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The effect of neurospecific knockdown of candidate genes for locomotor behavior and sound production in *Drosophila melanogaster*

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Abbreviations: BDSC, Bloomington *Drosophila* Stock Center; CNS, central nervous system; CPG, central pattern generator; CS, Canton-S strain; FITC, fluorescein isothiocyanate; Gal4, yeast transcription activator protein Gal4; GFP, green fluorescent protein; IPI, interpulse interval; LA, locomotor activity; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; *PdL*, *Ponce de Leon* P element construct; RNAi, RNA interference; shRNA, small hairpin RNA; TRiP, Transgenic RNAi Project; UAS, upstream activation sequence; VDRC, Vienna *Drosophila* RNAi Center

Molecular mechanisms underlying the functioning of central pattern generators (CPGs) are poorly understood. Investigations using genetic approaches in the model organism *Drosophila* may help to identify unknown molecular players participating in the formation or control of motor patterns. Here we report *Drosophila* genes as candidates for involvement in the neural mechanisms responsible for motor functions, such as locomotion and courtship song. Twenty-two *Drosophila* lines, used for gene identification, were isolated from a previously created collection of 1064 lines, each carrying a P element insertion in one of the autosomes. The lines displayed extreme deviations in locomotor and/or courtship song parameters compared with the whole collection. The behavioral consequences of CNS-specific RNAi-mediated knockdowns for 10 identified genes were estimated. The most prominent changes in the courtship song interpulse interval (IPI) were seen in flies with *Sps2* or *CG15630* knockdown. Glia-specific knockdown of these genes produced no effect on the IPI. Estrogen-induced knockdown of *CG15630* in adults reduced the IPI. The product of the CNS-specific gene, *CG15630* (a predicted cell surface receptor), is likely to be directly involved in the functioning of the CPG generating the pulse song pattern. Future studies should ascertain its functional role in the neurons that constitute the song CPG. Other genes (*Sps2, CG34460*), whose CNS-specific knockdown resulted in IPI reduction, are also worthy of detailed examination.

Introduction

Motor output from animal nervous systems is formed by regulatory and trigger signals from sensory and integrative structures, and is dependent on the intrinsic properties of motor neurons and their interactions.[1] Motor output may manifest as a brief burst of pulses (e.g., knee jerk) or a continuous rhythmically organized sequence of pulses (e.g., wing beats). The second type of activity is attributed to central pattern generators (CPGs) in the nervous system, either single-cell pacemakers or ensembles of interacting neurons creating rhythmic output. Pacemaker rhythmic activity is created by oscillations in membrane permeability for sodium, potassium and calcium ions, but elucidation of the molecular mechanisms controlling these oscillations is not yet complete.[2-4]

Drosophila melanogaster is a model organism with a simple nervous system. A powerful arsenal of genetic techniques has been assembled over the last hundred years of investigating the fruit fly. These advantages provide a system for discovering the molecular basis of rhythmic activity generation. Several new molecular components participating in motor functions, including CPG operation and regulation, have been described in *Drosophila*.[5,6] However, the data in this field are fragmentary, and the approach of forward genetics, i.e., genetic screening for impairments of motor functions, may be very helpful in identifying genes involved in CPG functioning.

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Many behaviors may be studied to investigate CPGs in *Drosophila* (e.g., walking, flight and sound production in adult flies or crawling in larvae). We chose walking and male courtship song because of the relative ease of performing mass testing of flies using simple automated techniques (see Methods).

The locomotor activity of single flies, as revealed by 'open-field' observations, is characterized by 2 different behavioral components, the 'amount' and 'speed' of movement.[7] The 'amount' is determined by run initiation frequency and run duration. CPG malfunction can be reflected in both parameters. For example, mutation of the *na* gene, encoding a Na^+ leak channel known to regulate pacemaker activity, makes flies walk in fits and starts, taking a few steps and then stopping before starting again.[4,5,8] This motor phenotype would be characterized by high run initiation frequency and low run duration. Alteration of run speed may indicate changes in the motor pattern of leg coordination, which could be derived from defective CPG regulation. Mendes et al.[9] reported that flies increasingly use tetrapod and non-canonical combinations instead of a tripod gait as they decrease their speed. Hence, an absent tripod gait in mutant lines could lead to a reduction in run speed.

The impulse component of the male *D. melanogaster* courtship song has a rhythmic repetitive structure of sound pulses varying from 2 to 50 pulses per train.[10] The time between pulses is known as the interpulse interval (IPI), whose value is inversely correlated with the activation level of neurons of the putative song CPG.[11] Thus, in contrast to locomotor parameters, the IPI pattern directly reflects the functioning of the song CPG.

Genetic studies of locomotor behavior in *Drosophila* have a long history starting with selection experiments[12-17] and later with linkage analysis.[7,18,19] To identify candidate genes affecting locomotor behavior, quantitative trait loci analysis[21,22] and whole genome analysis of differences in expression levels between selection lines[22] were used. With the same aim, Strauss[23] performed a screen for ethyl methanesulfonate induced X-linked locomotor mutants. However, to date, *Drosophila* locomotor behavior in terms of CPG functioning has not been investigated and no genetic screen for autosomal genes affecting various locomotor parameters has been coupled with simultaneous investigation of courtship song parameters.

A great number of genetic studies of *Drosophila* courtship song, including genetic screens and analysis of numerous mutants, have been performed (for a review see ref. 24, and for recent advances in this field see ref. 25); however, the present paper shows that the list of genes having possible involvement in song CPG is not complete.

Here we report the identification of 22 *Drosophila* genes as candidates for involvement in the neural mechanisms responsible for motor functions. The 22 lines used for gene identification were isolated from a previously created collection of 1064 lines carrying a single random P element insertion (*PdL* transposon) in one of the autosomes. These 22 lines displayed extreme deviations in locomotor and/or courtship song parameters compared with the whole collection (the details and results of the behavioral screening will be published elsewhere).

The behavioral consequences of CNS-specific knockdowns of 10 identified genes were estimated using local RNA interference (RNAi) under the control of various GAL4 drivers (*elav/nrv2/appl/tsh/repo-GAL4*) in the Gal4/UAS system. Tissue-specificity and efficiency of RNAi were confirmed by GFP fluorescence localization in the embryo and quantitative assessment of target gene expression in imago (for genes *CG15630*, *Sps2* and *Mef2*).

RNAi of each analyzed gene led to deviation of one or more parameters of locomotor behavior or courtship song. The results greatly depended on the suppressed gene and the CNS-specific driver used. The most prominent changes in the courtship song IPI, directly reflecting CPG functioning (motor pattern of wing vibration), were seen in flies with *Sps2* and *CG15630* RNAi. Absence of courtship song impairments in flies with glia-specific *CG15630* knockdown and IPI reduction following estrogen-induced *CG15630* RNAi at adult stage indicates that *CG15630* codes for a product, which is necessary for the correct functioning of courtship song CPG neurons in adult flies.

Results

Identification of candidate genes in mutants with motor phenotypes

Location of the *PdL* transposon sequence and its direction were determined for selected lines with motor phenotypes. Of the 22 lines analyzed, each had a unique *PdL* transposon insertion site (**Table 1**). Insertion site locations were as follows: in 15 lines in non-coding regions of introns or exons of the genes, *Cf2*, *Dgp-1*, *Ext2*, *lola*, *MESR4*, *Map205*, *Mef2*, *Sps2*, *Treh*, *wdp*, *yps*, *CG1943*, *CG5807*, *CG6746* and *CG15630*; in 2 lines in coding regions of the genes, *jumu* and *CG8708*; in one line in the cluster of transposons in the pericentromeric region (*no gene*); in 4 lines at various distances from the genes, *drl*, *jing*, *Hsr*ω and *CG34460*.

Locomotor behavior in flies with CNS-specific knockdown of candidate genes

Parameters of locomotion were assessed in flies with suppressed CNS expression in 10 candidate genes (*Sps2*, *CG15630*, *Dgp-1*, *CG6746*, *CG8708*, *CG34460*, *Mef2*, *lola*, *jing* and *drl*). The selection of target genes was based on the availability of RNAi lines with *attP* landing sites from VDRC and TRiP collections. The GAL4/UAS system for post-transcriptional silencing of a target gene by means of local RNAi was used. The experimental flies were derived by crossing the VDRC (Vienna *Drosophila* RNAi Center) or TRiP (Transgenic RNAi Project) lines, which carry transgenes coding for target gene-specific interfering RNA under a UAS element, with lines carrying GAL4 CNS-specific drivers (*elav/nrv2/appl/tsh-GAL4,* **Table 2**). GAL4 control flies were derived from crosses of the same GAL4 lines with host strains for VDRC and TRiP RNAi lines, #60100 and #36303 respectively, which did not carry an interfering RNA transgene (see Materials and Methods). To exclude from analysis the motor deviations caused by genetic background differences between

Table 1. Genomic location and orientation of PdL insertions in the mutant lines. Line ID - laboratory identifier of the mutant line. Designations in the second column are: XX:YYYYYYYY[+/-], where XX indicates the chromosome arm; YYYYYYYY indicates the *Drosophila* genome nucleotide number downstream of the *PdL* insertion; [+/-] indicates forward (5′-3′) or reverse (3′-5′) orientation of the *PdL* transposon in the chromosome. Genomic location and orientation are determined according to Flybase[51] data released September 7th, 2010 (Dmel Release 5.30), sequenced strain y^1; cn^1 bw^1 sp^1 (Bloomington # 2057)

Line ID	*PdL* genomic position and orientation	Candidate gene affected	Known or predicted gene products	Allele
3724	2L:4882790[+]	*Cf2*	transcription factor	$Cf2^{3724}$
663	2R:5842843[-]	*Mef2*	transcription factor (predicted)	$Mef2^{663}$
5282b	2R:6429208[+]	*lola*	transcription factor (predicted)	$lola^{5282b}$
5493	3R:6182222[+]	*jumu*	transcription factor (predicted)	$jumu^{5493}$
3494	2R:2389393[-]	*jing*	transcription factor	$jing^{3494}$
2169	2R:13435745[+]	*MESR4*	unknown	$MESR4^{2169}$
843K	2R:14059049[+]	*Dgp-1*	translation factor (predicted)	$Dgp\text{-}1^{843K}$
7081	3L:12118218[-]	*yps*	translation repressor	yps^{7081}
3404з	2L:4793956[+]	*CG15630*	cell surface receptor (predicted)	$CG15630^{3404з}$
3979a-s2	2R:18199553[-]	*wdp*	cell surface receptor (predicted)	$wdp^{3979a\text{-}s2}$
2248	2L:19190341[-]	*drl*	receptor tyrosine kinase (predicted)	drl^{2248}
3328b	3R:20639718[-]	*CG5807*	lipocalin-1 cell surface receptor (predicted)	$CG5807^{3328b}$
5433-t3	3R:17122251[+]	*Hsrω*	regulatory RNA (predicted)	$Hsr\omega^{5433\text{-}t3}$
6225a	2L:20116133[+]	no gene	piRNA (predicted)	no gene
3389	2L:12002261[-]	*CG6746*	3-hydroxyacyl-CoA dehydratases (predicted)	$CG6746^{3389}$
5769	2L:10342643[+]	*Sps2*	monoselenphosphate synthetase (predicted)	$Sps2^{5769}$
5180-t3	3R:2901187[+]	*CG1943*	unknown	$CG1943^{5180\text{-}t3}$
4653	2R:4047132[+]	*CG8708*	β-1,3-galactosyltransferase	$CG8708^{4653}$
3290	2R:12716378[-]	*CG34460*	unknown	$CG34460^{3290}$
5567a	3R:27894136[-]	*Map205*	microtubule binding protein	$Map205^{5567a}$
6387з-s2	2R:12036471[+]	*Ext2/ CG10731*	glycosyltransferase (predicted)/ATP synthase subunit s	$Ext2^{6387з\text{-}s2}$/ $CG10731^{6387з\text{-}s2}$
4262*c*-s2	2R:16964321[+]	*Treh*	trehalase	$Treh^{4262c\text{-}s2}$

control and experimental lines and probable nonspecific leaking from RNAi transgene we tested UAS-RNAi control flies originated from crosses between wild-type strain Canton-S (CS_{BDSC}) and RNAi line for each gene (**Table2**). The crosses between CS_{BDSC} and host controls for RNAi lines (60100 and 36303) were used for comparison.

For VDRC and TRiP lines, comparison of UAS-RNAi controls with $CS_{BDSC}\times60100$ and $CS_{BDSC}\times36303$ host line hybrids respectively (see CS in **Figs. 1**, **2**) showed own effect of UAS-RNAi transgene insertions. It was manifested as a decrease in run frequency (4 VDRC lines) and run speed (TRiP line for gene *lola*) and as an increase in run duration (2 other VDRC

Table 2. Collection and FlyBase identification numbers (ID) of transgenic flies used in experiments. w* - unspecified allele

Stock type	Collection ID	Transgene	FlyBase ID	Genotype
Gal4 lines	8760	*elav-GAL4*	FBst0008760	*w[*]; P{w[+mC] = GAL4-elav.L}3*
	8765	*elav-GAL4*	FBst0008760	*P{w[+mC] = GAL4-elav.L}2/CyO*
	6794	*nrv2-GAL4*	FBst0006794	*w[*]; P{w[+mC] = nrv2-GAL4.S}8 P{w[+mC] = UAS-GFP.S65T}eg[T10*
	30546	*appl-GAL4*	FBst0030546	*P{w[+m*] = Appl-GAL4.G1a}1, y[1] w[*]; Mlf[Delta10]/CyO*
	3040	*tsh-GAL4*	FBst0003040	*y[1] w[1118]; P{w[+mW.hs] = GawB}tsh[md621]/CyO; P{w[+mC] = UAS-y.C}MC1/TM2*
	25038	*GAL4.ER*	FBst0025038	*w[*]; P{w[+mC] = hs-GAL4.ER}ER156*
	7415	*repo-GAL4*	FBst0007415	*w[1118]; P{w[+m*] = GAL4}repo/TM3, Sb[1]*
TriP RNAi lines	28699	*UAS-Mef2*	FBst0028699	*y1 v1; P{TRiP.JF03115}attP2*
	26714	*UAS-lola*	FBst0026714	*y1 v1; P{TRiP.JF02254}attP2*
	27024	*UAS-jing*	FBst0027024	*y1 v1; P{TRiP.JF02345}attP2*
	29602	*UAS-drl*	FBst0029602	*y1 v1; P{TRiP.JF03281}attP2*
VDRC RNAi KK lines	105268	*UAS-Sps2*	FBst0477096	*y,w[1118];P{attP,y[+],w[3`]}*
	107797	*UAS-CG15630*	FBst0479610	*y,w[1118];P{attP,y[+],w[3`]}*
	109410	*UAS-Dgp-1*	FBst0481099	*y,w[1118];P{attP,y[+],w[3`]}*
	103625	*UAS-CG6746*	FBst0475483	*y,w[1118];P{attP,y[+],w[3`]}*
	102288	*UAS-CG8708*	FBst0474157	*y,w[1118];P{attP,y[+],w[3`]}*
	110120	*UAS-CG34460*	FBst0481705	*y,w[1118];P{attP,y[+],w[3`]}*
host strain for TRiP RNAi lines	36303	No	FBst0036303	*y[1] v[1]; P{y[+t7.7] = CaryP}attP2*
host strain for VDRC KK RNAi lines	60100	No	No	*y,w[1118];P{attP,y[+],w[3`]}*
GFP line	32202	*UAS-GFP*	FBst0032202	*w[*]; P{w[+mC] = 10XUAS-IVS-GFP-WPRE}attP2*
CS_{BDSC}	1	No	FBst0000001	*Wild type*

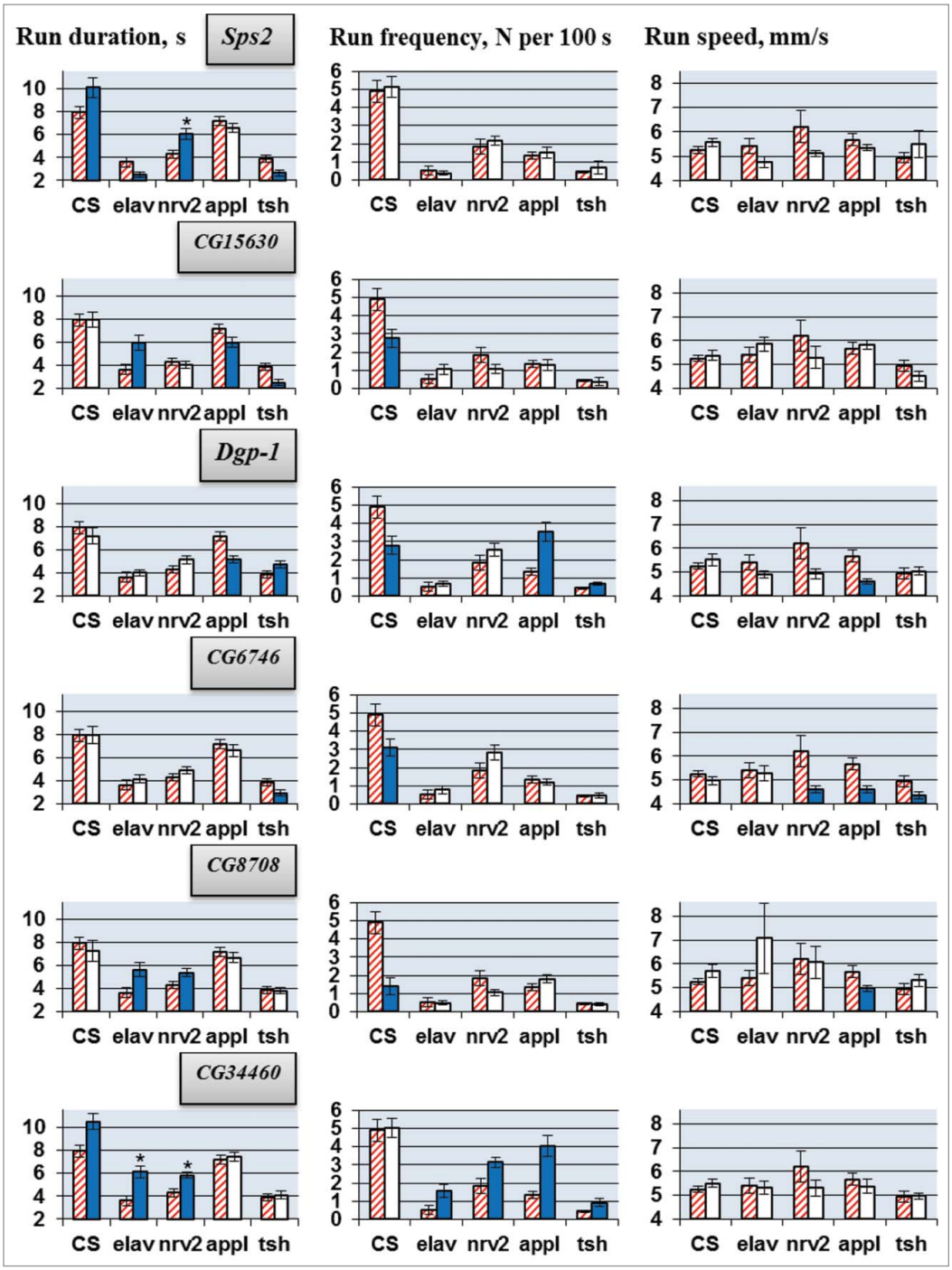

Figure 1. Locomotion parameters in flies with tissue-specific knockdown of the candidate genes (VDRC RNAi lines). GAL4/UAS-RNAi flies with spatially restricted knockdown (second column for each driver) derived by crossing *elav/nrv2/appl/tsh-GAL4* drivers with VDRC RNAi lines. GAL4 controls without UAS-RNAi transgene (first, hatched columns) originate from cross of the same GAL4 drivers with host strain #60100 and are specific for each GAL4 driver. Also presented are locomotion parameters in UAS-RNAi controls descended from cross of CS_{BDSC} with VDRC RNAi line for a gene indicated (CS, second columns) and in flies derived by crossing CS_{BDSC} with host strain #60100 (CS, first, hatched column). Mean values with standard errors are shown. N = 40 for each data point. Significant difference from a corresponding control is indicated by filling (2-sided randomization test, 10,000 iterations, $P < 0.05$). Comparisons excluded from consideration (see text) are marked with asterisk. For details of genotypes see Methods and **Table 2**.

lines) and run speed (TRiP line for gene *jing*). For that reason, the knockdown effects coinciding with own effect of UAS-RNAi insertion (marked with asterisk in **Figs. 1**, **2**) must be excluded from consideration. RNAi under *tsh-GAL4* control caused lethality for *Mef2* and *lola* and gross morphological wing defects for *jing* (**Fig. 2**).

RNAi of each candidate gene led to deviations in one or more locomotor parameters (**Figs. 1**, **2**). The results depended greatly on the gene being suppressed and the CNS-specific driver used. Run duration and run frequency were especially variable. Under different drivers, RNAi of a target gene often caused opposite changes in these parameters, while RNAi under some drivers produced no effect. Run speed was more consistent. For all candidate genes, RNAi led to either no changes or to a decrease in run speed. For *CG6746*, 3 different drivers produced a decrease in run speed, while for all other genes this effect was only observed for one of the 4 drivers.

In general, we can conclude that all selected candidate genes are involved in the neural processes responsible for locomotion. However, their roles are determined by unique spatiotemporal patterns of gene expression in various neurons. This explains why the RNAi effects are strongly dependent on the GAL4 driver, which specifies the location, stage and intensity of gene suppression.

Courtship song parameters in flies with CNS-specific knockdown of candidate genes

Parameters of courtship song were assessed by CNS-specific RNAi knockdown of the candidate genes, *Sps2*, *CG15630*, *CG6746*, *CG34460* and *Mef2*, which showed significant deviations in locomotor behavior and, according to the literature and bio-informational data, had more perspectives to be considered as a molecular players in CPG functioning. For all genes no own effect of UAS-RNAi transgene insertions was observed (**Fig. 3**). As for locomotion, deviations in courtship song parameters varied according to different 'driver - target gene' combinations. Similar to locomotion parameters, opposite changes occurred in pulse train duration and IPI according to the different GAL4 drivers used for RNAi. We paid special attention to IPI because this parameter directly reflects CPG functioning (motor pattern of wing vibration). Knockdown of all candidate genes with at least one GAL4 driver led to IPI deviations. The most prominent changes were seen in flies with RNAi of *Sps2* or *CG15630*. Suppression of either gene resulted in IPI reduction.

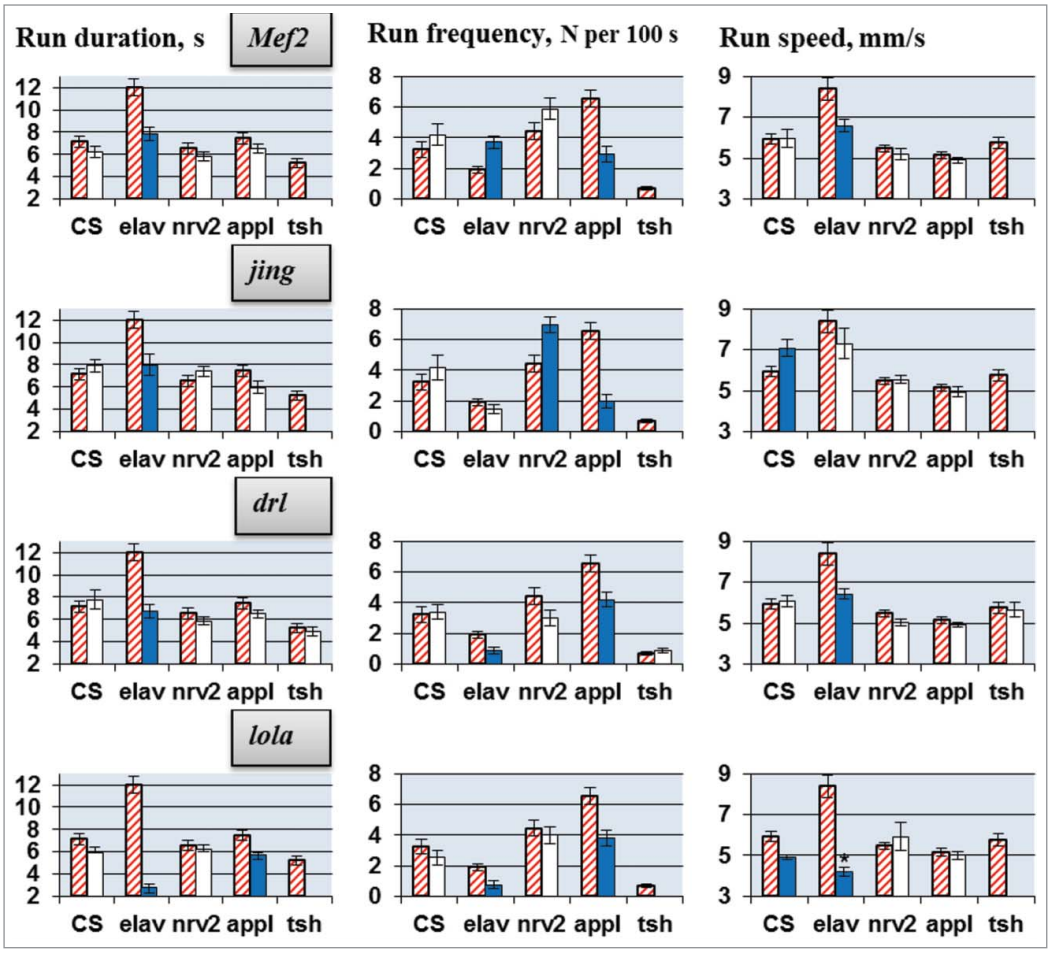

Figure 2. Locomotion parameters in flies with tissue-specific knockdown of the candidate genes (TRiP RNAi lines). Flies with spatially restricted knockdown derived by crossing *elav/nrv2/appl/tsh-GAL4* drivers with TRiP RNAi lines. The host strain #36303 was used for all control crosses. Absence of a value indicates lethality or gross morphological effects of the knockdown. Other explanations, see legend for **Figure 1**.

***Sps2*, *CG15630* and *Mef2* expression levels in flies with tissue-specific knockdown**

In many cases, behavioral deviations had opposite directions under control of different GAL4 drivers (**Figs. 1–3**). We chose 3 genes, most perspective for further investigations. The quantitative assessment of *Sps2*, *CG15630* and *Mef2* expression levels at the imago stage showed a reduction in the expression of each target gene (**Fig. 4**). However, the magnitude of suppression varied according to different 'driver - target gene' combinations. The differences in suppression levels are determined by the extent of concordance between endogenous expression patterns of candidate genes and drivers. For example, RNAi of *CG15630* under *elav-GAL4* leads to more pronounced suppression compared with *Sps2* and *Mef2*. Indeed, according to the high-throughput data of Chintapalli et al.[26] (cited in FlyAtlas[27]), the expression pattern of *CG15630* in different adult tissues is more similar to the *elav* expression pattern than that of *Sps2*.

We also analyzed GFP fluorescence under the control of *elav/appl/nrv2/tsh-GAL4* drivers at embryo stage 13–16 to confirm tissue-specificity of expression patterns. All drivers showed expression patterns consistent with previously published results.[28-31] *elav-* and *appl-GAL4* produced GFP fluorescence in the brain and ventral ganglion, while *tsh-GAL4* expression was mainly in the ventral ganglion (data not shown).

Locomotor and courtship song parameters in flies with glia-specific *Sps2* or *CG15630* knockdown

For each candidate gene, the effect of RNAi on motor activity was strongly dependent on the CNS-specific GAL4 driver used (see the run and pulse train durations in *Sps2* knockdowns, **Figs. 1**, **3**). *elav* and *nrv2* are both reported to be expressed in neurons and glia.[32,33] Conversely, the *appl* driver was used for selective expression in neuronal structures but not in glia.[34] Therefore, we tested flies with RNAi under control of the glia-

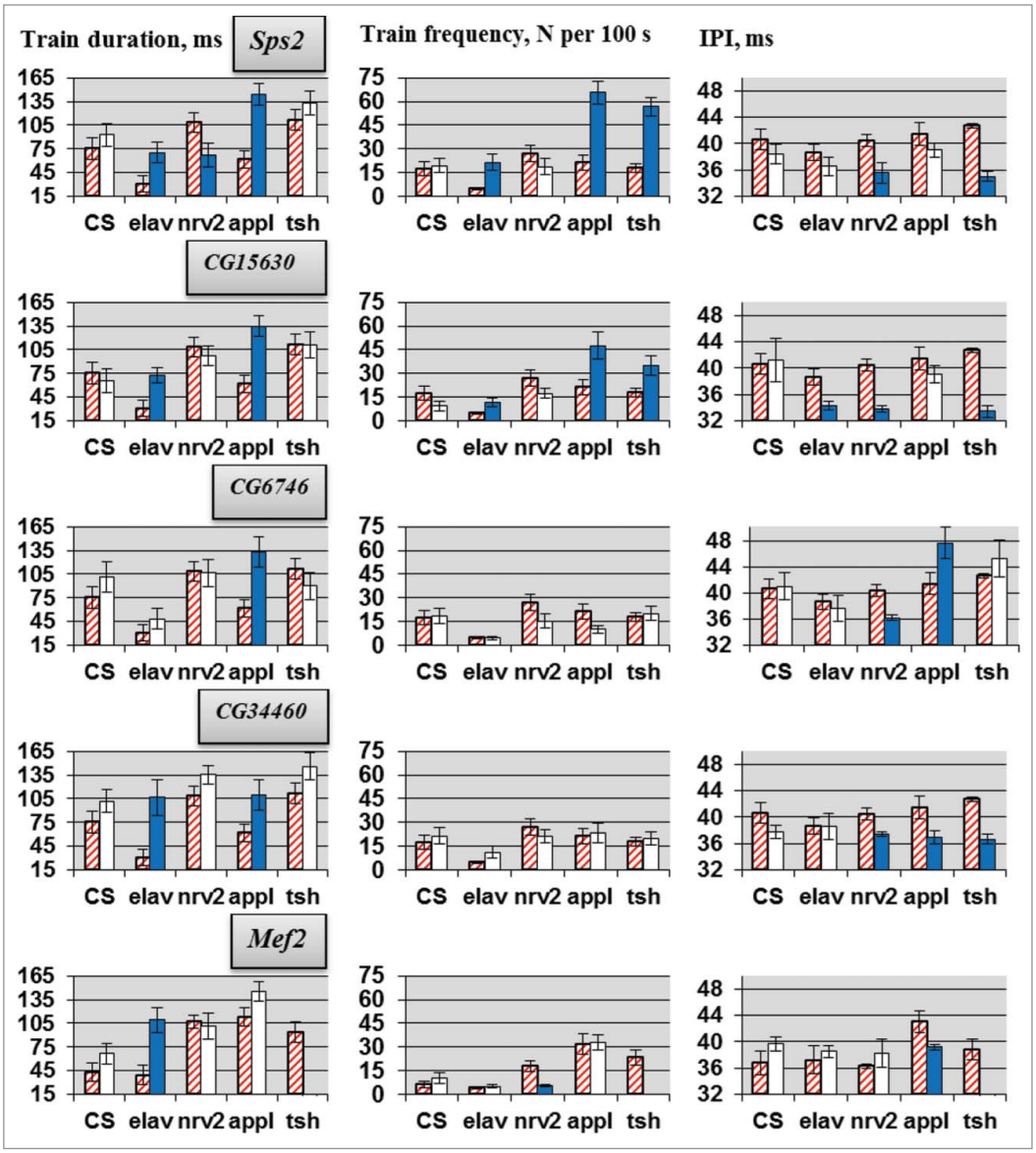

Figure 3. Courtship song parameters in flies with tissue-specific knockdown of the candidate genes. Flies with tissue-specific knockdown derived by crossing *elav/nrv2/appl/tsh-GAL4* with VDRC/TRiP RNAi lines. N = 20 for each data point. Explanations are presented in legends for **Figures 1 and 2**.

specific *repo-GAL4* driver to determine the consequences of glial suppression of the candidate genes. We revealed that knockdown of *Sps2* or *CG15630* in glia has no significant effect on either locomotor and courtship song parameters, except reduction in run frequency for *CG15630* knockdown (**Fig. 5**). Importantly, it was not observed for neurospecific drivers but was revealed as nonspecific alteration in flies from cross of CS_{BDSC} with RNAi line for *CG15630* (**Fig. 1**). Thus, the effect of *CG15630* RNAi on motor functions seems to be related to underexpression of *CG15630* in neurons rather than in glia.

Tissue-specificity of *repo-GAL4* driver expression was assessed by observing *UAS-GFP* expression in stage 13–16 embryos (data not shown). The *repo-GAL4* expression pattern was consistent with previously described results.[35]

Locomotor and courtship song parameters in flies with estrogen-induced knockdown of *CG15630* in adults

CNS-specific RNAi of *CG15630* caused significant deviations in locomotor (**Fig. 1**) and courtship song (**Fig. 3**) parameters, the most prominent being for IPI. This may result from RNAi effects at either developmental or imago stages. Therefore, using the estrogen-induced *GAL4* driver (*GAL4.ER*), we tested motor activity in flies with *CG15630* RNAi turned on at the imago stage.

Knockdown of *CG15630* in adult flies resulted in deviations of both locomotor and courtship song parameters: increasing the run duration and decreasing the run frequency and IPI (**Fig. 6**). Similar effects were observed for RNAi under some of the constitutive drivers (**Figs. 1**, **3**). Neither estrogen-induced nor constitutive RNAi of *CG15630* changed the run speed (**Figs. 1**, **6**).

Given that *CG15630* is expressed predominantly in the CNS[26,27] and that *GAL4.ER* drives expression in the CNS,[36] and taking into account the conclusions in the previous section, we can assert that neuronal expression of *CG15630* at the imago stage is required for the neural mechanisms that determine run duration and the mechanisms responsible for formation of pulse song rhythmicity (IPI). It is very probable that *CG15630* RNAi influences pulse song patterning through participation of the gene product in the molecular mechanism of song CPG.

Estrogen-induced knockdown of *CG15630* did not lead to changes in train duration or frequency (**Fig. 6**), changes that were produced by some of the non-induced CNS-specific knockdowns (**Fig. 3**). Therefore, it is likely that, in the latter case, deviations in these parameters were caused by developmental abnormalities.

CG15630 suppression in flies with estrogen-induced knockdown was confirmed by quantitative assessment of gene

expression in adults, mean expression ratio (knockdown/control) was 0.649, standard error ±0.044.

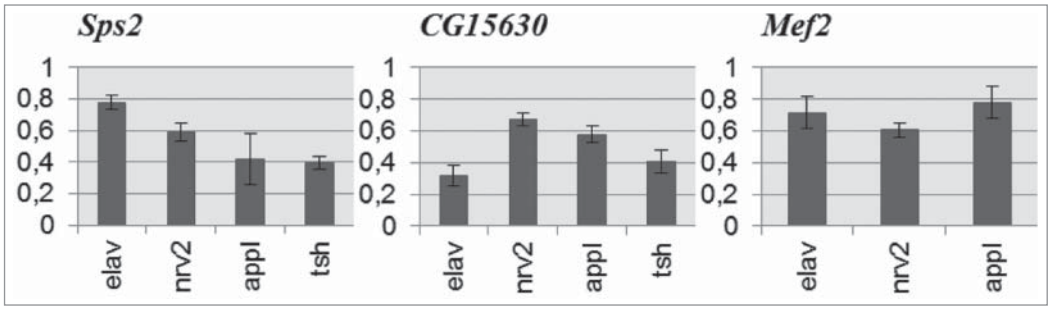

Figure 4. Expression level of *Sps2*, *CG15630* and *Mef2* genes in RNAi-mediated knockdowns relative to control. Mean expression ratios (knockdown/control) with standard errors are shown. Expression levels of all candidate genes are reduced in all experimental samples (2-sided randomization test, $p < 0.05$, REST 2009 software).

Discussion

The genes identified as candidates for involvement in motor functions (**Table 1**) encode 8 transcription or translation factors, 4 cell surface receptor components, 2 regulatory RNAs, a microtubule binding protein and various metabolic enzymes. To determine CNS-specific effects of selected genes on motor functions, we assessed locomotor (10 genes) and courtship song (5 genes) parameters in flies with tissue-specific knockdowns.

Comparison of cross between the wild-type strain CS_{BDSC} and host strain (60100 or 36303) with crosses between CS_{BDSC} and RNAi lines (**Fig. 1**, 2, the first pair of columns on each graph) revealed several differences in locomotion parameters. The descendants of 2 TRiP RNAi lines demonstrated deviations in run speed, while descendants of VDRC RNAi lines showed decreased run frequency or increased run duration. These deviations could be caused either by own effect of UAS-RNAi transgenes or by difference in genetic background between the host strains and RNAi lines. Recently Green et al.[37] reported that transgenic RNAi-inducing lines from the VDRC libraries are subject to dominant phenotypic effects. Specifically, on crossing 39 randomly selected KK lines to the panneuronal driver *elav-GAL4c155*, 9 of them produced F1 progeny unable to properly inflate their wings. They found that the main integration site for pKC26 vector (carrying the shRNA sequences) in the VDRC KK library is the non-annotated pKC43 target (occupied in all 39 lines tested), whereas only the 9 lines displaying the *elav-GAL4c155*-dependent non-inflating wing phenotype were found to have a pKC26 integration into the annotated pKC43 insertion. In our experiments we did not observe any gross morphological or wing defects in experimental flies generated by crossing GAL4 and VDRC RNAi lines.

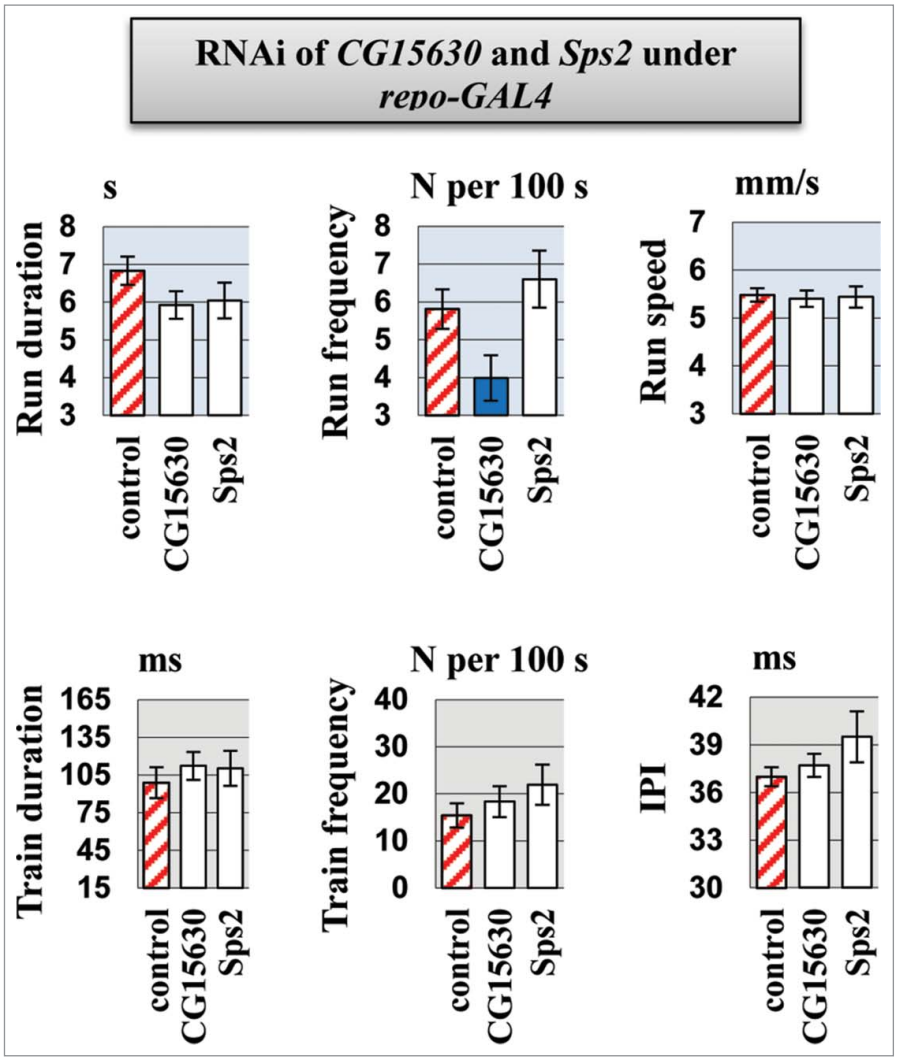

Figure 5. Motor parameters in flies with knockdown of *CG15630* under the glia-specific repo-Gal4 driver. Glia-specific knockdown flies derived by crossing repo-GAL4 driver with VDRC RNAi lines #107797 for *CG15630* and #105268 for *Sps2*. Control flies derived by crossing repo-GAL4 driver with host line #60100. Mean values and standard errors are shown. N = 40 for locomotor parameters, N = 20 for courtship song parameters. Significant difference from a corresponding control is indicated by filled columns (2-sided randomization test, 10,000 iterations, $p < 0.05$).

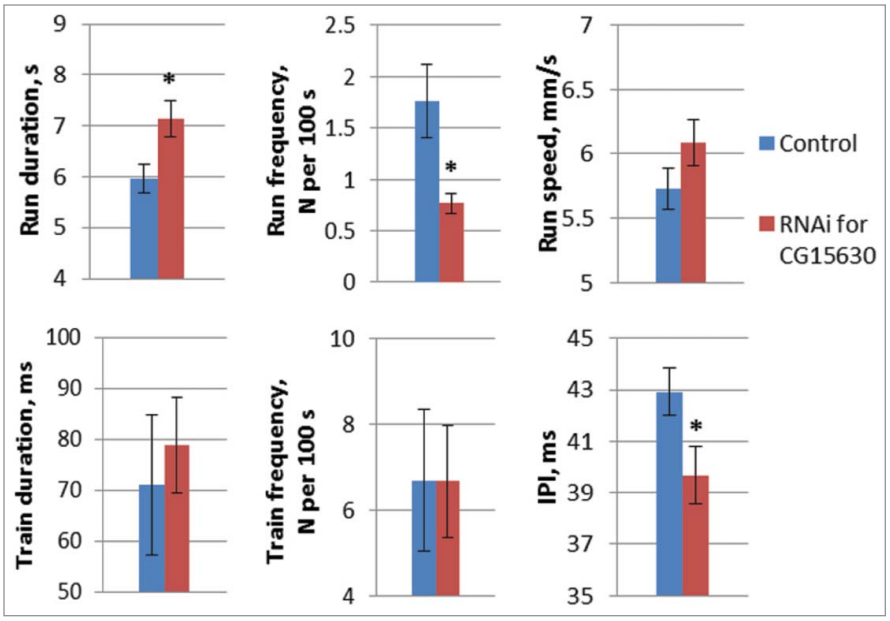

Figure 6. Motor parameters in flies with estrogen-induced knockdown of *CG15630* in adults. Estrogen-induced knockdown flies derived by crossing GAL4.ER driver with VDRC RNAi line #107797 for CG15630. Control flies derived by crossing GAL4.ER driver with host line #60100. Both experimental and control flies were kept on estrogen-containing medium from the moment of imago eclosion. Mean values and standard errors are shown. N = 40 for locomotor parameters, N = 20 for courtship song parameters. Significant differences between RNAi and control data are marked with asterisks (2-sided randomization test, 10,000 iterations, $P < 0.05$).

RNAi of *lola* and *drl*, under the control of *elav/appl-GAL4* drivers, repressed locomotion (run duration and frequency, **Fig. 2**). *lola* and *drl*, code for a transcription factor and a receptor tyrosine kinase, respectively, and are important determinants of motor activity. Feminization of cells expressing *lola* produced a 'no song' phenotype,[25] and *drl* was identified to affect locomotor reactivity in a quantitative trait loci study.[21] At the same time, mutations in these genes led to structural defects in the adult brain.[38-40] Therefore, the probable explanation of locomotion reduction in *lola* and *drl* RNAi knockdowns is the disturbance of regulatory inputs from the brain to the locomotor CPG, but not a malfunction of the latter.

Knockdown of 2 other candidate genes encoding transcription factors, *Mef2* and *jing*, also reduced run duration, but only under *elav-GAL4* control; different drivers produced opposite deviations in run frequency. Contra-directional deviations, depending on the driver used, were also found for run duration in flies with RNAi of *CG15630* and *Dgp-1* (**Fig. 1**, increase in run duration in *Sps2* knockdown under *nrv2-GAL4* is not taken into consideration due to coincidence of its effect with own effect of UAS-RNAi transgene insertion). Contrary effects of *elav-GAL4* and *appl-GAL4* on physiological parameters were reported earlier. Simonsen et al.[41] noted that enhanced expression of *Atg8a* (*autophagy-related* gene) under *appl-GAL4* control produced a dramatic extension of adult longevity, but under *elav-GAL4* control the lifespan was, conversely, reduced. The authors suggested that this difference may be related to a difference in age specificity of gene expression under these drivers, suggesting that the timing of *Atg8a* expression in the aging CNS is critical for its ability to extend longevity.

The designers of *nrv2-GAL4* [31] revealed a few notable differences between patterns of *nrv2-GAL4*- and *elav-GAL4*-directed GFP fluorescence that could explain contrary effects of the drivers. Fluorescence was not seen in many peripheral sensory neurons in the *nrv2-GAL4/GFP* flies in late embryo and larval stages, in developing photoreceptor cell neurons and in the third-instar larval eye disks, fluorescence that was observed in the *elav-GAL4/GFP* flies. Moreover, Dumstrei et al.[42] expressed a target gene *(DE-cad^{ex})* specifically in cortical glia of the larval brain using the *nrv2-Gal4* driver and specifically in cortical neurons using the *elav-GAL4* driver.

Unlike for run duration and frequency, RNAi knockdowns caused only decreases in run speed. Almost all mutants with structural defects of the central complex walked more slowly than wild-type flies.[43] Strauss et al.[44] reported that reduced walking speed in *nob* flies, which have a medially lesioned protocerebral bridge, was caused by their inability to increase step length concomitant with an increase in stepping frequency. In contrast, *nob* swing phases were of normal duration and frequency throughout the whole frequency range, because timing is most probably a function of the thoracic ganglia. Accordingly, we did not observe (with the exception of *CG6746*) speed alterations in RNAi flies under the control of *tsh-Gal4*, which is expressed in the thoracic segments,[45] predominantly in the CNS[26,27] where the insect locomotor CPG is most likely situated.[46,47]

Compared with locomotion, deviations in the courtship song parameters were more uniform under the different GAL4 drivers (**Fig. 3**). Contra-directional alterations in pulse train duration were observed only in males with *Sps2* RNAi, while IPI was oppositely changed only in males with *CG6746* RNAi. It should be noted that *CG6746* suppression under *tsh-Gal4* control did not significantly influence IPI. Therefore, it seems likely that the product of this gene (a predicted 3-hydroxyacyl-CoA dehydratase) does not participate in the functioning of the song CPG,[48] while products of the genes, *Sps2* (a predicted monoselenphosphate synthetase), *CG15630* (see below), *CG34460* (product unknown) and, probably, *Mef2* (a predicted transcription factor) do. The conclusion for *Mef2* is uncertain because the *tsh-Gal4/Mef2* combination is lethal. The most prominent IPI changes were observed for *CG15630*, a CNS-specific gene[26,27] that encodes a protein-binding protein, with immunoglobulin-like and fibronectin type III domains (accession number Q9VR25).[49] In contrast to other drivers, *CG15630* RNAi under *appl-Gal4* control did not produce a significant IPI reduction. Because *appl* is not expressed in glia, it is possible that IPI reduction in *CG15630* RNAi flies is associated with abnormality of glial cells

but not neurons. However, *CG15630* RNAi under glia-specific *repo-Gal4* control does not affect courtship song parameters, indicating neuronal effects of the knockdowns on impulse song.

In principle, changes in courtship song parameters may result from both developmental effects of RNAi and malfunction of the neural structures determining song CPG activity in adults. Conditional estrogen-induced *CG15630* RNAi in adults causes IPI reduction (**Fig. 6**), suggesting direct involvement of *CG15630* in the functioning of the song CPG. Absence of alterations in train frequency and duration following RNAi under *GAL4.ER* control indicates that deviations of these parameters in flies with gene suppression under other drivers (**Fig. 3**) are possibly associated with developmental defects. However, it cannot be excluded that the difference between consequences of induced and persistent *CG15630* RNAi is determined by the distinctive expression pattern of *GAL4.ER*.

The effects of *CG15630* RNAi driven by *GAL4.ER* on courtship song production are similar to those driven by *tsh-* and *nrv2-Gal4* (**Fig. 3**). *tsh* is predominantly expressed in the thoracic-abdominal ganglion, so *tsh-Gal4*-driven RNAi can directly affect CPG functioning but not brain regulatory mechanisms. The expression of the *nrv2-Gal4* driver is controlled by transcriptional regulatory elements present in the flanking DNA of the *Drosophila* Na^+,K^+-ATPase β-subunit gene, *Nervana2*.[31] In imagoes, *nrv2-Gal4*-driven expression is more pronounced than that driven by *appl-* and *elav-Gal4*.[50,51] Together, these data indicate that *CG15630* is an important genetic determinant whose expression in adults is necessary for proper functioning of the courtship song CPG. Von Philipsborn et al.[11] discovered a class of *fru* neurons (vPR6) in *Drosophila* mesothoracic ganglia whose activity was both necessary and sufficient for robust song production. In addition, they reported that the level of vPR6 activity had tight inverse coupling with IPI. Therefore, IPI reduction in flies with *CG15630* RNAi under *tsh-Gal4*, *nrv2-Gal4* and *GAL4.ER* control may be related to reduced activity of vPR6. Future studies will ascertain the role of the *CG15630* gene product in the functioning of vPR6 and other *fru* neurons, which comprise the courtship song CPG.[48]

Conclusions

The product of the CNS-specific gene, *CG15630* (a predicted cell surface receptor), is likely to be directly involved in the functioning of the CPG generating the pulse song pattern. Future studies should ascertain its functional role in the neurons that produce the song CPG. Other genes (*Sps2*, *CG34460*), whose CNS-specific knockdown resulted in IPI reduction, are also worthy of detailed examination.

Materials and Methods

Drosophila stocks

The 22 lines used for gene identification were isolated from a collection previously created by the authors. This consisted of 1064 lines carrying a single random P element insertion (*PdL* transposon) in one of the autosomes. The 22 lines displayed extreme deviations in locomotor and/or courtship song parameters compared with the whole collection (the results of behavioral screening will be published elsewhere).

Tissue-specific suppression of the candidate genes was achieved by synthesis of interfering RNA using the GAL4/UAS system.[52] The GAL4/UAS flies were produced by crossing females of GAL4 lines carrying various GAL4 transgenes under CNS specific promotors (drivers) and males of RNAi lines carrying interfering RNA transgenes under UAS (upstream activation sequence) control (**Table 2**). All GAL4 lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC), including lines with various CNS-specific drivers: *elav-GAL4*, *nrv2-GAL4*, *appl-GAL4*, *tsh-GAL*4, *GAL4.ER* and the glia-specific driver, *repo-GAL4*. phiC31-based RNAi lines with site specific insertion[53] of shRNA sequences within the vector were obtained from the Vienna *Drosophila* RNAi Center (VDRC KK lines) and the Transgenic RNAi Project (TRiP lines). The GAL4 controls, specific for each driver, were produced by crossing the corresponding GAL4 line and the line used for creating RNAi lines, without an interfering RNA transgene (**Table 2**, host strains for VDRC KK and TRiP RNAi lines, #60100 and #36303 respectively). Additionally, we tested flies from crosses between wild-type strain Canton-S (CS_{BDSC}) and RNAi line for each gene (**Table 2**), as well as hybrids between CS_{BDSC} and host controls for RNAi lines, 60100 and 36303. In GFP fluorescence experiments, *UAS-GFP* stock from BDSC was used (**Table 2**).

Females of the outbred wild-type strain Canton-S (CS_{PIF}) have been maintained as mass-bred stock for several decades in the Pavlov Institute of Physiology in Koltushi (St. Petersburg) and were used as a standard opponent for male courtship in experiments with courtship song registration.

Experimental conditions

Flies were raised and kept on standard yeast-raisin medium at 25°C and under a 12-h light/dark cycle. For the locomotion test, experimental males were collected using CO_2 anesthesia soon after eclosion and kept in groups of 30 flies in culture vials with yeast-raisin medium for 3 d until the experiment was performed. For courtship song registration, males were collected without anesthesia and kept individually. Females (CS_{PIF}) were collected as virgins (10–12 flies per vial). A day before the experiment, 10–12 3-day-old females were brought together with 15–18 3-day-old males (CS_{PIF}) in one vial and left to mate for 18–22 h. Behavioral assays were performed at 25°C between 9 a.m. and 6 p.m.

Hormone treatment

β-estradiol (Sigma-Aldrich International GmbH, cat. # E8875) was dissolved in dimethyl sulfoxide (Sigma-Aldrich International GmbH, cat. # D5879) at a concentration of 260 mg/ml and mixed 1:3 (vol/vol) with wet yeast paste. From the moment of eclosion, males were maintained in culture vials with yeast-raisin medium and a drop of yeast (with or without hormone), refreshed daily.

Locomotion and courtship song assays and analysis

Individual males were freely moving in Perspex experimental chambers (15 mm diam., 5 mm high), which had transparent Perspex covers and a lateral entry (3 mm diam.) with stopper. Before locomotion recording, flies were agitated by chamber shaking. The coordinates of 20 individual male flies were simultaneously recorded for 1 h at a frequency of 10 Hz using 2 web cameras and *Drosophila tracks* software (© N.G. Kamyshev). Data processing was performed by the analysis module of *Drosophila tracks*. The percentage of time spent in locomotion (LA index), run frequency (number of runs per 100 s), run duration (s), run speed (mm/s) and other parameters of locomotor behavior were calculated for each fly. The flies were tested in duplicate (2×20 males).

Courtship song was recorded in a soundproof room for 5 min from 4 males simultaneously, each male being placed together with a fertilized CS female in a Perspex chamber with a latticed bottom on top of a microphone in a foam box, which functioned as an anechoic chamber. The sound signal was filtered, cutting off frequencies below 100 and above 800 Hz, processed with an analog-to-digital converter at 11025 Hz and saved as a sound file. The software *Drosophila courtship song analysis* (*DCSA*, © N.G. Kamyshev) autodetects the pulse song in sound recordings and allows manual editing of the results for automatic recognition. The *DCSA* analysis module performed primary data processing and created data tables for experimental groups. The calculated parameters involved percentage of time spent in pulse song (pulse song index), the frequency of pulse trains (trains per 100 s), number of pulses in a train, train duration (ms), intertrain interval (ms) and interpulse interval within a train (IPI, ms).

Statistical comparisons were made using the randomization test at significance level $\alpha = 0.05$.[54] We did not use the Bonferroni method to adjust statistical significance for the number of tests that have been performed in the study because we share the opinion that it creates more problems than it solves.[55]

Mapping of the P element insertion site by inverse PCR and DNA sequencing

Genomic DNA was prepared from 20 adult flies as described in Kim et al.[56] Genomic DNA equivalent to 2 adult flies was digested with TaqI (SibEnzyme Ltd., cat. #E133) or BstKTI (SibEnzyme Ltd., cat. #E119) and ligated with T4 DNA ligase (SibEnzyme Ltd., cat. #E319). The ligation mixture was used as a template for PCR. HSP (5′CTGCAGATTGTTTAGC TTGTTC3′) and IRS (5′CGGGACCCACCTTATGT TAT3′) served as PCR primers (Beagle). The PCR products were run on agarose gels, eluted, and sequenced with the HSP primer using an Applied Biosystems 3130 Genetic Analyzer (USA).

Real-time reverse transcription PCR analysis

Target gene expression levels were assessed using real-time reverse transcription PCR. Total RNA was isolated from 30 adult males using TRIzol,[57] treated with DNaseI and reverse transcribed by M-MuLV (SibEnzyme Ltd., cat #E317) reverse transcriptase and random hexamer primer. Quantitative real-time PCR was performed on a StepOnePlus (Applied Biosystems, Inc., USA) using EvaGreen® fluorescent dye (Biotium, Inc., BT-31000). Baseline and cycle threshold values were determined by automated threshold analysis with StepOne software v.2.0 (Applied Biosystems, USA). Both non-template and non-reverse transcribed RNA samples were used as negative controls for each sample. Expression levels of the *RpL32* transcript were used as an internal control, and relative mRNA levels were calculated using the comparative ΔCt method. Primers used (Beagle) were *CG15630*-RA (5′-ATTCGTTGAGATTCTCG-CAATGCG-3′ / 5′-CGGCGATTTCCAATGGAGCT-3′), *Sps2*-RA (5′-TGAGAAGGAACGCGACGTTGTG-3′ / 5′-GC TGCGCGTTTGACGGTAGTATTAT-3′), *Mef2*-RA (5′;-TGT ACCAGTACGCCAGCACCGA-3′ / 5′-TTGTACTTGGCC TCCGTTCGC-3′) and *RpL32*-RA (5′-TATGCTAAGCTG TCGCACAAATGGC-3′ / 5′-GTTCTGCATGAGCAGGACC TCCA-3′).

Data were statistically analyzed using the randomization test at a significance level of $\alpha = 0.05$ using the free software REST 2009.[58]

Wide field fluorescence microscopy

GAL4 driver expression patterns were assayed in GFP fluorescence experiments (at 25°C). Tissue-specific GAL4 lines were crossed with the *UAS-GFP* line. Embryos from the cross were dechorionated with 50% bleach and mounted in a 1:1 mixture of glycerol/PBS in a small viewing chamber constructed of a filter paper support on a standard coverslip. Samples were observed with a Mikmed 2 fluorescence microscope (LOMO, Russia) under Hg illumination using standard FITC fluorescence filters. Digital images were recorded using a C*X*05 Baumer Optronic camera (Switzerland). Image files were processed using PHOTOSHOP (Adobe Systems).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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