to 14 cells per hemiganglion. This numerical variability affected all of the adult stages. Several examples are shown in the Figure 1 and Table 2. It is worth noting that within the same individual we found a very high variability in the number of ABLK between the right and left hemiganglion (Fig. 1B, 1F, Table 2), whereas others did not exhibit this (Fig 1D, 1E, Table 2). Due to the morphological changes that occur in the ganglion during metamorphosis, it was not possible to assign a segmental identity to the ABLKs observed in the adult period.

After embryonic neurogenesis, all but four NBs of the A1 and three NBs of the A2-7 abdominal segments undergo apoptosis. These NBs resume proliferation in the early third instar larva (Truman and Bate, 1988). These three NBs can be identified by their position in the ganglion as ventro-medial (vm), ventro-lateral (vl) and dorso-lateral (dl)NBs. More recently, these NBs have been correlated to NB 5.2, NB 5.3 and NB 3.5, respectively (Birkholz et al., 2015; Lacin and Truman 2016).

In a previous study we concluded that ABLKs observed in the adult were generated in two stages, i.e.: the seven ABLKs per hemiganglion detected in the early larva are generated in embryonic neurogenesis, and the additional ABLKs observed in the adult are generated in postembryonic neurogenesis (Estacio-Gómez et al. 2013). The ventral lateral NB (vINB) was identified as progenitor of the postembryonic ABLKs (Estacio-Gómez et al., 2013). In order to identify which of the adult ABLKs were generated in postembryonic neurogenesis, we labeled the lineage of postembryonic neuroblasts with a Flip-out cassette (*wor-Gal4 UAS-flp Act5C>stop> β -galactosidase tubGal80^{ts}*). This enabled us to label the neurons generated in the larval neurogenesis. In the adult ganglions, we observed 7 unlabeled ABLKs per hemiganglion. This result confirmed that the ABLKs generated during embryogenesis remain in the ganglion and maintain the expression of the LK, the remainder of the ABLKs expressed the reporter, which indicated that they were generated during postembryonic neurogenesis (Fig.2A,A').

While the number of unlabeled ABLKs was fixed, the number of those which had been labeled was very variable, both between individuals and between the left and the right hemiganglion of the same individual. These numbers ranged from 1 to 7, with an average of 4 per hemiganglion. These results confirm that the variability in the number of ABLKs in the adult is due to the differences in the number of ABLKs generated during the postembryonic neurogenesis.

The postembryonic ABLKs start to express LK in pupal stages.

To identify the stage at which postembryonic ABLKs undergo terminal differentiation and express LK, we examined the expression of LK at different pupal stages. The number of LK positive cells increased progressively from 7 in the late third instar larva up to an average of 11.3 at 12-24h after puparium formation (APF) (Fig. 3A,B,C,G). At 72h APF the average number was 10.6 cells (Fig. 3D,E,F,G). In contrast to what was observed in the larvae and the adults, the number of ABLKs detected by GFP (*Ik-Gal4 UAS-GFP*) and by immunostaining was not coincident in the pupae. At the early pupal stage (8-12h APF) several ABLKs were LK positive but lacked GFP expression (Fig. 3C,D), whereas at the late pupal stage (48-96hAPF) several ABLKs were GFP positive and LK negative (Fig 3E). At the time of hatching, all the ABLKs expressed both GFP and LK, although in different intensities (see Fig 1). A possible explanation for these results is that at the beginning of differentiation GFP expression is delayed with respect to LK expression, later GFP expression is more stable but the LK content in the cells varies due to secretory pulses of neuropeptide before neuron maturation.

We next wanted to know in which segments the postembryonic ABLKs are generated. At the early pupal stage it is still possible to identify the different abdominal segments, but there are no molecular markers that facilitate an unambiguous identification of segments in the VNC in the late pupa. We observed that most of these ABLKs were generated in the anterior-most segments of the abdomen. Thus the A1 hemineuromere showed 1 or 2 ABLKs, A2 and A3 hemineuromeres showed 3 or 4 ABLKs at 12-24h APF, which was the highest number of ABLKs found in any postembryonic hemineuromere (Fig 3C,D). The A6 hemineuromere had 1 or 2 ABLKs, whereas the A7 always had only 1 ABLK. From 24h APF onwards it was difficult to assign segments to each of the ABLKs. Noticeably, the number of ABLKs per hemineuromere was very variable among pupae of the same age and between the left and right hemineuromeres of the same segment (Fig. 3B,C,D,E). We also detected LK immunostaining in two pairs of peripheral neurons located in the abdominal nerves (Fig. 3F). These 'peripheral' neurons were also observed in the adults.

These results indicate that the variability in the number of ABLKs is due to their generation during postembryonic neurogenesis, since the 7 ABLKs per hemiganglion generated in the embryo remain unchanged.

Variation in the expression of Bx-C genes controls the number of ABLKs

Hox proteins, a conserved group of homeodomain transcription factors, function in the morphological diversification of segments along the anteroposterior body axis (Mann and Morata 2000). *Drosophila* Hox genes are grouped in two complexes: the Antennapedia-Complex, that specify the segments in the head and anterior thorax, and the Bx-C that specify

the segments of the posterior thorax and abdomen. Within their role as regulators of segment identities, the Hox genes contribute to specification, programming cell death and survival of neuroblasts in the developing *Drosophila* CNS (reviewed in Estacio-Gómez and Díaz-Benjumea, 2014; Technau et al., 2014).

Our previous results showed that the genes of the Bx-C: *Ultrabithorax (Ubx)*, *abdominal A (abd-A)* and *Abdominal-B (Abd-B)* are required to specify the embryonic and postembryonic pattern of the ABLKs (Estacio-Gómez et al. 2013). However, this analysis was mainly carried out in first instar larvae. To test the relationship of the Bx-C genes with the variability in number of ABLKs, we first looked at the expression of the Bx-C genes in the ABLKs of third instar larvae, adults and pupae. We then tested the effect of removing or misexpressing Bx-C genes on the number of adult ABLKs.

In the first instar larva all ABLKs express Ubx (Estacio-Gómez et al., 2013). In third instar larvae and early pupae Ubx expression is maintained in the ABLKs of A1-6 segments but is lost in the A7 segment. From 48h APF onwards all observed ABLKs express Ubx (Figs. 4A,B; 5A,B). The 'peripheral' LK expressing neurons reported above were also Ubx positive (results not shown).

In the first instar larvae Abd-A was expressed in all ABLKs of A2-7 segments and absent from A1 segment (Estacio-Gómez et al., 2013). From the third instar larvae onwards, this pattern was maintained in A1-6 segments but AbdA expression is lost in the A7 segment. In the adult, we observed that the posterior-most and several of the anterior-most ABLKs, did not express Abd-A (Fig. 4C,D). We assumed that these ABLKs corresponded to the A7 and A1 segments respectively, because we observed the absence of Abd-A in the A1 and A7 segments from 24h to 48h APF (Fig. 5C,D). The number of anterior ABLKs that did not express Abd-A varied both between individuals and within the same individual, ranging from between 1 and 2 per hemiganglion (Fig. 4C,D; Fig. 5C,D).

Abd-B was not expressed in any ABLK in the first instar larvae (Estacio-Gómez et al., 2013), but from the late third instar onwards the posterior-most pair of ABLKs, which we assumed corresponded to the A7 segment, expressed Abd-B (Fig., 4E,F; Fig. 5E,F). These results are summarized in Figure 6.

It is worth noting that firstly, the expression of Ubx and Abd-A was always coincident except in the A1 and A7 segments that were Ubx [+] and AbdA [-], and secondly, that the expression of Abd-B was mostly complementary to Ubx, although in both late pupae and adults we observed coexpression in A7 segment (Fig. 6).

To test whether the levels of expression of Ubx, Abd-A and Abd-B were responsible for the variation observed in the number of ABLKs, we examined the expression of LK in adults in which the level of expression of the Bx-C genes had been artificially reduced or increased. The expression of *Ubx* and *abd-A* was knocked down by misexpressing *Ubx:RNAi* and *abd-A:RNAi* (*elav-Gal4>UAS-Ubx:RNAi*, *UAS-abd-A:RNAi tub-Gal80ts*) from the first instar larva onwards. In adults, we observed a significant reduction in the number of ABLKs per hemiganglion (Fig. 7A,E). Next we knocked down the expression of *Abd-B* by misexpressing *Abd-B:RNAi* (*elav-Gal4>UAS-Abd-B:RNAi tub-Gal80ts*) and found a significant increase in the number of ABLKs

per hemiganglion in adults (Fig. 7B,E). The heterozygosis for the null allele *Abd-B[M1]* also increased the number of ABLKs (Fig. 7D, E). Conversely, those flies carrying three copies of Bx-C (*DpP(3;1)115;Dp(3;3)P5/TM6*) showed a higher number of ABLKs per hemiganglion (Fig. 7C,E). The individual misexpression of *abd-A:RNAi* and *Ubx:RNAi* did not show any phenotype. These results suggest that the level of expression of members of Bx-C determines the number of postembryonic ABLKs. Therefore an increase in the level of both Ubx and Abd-A resulted in an increase in the number of ABLKs. However, on the contrary an increase in the level of Abd-B resulted in a reduction in the number of ABLKs.

The next step was to investigate whether the level of expression of the Bx-C genes varied between individuals, and whether this variation correlates with the variation in the number of ABLKs observed. Therefore, we quantified the level of Ubx expression in the third instar larvae, pupae and adults. We found a notable variability in the level of Ubx expression in the ABLKs among individuals (Fig. 8A). Strikingly, we observed that Ubx expression varied strongly between the right and left hemiganglia of the same individual (Fig. 8B). No correlation was observed with the number of ABLKs. The anti-Abd-A antibodies available were not good enough to accurately measure its level of expression.

These results suggest that the variability in the level of expression of the Bx-C genes determines the number of the ABLKs found in both the pupae and adults.

Effects of the ecdysone hormone on the number of ABLKs

Several studies have implicated ecdysone as a central endocrine regulator that initiates genetic programs for the reorganization processes in the insect nervous system during metamorphosis (Truman et al., 1993). These processes include neuron remodeling (Schubiger et al., 2003), apoptosis (Winbush and Weeks, 2011), terminal differentiation of neurosecretory cells (Veverlytsa and Allan, 2012) and NB proliferation (Homem et al., 2014). Ecdysone is a steroidal hormone that exerts its actions via three isoforms of ecdysteroid receptor: EcRB1, EcRB2 and EcRA (Riddiford et al, 2001). The EcRB isoforms are evident during the last larval instar and the EcRA isoform at the outset of metamorphosis (Riddiford et al., 2001).

To study the possible role of ecdysone in ABLK development, we first performed coexpression assays with the EcR isoforms and LK and then quantified the numbers of ABLKs in those adults carrying mutations for the different isoforms. The *EcR^{K06210}* (EcR-lacZ) line reproduces the pattern of expression of the gene but it is not possible to distinguish between the different receptor isoforms. All ABLKs expressed *EcR-lacZ* from the third instar larva to the adult (Fig. 9A). Interestingly, when antibodies that allowed the identification of the different isoforms were used, we found that these alternated in their expression. *EcRB1* was expressed intensely in the larval ABLKs but not in adults, whereas *EcRA* was robustly present in the adult ABLKs but much less so in the larva (Fig. 9B,C). A specific antibody against the EcRB2 isoform is not available. This complementary pattern of expression has been observed in other tissues. *EcRA* is predominantly expressed in most tissues at pupariation, whereas *EcRB1* is predominant in the larval stage (Talbot et al, 1993).

The distinct temporal expression profiles of each EcR isoforms suggest that each one exhibits developmental stage specific functions. Consistent with this fact, from all allelic

mutants tested we observed that the *EcR*³¹/*EcR*^{K06210} mutant flies, which had affected the EcRB1 isoform, presented an elevated number of ABLKs in the third instar larva but a wild type number in the adults (Fig.9E,F). Moreover, *EcR*¹¹²/*EcR*^{M554fs} mutant flies, which had affected the EcRA isoform, showed a disorganized aspect in the third instar larva: lower number of ABLKs as well as defects in the axons and dendrites in the third instar larva (Fig.9D,F), and were lethal to the pupal stage. Therefore, these results suggest that these two EcR isoforms play different roles. EcRA appears to be involved in morphological remodeling and maintenance, albeit this was only observed in the larval ABLKs. By contrast, EcRB1 participates either in programmed cell death (PCD) or in the regulation of the exit of quiescence of the progenitor NB. The wild-type number of adult ABLKs observed in these *EcRB1* mutants points towards the second option as the more likely scenario, suggesting that the lack of EcRB1 in the NB anticipates the postembryonic program, which usually occurs after the exit of quiescence exit. To verify this hypothesis we checked apoptosis by *Drosophila* Caspase 1 antibody (Dcp1) in third instar larvae of EcR mutants and wild type flies. There were no differences in the number of cells which expressed this death marker (results not shown). This fact reinforces the exit of quiescence option.

To investigate further the loss of *EcRB1* function, we examined the effect of overexpressing a dominant negative version of this isoform with a panneuroblast driver (*wor-Gal4 >UAS-EcRB1^{DN}*). Consistent with the phenotype observed in the *EcRB1* mutants, we observed that the number of ABLKs increased in third instar larvae. However, ABLK number decreased in these adults (Fig.9F). Although we do not have a plausible explanation for the adult phenotypes, these results are still compatible with the proposed role of EcRB1 in quiescence.

We further determined whether overexpression of the EcRB1 and EcRA isoforms had an effect on the number of ABLKs. The overexpression of these isoforms either with a panneuroblast driver or with a panneural driver, were lethal in late larvae. These larvae showed no difference in the number of ABLKs compared to the wild-type individual. These data would rule out the role of EcRB1 in PCD.

Together, these results suggest two roles for ecdysone during the development of the ABLKs: firstly, as temporal regulator of the entry into quiescence of the ABLK-progenitor NB and secondly, as a terminal differentiation factor of the ABLKs. Although the results were not conclusive, they suggest that the variation in the number of ABLKs observed in adults could be determined by individual differences in the release of ecdysone and/or by the differential expression of the different EcR isoforms.

External factors associated with ABLK function do not always affect their number

In the above experiments, we manipulated the intrinsic factors related to the specification and terminal differentiation of ABLKs. We next wanted to test whether the detection of ABLKs by immunostaining was affected by storage and release of neuropeptide by the neurons due to its activity. LK is a diuretic hormone that modulates water homeostasis. Liu et al., (2015) demonstrated that desiccation affects the level of LK expression in the ABLKs. To test the effect of the diuretic requirements in the detection of LK, we analyzed the variation in the number of ABLKs in 8-day old adults exposed to different wet-air conditions (30 and 99%

RH) along their life), or exposed to different sorbitol concentrations in their food. A high-humidity environment would increase the diuresis. Sorbitol is a molecule that is not used as a nutrient but contributes to diuresis, so a higher concentration in the hemolymph would lead to higher water elimination; therefore, we expected that in both cases the LK requirement would be increased. Our results only showed any significant difference in the number of ABLKs, at the highest sorbitol molarity, their number decreased and the intensity of the LK immunolabeling also decreased (Fig. 10A,B). One explanation for this is that in the extreme molarity the depletion of LK within cells avoids its detection, but in the intermediate variable molarities or high-humidity environment the appearance and the number of ABLKs do not change. These results suggest that both neuropeptide production and secretion could be affected by dehydration, but this does not modify the number of ABLKs.

To induce inactivation or an excess of activation of the LK neurons *in vivo*, we constitutively expressed either a hyperpolarizing potassium channel Kir2.1 (*UAS-Kir2.1*), or a depolarizing sodium channel NaChBac (*UAS-NaChBac*) with a *LkGal4* driver from embryo to 8 days-old adults. We found that overexpression of *NaChBac*, resulted in a lower number of ABLKs (Fig. 10C,C',D); while the overexpression of Kir2.1 did not show the phenotype. Notably, in both cases we observed a decrease in the level of expression of LK, although the number of ABLKs, detected by the expression of *UAS-GFP*, was unaltered (Fig 10D). These experiments suggest that the number of ABLKs is not affected by these external factors, although either the production or the storage of neuropeptide in ABLKs was affected by the activity of the neurons.

Finally, humidity has been linked to starvation resistance in *Drosophila* (Parkash et al., 2014). We tested whether the number of ABLKs are affected by nutrient restriction. To this end we transferred second instar larvae of wild type genotypes from food with 20% sucrose and 5% protein to food containing only 20% sucrose. After a week the undernourished larvae were again transferred to food with sucrose and protein until adults. Controls were the flies fed with 20% sucrose and 5% protein. No differences in the number of ABLKs were observed among these two groups (Fig 10 E,F). Then nutrient restriction did not to alter the development of ABLKs.

Although we did not found any changes in the short term in the number of ABLKs at different wet-air conditions (Fig. 10B) or after nutrient restriction, we wanted to investigate the adaptive long term effects of external factors. Therefore, we examined three different populations of starvation-resistant flies (kindly provided by Dr Allen G Gibbs), which had been generated through experimental selection for about 100 generations (Masek et al., 2014). We compared the number of ABLKs in three starvation-resistant populations (SR populations A, B and C) with three replicated populations of fed control flies (F_{SR} populations, all derived from the same wild-type populations). Our results showed an increased number of ABLKs in the three SR populations, compared to F_{SR} three populations. The SRA and SRC populations had 12 ABLK neurons per adult and the SRB had 11 ABLKs neurons (Fig. 11A,D). Interestingly, we also observed low levels of LK immunolabeling in several of the ABLKs (Fig. 11A).

In the next stage of this investigation, we examined three long-term selected populations of desiccation resistant flies (DR populations A, B and C) which were obtained

through artificial selection through about 200 generations (Slocumb et al., 2015). (Also provided by Dr Allen G. Gibbs). We observed a significant reduction in the number of ABLKs in two of the three populations. The DRA and DRC populations, had a lower number of ABLKs per hemiganglion (9.7 and 10.1, respectively) than their hydrated control flies (F_{DR} populations), F_{DRA} and F_{DRC} , which showed, 10.8 and 11.0 ABLKs respectively (Fig 11B,C,D). These results suggest that nutrient deprivation or a prolonged desiccated environment can affect the number of ABLK neurons.

Physiological consequences of different numbers of ABLKs.

Leucokinin regulates water balance and food intake as tested by capillary feeding (CAFE) assays in several studies (Al-Anzi et al., 2010, Cognigni et al., 2011, Liu et al., 2015). These functions are affected by the LK production and release, which are reflected in the intensity of LK label in the ABLK cells (Luo et al., 2014, Liu et al. 2015). The desiccation resistance assay has shown that a decrease in the production or release of LK increases drought resistance. The CAFE experiments have also shown that a low level of LK production decreases food intake (Al-Anzi et al., 2010, Cognigni et al., 2011, Liu et al., 2015).

To investigate the physiological effect of the variation in the number of ABLKs, we monitored drought resistance and performed CAFE assays in 4 day-old adult flies with a significantly high or significantly low number of ABLKs (Fig. 12). We used flies with the genotypes *elav-Gal4>UAS-abd-A:RNAi UAS-Ubx:RNAi* (Experiment 1) or *elav-Gal4>UAS-Abd-B:RNAi* (Experiment 2) which show a significantly low (4.15 ± 0.5) and high (12.69 ± 0.2) number of ABLKs respectively (see preceding results). In Experiment 1 the flies showed a lower resistance to drought conditions. While in Experiment 2, we observed an increase in desiccation resistance (Fig. 12A). Nevertheless, these results were controversial because it would be expected that the highest resistance should have been found in those flies with low LK production, i.e. with the lowest number of ABLKs. However, the CAFE provided the expected results, the flies from Experiment 1 fed less than their control relatives, and the flies from Experiment 2 displayed an increase in feeding compared with their controls. In both cases the differences were significant after 48 h (Fig. 12B).

These findings provide evidence that the number of ABLKs affects both the diuretic and feeding functions. However, it is necessary to further investigate the unexpected results observed in the desiccation resistance.

DISCUSSION

Plasticity in development enables individuals with the same genotypes to show different phenotypes in response to a variation in either the external or internal environmental conditions during development. One of the most disruptive conditions during development is nutrient restriction. Many organisms undergo a decline in cell proliferation and growth rate leading to undersized adults. For example, *Drosophila* flies only achieve half of their normal size when nutrients are restricted. Tissues such as eyes or wings undergo a reduction in cell numbers (Brogiolo et al., 2001, Puig et al., 2003), but although the larva stops growing, the

CNS continues proliferate at its normal rate under fasting conditions, this ensures a normal central nervous system function (Cheng et al., 2011). In addition to this brain-sparing phenomenon, it has been shown that the *Drosophila* mushroom body neurons are highly plastic, because the number of neurons in each class can change in response to varying growth conditions (Lin et al., 2013). It has also been reported that optic lobes can reduce the number of neurons in poorly fed larvae while neuronal diversity is protected; as a consequence brain size is different in the individuals but it retains its normal visual function (Lanet et al., 2013).

Although it has been reported that the lineage of postembryonic NBs of the thoracic and abdominal segments could be affected by extrinsic factors (Maurange and Gould, 2005), there is little information about the precise effects of these factors on NB proliferation. In this work, we have described the variability in the number of ABLK neurons arising from the postembryonic abdominal vl NB among individuals exposed to the same conditions. This variation is evident both among individuals and between the right and left hemiganglion of the same individual. We have identified the Bx-C genes and the Ecdysone hormone as the two factors which determine this plasticity and explore the possible adaptive roles in which this variability participates.

Bx-C genes play a role in setting the number of ABLK cells.

Hox genes play important roles at various steps through segment specific divergence of serial homologous NB lineages. These roles include cell specification (Neuroectoderm, NBs, or progeny), control of PCD (NB or progeny) or control of cell proliferation (NBs) (Rogulja-Ortmann and Technau, 2008).

We have previously reported that Bx-C genes are required to specify the embryonic and postembryonic ABLKs (Estacio-Gómez et al, 2013). Our results suggested that Ubx and Abd-A are redundantly required to activate the ABLK fate in segments A2-A7 and only Ubx in segment A1; whereas Abd-B represses the fate of ABLK in the A8 segment during embryonic neurogenesis and in the A5-A8 segment during larval neurogenesis (Estacio-Gómez et al., 2013). In addition, in this work we have shown that distinct levels of expression of the Bx-C genes result in both, a variable number of postembryonic ABLKs in each segment, and a variable number of ABLKs per hemisegment. Our results show that the dynamic expression pattern of Hox genes along larval and pupal stages is critical for the specification of ABLKs, and also the different behaviour of the different segments, i.e. the A2-A4 segments where the highest number the ABLK cells are specified, ranging from 2 to 4 postembryonic ABLKs per hemisegment.

There is no data regarding the variability in the level of expression of Hox genes in a single neuron type of the same individual or in different individuals, but there are several studies that identify factors that affect the level of expression of Hox genes in the same individual. Rogulja-Ortmann et al. (2008), described that motoneurons from NB2-4 and NB 7-3 showed different levels of Ubx expression through the embryonic stages 14 to 16, in segments T3- A6. A high level of Ubx was necessary and sufficient to induce apoptosis in the posterior but not in the anterior segments. Their results indicate that the level of Ubx expression

contributes to segment diversity by regulating cell numbers in a segment-specific manner. Rogulja-Ortmann et al., also reported that the modulation of Hox RNA processing by ELAV (pan-neural RNA binding protein) regulates Hox expression and activates local programmes of neural differentiation, as described previously in which Ubx level is involved (Rogulja-Ortmann et al., 2014). Recently, Mellert et al. (2016) have found pathfinding variability in the lineage NB6-1. They think that Ubx specifies a cellular state that is developmental variable.

Alternatively, differential regulation of Ubx expression might be affected via micro RNAs (Garaulet et al., 2014). These authors have studied the effects of the microRNAs previously identified within the Bx-C. They observed substantial de-repression of Ubx in the larval Ventral Nerve Cord (VNC) of *mir-lab-4* mutants. As a consequence of Ubx de-repression, Ubx and Abd-A expanded in the most posterior segments. All of these micro RNAs regulate different levels of Ubx and Abd-A, which would affect the specification of the ABLKs.

Ecdysone induces terminal differentiation of ABLKs and in the exit of NB quiescence.

The ecdysone-induced cascade plays a critical role in the metamorphic changes. This cascade promotes postembryonic neurogenesis, morphological-remodeling, transdifferentiation and temporary-tuned differentiation (Veverlytsa and Allan, 2013). We tested two of these roles in ABLKs terminal differentiation. Firstly, the embryonic ABLKs need ecdysone to remodel their neurites before pupariation. In the *EcRA* mutant, the axon does not reach its target and the neurons are LK depleted as observed. A similar morphological errors was observed in the differentiation of the FMRF neurons in the *EcRB1* mutants, where the axonal regrowth was inhibited (Schubiger et. al, 2003). Secondly, the embryonic NBs need ecdysone to exit quiescence before postembryonic neurogenesis. Postembryonic vLNBS generate new neurons in larvae that are recruited together with the embryonic ABLKs. These postembryonic neurons undergo terminal differentiation at pupariation. In individuals in which the ecdysone pathway is disrupted, quiescence is shorter and neurogenesis and terminal differentiation of ABLKs occurs precociously. Probably, some factors inhibited by the hormone nuclear cascade are responsible for this advance. An elegant study carried out on NBs from *Drosophila* brain demonstrated that ecdysone signalling is essential for NBs to transit from proliferating to the cell-cycle exit (Homen et al., 2014). When either the *EcRDN* or *EcRRNAi* forms were expressed in NBs, these remained mitotically active and lived longer, resulting in an increased number of neuronal progeny.

Although in our study, the *EcR* mutant adults did not show any differences in the number of ABLKs, they showed a higher number of ABLKs in the third instar larva, which proves that ABLK terminal differentiation depend on ecdysone activity. Probably, the variability in ecdysone secretion between the individuals or within an individual can be responsible for the variability in the number of ABLKs observed. Among the mechanisms that could explain this variability are those implicated in the positive feedback regulation of *EcR* transcription such as miR-14, which suppresses *EcR* expression whereas ecdysone acts via *EcR* to downregulate miR-14 expression. In addition, other *EcR* coactivators/corepressors can provide mechanisms that would explain this variability (for a review of ecdysone feedback regulation see Yamanaka et al., 2013).

Cell number versus size in the ABLKs. Different strategies to dehydration and starvation resistance

Studies by Dr. Nässel's group have shown that insulin receptor-mediated signaling can selectively regulate the size of peptidergic neuroendocrine cells during development and in the adult organism (Luo et al., 2014; Liu et al., 2015). This may provide a mechanism for the level of plasticity in secretory neurons. In particular, the modified size of the ABLKs provoked by either *InR* overexpression or knockdown had an effect on their desiccation resistance and feeding behavior, when compared with the control parents, but the effects were the same i.e. diminished LK release. A similar result was obtained with the overexpression of *OrK1*, which inactivated LK secretion. These effects increased desiccation resistance and decreased food intake. Therefore, the level of LK in the hemolymph is not determined by the cell size, but by the level of its secretion. Park et al. (2014) have also demonstrated that the production and secretion of insulin in IPC neurons was independently regulated.

Recently, Cannell et al. (2016) have found that starvation increases gene expression of epithelial Leucokinin Receptor (LKR), which affects Malpighian tubules, while this LKR expression decreases when dessication increases although the difference in this last case with wild type is not significant. The other diuretic hormone DH₄₄ studied by that authors, has parallel changes in dessication and starvation conditions to LK also through DH₄₄R in Malpighian tubules, but in these case the differences in DH₄₄R expression during desiccation conditions were significant and were not in starvation. They propose that the Malpighian tubules are critical not only for water body regulation but also for detoxification when the increased lipid mobilization due to starvation stress produces waste metabolites. All of these changes are observed in the short time of the individual life and would explain the different requirements of LK secretion in an individual.

Our data reveal a "sloppy" regulation of the number of ABLKs in the postembryonic development of each individual or exactly of each side of individual. The Bx-C genes and ecdysone signaling are involved in that chancy characteristic. Other postembryonic lineages have been found very recently which present wiring variation and depend on Ubx (Mellert et al, 2016), the authors propose that "Ubx at low or moderate levels, determine an epigenetic state in which developmental gene networks are unstable leading to the variable developmental outcome". In our case the variable level of Ubx expression would be responsible of number of postembryonic ABLK neurons.

Variability in the number of ABLKs does not necessary mean a disruption in the production or secretion of the neuropeptide itself. Only when food or water is scarce for a prolonged period, compensatory mechanisms drive either a higher number of ABLKs to face food deprivation or a lower number of ABLKs to compensate for the lack of water, which would produce an increase and decrease in LK release respectively. The starvation resistance observed in our experiments could be a consequence of increased LK secretion, produced by evolutionary selected individuals with a higher numbers of ABLKs. Therefore, it would be the number and not the size of the ABLKs behind the plasticity mechanism that allows the organism to adapt to different environmental conditions.

Only those NB lineages which vary in number of cells or connectivity without disrupting basic functions, as some of the postembryonic ones, are prone to present adaptive mechanisms to extreme environmental conditions.

ACKNOWLEDGEMENTS

We are grateful to Ernesto Sánchez-Herrero for providing the Bx-C fly strains. We are indebted to Allen G. Gibbs for providing starvation and desiccation resistant flies. We would also like to thank Bloomington Drosophila Stock Center for providing fly stocks and Beatriz Fraile for her technical assistance. We would also like to express our thanks to the Confocal Microscopy Service of CBM-SO for technical image assistance. We particularly get into debt to anonymous reviewers whose suggestions were very helpful.

CONFLICT OF INTEREST STAMENT

The authors declare no conflicts of interest.

ROLE OF THE AUTHORS

All the authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: FJDB and PH. Acquisition of data: JAR, MMS, AEG, MM and PH. Analysis and interpretation of data: MMS, AEG, FJDB and PH. Drafting of manuscript: PH. Critical revision of the article: All the authors. Obtaining funding: FJDB. Study supervision: FJDB and PH.

LITERATURE CITED

Abrous DN, Koehl M, Le Moal M. 2005. Adult neurogenesis: from precursors to network and physiology. *Physiol Rev* 85:523-569.

Al-Anzi B, Armand E, Nagamei P, Olszewski M, Sapin V, Waters C, Zinn K, Wyman RJ, Benzer S. 2010. The leucokinin pathway and its neurons regulate meal size in *Drosophila*. *Curr Biol* 20:969-978.

Birkholz O, Rickert C, Nowak J, Coban IC, Technau GM. 2015. Bridging the gap between postembryonic cell lineages and identified embryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster*. *Biology Open* 4: 420-434.

Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr Biol* 11:213-221.

Cannell E, Dornan AJ, Halberg KA, Terhzaz S, Dow JAT, Davies SA. 2016. The corticotrophin-releasing factor-like diuretic hormone 44 (DH44) and kinin neuropeptides

modulate desiccation and starvation tolerance in *Drosophila melanogaster*. *Peptides* 80:96-107.

Cantera R, Nässel DR. 1992. Segmental peptidergic innervations of abdominal targets in larval and adult dipteran insects revealed with an antiserum against leucokinin I. *Cell Tissue Res* 269:459-471.

Cheng LY, Bailey AP, Leever SJ, Ragan TJ, Driscoll PC, Gould AP. 2011. Anaplastic Lymphoma kinase spares organ growth during nutrient restriction in *Drosophila*. *Cell* 146:435-447.

Cognigni P, Bailey AP, Miguel-Aliaga I. 2011. Enteric neurons and systemic signals couple nutritional and reproductive status with intestinal homeostasis. *Cell Metab* 13:92-104.

De Haro M, Al-Ramahi I, Benito-Sipos J, López-Arias B, Dorado B, Veenstra JA, Herrero P. 2010. Detailed analysis of leucokinin-expressing neurons and their candidate functions in the *Drosophila* nervous system. *Cell Tissue Res* 339:321-336.

Estacio-Gómez A, Díaz-Benjumea FJ. 2014. Roles of Hox genes in the patterning of the central nervous system of *Drosophila*. *Fly* 8:26-32.

Estacio-Gómez A, Moris-Sanz M, Schäfer AK, Perea D, Herrero P., Díaz-Benjumea F. 2013. Bithorax-complex genes sculpt the pattern of leucokineric neurons in the *Drosophila* central nervous system. *Development* 140:2139-2148.

Fernández-Hernández I, Rhiner C, Moreno E. 2013. Adult neurogenesis in *Drosophila*. *Cell Reports* 3: 1857-1865.

Frank CA, Wang X, Collins CA, Rodal AA, Yuan Q, Verstreken P, Dickman DK. 2013. New approaches for studying synaptic development, function, and plasticity using *Drosophila* as a model system. *J Neurosci* 33:17560-17568.

Garaulet DL, Castellanos MC, Bejarano , Sanfilippo P, Tyler DM, Allan DW, Sánchez-Herrero E, Lai EC. 2014. Homeotic function of *Drosophila* Bithorax-Complex miRNAs mediates fertility by restricting multiple Hox genes and TALE cofactors in the CNS. *Dev Cell* 29:1-14.

Gonzalez-Reyes A, Morata G. 1990. The developmental effect of overexpressing a Ubx product in *Drosophila* embryos is dependent on its interactions with other homeotic products. *Cell* 61:515-522.

Herrero P, Magariños M, Torroja L, Canal I. 2003. Neurosecretory identity conferred by *apterous* gene: Lateral Horn leucokinin neurons in *Drosophila*. *J Comp Neurol* 457:123-132.

Homen CCF, Steinmann V, Burkard TR, Jais A, Esterbauer H, Knoblich JA. 2014. Ecdysone and mediator change energy metabolism to terminate proliferation in *Drosophila* neural stem cells. *Cell* 158:874-888.

Ja WW, Carvalho GB, Mak EM, de la Rosa NN, Fang AY, Liong JC, Brummel T, Benzer S. 2007. Prandiology of *Drosophila* and the CAFE assay. *PNAS* 104:8253-8256.

- Kowi M, Doe CQ. 2013. Temporal fate specification and neural progenitor competence during development. *Nature Rev Neurosci* 14:823-838.
- Lacin H, Truman JW. 2016. Lineage mapping identifies molecular and architectural similarities between the larval and adult *Drosophila* central nervous system. *eLIFE* 5:e13399.
- Lanet E, Gould AP, Maurange C. 2013. Protection of neuronal diversity at the expense of neuronal numbers during nutrient restriction in the *Drosophila* visual system. *Cell Reports* 3:587-594.
- Lin S, Marin EC, Yang CP, Kao CF, Apenteng BA, Huang Y, O'Connor MB, Truman JW, Lee T. 2013. Extremes of lineage plasticity in the *Drosophila* Brain. *Curr Biol* 23:1908-1913.
- Liu Y, Luo J, Carlsson MA, Nässel DR. 2015. Serotonin and insulin-like peptides modulate leucokinin-producing neurons that affect feeding and water homeostasis in *Drosophila*. *J Comp Neurol* 523:1840-1863.
- Luo J, Liu Y, Nässel DR. 2013. Insulin/IGF-regulated size scaling of neuroendocrine cells expressing the bHLH transcription factor *Dimmed* in *Drosophila*. *PLOS Genetics* 9:e1004052.
- Mann RS, Morata G. 2000. The developmental and molecular biology of genes that subdivide the body of *Drosophila*. *Ann Rev Cell Dev Biol* 16:243-271.
- Masek P, Reynolds LA, Bollinger WL, Moody C, Mehta A, Murakami K, Yoshizawa M, Gibbs AG, Keene AC. 2014. Altered regulation of sleep and feeding contributes to starvation resistance in *Drosophila melanogaster*. *J Exp Biol* 217:3122-3132.
- Maurange C, Gould AP. 2005. Brainy but not too brainy: Starting and stopping neuroblast divisions in *Drosophila*. *Trends Neurosci* 28:30-36.
- Mellert DJ, Williamson WR, Shirangi TR, Card GM, Truman JW. 2016. Genetic and environmental control of neurodevelopmental robustness in *Drosophila*. *Plos One* 11(5):e0155957.
- Monier B, Astier M, Semeriva M, Perrin L. 2005. Steroid-dependent modification of hox function drives myocyte reprogramming in the *Drosophila* heart. *Development* 132:5283-5293.
- Nässel DR. 2002. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol* 68:1-84.
- Nässel DR, Cantera R, Karlsson A. 1992. Neurons in the cockroach nervous system reacting with antisera to the neuropeptide leucokinin I. *J Comp Neurol* 322:45-67.
- Park S, Alfa RW, Topper SM, Kim GES, Kockel L, Kim SK. 2014. A genetic strategy to measure circulating *Drosophila* insulin reveals genes regulating insulin production and secretion. *PLOS Genetics* 10:e1004555.
- Parkash R, Ranga P, Aggarwai DD. 2014. Developmental acclimation to low or high humidity conditions affect starvation and heat resistance of *Drosophila melanogaster*. *Comp Biochem Physiol A Mol Intg Physiol* 175:46-56.

- Puig O, Marr MT, Rhf ML, Tjian R. 2003. Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* 17:2006-2020.
- Riddiford LM, Cherbas P, Truman JW. 2001. Ecdysone receptors and their biological actions. *Vitamins and Hormones* 60:1-78.
- Rogulja-Ortmann A, Technau GM. 2008. Multiple roles for Hox genes in segment-specific shaping of CNS lineages. *Fly* 2: 316-319.
- Rogulja-Ortmann A, Renner S, Technau GM. 2008. Antagonistic roles for Ultrabithorax and Antennapedia in regulating segment-specific apoptosis of differentiated motoneurons in the *Drosophila* embryonic central nervous system. *Development* 135:3435-3445.
- Rogulja-Ortmann A, Picao-Osorio J, Villava C, Patraquim P, Lafuente E, Aspden J, Thomsen S, Technau GM, Alonso CR. 2014. The RNA-binding protein ELAV regulates Hox RNA processing expression and function within the *Drosophila* nervous system. *Development* 141: 2046-2056.
- Sanchez-Herrero E, Vernós I, Marco R, Morata G. 1985. Genetic organization of *Drosophila* bithorax complex. *Nature* 313:108-113.
- Schubiger M, Tomita S, Sung C, Robinow S, Truman JW. 2003. Isoform specific control of gene activity in vivo by the *Drosophila* ecdysone receptor. *Mech Dev* 120:909-918.
- Slocumb ME, Regalado JM, Yoshizawa M, Neely GG, Masek P, Gibbs AG, Keene AC. 2015. Enhanced sleep is an evolutionarily adaptative response to starvation stress in *Drosophila*. *PLOS One* 10: e0131275.
- Sprecher SG, Desplan C. 2008. Switch of *rhodopsin* expression in terminally differentiated *Drosophila* sensory neurons. *Nature* 454:533-537.
- Struhl G, Basler K. 1993. Organizing activity of wingless protein in *Drosophila*. *Cell* 72:527-540.
- Talbot WS, Swyryd EA, Hogness DS. 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73:1323-1337.
- Technau GM, Rogulja-Ortmann A, Berger C, Birkholz O, Rickert C. 2014. Composition of a neuromere and its segmental diversification under the control of Hox genes in the embryonic CNS of *Drosophila*. *J Neurogenetics* 28::171-180.
- Terhzaz S, O'Connell FC, Pollock VP, Kean L, Davies SA, Veenstra JA, Dow JA. 1999. Isolation and characterization of a leucokinin-like peptide of *Drosophila melanogaster*. *J Exp Biol* 202: 3667-3676.
- Terriente-Felix J, Magariños M, Diaz-Benjumea FJ. 2007. Nab controls the activity of the zinc-finger transcription factors Squeeze and Rotund in *Drosophila* development. *Development* 134:1845-1852.

- Truman JW, Bate M. 1988. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev Biol* 125:145-157.
- Veverytsa L, Allan DW. 2012. Temporally tuned neuronal differentiation supports the functional remodeling of a neuronal network in *Drosophila*. *PNAS* 109:E748-E756.
- Veverytsa L, Allan DW. 2013. Subtype specific neuronal remodeling during *Drosophila* metamorphosis. *Fly* 7:78-86.
- Winbush A, Weeks JC. 2011. Steroid-triggered, cell-autonomous death of a *Drosophila* motoneuron during metamorphosis. *Neural Dev* 6:1-15.
- Yamanaka N, Rewitz KF, O'Connor MB. 2013. Ecdysone control of developmental transitions: Lessons from *Drosophila* research. *Ann Rev Entomol* 2013:497-516.
- Yau SY, So KF. 2014. Adult neurogenesis and dendritic remodeling in hippocampal plasticity: Which one is more important? *Cell Transplantation* 23:471-479.
- Zeidler MP, Tan C, Bellaiche Y, Cherry S, Häder S, Gayko U, Perrimon N. 2004. Temperature-sensitive control of protein activity by conditionally splicing inteins. *Nat Biotechnol* 22:871-876.
- Zhao C, Deng W, Gage FH. 2008. Mechanisms and functional implications of adult neurogenesis. *Cell* 132:645-660.

Legends of figures

Figure 1. Distribution and number of Abdominal Leucokinin Neurons (ABLKs) in the ventral ganglia of third instar larva and adult of *Drosophila*. A: Seven ABLK neurons per hemiganglion are LK-immunodetected in all larvae. B: Eleven ABLKs per hemiganglion are visualized with LK antibody in the adult nerve cord. C: Scheme showing the distribution of ABLKs in the abdominal ganglion of CNS. D, E: Ventral ganglia of four day old adults with different ABLK number between individuals and among hemiganglia. Colocalization of anti-LK (magenta) and *lk-Gal4>UAS-GFP* (green) is visualized in all ABLKs. F: Ventral ganglion of the adult with the maximum number of ABLKs found in wild type.

Figure 2. Postembryonic origin of adult ABLKs. A: Result of expressing β -galactosidase in adults with *wor-G4>UAS flpAct5C>stop> β -galactosidase tubGal-80ts*. The temperature was changed from 17°C to 29°C at second instar larva. Expression of LK (red) and β -galactosidase (green) are only coincident in the ABLKs originated during larval neurogenesis (boxes). A': Same picture as A, but visualized by LK immunoreaction. White arrowheads indicate the ABLKs of boxes in A. B: Result of expression in third instar larva of β -galactosidase from the same experiment in A. Note embryonic ABLKs do not express β -galactosidase (detail in box) and are clustered next to six β -galactosidase postembryonic neurons. C: Characterization of LK lineage in third instar larva. Neuroblasts are visualized with a specific neuroblast marker anti-Deadpan

(in magenta), neurons with a specific neuron marker anti-Elav (in green). Embryonic ABLK (anti-LK in blue) belongs to one of the Abdominal larval NB lineage. D: NB and neurons close to ABLK (magenta) in third instar larva express the nervous system marker Nab as *nab-Gal4>GFP* shows (green). Scale bar=20µm in A.

Figure 3. Dynamics of Leucokinin expression from third instar larva to adult. All images show anti-Lk (blue) and GFP expression from *Lk-Gal4>GFP* (green). A: ABLKs from wandering larva. B: ABLKs from 8h after puparium formation (APF) different number have arisen per hemineuromere. Note some ABLKs from anterior segments are not GFP (small arrows). C: ABLKs from 12 h APF. As in previous image new ABLKs are arising but a different number visualized in the two hemineuromeres of each neuromere. D: ABLKs from 24h APF, this stage presents the highest number of ABLKs during metamorphosis. E: ABLKs from 48 h APF. As shown (arrowheads), some ABLKs are GFP-labeled but not LK-immunodetected. F: ABLKs from 96 APF. This stage shows the same distribution of ABLKs as the eclosed adults, included the peripheral neurons located in nerves (arrows). G: Histogram showing the number of ABLKs per hemiganglion found in the different developmental phases.

Figure 4: Bithorax-Complex(Bx-C) gene expression in the ABLKs from third instar larva and adult. All images show Bx-C genes (magenta) and GFP (green) from *LK-Gal4>GFP*. A,C and E are images of the abdominal ganglion of larva and B,D and F are images of an adult. In every image the insets show details of Bx-C expression in the ABLKs marked with arrows. A: All ABLKs except the most posterior segment (A7) show Ubx expression. B: In adults all ABLKs express Ubx including the most posterior per hemiganglion. C: AbdA is not expressed in ABLKs of A1 and A7. D: Absence of AbdA is extended to more than one anterior and one posterior ABLK in adult. E: AbdB is only expressed in ABLK of A7 segment. F: In adults AbdB also is expressed only in the last ABLK. Scale bar=20µm in A, B.

Figure 5: Bithorax-Complex (Bx-C) gene expression in the ABLKs from 24 and 48 h after puparium formation (APF). All images show Bx-C gene (magenta) and GFP (green) from *Lk-Gal4>GFP*. A,C and E are images of the abdominal ganglion of a 24h APF and B,D and F are images of a 48h APF. In every image the insets show details of Bx-C expression in the ABLKs marked with arrows. A: All the ABLKs except the most posterior segment (A7) show Ubx expression. B: In a 48h APF all ABLKs express Ubx including the most posterior per hemiganglion. C: AbdA is not expressed in ABLKs of A1, and A7. D: Absence of AbdA is extended to more than one anterior and more than one posterior ABLK in a 48h APF. E: AbdB is only expressed in ABLK of the A7 segment. F: In a 48h APF AbdB is also only expressed in the last ABLK.

Figure 6: Diagram depicting the changes in Bx-C gene expression within ABLKs throughout development. Ubx expression disappears in the correspondent ABLK of A7 from L3 until early pupa. AbdA expression in L1 is present in all ABLKs except for the A1. At L3 AbdA also becomes absent in ABLKs of A7. Afterwards, this pattern of expression is maintained until adulthood, i.e. the ABLKs of A1 and A7 segments are AbdA-negatives. AbdB expression is absent in all ABLKs of L1, after that it is constantly expressed exclusively in the correspondent ABLK of segment A7.

Figure 7: Effect of Bx-C gene expression alterations in adult ABLK number. A: *elav-Gal4>UAS-abdA:RNAi,UAS-Ubx:RNAi, tubGal80^{ts}* reduces the ABLK number. B: *elav-Gal4>UAS-AbdB:RNAi, tub-Gal80^{ts}* increases the number of ABLKs. C: Three Bx-C copies increase the number of ABLKs. D: Mutant *AbdB* in heterozygosis increases the number of ABLKs. E: Histogram showing the number of ABLKs per hemiganglion found in the different genotypes (the number of individuals in parenthesis), (* $p<0.05$, ** $p<0.005$, *** $p<0.001$ Student's T-test). Scale bar=20 μ m in A.

Figure 8: Quantification of Ubx immunofluorescence in ABLKs of different wild type individuals. A: Four different adults show different amounts of Ubx between the ABLK of equivalent position and between the right-left side. B: Four different larvae show different amounts of Ubx between ABLKs of equivalent segments and between the right-left side.

Figure 9: Expression of Ecdysone Receptor (EcR) in ABLKs from larva and adults and effects on the ABLKs of mutants of EcR. A: *EcRlacZ* transgenic construction shows that all ABLK (magenta) in third instar larva express EcR (green). The inset shows details of the lacZ expression in ABLKs marked with red ellipsoids. B: Adults ventral ganglia have no expression of the EcRB1 isoform because anti-EcRB1 (magenta) cannot be detected clearly. ABLKs have no EcRB1 expression. C: Anti-EcRA isoform (magenta) can be detected in adult ventral ganglia and colocalization to ABLKs is generalized. The insets show EcRA expression details of ABLKs marked with green ellipsoids. D: Failed ABLK number (arrows) and axon pathfindings (arrowheads) on larva CNS can be observed in mutants of the EcRA isoform (*EcR¹¹²*). E: A higher number of ABLKs is observed in larva mutants of EcRB1 isoform (*EcR³¹*). F: The wild type phenotype is observed in the number of ABLKs of adult transheterozygotes of EcRB1 mutants (*EcR³¹/EcR^{k0612}*). G: Histograms showing the effects of EcR mutations in ABLK number of larvae and adults, and the effect of Insuline Receptor mutations in ABLKs of adults. (The number of individuals studied in parenthesis), (* $p<0.05$, ** $p<0.001$, *** $p<0.0001$ Student's T-test). Scale bar=20 μ m in B.

Figure 10: Effect of short-term changes in extrinsic factors on the number of ABLKs in adult flies. A: Very High sorbitol concentration (2.4M) in the food produced a lower number of ABLKs and depletion of LK. B: No significant differences are observed when the ambient humidity is changed or osmotic stress is provoked by sorbitol, only the highest sorbitol concentration significantly reduce the number of ABLKs ($p<0.05\%$ Student's T-test). C,C': Overexpression of depolarizing sodium channel (NaCh) does not reduce the number of GFP expressed number of ABLKs, but reduced the number of LK immunodetected. The arrowheads show the GFP positive ABLKs are not LK positives. D: The depolarizing effect of ABLKs is the only factor which changed significantly the number of ABLKs LK immunolabeled ($p<0.001$ Student's T-test). Scale bar=20 μ m in A. E,F: Nutrition restriction during larvae stage do not affect the ABLKs number as can be observed when compared *yw* flies reared on standard food (E) and on restricted nutritive medium (F). G: No significant differences are observed when the nutrition restriction are applied. ($p<0.001\%$ Student's T-test)

Figure 11: Effects of long-term changes in extrinsic factors on the number of ABLKs in adult flies. A: Starvation resistant selected flies show a higher number of ABLKs than not selected flies. B: The wild type phenotype is observed in flies not selected to desiccation

resistance. C: Desiccation resistant selected flies show a lesser number of ABLKs than not selected flies. D: Histograms showing significant differences in the ABLK number in flies selected during generations to starvation resistance or desiccation resistance, except the DRB population (* $p < 0.01$, $p < 0.001$ Student's T-test). Scale bar=20 μ m in C.

Figure 12: Functional effects of manipulations of ABLK number in adult flies. A: Genotypes with a lower number of ABLKs show weaker resistance to dry starvation than their relatives. *elav-G4>UAS-abdA:RNAi,UAS-Ubx:RNAi tub-Gal80ts* compared with *elavG4;+/CyO;+/TM6*, and *elavG4>+/TM6* compared with *elav-G4>UAS-AbdB:RNAi tub-Gal80ts*. B: Flies of genotypes with a higher number of ABLKs show a significant increase in food intake as monitored by capillary feeding (CAFE) compared with their relatives. *elav-G4>UASAbdB:RNAi tub-Gal80ts* compared with *elavG4>+/TM6*, and *elav-G4;+/CyO;+/TM6* compared with *elav-G4>UAS-abdA:RNAi,UAS-Ubx:RNAi tub-Gal80ts*. (** $p < 0.001$, * $p < 0.01$, $n=10$ flies for each genotype with three replicates, two-way ANOVA).

TABLE 1

Antibodies Used for Immunocytochemistry

Antibody	Immunogen	Source/RRID	Dilution	Specificity
Mouse anti-ABD-B	Fusion protein from <i>Drosophila</i> resultant of all the Abd-B transcripts.	DHSB, Iowa, USA, Cat#1A2.E9 RRID:AB_2567205	1:20	Immunohistochemical analysis of <i>Drosophila</i> embryos
Mouse anti-UBX	Ultrabithorax 5'exon from <i>Drosophila</i> protein (107 aminoacids)	DHSB, Iowa, USA , Cat#FP3.38 RRID:AB_10805300	1:20	Tested in S2 cells of <i>Drosophila</i>
Mouse anti-EcR	Ecdysone Receptor fragment protein (aa 335-393) from <i>Drosophila</i>	DHSB, Iowa, USA Cat#DDA2.7 RRID:AB_528209	1:10	Tested in S2 cells of <i>Drosophila</i>
Mouse anti-EcR1	Ecdysone Receptor fragment protein (aa 68-222) from <i>Drosophila</i>	DSHB, Iowa, USA Cat# AD4.4 , RRID:AB_528215	1:10	Tested in S2 cells of <i>Drosophila</i>
Mouse anti-EcRA	Ecdysone Receptor fragment protein (aa 15-152) from <i>Drosophila</i>	DSHB, Iowa, USA Cat# 15G1a, RRID:AB_528214	1:10	Tested in S2 cells of <i>Drosophila</i>
Mouse anti- β -galactosidase	β -galactosidase purified	Promega, Madison USA Cat# Z3781 RRID: AB_430877	1:300	<i>E. coli</i> β -gal near the C-terminal end
Rabbit anti- β -galactosidase	<i>E. coli</i> β -galactosidase	ICN Pharmaceuticals, Costa Mesa, CA, USA Cat# 55976 RRID:AB_2313707	1:2500	<i>E. coli</i> β -galactosidase
Rat anti-Leucokinin	Lk <i>Drosophila</i> peptide coupled to keyhole limpet hemocyanin (KLH)	Lab generated (Estacio-Gómez) RRID:AB_2567574	1:1000	Immunohistochemical analysis of <i>Drosophila</i> CNS. (Estacio-Gómez et al., 2013)
Rabbit anti-Leucokinin	Lk I-peptide conjugated to Bovine Serum albumin (BSA)	Lab generated (Nässel)	1:2000	Preabsorption in ELISA, dot-blot (Nässel et al., 1992)
Rabbit anti-Dcp1	22kDa amino terminal fragment of cleaved Dcp-1 <i>Drosophila</i> protein (Death-caspase 1)	Cell Signaling Technology Cat#9578 RRID:AB_2567931	1:200	Tested in S2 cells of <i>Drosophila</i>

TABLE 2

Individual number of ABLKs per left and right hemiganglion of Ventral Nerve Cord in different developmental stages.

3rd instar larvae		0-4h APF		4-8h APF		8-12h APF		12-24h APF		48h APF		72hAPF		0 day old		4days old		30 days old	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
7	7	6	7	9	9	10	11	11	12	12	12	13	10	11	11	10	13	11	8
7	7	8	6	8	7	9	9	9	8	11	12	12	11	11	11	12	10	11	12
7	7	7	7	10	9	14	14	11	10	11	13	12	11	11	10	12	11	8	11
7	7	6	7	8	8	14	12	11	11	10	11	12	12	11	12	12	10	8	10
7	7	7	7	7	7	14	14	9	10	8	13	7	10	11	10	11	11	11	11
7	7	7	7	11	9	13	12	10	12	11	12	10	11	11	11	11	11	13	12
6	7	7	7	12	7	11	14	9	12	10	13	9	9	11	12	11	11	10	11
7	6					9	11	16	11			10	10	12	12	11	7	11	12
7	7					9	11	11	12			11	10	11	11	9	11	11	10
7	7					10	11	12	11			13	12	11	11	10	11	10	10
7	7					8	10	14	13			11	12	10	11	11	12	10	9
6	7					11	10	12	11									11	12
7	7					10	11	12	14									10	10
7	6					11	11												
7	7

Figure 1. Distribution and number of Abdominal Leucokinin Neurons (ABLKs) in the ventral ganglia of third instar larva and adult of *Drosophila*. A: Seven ABLK neurons per hemiganglion are LK-immunodetected in all larvae. B: Eleven ABLKs per hemiganglion are visualized with LK antibody in the adult nerve cord. C: Scheme showing the distribution of ABLKs in the abdominal ganglion of CNS. D, E: Ventral ganglia of four day old adults with different ABLK number between individuals and among hemiganglia. Colocalization of anti-LK (magenta) and *Ik-Gal4>UAS-GFP* (green) is visualized in all ABLKs. F: Ventral ganglion of the adult with the maximum number of ABLKs found in wild type

199x299mm (300 x 300 DPI)

Figure 2. Postembryonic origin of adult ABLKs. A: Result of expressing β -galactosidase in adults with $wor-G4>UAS\ flpAct5C>stop>\beta$ -galactosidase $tubGal-80ts$. The temperature was changed from 17°C to 29°C at second instar larva. Expression of LK (red) and β -galactosidase (green) are only coincident in the ABLKs originated during larval neurogenesis (boxes). A': Same picture as A, but visualized by LK immunoreaction. White arrowheads indicate the ABLKs of boxes in A. B: Result of expression in third instar larva of β -galactosidase from the same experiment in A. Note embryonic ABLKs do not express β -galactosidase (detail in box) and are clustered next to six β -galactosidase postembryonic neurons. C: Characterization of LK lineage in third instar larva. Neuroblasts are visualized with a specific neuroblast marker anti-Deadpan (in magenta), neurons with a specific neuron marker anti-Elav (in green). Embryonic ABLK (anti-LK in blue) belongs to one of the Abdominal larval NB lineage. D: NB and neurons close to ABLK (magenta) in third instar larva express the nervous system marker Nab as $nab-Gal4>GFP$ shows (green). Scale bar=20 μ m in A.

199x300mm (300 x 300 DPI)

Figure 3. Dynamics of Leucokinin expression from third instar larva to adult. All images show anti-Lk (blue) and GFP expression from Lk-Gal4>GFP (green). A: ABLKs from wandering larva. B: ABLKs from 8h after puparium formation (APF) different number have arisen per hemineuromere. Note some ABLKs from anterior segments are not GFP (small arrows). C: ABLKs from 12 h APF. As in previous image new ABLKs are arising but a different number visualized in the two hemineuromeres of each neuromere. D: ABLKs from 24h APF, this stage presents the highest number of ABLKs during metamorphosis. E: ABLKs from 48 h APF. As shown (arrowheads), some ABLKs are GFP-labeled but not LK-immunodetected. F: ABLKs from 96 APF. This stage shows the same distribution of ABLKs as the eclosed adults, included the peripheral neurons located in nerves (arrows). G: Histogram showing the number of ABLKs per hemiganglion found in the different developmental phases.

199x289mm (300 x 300 DPI)

Figure 4: Bithorax-Complex(Bx-C) gene expression in the ABLKs from third instar larva and adult. All images show Bx-C genes (magenta) and GFP (green) from LK-Gal4>GFP. A,C and E are images of the abdominal ganglion of larva and B,D and F are images of an adult. In every image the insets show details of Bx-C expression in the ABLKs marked with arrows. A: All ABLKs except the most posterior segment (A7) show Ubx expression. B: In adults all ABLKs express Ubx including the most posterior per hemiganglion. C: AbdA is not expressed in ABLKs of A1 and A7. D: Absence of AbdA is extended to more than one anterior and one posterior ABLK in adult. E: AbdB is only expressed in ABLK of A7 segment. F: In adults AbdB also is expressed only in the last ABLK. Scale bar=20μm in A, B.

199x289mm (300 x 300 DPI)

Figure 5: Bithorax-Complex (Bx-C) gene expression in the ABLKs from 24 and 48 h after puparium formation (APF). All images show Bx-C gene (magenta) and GFP (green) from *Ik-Gal4>GFP*. A, C and E are images of the abdominal ganglion of a 24h APF and B, D and F are images of a 48h APF. In every image the insets show details of Bx-C expression in the ABLKs marked with arrows. A: All the ABLKs except the most posterior segment (A7) show Ubx expression. B: In a 48h APF all ABLKs express Ubx including the most posterior per hemiganglion. C: AbdA is not expressed in ABLKs of A1, and A7. D: Absence of AbdA is extended to more than one anterior and more than one posterior ABLK in a 48h APF. E: AbdB is only expressed in ABLK of the A7 segment. F: In a 48h APF AbdB is also only expressed in the last ABLK.

201x289mm (300 x 300 DPI)

Figure 6: Diagram depicting the changes in Bx-C gene expression within ABLKs throughout development. Ubx expression disappears in the correspondent ABLK of A7 from L3 until early pupa. AbdA expression in L1 is present in all ABLKs except for the A1. At L3 AbdA also becomes absent in ABLKs of A7. Afterwards, this pattern of expression is maintained until adulthood, i.e. the ABLKs of A1 and A7 segments are AbdA-negatives. AbdB expression is absent in all ABLKs of L1, after that it is constantly expressed exclusively in the correspondent ABLK of segment A7.

199x289mm (300 x 300 DPI)

Figure 7: Effect of Bx-C gene expression alterations in adult ABLK number. A: *elav-Gal4>UAS-AbdA:RNAi,UAS-Ubx:RNAi, tubGal80ts* reduces the ABLK number. B: *elav-Gal4>UAS- AbdB:RNAi, tub-Gal80ts* increases the number of ABLKs. C: Three Bx-C copies increase the number of ABLKs. D: Mutant AbdB in heterocygosis increases the number of ABLKs. E: Histogram showing the number of ABLKs per hemiganglion found in the different genotypes (the number of individuals in parenthesis), (* $p<0.05$, ** $p<0.005$, *** $p<0.001$ Student's T-test). Scale bar=20μm in A.

199x289mm (300 x 300 DPI)

Figure 8: Quantification of Ubx immunofluorescence in ABLKs of different wild type individuals. A: Four different adults show different amounts of Ubx between the ABLK of equivalent position and between the right-left side. B: Four different larvae show different amounts of Ubx between ABLKs of equivalent segments and between the right-left side.

199x289mm (300 x 300 DPI)

Figure 9: Expression of Ecdysone Receptor (EcR) in ABLKs from larva and adults and effects on the ABLKs of mutants of EcR. A: EcRlacZ transgenic construction shows that all ABLK (magenta) in third instar larva express EcR (green). The inset shows details of the lacZ expression in ABLKs marked with red ellipsoids. B: Adults ventral ganglia have no expression of the EcRB1 isoform because anti-EcRB1 (magenta) cannot be detected clearly. ABLKs have no EcRB1 expression. C: Anti-EcRA isoform (magenta) can be detected in adult ventral ganglia and colocalization to ABLKs is generalized. The insets show Ecra expression details of ABLKs marked with green ellipsoids. D: Failed ABLK number (arrows) and axon pathfindings (arrowheads) on larva CNS can be observed in mutants of the EcRA isoform (EcR112). E: A higher number of ABLKs is observed in larva mutants of EcRB1 isoform (EcR31). F: The wild type phenotype is observed in the number of ABLKs of adult transheterozygotes of EcRB1 mutants (EcR31/EcRk0612). G: Histograms showing the effects of EcR mutations in ABLK number of larvae and adults, and the effect of Insuline Receptor mutations in ABLKs of adults. (The number of individuals studied in parenthesis), (* p < 0.05, ** p < 0.001, *** p < 0.0001 Student's T-test). Scale bar = 20 μ m in B.

Figure 10: Effect of short-term changes in extrinsic factors on the number of ABLKs in adult flies. A: Very High sorbitol concentration (2.4M) in the food produced a lower number of ABLKs and depletion of LK. B: No significant differences are observed when the ambient humidity is changed or osmotic stress is provoked by sorbitol, only the highest sorbitol concentration significantly reduce the number of ABLKs ($p < 0.05$ Student's T-test). C, C': Overexpression of depolarizing sodium channel (NaCh) does not reduce the number of GFP expressed number of ABLKs, but reduced the number of LK immunodetected. The arrowheads show the GFP positive ABLKs are not LK positives. D: The depolarizing effect of ABLKs is the only factor which changed significantly the number of ABLKs LK immunolabeled ($p < 0.001$ Student's T-test). Scale bar = $20\mu\text{m}$ in A. E, F: Nutrition restriction during larvae stage do not affect the ABLKs number as can be observed when compared yw flies reared on standard food (E) and on restricted nutritive medium (F). G: No significant differences are observed when the nutrition restriction are applied. ($p < 0.001$ Student's T-test)

199x289mm (300 x 300 DPI)

Figure 11: Effects of long-term changes in extrinsic factors on the number of ABLKs in adult flies. A: Starvation resistant selected flies show a higher number of ABLKs than not selected flies. B: The wild type phenotype is observed in flies not selected to desiccation resistance. C: Desiccation resistant selected flies show a lesser number of ABLKs than not selected flies. D: Histograms showing significant differences in the ABLK number in flies selected during generations to starvation resistance or desiccation resistance, except the DRB population (* $p < 0.01$, $p < 0.001$ Student's T-test). Scale bar = $20\mu\text{m}$ in C.

199x289mm (300 x 300 DPI)

Figure 12: Functional effects of manipulations of ABLK number in adult flies. A: Genotypes with a lower number of ABLKs show weaker resistance to dry starvation than their relatives. elav-G4>UAS-abdA:RNAi,UAS-Ubx:RNAi tub-Gal80ts compared with elavG4>+/CyO;+/TM6, and elavG4>+/TM6 compared with elav-G4>UAS-AbdB:RNAi tub-Gal80ts. B: Flies of genotypes with a higher number of ABLKs show a significant increase in food intake as monitored by capillary feeding (CAFE) compared with their relatives. elav-G4>UASAbdB:RNAi tub-Gal80ts compared with elavG4>+/TM6, and elav-G4>+/CyO;+/TM6 compared with elav-G4>UAS-abdA:RNAi,UAS-Ubx:RNAi tub-Gal80ts. (**p<0.001, *p<0.01, n=10 flies for each genotype with three replicates, two-way ANOVA).

199x289mm (300 x 300 DPI)

141x105mm (72 x 72 DPI)

Graphical abstract text:

The authors explore the genetic, hormonal and environmental factors causing variability in the number of abdominal leucokinin neurons in adults of *Drosophila*. This variability arises in postembryonic neurogenesis and affects not only different individuals but also the right and left side of the nervous system.